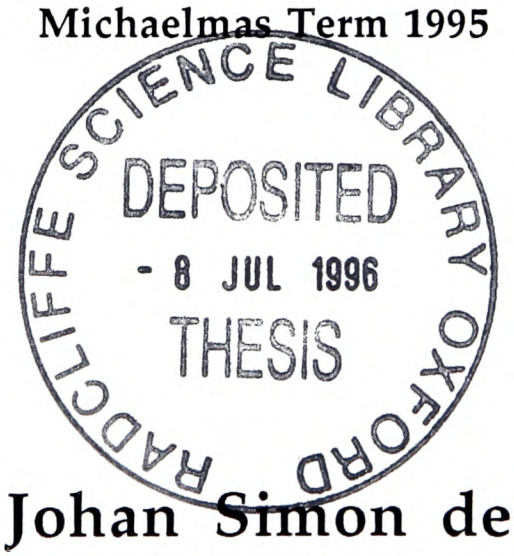
Cytokine and Growth Factor Regulation of Murine Macrophage Scavenger Receptor Expression and Function

A thesis submitted for the degree of Doctor of Philosophy in the

University of Oxford

Michaelmas Term 1995



Willem Johan Simon de Villiers

Sir William Dunn School of Pathology

and

Wolfson College

Abstract

Cytokine and Growth Factor Regulation of Murine Macrophage Scavenger Receptor Expression and Function

Willem Johan Simon de Villiers, Wolfson College
Submitted for the degree of D.Phil., Michaelmas Term, 1995.

The macrophage (Mϕ) foam cell in the atherosclerotic plaque micro-environment is subjected to cytokines and growth factors secreted by smooth muscle cells (SMCs), endothelial cells (ECs), platelets and lesional Mϕ themselves. This thesis examines the effects of some of these soluble factors on a murine macrophage membrane molecule, the macrophage scavenger receptor (MSR), considered pivotal in foam cell formation and atherogenesis. Macrophage Colony-stimulating factor (M-CSF) enhances MSR expression and functional activity (including MSR-dependent adhesion) in elicited peritoneal Mϕ markedly and selectively. The T lymphocyte products, Th1 (interferon-gamma(IFN-γ)) and Th2 (chiefly interleukin-4 (IL-4)) cytokines, have divergent effects with IL-4 upregulating and IFN-γ either maintaining or downregulating MSR status. IL-4 induced MSR microheterogeneity is due to changes in N-linked glycosylation, specifically sialylation and may be physiologically significant. In contrast to interleukin-10 (IL-10), transforming growth factor-beta (TGF-β) inhibits MSR upregulation, also when produced endogenously. TGF-β is as potent an inhibitor of MSR function as M-CSF is a stimulator. A cleaved truncated soluble form of MSR which lacks the cytoplasmic domain is present, by immunochemical assays, in culture supernatants. M-CSF increases soluble MSR release *in vitro* which is functionally active. Following upregulation of MSR surface expression in transfected CHO cells by prolonged culture, a modest MSR-dependent contribution to adhesion becomes apparent. To determine a possible adhesion-promoting region in the MSR, the binding site of mAb 2F8 was mapped using a series of MSR truncation mutants, and localized to residues 183 to 197 in the proximal α-helical coiled-coil domain. Morphological evidence, obtained by confocal and electron microscopy, supports an adhesion role for the MSR in primary Mϕ and transfected CHO cells. MSR expression is prominently directed to the adherent surface and its distribution is restricted to cellular contact areas with the substratum. Organs and atherosclerotic lesions from mice deficient in M-CSF (osteopetrotic) and apolipoprotein E were examined to determine the effects of M-CSF on Mϕ phenotype (including MSR expression) and lesion development *in vivo*. Though severely hypercholesterolemic, doubly deficient mice are protected against atherosclerosis and exhibit fewer Mϕ and low MSR expression on remaining M-CSF independent populations. Prominent hepatic lipid accumulation suggests a crucial M-CSF dependent role for Kupffer cells in lipoprotein uptake, transfer to hepatocytes and biliary excretion of cholesterol. Regulation of MSR activity may therefore be important for the recruitment of Mϕ into the arterial wall and, at the post-endothelial stage, to anchor Mϕ at specific locations, thus favouring foam cell formation.

ACKNOWLEDGEMENTS

I am endebted to my supervisor, Prof. Siamon Gordon, for his tireless enthusiasm, guidance and support, both scientific and pastoral. He has been a mentor in the true sense of the word and I value his friendship. I also thank Prof. Sir Henry Harris and Prof. Herman Waldmann for the opportunity to work in the Sir William Dunn School of Pathology.

Many members (past and present) of the Gordon macrophage "family" contributed to a most enjoyable time spent in Oxford. Rosangela da Silva attempted my initial scientific and technical education, and the subsequent collaboration with Iain Fraser and Anthony Doyle was a pleasure. The assistance of Derralynn Hughes is also gratefully acknowledged. Discussions with Peter Gough and David Greaves, as the other members of the "scavenger club", were always helpful and provocative. Andrew McKnight freely dispensed excellent advice on scientific matters, and also opinions on a staggering number of other topics, including football and Scotland. The bustling presences of Alison Macfarlane, Signe Balch and Luisa Martinez-Pomares were always encouraging, and Rick Haworth (for collaborating on the MSR-deficient mice) and Nick Platt (for general assistance and as a fellow member of the mostly victorious Dunn School cricket team) are also thanked. Illuminating scientific and philosophical discussions were held with fellow South Africans Satish Keshav and Mike Stein (my opponent in some epic squash games). Peter Tree is thanked for his help in animal experiments and his love for rugby union and Laurence Turley for general technical support and patience with radioactive matters.

Mike Hollinshead (confocal and electron microscopy) and Liz Darley (immunohistochemistry) were always enthusiastic and supportive, and Harry Edwards and Lance Tomlinson from Photography were superb in their professionalism. I also thank Dr. Derek Jewell (and his dedicated staff) for affording me the opportunity of some clinical work in the Gastroenterology Department at the John Radcliffe Hospital.

The financial support of the Nuffield Medical Dominion Trust and the British Heart Foundation is gratefully acknowledged, and Wolfson College is thanked for affordable family accommodation conveniently located near schools.

Finally, a heartfelt word of gratitude is due to my wife, Catherine, for her enduring support and to my wonderful three children, Katusha, Braam and Gera, for their love and keeping the noise levels down at crucial times.

ABBREVIATIONS

AcLDL	Acetylated Low Density Lipoprotein
aFGF	acidic Fibroblast Growth Factor
ADP	Adenosine Diphosphate
ADCC	Antibody Dependent Cellular Cytotoxicity
Ag	Antigen
AGE	Advanced Glycosylation Endproducts
ApoE	Apolipoprotein E
APC	Antigen Presenting Cell
APU	Arbitrary Phosphorimager Units
BCG	Bacille Calmette-Guérin
bFGF	basic Fibroblast Growth Factor
BgPMϕ	Biogel-elicited Peritoneal Macrophage
BMDMϕ	Bone Marrow-Derived Macrophage
βME	2-Mercaptoethanol
BP	Bacteriologic Plastic
b.p.	Base Pair
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	complementary Deoxyribonucleic Acid
CHO	Chinese Hamster Ovary (cell)
CR3	Complement Receptor type 3
DAB	Di-amino Benzidine
DEAE-	Diethylaminoethyl-
DiI-	1,1'-dioctadecyl-1-3,3,3',3'tetramethylindo-carbocyanine perchlorate-
DMEM	Dulbecco's Modification of Eagle Medium
DMSO	Dimethyl Sulphoxide
dNTP	Deoxynucleotide Triphosphate
EC(s)	Endothelial Cells
ECM	Extracellular Matrix
EDTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscope
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting/Scanning
FcR	Receptor for Fc portion of immunoglobulin
FCS	Fetal Calf Serum
fig	Figure
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor

gp	Glycoprotein
GPI-	Glycosylphosphatidylinositol-
h	Hour/s
HB-EGF	Heparin-binding Epidermal Growth Factor
HEL	Human Erythroleukaemia
HI	Heat Inactivated
HRP	Horseradish Peroxidase
HS	Human Serum
ICAM	Intercellular Adhesion Molecule
IDL	Intermediate Density Lipoprotein
Ig	Immunoglobulin
IGF-1	Insulin-like Growth Factor-1
IL-*	Interleukin-
IL-1ra	Interleukin-1 Receptor Antagonist
IFN	Interferon
i.p.	Intra-peritoneal
i.v.	Intravenous
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
kb	Kilobase
kDa/K	Kilodalton
L/N	Lymph node
LAD	Leukocyte Adhesion Deficiency
LBP	LPS-binding protein
LDL	Low Density Lipoprotein
LFA-1	Leukocyte Function Antigen-1
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MCP	Monocyte Chemoattractant Protein
M-CSF	Macrophage Colony-Stimulating factor
MEM	Minimum Eagle's Medium
Mϕ	Macrophage
MHC	Major Histocompatability Comples
min	Minute(s)
MIP-1α/β	Macrophage Inflammatory Protein -1α/β
MM-LDL	Minimally Modified-Low Density Lipoprotein
MMR	Macrophage Mannose Receptor
M_r	Apparent Molecular Weight
mRNA	Messenger Ribonucleic Acid
MSR	Macrophage Scavenger Receptor
ND	Not Done
NK	Natural Killer

NMS	Normal Mouse Serum
NO	Nitric oxide
NP-40	Nonidet-P40
O.D.	Optical Density
OxLDL	Oxidized Low Density Lipoprotein
PAGE	Polyacrylamide Gel Electrophoresis
PBA	Phosphate Buffered Saline plus Albumin plus Azide
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PECAM-1	Platelet-Endothelial Cell Adhesion Molecule-1
PFA	Paraformaldehyde
PMN(s)	Polymorphonuclear cells (neutrophils)
PMSF	Phenylmethylsulfonyl fluoride
Poly (I),(C)	Polyinosinic, Polycytidylic acid
r.p.m.	Revolutions Per Minute
RAGE	Receptor for Avanced Glycosylation Endproducts
RANTES	Regulated on Activation, Normal; T Expressed and Secreted
RT	Reverse Transcription
RPMϕ	Resident Peritoneal Macrophage
s.d.	Standard Deviation
s/c	Subcutaneous
SDS	Sodium Dodecyl Sulfate
Sn	Sialoadhesin
SMC(s)	Smooth Muscle Cells
Taq	*Thermus Aquaticus* DNA Polymerase
TCP	Tissue culture Plastic
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor-β
Th	T-Helper
TNF	Tumour Necrosis Factor
TPMϕ	Thioglycollate-Elicited Peritoneal Macrophage
Tris	Tris[hydroxymethyl]aminomethane
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VLA	Very Late Antigen
VLDL	Very Low Density Lipoprotein
vs.	Versus
WGA	Wheat Germ Agglutinin

CONTENTS

Chapter One: General Introduction **Page**

1.1 Macrophage activation and phenotypic heterogeneity 1
1.2 The role of Mϕ in atherogenesis 3
1.2.1 Historical background: morphologic evidence of inflammation in human and experimental lesions 3
1.2.2 Monocyte involvement in early atherogenesis 4
1.2.3 Mechanisms underlying monocyte recruitment in atherosclerosis 5
1.2.4 Foam cell formation 9
1.2.5 Foam cell lipid metabolism 12
1.2.6 Activation and mediator production by Mϕ in atherosclerotic lesions 14
1.2.7 Regulators of smooth muscle proliferation 15
1.2.8 Inhibition of smooth muscle proliferation 17
1.2.9 Matrix deposition and remodelling 18
1.2.10 Mϕ as regulators of coagulation, thrombosis, and fibrinolysis 19
1.2.11 Are Mϕ the good or bad guys in atherogenesis? Problems and avenues for future research 20
1.3 Macrophage scavenger receptor (MSR) 21
1.3.1 Scavenger receptor family 21
1.3.2 MSR: Protein structure 22
1.3.3 MSR: Gene structure 27
1.3.4 MSR: Expression *in vivo* 28
1.3.5 Regulation of MSR expression and function 30
1.3.6 MSR: Ligands 31
1.3.7 MSR: Functions 34
1.3.7 MSR: Studied presented in this thesis 36

Chapter Two: Materials and Methods

2.1 Animals 39
2.2 Media and sera 39
2.3 Reagents 39
2.4 Cells 40
2.4.1 Primary cells 40
2.4.2 Cell lines 41

2.4.3 Transfected cell lines 42
2.5 Antibodies 42
2.5.1 Primary antibodies, monoclonal 42
2.5.2 Primary antibodies, polyclonal 43
2.5.3 Second antibodies 44
2.6 Cytokines and growth factors 44
2.7 Immunocytochemical techniques 45
2.7.1 Immunocytochemistry 45
2.7.2 Immunohistochemistry 45
2.7.3 Immunofluorescence 46
2.7.4 Flow cytometry 46
2.8 Cell lysates and immunoprecipitation 47
2.8.1 Lysis buffer 47
2.8.2 Immunoprecipitation 47
2.9 SDS-PAGE and blotting 47
2.9.1 SDS-PAGE and membrane transfer 47
2.9.2 Immunoblotting 48
2.10 Labeling of soluble and membrane-bound proteins 49
2.10.1 Metabolic labeling 49
2.10.2 Surface biotinylation 49
2.10.3 Covalent cross-linking of AcLDL-binding protein in serum and culture supernatants 50
2.10.4 Poly G-sepharose MSR binding assay 50
2.11 Reverse transcription (RT)-PCR analysis 50
2.12 Cell adhesion assay for Mϕ adhesion to plastic surfaces 51
2.13 Acetylated low density lipoprotein uptake assays 52
2.13.1 Uptake of AcLDL by adherent cells 52
2.13.2 Quantitation of AcLDL uptake by flow cytometry 52
2.14 Confocal and electron microscopy 52

Chapter Three: M-CSF selective enhances MSR expression and function

3.1 Introduction 54
3.2 The effects of M-CSF on MSR expression 56
3.3 The effects of M-CSF on MSR function 60
3.4 Effects of M-CSF on cell lines and human monocytes 61
3.5 Discussion 62

Chapter Four: The regulation of MSR expression and function by Th1 and Th2 cytokines

4.1 Introduction 66

4.2 The effects of Th1/Th2 cytokines on MSR protein and mRNA expression 69
4.3 MSR post-translational modification by Th1/Th2 cytokines 73
4.4 Th1/Th2 effects on MSR glycosylation 74
4.5 The effects of Th1/Th2 cytokines on MSR function 76
4.6 Tissue expression of MSR reveals size heterogeneity 78
4.7 Discussion 79

Chapter Five: The regulation of MSR expression and function by TGF-β, IL-10 and chemokines

5.1 Introduction 82
5.2 The effects of TGF-β and IL-10 on MSR expression and function 83
5.2.1 Protein and mRNA expression 85
5.2.2 The effects of TGF-β and IL-10 on MSR function 88
5.2.3 The effects of TGF-β and IL-10 on RAW 264.7 cells and M-CSF stimulated BgPMϕ 89
5.2.4 The effects of endogenous TGF-β on MSR 91
5.2.5 Discussion 94
5.3 The effects of C-C chemokines and other cytokines and growth factors on MSR expression and function 97
5.3.1 The role of C-C chemokines in atherosclerosis and effects on MSR 97
5.3.2 The effects of other cytokines and growth factors on MSR 99

Chapter Six: MSR exists as a truncated soluble form *in vitro*

6.1 Introduction 102
6.2 Biochemical evidence for a soluble form of MSR 104
6.3 Bioactivity of soluble MSR 107
6.4 Discussion 109
6.4.1 Physiological significance of soluble MSR 109
6.4.2 Enzymes involved in membrane receptor cleavage 111

Chapter Seven: The expression and function of MSR transfected into Chinese Hamster Ovary cells

7.1 Introduction 115
7.2 MSR expression and function in CHO transfectants 116

7.2.1 Biosynthesis of MSR in CHO transfectants 116
7.2.2 Cell surface vs. intracellular expression of MSR 117
7.2.3 Upregulation of MSR expression and function in transfected CHO cells 119
7.2.4 Further upregulation of MSR expression and function in CHO transfectants 121
7.2.5 Discussion 122
7.3 Localization of the MSR binding site of mAb 2F8: implications for a functional adhesion-promoting site 123

Chapter Eight: Macrophage phenotype, including MSR expression, in mice deficient in both M-CSF (op) and apolipoprotein E

8.1 Introduction 127
8.2 Results 130
8.2.1 Mϕ phenotype in atherosclerotic lesions in op2/E0, op1/E0 and op0/E0 mice 130
8.2.2 Myocardial interstitial Mϕ 133
8.3 Discussion 136

Chapter Nine: MSR cell biology: morphological studies of expression

9.1 Introduction 144
9.2 Morphology of MSR expression in M-CSF treated BgPMϕ 146
9.3 Morphology of MSR expression in CHO MSR II MAC cells 149
9.4 Discussion 152

Chapter Ten: Conclusions and future directions

10.1 Summary of main findings 154
10.2 Does the MSR play a role in Mϕ adhesion *in vivo*? 157
10.3 Identification of the MSR adhesion-promoting ligand 157
10.4 Characterization of Mϕ phenotypes in atherosclerotic lesions in humans and in mouse models 158
10.5 Signalling through the MSR relevant to atherogenesis 159

References 161

Chapter One

General Introduction

1.1 Macrophage activation and phenotypic heterogeneity

Monocytes and macrophages (Mϕ) were once thought of as solely phagocytic, but are now known to have a large repertoire of important and diverse functions, including induction of acute phase responses, regulation of haemopoiesis, activation of the immune and coagulation systems, killing of organisms, and tissue remodeling and repair. The important role of Mϕ in such seemingly divergent processes as atherosclerosis and host defence against infection and malignancy, makes Mϕ biology a fascinating area for research in cell biology and immunology.

Most mature, resident tissue Mϕ are derived from circulating monocytes arising from bone marrow progenitors, but local non-inflammatory Mϕ production may continue in the spleen and lung (alveolar Mϕ). Resident tissue Mϕ are recruited from the bone marrow at a steady state throughout adult life and are distributed in liver (Kupffer cells), spleen, lymph nodes, as well as central nervous system, endocrine organs, gut and kidney. These cells are relatively long-lived and perform poorly-defined trophic and homeostatic functions (Fraser and Gordon 1993). Using the rat mAb F4/80 as a marker for this population, resident Mϕ are, for example, detected in association with vascular endothelium (sinusoidal ECs in liver and adrenal gland) and the numerous close contacts may suggest a Mϕ regulatory role (Austyn and Gordon 1981; Gordon *et al.* 1992). Resident Mϕ are capable of regional differentiation and modulation and may achieve considerable phenotypic heterogeneity depending on their specific micro-environment.

It is useful to distinguish resident tissue Mϕ from elicited Mϕ which arise from the enhanced inflammatory recruitment of monocytes from bone marrow to circulation, and circulation to sites of injury. The nature of these stimuli may contibute further to Mϕ functional heterogeneity. Factors such as the C-C chemokines, including MCP-1, RANTES, and MIP-1α may be more selectively chemotactic for monocytes alone and explain the Mϕ predominance in chronic inflammatory lesions (Cook *et al.* 1995; Schall 1995). Mϕ elicited in

response to sterile stimuli (such as Bio-Gel polyacrylamide beads or thioglycollate broth) differ phenotypically from those recruited by infectious and other agents (BCG) and activated by antigen-specific, T lymphocyte-dependent mechanisms. There is considerable variation in Mϕ activation, depending on the particular target, plasma membrane trigger (LPS) and ligation of specific receptor (Fc receptor). Functional activities may differ with regard to cytocidal and regenerative abilities to produce toxic oxygen radicals or nitric oxide (NO), or in secreting proteases and cytokines. Cytokines produced by CD4+ Th1- and Th2-type lymphocytes, as well as other cells, have profound effects on recruited Mϕ phenotype and function, resulting in a spectrum of activation states (table 1.1).

The prototypic Th1 mediator of Mϕ activation, IFN-γ, represents an extreme favouring cellular immunity and cytotoxicity, whilst the other extreme is represented by Th2- and Mϕ-derived IL-10, which inhibits cellular inflammatory actions. The Th2 products IL-4 and IL-13 share a common receptor subunit and have broadly similar activities, producing an alternatively activated mixed, intermediate phenotype. In contrast, the non-activating cytokines, M-CSF and GM-CSF, have little effect on MHC class II expression or cytotoxicity, but stimulate proliferation, adhesion, endocytosis and proteolysis.

Table 1.1 Spectrum of Mϕ activation by cytokines
(adapted from (Gordon *et al.* 1995))

Marker	**IFN-γ**	**IL-4/IL-13**	**M-CSF/GM-CSF**	**IL-10**
Growth	-	+	++	0
MHC II	++	+	+/-	0/-
Pro-inflammatory cytokines	++	0/-	0/-/+	-
Respiratory burst	++	0	0/+	-
Nitric oxide	++	-	0/+	-
Urokinase	++	ND	+	ND
FcγR	+	+/-	+	+
Mannose receptor	--	++	+/0	+/0
Sialoadhesin	-	--	0/+	0
IL-1 RA	+	++	+	+
Overall	**Activation**	**Alternative activation**	**Non-activation**	**Deactivation**

MHC II, Major Histocompatibility Complex Class II antigens; FcγR, Fc receptor for IgG; IL-1 RA, IL-1 receptor antagonist; -, decrease; +, increase; 0, unchanged; ND, not done.

The ability of Mϕ to respond in such a varied fashion to a variety of stimuli depends on their expression of a large repertoire of cell surface receptors in a controlled and coordinated manner. Mϕ membrane receptors modulate Mϕ activities including growth, differentiation, activation, chemotaxis, adhesion to endothelium, migration through tissues, recognition, and effector functions such as endocytosis and secretion. They are therefore of important physiological and pathological significance, influencing processes such as host defence, inflammation and repair.

Atherosclerosis is the principal cause of heart attacks, stroke and gangrene of the extremities and is responsible for 50% of all mortality in the United States, Europe and Japan (Ross 1993). It remains an extremely difficult disease to study because of its chronicity and complex pathology; but the concept of atherosclerosis as a chronic inflammatory disease process has gained increasing prominence recently. Mϕ are present at all stages in atherosclerosis and have moved centre stage in atherosclerosis research. The Mϕ-specific membrane molecule, the macrophage scavenger receptor (MSR), is thought to play a pivotal role in foam cell formation and atherogenesis (Krieger and Herz 1994). MSR activity levels may be crucial in determining the extent of disease; this thesis is concerned with *in vitro* studies on the regulation of murine MSR expression and function by various cytokines and growth factors present in the atheromatous plaque micro-environment.

1.2 The role of Mϕ in atherosclerosis

1.2.1 Historical background: morphologic evidence of inflammation in human and experimental lesions

Atherosclerosis is no exception to the paradigm that scientific progress reflects cyclical episodes of rediscovery. Virchow noted in 1862 that atheromatous lesions exhibit morphological evidence of inflammation, coining the term *endarteritis chronica sine nodosa* (Schwartz and Valente 1995). The frequent granulomatous foci and the prominent adventitial lymphocytic infiltration of advanced lesions bear witness to his early observations. In 1913, Anitschkow, an early pioneer of experimental atherosclerosis, illustrated Mϕ-derived foam cells in the aortas of cholesterol-fed rabbits. Despite intermittent descriptions implicating Mϕ in atherogenesis, it was not until the observations of Poole and Florey from the Dunn School in Oxford in 1958 (Poole and Florey 1958) that the association between monocyte-Mϕ and the

development of arterial lesions in the cholesterol-fed rabbit was clearly explained.

The significance of these studies eluded the main stream of science, partly because of the dominant interest in intimal smooth muscle cells (SMC) as the principal progenitor of plaque foam cells. The controversy over the origin of foam cells in atherosclerotic lesions yielded to the application of modern methodology for cell typing (Fowler *et al.* 1979; Aqel *et al.* 1985; Klurfeld 1985; Jonasson *et al.* 1986; Tsukuda *et al.* 1986). Although SMC can also take up lipid, the majority of plaque foam cells in all stages of human and experimental atherosclerosis are clearly of monocyte-Mϕ lineage.

Both the early and later stages of atherosclerosis meet many of the criteria defining inflammation as a process characterized by localized tissue accumulation of blood constituents including plasma and plasma proteins (e.g. lipoproteins, fibrinogen, immune complexes), together with the interstitial or extravascular accumulation of blood cells (e.g. monocytes, lymphocytes, platelets) (Libby and Clinton 1993). The presence of chronically stimulated T cells within lesions, and the widespread expression of MHC class II antigens by lesional Mϕ indicate that Mϕ can participate decisively in the local immune response that occurs during atherogenesis. Recent evidence suggests that certain epitopes of modified lipoproteins (OxLDL) might function as neoantigens and that T lymphocytes in the atherosclerotic plaque are involved in a T cell-dependent, autoimmune response (Stemme *et al.* 1995). Advanced atherosclerotic lesions have one other inflammatory feature which is the frequent presence of pleiomorphic granulomatous foci adjacent to the extracellular necrotic plaque lipid, often containing polykaryons, and sometimes apparently eroding into the underlying media. Such areas probably represent foreign body granulomata, developing as a chronic inflammatory response to components of the extracellular necrotic lipid core and cellular debris.

1.2.2 Monocyte involvement in early atherogenesis

Monocyte adhesion to the luminal surface of the endothelium and penetration into the intima was recognized as the earliest phases of experimental atherogenesis in rabbits (Poole and Florey 1958). This was also a consistent response to atherogenic diets in other species studied including swine, rats and nonhuman primates (Joris *et al.* 1983; Faggiotto *et al.* 1984; Gerrity *et al.* 1985; Rosenfeld *et al.* 1987). The fatty streak, often a precursor to established atherosclerotic lesions, contains intimal accumulations of monocytes that had been transformed into

lipid-laden foam cells. Recent data from the multicenter PDAY study indicate that, in humans age 15-34, there is preferential recruitment of leukocytes in atherosclerosis-susceptible regions of thoracic aorta which may not progress. Some 60-80% of these cells are monocytes, the remainder being T- and B-lymphocytes in relatively equal numbers (Wissler 1994). Thus there is good correlation between human and animal model data regarding the preferential occurrence of monocytes in susceptible regions, which suggests that recruitment mechanisms in animal models and in tissue culture may also be present in human atherogenesis. As atherosclerotic lesions progress, the expanded intima develops a rich network of newly formed microvessels which tend to occur in regions rich in Mϕ (Brogi *et al.* 1994). In the latter stages of lesion development, the luminal surface of these microvessels provides a large surface area for potential monocyte recruitment into the evolving lesion.

1.2.3 Mechanisms underlying monocyte recruitment in atherosclerosis

Recruitment of leukocytes from the circulation involves their binding to, and transmigration through the vascular EC layer in a process involving a cascade of multiple receptor-ligand interactions. While large numbers of adhesion molecules are implicated in this complex series of interactions (Springer 1990; Bevilacqua 1993; Hogg and Landis 1993), the recruitment process may be broken down into three distinct phases. In the first instance, leukocytes leave the axial stream of blood flow, marginate towards the vessel wall and roll along the EC layer. This reversible binding under flow conditions is mediated by the selectin family of cell adhesion molecules (Lawrence and Springer 1991; Lasky 1992). During the second phase, activation of the leukocyte integrins occurs, rendering them capable of arresting leukocyte motion and strengthening the leukocyte-endothelial interaction (Hynes 1992). The third phase involves transmigration of leukocytes through EC junctions into the subendothelial space (diapedesis) in a process largely dependent on the PECAM-1 (CD31) adhesion molecule (Muller *et al.* 1993).

An overview (see the above reviews and (Carlos and Harlan 1994) for more complete accounts) will be given of the involvement of these adhesion molecules in monocyte recruitment in chronic inflammation and, especially atherogenesis. Because of the predictably focal nature of both human and experimental atherosclerotic lesions, there is now a consensus that lesions develop preferentially at sites of low

hemodynamic shear stress, or lesion-prone areas such as at bifurcations of major vessels . At such sites blood-monocyte recruitment to the intima is markedly augmented by even short periods of hypercholesterolemia. Further, it is likely that at such low-shear sites, there exist microdomains of reversing blood flow where blood monocytes have a prolonged residence time of contact with endothelium.

The **selectins** (L-, E-, and P-selectin) are transmembrane proteins that contain an N-terminal, Ca^{2+}-dependent lectin domain. L-selectin is the only family member expressed by monocytes and binds i) to an as yet unidentified cytokine-induced counter-structure on non-lymphoid endothelium (X-LAM) (Spertini *et al.* 1991; Berliner *et al.* 1995); ii) to sialylated, fucosylated, and sulfated carbohydrate structures expressed on murine glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1); iii) to CD34 on high endothelial venules in murine peripheral lymphoid tissue; and iv) to the murine mucosal lymphoid addressin, MadCAM-1 (Carlos and Harlan 1994).

The carbohydrate ligands (sialylated, fucosylated moieties, including sialyl Lewis^x (SLe^x,CD15s)) recognized by E- and P-selectins on ECs are however expressed by monocytes (Larsen *et al.* 1990; Carlos *et al.* 1991; Moore *et al.* 1992). Disruption of the gene for P-selectin in mice resulted in an absence of leukocyte rolling, and delayed recruitment of leukocytes to peritoneal cavity inflammatory stimuli (Mayadas *et al.* 1993). The importance of the selectins in humans was illustrated by the recent description of a leukocyte adhesion deficiency (LAD) syndrome, termed LAD-2 (to distinguish it from the leukocyte integrin deficiency LAD-1 (Etzioni *et al.* 1992). These patients present with recurrent infections and are deficient in expressing selectin carbohydrate ligands. Their leukocytes are unable to roll or adhere under shear forces on endothelia expressing E- and P-selectin, but still maintain the ability to adhere and migrate under static conditions in an integrin-dependent fashion. Endothelial cells express E- or P-selectin only after stimulation by specific biological response modifiers such as thrombin, LPS, IL-1, TNF-α and phorbol esters, and these molecules interact preferentially with granulocytes rather than mononuclear cells. Selectin-mediated rolling is a reversible process which provides the leukocyte with an opportunity to be exposed to regulatory molecules presented by ECs, resulting in activation and transmigration.

Monocytes and Mϕ express at least ten members of the **integrin** family (Hogg 1992) of heterodimeric, divalent cation-dependent adhesion molecules. Integrins consist of noncovalently associated α and β chains, and are classified into subgroups on the basis of shared β

chains. These molecules have a promiscuous range of ligand specificities, but play a major role in the interaction of leukocytes with vascular endothelium (Rosen 1990). This critical role is demonstrated by the clinical syndrome, LAD-1, in which the β_2 integrins (LFA-1(α_L; CD11a/CD18), CR3 (α_M; CD11b/CD18) and p150,95 (α_X; CD11c/CD18)) are defective (Anderson and Springer 1987; Kishimoto *et al.* 1987). The integrins on resting circulating leukocytes have a low avidity for their ligands; but chemotactic factors (e.g endotoxin, PAF, IL-8, chemokines, C5a fragment of complement, fMLP), acting on a range of surface receptors, are capable of both enhancing integrin expression on the cell surface and rapidly increasing their avidity state. These activating factors may be presented to leukocytes by structural molecules on the EC surface, thus limiting their activity to the site of inflammation, and preventing their dilution in the blood stream (Tanaka *et al.* 1993). The β_1 integrins, VLA-4, -5, and -6, are expressed at moderate to high levels on monocytes, as opposed to the low levels of VLA-1, -2, and -3 (Hemler 1990). VLA-4 and -5 are well characterized as fibronectin receptors and may mediate Mϕ recruitment to inflammatory foci (Brown *et al.* 1989; Holers *et al.* 1989; Wayner *et al.* 1989). The $\alpha_4\beta_7$ integrin interacts with MadCAM-1 expressed on HEV in murine mucosal lymphoid tissues (Carlos and Harlan 1994).

During the process of leukocyte adhesion, integrins also bind members of the **immunoglobulin superfamily**, such as ICAM-1 and -2, and VCAM-1 (Williams and Barclay 1988; Springer 1990). VCAM-1 is expressed on various cell types, including vascular endothelium and interacts with VLA-4, an $\alpha_4\beta_1$ integrin expressed particularly on the surface of mononuclear leukocytes. Because of this selectivity, VCAM-1 merits particular attention in relation to monocyte recruitment during atherogenesis. ECs over nascent fatty streaks in rabbits with diet-induced or genetically determined hypercholesterolemia express VCAM-1 focally (Cybulsky and Gimbrone 1991). Endothelium in lesion-prone areas of the rabbit aorta expresses VCAM-1 as early as 1 week after initiating a hypercholesterolemic diet, before intimal Mϕ accumulate (Li *et al.* 1993). Similar findings emerged from studies of rabbits with experimentally induced diabetes mellitus with or without concomitant hypercholesterolemia (Richardson *et al.* 1994). In advanced human atheroma, microvascular endothelial cells express VCAM-1, supporting the possibility that these neovascular channels provide a portal for entry of mononuclear cells into the established lesion (O'Brien *et al.* 1993). Lysophosphatidylcholine, a component of modified lipoproteins, has been shown to induce expression of VCAM-1 mRNA by human ECs *in vitro* (Collins 1992; Neish *et al.* 1992). Oxidatively modified or non-

enzymatically glycated lipoproteins may thus trigger local cytokine expression (IL-1, TNF-α and IL-4) which could elicit VCAM-1 expression during atherogenesis *in vivo* (Kume *et al.* 1992; Kume and Gimbrone 1994).

Almost certainly, molecules other than VCAM-1 contribute to this phase of monocyte adhesion. ICAM-1 (CD54) expression on ECs is upregulated on inflammatory activation (Dustin and Springer 1991) and reported ligands include LFA-1, CR3, and leukosialin (CD43) (Makgoba *et al.* 1988; Diamond *et al.* 1990; Rosenstein *et al.* 1991), none of which show selectivity for mononuclear cells. It is noteworthy that binding of monocytes to activated endothelium appears (*in vitro* at least) to be more dependent on the LFA-1-ICAM-1 interaction, than on the VLA-4-VCAM-1 interaction (Beekhuizen *et al.* 1992).

Once monocytes attach to luminal ECs, be they macro- or microvascular, **chemoattractant stimuli** may, subsequent to integrin activation, promote their directed transendothelial migration to the intima and accumulation within lesions. Potential monocyte chemoattractants include plasma-derived OxLDL, thrombin and C5a; matrix-derived fibronectin, collagen and elastin peptides as well as cell-derived molecules such as MCP-1, -2, -3, RANTES, MIP-1α and MIP-1β (Valente *et al.* 1995). TGF-β, PDGF, and M-CSF have been shown not only to be produced by arterial wall cells but also to be chemotactic for monocytes. A switch to monocyte-specific chemokines of the C-C type (e.g. MCP-1 and RANTES) (Schall 1995) would explain the monocyte predominance in chronic inflammatory responses (Kasama *et al.* 1993). The best studied chemoattractant in this regard in atherogenesis is MCP-1 (also known as JE, the mouse homologue of MCP-1). Vascular ECs, SMC, and Mϕ can inducibly express MCP-1 *in vitro* (Rollins *et al.* 1990; Rollins and Pober 1991) and plasma LDL that has undergone a minimal form of oxidative modification (MM-LDL) is a potent inducer of MCP-1 and M-CSF in vascular ECs (Cushing *et al.* 1990; Rajavashisth *et al.* 1990). *In situ* observation in human and experimental atherosclerotic lesions, but not normal vascular tissue, indicates expression of MCP-1 (Nelken *et al.* 1991; Yla-Herttuala *et al.* 1991). All these data suggest an important role for MCP-1 during atherogenesis (shown diagrammatically in Fig. 1.1), but the precise links between hypercholesterolemia and other pro-atherogenic stimuli and the expression of monocyte adhesion molecules and chemoattractants by vascular cells *in vivo* remain incompletely defined.

During the **transmigration phase** of leukocyte recruitment, cells extravasate between tightly apposed endothelial cells. Recent evidence suggests PECAM-1 (CD31) is essential for transmigration and interacts at

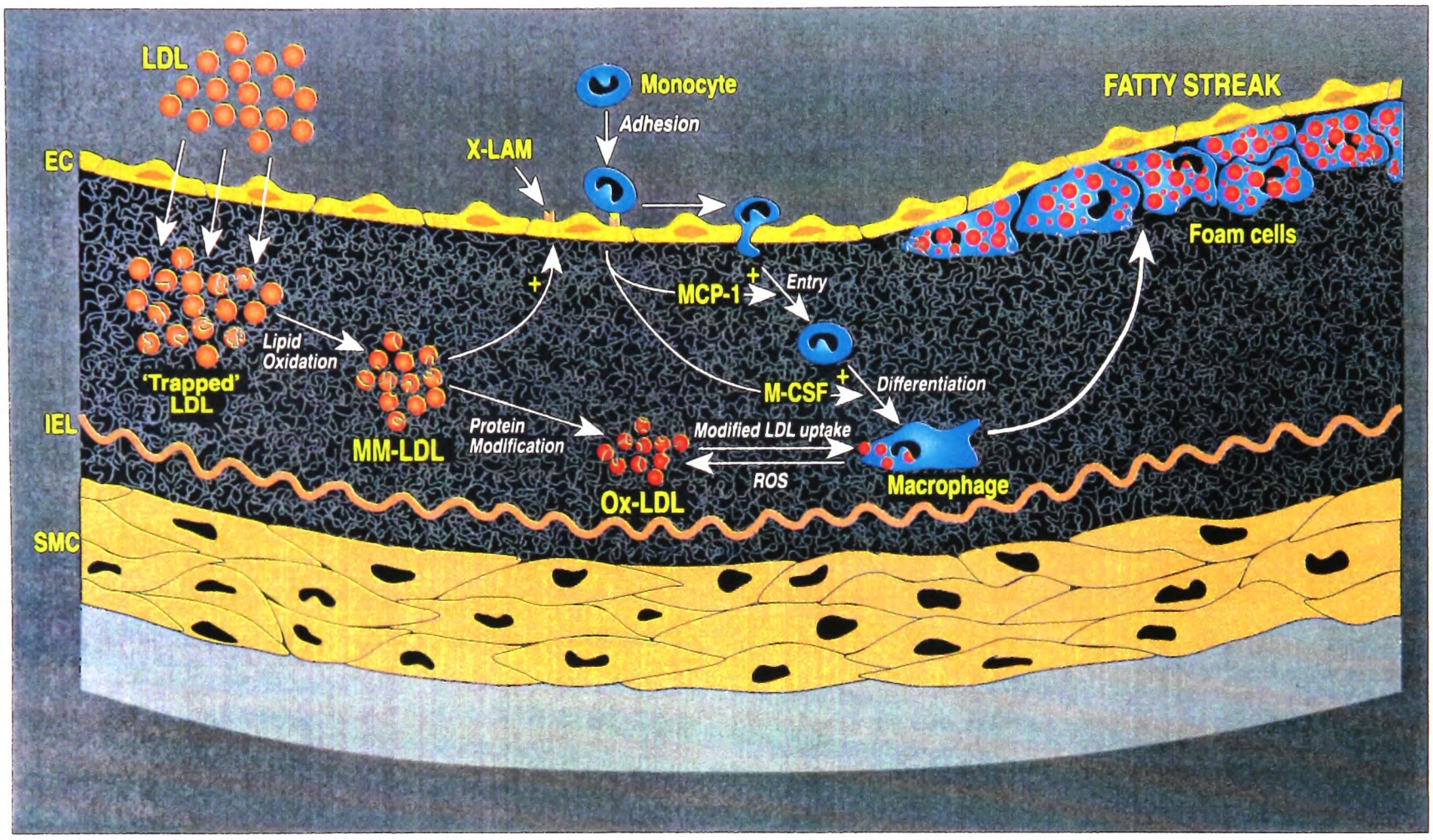


Fig. 1.1 **Diagrammatic representation of early atherosclerosis** (adapted from Berliner *et al*, 1995)

Low-density lipoproteins accumulate in the artery wall, in a process accelerated by hyperlipidaemia, and become minimally oxidized due to reactive oxygen intermediates (ROI) produced by vascular cells. Minimally oxidized LDL (MM-LDL) potently induces inflammatory genes, such as adhesion molecules on ECs for monocytes (X-LAM), and the secretion of MCP-1, and M-CSF by ECs. As a result, monocytes are recruited from the circulation to the artery wall, where they proliferate and differentiate into Mϕ. MM-LDL undergoes further protein modification with time, becoming highly oxidized (OxLDL), and are taken up by Mϕ via the MSR to transform them into lipid-laden foam cells. EC, endothelial cells; IEL, internal elastic lamina; SMC, smooth muscle cells; +, upregulation.

a stage distal to that mediated by the integrins (Muller *et al.* 1993). PECAM-1 is a member of the Ig superfamily that is expressed on ECs, monocytes and neutrophils (Newman *et al.* 1990; Stockinger *et al.* 1990). In confluent EC monolayers, PECAM-1 is concentrated at the intercellular junctions. Antibodies to PECAM-1 as well as recombinant soluble PECAM-1 can block the transendothelial migration of leukocytes at intercellular junctions; this inhibition is thought to represent a homophilic adhesion event (Muller *et al.* 1993).

Other monocyte-specific adhesion molecules may also have a role in determining selectivity of recruitment in chronic inflammation and atherogenesis. These include CD14, a GPI-anchored glycoprotein expressed predominantly by monocyte-Mϕ (Goyert *et al.* 1988). Functionally, it has been well characterized as a receptor for the complex between bacterial endotoxin/LPS and its serum binding protein (LBP) (Wright *et al.* 1989; Wright *et al.* 1990). It has also been reported to contribute to the adhesion of monocytes to endothelium stimulated by inflammatory mediators, but not to unstimulated endothelium (Beekhuizen *et al.* 1991). Endoglin is a disulfide-linked homodimer expressed on ECs and mature Mϕ, but not on monocytes (Lastres *et al.* 1992). The presence of an Arg-Gly-Asp (RGD) motif in the extracellular domain suggests it may be a counter-receptor for integrins and endoglin has been postulated to contribute to the post-endothelial migration of Mϕ (Gougos and Letarte 1990).

The regulation of MSR expression and function is the subject of this thesis. A novel adhesion function for this Mϕ-specific molecule was demonstrated when a MSR-specific mAb inhibited the divalent cation-independent adhesion of Mϕ to serum-coated plastic surfaces (Fraser *et al.* 1993). This is in addition to its traditional role as an endocytic molecule mediating the uptake of a wide range of chemically modified ligands (Krieger and Herz 1994). A role is proposed for the MSR in Mϕ recruitment and retention within the tissue micro-environment, presumably in the post-endothelial phase following transmigration.

1.2.4 Foam cell formation

Having entered the arterial intima, many mononuclear phagocytes differentiate into lipid-laden foam cells, a specialized type of Mϕ. The receptor-specific pathways by which unregulated intracellular cholesteryl ester deposition in Mϕ results in the formation of foam cells have been the focus of considerable efforts to define the mechanisms of atherogenesis.

Foam cell formation does not require the presence on Mϕ of the

classical LDL receptor which mediates the uptake of native LDL. This was shown by the premature development of typical lipid-laden atherosclerotic plaques in patients with familial hypercholesterolemia (FH), an autosomal dominant disease in which the LDL receptor is either absent or defective in function. LDL downregulates the expression of its own receptor efficiently enough to prevent the accumulation of cholesteryl esters found in foam cells (Brown and Goldstein 1986).

An alternative or scavenger receptor-mediated endocytic pathway was thus proposed (Goldstein *et al.* 1979) and subsequently proven (Goldstein *et al.* 1979; Brown and Goldstein 1983; Steinberg *et al.* 1989) to modulate the formation of Mϕ foam cells. These studies demonstrated the presence of a high affinity binding site for acetylated LDL (AcLDL), but not for native LDL, on murine peritoneal Mϕ, which mediated the uptake and degradation of this modified lipoprotein, resulting in massive intracellular cholesterol deposition. The AcLDL (MSR) receptor was not subjected to downregulation by cellular accumulation of cholesterol. MSR types I and II were subsequently cloned and sequenced from a bovine lung library, using oligonucleotide probes derived from peptide sequences from purified receptor (Kodama *et al.* 1988; Kodama *et al.* 1990; Rohrer *et al.* 1990) and were characterized as predominantly Mϕ-specific membrane molecules which defined members of a new family of transmembrane proteins (Freeman *et al.* 1990; Krieger and Herz 1994). Circulating monocytes do not express the MSR which can be induced upon differentiation into Mϕ *in vitro* (Naito *et al.* 1991, Naito *et al* 1992, Geng *et al* 1994). Foam cells within human atheromatous lesions express both types I and II MSR (Matsumoto *et al.* 1990; Naito *et al.* 1992; Luoma *et al.* 1994). *In vivo*, oxidative modification represents a plausible pathway whereby lipoprotein particles (OxLDL) attain the ability to bind MSR, a property modeled *in vitro* by acetylation (Steinberg *et al.* 1989).

The existence of additional receptors that bind OxLDL was suggested by the phenomenon of non-reciprocal cross-competition between AcLDL and OxLDL (Arai *et al.* 1989; Sparrow *et al.* 1989; Freeman *et al.* 1991). Non-reciprocal cross-competition occurs when one ligand competes efficiently for the binding of a second ligand, whereas the second ligand does not, or only partially, competes for the binding of the first. The presence of multiple binding sites on a single receptor, or the existence of multiple receptors, may explain these observations. Expression cloning has identified additional Mϕ membrane molecules as possible interaction sites for OxLDL, namely the FcγRII-B2 receptor for IgG aggregates (CD32) (Stanton *et al.* 1992), CD36 (Endemann *et al.* 1993)

and SR-B1 (a new member of the CD36 family of membrane proteins or class B scavenger receptors) (Acton *et al* 1994).

The clearance of immune complexed lipoproteins through Mϕ FcγR may contribute to foam cell development. Recently FcγRI and FcγRII on adherent human monocytes *in vitro* were shown to enhance metabolism of LDL aggregates (Morganelli *et al.* 1995). In transfection studies however, the FcγRII-B2 receptor (M_r of 50K) bound OxLDL, but recognition was not coupled with uptake (Stanton *et al.* 1992). In contrast, both CD36 and SR-B1 recognized and internalized AcLDL and OxLDL (Endemann *et al.* 1993, Acton *et al* 1994). Interestingly, SR-B1 also binds native LDL (Acton *et al* 1994) and its possible important role in lipid metabolism as an HDL receptor was recently demonstrated by its internalization of this ligand (Acton *et al* 1996).

CD36 and SR-B1 are not Mϕ-specific molecules, in contrast to MSR type I and II (or class A scavenger receptors (SR-AI and SR-AII); but are expressed in a variety of cells and tissues, including Mϕ and monocytes, epithelial cells, ECs, platelets, adipocytes, lung and liver (Acton *et al* 1994, Rigotti *et al.* 1995). CD36 has also been demonstrated in human atherosclerotic lesions (van der Wal *et al.* 1992). A recent report shows that binding and uptake of OxLDL by monocyte/Mϕ from patients totally lacking CD36 is reduced by 40% (Nozaki *et al.* 1995). A classification of the expanding scavenger receptor family of membrane molecules, their ligands and proposed functions is provided in table 1.3. In this thesis for matters of convenience the class A scavenger receptors (SR-AI and SR-AII) are referred to as MSR type I and II respectively.

Another example of an OxLDL-binding Mϕ membrane protein is a partially purified and characterized 94-97K protein that occurs on murine peritoneal Mϕ, on rabbit aortic foam cells and carrageenan granulomas (Ottnad *et al.* 1995), and on rat Kupffer cells (de Rijke and van Berkel 1994). This protein also binds phosphatidylserine-rich liposomes, suggesting a role in the clearance of senescent erythrocytes (Sambrano *et al.* 1994; Sambrano and Steinberg 1995). Following further purification from murine peritoneal Mϕ and the Mϕ-like cell line RAW 264.7, it has recently been proposed to be macrosialin (Holness *et al.* 1993; Ramprasad *et al.* 1995), the mouse homologue of CD68 (Holness and Simmons 1993). The very low level of macrosialin (a Mϕ-specific late endosomal integral membrane glycoprotein) expressed on the cell surface cautions against a direct role for this molecule in OxLDL uptake (Rabinowitz and Gordon 1991; Rabinowitz *et al.* 1992). This does not exclude a massive recycling flux and efficient role in internalization. Ultimate assessment of the relative importance of the several candidate "OxLDL receptors" may depend on the use of "knockout" experiments.

The LDL-receptor related protein (LRP) is, like the MSR, a multiligand receptor, and mediates cellular uptake of apoE-containing, remnant-like lipoproteins (Krieger and Herz 1994). It is primarily expressed in liver parenchymal cells (with some presence in Kupffer cells), the brain, and the placenta, but has also been defined as a monocyte differentiation antigen (Moestrup *et al.* 1990; Krieger and Herz 1994). In addition to its role in normal lipoprotein metabolism, LRP may participate in the uptake of lipoprotein cholesterol into Mϕ during atherogenesis. Cholesterol accumulation induces cultured Mϕ to synthesize and secrete large amounts of apoE (Basu *et al.* 1981). An autocatalytic cycle can be envisaged whereby LRP on the surface of Mϕ internalizes lipoproteins that had been enriched by apoE in the subendothelial space of atheromatous plaques. This would be similar to the secretion/recapture mechanism proposed for the hepatic uptake of chylomicron remnants (Brown *et al.* 1991). Interestingly, LRP is present in human atherosclerotic lesions (Luoma *et al.* 1994) and expression *in vitro* is increased by M-CSF, one of many cytokines present in atherosclerosis (Hussaini *et al.* 1990).

1.2.5 Foam cell lipid metabolism

Intimal foam cells are not mere passive bystanders in the developing plaque but may themselves modify lipoproteins to form derivatives that modulate lesion formation. These cells also participate in local lipid metabolism by sequestering, processing, and exporting lipids. As alluded to above, apoE secreted by lesional Mϕ may be important in reverse cholesterol transport.

Mice rendered apoE deficient by gene targeting have markedly elevated plasma cholesterol concentrations and a propensity to develop atherosclerotic lesions with a complex morphology (Plump *et al.* 1992; Zhang *et al.* 1992). This enhanced atherogenesis accompanies a striking rise in "remnant"-type lipoprotein particles in the blood of these apoE-deficient mice. Conversely, the overexpression of apoE in transgenic mice reduces diet-induced hypercholesterolemia (Shimano *et al.* 1992). Exogenous administration of apoE also inhibits lesion formation in hypercholesterolemic rabits (Yamada *et al.* 1992). Transgenic mice expressing human apoE in the arterial wall under control of the mouse H2 Ld (MHC class I antigen) promoter have been demonstrated to be resistant to diet-induced atheroma formation, strongly suggesting that apoE enhances cholesterol efflux from the aortic wall into plasma (Shimano *et al.* 1995). Factors that augment apoE expression in foam cells such as M-CSF may thus retard progression or promote regression

of atherosclerosis (Clinton *et al.* 1992; Inoue *et al.* 1992). ApoE is synthesized in many tissues, but the dominant source appears to be the liver (Shimano *et al.* 1992). Although Mϕ synthesize apoE, their specific role in the control of plasma cholesterol concentrations and in the development of atherosclerosis has not been previously defined.

This problem has been elegantly addressed by using bone marrow transplantation in irradiated apoE-deficient mice (Boisvert *et al.* 1995; Linton *et al.* 1995). The bone marrow transplantation of cells derived from apoE +/+ mice into apoE -/- mice led to the synthesis of apoE mRNA in many of the tissues normally containing Mϕ (spleen, lung, small intestine, kidney, liver and brain) and the appearance of apoE protein in plasma; providing evidence for reconstitution in these deficient mice. Although plasma concentrations of apoE in transplanted mice were only 12.5% compared with normal apoE mice, this was sufficient to decrease serum cholesterol concentrations markedly mainly due to reductions in VLDL, IDL, and LDL. ApoE -/- mice receiving Mϕ from apoE +/+ mice were fed an atherogenic diet and proved resistant to the development of hypercholesterolemia. Apo E -/- mice given apoE -/- cells developed atherosclerosis in which the lesions were raised and consisted of a fibrous cap covering a lipid-rich core. Such atherosclerotic lesions were virtually absent in the apoE -/- mice that were given cells from apoE +/+ mice. The apoE synthesized by bone-marrow-derived cells (Mϕ) thus has a profound effect on plasma lipoprotein concentrations and atherogenesis.

In addition to apoE, Mϕ produce another apolipoprotein, serum amyloid A_3, a component of certain HDL particles (Meek *et al.* 1992). The hepatic- and Mϕ-derived SAA_3 content of HDL particles may influence their metabolism, as was suggested by the association of increased hepatic SAA_3 levels with lesion development in C57BL/6 mice fed an atherogenic diet (Liao *et al.* 1993).

Lesional Mϕ can also express 15-lipoxygenase (Yla-Herttuala *et al.* 1990), an enzyme that may catalyze oxidative modification of intramural lipoproteins by fatty acid peroxidation. These products may crosslink covalently to the apoB moiety of LDL and become suitable substrates for scavenger receptor uptake pathways (Ross 1993); this was supported by recent gene transfer studies in which 15-lipoxygenase expressed in rabbit iliac arteries resulted in the appearance of oxidized lipid-protein adducts similar to OxLDL (Yla-Herttuala *et al.* 1995). In addition, locally acting mediators such as MCP-1 and M-CSF (Liao *et al.* 1991) may be elicited. Within human atheroma, Mϕ also produce lipoprotein lipase (Yla-Herttuala *et al.* 1991; O'Brien *et al.* 1992), an enzyme important in the remodeling of lipoprotein particles. These observations broaden the

traditional concept that LPL acts endovascularly at the luminal surface, and emphasize the potential ongoing metabolism of lipoprotein constituents within the arterial wall itself during atherogenesis.

1.2.6 Activation and mediator production by Mϕ in atherosclerotic lesions

Mϕ are the principal inflammatory mediators in the atheromatous plaque micro-environment and intimal foam cells may be considered awash in a broth of cytokines, growth factors, and other mediators that vary in spatial and temporal distribution during lesion formation (fig. 1.2). It is important to determine which of the vast array of bioactive substances secreted by activated Mϕ are actually produced by Mϕ in atherosclerotic lesions; this would increase our understanding of the functional significance of Mϕ in atherogenesis. Recent *in situ* hybridization studies of gene expression by Mϕ in human and rabbit lesions have demonstrated their heterogeneity and diverse functional properties (table 1.2). Such heterogeneity appears the rule rather than the exception during atherogenesis.

For example, replication of Mϕ occurs much more commonly in advanced atherosclerotic lesions than had been previously appreciated (Gordon *et al.* 1990; Rosenfeld and Ross 1990), but only a fraction of the Mϕ present within a lesion divide at any given time in the life history of that particular lesion. Similarly, only subpopulations of lesional Mϕ express a variety of other functions during any given 'slice in time' of atheroma sampling. Some of this functional heterogeneity may reflect the residence time of a Mϕ within a lesion. The particular spectrum of foam cell functions expressed by a given lesional Mϕ may depend upon the local micro-environment of stimulatory or inhibitory cytokine signals. Alternatively, regional location of a Mϕ may determine its functional state. For example, within the "necrotic core" of advanced atherosclerotic plaques, apoptosis of Mϕ may occur. This cell death may result from accumulations of toxic concentrations of modified lipoprotein derivatives, of NO, or local depletion of the "survival factor" M-CSF.

To fully comprehend functional heterogeneity, the studies in table 1.2 have to be systematically repeated using cross sections of lesions of varying severity as well as from different anatomic locations. Furthermore, despite the fact that the Mϕ gene products studied *in vivo* have known *in vitro* effects on the activation, proliferation, or lipid metabolism of artery wall cells, there is little evidence overall that their expression by lesional Mϕ *in vivo* results in changes in cellular

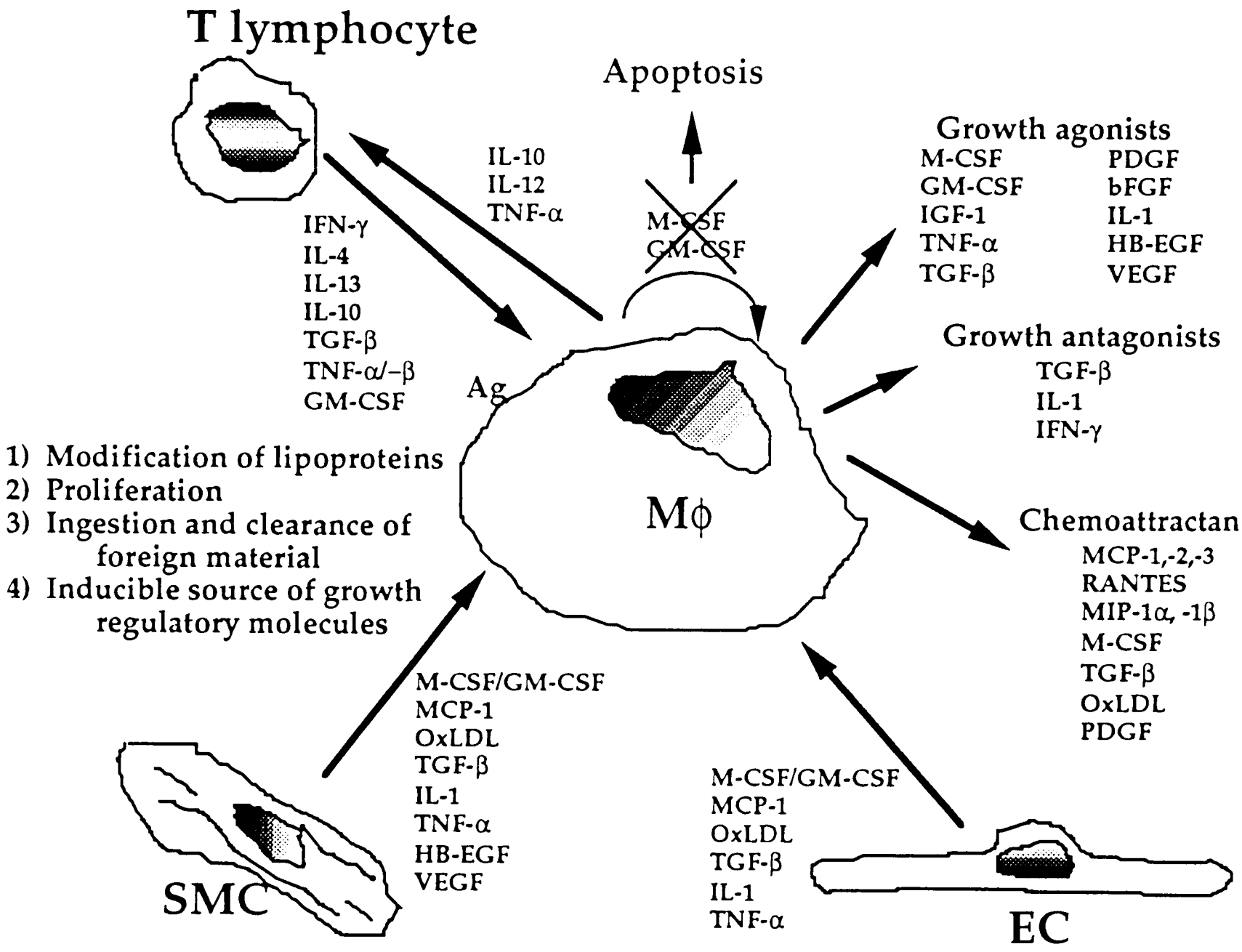


Fig. 1.2 Activator and mediator production by Mϕ in atherosclerosis
(adapted from Ross, 1993)

The possible immune response during atherogenesis is depicted by the relationship between T lymphocytes and Mϕ. All of the vascular cells, namely ECs, SMCs and T lymphocytes, can produce growth factors which interact with Mϕ affecting proliferation and activation, maintaining cell viability, and preventing apoptosis and cell death. SMCs and ECs secrete chemoattractants for Mϕ, including MCP-1 and OxLDL, and factors that affect Mϕ metabolism, such as IL-1 and TNF-α.

behaviour or affects atherogenesis.

In the following sections some of the effects of Mϕ-derived mediators in the atheromatous plaque will be selectively highlighted by functional grouping. Although many of these mediators may play pathogenic roles in atherosclerosis, some may exert protective functions.

1.2.7 Regulators of smooth muscle proliferation

Smooth muscle cell migration and proliferation are likely to contribute to the formation of arterial hyperplastic diseases, including atherosclerosis. Mϕ have been shown to be a source of paracrine stimulators of SMC proliferation. These include:

i) forms of platelet-derived growth factor (PDGF), a family of mediators capable of stimulating smooth muscle proliferation *in vitro* and migration of smooth muscle cell *in vivo* (Shimokado *et al.* 1985; Martinet *et al.* 1986; Ferns *et al.* 1991)

ii) members of the epidermal growth factor family of smooth muscle mitogens, including transforming growth factor-α (TGF-α) (Madtes *et al.* 1988); and a newly recognized heparin-binding epidermal growth factor-like molecule (HB-EGF) (Higashiyama *et al.* 1991).

iii) fibroblast growth factors. Mϕ in lesions may produce both "classical" forms of fibroblast growth factor (FGF), acidic (Brogi *et al.* 1994) and basic FGF (or FGF1 and 2) (Baird *et al.* 1985; Joseph-Silverstein *et al.* 1988) and stimulate proliferation of ECs as well as SMCs during different phases of atherogenesis. Recently high levels of acidic FGF have been detected in association with Mϕ in regions of neovascularization of advanced human atheroma (Brogi *et al.* 1994). *In vitro* studies confirmed that Mϕ-like cells (THP-1 cells exposed to phorbol esters for several weeks) express the gene for acidic FGF.

iv) M-CSF, a product of Mϕ and other cells within atheromatous lesions, for example, is required for survival of mononuclear phagocytes, and renders them more responsive to mitogens such as IL-3 or GM-CSF (Clinton *et al.* 1992; Rosenfeld *et al.* 1992). Colony stimulating factors were originally described as factors that supported the survival, proliferation, or development of various classes of bone-marrow-derived cells. Under some circumstances, SMCs can also express c-*fms*, the receptor for M-CSF which then, in addition to altering a number of Mϕ functions potentially relevant to atherosclerosis, may modulate SMC replication (Shimada *et al.* 1992; Inaba *et al.* 1993; Inaba *et al.* 1993).

v) IL-1α and β, like the colony-stimulating factors, were initially thought to act on leukocyte targets exclusively. IL-1 produced in large

Table 1.2 Mϕ gene expression in atherosclerotic lesions

Gene	Location	Lesion type	Species	Study
Tissue factor	Carotid artery, Internal mammary artery, saphenous vein	Normal, advanced plaque	Human	(Wilcox *et al.* 1989)
TNF	Femoral artery, carotid artery	Atheroma	Human	(Barath *et al.* 1990) (Tipping and Hancock 1993)
15-lipoxygenase	Aorta	Fatty streak	Human, rabbit	(Yla-Herttuala *et al.* 1990)
PDGF-B	Carotid artery		Monkey, human	(Ross *et al.* 1990)
MCP-1	Carotid artery	Aorta, DIT, FS, Atheromatous plaques,	Human, rabbit	(Nelken *et al.* 1991) (Yla-Herttuala *et al.* 1991) (Takeya *et al.* 1993)
Stromelysin	Coronary artery	Normal, atheromatous plaques	Human	(Henney *et al.* 1991)
IL-1	Aorta	DIT, advanced lesions	Human, rabbit, monkey	(Clinton *et al.* 1991) (Moyer *et al.* 1991) (Tipping and Hancock, 1993)
MSR	Aorta,	Fatty streak	Human	(Yla-Herttuala *et al.* 1991) (Takahashi *et al.* 1992) (Luoma *et al.* 1994)
LPL	Aorta, coronary artery	Fatty streak	Human, rabbit	(Yla-Herttuala *et al.* 1991) (O'Brien *et al.* 1992)
ApoE	Aorta	DIT, FS, advanced lesions	Human, rabbit	(Salomon *et al.* 1992) (Rosenfeld *et al.* 1993)
CD59	Carotid artery, coronary artery, saphenous vein	Fatty streak, fibrous plaque	Human	(Seifert *et al.* 1992)
ICAM-1	Aorta	Fatty streak, fibrous plaques	Human	(Printseva *et al.* 1992)
M-CSF	Aorta	Fatty streak	Human, rabbit	(Clinton *et al.* 1992) (Rosenfeld *et al.* 1992)

CD14	Aorta, coronary artery, carotid artery	DIT, FS, atheroma	Human	(van der Wal *et al.* 1992)
CD36	Aorta, coronary artery, carotid artery	DIT, FS, atheroma	Human	(van der Wal *et al.* 1992)
CR3	Aorta, coronary artery, carotid artery	DIT, FS, atheroma	Human	(van der Wal, *et al.* 1992)
PAI-1	Carotid artery, coronary artery, saphenous vein	Advanced lesions	Human	(Lupu *et al.* 1993)
TGF-β	Carotid artery		Monkey, human	(Ross *et al.* 1990)
GM-CSF	Carotid artery		Monkey, human	(Ross, *et al.* 1990)
Connexin-43	Carotid artery	DIT, FS	Human	(Polacek *et al.* 1993)

TNF, tumor necrosis factor; PDGF, platelet-derived growth factor; MCP, monocyte chemotactic protein; IL, interleukin; DIT, diffuse intimal thickening; FS, fatty streak; LPL, lipoprotein lipase; ICAM, intercellular adhesion molecule; M-CSF, macrophage-colony stimulating factor; PAI, plasminogen activator inhibitor.

quantities by activated Mϕ may also however act as signaling triggers and elicit the production of endogenous growth factors (PDGF-AA, HB-EGF and prostanoids) that may amplify mitogenic effects in fibroblasts and vascular SMCs (Libby *et al.* 1988; Raines *et al.* 1989; Gay and Winkles 1991).

1.2.8 Inhibition of smooth muscle proliferation

Mϕ not only produce stimulators of SMC proliferation, but elaborate growth inhibitors as well. For example, Mϕ produce prostaglandins such as prostaglandin E_2. Such prostanoids can inhibit proliferation of vascular cells by raising intracellular levels of cyclic AMP (Libby *et al.* 1988). The nitric oxide radical (NO) is produced following Mϕ expression of the inducible form of the nitric oxide synthase (Nathan and Sporn 1991; Lyons *et al.* 1992); and has microbicidal actions important in host defences against infectious agents. In the context of atherogenesis, NO may inhibit smooth muscle proliferation by increasing intracellular levels of cyclic GMP (Garg and Hassid 1989). Mϕ-derived TNF-α and IL-1, although they promote proliferation of SMCs, inhibit the replication of ECs.

TGF–β also inhibits SMC proliferation under many experimental conditions *in vitro* (Owens *et al.* 1988; Battegay *et al.* 1990; Bjorkerud

1991). A flurry of recent studies have indicated that lipoprotein (a), an important risk factor for atherosclerosis, acts to inhibit plasminogen activation *in vivo*. A consequence of this is reduced activation of TGF-β, causing increased SMC proliferation (Grainger *et al.* 1993; Grainger *et al.* 1994; Grainger *et al.* 1995). The effects of TGF-β on MSR expression and regulation are discussed in Chapter 5. Interestingly, TGF-β potently stimulates synthesis of interstitial forms of collagen by SMCs (Liau and Chan 1989; Amento *et al.* 1991). Thus, although it may limit the division of SMCs, it can promote accumulation of extracellular matrix during evolution of the atheromatous plaque.

1.2.9 Matrix deposition and remodeling

Metabolism of the extracellular matrix has received surprisingly little attention in recent atherosclerosis research, although the matrix makes up the bulk of most complicated atherosclerotic lesions. Remodeling of the extracellular matrix doubtlessly contributes to lesion evolution by permitting migration of SMCs during intimal thickening and of ECs during intralesional angiogenesis. Rupture of coronary atheroma precipitates most acute myocardial infarctions. Plaque ruptures occur predictably at sites of thinning of the lesion's fibrous cap; these areas are frequently associated with Mϕ accumulation (Lendon *et al.* 1991).

Foam cells may contribute to this lability of the fibrous cap as Mϕ produce in a regulatable fashion enzymes of the matrix metalloproteinase (MMP) family that are capable of degrading components of the extracellular matrix (Xie *et al.* 1994). These enzymes depend upon a co-ordinated zinc atom for their common catalytic activity, share structural similarity, and exist as pro-enzymes or zymogens requiring limited proteolysis for activation. Mϕ elaborate interstitial collagenase (MMP-1), which degrades types I and III collagen, the most abundant forms of this matrix protein in atherosclerotic lesions (Welgus *et al.* 1990; Welgus *et al.* 1992). Elastinolytic MMPs (such as matrilysin) as well as cysteine proteinases (cathepsins B, L, and S) are also secreted by Mϕ (Reddy *et al.* 1995). Other MMPs produced by Mϕ include the 72K (MMP-2) and 92K (MMP-9) gelatinases which are capable of degrading basement membrane collagen types IV and V found in the vascular intima as well as further hydrolysing collagen peptides (Campbell *et al.* 1991). Cytokines and LPS can modulate Mϕ expression of matrix metalloproteinases both positively and negatively (Corcoran *et al.* 1992; Lacraz *et al.* 1992; Xie *et al.* 1994). In addition to producing these hydrolytic enzymes, Mϕ produce tissue inhibitors of metalloproteinases

(TIMPs), endogenous antagonists of these enzymes (Campbell *et al.* 1991).

Gene expression of stromelysin, an enzyme capable of activating the zymogens of the MMP family, was localized to Mϕ-enriched areas in human atherosclerotic lesions (Henney *et al.* 1991). Immunoreactive studies colocalized interstitial collagenase (MMP-1), the 72K gelatinase (MMP-2), stromelysin (MMP-3), and the 92K gelatinase (MMP-9) with lesional Mϕ in human atherosclerotic tissue (Galis *et al.* 1995). Whether foam cells, as specialized Mϕ, actually produce MMPs or whether the immunoreactivity represents phagocytosed MMPs derived from other sources, remains unclear. A recent study shows lipid-laden Mϕ from rabbit atheromas secrete interstitial collagenase (MMP-1), stromelysin (MMP-3), and the 92K gelatinase (MMP-9) (Galis *et al.* 1995). Thus, Mϕ within the atheroma possess a broad and tightly regulated complement of enzymes that can remodel the matrix of the lesion, and might contribute to its focal weakening at sites prone to plaque rupture.

1.2.10 Mϕ as regulators of coagulation, thrombosis, and fibrinolysis

One of the consequences of plaque rupture is acute thrombosis. Mϕ-derived proteins that modulate blood coagulation and fibrinolysis participate in local clotting and contribute to lesion evolution, as well as the transition from the chronic to the acute stages of atherosclerosis. These proteins include factors II, VII, IX, X, XIII, thromboplastin, prothrombinase, t-PA and u-PA (Schwartz and Valente 1995). In atherosclerotic plaques lesional Mϕ contain abundant tissue factor, an initiator of the so-called "extrinsic" pathway of blood coagulation (Wilcox *et al.* 1989).

Mϕ also produce an endogenous antagonist of plasminogen activator, plasminogen activator inhibitor-1 (PAI-1) (Schwartz and Bradshaw 1992). This protein inhibits the endogenous urokinase or tissue-type plasminogen activators associated with Mϕ and impedes fibrinolysis; thus stabilizing any clot that may form in response to tissue factor. As in the case of several of the other regulatory systems described above, the Mϕ has an entire complement of activities which can regulate local tendencies for blood clots to form and lyse (Hamilton *et al.* 1991).

1.2.11 Are Mϕ the good or bad guys in atherogenesis? Unresolved problems and possible avenues for future research

The modified lipoprotein oxidation hypothesis would argue that Mϕ have a mostly deleterious role in atherogenesis. Foam cell formation results from the unregulated uptake of atherogenic oxidatively modified LDL molecules; but Mϕ themselves may contribute to LDL oxidation through release of active oxygen species. Oxidized LDL is cytotoxic to other arterial wall cells in culture and the continued generation of free radicals, and secretion of metalloproteases threaten plaque integrity. Based on this, intervention strategies should be targeted at i) the prevention of lipoprotein oxidation, ii) the reduction of MSR-mediated uptake of modified lipoproteins by Mϕ, or iii) the prevention of recruitment of monocytes into the arterial wall. This reasoning has gained credence by studies showing reduction of atherosclerosis in animal models treated with antioxidants (Carew *et al.* 1987; Kita *et al.* 1987; Steinberg *et al.* 1989; Steinberg 1995).

On the other hand, under certain circumstances Mϕ may have a protective or beneficial role, contributing to physiological intimal debridement, and protecting the vessel wall from toxic anionic macromolecules. It may be argued that fatty streaks constitute the precursors of clinically significant atherosclerotic plaques, and monocytes are recruited into the arterial wall under atherogenic conditions as a defence mechanism; Mϕ then remove lipid leading to lesion regression. This reasoning suggests an alternative therapeutic strategy to promote physiological monocyte recruitment mechanisms, particularly in the young.

In a recent study the role of monocyte/Mϕ in atherogenesis was examined using mice deficient for both M-CSF (op mutation) and apoE proteins (Smith *et al.* 1995); these animals were protected against atherosclerosis in the face of elevated cholesterol levels. Immunohistochemical characterization of Mϕ phenotypes was performed on tissues and atherosclerotic lesions from these animals and the results are reported in Chapter Eight.

The regulation of the various Mϕ functions has become increasingly better understood *in vitro*. The *in vivo* environment of the Mϕ and foam cell however remains extremely complex, with multiple stimuli acting concomitantly. Considerable caution has to be exercised when extrapolating studies on Mϕ and cell lines in culture to the behaviour of foam cells within atheroma; and extrapolation to atherogenesis in the intact animal, and humans in particular, remains speculative.

Possible avenues for Mϕ research in atherosclerosis are listed below, each of which has important implications for the development of innovative therapeutic interventions:

i) Are there specific subpopulations of monocytes involved?

ii) Which mechanisms are dominant in monocyte-endothelium attachment and directed migration to the subendothelial space?

iii) Are intimal Mϕ a major source of reactive oxygen species?

iv) What is the contribution of lipoprotein phagocytosis to foam-cell formation?

v) Is foam cell death necrotic or programmed, i.e. apoptotic?

vi) What are the pathobiological implications of monocyte-Mϕ activation/differentiation?

vii) What are the biologic implications of retarding intimal monocyte-Mϕ recruitment?

viii) Do Mϕ participate in plaque regression?

ix) Do intimal/adventitial lymphocytes modulate the role of Mϕ in atherogenesis?

1.3 Macrophage scavenger receptor (MSR)

The role of unregulated scavenger receptor-mediated lipid endocytosis in foam cell formation and atherosclerosis has attracted great interest since the initial description of these receptors in 1979 (Goldstein *et al.* 1979) and exponentially so, since the isolation and cDNA cloning of MSR types I and II in 1990 (Kodama *et al.* 1988; Kodama *et al.* 1990). Recent reviews deal extensively with MSR biology (Kurihara *et al.* 1991; Krieger *et al.* 1993; Freeman 1994; Krieger and Herz 1994) and this overview will set the background for the work presented in this thesis, and will focus on the regulation of MSR expression and function, especially its recently identified role in adhesion, as well as on data published recently.

1.3.1 Scavenger receptor family

Macrophage scavenger receptors are trimeric, integral membrane glycoproteins that bind an unusually diverse array of ligands. As discussed in section 1.2.4 the complex binding of OxLDL and AcLDL to Mϕ and ECs indicates at least three different classes of modified LDL receptors; receptors that recognize either AcLDL or OxLDL or both of these ligands (Krieger and Herz 1994). Broad ligand specificity, including both lipoprotein and nonlipoprotein ligands, is a central characteristic of

the scavenger receptor family members which have recently been defined based on these distinctive binding properties and primary sequences (Acton *et al.* 1994; Pearson *et al.* 1995); and which are suggested to be involved in a wide variety of biological processes (table 1.3).

Other molecules which may functionally, but not structurally, be considered as scavenger receptors due to their broad ligand specificities for non-native proteins, are the α_2-macroglobulin receptor/LDL-receptor related protein (LRP) (Krieger and Herz 1994) and the receptor for advanced glycosylation end products (RAGE). The possible role of LRP in foam cell formation was discussed in section 1.2.3, whilst RAGE has been described to mediate the uptake of matrix and tissue proteins which had been non-enzymatically modified by glucose (Vlassara *et al.* 1986). RAGE is distinct from the MSR (Radoff *et al.* 1990), but the presence of AGE-modified proteins in atherosclerotic lesions (Lee *et al.* 1993; Kume *et al.* 1995) and the glycosylation of arterial components in diabetes mellitus and ageing suggest a role for RAGE in tissue remodelling and atherogenesis (Vlassara *et al.* 1992).

1.3.2 MSR protein structure

Complementary DNAs encoding two forms of the MSR, designated type I and type II, have now been isolated from bovine (Kodama *et al.* 1990; Rohrer *et al.* 1990), mouse (Freeman *et al.* 1990; Ashkenas *et al.* 1993), human (Matsumoto *et al.* 1990), and rabbit (Bickel and Freeman 1992) sources. They are highly conserved, with sequence identities in interspecies pairwise comparisons varying from 64-81% for the type I receptors and 60-81% for the type II receptors (see (Ashkenas *et al.* 1993) for detailed comparisons). No structural data exist for the receptor as yet, but a six domain structure has been predicted (fig. 1.3). The original structure predicted in the cloning of the bovine proteins is evident in the other three species, but revisions in the original assignment of residues have been made in the demarcation between domains III and IV, because of species differences involving those regions. The type I and type II receptors are elongated homotrimeric integral membrane proteins varying in length from 451-454 amino acids; domain VI confers type specificity, with the type II proteins possessing a much shorter (6-17 amino acids) carboxyl-terminal region that is not conserved among the species.

Fig. 1.3 **MSR: Predicted Quaternary Protein Structure**

The structures of type I and type II murine MSR (as predicted from the derived amino acid sequences) are illustrated in cartoon form. Domains I-VI are named, and important features of each are depicted in the boxes situated between the two receptor types. Cysteine residues are indicated by the filled circles. aa = amino acids. TM = transmembrane domain. SRCR = Scavenger Receptor Cysteine Rich repeat. Note the species differences at the C-terminal of the type II receptor.

Diagrams adapted from Rohrer *et al,* 1990; Ashkenas *et al,* 1993.

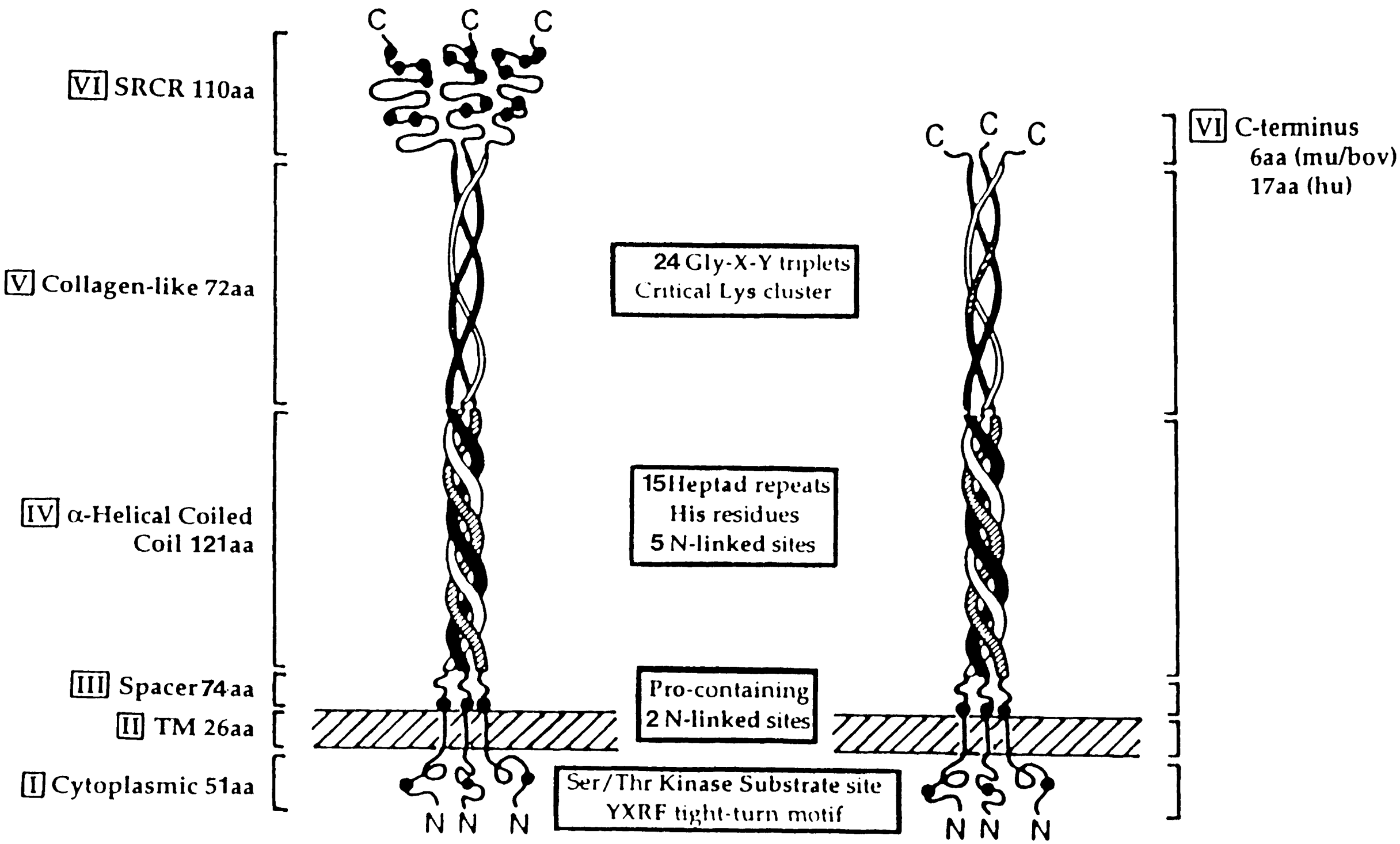

Type I (454aa)
Type II (350aa)
VI SRCR 110aa
V Collagen-like 72aa
IV α-Helical Coiled Coil 121aa
III Spacer 74aa
II TM 26aa
I Cytoplasmic 51aa
C C C
N N N
24 Gly-X-Y triplets
Critical Lys cluster
15 Heptad repeats
His residues
5 N-linked sites
Pro-containing
2 N-linked sites
Ser/Thr Kinase Substrate site
YXRF tight-turn motif
VI C-terminus
6aa (mu/bov)
17aa (hu)

Table 1.3 The scavenger receptor family

	Expression	Ligands		Functions
		Lipoprotein	Non-lipoprotein	
Class A				
SR-AI (MSR type I)[1] **SR-AII (MSR type II)** [1]	Mϕ PMA-treated SMCs and fibroblasts	AcLDL OxLDL	M-BSA poly (G), poly (I) others (table1.5)	Atherogenesis Innate host defence Adhesion Phagocytosis
SR-AIII (MARCO) [2]	Mϕ in murine spleen, lymph nodes	AcLDL	bacteria	Host defence
Class B (CD36 family of membrane proteins)				
CD36 [3,4]	Mϕ/ monocyte ECs platelets adipocytes epithelial cells	OxLDL AcLDL	thrombospondin collagen LCFA Pl. falciparum-infected RBCs PS/PI liposomes	Adhesion LCFA transporter Signal transduction Mϕ receptor for senescent neutrophils
SR-B1 [4,5]	adipocytes liver lung	AcLDL OxLDL LDL HDL	M-BSA PS/PI liposomes	Lipid metabolism HDL receptor
Class C				
dSR-CI [6]	Drosophila melanogaster embryonic Mϕ	AcLDL	M-BSA poly (G), poly (I) dextran sulfate laminarin	Adhesion Host defence Clearance of apoptotic and senescent cells

LCFA = long chain fatty acids; Pl. = Plasmodium; RBCs = red blood cells; PS = phosphatidyldserine; PI = phosphatidylinositol; MARCO = Mϕ receptor with a collagenous domain

References: 1. (Krieger and Herz 1994) 2. (Elomaa *et al.* 1995)
3. (Endemann *et al.* 1993) 4. (Rigotti *et al.* 1995)
5. (Acton *et al.* 1994) 6. (Pearson *et al.* 1995)

Domain I (residues 1-51 murine sequence) is the MSR **cytoplasmic tail** containing the N-terminal part of the polypeptide, which orientates the MSR as a type II transmembrane protein (Schneider *et al.* 1984). No signal sequence peptide (Gierasch 1989) has been identified. The cytoplasmic tail would, by analogy to other endocytic receptors, be expected to contain signals that direct the receptor-ligand complex to a lysosomal degradation pathway. Interestingly, the MSR does not contain the Asn-Pro-X-Tyr (NPXY) (X represents any amino acid) internalization sequence as reported for the LDL, and other endocytic receptors (Chen *et al.* 1990). A Tyr-X-Arg-Phe (YXRF) tight turn conformational motif (as reported for the transferrin receptor (Collawn *et al.* 1990)) at residues 12-15 has been suggested as the structural motif required for MSR endocytosis (Kurihara *et al.* 1991). Deletion of cytoplasmic tail residues VKFDARS (positions 21-27), conserved in all four species of the receptor, results in a dramatic decrease in receptor internalization (Freeman 1994); more detailed studies will be required to determine whether these amino acids constitute the scavenger receptor's internalization sequence. A conserved protein kinase substrate site (Arg-X-X-Ser/Thr) (Edelman *et al.* 1987) of unknown function is located in the middle of this domain.

Domain II (residues 52-77) is the **transmembrane domain** and contains a single stretch of 26-28 hydrophobic residues. It has been suggested that a conserved proline residue at position 67 may affect secondary structure, and/or play a role in the packaging of trimers in the membrane (Ashkenas *et al.* 1993).

Domain III (residues 78-151) is a juxtamembrane **spacer domain** which includes two of the MSR's seven potential N-linked glycosylation sites. The MSR trimer is assembled through the association of a monomer with a covalently linked dimer. Using a site-directed mutagenesis technique, it was demonstrated that dimers form via a disulfide linkage involving a conserved cysteine at position 83 in the spacer domain (Penman *et al.* 1991). This may however be an *in vitro* isolation artefact and is not thought to play a role in cellular MSR trimerisation as substitution of Cys83 to Gly83 did not interfere with trimerization, intracellular transport, or endocytic activity. Two cysteines within this region of the murine MSR may similarly be responsible for artefactual covalent crosslinking into trimers (Ashkenas *et al.* 1993). Although the preponderance of evidence indicates that the functional form of the receptor is a trimer, monomers have been suggested to bind ligand (Via *et al.* 1992). The coiled-coil structures in domains IV and V may facilitate the association of monomers in both covalent and non-covalent linked trimers.

Domain IV (residues 152-272) is an **α-helical coiled-coil structure** (Cohen and Parry 1990) which consists of multiple seven amino acid repeats. These repeats contain appropriately spaced amino acids with hydrophobic side chains that interact to form a three-stranded coil with an interhelical core. The heptad repeats are interrupted by a skip residue (Asn or Ser) at position 204, and this, supported by an intron-exon boundary, prompted division of the domain into subdomains IVa (residues 152-211) and IVb (residues 212-272) (Ashkenas *et al.* 1993). The remaining five sites for N-linked glycosylation are all located in this domain and data suggests that six out of the total of seven sites are, in fact, glycosylated (Resnick *et al.* 1993). This domain has been strongly implicated in trimer assembly during MSR biosynthesis and a conserved histidine residue at position 260 is essential for ligand dissociation within the endosomal compartment and receptor recycling (Mellman *et al.* 1986; Doi *et al.* 1994). Protonation of His260 in the acidic endosome is suggested to disrupt the hydrogen bonds with Lys256 leading to conformation change and ligand dissociation.

Domain V (residues 273-344) or **collagenous domain** contains 24 Gly-X-Y triplets (where X, Y represents any amino acid). Lysines or prolines are located in the Y position of 50% or more of the triplets in each species in the MSR. Hydroxylation of prolines and lysines in mammalian collagens occurs in the ER and serves to stabilize the collagen trimeric helical structure. Alterations in MSR mobility following treatment with the hydroxylase inhibitor, α,α′-dipyridyl has similarly provided evidence for MSR hydroxylation in the ER (Penman *et al.* 1991).

The triplets are predicted to be neutral or positively charged at physiological pH (Kodama *et al.* 1990; Rohrer *et al.* 1990), with clusters of positively charged residues (lysines and arginines at X and Y positions) being highly conserved across species (Ashkenas *et al.* 1993). As the one common feature of the diverse array of SR ligands is their polyanionic charges, the presence of many positively charged residues in the collagen domain made it an attractive target for deletional and substitutional mutagenesis.

Mutagenesis experiments have provided evidence that the collagenous domain is both necessary and sufficient to account for the broad ligand specificity of the MSR, and have given rise to the descriptive term "molecular flypaper" (Krieger 1992; Krieger *et al.* 1993). Truncation of the collagenous domain to eight (bovine) or five (murine) of the 24 Gly-X-Y repeats abolished MSR ligand binding without affecting synthesis and cell surface expression (Acton *et al.* 1993). Replacement of one or more of four conserved lysine residues in

domain V (positions 327 ; 334 ; 337 ; 340) with alanine showed that substitution of Lys337 alone abolished degradation of AcLDL, but that more than two Lys residues required substitution before OxLDL degradation could be abolished (Doi *et al.* 1993). This use of different lysine residues for binding of different ligands may explain the phenomenon of non-reciprocal cross-competition. A three-dimensional computer graphic model for the collagenous domain was generated that predicts that the lysine at position 337 resides in a coiled groove structure that facilitates ligand-receptor interactions. Additional studies of other mutants will be required to test this model, although direct studies of purified protein will ultimately be needed to fully understand the structure of the receptor in this region.

Receptor mutants containing truncated collagenous domains have also been co-expressed with wild-type receptors and found to reduce the activity of the wild-type protein (Acton *et al.* 1993; Dejager *et al.* 1993). This dominant negative phenomenon suggests that the mutant receptor monomers associate with the wild-type receptor monomers to form a dysfunctional oligomer. Binding of ligands to the MSR is also influenced by other residues and domains of the receptor. Conserved negatively charged residues in domain VI may contribute to the specificity of ligand binding by means of charge repulsion effects (Krieger *et al.* 1993). The SRCR in domain VI of type I receptors (see below) appears to interfere with the ability of endotoxin to inhibit binding of some MSR ligands (Ashkenas *et al.* 1993).

Domain VI (residues 345-454) confers MSR type specificity. The type II receptor contains a truncated C-terminal domain of six (murine and bovine) or 17 (human) residues. In contrast, the type I MSR's domain VI is subdivided into hinge (domain VIa ; residues 345-352) and **scavenger receptor cysteine-rich (SRCR)** (domain VIb ; residues 353-454) regions. The SRCR has been designated a new cysteine rich superfamily based largely on the spacings between the six cysteine residues (Freeman *et al.* 1990). The functional significance of the SRCR in the MSR is unclear; it does not play a critical role in MSR-ligand interactions as the SRCR-deficient MSR type II binds known SR ligands as well or better than MSR type I (Rohrer *et al.* 1990). Recent studies with transfected murine MSRs in CHO cells indicate however that the SRCR domain may subtly influence the binding of ligands to the collagenous domain of MSR (Ashkenas *et al.* 1993).

A number of proteins have now been shown to contain one or more SRCR domains (reviewed in (Resnick *et al.* 1994)). These include (number of SRCR repeats indicated in brackets) MSR type I (1) (Rohrer *et al.* 1990), the soluble cyclophilin C or Mac-2 binding proteins (1)

(Friedman *et al.* 1993; Koths *et al.* 1993), sea urchin speract receptor (4) (Dangott *et al.* 1989), complement control protein factor I (1) (Goldberger *et al.* 1987), bovine WC1 protein (11) (Wijngaard *et al.* 1992), CD5 (3) (Jones *et al.* 1986), and CD6 (3) (Aruffo *et al.* 1991). A definitive role for this domain has not been demonstrated, but on the basis of its cross-species conservation, it has been postulated to be a primitive ligand binding/recognition domain. For example, 91% of the extracellular portion of the speract receptor is composed of repeated SRCR domains, making it likely that these domains participate in the binding of the secreted egg peptides recognized by that receptor (Dangott *et al.* 1989). Recent data indicating that the membrane-proximal SRCR domain of CD6 contains the activated leukocyte cell adhesion molecule (ALCAM) binding site, is the first direct evidence for the interaction of an SRCR domain with a ligand; this interaction mediates thymocyte-thymic epithelium cell binding (Whitney *et al.* 1995).

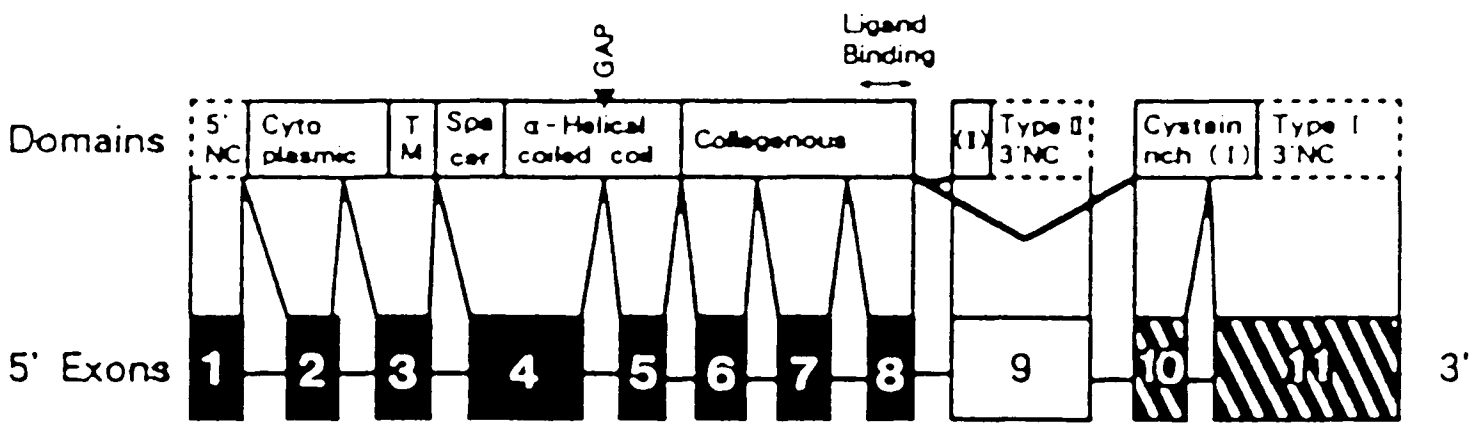
1.3.3 MSR: Gene Structure

The MSR type I and type II proteins arise from transcripts that are differentially spliced from a single gene that maps to chromosome 8 in both the mouse and the human (Freeman *et al.* 1990; Matsumoto *et al.* 1990). The human MSR gene was isolated from phorbol ester-induced THP-1 cells, and localized to band p22 on chromosome 8 (close to the LPL gene) (Emi *et al.* 1993). MSR gene structure and the relationship between exons and protein domains are illustrated in figure 1.4. The gene consists of 11 exons with the first eight encoding the common portions of the type I and type II receptors. To create the type II receptor, exon 8 is spliced to exon 9; the type I receptor arises from a splicing event that links exon 8 to exons 10 and 11. As expected, the mouse gene is similarly organized (Aftring and Freeman 1995).

The Mϕ-restricted expression of MSR makes its promoter region a potential tool for the targeting of genes to Mϕ. The DNA sequences upstream of the bovine, human, and mouse genes are very similar in the 300 base pairs proximal to the transcription start site (Moulton *et al.* 1992; Emi *et al.* 1993; Aftring and Freeman 1995). The bovine and mouse promoters do not appear to have the usual TATA element located approximately 30 bp upstream of the transcription start site. Recent results suggest that 291 bp of the proximal MSR promoter (-245 to +46), in concert with a 400-bp upstream enhancer element, are sufficient to direct Mϕ-specific expression of a human growth hormone reporter in transgenic mice (Horvai *et al.* 1995). These regulatory elements, which contain binding sites for PU.1, AP-1, and cooperating ets-domain

MACROPHAGE SCAVENGER RECEPTOR

(Gene Structure: Emi *et al* , *J. Biol. Chem.* 268:2120, 1993)



Human : 8p22
Mouse: 8p

Fig. 1.4 MSR: Gene Structure

The structure of the human MSR gene, located on chromosome 8 is illustrated in cartoon form. Exons are numbered 1-11, and their relationship to the encoded protein domains is indicated. Alternative splicing between exons 8 and 9, and between exons 8 and 10/11 generate mRNAs specific for types II and I MSR respectively. Note the mapping of the ligand binding domain to the 3' end of the collagenous domain.

transcription factors, are also sufficient to maintain regulation during *in vitro* differentiation of bone marrow progenitor cells in response to M-CSF. Mutation of the PU.1 binding site severely impairs transgene expression, consistent with a crucial role for PU.1 in MSR gene expression regulation. Another study shows that a 127 bp promoter fragment (-67 to +50) confers cell-specific transcription and the activity appears to be mediated by factors that recognize AP-1 and GATA elements (Aftring and Freeman 1995). This is in contrast with the predicted absence of an AP-1 binding site from the human MSR gene sequence (Emi *et al.* 1993); which may emphasize the importance of combinatorial interactions of relevant transcription factors.

AP-1/Ets elements are known as Ras responsive elements, since they mediate activation signals transduced through the GTP-binding protein Ras, an early signaling intermediate of the AP-1 signal transduction pathway. Recent data define the AP-1/Ets composite sites in the MSR promoter as oxidant responsive elements and indicate their functional cooperation to mediate H_2O_2-mediated transcription (Roebuck *et al.* 1995). This would also be consistent with the transcriptional stimulation of MSR gene expression by phorbol esters which occurs after a lag phase, suggesting new protein synthesis, in contrast to that seen with early response genes, (Moulton *et al.* 1992). As expected from the failure of cholesterol to downregulate MSR activity, no sterol response elements (as defined for the LDL receptor (Brown and Goldstein 1986)) were detected in the MSR gene. Circulating monocytes express little or no MSR activity until they penetrate into the arterial intima and differentiate into Mϕ (Naito *et al* 1991, Naito *et al* 1992, Geng *et al* 1994), and it is likely that studies of the regulation of the MSR gene *in vitro* and *in vivo* will shed considerable light on the modulation of gene expression occurring in those Mϕ found in developing atherosclerotic plaques. To date, there is only one report of a microsatellite polymorphism adjacent to the human MSR gene (Schmidt *et al.* 1994); preliminary human genetic studies have not been informative thus far (Cambien, personal communication).

1.3.4 MSR expression *in vivo*

Studies of MSR expression *in vivo* have been limited by the availability of specific reagents. Functional expression of scavenger receptors has been determined by assessing the uptake of DiI-AcLDL (Nagelkerke *et al.* 1983; Pitas 1990), with all the inherent problems of non-specificity. This method is still employed in species where no reagent is available (Van Berkel *et al.* 1991).

MSR protein expression has been detected by immunohistochemistry in bovine (species-specific mAb IgD1) (Naito *et al.* 1991), human (polyclonal type-specific anti-human peptide antibodies) (Naito *et al.* 1992; Geng *et al.* 1994), and mouse (species-specific mAb 2F8) (Hughes *et al.* 1995) tissues. In both cow and human, MSR was detected at high levels on Kupffer cells and alveolar Mϕ, and less prominently in lymphatic sinuses, splenic red pulp, dermal Mϕ, adrenal glands, kidneys, endometrium and testis. The two types of MSR were coexpressed in the human organs studied. Both types of MSR were also recently demonstrated on high ECs of postcapillary venules in human peripheral lymph nodes (Geng and Hansson 1995). Cell type analysis in the mouse showed Mϕ-restricted MSR expression (apart from sinusoidal ECs in the liver and high endothelial venules of lymph nodes) in the red pulp and marginal zone of spleen, Kupffer cells, medulla of the thymus and subcapsular region of the lymph nodes, alveolar Mϕ, and lamina propria Mϕ in the gut (Hughes *et al.* 1995). Frozen section adhesion assays suggested MSR-dependent adhesion of Mϕ correlated with MSR antigen expression in tissues; this indicates the presence of an endogenous MSR ligand within tissues. It was proposed that MSR may serve a homing or retention function for Mϕ localization within ligand-rich tissues.

Whether any differential expression of MSR types occurs *in vivo*, is still unclear. Atherosclerotic lesions in humans and rabbits contain both types, as assessed by *in situ* hybridization (Matsumoto *et al.* 1990; Kurihara *et al.* 1991; Luoma *et al.* 1994). The results of MSR immunoblots from murine tissues presented in Chapter 4 of this thesis, and MSR expression in two murine Mϕ-like cell lines, P388D1 (Ashkenas *et al.* 1993) and RAW 264.7 (Fraser 1993), indicate the type II MSR as the predominant isoform. In contrast, preferential expression of MSR type I (by RT-PCR analysis) was shown during monocyte/Mϕ differentiation and foam cell formation (Geng *et al.* 1994).

The "scavenger receptor activity" exhibited by rat liver ECs and Kupffer cells does not appear to be mediated by MSR type I or II, but rather by a 95K OxLDL-binding membrane protein (de Rijke and van Berkel 1994). Species differences may also account for MSR expression detected in rabbit SMCs and fibroblasts *in vitro* (Pitas 1990; Dejager *et al.* 1993; Dejager *et al.* 1993) and by SMCs in atherosclerotic lesions *in vivo* (both protein and mRNA) (Li *et al.* 1995). The MSR antigen was not detected on bovine fibroblasts, ECs and SMCs (Naito *et al.* 1991). Similarly, MSR expression in mice appear predominantly Mϕ-specific and in human atherosclerotic lesions, expression was confined to Mϕ and not intimal SMCs (Yla-Herttuala *et al.* 1991; Naito *et al.* 1992; Luoma

et al. 1994).

1.3.5 Regulation of MSR expression and function

Although the MSR is not regulated by the intracellular cholesterol content, numerous non-sterol regulators have been proposed. However, these data, all of which have been obtained from cultured cells, are contradictory. The interpretation of the studies describing regulation of MSR expression and activity has been complicated by: i) the nature of the assays (functional uptake vs. mRNA expression vs. protein expression), ii) the types of cell used (monocytes vs. different Mϕ populations vs. SMCs vs. ECs), iii) differences in sources and purities of cytokines used to treat cells, and iv) differences between species (murine vs. human vs. bovine). The characterization of MSR regulation expression has also been hampered by the lack of specific reagents. The species-specific 2F8 mAb to the murine MSR was recently isolated in this laboratory (Fraser *et al.* 1993). The mAb inhibited the divalent cation-independent adhesion of murine Mϕ *in vitro*, thereby defining a novel adhesion function for the MSR. This MSR-specific reagent additionally allowed reliable regulatory studies on MSR protein expression to be performed (de Villiers *et al.* 1994; de Villiers *et al.* 1994; de Villiers *et al.* 1995). Table 1.4 shows a summary of the major studies published to date describing factors affecting Mϕ MSR regulation.

It should be noted that dexamethasone has been described as both up- and down-regulating MSR activity, an example of the difficulties alluded to above. The inhibitory effect of bacterial LPS on MSR function (Van Lenten *et al.* 1985) appears to be mediated via autocrine TNF α produced by the Mϕ (Van Lenten and Fogelman 1992). Most of the studies have concentrated on cytokines and regulatory molecules produced by the cells located within the vascular wall and/or in atherosclerotic lesions. Few, if any have considered the regulation of MSR *in vivo* at its many, and apparently highly regulated non-atherosclerotic locations.

To add to the confusion, recent reports show contrasting effects of soluble factors on MSR regulation in rabbit SMCs, suggesting a species and/or cell type difference. MSR expression and activity *in vitro* was increased by TNF-α and IFN-γ (Li *et al.* 1995). Platelet secretion factors, containing PDGF-BB, EGF, and TGF-β) similarly upregulated MSR activity and mRNA expression, and TGF-β was predominantly responsible for the MSR upregulatory activity in rabbit SMCs (Gong and Pitas 1995).

Table 1.4 Factors regulating MSR activity/expression

REGULATORY FACTORS	REFERENCES
<u>UPREGULATION</u>	
•Monocyte maturation (phorbol ester; adherence)	(Fogelman *et al.* 1981)
•Serum component(s)	(Fogelman *et al.* 1981)
•Dexamethasone	(Hirsch and Mazzone 1986)
•Monocyte conditioned-medium	(Aviram 1989)
•Arterial SMC conditioned-medium	(Aviram 1989)
•Fibroblast conditioned-medium	(Aviram 1989)
•1,25-dihydroxyvitamin D_3	(Jouni and McNamara 1991)
•M-CSF	(Ishibashi *et al.* 1990) (de Villiers *et al.* 1994)
•Chemotactic S100 peptide (CP-10)	(Lau *et al.* 1995)
<u>DOWNREGULATION</u>	
•Lymphocyte conditioned-medium	(Fogelman *et al.* 1982)
•LPS/endotoxin	(Van Lenten *et al.* 1985)
•"Platelet-derived products" (serotonin, ADP, fibrinogen, fibronectin, PDGF)	(Aviram 1989)
•TGF-β1	(Bottalico *et al.* 1991)
•Protein kinase C inhibitors	(Akeson *et al.* 1991)
•Poly I:C	(de Whalley and Riches 1991)
•IFN-γ	(Fong *et al.* 1990; Geng and Hansson 1992)
•Dexamethasone	(Moulton *et al.* 1992)
•TNF-α	(Van Lenten and Fogelman 1992)
•Prostacyclin agents	(Kowala *et al.* 1993)

1.3.6 <u>MSR: Ligands</u>

A number of complex characteristics are exhibited by ligands binding to MSR types I and II:

i) While the MSR exhibits broad ligand binding specificity and all MSR ligands are polyanionic, not all polyanions will bind to the MSR. This suggests a complex steric relationship between negative charges on ligand and positive charges on receptor. The range of ligands reported to bind to MSR (and chemically related non-ligands) are indicated in table 1.5.

ii) Binding of ligands to MSR is both temperature (maximal at

37°C) and pH-dependent but divalent cation-independent (Goldstein *et al.* 1979; Naito *et al.* 1991; Ashkenas *et al.* 1993; Doi *et al.* 1993; Resnick *et al.* 1993).

iii) There are both high- and low-affinity binding of AcLDL (Ashkenas *et al.* 1993).

iv) The modified lipoproteins OxLDL and AcLDL exhibit non-reciprocal cross-competition. This observation in CHO cells expressing only the type I or type II MSR was unexpected (Freeman *et al.* 1991; Ashkenas *et al.* 1993) and could suggest the presence of multiple binding sites on a single receptor or the existence of multiple receptor conformations with different binding properties (Krieger and Herz 1994).

v) MSR species-dependent (bovine vs murine) and type-dependent (type I vs type II) differences exist in ligand binding (Ashkenas *et al.* 1993). The ReLPS form of endotoxin inhibits the high-affinity, but not low-affinity, binding of AcLDL to bovine type II and murine type I MSR, but has no effect on AcLDL binding to bovine type I receptors. This may be the first indication of a subtle influence of the SRCR domain in type I MSR on ligand binding.

It should be noted that few of the described ligands are found *in vivo*, and several groups are actively searching for more "physiological ligands." Anionic phospholipids, such as phosphatidylserine and phosphatidylinositol, are no longer considered ligands for the MSR (Rigotti *et al* 1995). The study of ligand specificities of MSR type I and II transfected into CHO cells will provide more rigorous analysis of MSR-specific ligand interactions.

The binding of polynucleotides to the MSR is dependent on the ability of the nucleotides to form base-quartet stabilized four-stranded helices (Pearson *et al.* 1993). The spatial distribution of the negatively charged phosphates in polynucleotide quadraplexes provide a charged surface complementary to the MSR collagenous ligand binding domain. As poly I and poly G are able to form such helices while poly C and poly A cannot, these data provide a potential explanation for the selective recognition of polyribonucleic acids by MSR.

The first ligand used in MSR characterization, Ac-LDL, was generated by acetic anhydride charge-neutralization of lysine residues found in the apolipoprotein B moiety of LDL (Goldstein *et al.* 1979). It has now been demonstrated that derivatization of apolipoprotein B lysines by oxidation products of unsaturated fatty acids also results in the MSR recognition of these modified LDLs (Steinbrecher *et al.* 1989; Zhang *et al.* 1993). Derivatization of the lysines alone, without oxidation of other components of the LDL, appears to be sufficient to shift binding to the MSR and away from the LDL receptor. This work represents a useful

Table 1.5: MSR ligands, and their negative controls (adapted from Krieger and Herz 1994)

	LIGANDS (all polyanions)	**NON-LIGANDS** (polyanions and polycations)
Modified proteins	AcLDL, OxLDL, maleylated-LDL (M-LDL), M-HDL, M-BSA	Native LDL, methylated LDL, native BSA Acetylated proteins, including albumin, transferrin, histones
Polyribonucleotides	Poly I Poly G	Poly A, Poly C, Poly U, ss and dsDNA
Polysaccharides	Dextran sulfate Fucoidan Carrageenan	Heparin, Yeast mannan, Chondroitin sulfate, Polysialic acid
Collagen metabolites	PINP/PIIINP (2) Glycated collagen type IV (3)	Native collagen
Others	Polyvinyl sulfate Lipopolysaccharide Crocidolite asbestos Lipoteichoic acid (4) ?Oxidized erythrocytes (5) Sialosaccharide chains on glycophorin on damaged RBCs (6) Unopsonized environmental dust - titanium dioxide - iron oxide - α-quartz (7)	Polyphosphates

Added references: 1. Nishikawa et al 1990 2. (Melkko *et al.* 1994)
3. (El Khoury *et al.* 1994) 4. (Dunne *et al.* 1994)
5. (Sambrano *et al.* 1994) 6. (Beppu *et al.* 1995)
7. (Kobzik 1995)

advance in the development of a much needed reagent in the field, i.e. a well characterized Ox-LDL ligand for the MSR. A similarly burningquestion is, as recently defined, the identity of the MSR ligand (seemingly non-lipoprotein) in tissues which promotes Mϕ adhesion *in vivo* (Hughes 1994; Hughes *et al.* 1995).

1.3.7 MSR: Functions

The best characterized *in vitro* and *in vivo* role for the MSR is in atherogenesis (as discussed). This does not however explain its widespread tissue distribution or broad ligand specificity; and the MSR has been hypothesized to be involved in other Mϕ-associated processes such as adhesion and host defence (Krieger and Herz 1994).

The dual functions of the murine MSR as both an adhesion and endocytic molecule became apparent in reports published from this laboratory (Fraser *et al.* 1993; Hughes *et al.* 1995). Supporting evidence for an adhesion role followed with the demonstration that the MSR mediates the adhesion of human monocytes to non-enzymatically glycated collagen (El Khoury *et al.* 1994). The MSR was thus proposed to contribute to the adherence of Mϕ to glycated arterial basement membranes and the accelerated development of diabetic vascular lesions. This would be consistent with the uptake of advanced glycation end products by the MSR *in vitro* (Araki *et al.* 1995) as well as the colocalization of these products with MSR-positive Mϕ in human atherosclerotic lesion (Kume *et al.* 1995). A recent study also shows the increased MSR-mediated (as inferred from inhibition by poly I and dextran sulfate) adhesion of Mϕ to oxidized mesangial matrix, suggesting a role in Mϕ accumulation and retention in the mesangium and the development of glomerulosclerosis (Mattana *et al.* 1995).

Two low M_r membrane-associated proteins (gp18 and gp30), which are ubiquitously expressed in tissues, display scavenger receptor-like properties, binding a broad range of ligands, including maleylated albumin and dextran sulfate, but not modified lipoproteins (Schnitzer *et al.* 1992). Previous work in our laboratory showed that MSR-dependent adhesion of murine Mϕ to TCP requires serum, while Mϕ adhesion to frozen tissue sections occurs without the addition of exogenous serum (Hughes 1994). This suggests the presence of MSR adhesion-promoting ligand(s) within tissues itself as well as in serum. An obvious MSR-tissue ligand is not apparent among the list of well-characterized ligands. Interestingly, the ligand seems non-lipoprotein in nature as OxLDL and AcLDL proved ineffective in ligand substitution adhesion assays, and Mϕ adhesion proceeded normally in the presence of delipidated sera. Pulmonary surfactant protein (SP-A) and serum amyloid P proteins have been identified as possible physiological MSR ligands which could mediate Mϕ adhesion and need further characterization (Hughes 1994).

The MSR has been implicated as an ancient cell surface recognition system in first line non-clonal innate host defence (Krieger and Herz 1994). According to this reasoning, the MSR contains two

domains, the SRCR and collagenous, both of which organisms may have employed in innate host defence prior to the development of a sophisticated and specific immune system. The MSR thus forms part of a family of "pattern recognition molecules" (of which the collectins constitute other members) which recognize non-self constituents of pathogens such as mannosylated residues, glycans, LPS and dsRNA (Janeway 1992; Sastry and Ezekowitz 1993). Indirect experimental evidence in support of this hypothesis of the MSR as an ancient "anti-inflammatory receptor" include:

i) the inhibition by MSR ligands of LPS binding and clearance by Kupffer cells *in vitro* and *in vivo* (Hampton *et al.* 1991). This CD14-independent binding of LPS did not result in Mϕ activation, suggesting a possible anti-inflammatory role for the MSR;

ii) the involvement of MSR in the binding and clearance of lipoteichoic acid, a constituent of gram-positive bacterial cell walls (Dunne *et al.* 1994). The above may indicate a role for MSR in protection from septic shock during gram-negative or -positive sepsis; and

iii) the binding of crocidolite asbestos (involved in asbestosis and mesothelioma) by secreted forms of the MSR (Resnick *et al.* 1993).

The collagenous domain in MSR is structurally related to a number of secreted molecules known to contain collagen-like sequences. The collectins are multimeric proteins with lectin domains, and include C1q (Thiel and Reid 1989), pulmonary surfactant apoprotein (SP-A and SP-D) (Floros *et al.* 1986), serum mannose binding protein (Sastry *et al.* 1991), and conglutinin (Lachmann 1967). These molecules, like collagen itself, bind a wide range of polyanionic ligands and may be involved in first line host defence and clearance of debris, including pathogens, from the extracellular space (Sastry and Ezekowitz 1993). Consistent with this, the recently identified MARCO (Mϕ receptor with a collagen domain) protein was reported to bind bacteria and DiI-AcLDL (Elomaa *et al.* 1995). Interestingly, the bullous pemphigoid autoantigen BP180, another integral membrane protein with a collagenous domain, acts as an adhesion molecule in anchoring basal keratinocytes (via hemidesmosomes) to the cutaneous basement membrane (Gipson *et al.* 1993). Mutations in this hemidesmosomal transmembrane collagen have recently been reported as the cause of a form of epidermolysis bullosa, a blistering skin disorder, in humans (McGrath *et al.* 1995).

A role for the MSR in normal physiology has been suggested by: i) the MSR-mediated clearance of collagen propeptides, which are products of normal metabolism (Melkko *et al.* 1994); and ii) MSR recognition of acidic phospholipids (Nishikawa *et al.* 1990) and sialosaccharide side chains of glycophorin (Beppu *et al.* 1995) on oxidatively damaged

erythrocyte membranes, implicating the MSR in clearance of senescent red blood cells. Another novel physiologic function for the MSR is its involvement as a phagocytic receptor in the uptake of apoptotic thymocytes by thymic Mϕ (N. Platt and S. Gordon, personal communication).

1.3.7 MSR: Studies presented in this thesis

Following the description of its ability to inhibit the divalent cation-independent adhesion of Mϕ to TCP surfaces in the presence of FCS (Fraser *et al.* 1993), mAb 2F8 to the murine MSR has been used to initiate a number of studies directed at furthering the understanding of MSR expression *in vivo*, its biosynthesis and processing in the cell, and regulation of its expression *in vitro*.

To date, mAb 2F8 has only been shown to inhibit Mϕ adhesion *in vitro*. Attempts to inhibit Mϕ recruitment *in vivo* were unsuccessful in mouse models in which mAb 2F8 was administered via the i.v. route. The failure to detect intravenously injected mAb 2F8 in the tissues of mice using appropriate second antibody reagents suggests that the antibody itself may be rapidly cleared from the circulation and degraded in Mϕ by an MSR-dependent pathway (as would conventional MSR ligands). If this were indeed the case, mAb would not be available for inhibition of cell surface MSR, and alternative methods for determining its function *in vivo*, such as MSR-deficient mice egineered by embryonic stem cell recombinant technology will be useful. A colony of MSR-deficient mice was recently obtained from Dr. T. Kodama (Tokyo, Japan) and established at the Dunn School of Pathology, Oxford and preliminary results (Honda *et al.* 1995; Takahashi *et al.* 1995) are briefly reported in Chapter 10.2.

In an attempt to relate Mϕ adhesion *in vitro* to migration and adhesion processes *in vivo*, Mϕ were found to adhere to specific regions of frozen tissue sections of murine organs (Hughes *et al.* 1995). MSR-dependent (divalent cation-independent, mAb 2F8-inhibitable) adhesion occurred in areas where the endogenous expression of MSR was prominent, suggesting an adhesion role within organs *in vivo*. Similar assays also suggested a role in Mϕ adhesion during BCG infection where MSR expression is retained (Hughes 1994). In contrast, in the osteopetrotic mouse, which lacks M-CSF, the pathologic phenotype may be related to the reduction in MSR expression and Mϕ adhesive capacity (Hughes 1994).

The work presented in this thesis is briefly summarized:

i) In Chapter Three, M-CSF is demonstrated to enhance MSR

expression and function markedly and selectively in murine elicited peritoneal Mϕ. The results suggest a plausible mechanism whereby M-CSF production in the atheromatous plaque micro-environment could promote the recruitment and retention of Mϕ and subsequent foam cell formation.

ii) Chapter Four deals with the divergent effects of the archetypal Th1 (IFN-γ) and Th2 (chiefly IL-4) lymphokines on MSR expression and activity; with IL-4 serving as an upregulating stimulus and IFN-γ either maintaining or downregulating MSR status. Micro-heterogeneity in MSR structure is a major manifestation in these cytokine-mediated differences and results from changes in N-linked glycosylation, specifically sialylation. The functional significance of the microheterogeneity was investigated by assessing the sensitivity of MSR-mediated AcLDL uptake to inhibition by various MSR ligands, especially lipoteichoic acid.

iii) In Chapter Five the effects of TGF-β, IL-10, C-C chemokines and other soluble factors on MSR expression and activity are examined. In contrast to IL-10, TGF-β is identified as a potent dose-dependent inhibitor of MSR upregulation in M-CSF stimulated BgPMϕ. Endogenously produced TGF-β inhibits MSR function in BgPMϕ, a Mϕ-like cell line RAW 264.7 and foam cells *in vitro*. MCP-1 reproducibly enhanced BgPMϕ adhesion to TCP in a MSR-dependent fashion.

iv) Chapter Six contains immunochemical evidence for the presence in BgPMϕ culture supernatants of a cleaved soluble form of MSR which lacks the cytoplasmic domain. M-CSF increases release of this fragment *in vitro* and soluble MSR is shown to be functionally active.

v) In Chapter Seven studies with murine MSR cDNAs transfected into CHO cells are described. The expression and function of transfected type II MSR is upregulated by culture in a scavenger receptor-specific selection medium. The MSR contribution to CHO cell adhesion to TCP requires such upregulation to become apparent. To determine a possible adhesion-promoting region in the MSR, the binding site of mAb 2F8 was mapped using a series of MSR truncation mutants, and localized to the proximal α-helical coiled-coil domain.

vi) Chapter Eight examines the immunohistochemical Mϕ phenotype, including MSR expression, in tissues and atherosclerotic lesions in mice deficient in both M-CSF (op) and apolipoprotein E. These mice are protected from developing atherosclerosis in the face of markedly elevated plasma cholesterol levels (Smith *et al.* 1995).

vii) In Chapter Nine morphological evidence, obtained by confocal and electron microscopy, is presented for an adhesion role for

the MSR in primary Mϕ and transfected CHO cells. MSR expression is prominently expressed at the adherent surface and its distribution may be restricted to cellular contact areas.

viii) Chapter Ten deals with general conclusions and possible directions for future research.

Chapter Two

Materials and Methods

2.1 Animals

Animals were bred and housed at the Sir William Dunn School of Pathology, Oxford. **PO** (Outbred Swiss Pathology Oxford), **Balb/c** (inbred), **C57BL/6** (inbred), and **129 MSR-deficient** mice (obtained from Dr. T. Kodama, Tokyo, Japan) were used between five and seven weeks of age. Animals were handled and killed in accordance with guidelines issued by the Home Office.

2.2 Media and Sera

The following culture media were all obtained from Gibco BRL, UK: **RPMI 1640** ; Dulbecco's Modified Eagle Media (**DMEM**) ; Modified Eagle's Medium (**MEM**) (with and without L-methionine); **F-12 HAM Nutrient medium** (for CHO cells) and **OptiMEM**, a proprietary serum-free medium. All media were routinely supplemented with 2mM glutamine, 50μg/ml streptomycin and 50 IU/ml penicillin G. Where cells were required to spend time outside the CO_2 incubator (such as incubation in the cold prior to adhesion assays), media were also supplemented with 20mM Hepes (pH 7.3).

Fetal Calf Serum (**FCS**) (Biological Industries, Israel), and Horse Serum (**HS**) (Gibco BRL, UK) were heat inactivated at 56°C for 30 minutes, and filter sterilized (0.22μm pore size) prior to use at concentrations indicated in the text. Normal Mouse Serum (**NMS**) was prepared from mouse blood in this laboratory by P. Tree, and was heat inactivated and sterilized as above prior to use.

2.3 Reagents

Phosphate Buffered Saline (**PBS**) pH 7.4 was obtained in tablet form from Oxoid Ltd., UK, and was used without divalent cations. **PBA** consisted of PBS plus 0.25% (w/v) Bovine Serum Albumin (BSA) and 10mM NaN_3.

Tissue culture treated plastic (**TCP**) flasks, plates and dishes were from Nunc, Denmark, or from Falcon, Becton Dickinson Labware, USA. Bacteriologic plastic (**BP**) vessels were from Greiner Labortechnik, UK.

Heparin (5000 Units/ml) was from Flow Laboratories, UK, and was used for the collection of mouse plasma and blood cells.

Peptide-N-Glycosidase F (**N-Glycanase**), and the Sialidases (**Newcastle Disease Virus,** ***Arthrobacter ureafaciens*** and ***Clostridium perfringens***) were all from Oxford GlycoSystems, UK, and were used with the reaction buffers supplied with the enzymes. Digoxigenin-labeled **WGA** (wheat germ agglutinin), and the DIG Glycan differentiation kit (containing the digoxigenin-labeled lectins **GNA** (*Galanthus nivalis* agglutinin), **SNA** (*Sambucus nigra* agglutinin), **MAA** (*Maackia amurensis* agglutinin), **PNA** (Peanut agglutinin) and **DSA** (*Datura stramonium* agglutinin); Endoglycosidase F (**Endo-F**) and **Neuraminidase** (from *Vibrio cholerae*) were from Boehringer Mannheim, Germany, and were used according to the manufacturer's instructions or with incubation buffer E-1 from Oxford GlycoSystems.

1,25-Dihydroxy Vitamin D3 [used at 12nM final concentration], **Prostaglandin E_2** [used at 10μM final concentration] and **Leukotriene B4** [used at 50nM final concentration] were from Biomol, USA. **17β-Estradiol** [used at 10ng/ml final concentration], **Dexamethasone** [used at 10^{-6} M final concentration], **Indomethacin** [used at 1μM final concentration], **Probucol** [used at 50μM final concentration] and **α-Tocopherol** [used at 50μM final concentration] were from Sigma, UK.

Polyinosinic acid (**Poly I**), Polycytidylic acid (**Poly C**), **Polyvinyl sulfate, Fucoidan** (from *Fucus vesiculosis*) and **Lipoteichoic acid** were all from Sigma, UK. **Dextran sulfate** (MW~500 000) was from Pharmacia, Sweden. **Nocodazole** [10μM final concentration], **Brefeldin A** [10μg/ml final concentration] and **Cytochalasin D** [1μM final concentration] were all from Sigma, UK.

Alkaline phosphatase (from calf intestine) and **Collagenase** were from Boehringer Mannheim, Germany, and was used with the reaction buffer supplied by the manufacturers.

2.4 Cells

2.4.1 Primary cells

i) Peritoneal Mϕ were harvested by injecting 5-10 ml sterile PBS into the murine peritoneal cavity, agitating the abdominal contents by rubbing with a Pasteur pipette, and aspirating the lavaged cells. Elicited Mϕ populations were obtained by injecting 1ml of Brewer's complete thioglycollate broth (Difco Laboratories, Surrey) (for **TPMϕ**) (Johnson *et al.* 1978) or 1ml of a 1% (v/v) suspension of Bio-Gel P100 (fine) polyacrylamide beads (Bio-Rad, Richmond, USA) (for **BgPMϕ**) (Fauve *et*

al. 1983; Rabinowitz 1988) two to five days before harvesting, as indicated.

ii) Human monocytes. Peripheral blood mononuclear cells were prepared in standard fashion by centrifuging buffy coat cells over a cushion of Ficoll ("Ficoll-Paque"; Pharmacia LKB). Mononuclear cells remaining above the Ficoll were harvested and washed at low speed to remove residual Ficoll and platelets. Monocytes were subsequently isolated by adherence to gelatin providing a 95% pure monocyte population. Adherence may be reversed immediately by chelation of divalent cations in the medium, but after 48-60 hours the cells detach spontaneously (Collin *et al.* 1995). Following detachment monocytes were cultured on TCP in RPMI and 10% HS; with the addition of recombinant human M-CSF [1000 units/ml] (Cetus Corporation, USA) and 200nM PMA (Phorbol 12-myristate 13-acetate; Sigma, UK) for differentiation to adherent Mϕ and upregulation of MSR expression as described (Ishibashi *et al.* 1990).

iii) Bone Marrow cells were flushed from the femora of mice with PBS using a syringe and 19G needle. These plugs were either digested with 0.05% (w/v) collagenase and 0.002% (w/v) DNAse (both from Boehringer Mannheim, UK) for one hour at 37°C on a rotating wheel at 30 r.p.m. to obtain resident bone marrow Mϕ , or were mechanically disrupted by pipetting through a wide bore plastic pipette. For long term culture, cells were plated in RPMI plus 10% FCS and 10% HS plus 10^{-6} M Dexamethasone (Sigma, UK; diluted from 10^{-2} M stock in ethanol). Where indicated erythrocytes were subjected to hypotonic lysis by a five minute incubation in ammonium chloride solution (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA, pH 7.2) as described (Hoffman *et al.* 1980).

2.4.2 Cell Lines

Unless otherwise indicated, cell lines were obtained from stocks maintained at the cell bank, Sir William Dunn School of Pathology, Oxford, and were routinely tested for contamination by *Mycoplasma*.

i) Murine Mϕ-like cell lines used included **RAW 264.7** (Raschke *et al.* 1978) , **J774** (Ralph *et al.* 1975), and **P388D1** (Koren *et al.* 1975). These were cultured in RPMI plus 10% FCS on either bacteriologic (BP) or tissue culture plastic (TCP) surfaces. On BP, they could be readily harvested by incubation in PBS plus 5mM EDTA prior to collection by pipetting. On TCP, harvesting required the addition of 15mM lidocaine-HCl (Astra Pharmaceuticals, UK) to the PBS-EDTA (Rabinovitch and de Stefano 1976).

ii) Human THP-1, a non-adherent monocytic leukaemia cell line (Tsuchiya *et al.* 1980) was cultured in RPMI plus 10% FCS. Differentiation to adherent Mϕ-like cells was achieved by adding 200nM PMA (Phorbol 12-myristate 13-acetate; Sigma, UK) to the incubation medium as described (Hara *et al.* 1987).

2.4.3 Transfected Cell Lines

Chinese Hamster Ovary (CHO M1015) cells stably transfected with either **Type I (CHO M1015 + pJA28C5)** or **Type II (CHO M1015 + pJA22-1C8)** Murine Macrophage Scavenger Receptor (MSR) cDNAs (Ashkenas, J *et al.* 1993) were a generous gift of Dr M. Krieger, Cambridge, USA. Cells were grown in RPMI plus 5% FCS, and were harvested by incubating in PBS plus 5mM EDTA followed by pipetting to remove adherent cells.

In collaboration with Drs. M. Honda and T. Kodama, Tokyo, a series of murine MSR truncation mutants stably transfected into CHO cells were investigated (as described in Chapter Seven). These mutants include **#1 CHO-MSR II** expressing the complete MSR type II (350 aa); **#2 CHO-Δ22aa** (325 aa) lacking the C-terminal 22 amino acids containing the putative AcLDL binding site; **#3 CHO-Δcollagen-like domain** (277 aa) lacking the collagen-binding domain; and **#4-#8** a series of deletion mutants in the α-helical coiled-coil domain (249, 226, 197, 183 and 167 aa respectively). Truncated mutants were constructed in the expression vector pRC/CMV (Invitrogen, San Diego, CA) which contains *HindIII* and *XbaI* restriction sites as well as a neomycin resistance gene. The DNA sequences of all mutant genes were determined by the dideoxy method. Expression vectors containing the various mutants were transfected into CHO cells using the polybrene method. To isolate transfectants expressing the mutants, CHO cells were grown, selected and cloned by dilution plating in F-12 Ham nutrient medium supplemented with 5% (v/v) FCS, 100units/ml penicillin, 100μg/ml streptomycin, 2mM glutamine and 300 μg/ml geneticin (G418) for at least 6 weeks. Expression and functional studies were also performed on **#1 CHO-MSR II** cells that had been cultured in MSR selection medium for a prolonged period. This so-called **MAC medium** contains 3% (v/v) newborn calf lipoprotein-deficient serum (Sigma, UK), 0.5mg/ml geneticin (G418, Gibco, UK), 250μM **M**evalonate, 3μg protein/ml **A**cLDL, and 40μM **C**ompactin (all from Sigma, UK).

2.5 Antibodies

2.5.1 Primary Antibodies, Monoclonal

Unless otherwise indicated, all are rat mAb. Isotype is indicated where known.

Table 2.1. Monoclonal antibodies used in this thesis

Name	Specificity	Isotype	Reference
2F8	MSR (SR-AI and II)	Rat IgG2b	(Fraser *et al.* 1993)
F4/80	Macrophage	Rat IgG2b	(Austyn and Gordon 1981)
FA-11	Macrosialin (CD68) on Mϕ	Rat IgG2a	(Smith and Koch 1987)
5C6	CD11b subunit of CR3	Rat IgG2b	(Rosen and Gordon 1987)
3D6	Sialoadhesin on Mϕ	Rat IgG2a	(van den Berg *et al.* 1992)
8D2	CD44	Rat IgG2b	Doyle and Nath, unpublished
2.4G2	FcRII (CD32)	Rat IgG2b	(Unkeless 1979)
TIB120	MHC class II, IA/IE	Rat IgG2b	Bhattacharya *et al.* 1981
CAMPATH-1G	CDw52	Rat IgG2b	(Hale *et al.* 1987)
NLDC145	Interdigitating (dendritic cells)	Rat IgG2a	(Kraal *et al.* 1986)
GK 1.5	CD4	Rat IgG2b	Dialynas *et al.* 1983
53.6.72	CD8	Rat IgG2a	Ledbetter and Herzenberg 1979
	Talin	Rat IgG2b	Rees 1993
YL1/2	Tubulin	Rat IgG2a	Pharmingen

MAb 2F8 was digoxigenin-labeled using a DIG antibody labeling kit from Boehringer Mannheim, UK according to the manufacturer's instructions. Rhodamine-phalloidin [5μM] was from Molecular Probes, USA.

2.5.2 Primary Antibodies, Polyclonal

Rabbit anti-murine MSR cytoplasmic domain peptide (MTENQRLCPHEREDADC) antiserum (7.8mg/ml) was obtained from Dr. M. Krieger, Cambridge, MA (Ashkenas *et al.* 1993). **Rabbit anti-TGN38** (38K protein of the *trans*-Golgi network, **anti-protein disulfise**

isomerase (PDI), and **anti-transferrin receptor** polysera were obtained from Dr. D. Vaux (Oxford).

2.5.3 Second Antibodies

Goat anti-rat IgG (Alkaline Phosphatase- and Peroxidase-conjugated) antisera were from Sigma, UK. **Goat anti-rat IgG** (Rhodamine-conjugated F(ab')2) antiserum was from Chemicon, USA. **Goat anti-rat IgG** (Biotinylated) antiserum was from Vector laboratories, UK. **Rabbit anti-goat IgG** (Peroxidase-conjugated) antiserum was from Sigma, UK. **Mouse anti-rat IgG** (FITC-conjugated) antiserum was from Boehringer-Mannheim, USA. **Mouse anti-rat IgG** (FITC-conjugated F(ab')2) was from Jackson ImmunoResearch Laboratories, USA. **Goat anti-mouse IgG** (Peroxidase-conjugated) antiserum was from Sigma, UK. Horse radish peroxidase-conjugated **sheep anti-digoxigenin F(ab')2 fragments** were from Boehringer-Mannheim, UK. **Goat anti-mouse and goat anti-rat IgG immunogold conjugates** were from British Biocell, UK.

2.6 Cytokines

Purified recombinant murine proteins were gifts from the following (used at final concentration indicated in square brackets): **Interferon-γ** (IFN-γ) [100units/ml] from Dr F. Balkwill, ICRF London, UK. **IL-4** [5ng/ml] from Dr S. Gillis, Immunex Corporation, USA. **TNF-α** [500units/ml] from Dr E. Havell, Trudeau Institute, USA. **IL-10** [50units/ml] from Dr K. Moore, DNAX Research Institute, USA. **GM-CSF** [1000units/ml] from Dr A. Bernard, Glaxo Institute for Molecular Biology, Switzerland.

Murine **IL-13** [10ng/ml] (Minty *et al.* 1993) (originally known as **P600** (Brown *et al.* 1989)) was used as a tissue culture supernatant from transfected CHO cells. This, together with a negative control supernatant, **PSE**, was obtained from Dr A. Minty, Sanofi Elf Bio Recherches, France.

Purified recombinant **human M-CSF** [1000units/ml] was from Dr S. Aukerman, Chiron Corporation, USA. **Murine M-CSF** reagents used were either L-cell conditioned medium [15% of RPMI 1640 incubation medium] as a rich source of murine M-CSF (Stanley and Heard 1977) or murine M-CSF [±1000 units/ml] as supernatant from transfected insect cells obtained from Dr. J Schreurs, Chiron Corporation, USA. Recombinant Human **TGF-β** [0.5ng/ml] was from R&D Systems, UK, as were **PDGF-BB** [1ng/ml], murine **IL-3** [5ng/ml], murine **IL-6** [10ng/ml], and murine **IL-12** [5ng/ml]. The human cytokines are known to act on

murine cellular target cells.

Purified recombinant murine **MCP-1** [20ng/ml] and murine **RANTES** [20ng/ml]were obtained from Dr. B. Rollins (Dana Farber Cancer Institute, Boston, USA), and Dr. H. Rosen (MSD, New Jersey, USA) respectively. Recombinant murine **MIP-1α** [10ng/ml], and **MIP-1β** [200ng/ml], were from R&D Systems, UK.

Neutralizing murine mAb **anti-human M-CSF 5H4** was from Dr. J. Schreurs (Chiron Corporation, USA) and goat **anti-L cell M-CSF and M-CSF receptor** antisera as well as rabbit **anti-mouse Mϕ mannose receptor** antiserum were from Drs. E.R. Stanley (New York) and P. Stahl (St. Louis), respectively. Rabbit **anti-mouse RANTES** antiserum was a kind gift from Dr. H. Rosen (MSD, New Jersey, USA) and chicken **anti-TGF-β** (which cross-reacts with both human and mouse TGF-β) and goat **anti-murine IL-10** were obtained from R&D Systems, UK.

2.7 Immunocytochemical Techniques

2.7.1 Immunocytochemistry

Adherent cells were plated on 11mm diameter acid-washed glass coverslips in 24 well plates, and allowed to adhere at 37°C in complete medium. After washing with PBS, cells were fixed for 30 minutes at room temperature in PBS containing either 0.125% glutaraldehyde (Agar Aids, UK) or 3% Paraformaldehyde (BDH, UK). Unreacted groups were quenched for one hour with PBS plus 10% FCS, following which cells were stained immediately, or kept in PBA at 4°C until used.

Primary antibody (approx. 10μg/ml purified, or neat tissue culture supernatant) were incubated with the cells for one hour at room temperature in the presence of 5% normal goat serum. Antibody binding was detected with the appropriate biotinylated second antibody by the avidin-biotin peroxidase method (Hsu *et al.* 1981), using reagents from Vector laboratories, UK, with diaminobenzidine (Polysciences, UK) as the chromogen. Endogenous peroxidase activity was eliminated by incubating the cells for 20 minutes at room temperature in methanol with 0.3% H_2O_2. Counterstaining was with Mayer's haematoxylin. Stained cells were dehydrated and mounted in DePeX mountant (BDH, UK).

2.7.2 Immunohistochemistry

Organs (heart, aorta, lungs, liver, spleen, gut, kidneys, and skin) from apoE-deficient mice, and mice deficient in both M-CSF (op) and

apoE (op2/E0, op1/E0 and op0/E0 animals) were obtained from Dr. J. Smith (Rockefeller University). These animals were fed an atherogenic diet for 16 weeks, sacrificed and saline perfused. Organs were removed, placed in Tissue-Tek O.C.T. compound (Miles Scientific, UK), and snap frozen in iso-pentane cooled by dry ice. Sections were cut on a cryostat (10μm thick), stained with mAbs as described in Chapter Nine, and binding detected by the avidin-biotin peroxidase method detailed above (2.7.1).

2.7.3 Immunofluorescence

Cells were prepared as for immunocytochemistry (2.71). Where visualisation of intracellular antigen was required, cells were processed (antibody incubations and washes) in PBA containing either 0.1% (w/v) saponin (Sigma, UK), or 0.1% (v/v) Triton X-100 (Sigma, UK). Bound antibody was visualized with the appropriate second antibody conjugated with either FITC or Rhodamine. Cells were washed in distilled water, then mounted in Mowiol mountant (Hoechst, UK) and allowed to dry overnight in the dark. Cells were viewed under epifluorescence microscopy using standard rhodamine or FITC excitation and emission filters.

2.7.4 Flow Cytometry

Cells were harvested, washed in PBS and suspended in 50μl of antibody (10μg/ml purified antibody in PBS plus 5% NMS) for one hour at 4°C. Cells were washed three times in PBA then incubated for a further hour at 4°C in 50μl of FITC-conjugated second antibody diluted 1:40 in PBA plus NMS. Mouse anti-rat reagents (both monoclonal and polyclonal) consistently produced the least non-specific binding, especially when used as $F(ab')_2$ fragments. Cells were then washed once and resuspended in PBA for analysis on a FACScan® (Becton Dickinson & Co.). Excitation was with a 15mW Argon-Ion laser at 488nm, with FITC emission collected in the FL1 photomultiplier at 515-545nm. Cells and debris having low forward scatter (FSC) were routinely gated out of the analysis. Data were analyzed by the Lysys II software programme, and where numerical data are presented, geometric statistics were used. As described in the Results section "Surface" levels of antigen detected refer to molecules present on the plasma membrane, whilst "internal" levels include molecules detected after cell permeabilization in 1% saponin (Sigma) for 30min followed by fixation for 10min in 4% paraformaldehyde. "Internal" levels would be a reflection of "total"

antigen present intracellularly and on the plasma membrane following saponin disruption (Franek *et al* 1994). For visualisation of intracellular antigen, all steps were performed in the presence of 0.1% Saponin or 0.1% Triton X-100 (as above, 2.7.3) (Franek *et al* 1994).

2.8 Cell Lysates and Immunoprecipitation

2.8.1 Lysis Buffer

Cells were lysed prior to immunoprecipitation and immunoblotting by the same process. After washing five times in PBS, cells were lysed on ice in 150mM NaCl, 10mM EDTA, 10mM NaN_3, 10mM Tris (pH 8.0), 1mM PMSF, 5mM iodoacetamide and 1% Nonidet P-40 (NP-40) for 30-60 min. Organs (for tissue immunoblots) were rapidly frozen in liquid nitrogen and stored at -70°C. They were then weighed and Polytron disrupted in 50-100mg tissue/ml of lysis buffer and lysed for 1-2 hours. Cell and tissue lysates were spun for five minutes in a microfuge (13 000 r.p.m.) to remove nuclei, and then at 100 000 X g to remove debris. Supernatants were stored at 4°C for up to one week before use, or at -70°C for longer periods. Protein concentrations of lysates were measured using the Bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce Chemical Company, USA) using BSA to generate the standard curve.

2.8.2 Immunoprecipitation

All steps were performed at 4°C, or at room temperature using ice-cold reagents. Lysates were precleared by rotating overnight in the presence of Protein G-Sepharose (50µl/ml lysate of a 20% (v/v) slurry in lysis buffer) (Pharmacia LKB, Sweden). After incubation with purified antibody (25µg/ml lysate) for at least four hours, antigen was precipitated with Protein G-Sepharose. The precipitate was washed six times in 10mM Tris (pH 8.0) buffers: three times in 500mM NaCl, 0.5% Triton X-100, 0.5% Deoxycholate, 0.05% SDS; twice in 150mM NaCl, 0.5% Triton X-100, 0.5% Deoxycholate, 0.05% SDS; and once in 0.05% SDS. The final pellet was boiled in Laemmli gel buffer (0.0625 M Tris pH 6.8 ; 10% Glycerol; 2% SDS and 0.001% bromophenol blue) in the presence or absence of 5% (v/v) 2-Mercaptoethanol (βME), prior to separation by SDS-PAGE (Laemmli 1970).

2.9 SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Blotting

2.9.1 SDS-PAGE (Laemmli 1970) and Membrane Transfer

7.5%, 6% or 5-10% linear-gradient polyacrylamide separating gels (pH 8.8) were used. Stacking gels were 4% polyacrylamide (pH 6.8), and electrode buffer contained 25mM Tris, 90mM Glycine. Electrophoresis was at 30mA for three hours. Molecular weights were estimated by comparison with standards ranging from 14.3-200 kDa (Rainbow Markers, Amersham, UK).

For autoradiography (^{125}I), gels were fixed in 40% Methanol, 10% Acetic acid for at least 30 minutes prior to drying and exposure to film at -70°C. For fluorography (^{35}S), gels were impregnated in EN^{3}HANCE (Du Pont, USA) after fixing, then dried and exposed as above.

For blotting (Towbin *et al.* 1979), separated proteins from unfixed gels were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Germany) for three hours at 300mA in a 25mM Tris; 90mM Glycine buffer containing 20% methanol. Blotted membranes were then handled as indicated below.

2.9.2 Immunoblotting

For immunoblotting, cell input was 30µl per lane of a lysate made up of approximately 10^8 cells/ml lysis buffer or equal protein loads as measured using the Bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce Chemical Company, USA) using BSA to generate the standard curve. Blotted membranes were transferred to blocking buffer (PBS plus 3% (w/v) powder milk (Marvel Original) plus 0.1% Tween 20), and left for at least one hour at 4°C before proceeding. First antibody was diluted to 10µg/ml in blocking buffer and incubated on a rocking platform with the membrane for one hour at room temperature. Membranes were washed three times in blocking buffer, before adding peroxidase-conjugated anti-rat IgG antibody (1:1000 in blocking buffer) for a further hour. After three washes in PBS plus 0.1% Tween 20, antibody binding was visualized by chemiluminescence (ECL, Amersham, UK). Typical exposure times were less than 10 seconds. For tissue mAb 2F8 immunoblots, blotting was preceded by mAb 2F8 immunoprecipitation as detailed above, SDS-PAGE and transfer to nitrocellulose membranes. Digoxigenin-labeled mAb 2F8 was used as first antibody followed by peroxidase-conjugated sheep anti-digoxigenin $F(ab')_2$ fragments and

chemiluminescence detection.

2.10 Labeling of Soluble and Membrane-bound Proteins

2.10.1 Metabolic Labeling

Cells were plated at, or grown to confluence in 35mm TCP dishes in RPMI plus 10% FCS prior to metabolic labeling. They were washed in methionine-free MEM, preincubated in the same medium containing 10% dialysed FCS for one hour at 37°C, and then labeled with Tran^{35}S-label™ (70% ^{35}S-L-methionine ; ~15% ^{35}S-L-cysteine) or ^{3}H-galactose (ICN, UK). For pulse-chase experiments, cells were pulse-labeled with 500µCi/ml, and then chased in MEM plus 10% FCS for the times indicated. In other experiments, cells were labeled overnight (approximately 16 hours) with 50µCi/ml label in methionine-free MEM plus 10% dialysed FCS. After labeling, cells were washed five times with PBS, then lysed in the dish with lysis buffer, and prepared for immunoprecipitation and SDS-PAGE as above.

Quantification of protein bands on dried gels was by PhosphorImager and ImageQuant software analysis program (Molecular Dynamics, Inc., Sunnyvale, CA) and results expressed as a ratio of arbitrary PhosphorImager Units (APUs).

2.10.2 **Surface Biotinylation** (Harter and Mellman 1992)

Cells were grown in 35mM dishes (5 x 10^6/dish); and metabolically labeled overnight as desribed above. Monolayers were washed 6 times with ice-cold PBS. Freshly prepared membrane impermeable Biotinamidocaproate sulfo-N-Hydroxysuccinimide Ester (sulfo-N-HSS-Biotin) (Pierce, USA), at 1.5mg/ml in PBS, was added for 30min on ice. The biotin was quenched with 50mM glycine in PBS (six washes) and the cells lysed in standard NP-40 lysis buffer. Immunoprecipitations were carried out in two steps; i) mAb 2F8 followed by washes and 10 min boiling to dissociate antibody-antigen complexes. 50µl Octylglucoside (10%) (for quenching) and 360µl were added for a final volume of 500µl, and the eluted material split into two; ii) a second round of immunoprecipitation was performed with mAb 2F8 (20µg/ml) and 50µl 20% Gammabind-Sepharose (v/v) on the one sample, and the other sample was incubated with 50µl 20% (v/v) immobilized avidin (ImmunoPure, Pierce, USA) overnight prior to final washes and SDS-PAGE, fluorography and/or PhosphorImager analysis.

2.10.3 Covalent cross-linking of AcLDL-binding protein in serum and culture supernatants

Serum or culture supernatant (50µl) was incubated overnight at 4°C with ^{125}I-AcLDL (Biogenesis, USA) at a final concentration of 10ng/ml with or without 100-fold excess of cold AcLDL (Biogenesis, USA) or Poly I (Sigma, UK) to assess specific binding. Subsequently, Disuccinimidyl suberate (DSS) (Pierce, USA), freshly prepared in DMSO, was added to a final concentration of 1mg/ml and the sample incubated at 4°C for 45 min. Crosslinking reactions were terminated by the addition of 50 µl SDS-PAGE sample buffer (1% (wt/vol) SDS, 10% (vol/vol) glycerol, 62.5mM Tris-HCL, pH 6.8). Samples, to be tested under reducing conditions, were treated with dithiothreitol (DTT; 100mM) and boiled for 10 min. Cross-linked complexes were run on 6% SDS-PAGE and identified by autoradiography after 48 hour exposure. Iodinated AcLDL was assessed for self-association in all cross-linking experiments.

2.10.4 Poly G-sepharose MSR binding assay (Resnick *et al.* 1993)

Metabolically labeled medium (as described above) (750µl) was added to 250µl of buffer A (20 mM Tris-HCL, pH 8.0; 150mM NaCl; 1mM $CaCl_2$) containing 2mg/ml BSA and 0.01% (w/v) NaN_3 (buffer B). 25µl of a suspension of buffer A containing 50% (v/v) Sepharose beads coupled to Poly G (Sigma, UK) and the mixture placed on a rotator overnight at 4°C. The beads were then washed twice with buffer A, and adsorbed proteins eluted by boiling for 5 min gel loading buffer (reducing or non-reducing). The samples were subjected to 10% SDS-PAGE

2.11 Reverse transcription (RT)-PCR Analysis

For RNA isolation, BgPMϕ were incubated in 24-well tissue culture dishes at 5 x 10^5 cells/well and incubated with M-CSF for 24 and 48 hours. Total cellular RNA was extracted with RNAzol solution (Cinna/Biotex Laboratories, Houston,TX) and reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (BRL/Gibco, UK). The cDNA obtained served as a template for PCR using oligonucleotide pairs as follows: murine MSR (Ashkenas *et al.* 1993) i) common to both type I and II receptors (5′ primer: 5′-CCAAGTCCTTGCAGAGTCTG-3′; 3′-primer: 5′-GTCTGAGGTCGTTGGTGATG-3′; size of amplified fragment: 236 bp); ii)

Fig. 2.1 **Reverse transcription (RT)-PCR analysis: optimalization of PCR conditions**

PCR conditions were initial denaturation at 92°C for 3 min; annealing at 60°C for 1 min; extension at 72°C for 2 min and denaturation at 92°C for 1 min; x30 cycles. A final extension cycle was at 72°C for 5 min.

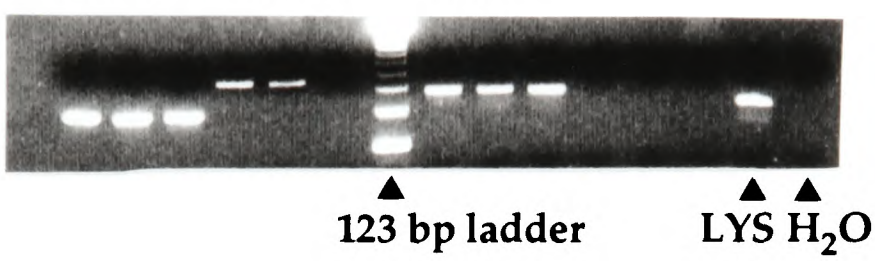
A. A $MgCl_2$ concentration of 1.5 mM was found optimal for amplification with all the primer pairs.

B. The amplified C-MSR, MSR-I and MSR-II fragments corresponded to their predicted sizes as per molecular weight markers. Restriction enzyme digests (not shown) were further proof of specificity.

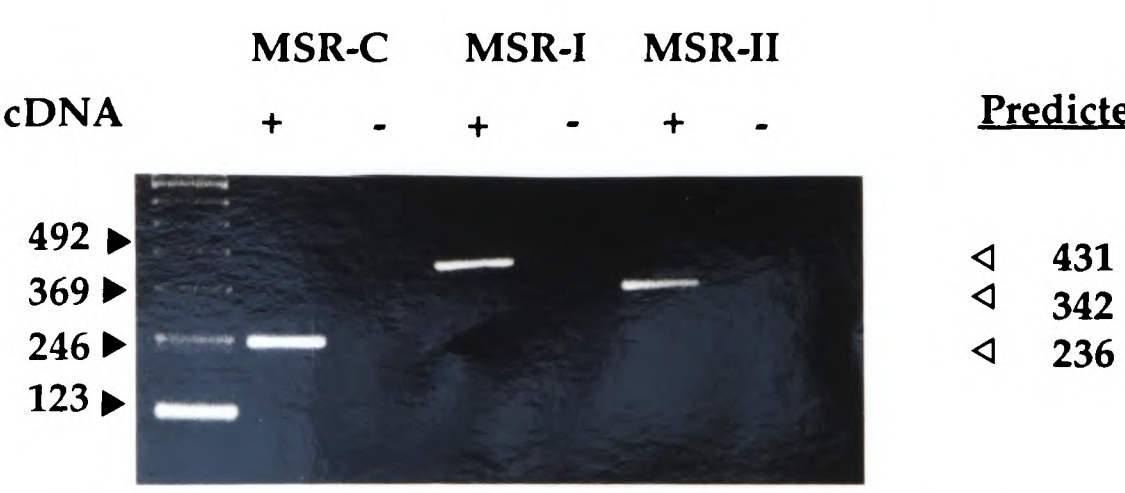
C. Primer specificity was further shown by amplification of C-MSR, MSR-I and MSR-II fragments from cDNA derived from RAW 264.7 Mϕ-like cell line and stably transfected CHO MSR I and CHO MSR II cells.

MSR OLIGONUCLEOTIDES

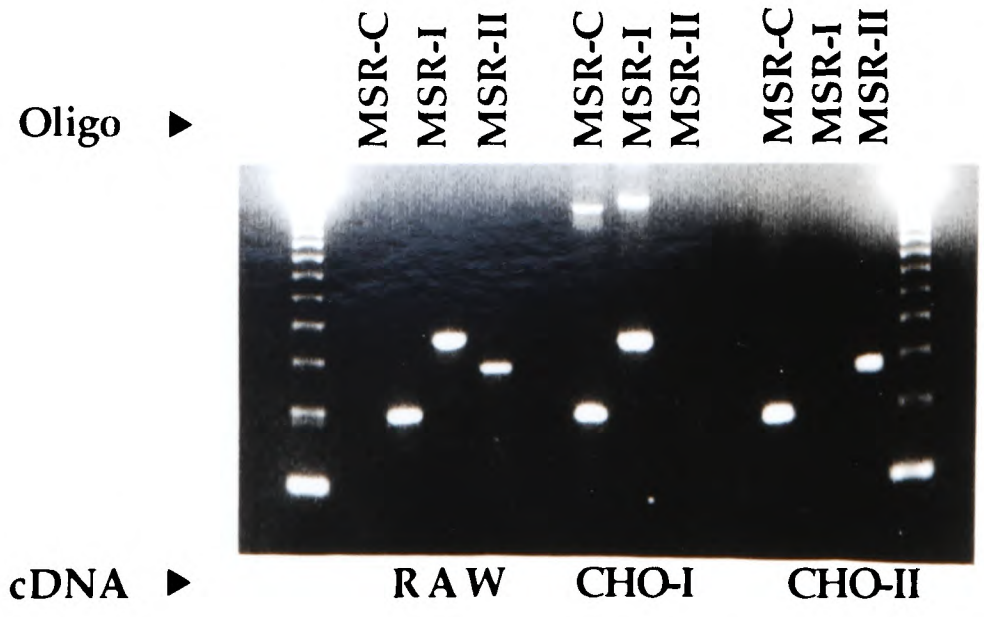
A. <u>Mg_{2+} Titration</u>: 1.5/2.0/2.5 mM $MgCl_2$; annealing temp. = 60°C; cDNA = Con A splenocytes



B. <u>Confirmation of Fragment Sizes;</u> 1.5 mM $MgCl_2$; annealing temp. = 60°C; cDNA = Con A splenocytes



C. <u>Specificity of Primers</u>: 1.5 mM MgCl2; Annealing temp. = 60°C; cDNA = RAW 264.7, CHO MSR I, CHO MSR II



specific for type I receptor (5′ primer: 5′-ACCAACGACCTCAGACTGAA-3′; 3′ primer: 5′-TAGACTCCGGCAGACAACTT-3′; size of amplified fragment: 431 bp); iii) specific for type II receptor (5′ primer: 5′-TGGTGCTCCAGGAATAAGAG-3′; 3′ primer: 5′-GCATGACACAGGAACCAATG-3′; size of amplified fragment: 342 bp) (fig. 2.1) and murine β – actin (5′ primer: 5′-TCAGAAGGACTCCTATGTGG-3′; 3′ primer: 5′-GTACGACCAGAGGCATACAG-3′; size of amplified fragment: 299 bp). PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and an UV transilluminator was used to view and photograph the gels. A 123-bp DNA ladder (BRL/Gibco, UK) was run in parallel to provide molecular weight markers. Specificity of the amplified bands was validated by their predicted size and restriction enzyme digests. Differences in intensity of PCR bands were confirmed by 10 x serial dilutions of cDNA samples to ensure comparability and that the plateau phase of amplification had not been reached. The results shown are representative of 3 independent experiments.

2.12 Cell Adhesion Assay for Mϕ Adhesion to Plastic Surfaces (Rosen and Gordon 1987; Fraser *et al.* 1993)

RAW 264 cells were grown on BP in RPMI plus 10% FCS, harvested with 5mM EDTA in PBS and washed in FCS-containing RPMI. Primary Mϕ were harvested and washed as described above (2.4.1). Cells were then suspended in Hepes-buffered RPMI plus 10% FCS and plated at a density of 1-3 X 10^5 cells/well in 96-well flat-bottomed TCP plates. EDTA or EGTA was added to a final concentration of 5mM where indicated. Potential inhibitors of adhesion (antibodies ; others as indicated) were added and incubated with the cells for 30 minutes at 4°C. Monoclonal Ab were used at a final concentration of 2μg/ml. Cells were then placed at 37°C for a further 90 minutes to allow adhesion to occur, then washed three times with PBS. In some assays, cell viability was assessed at each step by trypan blue exclusion. After observing adherent cells under phase contrast microscopy, cells were fixed in methanol to enable staining and quantitation. Cells were stained in 40% (v/v) Giemsa solution for 60 minutes, washed in tap water, dried, and the retained dye solubilized in methanol. Stain was quantitated by measuring absorbance at 450nm in an automated plate reader (Anthos HTII, Denley Instruments, UK). Definitive assays were performed in tripli- or qaudruplicate.

2.13 Acetylated Low Density Lipoprotein (AcLDL) Uptake Assays

2.13.1 Uptake of AcLDL by adherent cells

Cells to be tested were plated on glass coverslips in RPMI plus 10% FCS as described above (2.7.1). After overnight incubation at 37°C, cells were washed and placed in the same medium containing 10µg/ml AcLDL labelled with DiI (1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindo-carbocyanine perchlorate) (DiIAcLDL ; Biogenesis, UK) (Pitas *et al.* 1981). After three hours incubation at 37°C, cells were washed three times in PBS, mounted on microscope slides and viewed under epifluorescence microscopy using standard rhodamine excitation and emission filters.

2.13.2 Quantitation of AcLDL Uptake by Flow Cytometry

Uptake of DiIAcLDL was measured as described (Geng and Hansson 1992). Cells were plated in 24-well TCP plates at 10^6 cells/well in RPMI plus 10% FCS. After overnight incubation at 37°C, cells were washed and incubated in the same medium with 4µg/ml DiIAcLDL, plus either 10µg/ml mAb, or 400µg/ml unlabelled AcLDL (Biogenesis, UK). After three hours incubation at 37°C, cells were washed four times in PBA, and three times in PBS before harvesting cells by pipetting after incubation in PBS plus 5mM EDTA and 15mM lidocaine. Cells were fixed in 3% (w/v) paraformaldehyde in PBS, and subjected to flow cytometry as above (2.7.3). Emitted fluorescence was collected at 563-607nm (FL2 photomultiplier), and specific fluorescent intensity was calculated by subtracting autofluorescent intensity from fluorescent intensity of DiIAcLDL-labelled cells using geometric means derived from data analysis by Lysys II software.

2.14 Confocal microscopy and electron microscopy

Studies involving these imaging techniques were carried out under the supervision of (confocal microscopy) and in collaboration with (electron microscopy) Mr. M. Hollinshead.

Cells were prepared for confocal laser scanning microscopy (CLSM) by immunofluorescent staining as described (2.7.3). CLSM was performed using an Biorad MRC-1024, mounted on a Nikon Diaphot 200 microscope equipped with a 60:1 planapochromat NA 1.4 objective. A 15mW air-cooled Krypton-Argon ion laser at wavelengths 488nM, 568nM and 647nM was used. After selecting the correct focal plane

under direct scan, images were recorded; each image was derived from Kalman averaging of five scans. Series of 2 plane sections of 0.1 μM thickness were examined. Three-dimensional and vertical reconstructions were computed using COMOS software (Bio-Rad, UK).

For electron microscopy, cells were washed with ice-cold PBS and fixed in 4% PFA in Hepes for 10 min, followed by 8% PFA in Hepes for 50 min. The cells were postfixed in osmiumtetroxide, washed in water and then in 0.5% Mg uranyl acetate overnight at 4°C. The cells were then processed for Epon imbedding, ultra thin sectioning (110nM), and EM on a Zeiss EM 912 Omega Electron Microscope (Tooze and Hollinshead 1991). Cells were also incubated for varying times from 2 to 60 min with HRP sp. act. 1,362 U/mg (Serva Feinbiochemica, Heidelberg, Germany) at a concentration of 10mg/ml in medium at 37°C and 5% CO_2. Immediately after fixation, cells were washed with cacodylate buffer (200mM sodium cacodylate, pH 7.4) and then reacted with diaminobenzidine (DAB). The reaction was stopped by washing with cacodylate buffer, and the samples processed for EM.

For immunocytochemistry, immunogold labeling was with goat anti-rat IgG conjugated to 9nm colloidal gold particles. Postfixation, cells were scraped with a rubber policeman and spun to a pellet. The cell pellet was infiltrated with 2.1M sucrose and frozen in liquid nitrogen. Ultrathin sections were cut and labeled according to Tokuyasu (1980) and Griffiths *et al* (1984).

Chapter Three

M-CSF selectively enhances MSR expression and function

3.1 Introduction

Three colony stimulating factors (CSFs) induce the proliferation and differentiation of haematopoietic progenitor cells to Mϕ. These are M-CSF, which stimulates Mϕ survival, proliferation and differentiation exclusively; GM-CSF which acts on progenitor cells to give rise to both granulocytes and Mϕ; and IL-3, which stimulates primitive multipotent haematopoietic cells primarily, but also generates some Mϕ (Stanley 1994).

M-CSF is a homodimeric glycoprotein with homology to Stem Cell Factor (Witte 1990) and PDGF; and its effects are mediated by a dimeric high affinity cell surface receptor (product of the *c-fms* proto-oncogene) which has intrinsic tyrosine kinase activity (Yeung *et al.* 1987). The M-CSF receptor belongs to a growth factor receptor family (part of the immunoglobulin superfamily) that includes PDGF receptors α and β, and the Stem Cell Factor (*c-kit*) receptor (Sherr *et al.* 1991). The murine M-CSF gene is on chromosome 3 at the *op* locus (Gisselbrecht *et al.* 1989) and at least three different isoforms (N-terminus identical) of M-CSF arise by alternative splicing. Two of these forms are soluble, including a full length 130-160K chondroitin-SO4 proteoglycan (520 amino acids) and an intermediate 80-100K glycoprotein (406 amino acids); the smallest 68-86K form (224 amino acids) is a cell-surface membrane-spanning glycoprotein (Stanley 1994). Only the N-terminal 150 amino acids are required for *in vitro* biological activity (Kawasaki and Ladner 1990). Heterodimers may form between proteoglycan and circulating glycoprotein monomers; the physiological roles of these different forms are unknown and they all bind to a single class of *c-fms* receptor.

M-CSF is synthesized by many different cell types, including Mϕ themselves, following adherence and differentiation (Haskill, 1987; Horiguchi, 1986); the primary source of circulating M-CSF, however, are the ECs lining small blood vessels (Stanley 1994); Clearance of circulating M-CSF occurs mainly by sinusoidal Mϕ in the liver and spleen. In addition, M-CSF is synthesized locally by osteoblasts (Elford *et*

al. 1987) and epithelial cells of the female reproductive tract (Pollard *et al.* 1987). It is important to note (in view of its relevance to atherogenesis) that short-range regulation of Mϕ may occur due to both local synthesis of long-lived cell surface M-CSF and localized sequestration of secreted proteoglycan M-CSF (Price *et al.* 1992). Unlike the other CSFs, M-CSF is continuously present at biologically active concentrations in the serum of both mouse (≈ 12 ng/ml) and man (≈ 4 ng/ml) (Das *et al.* 1981; Hanamura *et al.* 1988) and is required for the survival of circulating monocytes and tissue Mϕ (Becker *et al.* 1987). M-CSF potentiates a number of inducible monocyte functions including phagocytic activity, microbial killing, tumor cytotoxicity, and enhances the synthesis of pro-inflammatory cytokines such as IL-1, TNF-α and IFN-γ (Moore *et al.* 1980; Wing *et al.* 1982; Moore *et al.* 1984; Warren and Ralph 1986).

The central role of the Mϕ in atherosclerosis suggests that M-CSF may be one of several cytokines and growth factors that interact to modulate adherence, infiltration, lipid metabolism, proliferation, and other aspects of monocyte activation during atherogenesis. M-CSF treatment in patients with haematological disease coincides with significant reductions in serum cholesterol levels (Motoyoshi and Takaku 1989). As M-CSF enhances acetylated low-density lipoprotein (AcLDL) uptake and degradation in human monocyte-derived Mϕ (Ishibashi *et al.* 1990), the MSR pathway has been non-specifically implicated in this observed reduction in serum cholesterol. Scavenger receptor activity is however also expressed by endothelial, Kupffer and liver parenchymal cells (Nagelkerke *et al.* 1983; Kume *et al.* 1991) as well as by phorbol ester induced fibroblasts and smooth mucle cells (Pitas 1990); molecules other than the Mϕ-specific MSR may mediate these properties. In terms of modified lipoprotein uptake, other Mϕ receptors such as the thrombospondin receptor (CD36) and the receptor for the Fc region of IgG (FcγRII-B2; CD32) are capable of mediating uptake of oxidized lipoprotein (Stanton *et al.* 1992; Endemann *et al.* 1993). Characterization of MSR regulation and expression has thus far been hampered by the lack of specific murine reagents.

Both human and rabbit atherosclerotic lesions contain immunoreactive M-CSF, as well as increased M-CSF mRNA (Clinton *et al.* 1992; Rosenfeld *et al.* 1992). Vascular endothelial cells and smooth muscle cells produce M-CSF in response to a range of stimuli, including modified low-density lipoproteins (Rajavashisth *et al.* 1990). In addition, a novel function for the MSR as an adhesion receptor was recently described (Fraser *et al.* 1993); this stemmed from the observation that a mAb, 2F8, to the murine MSR inhibited divalent cation-

independent Mϕ adhesion *in vitro*. Using this MSR-specific reagent, the studies detailed below show unequivocally that recombinant human M-CSF markedly and selectively enhances MSR expression and function in murine elicited peritoneal Mϕ.

3.2 The effects of M-CSF on MSR expression

To investigate the effects of M-CSF on MSR expression and function, primary murine Mϕ were treated with recombinant human M-CSF. Human M-CSF binds to the mouse M-CSF receptor but mouse M-CSF exhibits no or low binding to the human M-CSF receptor (Ralph *et al.* 1986). BgPMϕ were cultured in RPMI 1640 medium plus 10% fetal calf serum (FCS) as described. In an immunoblot analysis, the addition of M-CSF for 48 hours markedly upregulated the expression of MSR protein (fig. 3.1A and B). Similar results were obtained when BgPMϕ were cultured in OptiMEM (a serum-free proprietary medium) thereby excluding active soluble factors in FCS. Recombinant murine GM-CSF, which decreases serum cholesterol to a lesser extent when infused as an adjunct to chemotherapy (Nimer *et al.* 1988), caused a noticeable, but less marked increase (Fig. 3.1B). To exclude non-specific effects due to growth factor-induced proliferation, loading of lysates per well was corrected for total cellular protein concentration; in addition, it has previously been shown that M-CSF treatment of human monocyte-derived Mϕ increases cell size rather than cell number (Ishibashi *et al.* 1990). The observed increase in MSR protein would therefore be even more significant at a single cell level. Conditioned media from mouse L cells (a rich source of murine M-CSF (Stanley and Heard 1977)) or insect cells transfected with murine M-CSF cDNA similarly increased MSR expression and this effect was abrogated by the addition of species-specific antibodies to M-CSF (fig. 3.2). In some experiments, two MSR bands were present after immunoblotting with mAb 2F8; this may be due to the mAb at high concentrations (> 25μg/ml) binding to MSR dimer and trimer precursor. The increase in MSR expression was dose-dependent and plateaued at concentrations of 500 and 1000 units/ml M-CSF (fig. 3.3).

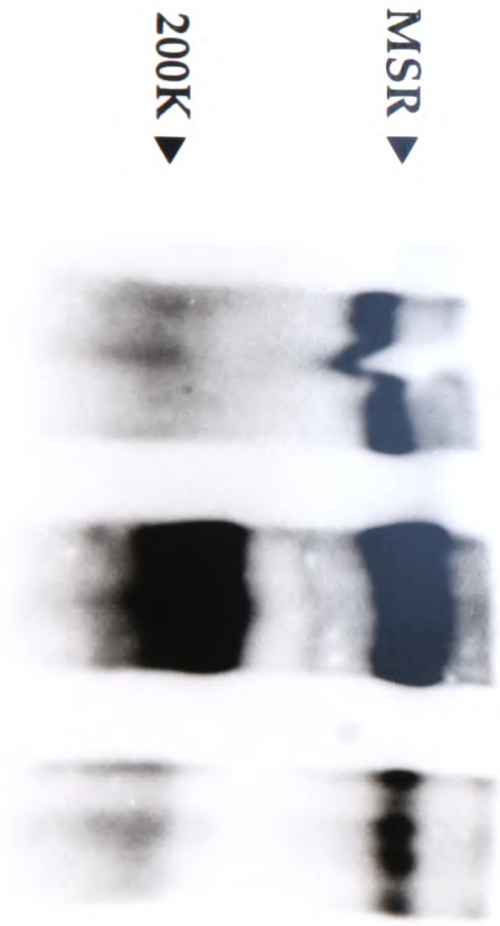
Immunoprecipitation of MSR from metabolically labelled cells confirmed the upregulation by M-CSF (fig. 3.4). The MSR is seen in its monomeric form as precipitates were subjected to SDS-PAGE under reducing conditions. The mAb 2F8 detects both type I and II isoforms of the MSR (Fraser *et al.* 1993). M-CSF increased MSR protein synthesis 2.3-fold (using PhosphorImager quantification) even after 48 hours of treatment, as is evident from the 0 hour chase point following a 30 minute labelling period with Tran^{35}S-methionine. The more

Fig. 3.1 (top) **M-CSF increases MSR protein expression**

For immunoblot analysis with mAb 2F8, BgPMϕ were treated for 48 hours with human M-CSF (1000units/ml) and murine GM-CSF (1000units/ml). Equal amounts of total cellular protein (20 μg) were subjected to SDS-PAGE (under non-reducing conditions) and transferred to nitrocellulose. Blots were probed with mAb 2F8, with antibody binding detected by peroxidase-conjugated second antibody and chemiluminescence. Results (**A** & **B**) are from two independent experiments. Arrowheads indicate Mr 200 kDa marker.

Fig. 3.2 (bottom)) **Murine M-CSF increases MSR protein expression specifically**

For immunoblot analysis with mAb 2F8, BgPMϕ were treated for 48 hours with murine M-CSF (culture supernatant from an insect cell transfectant at ≈ 1000units/ml) and mAb to murine M-CSF (10 μg/ml). Equal amounts of total cellular protein (20 μg) were subjected to SDS-PAGE (under non-reducing conditions) and transferred to nitrocellulose. Blots were probed with mAb 2F8, with antibody binding detected by peroxidase-conjugated second antibody and chemiluminescence. The result shown is representative of two independent experiments. Arrowheads indicate Mr 200 kDa marker.



CONTROL

murine M-CSF

α-murine M-CSF

B.

Control

M-CSF

GM-CSF

200

A.

Control

M-CSF

200

Fig. 3.3 (top) **M-CSF increases MSR protein expression in a dose-dependent manner**

For immunoblot analysis with mAb 2F8, BgPMϕ were treated for 48 hours with various concentrations of human M-CSF. Equal amounts of total cellular protein (20 μg) were subjected to SDS-PAGE (under non-reducing conditions) and transferred to nitrocellulose. Blots were probed with mAb 2F8, with antibody binding detected by peroxidase-conjugated second antibody and chemiluminescence. The result is representative of two independent experiments. Arrowheads indicate Mr 200 kDa marker.

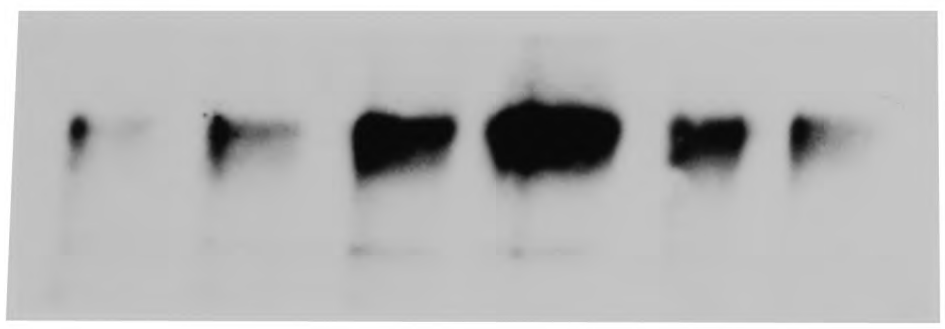
Fig. 3.4 (bottom) **M-CSF increases MSR protein synthesis**

BgPMϕ were treated *in vitro* for 48 hours with human M-CSF (1000 units/ml), metabolically labeled with Tran^{35}S-label™ for 30 minutes and chased for the indicated periods. Precipitates were adjusted to represent equivalent total protein concentrations, and separated by SDS-PAGE in the presence of β-mercaptoethanol. MSR precursor and monomer migrate at Mr ≈ 64 and ≈ 90K respectively. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Molecular weight standards in kDa are indicated by arrowheads.

M-CSF units/ml

0	10	10^2	$5x10^2$	10^3	10^4

MSR ▶



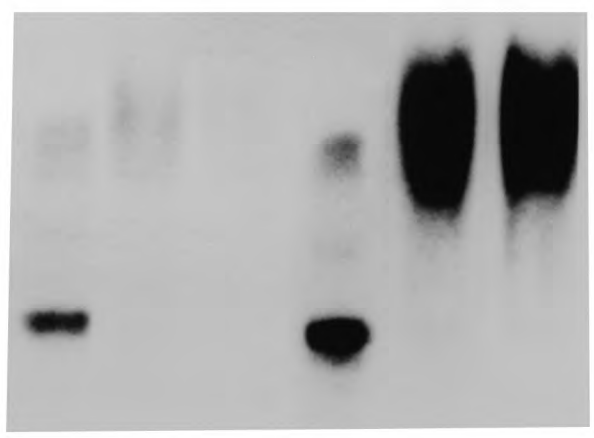
Treatment	Control			M-CSF		
Chase (hours)	0	12	36	0	12	36

200►

97.4►

69►

46►



impressive increases seen at 12 (4.5-fold) and 36 hours (6-fold) signify that, in addition to increasing MSR synthesis, M-CSF may also markedly prolonged the half-life of synthesized receptor. This was however not formally proven as the chase period was not continued for long enough to determine relative half-lives.

Expression of MSR at a single cell level as determined by flow cytometry (table 3.1) confirmed that M-CSF upregulated Mϕ cell surface expression of the receptor.

Table 3.1. M-CSF selectively increases MSR surface expression.

Treatment	*Surface*	*Internal*
Control	100±3	100±4
M-CSF	232±14*	118±8
GM-CSF	81±4	107±5

Effect of M-CSF (1000u/ml) and GM-CSF (1000u/ml) on expression of MSR by flow cytometry. BgPMϕ were treated *in vitro* for 48 hours as indicated and stained with either mAb 2F8 or CAMPATH-1G plus FITC-conjugated second antibody prior to analysis by FACScan® (Becton Dickinson & Co.) flow cytometry with (internal) or without (surface) saponin permeabilization. Results are depicted as percentage of control values and are the means ± s.d. of 3 experiments (each experiment in triplicate) in which specific fluorescence intensities were determined by subtracting geometric mean fluorescence obtained with CAMPATH-1G from that obtained with mAb 2F8. * $P < 0.01$ vs. Control.

The greater than 2-fold increase in MSR surface expression was not paralleled by the more modest 18% increase seen when intracellular (internal) levels of antigen were assessed by permeabilizing the cells with saponin prior to fixation. It was therefore possible that M-CSF may have redistributed the substantial intracellular pool of MSR to the cell surface. Indirect immunofluorescence studies with the mAb 2F8, although difficult to quantify, supported this hypothesis (fig. 3.5). Interestingly, GM-CSF had little effect on surface or internal MSR expression as assessed by flow cytometry which contrasts with the increase seen by immunoblotting. This inconsistency may be due to technical reasons as Tran^{35}S-methionine metabolic labeling to determine changes in MSR protein synthesis rate also showed no effect with GM-CSF.

The possible redistributive effect of M-CSF was investigated by biochemically quantifying the fraction of total MSR on the cell surface at steady state (table 3.2 and fig. 3.6).

Fig. 3.5 **MSR expression on M-CSF treated BgPMϕ as determined by immunofluorescence microscopy**

BgPMϕ were grown on 13 mm diameter acid-washed coverslips in 24 well plates in serum-containing medium and treated *in vitro* with recombinant human M-CSF (1000units/ml) for 48 hours. After washing with PBS, cells were fixed in PBS containing 3% (w/v) paraformaldehyde. The cells were permeabilized, where indicated, for visualization of intracellular MSR, by the addition of 0.1% (w/v) saponin to PBA in all antibody incubations and washes. Staining was with mAb 2F8 and FITC conjugated second antibody. Cells were photographed under epifluorescence microscopy oil immersion (objective lens = 100 x) with standard FITC excitation and emission filters. All exposures were of equivalent duration.

A. Untreated cells.

B. M-CSF treated cells.

C. Untreated cells, permeabilized.

D. M-CSF treated cells, permeabilized.

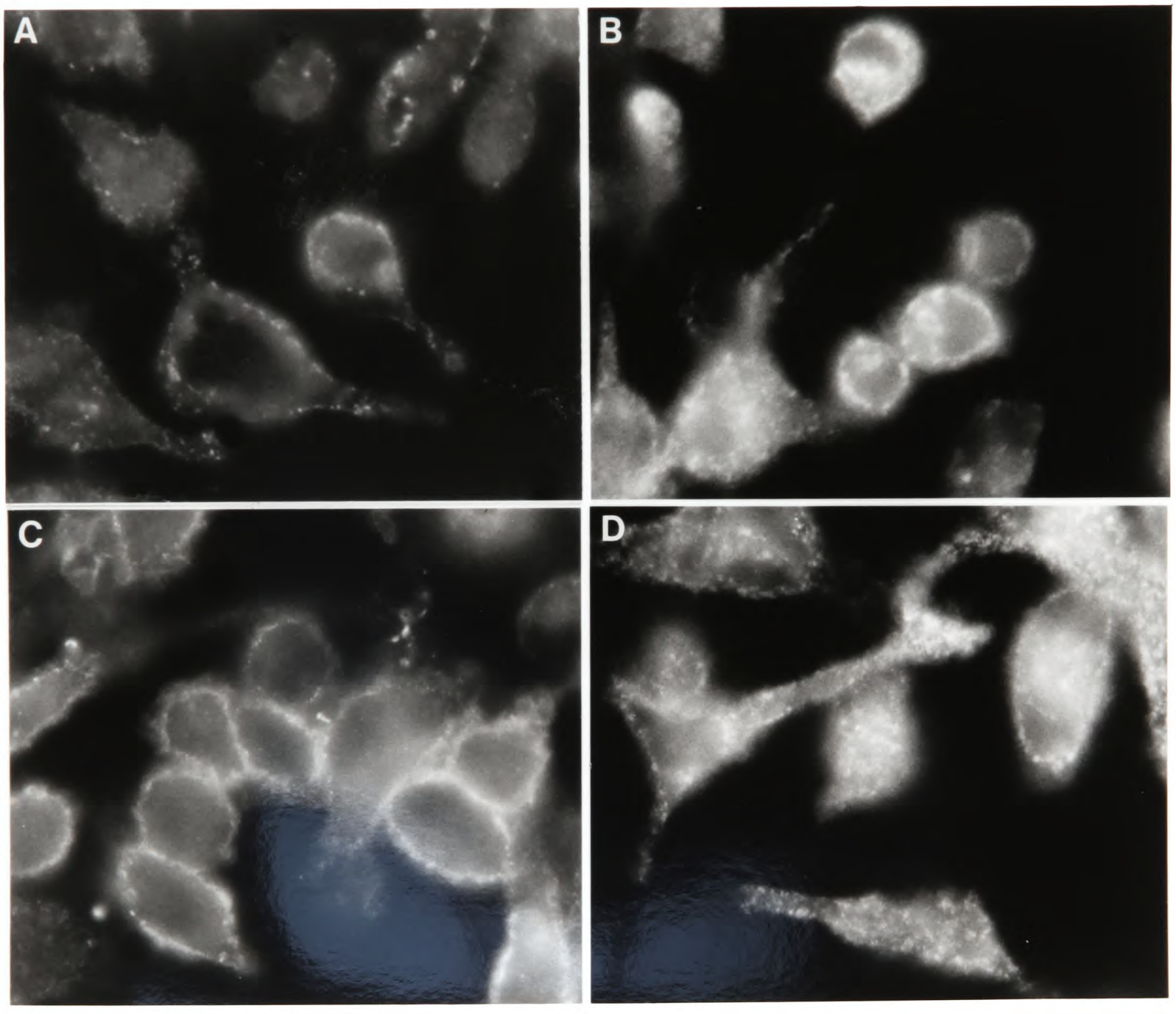

A
B
C
D

Table 3.2 Effect of M-CSF on cell surface distribution of MSR.

Treatment	*Total*	*Surface*
Control	23100±850	7100±650
% surface:total	31±3%	
M-CSF	115400±4500	94900±3600
% surface:total	82±4%	

BgPMϕ were metabolically labelled and surface biotinylated using sulfo-NHS-SS-biotin as described below. Quantification of protein bands on dried gels was done by PhosphorImager analysis and results expressed as a ratio of arbitrary PhosphorImager units (APUs). The values are the means ± s.d. from two independent experimants.

Cells were metabolically labelled to equilibrium followed by surface biotinylation, on ice, by the membrane-impermeant probe sulfo-NHS-SS-biotin (Harter and Mellman 1992). After cell lysis, total MSR was precipitated by mAb 2F8 and the resulting immunoprecipitate divided into two equal aliquots. Biotinylated "surface" MSR was precipitated from one aliquot using streptavidin-agarose and "total" MSR was precipitated from the other aliquot by a second round of mAb 2F8 immunoprecipitation. Total and biotinylated MSR were quantitated after SDS-PAGE by PhosphorImager analysis of dried gels. In these experiments M-CSF increased surface MSR expression 13-fold and total MSR protein synthesis nearly 5-fold. In addition, M-CSF treatment was associated with a shift of the cellular pool of MSR from a mainly intracellular location (69% of total) to a predominantly surface distribution (82% of total).

To exclude the possibility that the M-CSF effect on MSR expression represented a non-selective epiphenomenon of Mϕ differentiation, the action of M-CSF on several other Mϕ integral membrane proteins was analyzed (table 3.3).

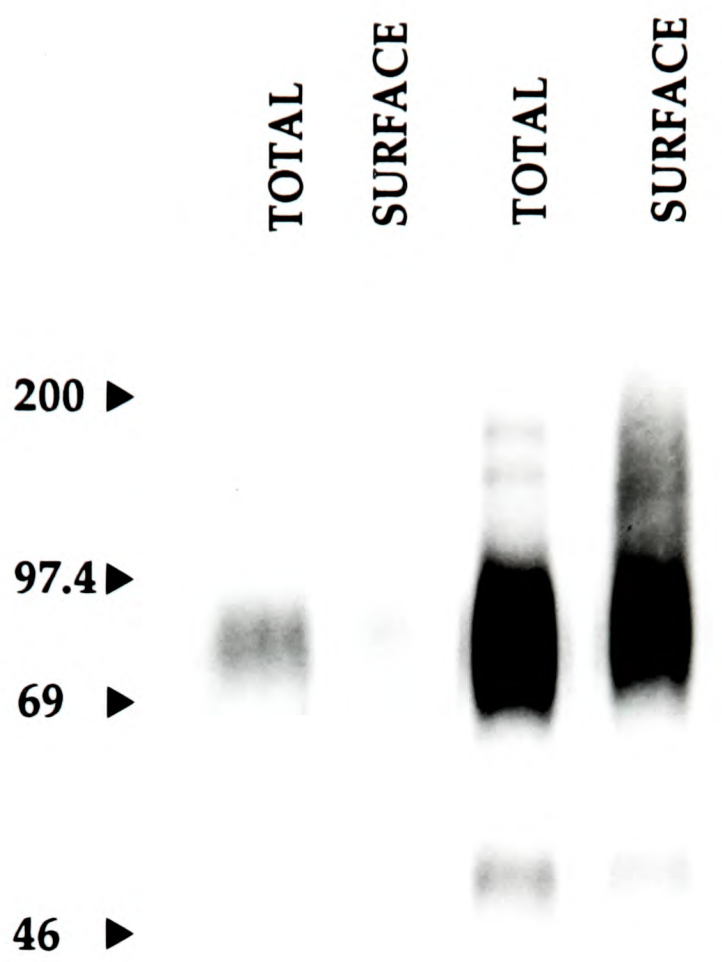
The expression of surface and internal pools of F4/80 (a Mϕ-specific differentiation antigen) (Austyn and Gordon 1981), type 3 complement receptor, MHC class II antigen and macrosialin (a Mϕ-specific late endosomal membrane molecule) (Rabinowitz and Gordon 1991) were analyzed by flow cytometry. M-CSF treatment elicited slight increases (statistically not significant) in surface CR3 (15%) and internal F4/80 (6%) levels only. The other antigens were either unchanged or decreased. Immunoblots of M-CSF treated BgPMϕ also showed no alteration in expression of macrophage mannose receptor (MMR) or class II MHC (not shown). These results point to a selective effect of M-CSF on MSR expression in an elicited primary Mϕ population.

Fig. 3.6 (top) **M-CSF enhances MSR protein surface expression**

BgPMϕ were treated *in vitro* for 48 hours with human M-CSF (1000 units/ml), and metabolically labelled with Tran^{35}S-label™ overnight. This was followed by surface biotinylation, on ice, by the membrane-impermeant probe sulfo-NHS-SS-biotin. After cell lysis, total MSR was precipitated by mAb 2F8 and the resulting immunoprecipitate divided into two equal aliquots. Biotinylated "surface" MSR was precipitated from one aliquot using streptavidin-agarose and "total" MSR was precipitated from the other aliquot by a second round of mAb 2F8 immunoprecipitation. Precipitates were adjusted to represent equivalent total protein concentrations, and separated by SDS-PAGE in the presence of β-mercaptoethanol. MSR precursor and monomer migrate at Mr ≈ 64 and ≈ 90K respectively. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Molecular weight standards in kDa are indicated by arrowheads. Total and biotinylated MSR were quantitated after SDS-PAGE by PhosphorImager analysis of dried gels and the values are from two independent experiments.

Fig. 3.7 (bottom) **M-CSF increases both type I and II MSR mRNA**

BgPMϕ were incubated with M-CSF for 24 and 48 hours. RT-PCR was done as described in Materials and Methods. Differences in intensity of PCR bands were confirmed by further 10-fold serial dilutions of cDNA samples to ensure comparability and that the plateau phase of amplification had not been reached. MSR-C represents transcripts common to both type I and II MSR. The results shown are representative of 3 independent experiments.


CONTROL M-CSF
TOTAL
SURFACE
TOTAL
SURFACE
200
97.4
69
46

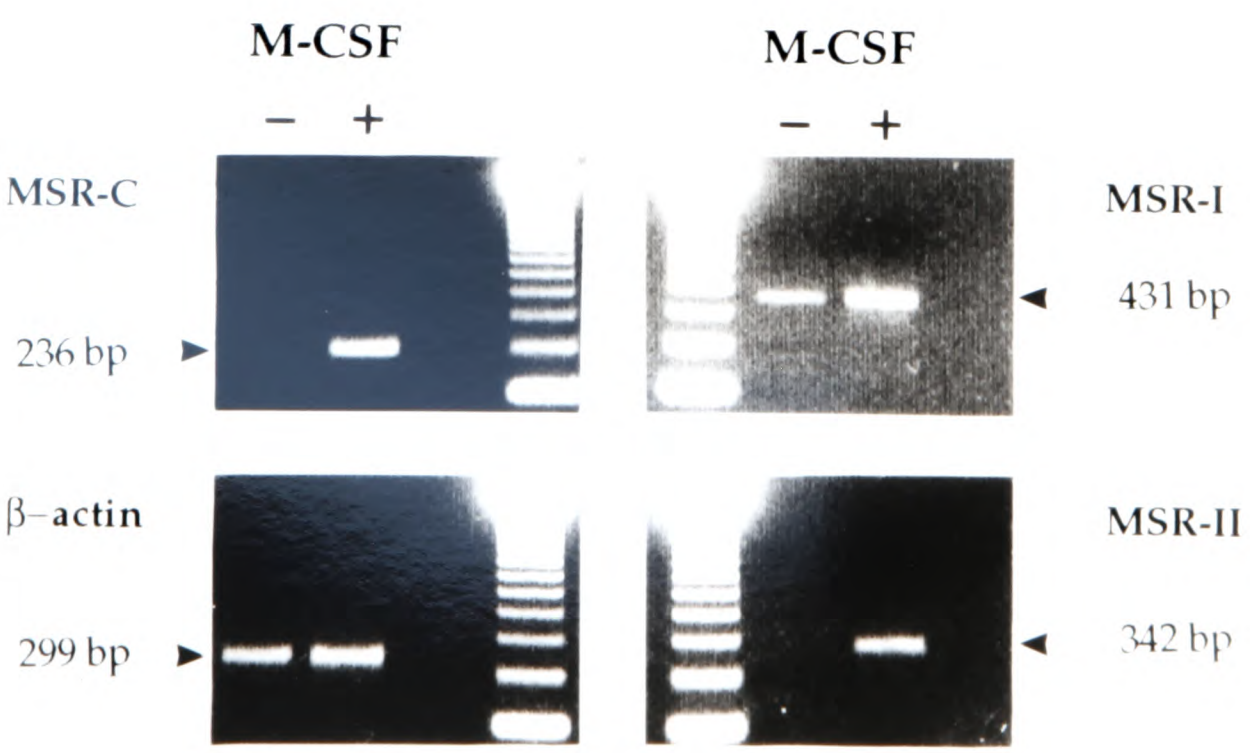

M-CSF
M-CSF
– +
– +
MSR-C
236 bp
MSR-I
431 bp
β–actin
299 bp
MSR-II
342 bp

Table 3.3 Effect of M-CSF on expression of Mϕ differentiation antigens by flow cytometry.

	Surface		*Internal*	
Treatment	Control	M-CSF	Control	M-CSF
MSR	60±5	117±8	177±9	208±11
F4/80 antigen	221±14	212±16	325±21	343±23
CR3	281±17	324±13	509±27	416±25
class II MHC	43±5	36±5	44±5	41±5
macrosialin	39±3	26±4	257±16	182±14

BgPMϕ were stained with the indicated mAb or its isotype control plus FITC-conjugated second antibody prior to analysis by flow cytometry. The specific monoclonal antibodies used were F4/80, 5C6 (CR3), TIB120 (class II MHC) and FA-11 (macrosialin). Results show the mean values (single experiment in triplicate) of specific fluorescence intensities determined by subtracting geometric mean fluorescence obtained with the isotype control from that obtained with the specific monoclonal antibody.

Two isoforms of MSR arise by alternative splicing of transcripts derived from a single gene (Emi *et al.* 1993; Freeman *et al* 1991). Both isoforms have been detected in atherosclerotic lesions and bind MSR ligands with equal affinities (Rohrer *et al* 1990; Naito *et al.* 1992; Emi *et al.* 1993). The scavenger receptor cysteine-rich (SRCR) domain forms the C-terminal of the type I receptor and confers MSR type specificity as the type II receptor contains a truncated C-terminal domain of six residues. A definitive role for the SRCR domain has not been demonstrated, but on the basis of its cross-species conservation, it is postulated to be a primitive ligand binding/recognition domain. Recently it has been shown to influence the binding of ligands to the collagenous domain of MSR subtly (Ashkenas *et al.* 1993). To investigate the point at which M-CSF mediates its upregulatory effect, we used a semi-quantitative RT-PCR method to assay MSR mRNA changes in response to M-CSF (fig. 3.7). This assay provides a sensitive measure of mRNA in Mϕ and control serial dilution experiments confirmed that for each set of primers the level of detection could be related to the initial cellular input used to prepare the cDNA. Following M-CSF treatment for 48 hours, there was a substantial increase in MSR transcripts (MSR-C) in general. In addition, levels of mRNA specific for both type I and II MSR mRNA were increased, excluding a selective effect for M-CSF on either

transcript. Although type I MSR transcript was present in untreated cells, type II MSR is more prevalent *in vivo* and *in vitro* (Ashkenas *et al.* 1993) and MSR-C transcripts were absent. Detection of type I message in the control cells may thus have to be explained by increased annealing affinity of the specific set of primers for the cDNA template. Similar results were obtained after 24 hours of M-CSF incubation (not shown). M-CSF therefore also upregulates MSR expression at a transcriptional level although post-transcriptional stabilization of MSR mRNA could not be excluded.

3.3 The effects of M-CSF on MSR function

As described, M-CSF may increase MSR expression at several different levels: message, protein synthesis, protein stability and shift of mature protein to the cell surface. The significance of these observed differences was tested by relating them to functional MSR studies. Traditionally, the MSR has been characterized as an endocytic receptor (Goldstein *et al.* 1979) with a broad ligand-binding specificity for a range of polyanions (including modified lipoproteins). The ability of treated BgPMϕ to endocytose and accumulate fluorescent AcLDL (DiI-AcLDL) was therefore tested (table 3.4).

Table 3.4 M-CSF enhances DiI-AcLDL uptake by BgPMϕ.

Treatment	*Expt . 1*	*Expt . 2*	*Expt . 3*
Control	314 ± 15	239 ± 14	166 ± 17
M-CSF	541 ± 17*	496 ± 34*	492 ± 32*
% increase	172%	208%	297%

M-CSF increases the uptake of DiI-AcLDL by BgPMϕ. Uptake of DiIAcLDL was measured as described. BgPMϕ were treated for 48 hours with human M-CSF and then exposed to medium containing 10μg/ml AcLDL labelled with DiI (1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindo-carbocyanine perchlorate) for 3 hours. Cells were fixed and subjected to FACScan® (Becton Dickinson & Co.) flow cytometry. Emitted fluorescence was collected at 563-607nm (FL2 photomultiplier), and specific fluorescent intensity was calculated by subtracting autofluorescent intensity from fluorescent intensity of DiIAcLDL-labelled cells using geometric means derived from data analysis by Lysys II software. Results show the mean values ± standard deviation (three independent experiments in triplicate). *$P < 0.001$ vs. Control.

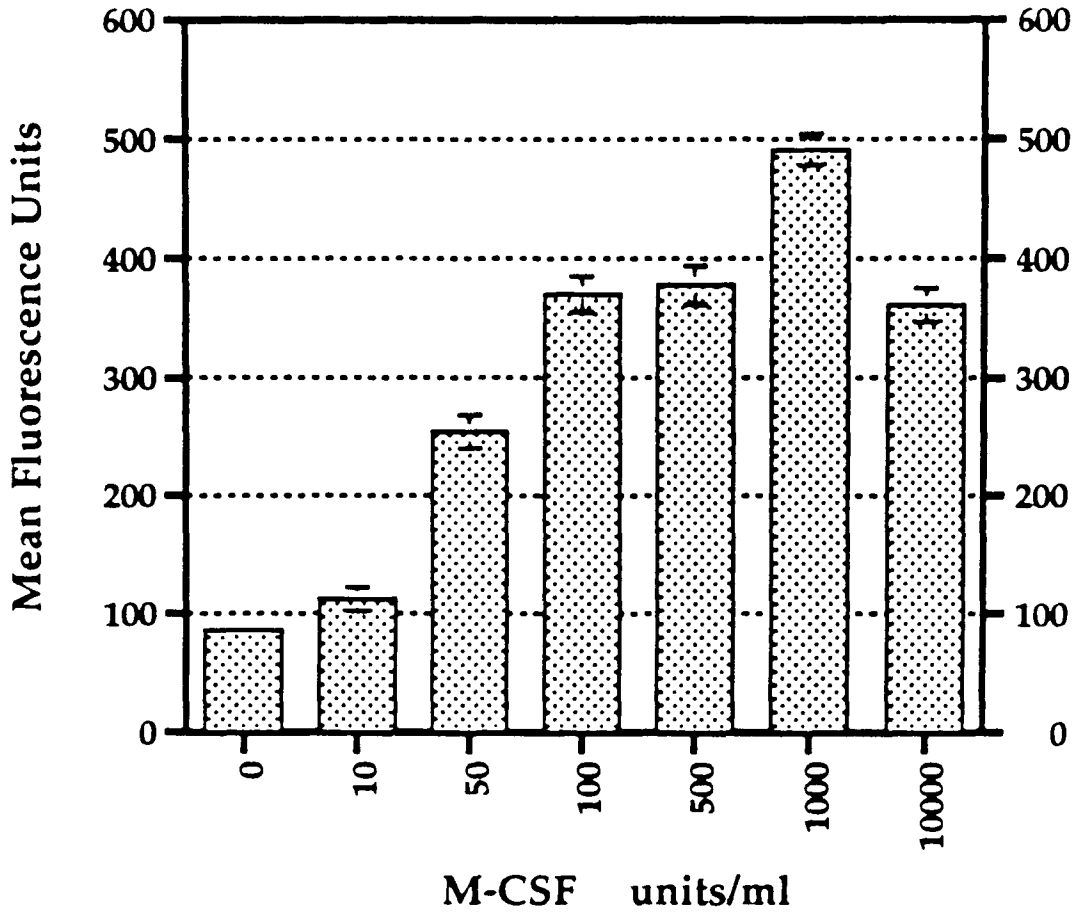
Fig. 3.8 (top) **M-CSF increases AcLDL uptake by BgPMϕ in a dose-dependent manner.**

BgPMϕ were treated for 48 hours with various concentrations of human M-CSF and then exposed to medium containing 10mg/ml AcLDL labelled with DiI (1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate). Cells were fixed and subjected to FACScan® (Becton Dickinson & Co.) flow cytometry. Emitted fluorescence was collected at 563-607nm (FL2 photomultiplier), and specific fluorescent intensity was calculated by subtracting autofluorescent intensity from fluorescent intensity of DiIAcLDL-labelled cells using geometric means derived from data analysis by Lysys II software. Results show the mean values ± standard deviation (two independent experiments in triplicate).

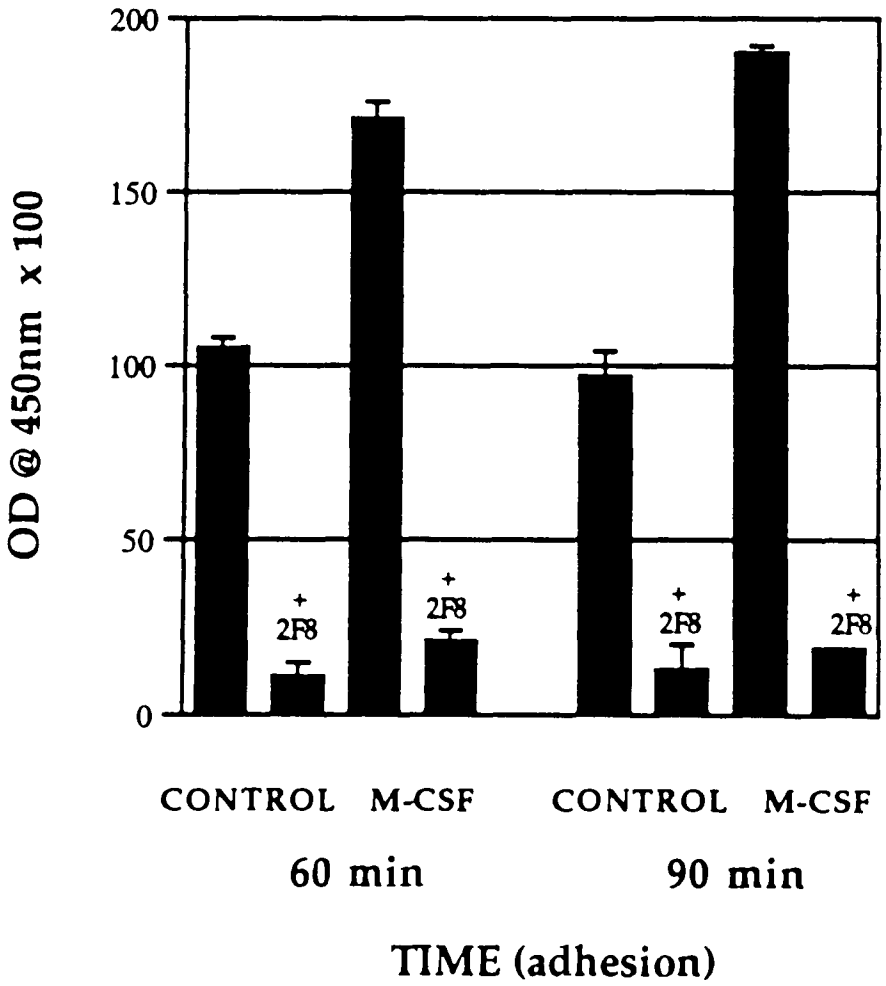
Fig. 3.9 (bottom) **M-CSF increases EDTA-resistant adhesion of Mϕ to tissue culture plastic**

BgPMϕ were cultured in RPMI plus 10% FCS on tissue culture plastic (TCP) surfaces in the presence or absence of M-CSF for 48 hours. Cell adhesion assay was performed as described. 2F8 monoclonal Ab was added at a final concentration of 2mg/ml. Results are derived from two experiments done in quadruplicate.

DiI-AcLDL UPTAKE AND M-CSF DOSE RESPONSE



M-CSF INCREASES EDTA-RESISTANT ADHESION OF BGPMΦ TO TCP



The M-CSF-mediated upregulation of murine MSR was associated with an enhanced capacity (1.7 to 3-fold) of treated Mϕ to endocytose AcLDL. AcLDL uptake has been regarded as a marker for MSR function; it has recently become evident that the thrombospondin receptor (CD36) on Mϕ may also mediate uptake of acetylated and/or oxidized lipoprotein (Acton *et al.* 1994; Nozaki *et al* 1995). This increase in MSR endocytic function was dose-dependent and plateaued at 1000 units/ml (fig. 3.8); significant effects were however evident at final concentrations of 50 to 500 units/ml M-CSF. This corresponds with the dose response seen when MSR expression was determined by immunoblotting (fig. 3.3).

A recently described novel function for the MSR is divalent cation-resistant (integrin-independent) adhesion (Fraser *et al.* 1993). The ability of M-CSF-treated BgPMϕ to adhere to serum-treated tissue culture plastic in an EDTA-independent manner was investigated (fig. 3.9). M-CSF increased adhesion at 60 min by 63% and at 90 min by 96%; this effect was completely abolished in the presence of mAb 2F8. The adherence of M-CSF-treated Mϕ *in vitro* was therefore markedly increased in a MSR-dependent (divalent cation-independent, 2F8-inhibitable) fashion. It is noteworthy that the extent of the increase seen in MSR surface expression following M-CSF treatment (13-fold) (Fig. 3.5 and Table 3.2) does not correlate with that detected by the functional assays - AcLDL uptake (3-fold increase) and MSR-dependent adhesion (2-fold increase). These inconsistencies may therefore reflect a relative lack of sensitivity on the part of the assays.

3.4 Effects of M-CSF on cell lines and human monocytes

The Mϕ-like cell line RAW 264.7 expresses functional MSR (predominantly the type II isoform) abundantly (Fraser *et al.* 1993). M-CSF treatment (1000 units/ml for 48 hours) had no further upregulatory effect on MSR expression (assessed by immunoblotting) or function (assessed by AcLDL uptake and MSR-dependent adhesion) (results not shown). This would suggest MSR to be constitutively upregulated in these cells; and enabled factors inhibiting MSR regulation to be investigated further (Chapter 5.2.3 and 5.2.4).

There are no mAbs to the human MSR currently available (polyclonal antisera to human types I and II MSR have however been described (Geng and Hansson1994)); attempts to extend the above findings to human cells were therefore limited to assays of scavenger receptor activity. This is known to be upregulated when human THP-1

monocytes, a non-adherent monocytic leukaemia cell line, differentiate to adherent Mϕ-like cells as a result of phorbol ester (200 nM PMA) treatment (Tsuchiya *et al.* 1980; Fogelman *et al.* 1981; Hara *et al.* 1987). Characterization of inducible THP-1 adhesion to TCP did not show any EDTA-resistant component; this contrasts with murine RAW 264.7 cells, and would suggest a lack of involvement by the MSR in inducible adhesion to TCP by THP-1 cells.

M-CSF was previously shown to enhance markedly the uptake and degradation of AcLDL (7.5-fold) and cholesterol esterification (24.1-fold) in human monocyte-derived Mϕ (Ishibashi *et al.* 1990). This increase in AcLDL uptake was confirmed in two experiments when treatment of adherent human monocytes with M-CSF (1000 units/ml for 48 hours) resulted in a moderate 1.6 to 1.8-fold increase in DiI-AcLDL uptake as assessed at single cell level by flow cytometry (results not shown). The published report contains important differences in experimental protocol including culture for 10 days, dose of M-CSF (25000units/ml), and incubation with AcLDL for a further 24 hours. There is also mention of considerable variability in M-CSF responsiveness among Mϕ obtained from different donors. In a TCP adhesion assay, no apparent difference was found between M-CSF-treated and control cells as far as EDTA-sensitive adhesion is concerned; and similar to THP-1 cells, human monocyte adhesion to TCP lacked an EDTA-resistant component (results not shown). The lack of any discernible EDTA-resistant component to adhesion persisted in further human monocyte experiments even when the cells were cultured for 7 days, followed by 200 nM PMA treatment for the final 24 hours, and the assessment of TCP adhesion after both 90 minute and 16 hour incubation periods. This experimental evidence casts doubt on a role for MSR in human monocyte adhesion and may imply a species-specific effect on murine Mϕ adhesion.

3.5 Discussion

In summary, the effect of M-CSF on the MSR pathway was studied using a recently developed mAb to murine MSR. M-CSF markedly and selectively increased MSR synthesis in murine Mϕ; post-translationally the receptor shifted to a predominantly surface distribution and its stability may also have been enhanced. Functionally M-CSF enhanced modified lipoprotein uptake and increased divalent cation-independent adhesion *in vitro*. It is hypothesized that regulation of MSR activity plays an important role in determining the extent of atherogenesis. These results suggest a plausible mechanism

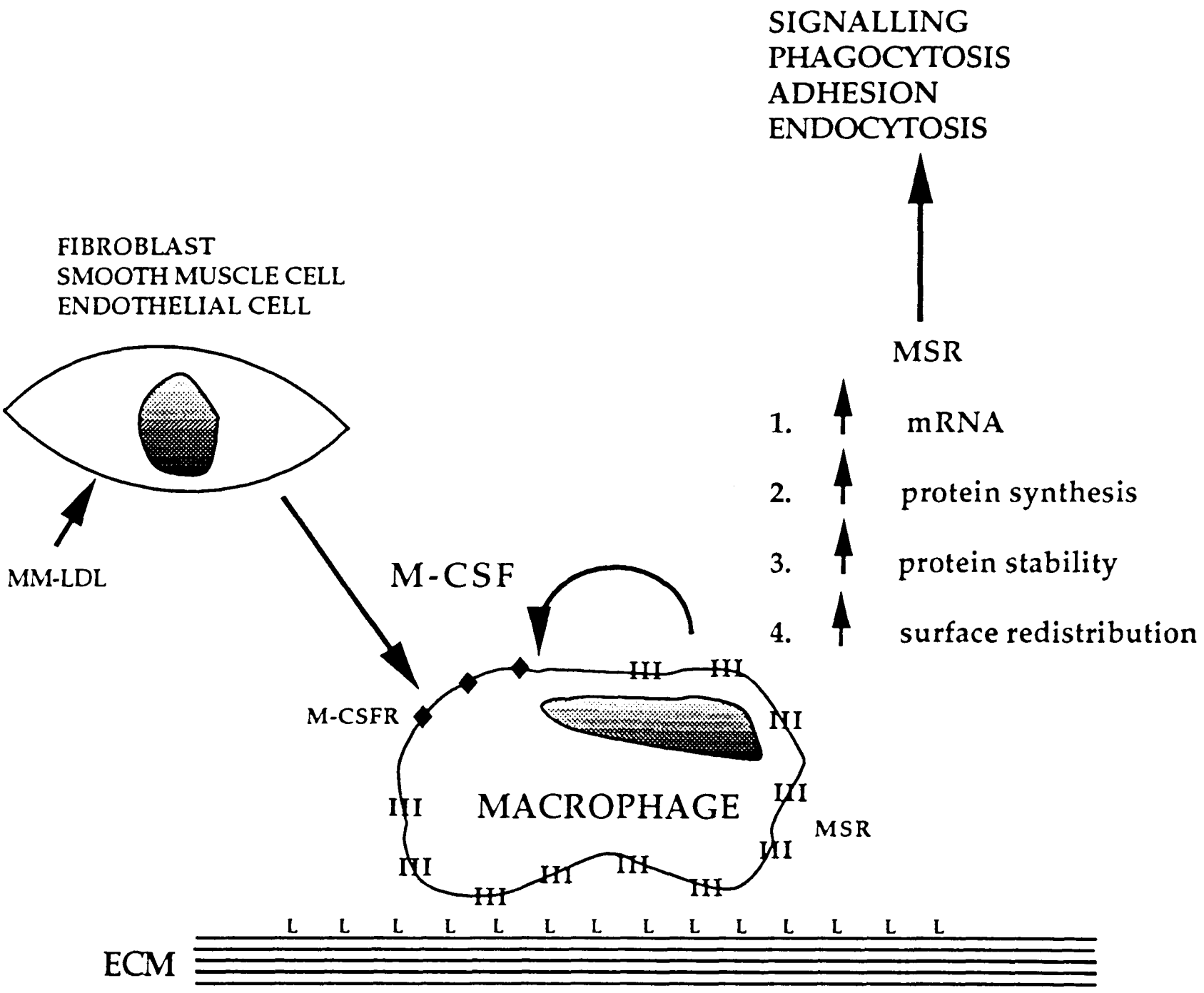


Fig. 3.10 **Diagram depicting the effects of M-CSF secretion by the different cell types in the atheromatous plaque on the Mϕ MSR pathway.**

MSR = III; M-CSFR = ◆; MM-LDL = minimally-modified LDL; L = MSR ligand(s); ECM = extracellular matrix.

whereby M-CSF production in the atheromatous plaque micro-environment could promote the recruitment and retention of mononuclear phagocytes and subsequent foam cell formation (fig. 3.10).

Several studies have demonstrated that M-CSF induces significant decreases in plasma cholesterol in normal rabbits, hypercholesterolemic LDL-receptor-defective Watanabe heritable hyperlipidemic (WHHL) rabbits, and non-human primates (Stoudemire and Garnick 1991; Schaub *et al.* 1994). Preliminary studies have also shown cholesterol decreases in both normo- and hypercholesterolemic humans (Motoyoshi and Takaku 1989; Sherman *et al.* 1995). A dose-dependent decrease in total cholesterol, almost entirely attributable to decreases in LDL-cholesterol, was similarly seen after M-CSF treatment of patients with homozygous familial hypercholesterolemia (FH) (Sherman *et al.* 1995). M-CSF treatment has also been associated with decreased foam cell development in Mϕ-rich carrageenan-induced granulomas in WHHL rabbits and with decreased progression and enhanced regression of atherosclerotic lesions in WHHL and cholesterol-fed New Zealand White (NZW) rabbits (Inoue *et al.* 1992; Schaub *et al.* 1994; Schaub *et al.* 1995).

Although the exact mechanism associated with the cholesterol-lowering effects of M-CSF is unknown, certain insights are available. Firstly, M-CSF enhances biliary excretion of cholesterol (Schaub *et al.* 1995) and much of the decrease is probably due to excretion by the liver; data presented in this chapter also suggest that MSR activity on Kupffer cells may be markedly upregulated which could contribute to increased modified lipoprotein uptake. Secondly, the fact that the decrease in cholesterol occurs in LDL-receptor-deficient animals and humans suggests a mechanism unrelated to this receptor. Hepatic LDL receptor function, when assayed *in vitro* by LDL binding, was also not markedly affected by M-CSF treatment (Stopeck *et al.* 1993). Thirdly, M-CSF-induced stimulation of reverse cholesterol transport is suggested both by an increase in biliary excretion of cholic acids and increases in HDL-cholesterol particle size (Parker *et al.* 1990). This is supported by the finding that M-CSF increases apolipoprotein E (apoE) expression in human monocytes (mRNA) (Clinton *et al.* 1992) and WHHL rabbit atherosclerotic lesions (protein) (Schaub *et al.* 1995). This apolipoprotein is an important mediator in reverse cholesterol transport. Fourthly, additional receptors important in cholesterol clearance may be implicated, and M-CSF was reported to increase expression of the apoE-binding protein, LDL-receptor-related protein (LRP), in Mϕ (Hussaini *et al.* 1990; Watanabe *et al.* 1994).

It is most likely that Mϕ are the target for M-CSF in developing

atheromatous lesions since *c-fms*, the M-CSF receptor, is absent from cultured EC or VSMCs (Clinton *et al.* 1992). Besides acting as a growth factor for the proliferation and differentiation of monocytic progenitors, M-CSF is also required for the survival and activation of mature monocytes and Mϕ (Hume *et al.* 1988). This maintenance role has led to speculation that M-CSF may be important for the long-term survival of Mϕ within lesions and that deficiency of local M-CSF production may lead to foam cell necrosis in advanced lesions (Rosenfeld *et al.* 1992). The effects of endogenously produced M-CSF by ECs, VSMCs and Mϕ within the vessel wall will, in the final analysis, depend on the specific forms of M-CSF expressed as well as the balance and coordination of the broader cytokine network.

Another consequence of M-CSF action in the arterial wall may be the activation of MCP-1 expression as reported *in vitro* in human umbilical vein endothelial cell (HUVEC); this was associated with an increase in the adhesive force between these cells and monocytes (Shyy *et al.* 1993). In this report the M-CSF enhanced adhesion was blocked by an antibody specific to MCP-1, and it appeared to act primarily on endothelium and not monocytes. The role of MCP-1 in atherosclerosis and its effects on MSR expression and function are discussed in Chapter 5.

In addition, M-CSF increased the synthesis and surface expression of two β_1 integrins, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ in BMDMϕ, resulting in increased calcium-dependent attachment to fibronectin (Shima *et al.* 1995). Interestingly, this was a differentiation-dependent phenomenon and may explain why prolonged M-CSF treatment of mature human monocytes did not cause increased surface expression of β_1 integrins (De Nichilo and Burns 1993). This differentiation-dependent phenomenon may also be relevant to the described inability to detect a MSR-dependent component to TCP adhesion in M-CSF treated human monocytes and THP-1 cells (section 3.4.); even though the endocytic uptake of DiI-AcLDL was moderately increased. Independent supporting evidence for a role for the MSR in human Mϕ adhesion include i) the divalent cation independent adhesion of human monocytes and Mϕ to surfaces coated with non-enzymatically glycated collagen IV as opposed to native collagen IV (El Khoury *et al.* 1994); this could suggest a role in the accelerated development of diabetic vascular lesions; and ii) abnormal Mϕ metabolism was also recently characterized in a normocholesterolemic kindred with planar xanthoma; monocytes in these patients express MSR prematurely and show increased adherence to artificial substrates (Giry *et al.* 1995).

MSR-mediated adhesion to TCP (EDTA-resistant, mAb 2F8-

inhibitable) *in vitro* is serum-dependent. The logical setting for MSR in the hierarchy of monocyte adhesion events would be following transmigration of monocytes into the subendothelial space, their differentiation into MSR-positive Mϕ, engagement by MSR of its as yet unidentified serum-derived ligand (possibly an extracellular matrix component), and subsequent retention of Mϕ within this micro-environment followed by foam cell development. The evidence that M-CSF significantly enhances MSR expression and function *in vitro* (and its production in the atheromatous plaque micro-environment) provides such a mechanism. The *in vivo* significance of M-CSF and Mϕ interactions and involvement in atherogenesis is further explored in murine models of atherosclerosis in Chapter 8.

Chapter Four

The regulation of MSR expression and function by Th1 and Th2 cytokines

4.1 Introduction

CD4+ T cells emerging from the thymus can become either inflammatory (Th1) cells, or helper (Th2) cells, and the final decision on which of these differentiation paths the cell will follow is made during their first encounter with antigen (Mosmann *et al.* 1986; Fitch *et al.* 1993; Thompson 1995). The factors determining this are not fully understood but may include the cytokines elicited by immunogens and the density of peptide:MHC ligands. Helper and inflammatory CD4+ T cell subsets release different but overlapping sets of cytokines, which define distinct actions in immunity. Helper (Th2) T cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 all of which activate B cells and humoral immunity; in contrast inflammatory (Th1) T cells secrete IL-2, IFN-γ, and TNF-β which activate Mϕ and lead to cell-mediated immunity. IFN-γ delivers the first sensitizing activation signal to the Mϕ and converts it into a potent effector cell; increasing its anti-bacterial activity by secretion of oxygen radicals and nitric oxide and amplifying the immune response by increasing the efficiency of presenting antigen to fresh naive T cells. Activated Mϕ may however seriously damage host tissues as a result of localised tissue distruction. Tight regulation of activity is required to allow a specific and effective deployment of this potent means of host defence, while minimising local tissue damage and energy consumption.

Mϕ and T cells interact to modulate the immune response by several mechanisms. Mϕ for their part play an essential role not only as antigen presenting cells but also as a source of cytokines that can regulate the emerging T cell response; they also serve as an important source of costimulatory molecules (B7-1 (CD80) and B7-2 (CD86) (Thompson 1995) which activate T and B cells efficiently. IFN-γ, as an archetypal Th1 cell cytokine, is the definitive Mϕ-activating molecule which causes increased expression of MHC molecules, increased cytotoxicity towards tumor cells and some parasites, as well as enhanced expression of

costimulatory molecules. It also induces the production of IL-12 by Mϕ which in turn is the determining factor in the development of Th1 responses (Hsieh *et al.* 1993). Th2 cells provide the balance to Mϕ activation by producing deactivating cytokines. IL-4, IL-10 and IL-13 comprise a triplet of defined Mϕ-deactivating cytokines and together with TGF-β, they each inhibit some but not all Mϕ functions (Bogdan and Nathan 1993). The effects of IL-4 and IL-13 on Mϕ *in vitro* are listed in Table 4.1. Deactivating cytokines can however also have potentiating effects: IL-4 and IL-13 may enhance antigen presentation and MMR expression in some Mϕ (Stein *et al.* 1992; De Waal Malefyt *et al.* 1993; Doyle *et al.* 1994; Zurawski and De Vries 1994). In addition, IL-13 which otherwise has similar properties to IL-4, affects Mϕ but not T cells, thus allowing finer control over Mϕ function without altering T cell development.

Table 4.1 ***In vitro*** **effects of Th1/Th2 cytokines on Mϕ**

	IFN-γ	IL-4	IL-13
IL-6, IL-1, IL-8, TNF-α	↑	↓	↓
IL-1ra, sIL-1r II	↑	↑	↑
IL-12	↑	↑↓	↑↓
MHC class II	↑	↑	↑
CD14	↑	↓	↓
FcγR	↑	↓	↓
Mϕ mannose receptor	↓	↑	↑
B7-1 + B7-2	↑	↓	↓
CD23 (FcεR)	↑	↓	↓
ADCC	↑	↓	↓
Nitric oxide	↑	↓	↓
Reactive oxygen intermediates	↑	↓↑	↓↑
Procoagulant activity	↑	↓	↓
APC function	↑	↓	↓

(based in part on (Minty 1995))

Recent work on Mϕ-T cell interactions has clouded the paradigm of activation/inactivation of Mϕ. Unlike T cells, in which definable subsets (Th1 and Th2) are associated with distinct and largely antagonistic immune responses, Mϕ function does not seem to be defined by unique pathways of differentiation. It may be more accurate

to think of Mϕ activation in terms of activation of specific functions rather than the conversion of the Mϕ to a different state. Mϕ can simultaneously release cytokines associated with Th1 (IL-12, TNF-α), Th2 (IL-1, IL-10) or both responses. This suggests that, rather than thinking of Mϕ as activated or deactivated, they should be considered multi-potent cells that can support a number of different responses depending on the local micro-environment.

Published work on the regulation of MSR expression has been heavily influenced by the potential role of this receptor in atherogenesis. Its broad ligand-binding specificity, which include as physiological ligands cell wall components of both gram-negative (endotoxin) (Hampton *et al.* 1991) and gram-positive (lipoteichoic acid) (Dunne *et al.* 1994) bacteria, also suggests a possible role in general host defence and innate immunity. The potential function of MSR as an adhesion receptor (Fraser *et al.* 1993), its distribution on defined Mϕ sub-populations throughout the normal adult body, and the high degree of conservation of receptor activity across species (Abrams *et al.* 1992), may all point towards a fundamental role for this receptor other than in atherogenesis.

In the atheromatous blood vessel the presence of T lymphocytes, together with numerous Mϕ, suggests there is not only an inflammatory reaction during atherogenesis, but also an immunological reaction. In terms of humoral immune responses, granular deposits of immunoglobulin and co-localised complement components, as well as high levels of expression of the receptors for C3b (CR1) and C3bi (CR3) on Mϕ, have been observed within atherosclerotic lesions, and not in non-atherosclerotic arteries (Seifert and Hansson 1989). Possible candidate antigens for such antibodies include oxidised LDL (Salonen *et al.* 1992) and certain stress proteins (Xu *et al.* 1992).

In addition to these humoral immune phenomena, it is now evident that T cells are among the earliest cells infiltrating the arterial intima in the initial stages in atherosclerosis. In the T cell population, 70% are CD4+, the remainder being CD8+; the majority are activated and express MHC class II HLA-DR and interleukin-2 receptor (IL2-R) Furthermore, T cells in fully developed atherosclerotic plaques express a low-molecular-weight form of the leukocyte common antigen (CD45R0), as well as the very late antigen-1 (VLA-1) integrin. (Stemme and Hansson 1994). The majority of CD3+ cells within the mononuclear cell infiltrates of atherosclerotic lesions bear TCRαβ molecules, but a considerable proportion also express TCRγδ. Rearrangement patterns of T cell receptor (TCR) genes are heterogeneous, i.e. T cells within plaques constitute a polyclonal population. It has been hypothesised that similar

to the mucosa associated lymphoid tissue (MALT), a vascular associated lymphoid tissue (VALT) may exist which monitors antigenic material that comes into contact with the vascular tree (Wick *et al.* 1992). Several questions still remain regarding the involvement of the immune system in atherogenesis: (1) are the humoral and cellular immune reactions of primary or secondary nature? (2) what are the antigens eliciting these immune responses? (3) what roles do inflammatory/immune reactions play in the chronological process of atherogenesis as opposed to classical risk factors? and (4) can animal models be developed that allow dissection of the role of immunological factors in atherogenesis?

The presence of large numbers of T lymphocytes in these lesions (Jonasson *et al.* 1986; Hansson *et al.* 1989), suggests a role for Th1 and Th2 lymphokines in the regulation of Mϕ function (including MSR expression and activity) at these sites. In this chapter the effects of the archetypal Th1 (IFN-γ) and Th2 (chiefly IL-4) lymphokines on MSR expression and function are examined.

4.2 The effects of Th1/Th2 cytokines on MSR expression

4.2.1 Protein and mRNA expression

Peritoneal Mϕ elicited by Bio-Gel acrylamide beads are known to be less activated and more responsive to cytokines than those elicited by thioglycollate broth (Stein and Gordon 1991; Stein *et al.* 1992), and have been used in most of the regulatory experiments described in this thesis. BgPMϕ were treated for 48 hours with IFN-γ, IL-4 and IL-13 and several experimental techniques were applied to characterize and quantify cytokine-mediated differences in MSR protein expression. Some of the initial work in this section was done in collaboration with I.P. Fraser, A.D. Doyle and P. Gough. P600, first discovered in 1987 (Cherwinski *et al.* 1987) and subsequently found to be the murine homolog of human IL-13 was used as a tissue culture supernatant, together with a negative control supernatant, PSE). Alhough IL-13 can be differentiated from IL-4 by its lack of direct effects on T cells, it closely resembles IL-4 in its actions on murine Mϕ with the generation of an "alternatively" activated phenotype (Doyle *et al.* 1994). This is indicated by the enhanced expression of MMR and MHC class II molecules, even though pro-inflammatory monokine release is inhibited. These similarities between IL-4 and IL-13 are explained by their receptors sharing a common gp-140 component important in signal transduction (Zurawski and De Vries 1994). Most studies on MSR expression and function

therefore focused on the archetypal Th2 cytokine, IL-4.

In the first instance, BgPMϕ were metabolically labeled overnight and the MSR immunoprecipitated with the 2F8 mAb (fig. 4.1). While IL-4 generally increased MSR protein synthesis as measured by ^{35}S-methionine incorporation after 48 hours cytokine treatment, IL-13 did so variably and IFN-γ had little effect. To quantify these differences protein bands on dried gels were analyzed by PhosphorImager and the ImageQuant software program (table 4.2). IL-4 increased MSR protein synthesis by 35% while IFN-γ treatment resulted in a 20% decrease. These differences were especially marked when reduced monomer bands at ≈ 80kD were analyzed.

Table 4.2 Effects of Th1/Th2 cytokines on MSR synthesis analyzed by PhosphorImager

Treatment	*Arbitrary PhosphorImager Units (APUs)*	*Percentage Control*
Control	32483 ± 562	
IL-4	50310 ± 723	+ 35%
IFN-γ	25986 ± 347	- 20%

BgPMϕ were treated *in vitro* for 48 hours and metabolically labeled. Quantification of protein bands on dried gels was done by PhosphorImager and results expressed as a ratio of arbitrary APUs. Results are the means (± s.d) from two experiments.

Immunoblot analysis did not show any marked differences in total MSR protein expression from cytokine-treated lysates (results not shown). This discrepancy could be due to i) an inherent lack of sensitivity in the western blotting method or ii) unchanged expression levels of total MSR. However, as the 2F8 mAb is most effective at blotting MSR in its non-reduced or mature trimer form, differences in monomer levels may be obscured. IL-4 did, as shown in fig 4.4, seem to increase mature trimeric MSR protein synthesis.

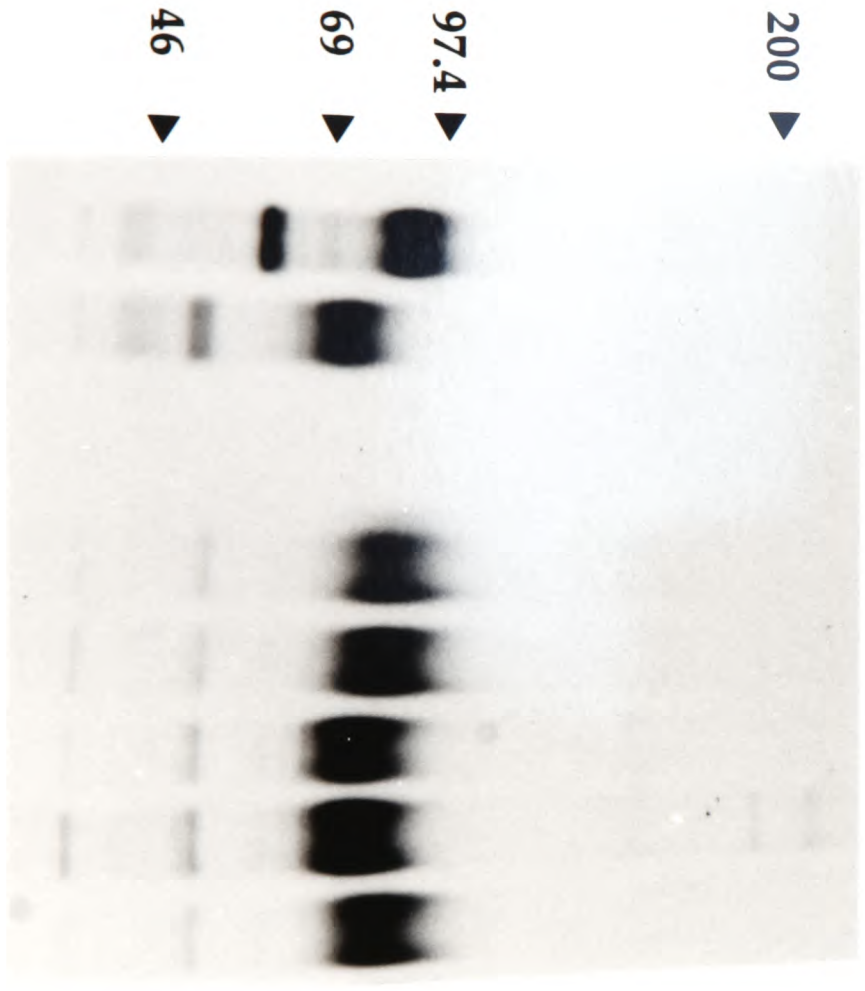
Work described in Chapter 3 showed a large intracellular pool of MSR in addition to that expressed on the plasma membrane (de Villiers *et al.* 1994). To determine whether IL-4 and IFN-γ affect the distribution of MSR within the cell, fluorescence immunocytochemistry of intact and permeabilized cells was attempted (fig 4.2). Although qualitative, compared to controls, IL-4 seemed to enhance and IFN-γ to decrease the intensity of MSR staining on the cell surface as well as the internal (permeabilized) pool. Cytokine-induced differences in cell shape made evaluation of the cellular distribution of MSR difficult; more

Fig. 4.1 (top) **Immunoprecipitation of MSR from Th1/Th2 cytokine-treated BgPMϕ**

Day five BgPMϕ were harvested and treated *in vitro* with the indicated cytokines for 48 hours (final concentrations: IL-4 = 5ng/ml; IL-13 = 10ng/ml; PSE = control supernatant; IFN-γ = 100units/ml). Cells were then metabolically labelled by overnight incubation with 50 μCi/ml Tran^{35}S, lysed and subjected to immunoprecipitation with mAb 2F8 as described. Immunoprecipitates were separated by 5-10% SDS-PAGE (equivalent cell protein per lane) in the presence of β-mercaptoethanol, fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C.

Fig. 4.3 **(bottom) Immunoprecipitation of MSR from Th1/Th2 cytokine-treated BgPMϕ - comparison to CHO MSR transfectants**

BgPMϕ were treated *in vitro* with the indicated cytokines for 48 hours (final concentrations: IL-4 = 5ng/ml; IL-13 = 10ng/ml; IFN-γ = 100units/ml). Cells were then metabolically labelled by overnight incubation with 50 μC Chapter Four lysed and subjected to immunoprecipitation with mAb 2F8 as described. In addition, CHO mSRI and mSRII transfectants were metabolically labelled and precipitated with mAb 2F8 as above. Immunoprecipitates were separated by 5-10% SDS-PAGE (equivalent cell protein per lane) in the presence of β-mercaptoethanol, fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C.


46
69
97.4
200
CHO MSR I
CHO MSR II
CONTROL
PSE
IL-4
IL-13
IFN-γ

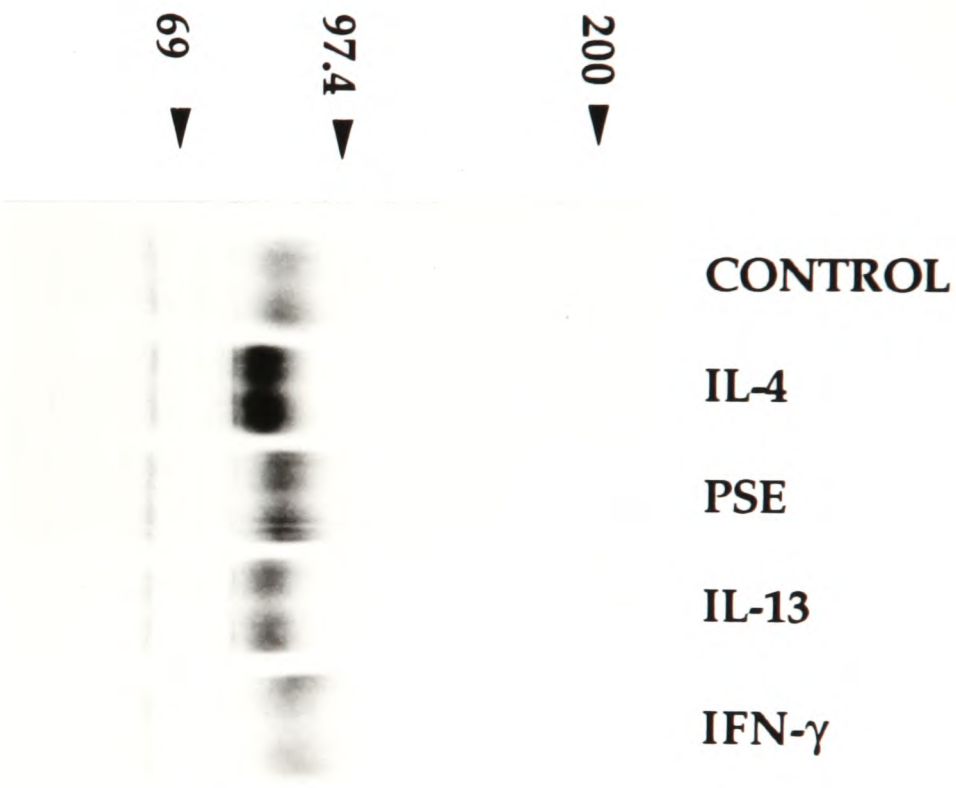

69
97.4
200
CONTROL
IL-4
PSE
IL-13
IFN-γ

Fig. 4.2 **MSR expression on Th1/Th2 cytokine-treated BgPMϕ as determined by immunofluorescence microscopy**

BgPMϕ were grown on 13 mm diameter acid-washed coverslips in 24 well plates in serum-containing medium and treated *in vitro* with the indicated cytokines for 48 hours (final concentrations: IL-4 = 5ng/ml; IFN-γ = 100units/ml). After washing with PBS, cells were fixed in PBS containing 3% (w/v) paraformaldehyde. The cells were permeabilized, where indicated, for visualization of intracellular MSR, by the addition of 0.1% (w/v) saponin to PBA in all antibody incubations and washes. Staining was with mAb 2F8 and FITC conjugated second antibody. Cells were photographed under epifluorescence microscopy oil immersion (objective lens = 100x) with standard FITC excitation and emission filters. All exposures were of equivalent duration.

A. Untreated cells.

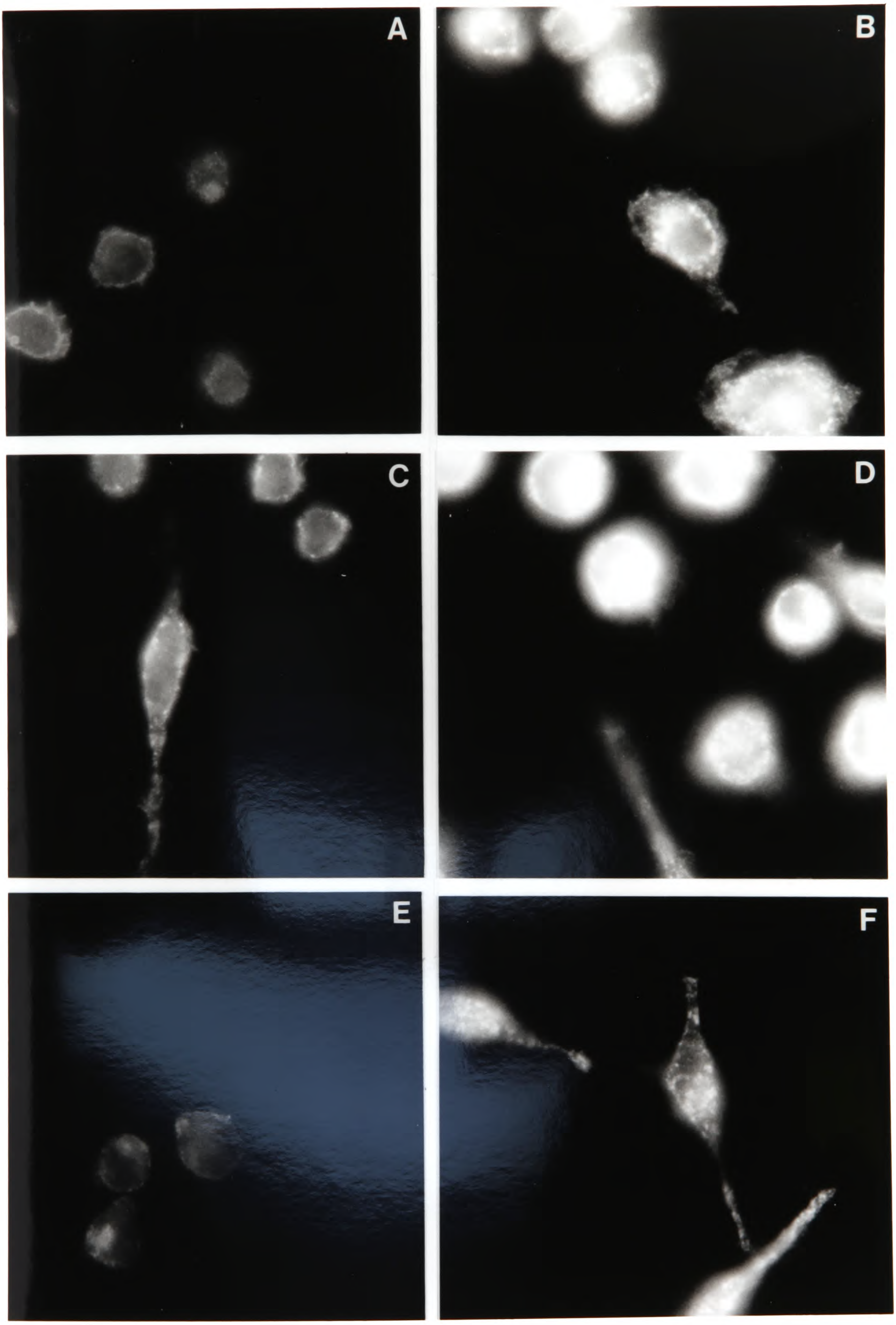
B. Untreated cells, permeabilized.

C. IL-4 treated cells.

D. IL-4 treated cells, permeabilized.

E. IFN-γ treated cells.

F. IFN-γ treated cells, permeabilized.


A
B
C
D
E
F

sophisticated imaging techniques such as confocal microscopy may be helpful. Expression of MSR at a single cell level was subsequently determined by flow cytometry (table 4.3) and the upregulatory effect of IL-4 on levels of MSR surface (101%) and internal (67%) antigen confirmed; IFN-γ had no apparent effect in this assay.

Table 4.3 Effect of Th1/Th2 cytokines on MSR expression by flow cytometry

Treatment	*Surface*	*Internal*
Control	100	100
IL-4	201± 14	167 ± 8
IFN-γ	105 ± 7	97 ± 5

BgPMϕ were treated in vitro for 48 hours as indicated and stained with either mAb 2F8 or CAMPATH-1G plus FITC-conjugated second antibody before flow cytometry. Results are depicted as percentage of control values and are the means ± s.d. of two experiments (each experiment in triplicate) in which specific fluorescence intensities were determined by subtracting geometric mean fluorescence obtained with CAMPATH-1G from that obtained with mAb 2F8.

MSR mRNA changes in response to cytokine treatment were investigated by semi-quantitative RT-PCR. No differences could be detected in either MSR mRNA as a whole (MSR-C) or mRNA specific for types I and II MSR (results not shown). No alteration in the levels or ratio of type I to type II mRNA therefore occurs during IL-4 and IFN-γ treatment. Any cytokine-induced changes in protein synthesis or expression are thus unlikely to be due to transcriptional upregulation or posttranscriptional stabilization of mRNA, and may be ascribed to post-translational changes.

In addition to differences elicited by metabolic labeling in levels of MSR synthesis, the most striking observation was a shift in the M_r on SDS-PAGE following treatment by the Th2 cytokines IL-4 and IL-13, when compared with control BgPMϕ, or those treated with IFN-γ. The MSR undergoes substantial post-translational processing (Penman *et al.* 1991; Krieger 1992; Via *et al.* 1992) and the increased mobility could be attributable to any of the following: i) class switching of MSR type at mRNA splicing level or preferential translation of type-specific mRNA, ii) differences in phosphorylation state of the MSR cytoplasmic domain, iii) alterations in the extent and level of N-glycosylation of the molecule's extracellular domain, and iv) variations in hydroxylation of the MSR's collagen-like domain.

The mobility shift was initially thought to be compatible with the

difference between the type I and type II forms of the MSR. RT-PCR analysis alluded to above seemed incompatible with a cytokine-induced effect on alternative mRNA splicing and type selection. On comparing the bands precipitated from the metabolically labeled BgPMϕ with those from the CHO MSR transfectants (fig 4.3), a cytokine-induced selective preference for the type I or II form of the MSR protein was also not apparent.

CHO cells and Mϕ have different glycosylation capabilities and the type I and II forms of the receptor expressed in these differing cell types could migrate very differently on SDS-PAGE. In order to address this problem more directly, BgPMϕ were treated with IL-4 or IFN-γ and then subjected to a short pulse label with Tran^{35}S in order to visualise the precursor (fig. 4.4). Pulse-chase experiments described in Chapter 7 done in collaboration with I.P. Fraser showed that a precursor at ≈ 64 K could be detected after a 30 minute pulse label, and that in CHO MSR transfectants, this precursor migrated differently in the case of the type I and II receptors. The multimerization of monomer that occurs during MSR synthesis makes samples run under reducing conditions easier to interpret. The cytokine treated BgPMϕ revealed MSR precursor bands of the same M_r at ≈ 60 K on the reduced gel, regardless of the cytokine used. Since the nascent polypeptide chains of type I and II MSR have predicted M_r of 49.6 and 38.2 K respectively (Ashkenas, 1993), the observed precursor bands probably represent core polypeptide plus cotranslationally added high-mannose oligosaccharide chains. IL-4 increased the amount of mature product (≈ 90 K) present at the end of the pulse period as well as that of heterogeneous intermediates (≈ 69 K); these may represent further complex N-linked oligosaccharide processing. It is evident that IL-4 increases the ratio of mature to precursor MSR monomer in comparison to control BgPMϕ, or those treated with IFN-γ. IL-4 therefore, in addition to increasing precursor synthesis slightly, may enhance post-translational processing to the mature form quite significantly. This effect would be in accordance with the RT-PCR results discussed previously. IL-4 also had no apparent effect on the mobility of the more mature form of MSR in these pulse labeling experiments; this may indicate the involvement of an IL-4 dependent step in more terminal post-translational processing of the molecule.

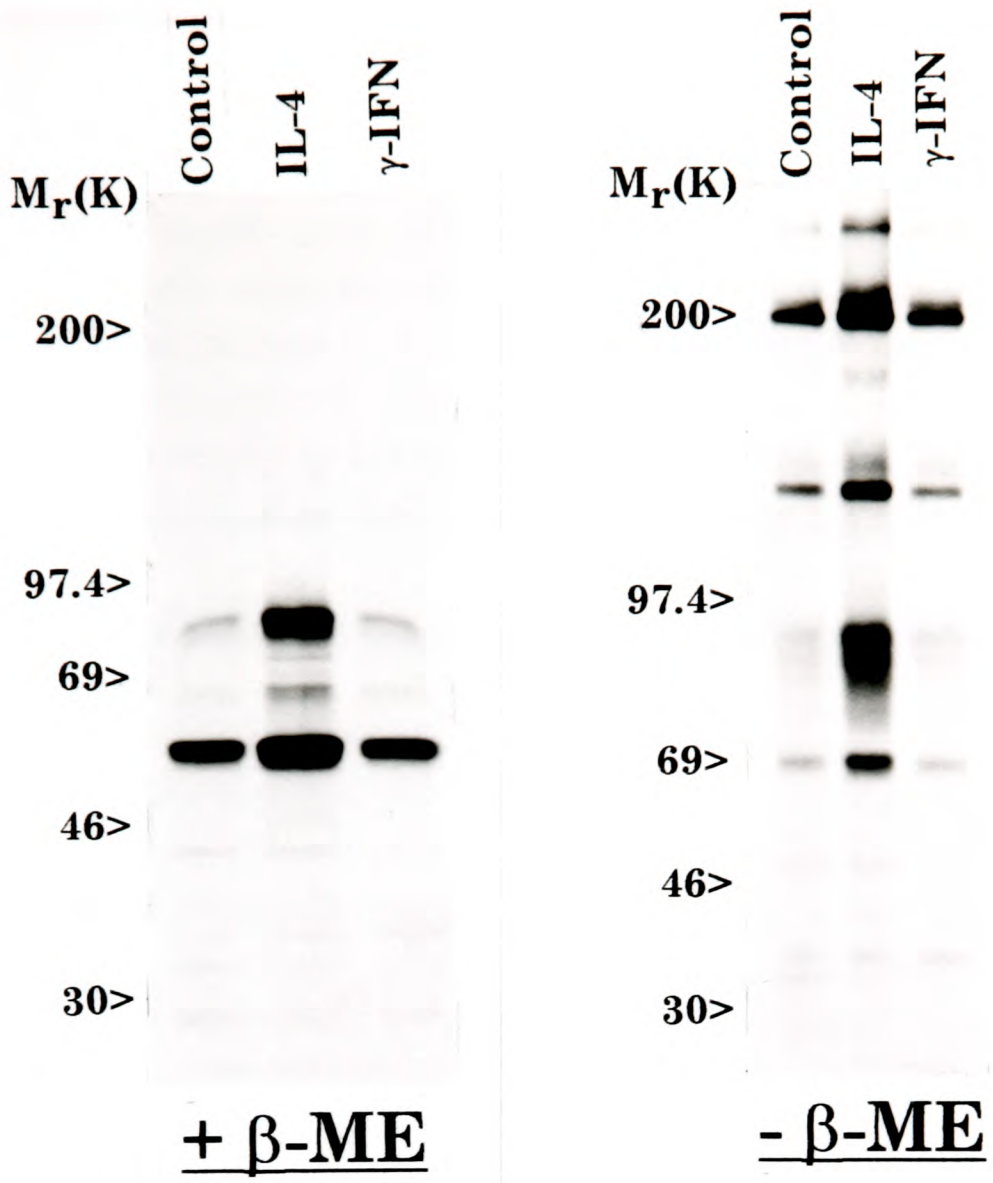
Interpretation of the non-reduced precipitates is complicated by the presence of monomer, dimer and trimer, each in precursor, intermediate and mature forms. Most evident is the MSR precursor band (≈ 200 K) and the relative reduction in intensity of the mature trimer band (≈ 240 K). Immature forms of dimers (≈ 130 K) and

Fig. 4.4 (top) **Immunoprecipitation of MSR from short pulse metabolically labeled Th1/Th2 cytokine-treated BgPMϕ**

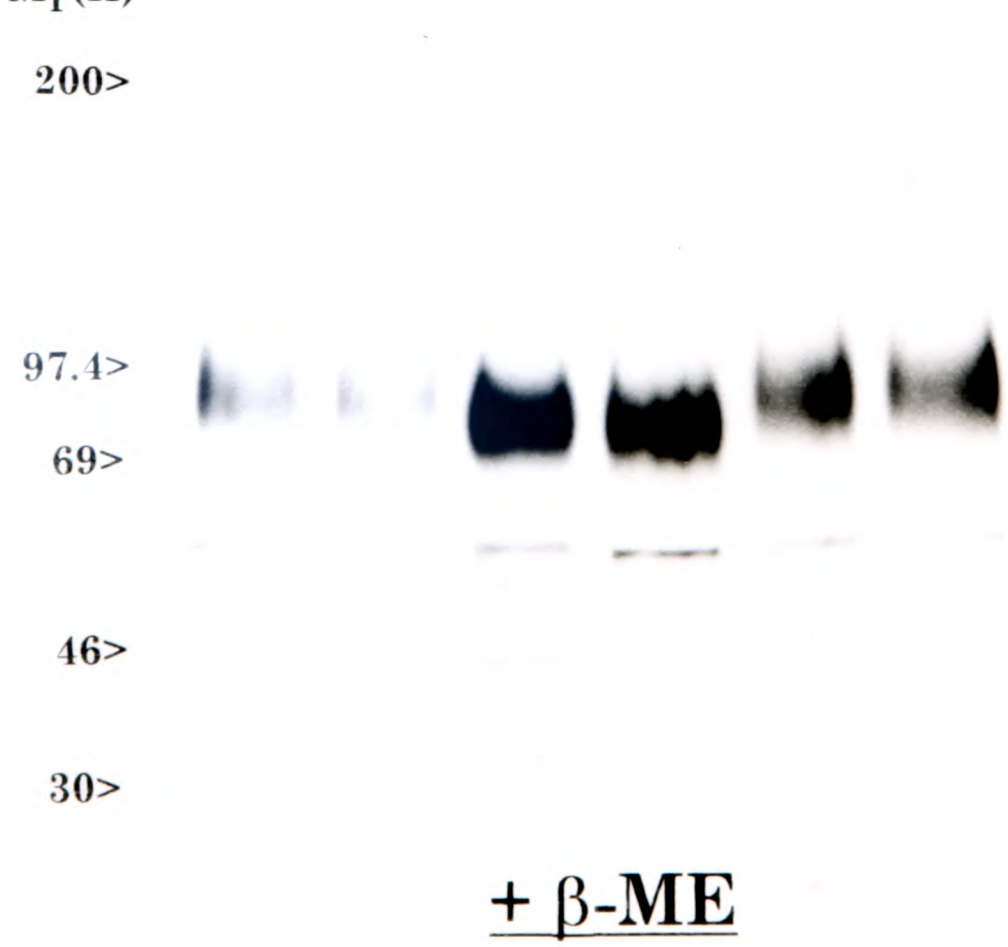
BgPMϕ were prepared and treated with IL-4, IFN-γ or in medium alone (Control) as described in fig. 4.1. After cytokine treatment, cells were incubated for 30 minutes with 500 μCi/ml Tran^{35}S-label, lysed and precipitated with mAb 2F8 as above. Immunoprecipitates were separated by 5-10% SDS-PAGE (equivalent cell protein per lane) in the presence (+βME) or absence (-βME) of β-mercaptoethanol, fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C.

Fig. 4.5 (bottom) **Alkaline Phosphatase treatment of MSR immunoprecipitates from Th1/Th2 cytokine-treated BgPMϕ**

BgPMϕ were prepared and treated as in fig. 4.1. After cytokine treatment, cells were incubated overnight with 50μCi/ml Tran^{35}S-label, prior to lysis and immunoprecipitation with mAb 2F8. Where indicated (" + phosphatase"), precipitates were treated with 20 units of calf intestinal alkaline phosphatase for 60 minutes at 37°C in the accompanying buffer. Untreated precipitates (" - phosphatase") were incubated at 37°C in buffer alone for the same time. Activity of the enzyme under these conditions was confirmed by observing a colour change when incubated with standard alkaline phosphatase substrate (p-Nitrophenyl phosphate). After phosphatase treatment, precipitates were separated by SDS-PAGE in the presence of β-mercaptoethanol as above. Equivalent input cell protein was loaded in each lane. Molecular weight standards are indicated by arrowheads - from the top down: 200; 97.4; 69 kDa.



	Control		IL-4		γ-IFN	
Alkaline Phosphatase	-	+	-	+	-	+



monomers (≈ 65 K) are also present. The increase in protein synthesis mediated by IL-4 is again seen, while the population of non-reduced MSR precursor bands display the same electrophoretic mobility, regardless of the cytokine used. These results therefore suggest that the microheterogeneity shown by MSR migration following Th1/Th2 cytokine treatment is likely to be due to post-translational modification of the core polypeptide, and not due to switching of receptor type.

4.3 MSR post-translational modification by Th1/Th2 cytokines

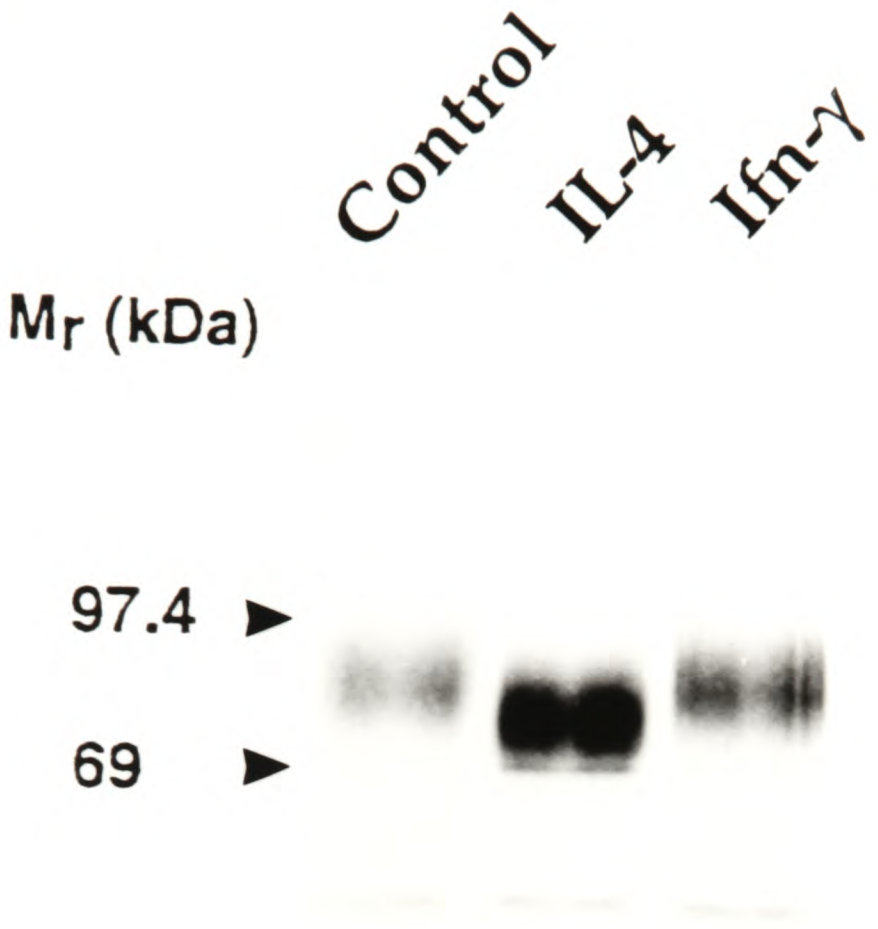
The MSR has a conserved consensus sequence for Ser/Thr phosphorylation (Edelman *et al.* 1987) in the middle of the cytoplasmic domain of the molecule (Kodama *et al.* 1990), but the use of this site has never been demonstrated. In order to determine if the IL-4/IFN-γ difference in MSR mobility was due to phosphorylation, MSR precipitated from metabolically labelled, cytokine-treated BgPMϕ was subjected to digestion with calf alkaline phosphatase as described (Pace *et al.* 1993) (fig. 4.5). In these experiments, no difference in the mobility of the mature MSR monomer could be detected after treatment with the phosphatase. This argues against altered IL-4-induced phosphorylation accounting for the difference in mature MSR mobility. Interestingly, in both the control and IL-4 treated cells, but not those treated with IFN-γ, phosphatase digestion seemed to decrease the mobility of the ≈ 60 K immature precursor band slightly, possibly indicating phosphorylation changes in the precursor. Antibodies to phosphoserine or phosphothreonine residues could be used to investigate this possibility further. In addition, MSR phosphorylation status following cytokine treatment should be investigated by ^{32}P-incorporation.

The MSR cDNA sequence predicts 7 possible N-linked, and no O-linked, glycosylation sites; six of these sites have been shown, by sequential N-glycanase digestion (Resnick *et al.* 1993), to be utilized. To investigate possible cytokine-induced changes in glycosylation, tunicamycin was initially used to inhibit MSR glycosylation during metabolic labelling of BgPMϕ. Tunicamycin inhibits N-linked glycosylation by preventing oligosaccharide side chains to be added onto the dolichol lipid carrier in the ER membrane (Elbein 1987). These results are not shown, as even at a relatively low dose of tunicamycin (2μg/ml), both total and 2F8-precipitable ^{35}S counts were significantly decreased compared with controls, casting doubt on cell viability. After lengthy exposure times (> three weeks), faintly visible bands appeared to consist mostly of mature, glycosylated MSR, with few obvious detectable lower M_r bands. This was probably due to an insufficient blocking

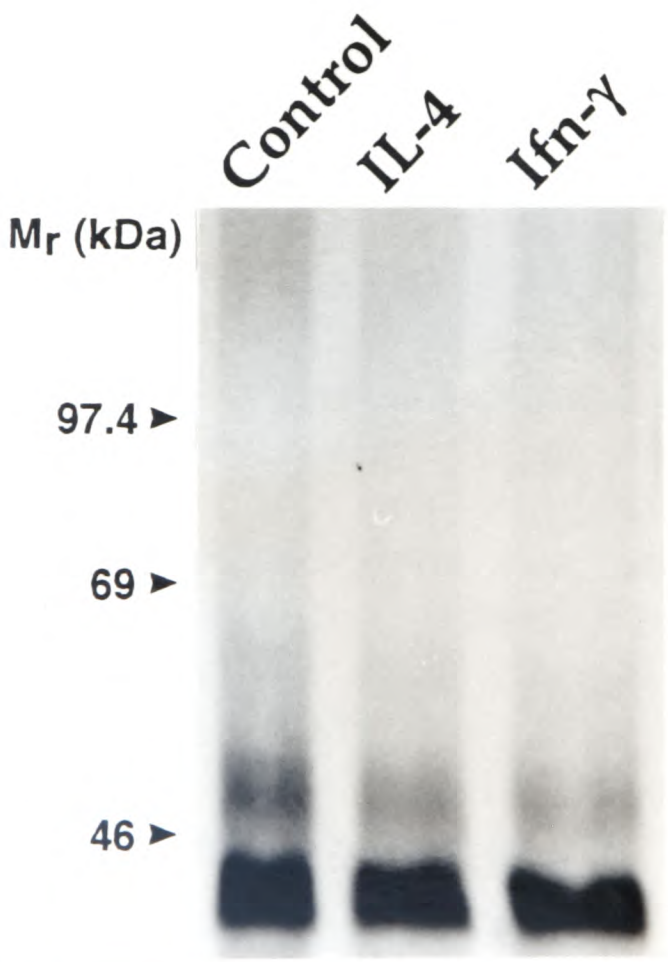
Fig. 4.6 **N-glycanase digestion of MSR immunoprecipitates from Th1/Th2 cytokine treated BgPMϕ**

BgPMϕ were prepared and treated as in fig. 4.1. After cytokine treatment, cells were incubated overnight with 50 μCi/ml Tran^{35}S-label, prior to lysis and immunoprecipitation with mAb 2F8. Precipitated antigen was eluted from the Protein G-Sepharose by boiling in the presence of SDS and β-mercaptoethanol, then incubated overnight at 37°C in the appropriate reaction buffer with 2 units N-glycanase. Octylglucoside was added to each reaction to prevent inactivation of the enzyme by SDS. After digestion, samples were boiled in gel loading buffer containing β-mercaptoethanol, and separated by 5-10% SDS-PAGE. The gel was fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Equivalent input cell protein was loaded in each lane. **A.** Untreated and **B.** N-Glycanase-treated samples.

A.



B.



- N-Glycanase Treatment of 2F8 I.P.
- Reducing SDS-PAGE

concentration of tunicamycin but could indicate the presence of a substantial pool of glycosylated dolichol at the start of the labeling period.

A more direct approach was therefore used to treat precipitated MSR (from cytokine treated Mϕ) with N-glycanase to observe the resulting differences in migration of deglycosylated monomer. As is clearly apparent from fig. 4.6 N-glycanase deglycosylation collapsed the MSR monomers to the predicted M_r of their core polypeptides. No difference in electrophoretic mobity was present in any of the treated cells, suggesting the induction by IL-4 of a selective change in MSR N-linked glycosylation.

Preliminary studies were also aimed at assessing possible differential hydroxylation of the collagenous domain (Penman *et al.* 1991). Collagenase treatment of precipitated MSR (from cytokine treated Mϕ) was used to ascertain relative resistance of hydroxylated collagenous domains to enzyme digestion. The IL-4 effect on MSR mobility was however maintained (results not shown). The effects of the hydroxylase inhibitor, α,α'- dipiridyl, on MSR mobility (Penman *et al* 1991) should however be studied to determine this definitively.

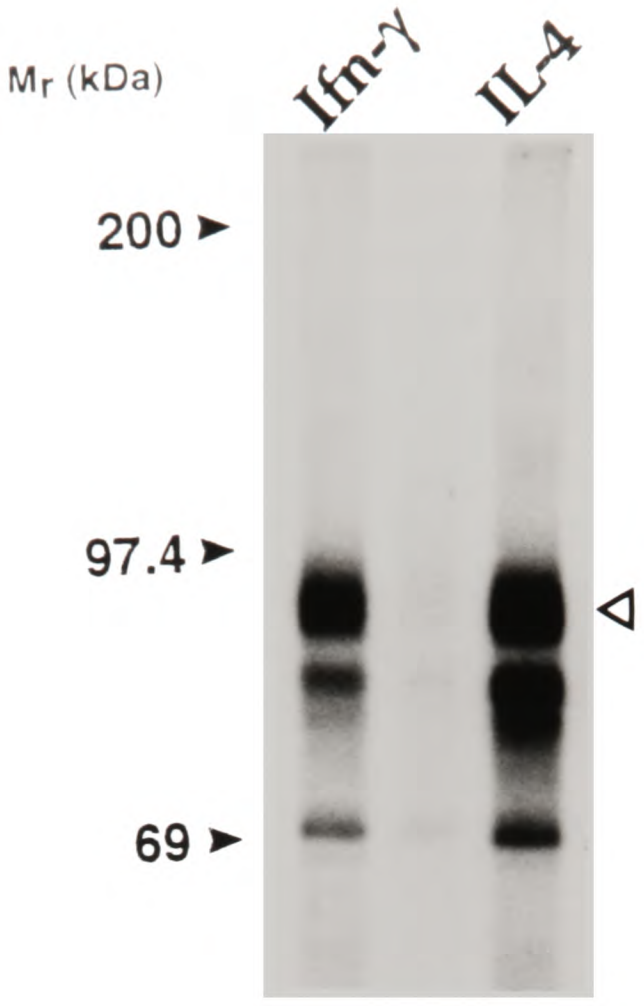
4.4 Characterization of Th1/Th2 effects on MSR glycosylation

Further attempts were made at characterizing the micro-heterogeneity in MSR glycosylation brought about by IL-4. The possibility that IL-4 affects MSR glycosylation non-selectively by virtue of its presence among countless other glycosylated proteins was analyzed by its action on other Mϕ integral membrane proteins. Neither macrosialin (FA-11 mAb) nor the Mϕ differentiation antigen, F4/80 (F4/80 mAb) showed any mobility difference in immunoprecipates from treated cells (fig. 4.7). Macrosialin is a Mϕ-restricted late endosomal, mainly intracellular, membrane glycoprotein (Rabinowitz and Gordon 1991). The protein has a 44 K core peptide which is heavily glycosylated to a mature glycoprotein of 87-110 K. During functional activation, such as inflammatory stimulation, this sugar component is modulated and the molecule expresses an alternative glycoform bearing poly-N-acetyllactosamine and, more abundantly, O-linked sialic acid sugar structures. The lack of any IL-4 effect on this extensively O-linked glycosylated molecule may suggest selectivity for N-linked sugars. The predominance of O-linked sugars may also have obscured changes resulting from IL-4 induced N-linked glycosylation. F4/80 is a Mϕ-restricted differentiation antigen which was recently cloned and predicted to be a G protein-linked transmembrane seven hormone

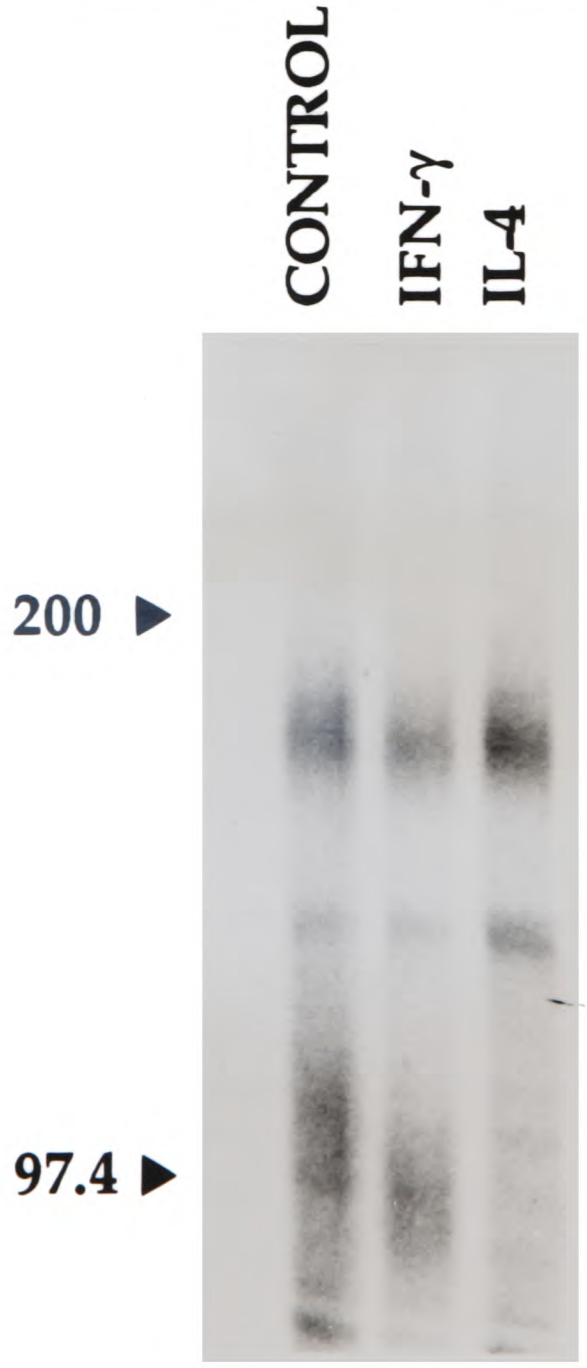
Fig. 4.7 **Immunoprecipitation of Mϕ-specific antigens, macrosialin and F4/80, from Th1/Th2 cytokine-treated BgPMϕ**

BgPMϕ were harvested and treated *in vitro* with the indicated cytokines for 48 hours (final concentrations: IL-4 = 5ng/ml; IFN-γ = 100units/ml). Cells were then metabolically labelled by overnight incubation with 50 μCi/ml Tran^{35}S, lysed and subjected to immunoprecipitation with mAbs FA-11 (macrosialin) and F4/80 (F4/80 antigen) as described. Immunoprecipitates were separated by 7.5% SDS-PAGE (equivalent cell protein per lane) in the presence of β-mercaptoethanol, fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. **A.** Macrosialin and **B.** F4/80.

A.



B.



receptor family member (Austyn and Gordon 1981; McKnight *et al* 1996). It has a core polypeptide of M_r 98.7 K; biochemical studies have revealed the presence of tritetra-antennary complex N-linked sugars on the mature protein (120-160 K) and 10 possible N-linked sites are predicted in the cDNA sequence. Although the many Ser and Thr residues in the sequence indicate potential O-linked sugar sites, this has not been supported by previous biochemical data (Starkey *et al.* 1987). The composition of the complex N-linked oligosaccharide chains may however differ between this molecule and the MSR accounting for the difference seen in IL-4 action on N-linked glycosylation. There is thus some limited evidence of a selective effect of IL-4 on MSR N-linked sugars.

To relate the IL-4-induced differential effect on MSR glycosylation micro-heterogeneity to compositional changes in its N-linked sugars the subsequent experimental methods were employed: lectin blotting, ^{3}H-galactose labeling of sugars and sialidase enzyme digestions of treated cells. Qualitative differences in lectin-binding abilities were assessed by blotting immunoprecipitated MSR from lysates with digoxigenin-labeled lectins such as wheat germ agglutinin (WGA), peanut agglutinin (PNA), MAA (*Maackia amurensis* agglutinin) and SNA (*Sambucus nigra* agglutinin). MAA was the only lectin which displayed faint, but definite binding to the MSR monomer (result not shown) signifying the presence of sialic acids linked α(2-3) to galactose; this binding did not however differ among cytokine-treated cells. The negative result seen with SNA indicates a relative lack of sialic acids linked α(2-6) to galactose. Similarly, when BgPMϕ were labeled with ^{3}H-galactose overnight, a differential cytokine-induced effect on near terminal galactose incorporation into the MSR complex N-linked sugars was not apparent. In this experiment MSR monomer bands remained very faint even after 6 weeks of autoradiography (results not shown).

In order to define any differences in terminal sialic acids of MSR further, denatured immunoprecipitates from cytokine-treated metabolically labeled BgPMϕ were treated with a number of sialidase enzymes (fig. 4.8). These sialidases share some common properties but are different in their substrate specificities, allowing selective and non-selective removal of terminal sialic acids. The loss of negatively charged sialic acids from the molecule leads to a paradoxical upward shift in mobility on SDS-PAGE. The sialidases from Newcastle Disease virus and less convincingly*Clostridium perfringens* (α 2-3 linkage specific) abrogate the IL-4-induced mobility shift of the MSR monomer, whilst the enzyme isolated from *Arthrobacter ureafaciens* (predominantly α 2-6 linkage specific) had little or no effect. In this single experiment the IL-

Fig. 4.8 **Sialidase digestion of MSR immunoprecipitates from Th1/Th2 cytokine treated BgPMϕ**

BgPMϕ were prepared and treated as in fig. 4.1. After cytokine treatment, cells were incubated overnight with 50μCi/ml Tran^{35}S-label, prior to lysis and immunoprecipitation with mAb 2F8. Precipitated antigen was eluted from the Protein G-Sepharose by boiling in the presence of SDS and β-mercaptoethanol, then incubated overnight at 37°C in the appropriate reaction buffer with sialidases isolated from Newcastle Disease virus (α 2-3 linkage specific), *Arthrobacter ureafaciens* (α 2-6 linkage specific) and *Clostridium perfringens* (α 2-3 and α 2-6 linkage specific). Octylglucoside was added to each reaction to prevent inactivation of the enzyme by SDS. After digestion, samples were boiled in gel loading buffer containing β-mercaptoethanol, and separated by 5-10% SDS-PAGE. The gel was fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Equivalent input cell protein was loaded in each lane. The sialidases from Newcastle Disease virus and *Clostridium perfringens* (α 2-3 linkage specific) abolish the IL-4-induced mobility shift of the MSR monomer, whilst the enzyme isolated from *Arthrobacter ureafaciens* (predominantly α 2-6 linkage specific) has little or no effect.

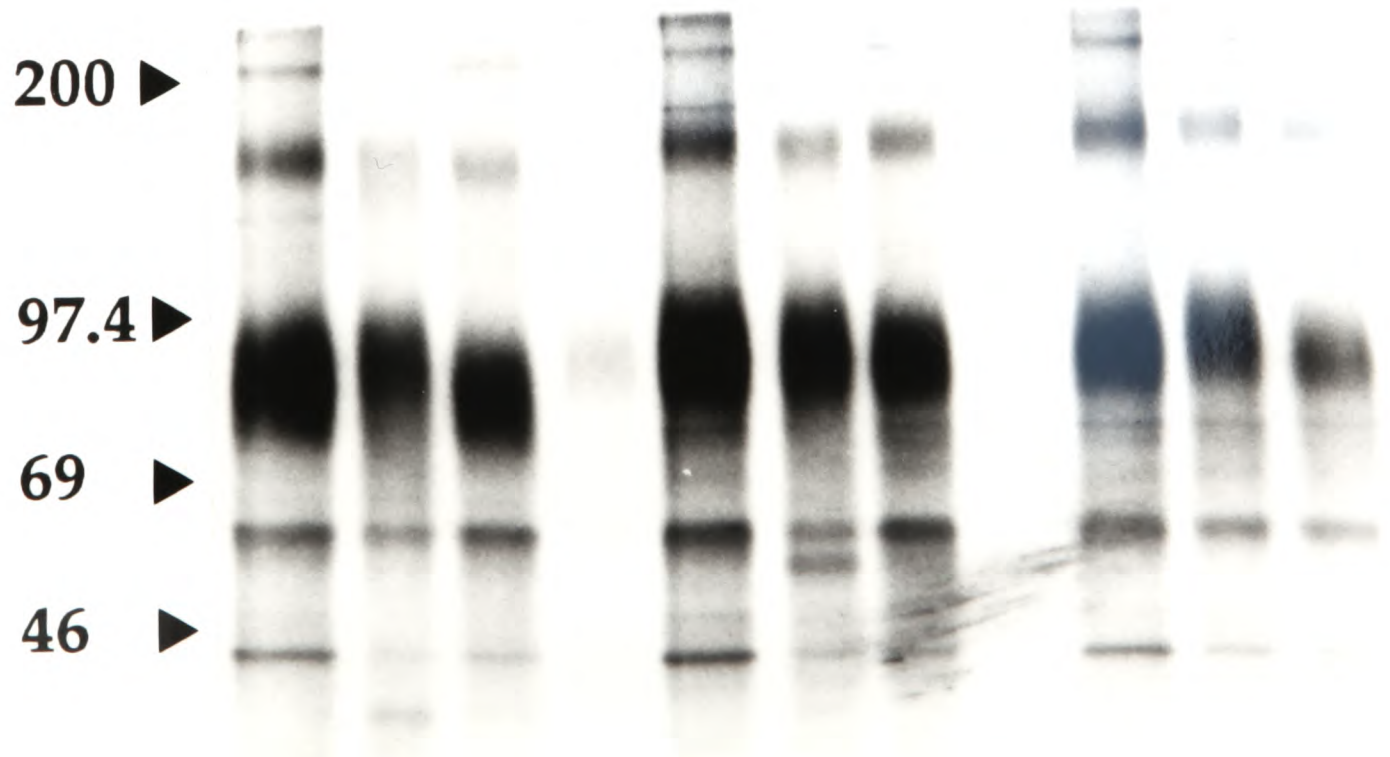
Sialidases:

Arthrobacter ureafaciens

Newcastle Disease Virus

Clostridium perfringens

CONTROL
IFN-γ
IL-4
CONTROL
IFN-γ
IL-4
CONTROL
IFN-γ
IL-4



4 upregulating effect on MSR synthesis was absent; repeat experiments would have to address this issue as well as include controls without sialidase additions.

Although inconclusive, there is some evidence suggesting IL-4 favours the preferential addition of terminal sialic acid α 2-3 linked to galactose or N-acetylgalactosamine. A possible mechanism for this may be the IL-4 selective upregulation of α 2-3 sialyltransferases or their activities. Semi-quantitative RT-PCR was used to investigate any transcriptional regulatory effect of IL-4 on sialyltransferases; this approach was however unsuccessful. Eleven sialyltransferase enzymes (7 of which show α 2-3 activity) have thus far been identified (van den Eijnden and Joziasse 1993) and of these, three cDNAs have been cloned; one α 2-6 and two α 2-3 sialyltransferases. Low stringency primers were designed from the available human and rat α 2-6 and α 2-3 sialyltransferase sequences for cross-reaction with murine enzymes, but were ineffective at amplification.

In conclusion, Th1/Th2 cytokine-induced differences in post-translational N-glycosylation result in microheterogeneity in the MSR. As yet inconclusive evidence suggests this may be due to IL-4 selectively favouring the addition of terminal sialic acids to MSR sugars in a α 2-3 linkage.

4.5 The effects of Th1/Th2 cytokines on MSR function

The significance of these differences in expression was tested by relating them to functional MSR studies. In the first instance, the ability of treated BgPMϕ to endocytose and accumulate fluorescent AcLDL (DiI-AcLDL) was assessed (table 4.4 and fig. 4.9).

Table 4.4: Effect of Th1/Th2 cytokines on uptake of DiI-AcLDL by BgPMϕ

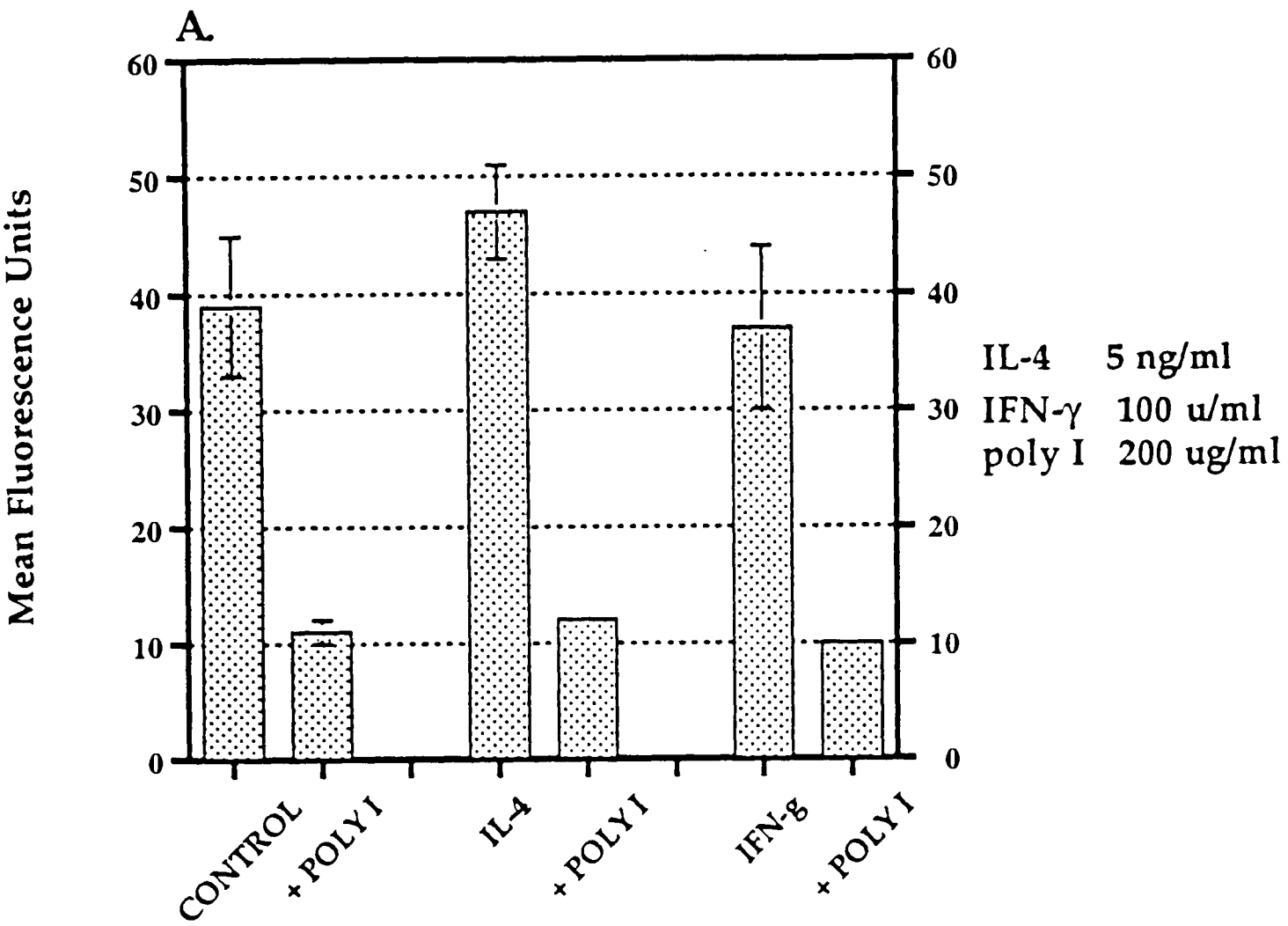
Treatment	*Specific fluorescent intensity*	*Percentage control*
Control	121 ± 2	100 ± 2
IL-4	233 ± 32	192 ± 2
IFN-γ	72 ± 1	60 ± 1

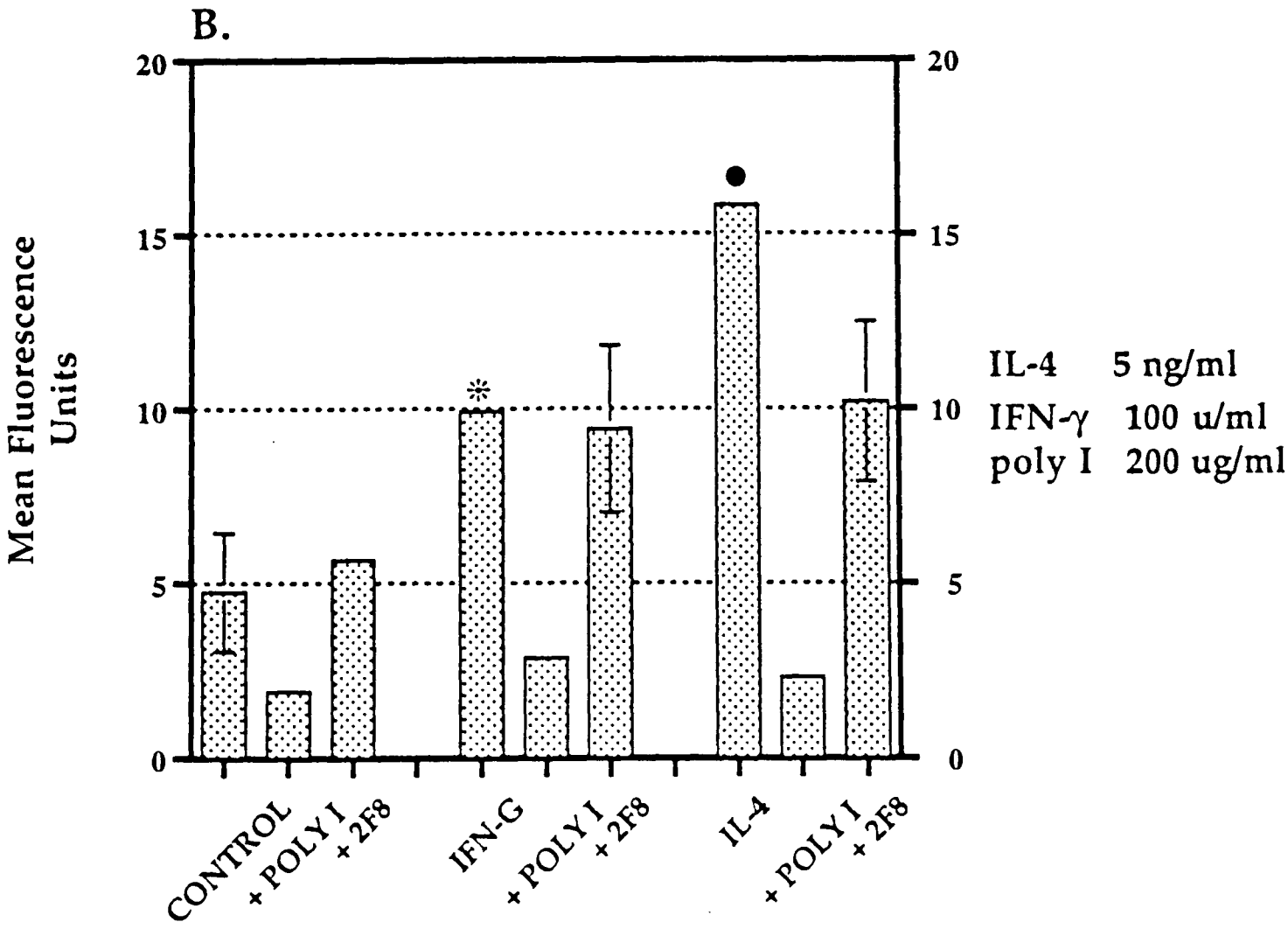
BgPMϕ were treated for 48 h and MSR-mediated uptake of DiI-AcLDL measured as described in Chapter 2. Specific fluorescent intensity was calculated by subtracting autofluorescent intensity from fluorescent intensity of DiI-AcLDL-labeled cells using geometric means ± s.d. derived from from data analysis by Lysys II software (Becton Dickinson). Results are from two experiments done in triplicate.

Fig. 4.9 **The effect of Th1/Th2 cytokines on DiI-AcLDL uptake by BgPMϕ**

BgPMϕ were harvested, plated in 24 well TCP plates (3 x 10^6 cells/well), and treated *in vitro* for 48 hours as indicated. After washing in PBS, cells were pre-incubated for 30 min in RPMI plus 10% FCS containing poly I (200 µg/ml) or mAb 2F8 (10 µg/ml) followed by the addition of DiI-AcLDL at 5 µg/ml. After incubation for 3 hours at 37°C, cells were washed, harvested using EDTA and lidocaine, and resuspended in PBA for analysis by flow cytometry (FL2 photomultiplier). Results (**A.** and **B.**) show the mean values (two independent experiments in duplicate) of specific fluorescence intensities obtained from subtracting geometric mean autofluorescence from the fluorescence obtained after uptake of DiI-AcLDL. * $P < 0.05$ vs. control; • $P < 0.01$ vs. Control

EFFECT OF Th1/Th2 CYTOKINES ON DiI-AcLDL UPTAKE IN BGPMϕ





In fig. 4.9A and B further examples are shown of DiI-AcLDL uptake in treated BgPMϕ. Although in fig. 4.9A IL-4 did not increase AcLDL uptake, the upregulatory effect of IL-4 is a constant finding in other inhibition experiments described later on. The down-regulatory effect of IFN-γ is however variable; and a decrease in uptake was most often seen when whole cell populations were analyzed. Flow cytometry revealed a bi-modal distribution of low (lower than control) and high (higher than control) fluorescence in some IFN-γ treated populations; determination of geometric means to assess AcLDL uptake may be inappropriate in these cases. In distinctly homogeneous fluorescent populations, IFN-γ increased diI-AcLDL uptake by up to two-fold, and was mid-range between control and IL-4 treated cells. This variability may reflect the heterogeneity of BgPMϕ as far as IFN-γ responsiveness is concerned. Poly I (200 μg/ml) inhibited AcLDL uptake efficiently, whilst the MSR mAb, 2F8, blocked uptake poorly in these primary Mϕ (values ranging from 64% to 118% of control). This lack of mAb 2F8 neutralizing activity is in accordance with work done on CHO cells stably transfected with MSR and described in Chapter 7, but differs from the partial inhibition of diI-AcLDL uptake described in the murine Mϕ-like cell line RAW 264.7 (Fraser *et al.* 1993). The reasons for this inconsistency are not quite clear, but possible explanations are dicussed in Chapter 7.

The ability of IL-4/IFN-γ treated BgPMϕ to adhere to serum-coated TCP in an EDTA-independent manner was investigated. It was postulated that IL-4 treatment, because of increased MSR surface expression, may also increase MSR-dependent (divalent cation-independent, mAb 2F8-inhibitable) adhesion. The potency of MSR as an adhesion molecule may also be indirectly enhanced by IL-4, as resolution of the carbohydrate-binding specificities of P- and E-selectin binding has shown terminal sialic acids linked $\alpha 2 \rightarrow 3$ to a galactose to be very powerful binding enhancers whereas $\alpha 2 \rightarrow 6$ sialic acid residues reduced binding affinity (Tyrell *et al.* 1991). No differences in MSR-dependent adhesion of cytokine-treated cells to TCP could however be detected (results not shown). This lack of a correlation between level of surface MSR expression and MSR-dependent adhesion does not support its proposed role in Mϕ adhesion; its role in adhesion may however be complex and the adhesion assay as a quantitative (instead of qualitative) measurement may lack robustness.

To attach functional relevance to Th1/Th2 cytokine-mediated effects on MSR expression and glycosylation, the sensitivity to inhibition of AcLDL uptake by various MSR ligands was investigated. From fig. 4.10

Fig. 4.10 **Immunofluorescence microscopy of DiI-AcLDL uptake by Th1/Th2 cytokine treated BgPMϕ - the effect of poly I inhibition**

BgPMϕ were grown on acid-washed coverslips in 24 well plates in serum-containing medium and treated *in vitro* with the indicated cytokines for 48 hours. After washing in PBS, cells were pre-incubated for 30 min in RPMI plus 10% FCS containing poly I (50 μg/ml) followed by the addition of DiI-AcLDL at 5 μg/ml. After incubation for 3 hours at 37°C, cells were washed and fixed in PBS containing 3% (w/v) paraformaldehyde. Cells were mounted in Mowiol mountant and allowed to dry overnight in the dark. Cells were photographed under epifluorescence microscopy oil immersion (objective lens = 100x) with standard DiI excitation and emission filters. All exposures were of equivalent duration.

A. Untreated cells.

B. Untreated cells + poly I.

C. IL-4 treated cells.

D. IL-4 treated cells + poly I.

E. IFN-γ treated cells.

F. IFN-γ treated cells + poly I.

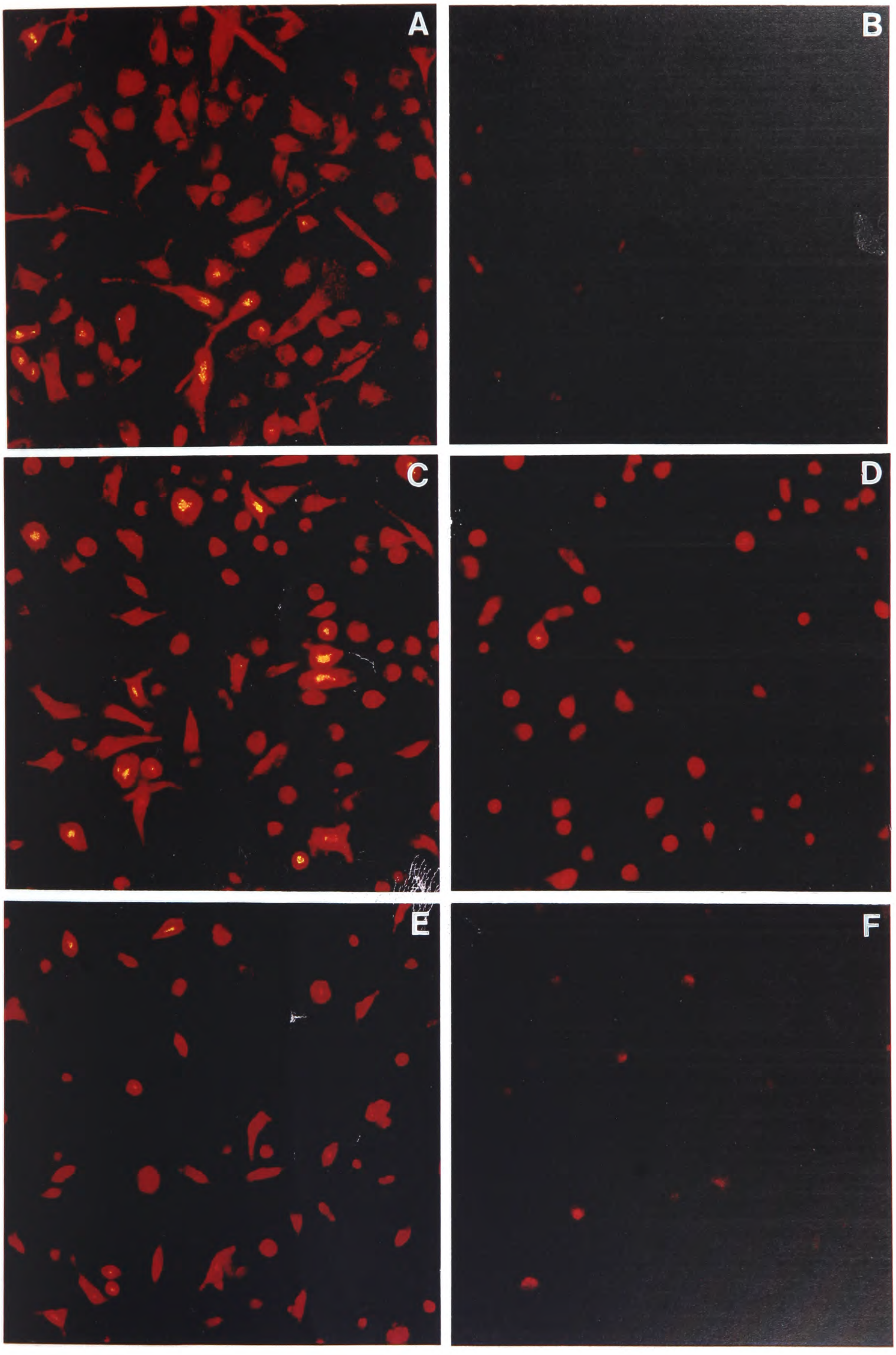

A
B
C
D
E
F

Fig. 4.11 **The inhibition by fucoidan of DiI-AcLDL uptake by Th1/Th2 cytokine treated BgPMϕ**

BgPMϕ were harvested, plated in 24 well TCP plates (3 x 10^6 cells/well), and treated *in vitro* for 48 hours as indicated. After washing in PBS, cells were pre-incubated for 30 min in RPMI plus 10% FCS containing fucoidan at the indicated concentrations followed by the addition of DiI-AcLDL at 5 μg/ml. After incubation for 3 hours at 37°C, cells were washed, harvested using EDTA and lidocaine, and resuspended in PBA for analysis by flow cytometry (FL2 photomultiplier). Results show the mean values (two independent experiments in duplicate) of specific fluorescence intensities obtained from subtracting geometric mean autofluorescence from the fluorescence obtained after uptake of DiI-AcLDL.

FUCOIDAN INHIBITION OF Di-AcLDL UPTAKE

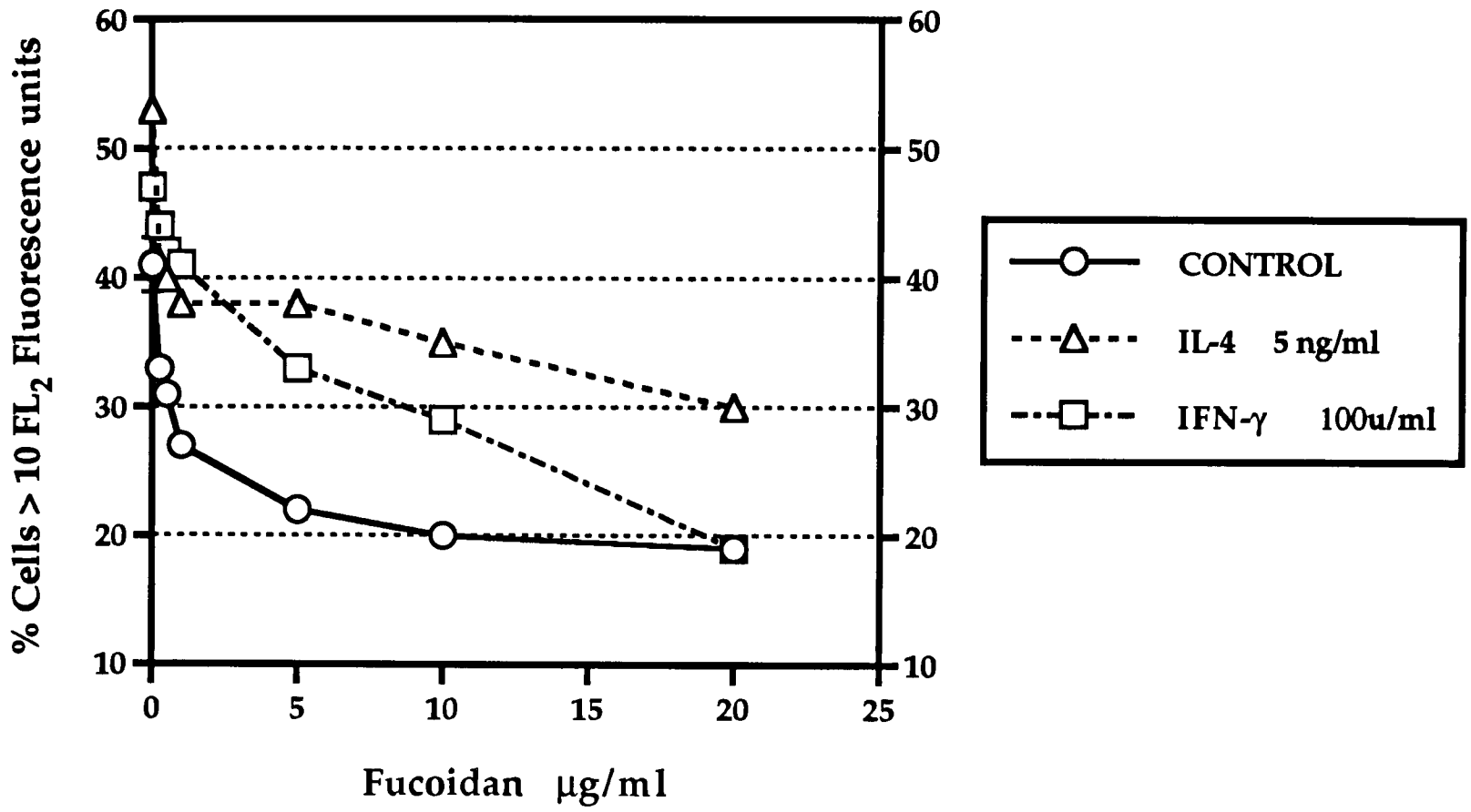
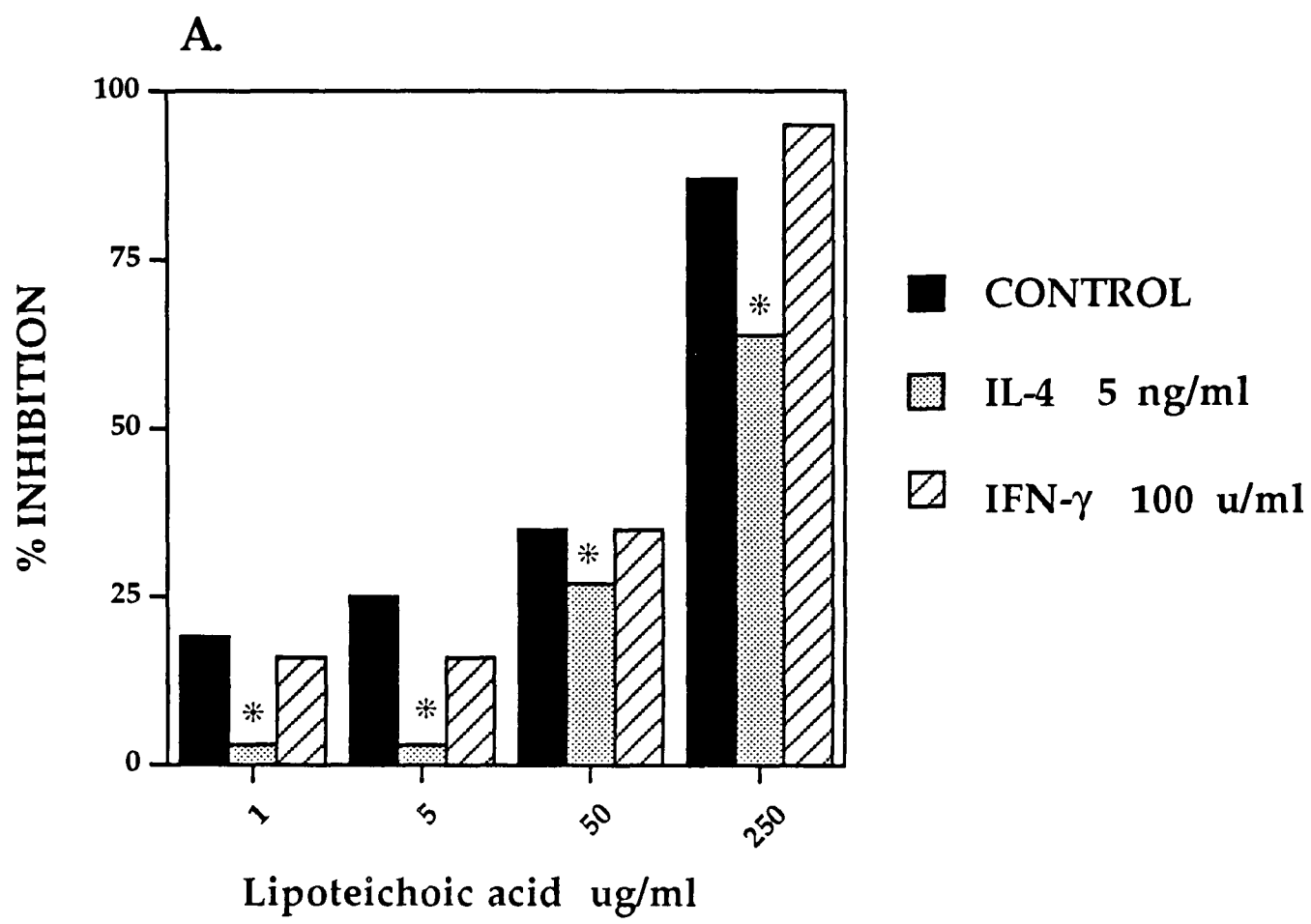
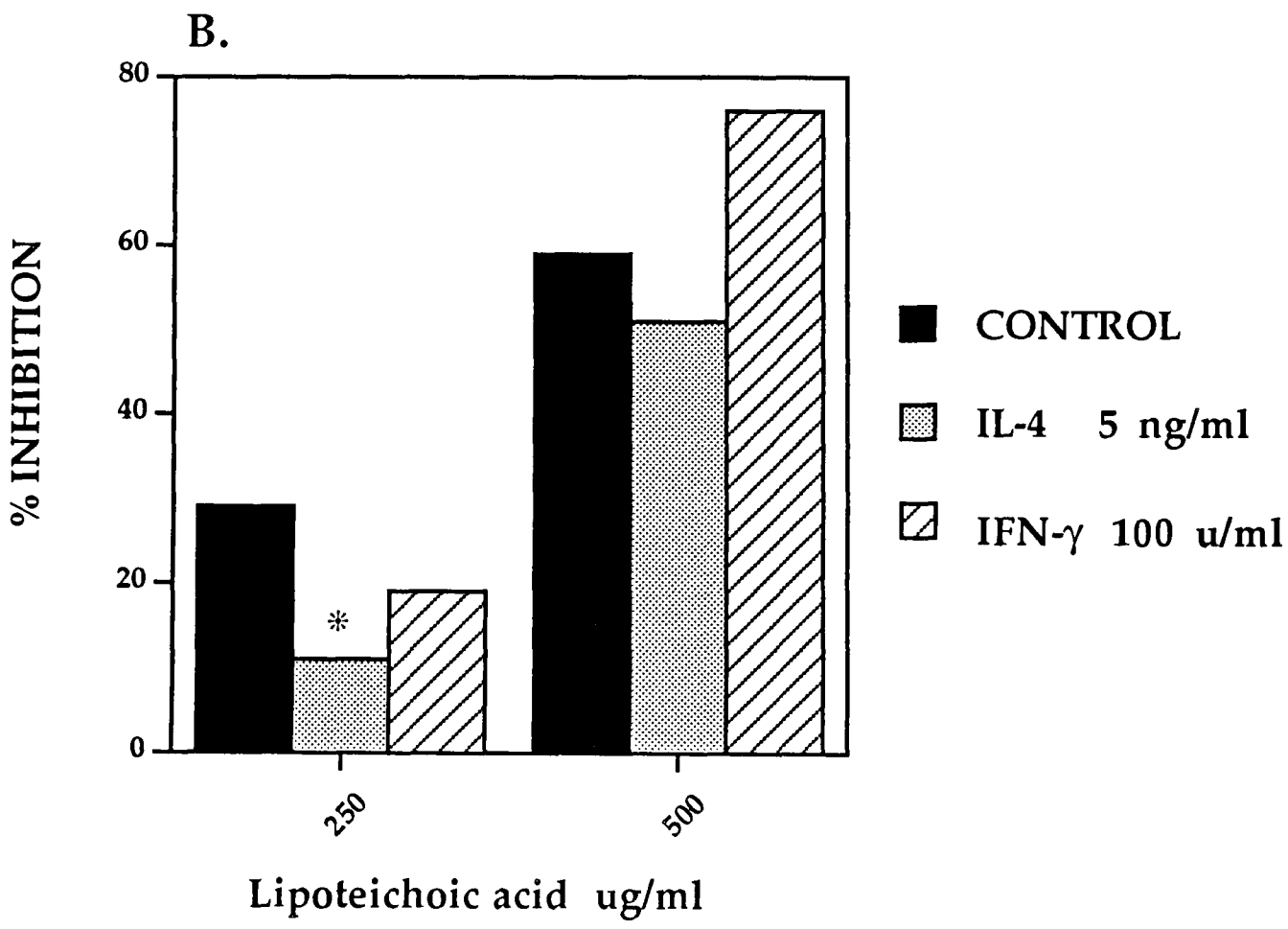


Fig. 4.12 **The inhibition by lipoteichoic acid of DiI-AcLDL uptake by Th1/Th2 cytokine treated BgPMϕ**

BgPMϕ were harvested, plated in 24 well TCP plates (3 x 10^6 cells/well), and treated *in vitro* for 48 hours as indicated. After washing in PBS, cells were pre-incubated for 30 min in RPMI plus 10% FCS containing lipoteichoic acid at the indicated concentrations followed by the addition of DiI-AcLDL at 5 µg/ml. After incubation for 3 hours at 37°C, cells were washed, harvested using EDTA and lidocaine, and resuspended in PBA for analysis by flow cytometry (FL2 photomultiplier). Results (**A.** and **B.**) are expressed as percentage inhibition of DiI-AcLDL uptake compared to cytokine-treated cells receiving no lipoteichoic acid. The values are derived from the mean (two independent experiments in duplicate) of specific fluorescence intensities obtained from subtracting geometric mean autofluorescence from the fluorescence obtained after uptake of DiI-AcLDL. * $P < 0.05$ vs. Control.

LIPOTEICHOIC ACID INHIBITION OF DiI-AcLDL UPTAKE BY IL-4 AND IFN-γ TREATED BgPMϕ





it is evident that IL-4 treated cells, incubated with poly I and then diI-AcLDL, were less prone to inhibition of AcLDL uptake than control and IFN-γ treated cells. Inhibition curves for fucoidan (fig. 4.11) and polyvinyl sulphate (not shown) similarly revealed less inhibition of diI-AcLDL uptake for IL-4 than for IFN-γ and control cells; the inhibition gradient for each cytokine treatment did not however appear different. The inhibition efficiency of lipoteichoic acid, a Gram-positive cell wall constituent and possible physiological MSR ligand (Dunne *et al.* 1994), was also assessed (fig. 4.12A and B). When the results were expressed as percentage inhibition of initial levels of AcLDL uptake, IL-4 treated BgPMϕ seemed to be more resistant to LTA inhibition. The difference between IL-4 and IFN-γ treated cells was more apparent at higher LTA concentrations (250 ug/ml) and 500 ug/ml (fig 4.12B). These inhibition studies, in general, did not yield impressive results and remain inconclusive. It still remains conceivable that IL-4 affects MSR glycosylation and, through steric interaction, its ligand binding properties; this could result in LTA being a less efficient ligand for the MSR endocytotic system. The effects of IL-4 on other "scavenger recptors" present on Mϕ can not however be dicounted.

4.6 Tissue expression of MSR reveals size heterogeneity

MSR expression *in vivo* in various murine tissues was examined by immunoprecipitating the antigen from organ lysates, which were corrected more or less for weight, followed by immunoblotting (fig. 4.13A and B). MSR was immunoprecipitated using mAb 2F8 which was also subsequently utilized as a digoxigenin-labeled primary antibody in Western blotting. Peroxidase-conjugated anti-digoxigenin $F(ab')_2$ fragments were used as secondary antibody and binding visualized by chemiluminescence. This approach was necessitated when excessive non-specific binding resulted from the direct blotting of organ lysates with mAb 2F8 and the use of peroxidase-conjugated anti-rat IgG as second antibody.

The liver expresses MSR strongly; this was somewhat surprising in view of the unimpressive positive immunohistochemical detection on Kupffer and sinusoidal endothelial cells previously described (Hughes *et al.* 1995). The expression seen in the large and small intestine (lamina propria Mϕ), spleen (red pulp and outer marginal zone Mϕ; marginal metallophils) and lymph node (subcapsular sinus and medullary cord Mϕ) corresponded with that reported, but the relative lack of expression in the bone marrow and lung (alveolar Mϕ) was unexpected. By size comparison to MSR types I and II transfected into CHO cells, the type II

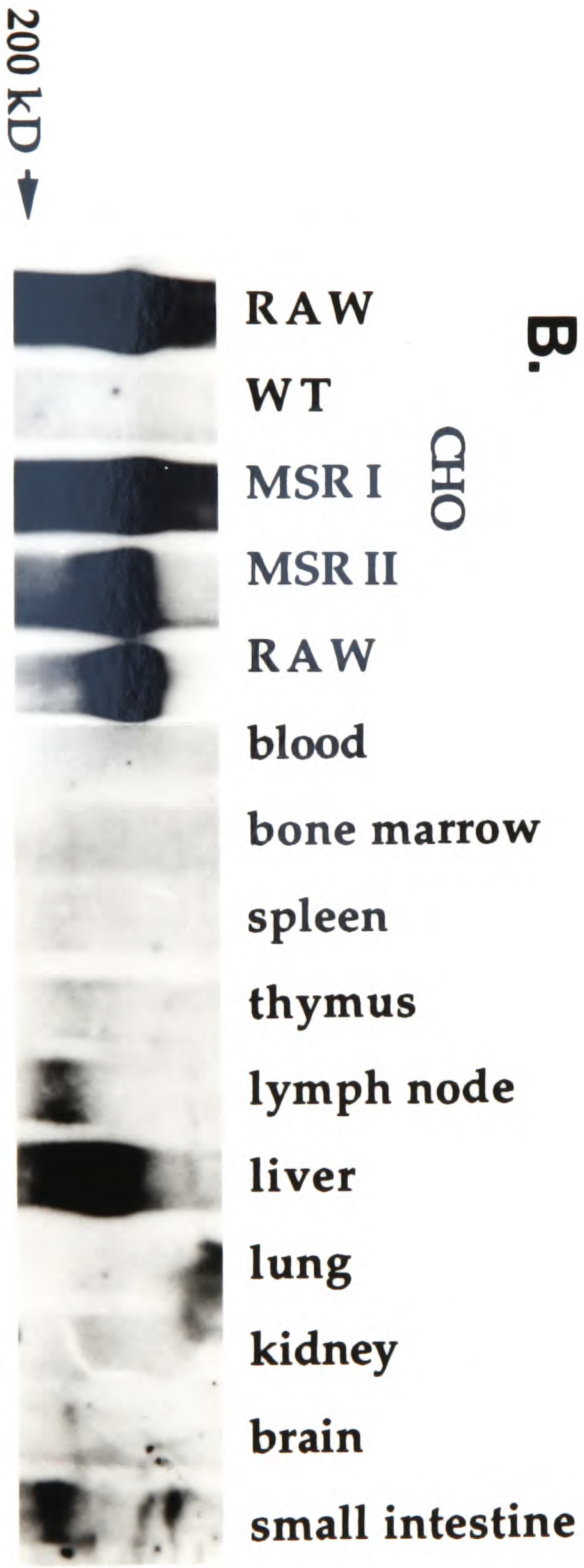
Fig. 4.13 A and B **Tissue-specific expression of MSR**

Prior to immunoprecipitation and immunoblotting, cells (RAW 264 and CHO transfectants) and murine tissues were lysed on ice using the same lysis buffer (150mM NaCl, 10mM EDTA, 10mM NaN_3, 10mM Tris (pH 8.0), 1mM PMSF, 5mM iodoacetamide and 1% Nonidet P-40). Organs were dissected from Balb/c mice, frozen in liquid nitrogen and kept at -70°C until required. Cells were suspended in lysis buffer (5×10^7 cells/ml) and tissues were homogenized in buffer (100mg/ml) by polytron. After 1 hour on ice lysates were spun for five minutes in a microfuge to remove nuclei, and then at 100 000 X g to remove debris. Supernatants were stored at 4°C before use and protein concentrations of lysates measured using the bicinchoninic acid (BCA) protein assay reagent kit. MSR was immunoprecipitated from cell and tissue lysates using mAb 2F8 and the samples separated by nonreducing 6% SDS-PAGE. Following transfer to nitrocellulose, digoxigenin-labeled mAb 2F8 was used as primary antibody for immunoblotting. Peroxidase-conjugated anti-digoxigenin $F(ab')_2$ fragments were used as secondary antibody and binding visualized by chemiluminescence. **A** and **B** depict results from two separate experiments.

A.


200 kD
CHO
RAW
WT
MSR I
MSR II
heart
lung
kidney
liver
spleen
small intestine
large intestine
muscle

B.


200 kD
CHO
RAW
WT
MSR I
MSR II
RAW
blood
bone marrow
spleen
thymus
lymph node
liver
lung
kidney
brain
small intestine

MSR protein seems to be the major form expressed *in vivo*. This agrees with the finding in the Mϕ-like cell lines RAW 264 and P388D1 (Via *et al.* 1992; Ashkenas *et al.* 1993; Fraser *et al.* 1993). Since the two types of MSR have non-identical binding properties (Ashkenas *et al.* 1993), the difference in expression levels may be of functional significance. Definitive determination of *in vivo* expression levels of the two types (by type-specific antibodies or in situ hybridization) would shed further light on this. The most striking finding, however, was the presence of even smaller forms of the MSR in small intestinal and lymph node tissues (two independent experiments). The existence of these isoforms of the MSR could be explained by tissue-specific differences in alternative splicing, thereby creating smaller variants, or in post-translational processing, resulting in tissue-specific glycoforms (perhaps increases in sialylation) and/or proteolytically cleaved surface receptors. The unexpectedly low levels of expression in bone marrow and lung will need further investigation; and a simple technical starting point could be to correct SDS-PAGE loading of organ lysates for protein concentrations rather than starting weight.

4.7 Discussion

In summary, MSR expression and function *in vitro* is, for the most part, divergently affected by the Th1/Th2 cytokines with IL-4 serving as an upregulatory stimulus and IFN-γ either maintaining or downregulating the receptor's status. Micro-heterogeneity in MSR structure is a major manifestation of these cytokine-mediated differences and results from changes in N-linked glycosylation, possibly sialylation. This micro-heterogeneity may be functionally relevant in affecting the sensitivity of MSR-mediated AcLDL uptake to inhibition by various MSR ligands, especially lipoteichoic acid. A further intriguing observation is the presence of structural heterogeneity in MSR expression *in vivo* in immunologically active locations.

It was previously reported that undefined supernatant from a stimulated Th2 clone had no effect on AcLDL degradation in resident murine peritoneal Mϕ (Fong *et al.* 1990). The contrast in findings may be explained by the use of different experimental systems, such as pure recombinant reagents rather than a crude mixture of cytokines from conditioned medium, and an elicited, more differentiated and cytokine-responsive peritoneal Mϕ population. IL-4 and IL-13 have been suggested to activate Mϕ in an alternative manner to classical activation by IFN-γ. This "alternatively" activated phenotype is characterized by enhanced expression of MMR and MHC class II (Stein *et al.* 1992; Doyle

et al. 1994). Upregulation of MSR expression and function should now be added as a further characteristic of this phenotype. The effects of IL-4 (and IL-13) on Mϕ would seem to be a dampening down of the immune/inflammatory response with the net result being the generation of a Mϕ phenotype which falls between the extremes of classical activation by IFN-γ and almost complete deactivation by IL-10 (Moore *et al.* 1993). These Mϕ would be in a reduced inflammatory state but have increased scavenging properties and abilities to curb microbial growth and present antigen while at the same time limiting damage to the host.

The results of studies describing regulation of MSR expression and activity by IFN-γ have been confusing. Interpretation has been complicated by differences in experimental approaches. Most recently, IFN-γ has been shown to decrease transcription of the MSR gene and AcLDL uptake in human monocyte-derived Mϕ (Geng and Hansson 1992). Studies with murine Mϕ, measuring AcLDL degradation following IFN-γ treatment, either showed no alteration (de Whalley and Riches 1991) or an inhibition of MSR functional activity (Fong *et al.* 1990). The authors of the latter report emphasise the minimal decrease in MSR protein levels (as measured by ligand binding studies) and postulate that IFN-γ primarily affects the rate of transport of MSR ligands to the lysosomes, without altering MSR expression levels. The findings described in this chapter that IFN-γ either maintained or minimally downregulated the MSR levels of expression and activity are consistent with these studies.

Changes in MSR glycosylation due to IL-4 and IL-13 could alter the structure of the spacer and α-helical coiled coil domains (i.e. those domains containing N-linked glycosylation sites) thereby subtly affecting ligand binding mediated by the adjacent collagenous domain of the receptor by steric interference. Experiments with lipoteichoic acid inhibition of AcLDL uptake suggest IL-4 does indeed affect MSR ligand binding properties. Although BgPMϕ adhesion remains unaffected, it is interesting to note (as described in Chapter 7) that the adhesion-mediating epitope of the MSR seems to be situated within the proximal glycosylated α-helical coiled coil domain rather than the collagenous domain. Some evidence was presented for the MSR to be sialylated (lectin blot) and IL-4 seemed to affect MSR α 2-3 linked sialic acids selectively. Analogous to this scenarion, modification of sialic acids has been described to regulate molecular and cellular adhesion (e.g. the binding by the bone marrow stromal Mϕ-restricted receptor, sialoadhesin, to specific sialylated glycoconjugates on sheep erythrocytes and haemopoietic cells (Crocker *et al.* 1991; Crocker *et al.* 1994)), to

increase the life span of sialoglycoconjugates and to influence the ligand specificity of sialic acid-specific receptors on mammalian cells and micro-organisms (e.g. the regulation of influenza C virus binding by 9-O-acetylated sialic acids) (Schauer 1991).

Different sialic acids occur in tissue-specific fashion and their relative amounts may vary according to the function and age of tissues. Thus, N-glycoloylneuraminic acid (Neu5Gc) or O-acetylated sialic acids are considered differentiation antigens in the rat intestine (Bouhours and Bouhours 1988), and in human colon cancer, Neu5Gc reappears and O-acetylated sialic acids are lost (Corfield *et al.* 1992). Although there is no direct supporting experimental evidence, it is tempting to speculate on the link beween IL-4 effect on Mϕ phenotype and MSR expression and function *in vitro* and the observed tissue heterogeneity. One can speculate that the smaller MSR forms in the small intestine and lymph nodes are differently glycosylated and sialylated; and that they act as markers for the significant presence of "alternatively" activated Mϕ in these locations. In these areas of continuous high antigenic load, tempering of the Mϕ inflammatory response may restrict self-damage. To answer these questions, MSR (and its sugars) will have to be purified from the relevant organs and biochemically and structurally characterized. Current studies are investigating the tissue-specific MSR forms in mice in which the IL-4, IFN-γ and IFN-γ receptor genes had been deleted by gene targeting and homologous recombination (Dalton *et al.* 1993; Huang *et al.* 1993; Kopf *et al.* 1993). The IL-4 "knockout" mice still, however, have IL-13 present which may, because of redundancy, mask the development of a significantly different Mϕ phenotype.

The production of IFN-γ and IL-4 by T lymphocytes in the atheromatous plaque micro-environment could influence the recruitment and retention of monocytes and subsequent accumulation of cholesteryl esters and foam cell formation. Apart from the modulation of MSR activity, other cellular processes important to atherogenesis such as initial monocyte recruitment, generation of modified LDL in the arterial wall and secretion of apolipoprotein E and reverse cholsterol transport may be affected. It has been reported that *in vivo* treatment with IFN-γ results in suppression of aortic atherosclerosis and arterial cholesterol deposition in cholesterol-fed rabbits (Wilson *et al.* 1990). The mechanism of this *in vivo* effect is unknown, but it has been suggested that IFN-γ (locally secreted by T lymphocytes in atherosclerotic lesions (Hansson *et al.* 1989)) down-regulates MSR expression and the transformation of Mϕ into foam cells. As discussed above, dissecting the mechanisms of IFN-γ regulation of MSR activity may be more complicated than initially described. In

addition, IFN-γ has also been reported to promote the adherence of Mϕ to the basement membrane glycoproteins laminin and type IV collagen (Shaw *et al* 1989).

Very litle work has been done on the role or presence of Th2 cytokines in atherosclerosis. As described in chapter 5, the actions of IL-10, another Th2 cytokine, differed from IL-4 and IL-13, and there was no apparent effect on MSR glycosylation and structural microheterogeneity. The upregulatory action of IL-4 (and IL-13) on MSR expression and AcLDL uptake may be considered pro-atherogenic. In support of a possible role in monocyte recruitment, IL-4 has been shown to induce expression of the integrin $\alpha_v\beta_3$ on BMDMϕ which enhances binding to its extracellular matrix ligand, vitronectin (Kitazawa *et al.* 1995). There is no *in vivo* evidence to suggest IL-4 promotes atherogenesis. Both IL-4 and IL-13 (in contrast to IFN-γ) enhance 15-lipoxygenase gene expression and enzyme activity; resulting in induction of an anti-inflammatory pathway by Th2 cytokines (Nassar *et al.* 1994). Several *in vitro* and *in vivo* studies showed a role for 15-lipoxygenase in the initiation of LDL oxidation in the arterial wall (Rankin *et al.* 1991; Yla-Herttuala *et al.* 1995). It may be argued that "alternatively" activated Mϕ (resulting from IL-4 and IL-13 actions) may have dualistic functions in atheromatous plaques: i) increasing LDL oxidation and scavenging abilities and ii) a protective or modulating role in which the chronic atherogenic inflammatory stimulus is down-regulated to limit tissue damage.

Chapter Five

The regulation of MSR expression and function by TGF-β, IL-10 and chemokines

5.1 Introduction

The terms growth factor and cytokine are often used interchangeably, although cytokines were originally considered to be mediators implicated in immunity and inflammation, and growth factors to be involved in cell proliferation and chemotaxis. These two roles are closely interrelated in atherosclerosis and the same mediator may sometimes act as an inflammatory and as a growth regulator, depending on its cellular target. None of these factors works alone in the atheromatous plaque micro-environment. It is probable that, through a network of cellular interactions, the release of one mediator leads to expression of another in a target cell that then either stimulates its neighbours in a paracrine or itself in an autocrine manner. Elucidation of their exact roles in atherogenesis provides opportunities for therapeutic exploitation.

Mϕ and foam cells secrete growth factors and cytokines (PDGF, M-CSF, TGF-β, IL-1 and TNF-α) which affect fibroproliferation directly or indirectly, and they also present antigen to T lymphocytes. The regulation of MSR function may contribute markedly to atherogenesis, both by influencing foam cell formation and by contributing to monocytes/Mϕ recruitment and retention (Fraser *et al.* 1993). The theme of MSR regulation by growth factors and cytokines is developed further in this chapter. The actions of the Mϕ deactivating cytokines, TGF-β and IL-10, are initially examined; this complements the work on IL-4 and IL-13 described in the previous chapter. In view of its possible role in adhesion, the effects on MSR function of chemotactic factors that mediate monocyte adherence and migration into the subendothelium are subsequently addressed. The actions of a number of other cytokines, growth factors, hormones and drugs are finally briefly summarized.

5.2 The effects of TGF-β and IL-10 on MSR expression and function

TGF-β is a 25K dimeric protein that not only regulates growth, but mediates far-ranging biologic processes including inflammation and host defence, in addition to development, tissue repair, and tumourigenesis. Released locally from platelet stores early in an inflammatory response, TGF-β is then generated by inflammatory cells themselves as part of the self-regulatory cytokine network. It both stimulates and inhibits the growth and differentiation of many different cell types; this is accounted for by the differential effect of TGF-β on resting and activated cells. Balance of TGF-β within an inflammatory lesion is critical as an excess is associated with fibrosis (Wahl, 1994; Massague, 1990).

IL-10 was originally identified as a cytokine synthesis inhibitory factor (CSIF) (Moore *et al.* 1993) and is secreted by Th1 and Th2 clones, as well as Mϕ and B cells; these cells also express IL-10 receptors. IL-10 is associated with the downregulation of IL-12 and IFN-γ and the development of a Th2-type immune response (D'Andrea *et al.* 1995). It is considered a general deactivator for Mϕ (Bogdan *et al.* 1991), and inhibits T cell proliferation, while supporting B cell growth. There are no studies showing increased IL-10 expression in atherosclerotic lesions; a potential role for IL-10 in modulating the atheromatous inflammatory response is however feasible, in view of the presence of numerous Mϕ and T lymphocytes in plaques and the high levels of autoregulating IL-10 produced by activated monocytes (de Waal Malefyt *et al.* 1991). The direct actions of TGF-β and IL-10 on Mϕ are documented in table 5.1.

TGF-β is generally not expressed in the normal artery, whereas it is upregulated in atherosclerotic plaques (Battegay *et al.* 1990); especially restenosis lesions following angioplasty (Nikol *et al.* 1992). Although TGF-β may be produced by all the cellular constituents of an atheroma (Ross, R 1993), atherosclerosis research has thus far neglected the possible impact of its actions on Mϕ (table 5.1) and has concentrated on its inhibition of VSMC migration and proliferation (Grainger *et al.* 1993) and potent stimulation of connective tissue formation. TGF-β also inhibits endothelial cell proliferation *in vitro;* in addition, it stimulates its own secretion (Ross 1993) and hemodynamic stimuli, such as fluid shear stress, induces endothelial TGF-β transcription and production (Ohno *et al.* 1995). TGF-β stimulates matrix formation by inducing gene expression for fibronectin, collagen types I, III, IV and V, tenascin,

Table 5.1 *In vitro* **effects of TGF-β and IL-10 on Mϕ**

	TGF-β	IL-10
Mϕ recruitment		
i) growth + differentiation	**bifunctional: immature - ↓ mature - ↑**	↓
ii) chemoattractant	↑	ND
iii) adhesion molecules	**LFA-1 VLA-3 VLA-5 CD31(PECAM-1)[1]** ↑	**CD11abc/CD18 CD14 CD54 (ICAM-1) CD58 (LFA-3)** ↔
Morphology	**"rounded" ↓ adherence to plastic**	**"rounded" ↓ adherence to plastic**
Phagocytosis		
i) CR3	↓	↔
ii) CD23 (FcεR2)	↓	↔
iii) FcγR	↑ **CD16 (FcγRIII)**	↑ **CD64 (FcγRI)**
iv) mannose receptor	ND	↔/↑
Sialoadhesin	ND	↔[2]
MHC class II	↓	↔/↓
B7-1 + B7-2	ND	↓
Pro-inflammatory cytokine production		
i) TNFα, IL-1, IL-6, IL-8	↓	↓
ii) IL-12	ND	↓
iii) IL-1ra, sIL-1r II	↑	↑
Respiratory burst	↓	↓
Nitric oxide	↓	↓
Extracellular Matrix formation and Proteolytic activity	↑ **uPAR**[3] ↑ **PAI-1/2** ↑ **TIMPs** ↓↑**collagenase** ↓ **tPA**	ND
Procoagulant activity	↓[4]	↓
apoE secretion	↓[5]	ND
5-lipoxygenase	↑[6]	ND
15-lipoxygenase	ND	↔[7]

Microbicidal activity	↓	↓
Tumouricidal activity	↔/↓	ND

(based for the main part on (Bogdan and Nathan 1993)); ND = not determined

1. (Lastres *et al.* 1994)
2. (Doyle *et al.* 1994)
3. (Lundgren *et al.* 1994)
4. (Jungi *et al.* 1994)
5. (Zuckerman *et al.* 1992)
6. (Brungs *et al.* 1995)
7. (Nassar *et al.* 1994)

thrombospondin, as well as glycosaminoglycans and proteoglycans (Massague 1990; Ross 1993); the expression of osteopontin and osteonectin, molecules associated with calcifying vascular cells is also enhanced (Watson *et al.* 1994). *In vivo* studies revealed that direct transfer of the TGF-β gene into porcine arteries stimulated fibrocellular hyperplasia (Nabel *et al.* 1993).

5.2.1 Protein and mRNA expression

BgPMϕ were treated for 48 hours with TGF-β and IL-10 and several experimental techniques used to characterize and quantify cytokine-mediated differences in MSR protein expression. Recombinant purified active human TGF-β, known to act on murine Mϕ, and murine IL-10 were used. Immunoblot analysis (fig. 5.1) was initially done as a screening assay; this showed a decrease in total MSR protein expression in TGF-β treated lysates, compared to control and IL-10 treated cells. This difference suggested divergent regulation of MSR expression by these two classical Mϕ deactivators. To quantify any cytokine-mediated differences, expression of MSR at a single cell level was determined by flow cytometry (table 5.2).

Table 5.2 Effect of TGF-β and IL-10 on MSR expression by flow cytometry

Treatment	*Surface*	*Internal*
Control	100	100
TGF-β	72±8*	42±4*
IL-10	52±6*	67±8*

BgPMϕ were treated in vitro for 48 hours as indicated and stained with either mAb 2F8 or CAMPATH-1G plus FITC-conjugated second antibody in the presence (internal) or absence (surface) of saponin before flow cytometry. Results are depicted as percentage of control values and are the means of two experiments (each experiment in triplicate) in

which specific fluorescence intensities were determined by subtracting geometric mean fluorescence obtained with CAMPATH-1G from that obtained with mAb 2F8. * $P < 0.05$ vs. Controls

TGF-β downregulated levels of MSR internal antigen to 42% relative to control, while IL-10 had a less marked effect (67%) in this assay. Cytokine-treated BgPMϕ were subsequently metabolically labeled overnight and MSR immunoprecipitated with 2F8 mAb. There were apparent differences in levels of MSR synthesis, and to quantify these effects the reduced monomer bands at ≈80kD were analyzed by PhosphorImager and the ImageQuant software program (table 5.3). The mAb 2F8 detects both type I and II isoforms of the MSR (Fraser *et al.* 1993) and, interestingly, TGF-β (42%) and IL-10 (52%) decreased MSR protein synthesis by a similar magnitude. In contrast to IL-4 and the findings described in Chapter 4, IL-10 as a Th2 cytokine had no effect on MSR microheterogeneity and the M_r of the reduced MSR monomer from treated cells remained similar to control BgPMϕ. Flow cytometry and protein synthesis studies showed TGF-β and IL-10 downregulated MSR expression to an equivalent degree; this contrasts with the Western blot analysis. This inconsistency is worrying and raises questions about assay sensitivity.

Table 5.3 Effects of TGF-β and IL-10 cytokines on MSR synthesis analyzed by PhosphorImager

Treatment	*Arbitrary PhosphorImager Units (APUs)*			*Percentage Control*
	1	**2**	**3**	
Control	13684	834569	22675	
TGF-β	8254	466115	13066	58 ± 2%
IL-10	6239	416103	ND	48 ± 3%

BgPMϕ were treated *in vitro* for 48 hours and metabolically labeled. Quantification of protein bands on dried gels was done by PhosphorImager and results expressed as a ratio of arbitrary APUs. Results are expressed as percentage of control (± s.d.) and are derived from three (TGF-β) and two (IL-10) independent experiments.

TGF-β decreased internal MSR antigen levels (table 5.2) to 42% of control, following saponin permeabilization of cells; this was not paralleled by the more modest decrease (72% of control) when surface

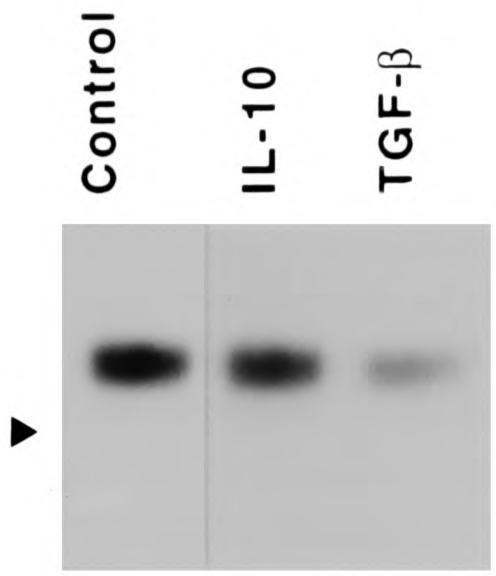
Fig. 5.1 (top - A.) **Immunoblot of MSR from TGF-β/IL-10 - treated BgPMϕ**

Day five BgPMϕ were harvested and treated *in vitro* with recombinant human TGF-β (5 ng/ml) and murine IL-10 (50units/ml) for 48 hours. Cells were lysed and protein concentration of lysates measured using the bicinchoninic acid Protein Assay Reagent kit. Equal amounts of total cellular protein (20 μg) were loaded, proteins separated by nonreducing 6% SDS-PAGE and transferred to nitrocellulose overnight. MAb 2F8 was diluted to 10 μg/ml followed by incubation with with peroxidase-conjugated anti-rat IgG antibody (1:1000) and detection by chemiluminescence.

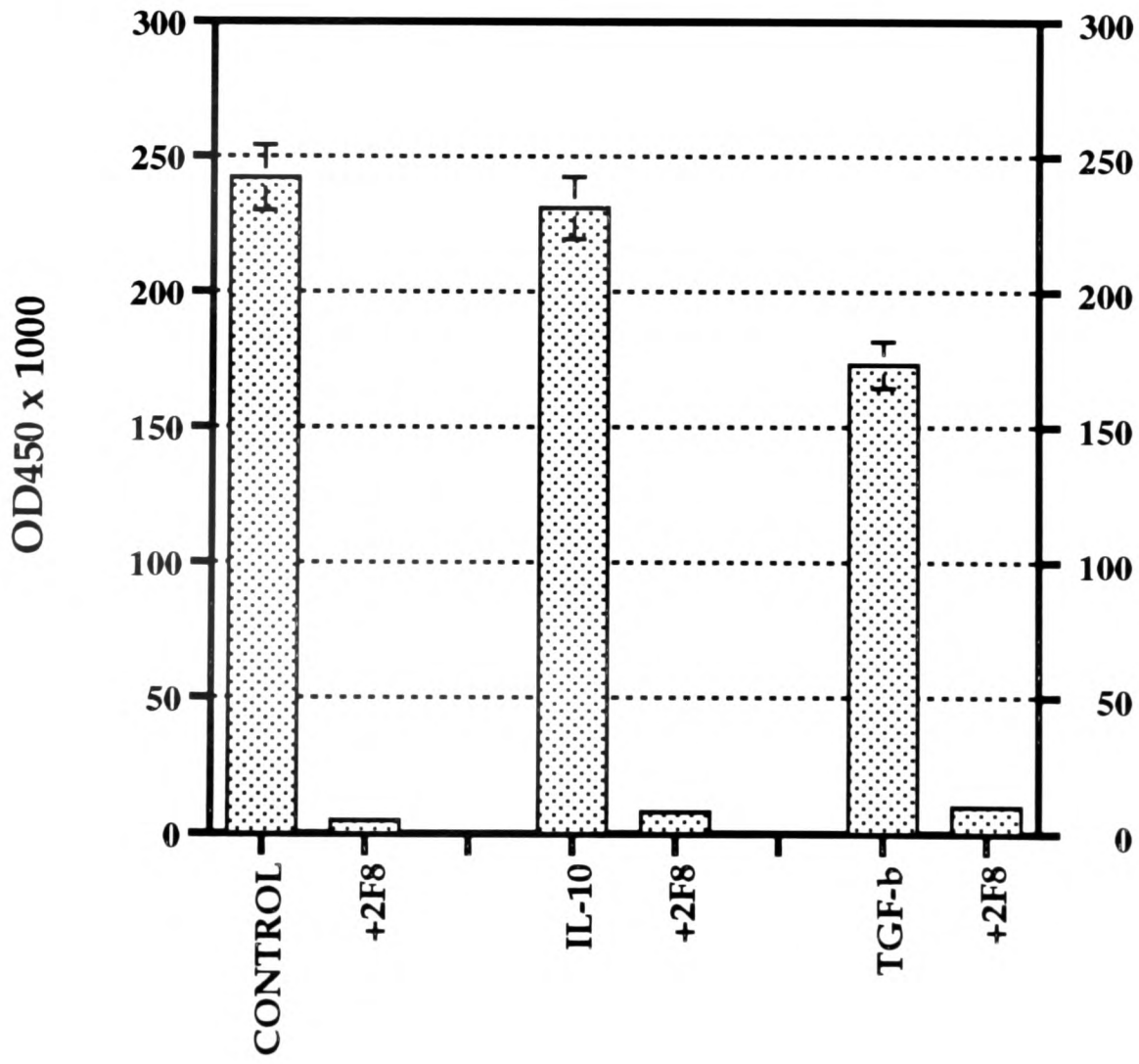
Fig. 5.2 (bottom - B.) **The effects of TGF-β/ IL-10 on MSR-dependent adhesion of BgPMϕ**

BgPMϕ were cultured in RPMI plus 10% FCS on tissue culture plastic (TCP) surfaces in the presence or absence of TGF-β (5ng/ml) and IL-10 (50units/ml) for 48 hours. Cell adhesion assay was performed as described in Chapter 2. EDTA was used at a final concentration of 5mM and mAb 2F8 was added at a final concentration of 2 μg/ml. After staining with Giemsa, plates were washed, dried, and the retained dye solubilised in methanol. Stain was quantified by measuring absorbance at 450nm in an automatic plate reader. Results (means ± s.d.) are derived from two experiments done in triplicate. $P < 0.05$ for TGF-β vs. Control.

A.



B. EDTA-RESISTANT ADHESION TO TCP



levels were assessed. Work described in Chapter 3 showed M-CSF treatment resulted in a shift of the cellular pool of MSR from a mainly intracellular location to a predominantly plasma membrane distribution (de Villiers *et al.* 1994). To determine whether TGF-β affects the distribution of MSR within the cell, the fraction of total MSR on the cell surface at steady state was biochemically quantified (table 5.4). This single experiment shows, that in addition to decreasing MSR protein synthesis, TGF-β is associated with a modest relative increase in the intracellular (61% of total) MSR pool compared to untreated controls (52% of total). If confirmed, this may suggest inhibition by TGF-β of MSR intracellular sorting and turnover. These steps, which determine functional MSR expression on the cell surface, may include processing in the Golgi apparatus, transport to plasma membrane, late endosomal ligand/receptor dissociation and receptor recycling to the cell surface.

Table 5.4 Effect of TGF-β on cell surface distribution MSR

Treatment	*Total*	*Surface*
Control	47 681	22 675
Percent surface/total	48%	
TGF-β	33 878	13 066
Percent surface/total	39%	

BgPMϕ were treated *in vitro* for 48 hours and metabolically labeled to equilibrium followed by surface biotinylation, on ice, by the membrane-impermeant probe sulfo-NHS-SS-biotin (Harter and Mellman 1992). After cell lysis, total MSR was precipitated by mAb 2F8 and the resulting immunoprecipitate divided into two equal aliquots. Biotinylated "surface" MSR was precipitated from one aliquot and "total" MSR was precipitated from the other aliquot by a second round of mAb 2F8 immunoprecipitation. Total and biotinylated MSR were quantitated after SDS-PAGE by PhosphorImager analysis of dried gels and results expressed as a ratio of arbitrary APUs.

The action of TGF-β and IL-10 on several other Mϕ integral membrane proteins was analyzed by flow cytometry (table 5.5) to assess the selectivity of the downregulatory action on MSR expression. Mϕ surface antigens other than MSR were unaffected by TGF-β downregulation; when intracellular (internal) levels were measured in permeabilized cells, TGF-β was also associated with a modest decrease in F4/80, CR3 and macrosialin levels. In addition to downregulating surface and internal MSR, IL-10 decreased surface levels of CR3 and

macrosialin, as well as internal macrosialin. Surprisingly, internal CR3 and class II MHC were increased, possibly indicating an IL-10 mediated block in intracellular transport of these antigens to the cell surface. These results suggest that TGF-β may be associated with a more selective downregulation of MSR expression than IL-10 which had a more general inhibitory effect on the Mϕ membrane proteins assayed.

Table 5.5 Effects of TGF-β and IL-10 on expression of Mϕ differentiation antigens by flow cytometry

	Surface			*Internal*		
Mϕ antigen	Contr	TGF-β	IL-10	Contr	TGF-β	IL-10
MSR	20	14	7	161	72	97
F4/80	25	28	ND	49	39	ND
CR3	15	19	9	59	33	72
Class II MHC	30	ND	27	45	ND	76
Macrosialin	20	20	14	303	266	231

BgPMϕ were stained with the indicated mAb or its isotype control plus FITC-conjugated second antibody before analysis by flow cytometry. The specific mAbs used were F4/80, 5C6 (*CR3*), TIB120 (*class II MHC*), and FA-11 (*macrosialin*). Results show the mean values (single experiment in triplicate) of specific fluorescence intensities determined by subtracting geometric mean fluorescence obtained with the isotype control from that obtained with the specific mAb.

MSR mRNA changes in response to TGF-β and IL-10 treatment were investigated by semi-quantitative RT-PCR. No differences could be detected in either MSR mRNA as a whole (MSR-C) or mRNA specific for types I and II MSR (results not shown) and the ratios of type I to type II mRNA levels remained unchanged. Cytokine-induced changes in MSR expression are thus most likely due to effects on posttranscriptional regulation.

5.2.2 The effects of TGF-β and IL-10 on MSR function

The significance of these differences in expression was tested by relating them to functional MSR studies. In the first instance, the ability of treated BgPMϕ to endocytose and accumulate fluorescent AcLDL (DiI-AcLDL) was assessed. Interestingly, neither TGF-β nor IL-10 (three separate experiments in triplicate) had any effect on AcLDL uptake in BgPMϕ relative to untreated controls (results not shown). In contrast,

TGF-β inhibited the MSR-dependent (divalent cation-independent, mAb 2F8-inhibitable) adhesion of treated BgPMϕ to serum-coated TCP by 28% (fig. 5.2); this figure corresponded surprisingly well to the decrease seen in surface MSR expression by TGF-β (table 5.2). These disparate effects of TGF-β on endocytic AcLDL uptake and MSR-dependent adhesion were surprising and hard to explain. A possible hypothesis may be differing sensitivities to regulation of the endocytic and adhesive properties of the MSR. The inability of IL-10 to influence MSR function, despite effecting a decrease in MSR expression, may similarly be a reflection of the functional reserve of the Mϕ scavenging system.

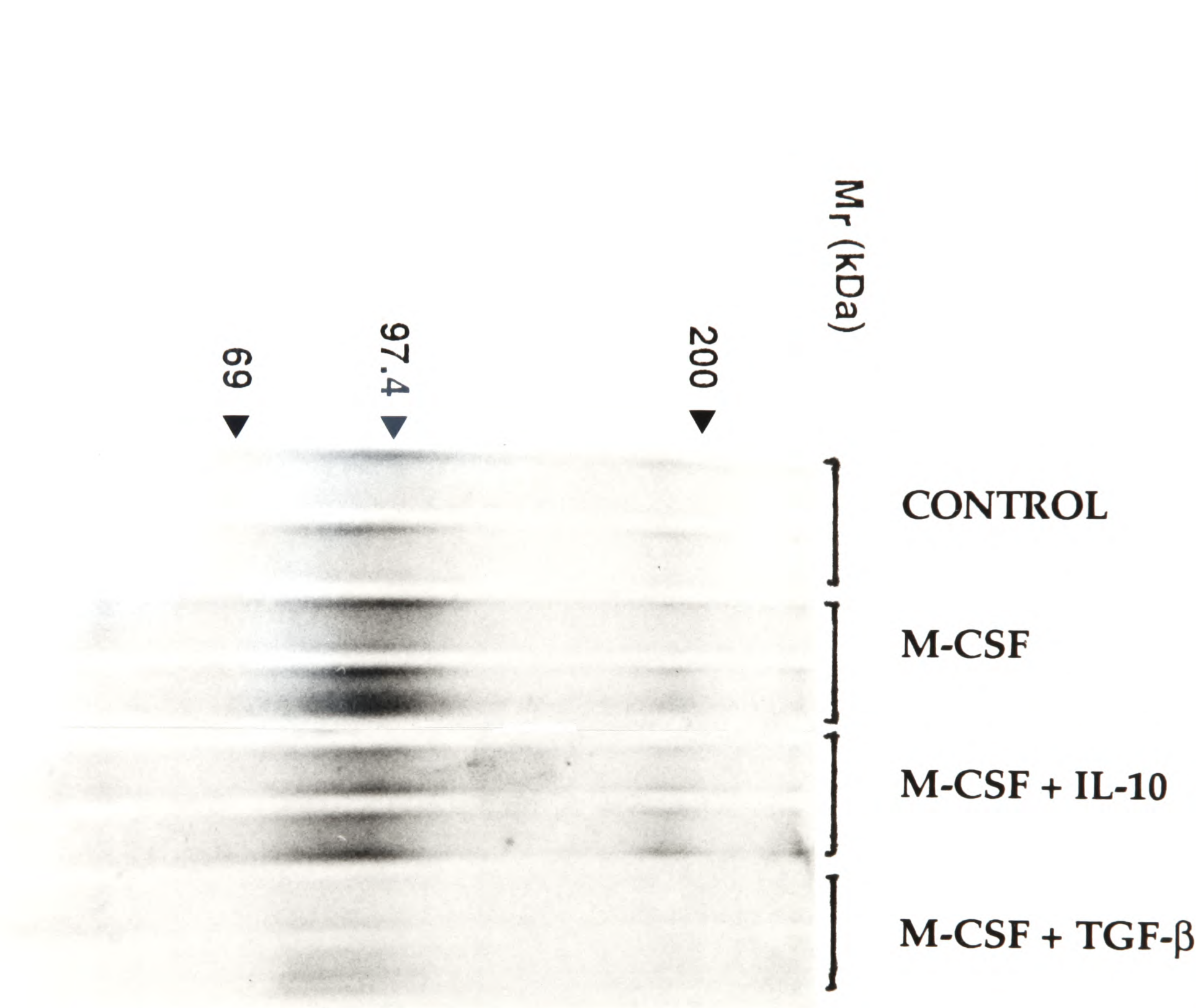
5.2.3 The effects of TGF-β and IL-10 on RAW 264.7 cells and M-CSF stimulated BgPMϕ

In general TGF-β and IL-10 had very modest effects on MSR function in unstimulated BgPMϕ. To gain further insight into possible inhibitory actions, other cell systems were explored. The Mϕ-like cell line RAW 264.7 expresses abundant MSR (predominantly the type II isoform) and takes up DiI-AcLDL avidly (Fraser *et al.* 1993). IFN-γ and LPS activate RAW 264.7 cells, and pre-treatment with TGF-β inhibits both their tumouricidal activity and ability to produce TNF-α, nitric oxide (NO) and prostaglandin E_2 in response to LPS (Haak-Frendscho *et al.* 1990; Hausmann *et al.* 1994; Reddy *et al.* 1994). The cell line also responds to mycobacterial cell wall components by producing pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Adams and Czuprynski 1994).

As described in Chapter 3, M-CSF selectively and potently upregulates MSR expression and function in BgPMϕ. M-CSF however had no further enhancing action on MSR expression in RAW 264.7 cells (assessed by immunoblotting), nor were AcLDL uptake or MSR-dependent adhesion further increased (results not shown). As MSR seems constitutively upregulated in RAW 264.7 cells, the possible inhibitory actions of the deactivating cytokines (TGF-β, IL-10 and IL-4) were examined in this system where any negative effects would presumably be more readily apparent. Flow cytometry, however, showed MSR surface and internal antigen levels to remain constant compared to control cells; AcLDL uptake and MSR-dependent adhesion were similarly unaffected (results not shown). MSR expression and function in RAW 264.7 cells therefore seem to be both upregulated and resistant to inhibitory cytokines; the underlying mechanisms for both these phenomena are unclear and need clarification.

Fig. 5.3 **Immunoprecipitation of MSR from BgPMϕ treated with M-CSF, M-CSF + TGF-β and M-CSF + IL-10**

Day five BgPMϕ were harvested and treated *in vitro* with the indicated cytokines for 48 hours (final concentrations: M-CSF = 1000units/ml; TGF-β = 5ng/ml; IL-10 = 50units/ml). Cells were then metabolically labelled by overnight incubation with 50 μCi/ml Tran^{35}S, lysed and subjected to immunoprecipitation with mAb 2F8 as described. Immunoprecipitates were separated by 7.5% SDS-PAGE (equivalent cell protein per lane) in the presence of β-mercaptoethanol, fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Duplicate samples are shown from a single experiment.


Mr (kDa)
200
97.4
69
CONTROL
M-CSF
M-CSF + IL-10
M-CSF + TGF-β

The effects of TGF-β and IL-10 on upregulated MSR expression in M-CSF treated BgPMϕ were next examined. It is apparent from fig. 5.3 that the M-CSF mediated increase in MSR synthesis is markedly inhibited by the addition of TGF-β compared to IL-10. PhosphorImager analysis was used to quantify these differences (table 5.6).

Table 5.6 Effects of TGF-β and IL-10 on M-CSF induced MSR synthesis

Treatment	*Arbitrary PhoshorImager Units (APUs)*	*Percentage Control*
Untreated	18804±2435	24
M-CSF	78627±8632	100
M-CSF + TGF-β	17129±2697	22
M-CSF + IL-10	62383±5328	79

BgPMϕ were treated *in vitro* for 48 hours and metabolically labeled. Quantification of protein bands on dried gels was done by PhosphorImager and the means of duplicates expressed as a ratio of arbitrary APUs. Results are expressed as percentage of M-CSF treated cells and are derived from a single experiment.

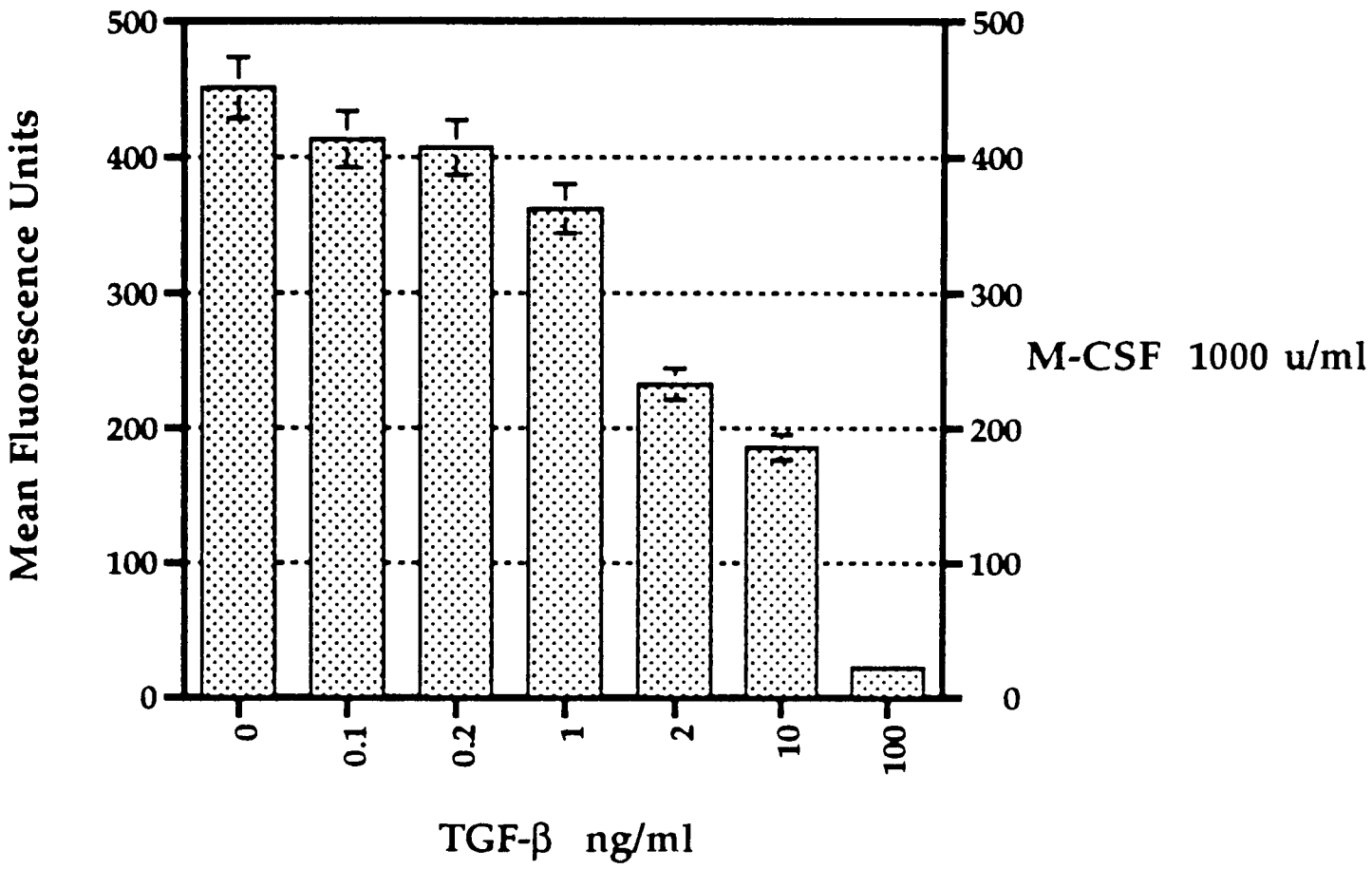
TGF-β was associated with a marked inhibition (78%) of the M-CSF induced increase in MSR synthesis, while the IL-10 effect (21%) was much less apparent. This suggests a significant difference between TGF-β and IL-10 as far as modulation of MSR expression is concerned; and may be an example where the differing abilities in overcoming an activating stimulus (M-CSF) have important implications for determining Mϕ phenotype. Thus, as far as expression is concerned, TGF-β, but not IL-10, readily inhibits M-CSF stimulated MSR. It is important to note that equal amounts of cellular protein were loaded before SDS-PAGE separation, as TGF-β has been reported to inhibit the growth response of murine peritoneal exudate Mϕ to M-CSF (Strassmann *et al.* 1988; Fan *et al.* 1992).

TGF-β was subsequently also shown to inhibit strongly the M-CSF-induced increase in DiI-AcLDL uptake in BgPMϕ in a dose-dependent manner (fig. 5.4A). Through a range of TGF-β concentrations, similar to that usually used in *in vitro* studies, percentage uptake of control decreased incrementally from 80% (1ng/ml) to 52% (2ng/ml) and 41% (10ng/ml). IL-10, in contrast, had no such inhibitory dose-dependent effect on AcLDL uptake (fig. 5.4B). These findings thus correlate with the MSR protein synthesis data

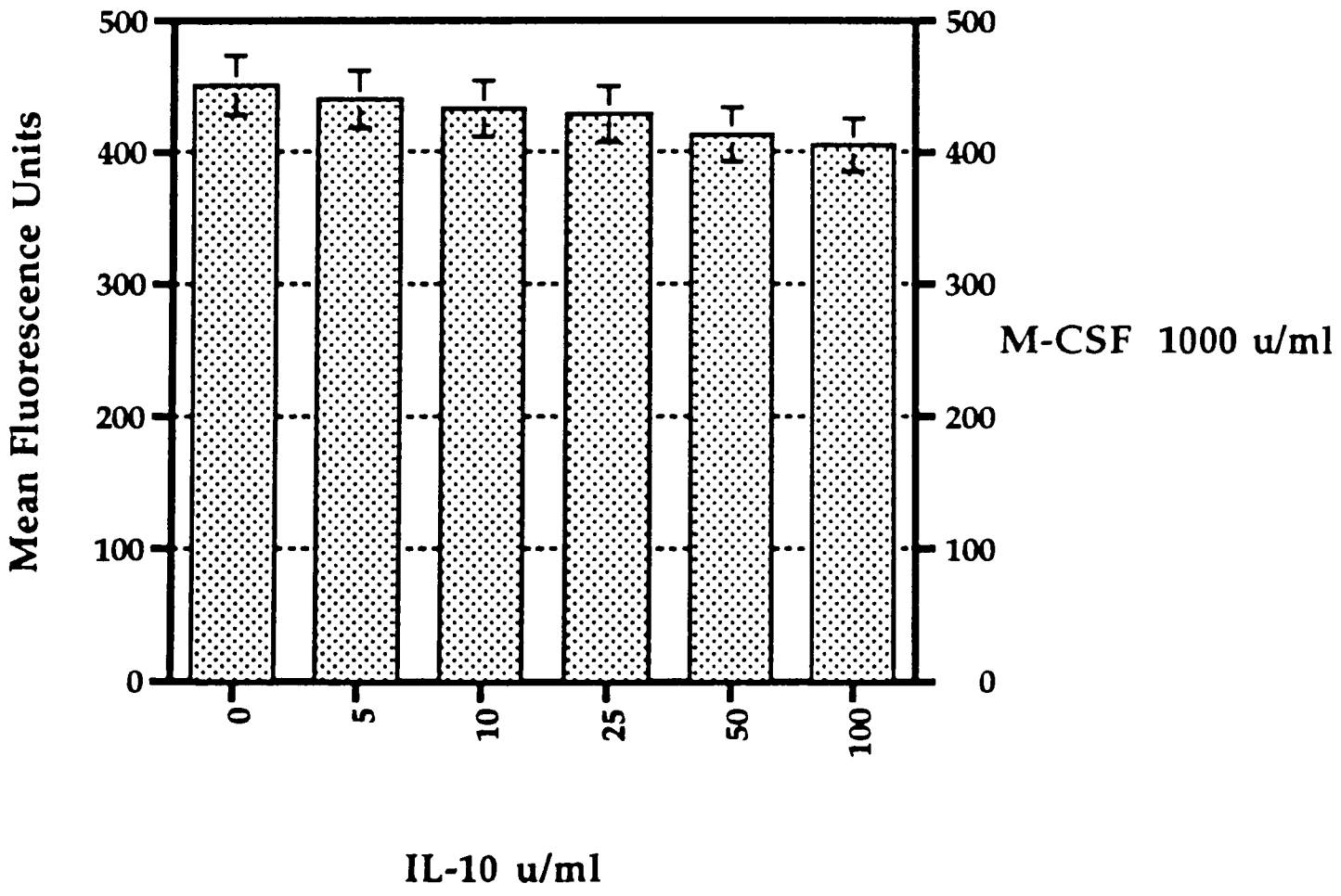
Fig. 5.4. **The inhibition of M-CSF induced DiI-AcLDL uptake by TGF-β (A) and IL-10 (B) in BgPMϕ**

BgPMϕ were harvested, plated in 24 well TCP plates (3 x 10^6 cells/well), and treated *in vitro* for 48 hours with M-CSF (final concentration = 1000units/ml) and the indicated doses of TGF-β and IL-10. After washing in PBS, DiI-AcLDL at 5 μg/ml was added. After incubation for 3 hours at 37 °C, cells were washed, harvested using EDTA and lidocaine, and resuspended in PBA for analysis by flow cytometry (FL2 photomultiplier). Results (**A. TGF-β** and **B. IL-10**) show the mean values (two independent experiments in duplicate) of specific fluorescence intensities obtained by subtracting geometric mean autofluorescence from the fluorescence obtained after uptake of DiI-AcLDL. $P < 0.05$ for TGF-β concentrations ≥ 1ng/ml.

TGF-β INHIBITION OF M-CSF INDUCED DiI-AcLDL UPTAKE



IL-10 INHIBITION OF M-CSF INDUCED DiI-AcLDL UPTAKE



described above, and support the notion of an upregulating stimulus being required to unmask the potent inhibitory action of TGF-β on MSR-mediated AcLDL uptake.

5.2.4 The effects of endogenous TGF-β on MSR

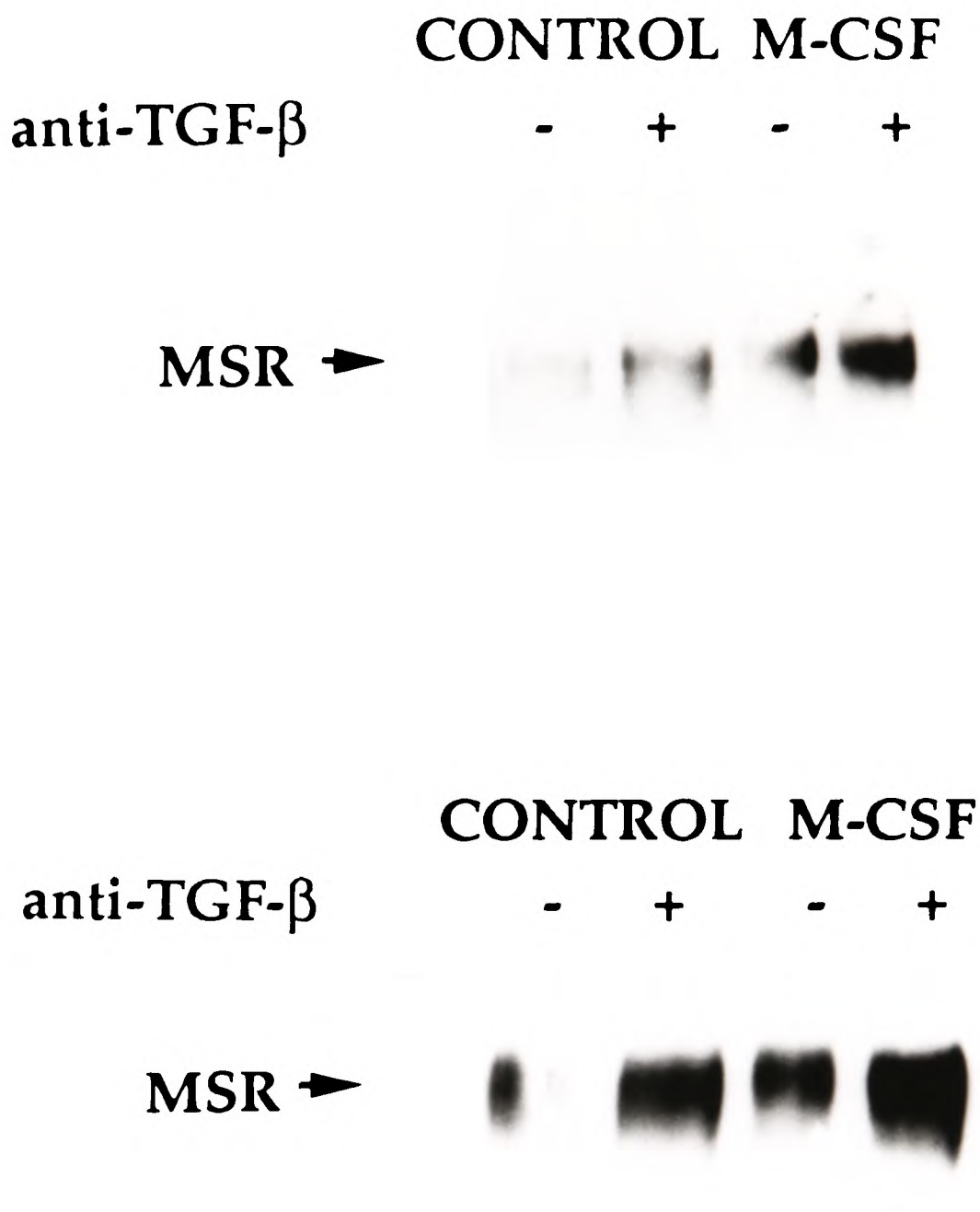
TGF-β and IL-10 not only act on Mϕ, but are produced by them as well. Resting human monocytes and alveolar and rat peritoneal Mϕ express TGF-β1 mRNA constitutively, although little or no TGF-β is produced without further stimulation (Assoian *et al.* 1987). Monocytes/Mϕ, stimulated with LPS *in vitro,* or isolated from synovium of rheumatoid arthritis patients, secrete significant amounts of latent, inactive or bioactive TGF-β protein, whereas mRNA levels remained unchanged (Wahl *et al.* 1990). Paracrine production and secretion of active TGF-β by Mϕ may control the activation of neighbouring Mϕ in the micro-environment and influence fibrosis during tissue repair (Wahl 1994) as well as SMC proliferation during atherogenesis (Ross 1993). The importance of Mϕ as a source of paracrine growth factors during tissue repair *in vivo* is demonstrated by the suppression of fibrosis in wound repair when monocyte infiltration is blocked (Wahl 1994). IL-10 does not affect TGF-β mRNA expression in LPS-stimulated human monocytes (Wahl *et al.* 1990), but effects on TGF-β secretion and activation have not yet been investigated.

The hypothesis was tested that endogenously produced active TGF-β and IL-10 influence the BgPMϕ phenotype; this could result in a constitutively deactivated state wherein any further inhibitory actions of added TGF-β and IL-10 on MSR regulation may be masked. Neutralizing antibodies (anti-murine IL-10 and anti-human TGF-β (which recognizes bioactive TGF-β and is known to be species cross-reactive)) were thus added to treated cells; CAMPATH-1G isotype antibody was used as mock treatment to control for signalling effects through Fc receptors. Immunoblot analysis showed a marked increase in total MSR protein following the addition of anti-TGF-β (fig. 5.5A); the suppressive effects of endogenous TGF-β was such, that once neutralized, MSR expression virtually reached those levels induced by M-CSF. The M-CSF induction of MSR was also further enhanced when the downregulatory action of endogenous TGF-β was removed. In contrast, anti-murine IL-10 had no enhancing effect on total MSR protein (fig. 5.5B); arguing against an important autoregulatory role for paracrine IL-10 in MSR regulation. Further investigations concentrated on the role of endogenous TGF-β in this system.

Fig. 5.5 **Immunoblot of MSR from BgPMϕ treated with M-CSF and anti-TGF-β and anti-IL-10 antibodies**

BgPMϕ were treated *in vitro* with recombinant human M-CSF (1000units/ml) and anti-TGF-β (5 μg/ml) or anti-IL-10 (10 μg/ml) for 48 hours. Cells were lysed and protein concentration of lysates measured using the bicinchoninic acid Protein Assay Reagent kit. Equal amounts of total cellular protein (20 μg) were loaded, proteins separated by nonreducing 6% SDS-PAGE and transferred to nitrocellulose overnight. MAb 2F8 was diluted to 10 μg/ml followed by incubation with peroxidase-conjugated anti-rat IgG antibody (1:1000) and detection by chemiluminescence. In **A.** examples from two independent anti-TGF-β experiments are shown and **B.** depicts the results from one anti-IL-10 experiment

A.


CONTROL M-CSF
anti-TGF-β - + - +
MSR
CONTROL M-CSF
anti-TGF-β - + - +
MSR

B.

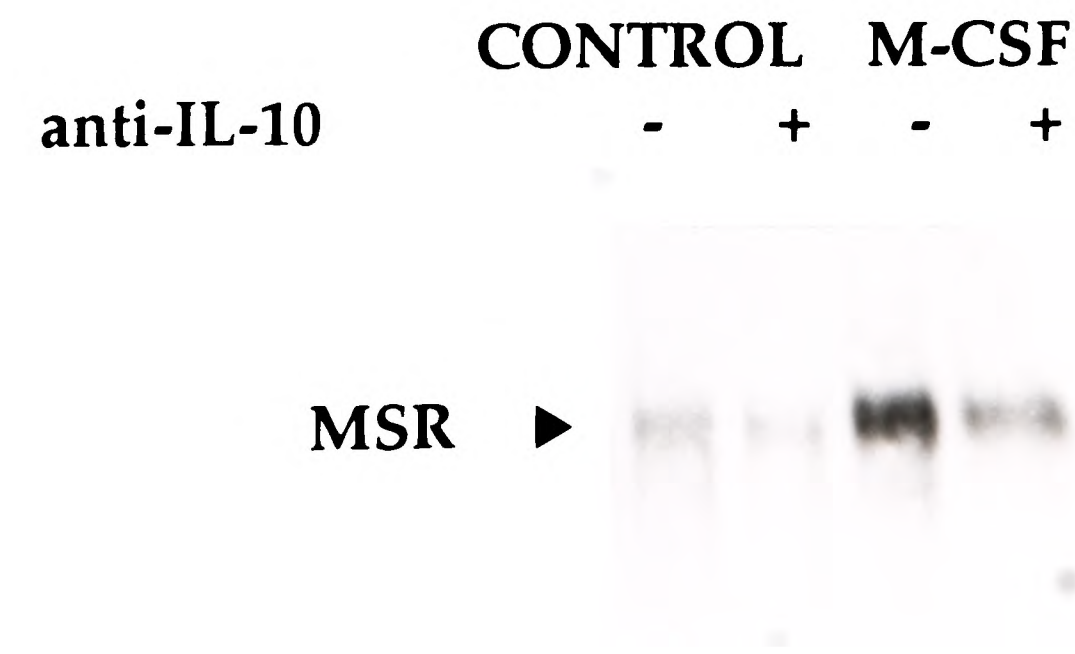

CONTROL M-CSF
anti-IL-10 - + - +
MSR

Fig. 5.6 (top) **Effects of M-CSF and anti-TGF-β on MSR expression by flow cytometry**

BgPMϕ were treated in vitro for 48 hours as indicated and stained with either mAb 2F8 or CAMPATH-1G plus FITC-conjugated second antibody before flow cytometry. Internal antigen levels were determined after cell permeabilization with saponin. Results are depicted as mean fluorescence units and are the means of two experiments (each experiment in triplicate) in which specific fluorescence intensities were determined by subtracting geometric mean fluorescence obtained with CAMPATH-1G from that obtained with mAb 2F8. * $P < 0.01$ vs. Surface Control; • $P < 0.01$ vs. Surface Control; # $P < 0.05$ vs Surface M-CSF; $ $P < 0.0001$ vs Internal Control; ¶ $P < 0.05$ vs Internal M-CSF.

Fig. 5.7 (bottom) **The effect of anti-TGF-β on M-CSF, IL-4 and IFN-γ induced DiI-AcLDL uptake by BgPMϕ**

BgPMϕ were plated in 24 well TCP plates (3×10^6 cells/well), and treated *in vitro* for 48 hours with M-CSF (1000units/ml), IL-4 (5 ng/ml), IFN-γ (100units/ml) and anti-TGF-β antibodies (5μg/ml). Controls received CAMPATH-1G (10μg/ml) as isotype control. After washing in PBS, DiI-AcLDL at 5 μg/ml was added. After incubation for 3 hours at 37°C, cells were washed, harvested using EDTA and lidocaine, and resuspended in PBA for analysis by flow cytometry (FL2 photomultiplier). Results show the mean values (two independent experiments in duplicate) of specific fluorescence intensities obtained by subtracting geometric mean autofluorescence from the fluorescence obtained after uptake of DiI-AcLDL. * $P < 0.01$ vs. Control; • $P < 0.05$ vs. M-CSF; # $P < 0.01$ vs. IL-4; § $P < 0.05$ vs. IFN-γ

Fig. 5.6

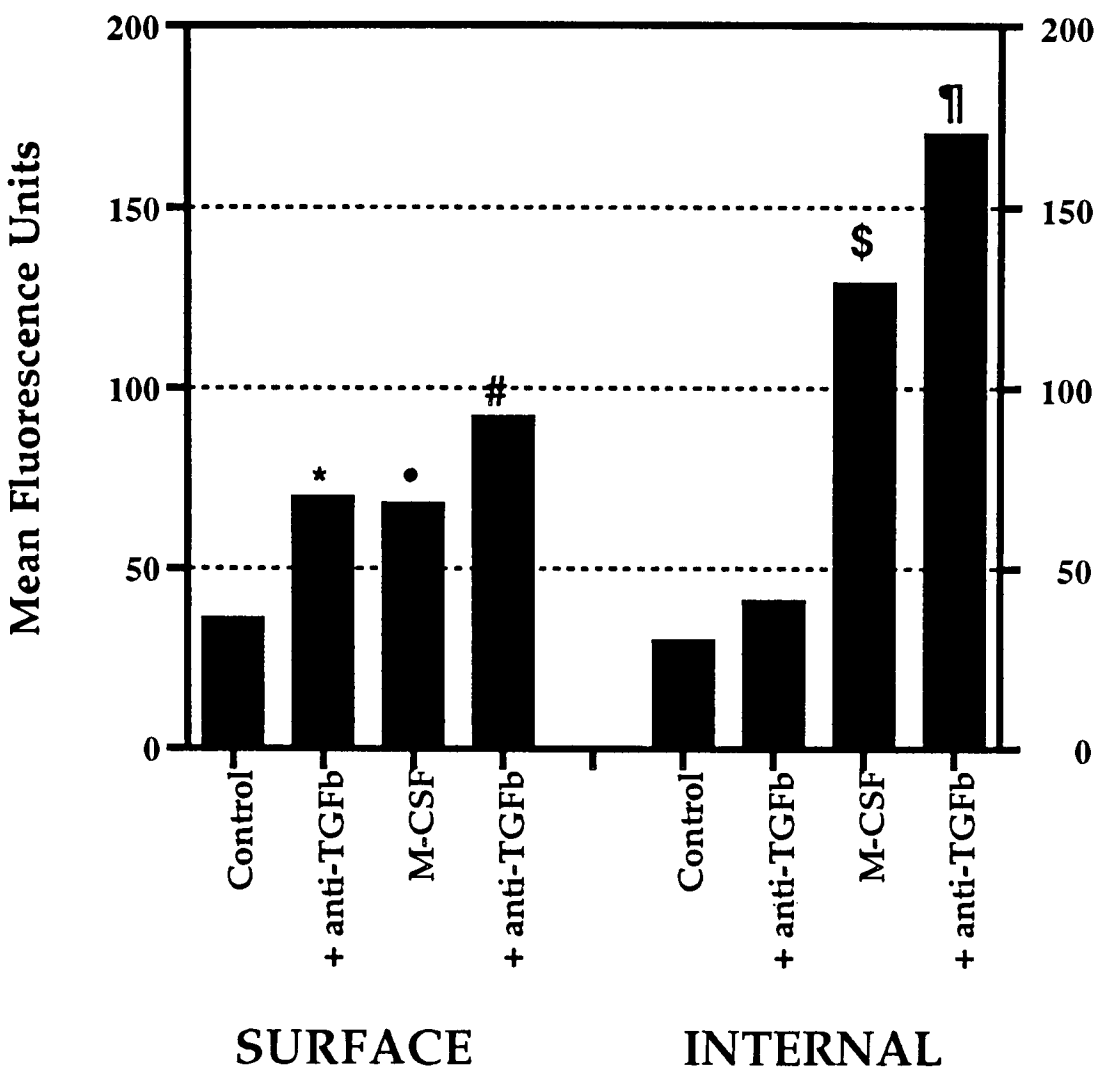
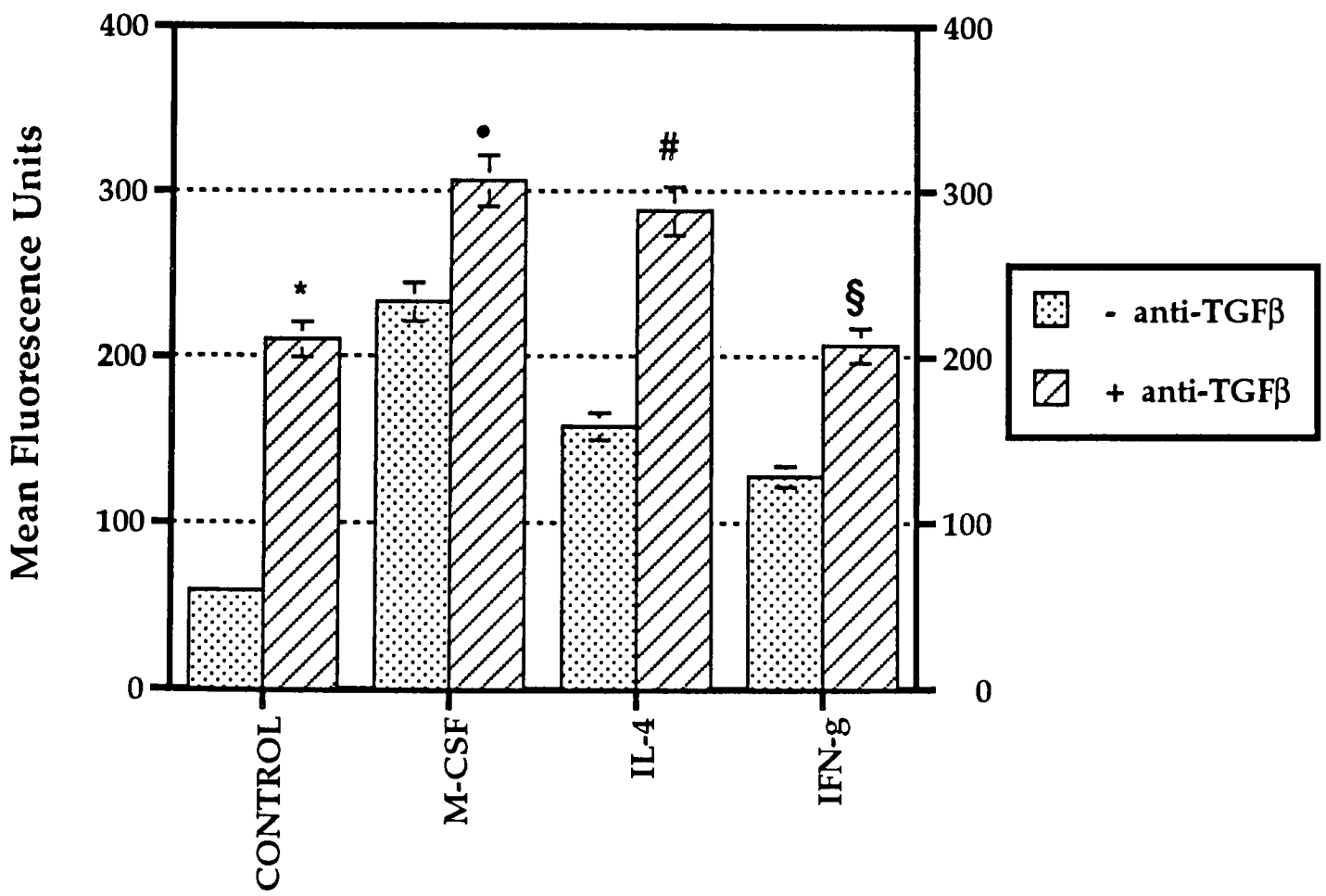

Effects of M-CSF and
anti-TGF-β on MSR levels
Mean Fluorescence Units
200
150
100
50
0
*
•
#
$
¶
Control
+ anti-TGFb
M-CSF
+ anti-TGFb
Control
+ anti-TGFb
M-CSF
+ anti-TGFb
SURFACE
INTERNAL

Fig. 5.7


THE EFFECT OF ANTI-TGFβ
ON DiI-AcLDL UPTAKE
Mean Fluorescence Units
400
300
200
100
0
*
•
#
§
- anti-TGFβ
+ anti-TGFβ
CONTROL
M-CSF
IL-4
IFN-g

The effect of anti-TGF-β on surface and internal MSR antigen was next assessed (fig. 5.6). The elevating action of anti-TGF-β was consistently present; and surface levels mirrored the result obtained with immunoblotting i.e. removal of endogenous TGF-β raised MSR levels to that induced by M-CSF. In this culture system TGF-β therefore proved as potent an inhibitor of MSR as M-CSF is an upregulator. There was only a modest increase in internal MSR on the addition of anti-TGF-β to control cells. This may suggest endogenous TGF-β has a primarily post-transcriptional role in affecting MSR cellular distribution, inhibiting transport to the plasma membrane. MSR immunoprecipitation by mAb 2F8 from metabolically labeled BgPMϕ after 48 hours of threatment, also showed only modest increases in MSR protein synthesis on addition of anti-TGF-β (table 5.7). This may suggest endogenous TGF-β has its maximal inhibitory action earlier on during the culture period prior to metabolic labeling, or alternatively, that the increase in surface expression is indeed a truly post-translational redistributive event.

Table 5.7: Effects of anti-TGF-β on M-CSF-induced MSR synthesis analyzed by PhosphorImager

Treatment	*Arbitrary PhosphorImager Units (APUs)*	*Percentage Control*
Control	32483	
+ anti-TGF-β	38221	+ 18%
M-CSF	44952	+ 38%
+ anti-TGF-β	50530	+ 56%

BgPMϕ were treated *in vitro* for 48 hours and metabolically labeled. Quantification of protein bands on dried gels was done by PhosphorImager and results expressed as a ratio of arbitrary APUs. Results are expressed as percentage of control and are derived from a single experiment.

The importance of endogenous TGF-β as a modulator of MSR function was underlined when DiI-AcLDL uptake was investigated (fig. 5.7). The inhibition of active TGF-β led to further increases in AcLDL uptake by BgPMϕ treated by M-CSF, IL-4 and IFN-γ. The increase noted in control cells confirms the potency of endogenous TGF-β as an inhibitor of MSR at a functional level. As discussed in Chapter 4, IL-4 upregulates MSR AcLDL uptake, and this action is further enhanced by removal of endogenous inhibitory TGF-β. Importantly, however, a

further increase in MSR-dependent (EDTA-resistant, mAb 2F8-inhibitable) adhesion of BgPMϕ to TCP surfaces could bot be elicited when anti-TGF-β was added to M-CSF treated cells (results not shown); this was another example of the difficulty in correlating MSR expression to MSR-mediated Mϕ adhesion and as already stated, could either reflect the sensitivity of the assay or differences in the extent of regulation of the endocytic and adhesive properties of the MSR.

In an attempt to mimic Mϕ in the atheromatous plaque micro-environment, BgPMϕ in culture were transformed into lipid-filled foam cells by adding 10-20% normal mouse serum to the medium for 24-48 hours; this results in the unregulated MSR-mediated uptake of lipoprotein and cholesterol (Brown and Goldstein 1983). MSR expression was analyzed by immunoblot (fig. 5.8), and in accordance with accepted dogma the addition of a lipid load did not upregulate MSR protein. Lipid-filled foam cells were however still responsive to MSR regulation and both the addition of M-CSF and inhibition of endogenous TGF-β increased total expression. When the constitutively upregulated MSR system in RAW 264.7 cells was examined, the addition of anti-TGF-β to cultures for 48 hours resulted in an increase in DiI-AcLDL uptake of 51 ± 15% relative to untreated cells (four separate experiments in collaboration with J. Major and D.R. Greaves). This is a further suggestion of both the potency of TGF-β as an inhibitor and the possible relevance of endogenous TGF-β as a physiological modulator of MSR function.

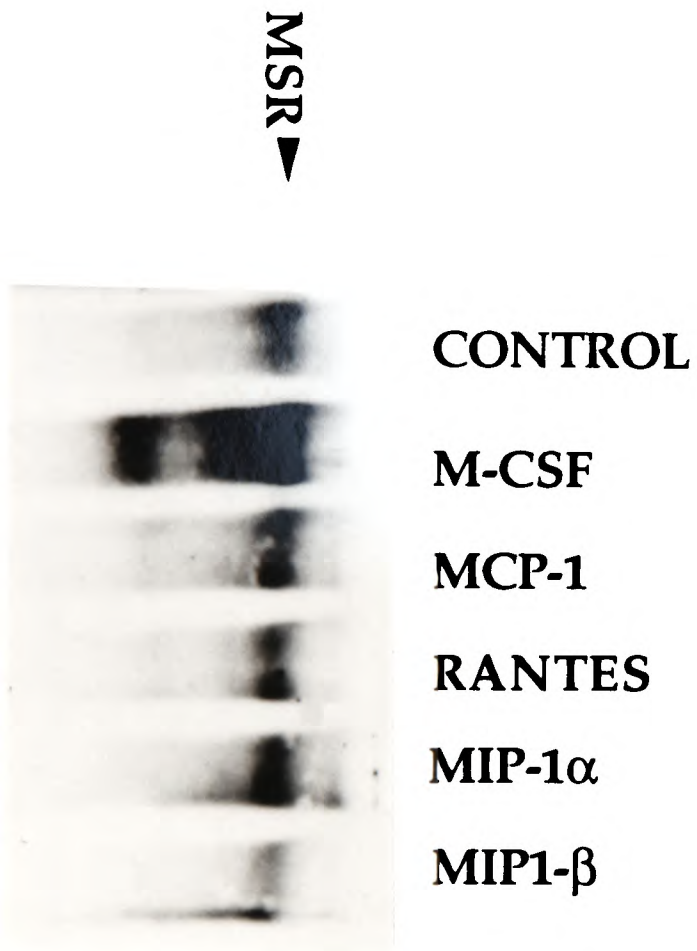
Previous studies have established the inducibility of SR expression in rabbit VSMCs (Pitas 1990) and its *in vivo* presence on VMSCs in atherosclerotic lesions in a rabbit model (Li *et al.* 1995). Surprisingly, IFN-γ increased SR expression (mRNA and protein) and AcLDL degradation in rabbit SMCs, in contrast to findings in murine and human Mϕ (Chapter 4 and Fong *et al.* 1990; Geng and Hansson 1992).To date, there has been no evidence for SR expression by VSMCs in human (Yla-Herttuala *et al.* 1991; Naito *et al.* 1992; Luoma *et al.* 1994) and murine (Chapter 8) atherosclerotic lesions. These apparently divergent patterns of expression and responses may point to important cell- and species-dependent differences in MSR regulation. Immunoblot analysis (fig. 5.8) of murine aortic SMCs treated with TGF-β and anti-TGF-β antibodies (in collaboration with Dr. D. Grainger, Cambridge), showed no MSR expression in these cells or any evidence of regulation by exogenous or endogenous TGF-β.

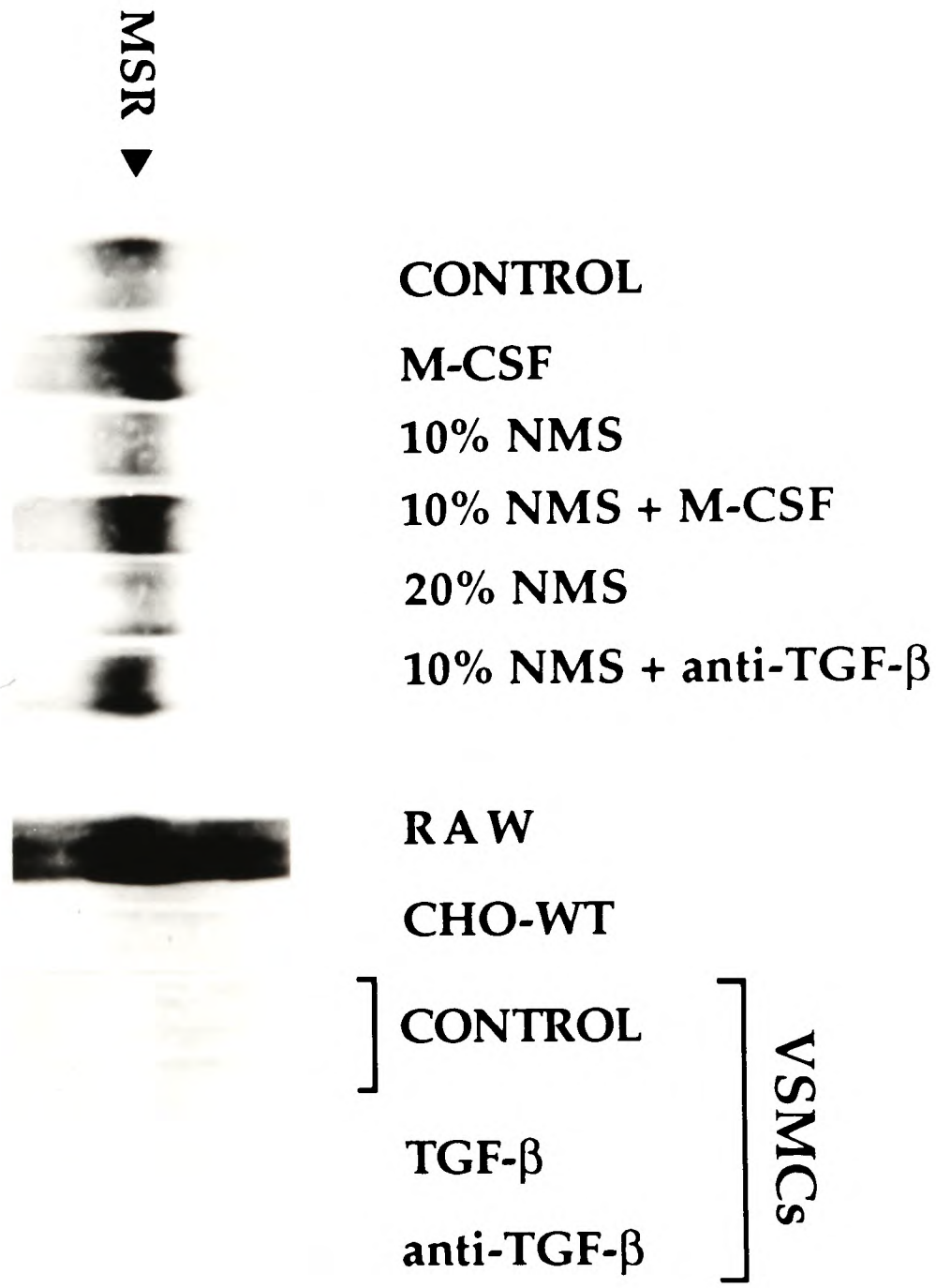
Fig. 5.8 **Immunoblot of MSR from foam cells treated with M-CSF and anti-TGF-β antibodies; and from aortic SMCs treated with TGF-β and anti-TGF-β antibodies**

BgPMϕ were treated *in vitro* with 10-20% normal mouse serum for 24 hours, followed by recombinant human M-CSF (1000units/ml) and anti-TGF-β (5 μg/ml) for 48 hours. Cells were lysed and protein concentration of lysates measured using the bicinchoninic acid Protein Assay Reagent kit. Equal amounts of total cellular protein (20 μg) were loaded, proteins separated by nonreducing 6% SDS-PAGE and transferred to nitrocellulose overnight. Aortic SMCs were derived from C57BL/6 mice by the explant technique and cultured for 48 hours with TGF-β (10 ng/ml) and neutralizing anti-TGF-β (25μg/ml). Cells were lysed as above and equal cell numbers loaded. MAb 2F8 was diluted to 10 μg/ml followed by incubation with with peroxidase-conjugated anti-rat IgG antibody (1:1000) and detection by chemiluminescence. The foam cell part is representative of two independent experiments.

Fig. 5.10 **Immunoblot of MSR from BgPMϕ treated with the C-C chemokines**

BgPMϕ were treated for 48 hours *in vitro* with murine MCP-1 (20ng/ml); murine RANTES (20ng/ml); murine MIP-1α (10ng/ml) and murine MIP1-β (200ng/ml). Cells were lysed and protein concentration of lysates measured using the bicinchoninic acid Protein Assay Reagent kit. Equal amounts of total cellular protein (20 μg) were loaded, proteins separated by nonreducing 6% SDS-PAGE and transferred to nitrocellulose overnight. MAb 2F8 was diluted to 10 μg/ml followed by incubation with peroxidase-conjugated anti-rat IgG antibody (1:1000) and detection by chemiluminescence. The result is representative of two independent experiments.


MSR
CONTROL
M-CSF
MCP-1
RANTES
MIP-1α
MIP1-β


MSR
CONTROL
M-CSF
10% NMS
10% NMS + M-CSF
20% NMS
10% NMS + anti-TGF-β
RAW
CHO-WT
CONTROL
TGF-β
anti-TGF-β
VSMCs

5.2.5 Discussion

The findings from these studies can be summarized as follows:
i) In unstimulated BgPMϕ, TGF-β and IL-10 decreased MSR expression, including protein synthesis, by about 50% without affecting mRNA levels. TGF-β may have favoured redistribution of MSR to a predominantly intracellular pool and was associated with a modest decrease in MSR-dependent adhesion. The decreases in MSR protein was not otherwise paralleled by changes in MSR function, specifically AcLDL endocytosis.
ii) In M-CSF stimulated BgPMϕ, TGF-β emerged as a potent inhibitor of MSR upregulation. This was also apparent when TGF-β inhibited AcLDL uptake in M-CSF stimulated cells in a dose-dependent manner. IL-10 had no effect in this system, showing a divergence in MSR regulation between these deactivating cytokines.
iii) Endogenously produced TGF-β, in contrast to IL-10, was shown to inhibit MSR expression and function markedly in BgPMϕ; suggesting an important autoregulatory role for TGF-β in MSR regulation. Inhibition of MSR by endogenous TGF-β was also present in a Mϕ-like cell line, RAW 264.7 and foam cells *in vitro*. There was no evidence for any MSR expression or its regulation by exogenous or endogenous TGF-β in murine aortic SMCs.

Recent *in vitro* and *in vivo* evidence has suggested the importance of active TGF-β as a key inhibitor of atherogenesis. TGF-β inhibits both the migration and proliferation of VSMCs in cell culture; these cells also produce TGF-β in a latent, inactive form which is activated proteolytically by the serine protease, plasmin (Grainger *et al.* 1993). Plasmin, in turn, is produced proteolytically from plasminogen by tissue plasminogen activator (tPA). Risk factors for developing atherosclerosis include elevated lipoprotein(a) (Lp(a)) and plasminogen activator inhibitor-1 (PAI-1) levels (Utermann 1989; de Bono 1994); these factors block the activation of latent TGF-β by competitively inhibiting tPA. PAI-1 and Lp(a) therefore promote human VSMC proliferation in culture by relieving the autocrine inhibition caused by active TGF-β. The inhibitory component of Lp(a) is apolipoprotein (a) (apo(a)), which has strong homology to domains in plasminogen. Evidence from a transgenic mouse model of atherosclerosis in which human (apo(a) is expressed, has provided *in vivo* support for a close correlation between accumulation of apo(a) on the vessel wall and inhibition of active TGF-β activation; at these sites VSMCs are activated and vascular lesions develop (Grainger *et al.* 1994). No mention was made of possible Mϕ

involvement. Interestingly, the concentration of active TGF-β was also found to be depressed in sera of patients with advanced atherosclerosis (Grainger *et al.* 1995). A model relating active TGF-β to atherogenesis and risk factors, incorporating the evidence regarding MSR regulation, is shown in fig. 5.9.

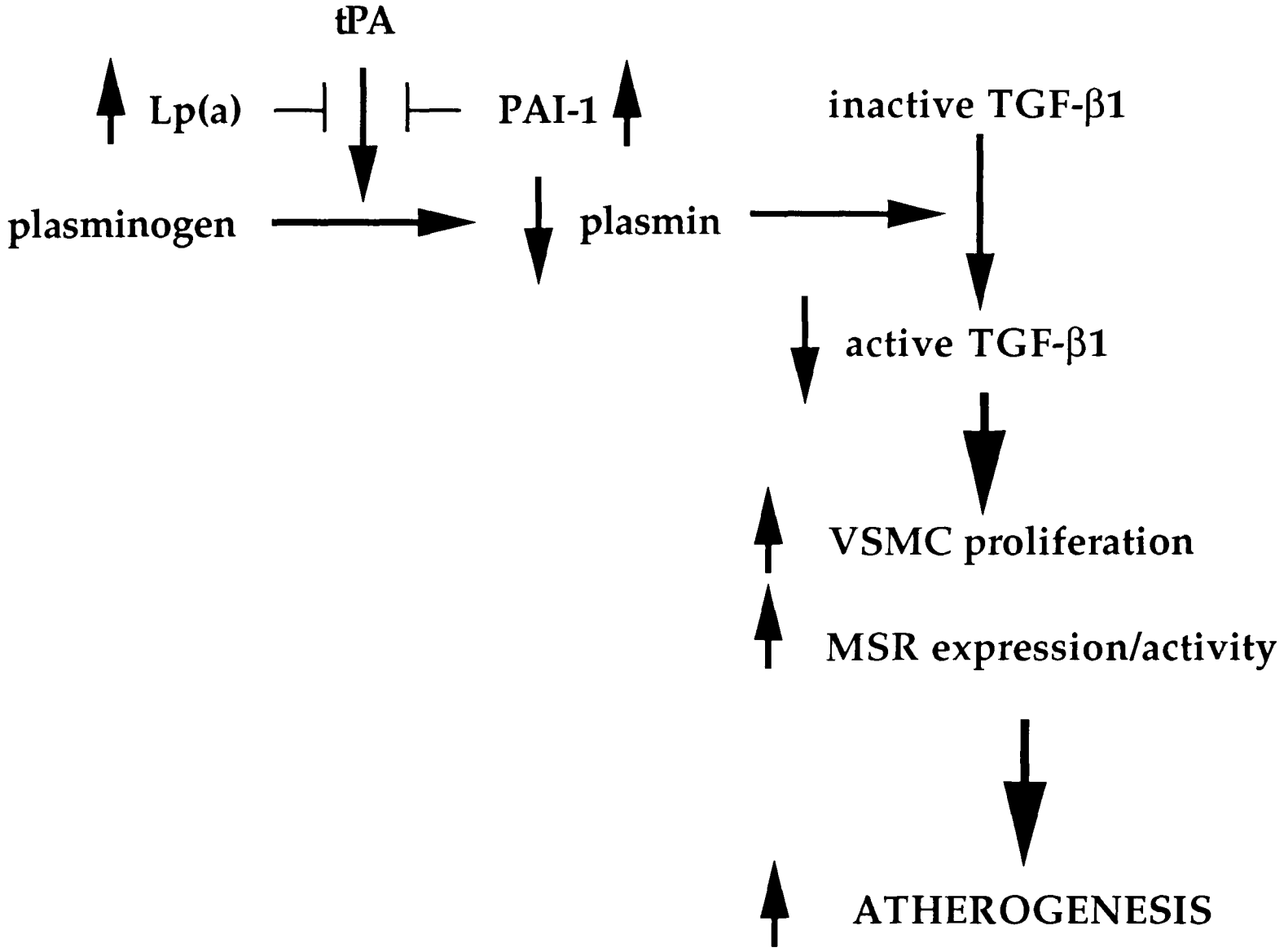


Fig. 5.9 Diagram relating active TGF-β to atherogenesis and risk factors.

The diagram illustrates the hypothesis that active TGF-β is a dominant inhibitor of VSMC migration and proliferation (shown by Grainger *et al*) as well as MSR expression and function (shown in this chapter) and therefore prevents the development of atherosclerosis.

MSR regulation by TGF-β has not been thoroughly investigated. Platelet secretory products (including serotonin, fibrinogen, fibronectin, and PDGF) have been reported to suppress MSR activity (Aviram 1989); but the effects were interpreted as due to changes in MSR affinity and not number. In a PMA-differentiated THP-1 human Mϕ-like cell line, TGF-β1 decreased undefined SR number (50% by Scatchard analysis), MSR mRNA (50%) and inhibited AcLDL degradation by 70% (Bottalico *et al.* 1991). A 2-fold decrease in cholesterol esterification following

AcLDL uptake was also noted in TGF-β treated human monocytes (Zuckerman *et al.* 1992). The physiological significance of our findings, in terms of atherosclerosis, depends on whether Mϕ foam cell formation is entirely a pathological event or, at least in the early stages of atherogenesis, a protective event (e.g. by scavenging harmful oxidized lipoproteins). In the former case, TGF-β1 mediated inhibition of MSR might be anti-atherogenic and, in accordance with the scheme in fig. 5.9, a decrease in active TGF-β1 secretion by arterial wall cells might accelerate progression of the disease. On the other hand, if foam cells are initially protective, an increase in active TGF-β1 secretion might contribute to the progression of atherosclerosis.

TGF-β, IL-4, IL-13 and IL-10 have complex actions on monocytes/Mϕ; none of them can be categorized as purely Mϕ deactivating since they also exert Mϕ activating effects. *In vitro*, these effects are strongly influenced by stimulation conditions (triggering signal, cytokine concentration, timing of cytokine addition), species (mouse vs. human vs. rabbit), source (blood vs. peritoneal vs. alveolar) and state of differentiation/activation of the Mϕ (resident vs. elicited vs. inflammatory). As described, there are important differences in the abilities of these cytokines to modulate MSR expression and function; IL-4 and IL-13 have generally upregulatory actions, IL-10 has no functional effects despite a decrease in MSR protein, and TGF-β is probably as potent an inhibitor as M-CSF is a stimulator of MSR. Disparities in the ability of TGF-β, IL-4 and IL-10 to modulate Mϕ phenotype are not unique to MSR regulation; but the most striking differences have been with regard to secretory Mϕ products, and not integral membrane proteins (Bogdan and Nathan 1993). These differences between TGF-β, IL-4 and IL-10 have become apparent in terms of spectrum of activity, extent of effect, requirement for stimulation, ability to overcome activating stimuli (as with MSR inhibition by TGF-β), and modes of action.

Mice with a genetically engineered TGF-β1 loss-of-function mutation develop excessive multi-organ inflammatory responses and die 2-3 weeks after birth (Shull *et al.* 1992; Kulkarni *et al.* 1993); their Mϕ phenotype, although not fully characterized, seems activated with increased secretion of IFN-γ, TNF-α and MIP1α. In view of the broader role envisaged for the MSR in general host defence and innate immunity, it is inviting to speculate on the association between uninhibited upregulated MSR expression due to a lack in TGF-β, its role as an adhesion receptor and the recruitment and retention of Mϕ in inflammatory lesions.

5.3 The effects of C-C chemokines and other cytokines and growth factors on MSR expression and function

5.3.1 The role of C-C chemokines in atherosclerosis and effects on MSR

The recruitment and transformation of circulating monocytes into foam cells requires adhesion, transmigration, and accumulation of lipid. Once the monocyte attaches to luminal endothelial cells, its transendothelial migration and entry into the subendothelial space and intima is mediated by a gradient of one or more locally generated chemotactic factors. Because the early atherosclerotic lesion is rich in monocyte-Mϕ, much attention has been focused on the identification of monocyte-specific chemoattractants produced by cells of the blood vessel wall, and their respective receptors.

The chemokines affect leukocyte chemotaxis and belong to a family of 6-12 K proteins characterized by a similarly spaced motif of four disulfide linked cysteine residues. They are subdivided into two main groups according to the position of the first two cysteines which are either separated by one amino acid (α group or C-X-C chemokines) or adjacent (β group or C-C chemokines) (Schall 1995). Generally, the C-X-C chemokines are chemotactic for and activate granulocytes, whereas the C-C family members act predominantly on monocytes; a degree of overlapping function or redundancy within each subgroup is however becoming more evident.

Other monocyte-specific chemoattractants include plasma- and connective tissue-derived proteins such as thrombin, proteolytic fragments of collagen, elastin and fibronectin (Valente *et al.* 1995). It is noteworthy that TGF-β, which has received considerably less attention, may be the most potent of all leukocyte chemoattractants (Murphy 1994). The chemotactic S100 peptide, CP-10, was also recently reported to enhance scavenger receptor and Mac-1 expression and cholesteryl ester accumulation in murine peritoneal Mϕ *in vivo* (Lau *et al.* 1995), suggesting a potential pathogenic role in stimulating monocyte recruitment and foam cell formation.

Monocyte chemoattractant protein-1 (MCP-1) and its murine equivalent, JE, are the best characterized members of the C-C subfamily (Rollins *et al.* 1990; Charo 1992) and stimulates directed migration of monocytes potently *in vitro*. Vascular endothelial cells, SMCs, and Mϕ can inducibly express MCP-1 *in vitro* (Cushing *et al.* 1990; Rollins *et al.* 1990; Rollins and Pober 1991) and there is *in vivo* evidence for expression in human and experimental atherosclerotic lesions (Nelken

et al. 1991; Yla-Herttuala *et al.* 1991). Plasma LDL that has undergone minimal oxidative modification (MM-LDL) increases monocyte adhesion to endothelial monolayers, induces MCP-1 expression (mRNA and protein), and increases transmigration of monocytes to the subendothelial spaces of co-cultures (Liao *et al.* 1991; Navab *et al.* 1994). These observations point to an important role for MCP-1 in intimal monocyte recruitment in atherogenesis. In some animal models of chronic inflammation, the infusion of neutralizing antibodies to MCP-1 inhibits monocyte migration to a site of tissue injury only partially; and more than one monocyte chemoattractant molecule may therefore modulate monocyte recruitment in inflammation. The actions of MCP-1 and other cell-derived members of the C-C chemokine subfamily, RANTES, MIP-1α and MIP-1β, (Schall 1995) on MSR expression and function *in vivo* were therefore investigated.

No change in MSR expression was apparent by immunoblot analysis after BgPMϕ in culture were treated for 48 hours with the respective C-C chemokines (fig. 5.10). MSR protein synthesis was subsequently assayed by metabolic labeling and mAb 2F8 immunoprecipitation (results not shown). PhosphorImager analysis revealed a 2.4-fold M-CSF mediated increase in this experiment, but no significant discernible effects by MCP-1, RANTES, MIP-1α or MIP-1β; there was also no additive or synergistic action when MCP-1 or RANTES were added to M-CSF in culture. These protein findings correlated with the lack of significant changes found when DiI-AcLDL uptake was assessed in treated BgPMϕ and compared with uptake in untreated and M-CSF induced cells (fig 5.11).

Interestingly, MCP-1, in contrast to the other C-C chemokines, reproducibly increased MSR-dependent adhesion to TCP (43%); similar in range to a M-CSF mediated increase (47%) (fig. 5.12). This was not the result of increased cell surface MSR as MCP-1 had no apparent effect on MSR cellular distribution as measured by flow cytometry (not shown). The increase in adhesion was maintained when neutralizing anti-murine M-CSF antibodies were added to the MCP-1 treatment; this was done to determine the specificity of the MCP-1 induced increase and to exclude it being an indirect effect due to autocrine M-CSF production by cultured Mϕ (Shyy *et al* 1993). It was shown in Chapter 3 that endogenous M-CSF did not have a marked upregulatory effect on MSR expression; this would make a contribution to increased MSR-dependent adhesion unlikely. The specificity of the MCP-1 stimulation could not be fully established because of lack of commercially available neutralizing anti-murine MCP-1 antibodies. This MCP-1 mediated increase in MSR-dependent BgPMϕ adhesion was puzzling, especially as

Fig. 5.11 **The effect of the C-C chemokines on DiI-AcLDL uptake by BgPMϕ**

BgPMϕ were plated in 24 well TCP plates (3 x 10^6 cells/well), and treated *in vitro* for 48 hours with with murine MCP-1 (20ng/ml); murine RANTES (20ng/ml); murine MIP-1α (10ng/ml) and murine MIP1-β (200ng/ml). After washing in PBS, DiI-AcLDL at 5 μg/ml was added. After incubation for 3 hours at 37°C, cells were washed, harvested using EDTA and lidocaine, and resuspended in PBA for analysis by flow cytometry (FL2 photomultiplier). Results show the mean values (two independent experiments in duplicate) of specific fluorescence intensities obtained from subtracting geometric mean autofluorescence from the fluorescence obtained after uptake of DiI-AcLDL.

Fig. 5.12 **The effects of C-C chemokines on MSR-dependent adhesion of BgPMϕ**

BgPMϕ were cultured in RPMI plus 10% FCS on tissue culture plastic (TCP) surfaces and treated *in vitro* for 48 hours with M-CSF (1000units/ml); MCP-1 (20ng/ml); MIP-1α (10ng/ml) and MIP1-β (200ng/ml). Cell adhesion assay was performed as described in Chapter 2. EDTA was used at a final concentration of 5mM and mAb 2F8 was added at a final concentration of 2 μg/ml. After staining with Giemsa, plates were washed, dried, and the retained dye solubilised in methanol. Stain was quantified by measuring absorbance at 450nm in an automatic plate reader. Results (means ± s.d.) are derived from two experiments done in triplicate. $P < 0.05$ for M-CSF and MCP-1 vs. Control.

Fig. 5.11

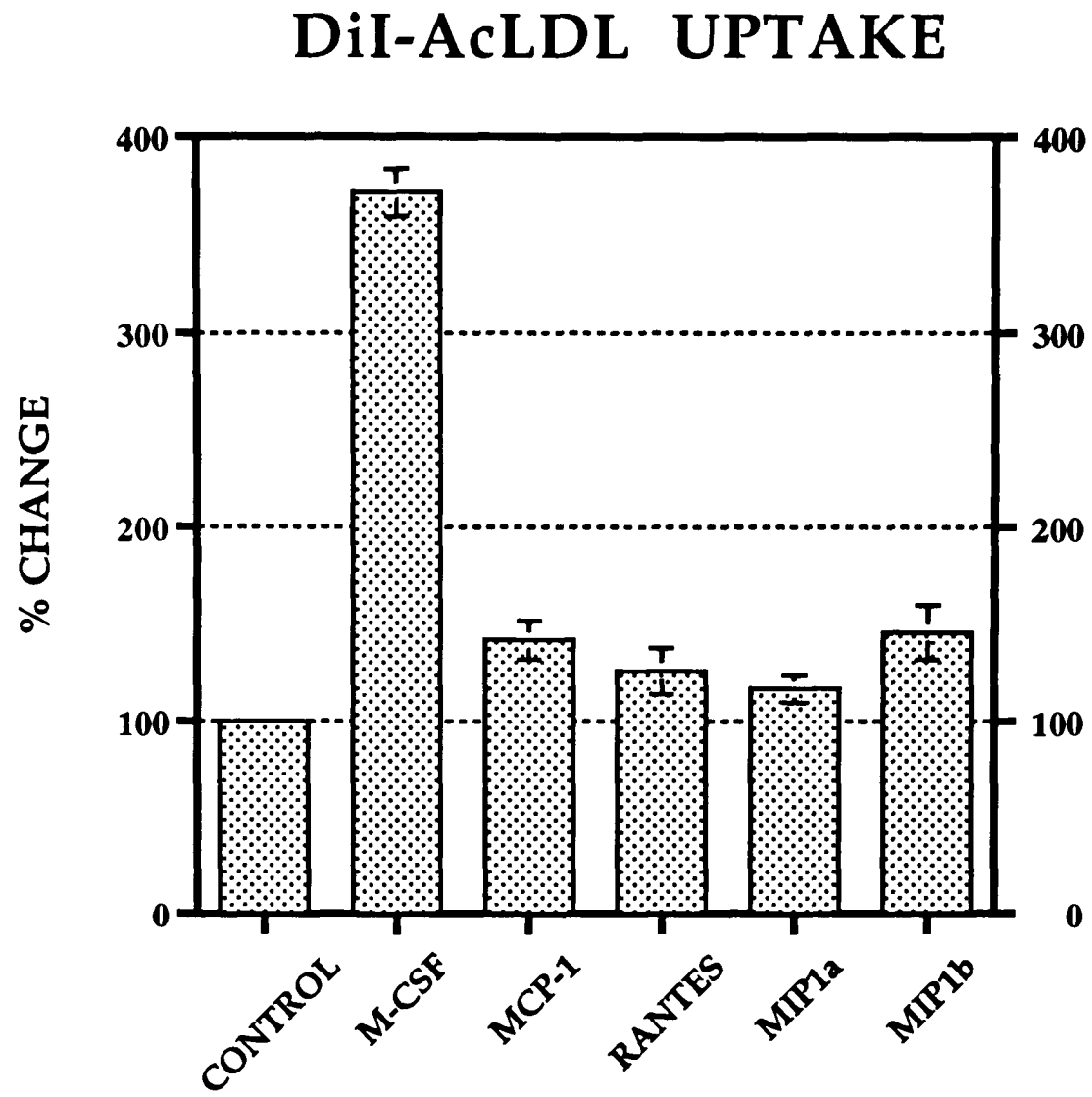
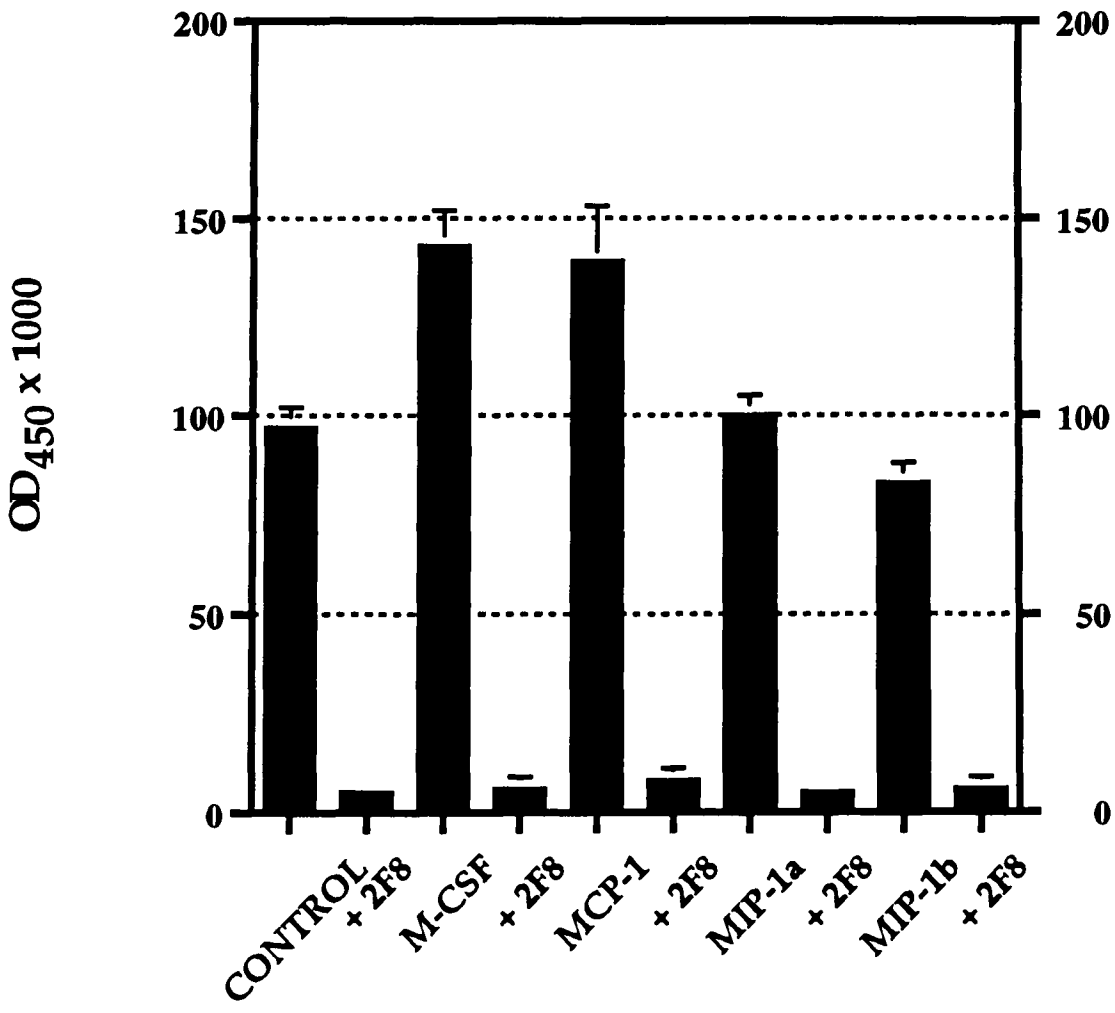


Fig. 5.12



no change in MSR surface expression could be documented. These results cast doubt on the quantitative (rather than qualitative) abilities of the MSR adhesion assay and will have to be investigated further.

Apart from the MCP-1 induced increase in adhesion, the C-C chemokines had little impact on MSR. The most obvious explanation for this may be that the role of the MSR in the hierarchy of monocyte adhesion is not so much one of recruitment (rolling, attachment, chemotactic activation, or transmigration) but rather, following these events, of Mϕ retention in inflammatory lesions by interacting with matrix and/or plasma-derived components. In such a scenario the chemokines would not be expected to have much of an effect.

In addition, autologous downregulation of chemokine receptors occurs (Valente *et al.* 1995), so that BgPMϕ may be relatively unresponsive to C-C chemokines. To date, cross-competition binding studies have identified three functionally distinct monocyte receptor specificities for the C-C chemokines (Murphy 1994); one is specific for MCP-1, one appears to be a shared receptor for MIP-1α and MIP-1β, and one a shared receptor for MIP-1α/RANTES/MCP-1. The genes for two C-C receptor proteins have recently been cloned; the first encodes a protein which binds MCP-1 specifically (Charo *et al.* 1994), and the second binds MIP-1β and RANTES with high affinity and MIP-1α and MCP-1 with low affinity (Neote *et al.* 1993). Both are seven transmembrane domain G-protein coupled receptors, and interaction with specific chemokine ligands leads to a pertussis toxin-sensitive pathway resulting in chemotaxis as a major biological response.

It is interesting to note that monocyte responsiveness to MCP-1 is labile and either reduced or absent in cells that have matured/differentiated into Mϕ (Valente *et al.* 1995); freshly isolated monocytes show a time-dependent specific loss of chemotactic reactivity and receptor-binding activity to MCP-1. On the other hand, MCP-1 (message and protein) expression is rapidly induced and maintained for several days after monocyte isolation (Cushing and Fogelman 1992). This loss of MCP-1 responsiveness and concomitant upregulation of MCP-1 expression in monocytes is not inconsistent with the primary function of MCP-1 and its receptor as important mediators of initial monocyte recruitment from the circulation to tissue.

5.3.2. The effects of other cytokines and growth factors on MSR

A number of other cytokines and growth factors have been implicated in atherogenesis by virtue of their upregulated expression in atherosclerotic lesions (Ross 1993). The effects on MSR biology of these

mediators, and other hormones, lipid mediators and drugs known to affect monocyte differentiation and adhesion, were investigated *in vitro* and the findings are briefly summarized in table 5.8. Following 48 hours treatment of BgPMϕ, changes in total MSR expression and function were initially screened for by immunoblot analysis and DiI-AcLDL uptake; more detailed investigations were done if these were thought significant.

Although some increase was seen in MSR expression following GM-CSF treatment, further studies involving protein synthesis, flow cytometry, RT-PCR and MSR-dependent adhesion to TCP showed no change; this suggests the MSR is not involved in the increase seen in GM-CSF induced monocyte adherence to endothelial cells (Elliott *et al.* 1990), or the decrease in serum cholesterol when GM-CSF is infused as an adjunct to chemotherapy (Nimer *et al.* 1988). In contrast to VSMCs (Inaba *et al.* 1992), BgPMϕ were unresponsive to PDGF-BB as far as MSR induction is concerned. Although the decrease in AcLDL uptake mediated by Vit. D_3 seemed impressive initially, this was inconsistent and not associated with any change in protein synthesis or mRNA levels. There are conflicting reports on the effects of dexamethasone on MSR regulation; the evidence in BgPMϕ is mostly in favour of downregulation, no change was seen in protein synthesis but MSR mRNA decreased. It is also noteworthy, with regard to 17β-estradiol, that no difference was seen in MSR levels in treated BgPMϕ from male and female animals.

The MSR upregulating trends shown by the pro-inflammatory cytokines IL-6 and IL-12 are interesting (in addition, IL-6 increased MSR mRNA and IL-12 internal MSR by flow cytometry) and should be investigated further, especially since the downregulation known to be mediated by TNF-α, another pro-inflammatory cytokine, was confirmed (Van Lenten and Fogelman 1992). Probucol and α-tocopherol also had potentially interesting effects, in that modest to moderate decreases in MSR function occurred along with stable MSR expression levels; these results would agree with their reported inhibition of monocyte adhesion and anti-atherogenic effects in general. It is worth noting that none of the mediators screened had as potent an upregulating action on MSR as M-CSF; similarly, none achieved the inhibitory potential of TGF-β. Some of the mediators described may have more marked effects when examined in systems where the contribution of endogenous TGF-β had been removed or M-CSF upregulation had occurred.

Table 5.8 The effects of various cytokines and growth factors on MSR

Treatment		MSR expression	MSR function		MSR regulation	Athero-sclerosis
	Final / ml	Western blot	AcLDL uptake	MSR-adhesion	References	References
Pro-inflammatory cytokines						
IL-6	10ng	↔/↑	42% ↑		ND	1
IL-12	5 ng	↔	35% ↑		ND	ND
TNF-α	100pg	↔	30% ↓		↓ 2	3
Growth factors						
GM-CSF	10^3 u	↑	↔	↔	↔/↑ 4,5	ND
Vit. D_3	12nM	↔/↑	52% ↓		↔ 6	-
IL-3	5ng	↔	ND		ND	ND
PDGF-BB	1ng	↔/↑	23% ↓	↔	↑ 7	8,9
Hormones and lipid mediators						
E_2	10ng	↔/↓	↔		ND	10
DXM	10μM	↔/↓	50% ↓		↑/↓ 11,12	-
PGE_2	10μM	↔	↔		ND	13
LTB_4	50nM	↔	↔		ND	13
Drugs						
Indo-methacin	1μM	↔/↓	29% ↓		ND	13
Probucol	50μM	↔	13% ↓	28% ↓	ND	14,15,16
α-tocopherol	50μM	↔	47% ↓	13% ↓	ND	17

E_2 = 17β–estradiol; PGE_2 = Prostaglandin E_2; DXM = Dexamethasone; Vit.D_3 = Dihydroxycholecalciferol($(OH)_2D_3$); LTB_4 = Leukotriene B_4; ND= not determined

References

1 (Salomon *et al.* 1992)
2 (Van Lenten and Fogelman 1992)
3 (Barath *et al.* 1990)
4 (Ishibashi *et al.* 1990)
5 (de Villiers *et al.* 1994)
6 (Jouni and McNamara 1991)
7 (Inaba *et al.* 1992)
8 (Pantazis *et al.* 1991)
9 (Inaba *et al.* 1993)
10 (Horowitz 1993)
11 (Hirsch and Mazzone 1986)
12 (Moulton *et al.* 1992)
13 (Kowala *et al.* 1993)
14 (Hara *et al.* 1992)
15 (Faulkner *et al.* 1993)
16 (Ferns *et al.* 1992)
17 (Faruqi *et al.* 1994)

Chapter Six

MSR exists as a truncated soluble form *in vitro*

6.1 Introduction

It was shown in Chapter 3 that M-CSF selectively upregulates MSR expression by a number of mechanisms, increasing message, protein synthesis, stability as well as redistributing MSR from an intracellular pool to increase functional surface expression. The production of soluble forms of MSR may represent another level of regulation of expression and function. Several Mϕ membrane molecules are enzymatically cleaved from the cell surface and then released into the extracellular medium in the form of soluble fragments. This process, commonly initiated by cell stimulation, may regulate the surface expression of such molecules, and may also be responsible for the production of their soluble forms *in vivo*. Evidence is provided in this chapter for the existence of a functional soluble form of the MSR. An overview is given of Mϕ membrane receptors that are cleaved from cells (table 6.1), and the general mechanisms and putative enzymes involved in this process, as well as the potential physiological significance of these events, with particular reference to soluble MSR, are discussed.

Table 6.1 Cleaved Mϕ membrane molecules: *in vivo* presence and physiological activity

Molecule	Function of membrane form	Presence of soluble form in body fluids	Physiological activity of soluble form	References
CD14	LPS receptor, adhesion	serum; ↑ in sepsis, urine of nephrotic patients	LPS response and clearance	(Bazil and Strominger 1991)
CD16	FcγRIII	serum; synovial fluid (↑ in RA), seminal fluid, saliva, urine	?	(Harrison *et al.* 1991)

CD23	FcεRII, adhesion	serum; ↑ in B-cell CLL	regulation of IgE synthesis, inhibition of monocyte migration	(Letellier *et al.* 1990)
CD25	IL-2R α chain	serum; ↑ in leukemias	binding of IL-2	(Waldmann and al 1991)
CD32	FcγRII	serum; ↑ in certain diseases	binding of IgG	(Fridman *et al.* 1993)
CD35	CR1	serum; ↑ in liver/renal failure, lymphoma and leukemia	? complement inhibitor	(Danielsson *et al.* 1994)
CD43	adhesion	serum	?	(Schmid *et al.* 1992)
CD44	adhesion	serum; ↑ in lymphoma	?	(Bazil and Horejsi 1992)
CD54	ICAM-1 (adhesion)	serum; ↑ in certain diseases	inhibition of cell-mediated cytotoxicity	(Gearing and Newman 1993)
CD55	DAF	serum; urine, saliva and tears	factor H-like activity	(Medof *et al.* 1987)
CD62-L	L-selectin (adhesion)	serum; ↑ in various diseases	inhibition of lymphocyte attachment to cytokine-activated endothelium	(Schleiffenbaum *et al.* 1992)
CD71	Transferrin receptor	serum	?	(Shih and al 1990)
CD106	VCAM-1 (adhesion)	serum; ↑ in various diseases	?	(Gearing and Newman 1993)
CD126	IL-6R α chain	serum; ↑ in HIV infection and MG	binding of IL-6	(Mullberg *et al.* 1993)
IL-3R		serum	?	(Mui *et al.* 1992)
TNF-α		serum	growth factor	(Kriegler *et al.* 1988)
TGF-α		serum	growth factor	(Pandiella and Massague 1991)

M-CSF		serum	growth factor	(Stein and Rettenmier 1991)
TNFR	TNF-R55 and TNF-R75	serum; ↑ in sepsis and various diseases	binding of TNF-α	(Leeuwenberg *et al.* 1994)
LDLR		serum	antiviral activity	(Fischer *et al.* 1993)
MHC class I		serum	?	(Demaria *et al.* 1994)

Abbreviations: RA, rheumatoid arthritis; B-CLL, B-cell chronic lymphocytic leukemia; CR1, complement receptor type 1; ICAM-1, intercellular adhesion molecule 1; DAF, decay-accelerating factor; VCAM-1, vascular cell adhesion molecule 1; HIV, human immunodeficiency virus; MG, monoclonal gammopathy; IL-6R, IL-6 receptor; IL-3R, IL-3 receptor; TNFR, tumor necrosis factor receptor; LDLR, low-density lipoprotein receptor; MHC, major histocompatibility complex.

6.2 Biochemical evidence for the existence of soluble MSR

The cleavage of membrane molecules from the surface of leukocytes and other cells by endogenous cellular enzymes, thereby releasing their soluble fragments into the extracellular medium, has been demonstrated *in vitro* by a combination of methods, including: i) detection of soluble fragments in the culture supernatant of surface-labeled cells; ii) comparison of the apparent molecular weight of the membrane form with the released soluble form; and iii) inhibition of the release of the soluble forms by enzyme inhibitors. A similar approach was followed in attempts to determine whether the MSR exists as a soluble form.

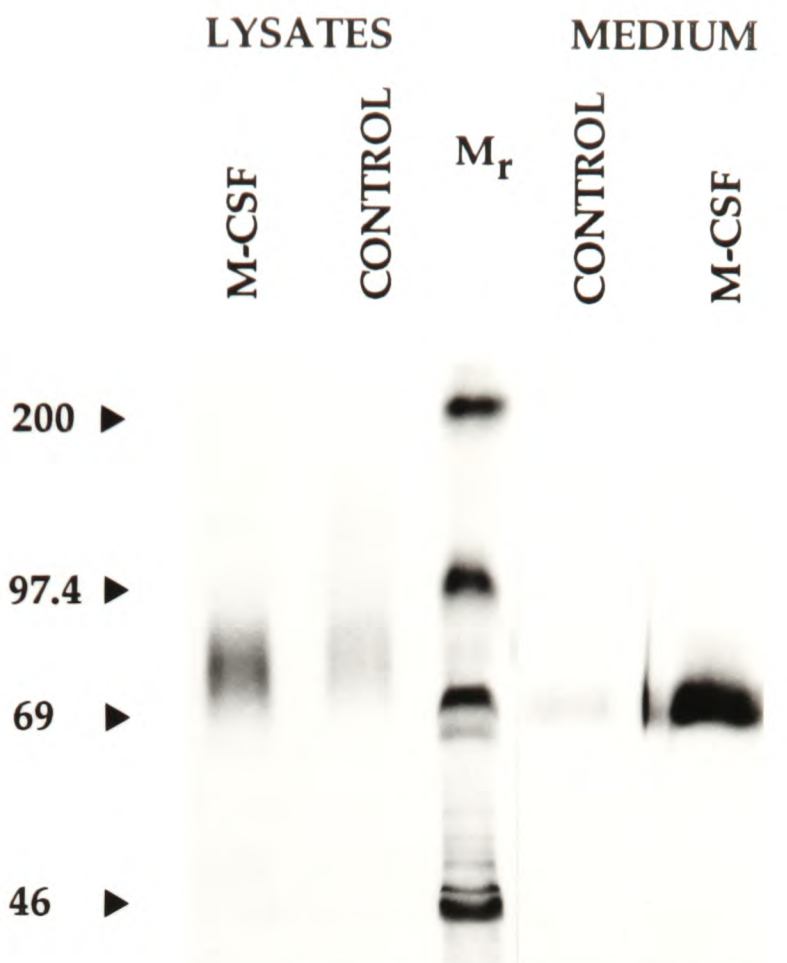
In the first instance, BgPMϕ were treated with a number of cytokines and growth factors for 48 hours and metabolically labeled with Tran^{35}S-methionine overnight. The culture supernatants of these labeled cells were then analyzed for the presence of soluble MSR by immunoprecipitation with mAb, 2F8. The results of these experiments are shown in figs. 6.1.- 6.2. The presence of a slightly smaller, less heterogeneous precipitable form of monomeric MSR in the medium of treated cells is clearly apparent. The question arose whether this soluble form of MSR represented enzymatic cleavage of membrane-bound molecules, MSR secretion, or non-specific membrane shedding during the labeling period.

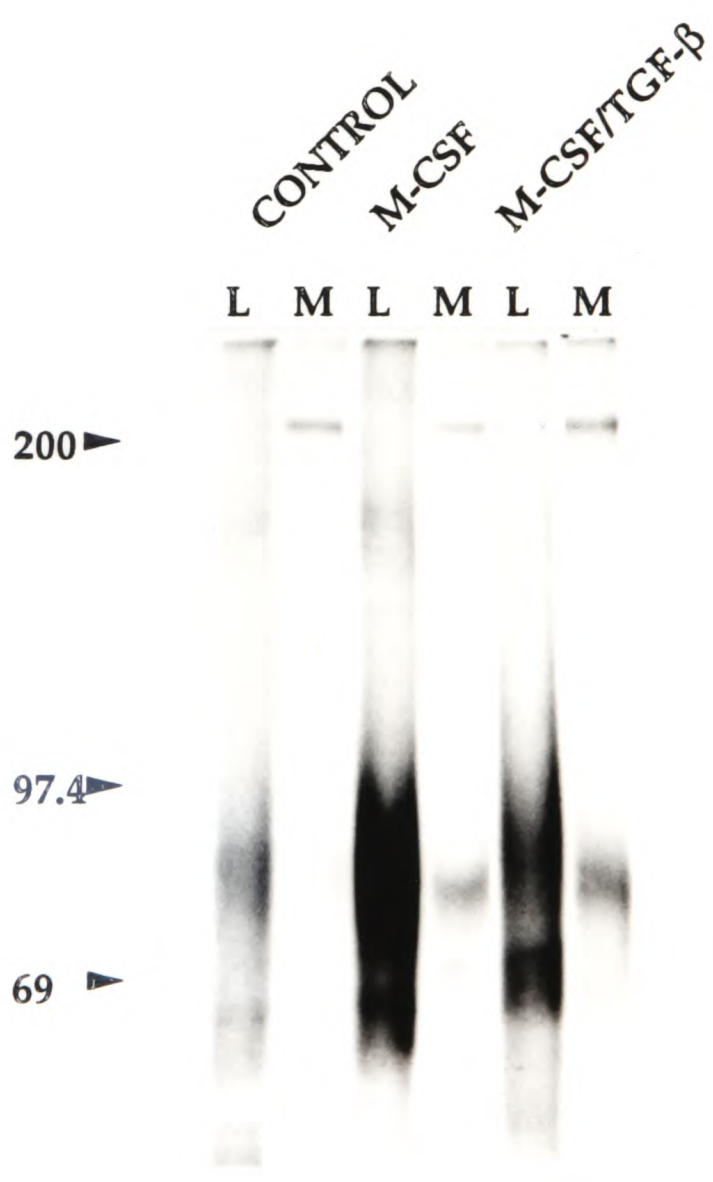
Non-specific membrane shedding was unlikely as supernatants were carefully cleared of cells and cellular debris by two successive centrifugation steps at 4°C, the first at 13000 rpm for 5 min and the

Fig. 6.1 (top) and 6.2 (bottom)

MSR protein is released into culture supernatants by labeled BgPMϕ and this is enhanced by M-CSF.

BgPMϕ were treated *in vitro* for 48 hours with human M-CSF (1000 units/ml) (fig. 6.1 and 6.2) and TGF-β (5ng/ml) (fig. 6.2), and metabolically labeled with Tran^{35}S-label™ for 16 hours. Cell lysates (in 500µl lysis buffer) and culture supernatants (in 1ml labeling medium) were cleared of cells and cellular debris by two successive centrifugation steps at 4°C, one at 13000 rpm for 5 min, and the second at 55000 rpm for 30 min. Immunoprecipitation with mAb 2F8 was performed as described. Lysate precipitates were adjusted to represent equivalent total protein concentrations, and samples separated by SDS-PAGE in the presence of β-mercaptoethanol. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Molecular weight standards in kDa are indicated by arrowheads.


LYSATES
MEDIUM
M-CSF
CONTROL
M_r
CONTROL
M-CSF
200
97.4
69
46


CONTROL
M-CSF
M-CSF/TGF-β
L M L M L M
200
97.4
69

second for 30 min at 55000 rpm. In addition, a consistent finding was the smaller M_r (by ≈10K) of the soluble form (sMSR) compared to monomeric membrane MSR (mMSR). This could be compatible with cleavage of an integral membrane bound form of the MSR, possibly within the membrane proximal region of the spacer domain (leaving behind 80-90 residues consisting of mainly the cytoplasmic (51 aa) and transmembrane (26 aa) domains). It could be argued that the smaller Mr of the soluble form represents a technical artifact due to MSR conformational changes in different immunoprecipitation buffers (lysis buffer for cell lysates and labeling medium i.e. methionine-free MEM and 10% dialyzed FCS for culture supernatants) and that this could result in variations in electrophoretic mobility. This is unlikely as samples were treated identically following pre-clearing with protein G and immunoprecipitation with mAb 2F8 and protein G-sepharose beads. Both sets of samples were then subjected to identical NaCl washes to diminish non-specific binding before resuspension in reducing gel loading buffer.

It is also clear, in general, that the amount of specific radioactivity (soluble labeled MSR) in the medium mirrored the effects of the specific growth factor/cytokine on MSR protein synthesis in BgPMϕ during the labeling period. Thus M-CSF, as a potent upregulator of MSR synthesis was associated with a markedly increased amount of soluble MSR. The kinetics of sMSR release was investigated by the detection, at indicated time points, of sMSR in culture supernatants of 30 min-pulsed Tran^{35}S-labeled control and M-CSF treated BgPMϕ (fig. 6.3). Immunoprecipitates were analyzed by 7.5% SDS-PAGE under reducing conditions followed by autoradiography and PhophorImager analysis. The release of soluble MSR by untreated BgPMϕ was rapid and the amount maximal after 4 h of chase; relative to this point the amount decreased at 12 h (83%), 24 h (42%) and 36 h (5%). In contrast, M-CSF treated cells, in addition to releasing more sMSR after 4 h of chase (144%) also sustained this release so that it was still readily apparent after 36 h of chase (12.4-fold more than untreated cells). This suggests that the upregulating effect of M-CSF on total MSR expression (cell-associated and released) is even more marked than when, as described in Chapter 3, only cell-associated expression is measured.

Additional evidence in favour of cleavage rather than non-specific membrane shedding causing sMSR was provided by the following experiments: i) macrosialin, an abundant late endosomal Mϕ-specific membrane glycoprotein could not be detected in metabolically labeled culture media from control and M-CSF treated cells; suggesting a selective release of MSR from the Mϕ cell surface (fig. 6.4); the Mϕ

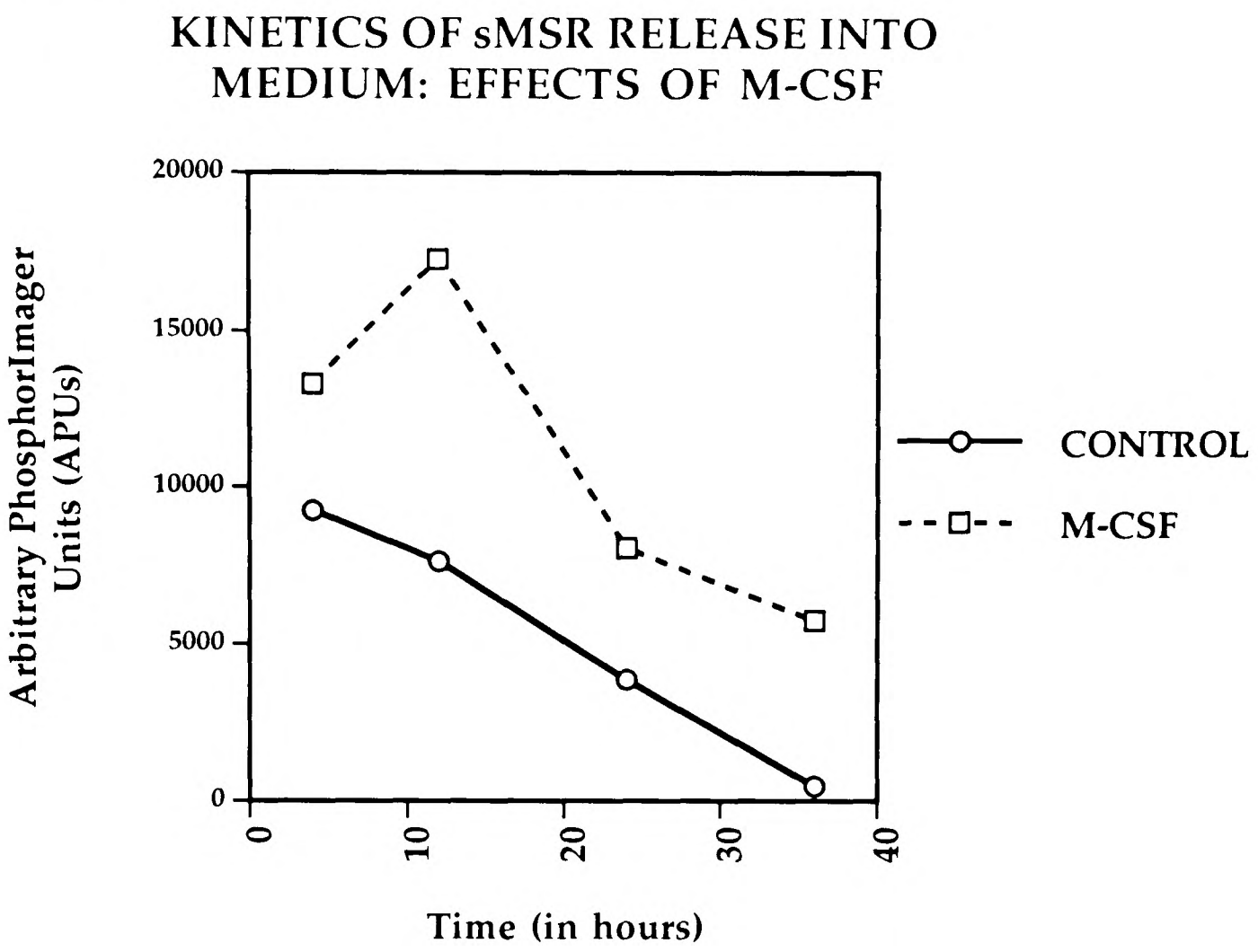
Fig. 6.3 (top) **The effect of M-CSF on the kinetics of sMSR release into culture supernatants by labeled BgPMϕ**

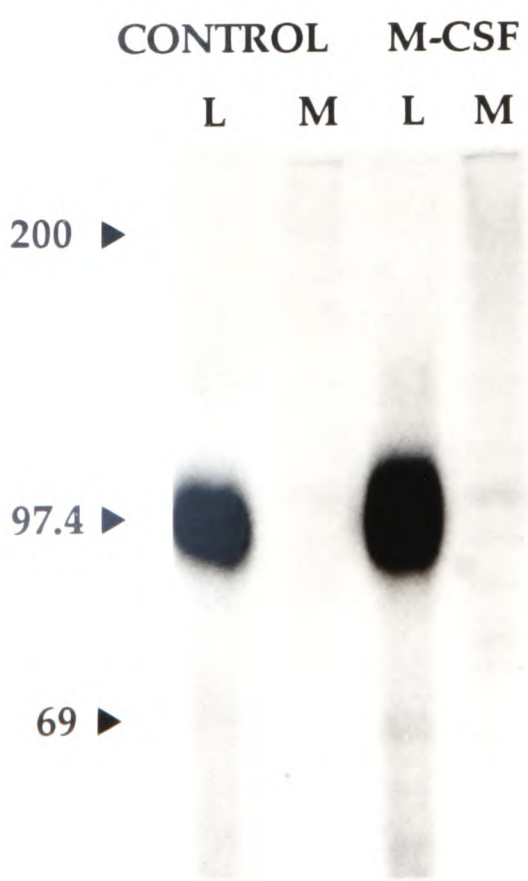
BgPMϕ (5×10^6 cells/well) were treated *in vitro* for 48 hours with M-CSF (1000units/ml), cultured in methionine-free medium for 30 min, and metabolically pulse-labeled with 500 µCi Tran^{35}S-label™ for 30 min and then chased for 4, 12, 24 and 36 hours. Culture supernatants (in 1ml labeling medium) were cleared of cells and cellular debris by two successive centrifugation steps at 4°C, one at 13000 rpm for 5 min, and the second at 55000 rpm for 30 min. Soluble MSR was detected by immunoprecipitation with mAb 2F8 as described and samples separated by SDS-PAGE in the presence of β-mercaptoethanol. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Molecular weight standards in kDa are indicated by arrowheads. Monomeric MSR was quantified at the indicated time points by PhosphorImager analysis and the intensity graphically displayed as Arbitrary PhosphorImager Units (APUs).

Fig. 6.4 (bottom)
Macrosialin, an abundant Mϕ-specific late endosomal membrane glycoprotein, is absent from the culture supernatants of M-CSF treated labeled BgPMϕ.

BgPMϕ were treated *in vitro* for 48 hours with human M-CSF (1000 units/ml) and metabolically labeled with Tran^{35}S-label™ for 16 hours. Cell lysates (in 500µl lysis buffer) and culture supernatants (in 1ml labeling medium) were cleared of cells and cellular debris by two successive centrifugation steps at 4°C, one at 13000 rpm for 5 min, and the second at 55000 rpm for 30 min. Immunoprecipitation was performed with mAb FA-11 (macrosialin), lysate precipitates were adjusted to represent equivalent total protein concentrations, and samples separated by SDS-PAGE in the presence of β-mercaptoethanol. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Molecular weight standards in kDa are indicated by arrowheads.

Fig. 6.3


KINETICS OF sMSR RELEASE INTO MEDIUM: EFFECTS OF M-CSF
Arbitrary PhosphorImager Units (APUs)
20000
15000
10000
5000
0
0
10
20
30
40
Time (in hours)
CONTROL
M-CSF


CONTROL
M-CSF
L
M
L
M
200 ▶
97.4 ▶
69 ▶

differentiation antigen F4/80 could also not be detected in supernatant (results not shown), ii) the soluble MSR form remained present when Mϕ were labeled under serum-free conditions using OptiMEM, a proprietary serum-free medium (results not shown); this would indicate the involvement of endogenous cellular enzymes, rather than enzymes present in serum; and iii) it is apparent from fig. 6.5 that when SDS-PAGE was run under incomplete reducing conditions, the soluble MSR also existed in a mature trimeric form in culture supernatants from labeled Mϕ subjected to various treatments. Trimeric sMSR differed markedly in size from trimeric membrane-bound MSR (≈ 30-40K), and this both confirmed the size difference between sMSR and mMSR and implied that in some cases, cleavage in the membrane proximal region of all three constituent MSR monomers may occur prior to release into the medium.

The most convincing evidence for enzymatic cleavage of MSR is shown in Fig. 6.6. It has been reported that L-selectin, on activation of granulocytes, is cleaved from the surface into the surrounding medium, leaving a 6-14K cytoplasmic domain as remnant (Kahn *et al.* 1994). This could be confirmed by using two types of specific antibodies, one raised against the cytoplasmic domain and the other against an extracellular portion of L-selectin. Both antibodies detect membrane-bound cellular L-selectin; but only the Ab against the extracellular portion detected the cleaved form of L-selectin in the medium. A similar principle was used in the experiment of which the result is shown in fig. 6.6. The mAb, 2F8, is presumed to recognize an extracellular portion of MSR. Evidence for this assumption is two-fold; i) the mAb was characterized using a functional adhesion inhibition screening assay (RAW 264.7 cells to TCP) and ii) immunoprecipitation of the antigen is possible following surface labeling with ^{125}I or biotin (Fraser *et al.* 1993). Polyclonal antiserum raised against a peptide sequence in the murine N-terminus cytoplasmic MSR domain (MTENQRLCPHEREDADC) was obtained as a kind gift from the Krieger laboratory in Cambridge, Massachusetts (Ashkenas *et al.* 1993). Both this PCS and mAb 2F8 recognized monomeric MSR from labeled cell lysates; in contrast, only mAb 2F8 precipitated MSR from labeled medium. This would suggest the absence of cytoplasmic intracellular domain from the soluble form of MSR. Alternate splicing of a single gene product has been described as producing types I and II MSR; both these forms do however contain cytoplasmic and transmembrane domains (Emi *et al.* 1993) which makes a secretory mechanism for sMSR release more unlikely; further splice variants have not however been ruled out. Soluble MSR is thus most likely to be a fragment cleaved from intact membrane-bound MSR, and probably

Fig. 6.5 (top)

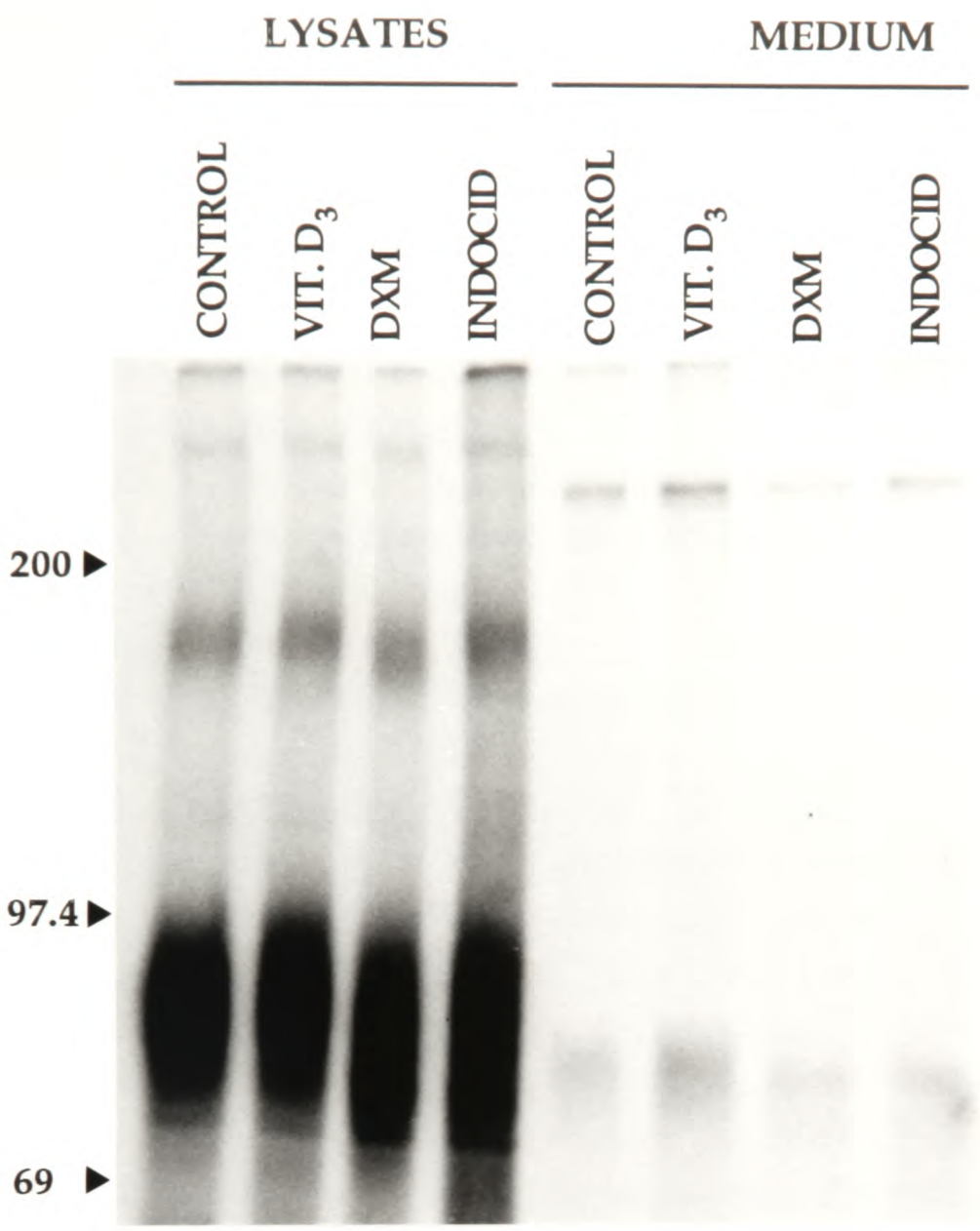
Soluble MSR is present in its trimeric form and differs in size from membrane trimeric MSR in culture supernatants of treated labeled BgPMϕ.

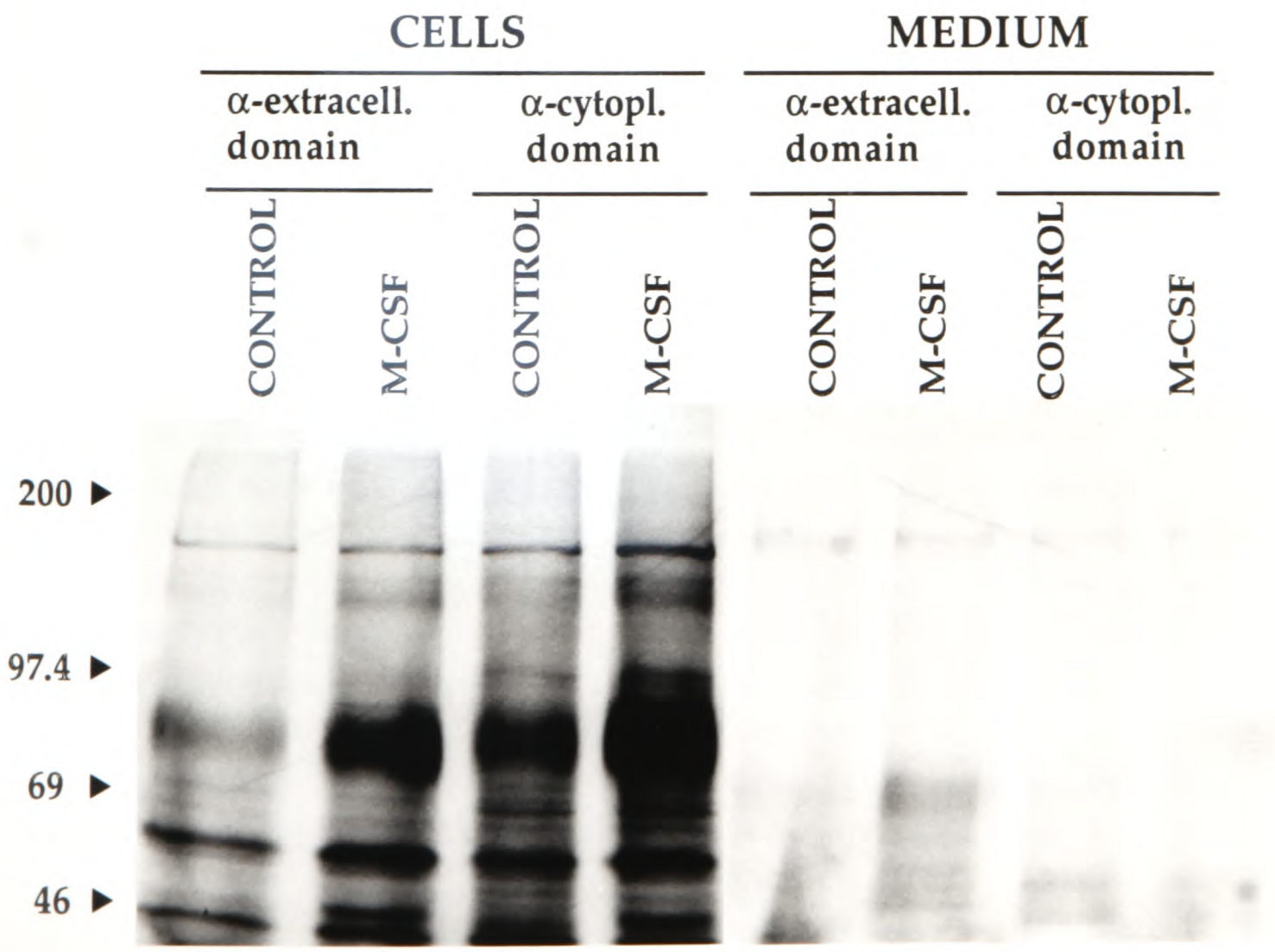
BgPMϕ were treated *in vitro* for 48 hours with Vit. D_3 (12nM), dexamethasone (10μM) and indomethacin (1μM) as part of a general screen for the effects of soluble mediators on MSR expression, and metabolically labeled with Tran35S-label™ for 16 hours. Cell lysates (in 500μl lysis buffer) and culture supernatants (in 1ml labeling medium) were cleared of cells and cellular debris by two successive centrifugation steps at 4°C, one at 13000 rpm for 5 min, and the second at 55000 rpm for 30 min. Immunoprecipitation was performed with mAb 2F8, lysate precipitates were adjusted to represent equivalent total protein concentrations, and samples separated by SDS-PAGE in the presence of β-mercaptoethanol. In this instance, mature trimeric MSR was incompletely reduced to monomers. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Molecular weight standards in kDa are indicated by arrowheads.

Fig. 6.6 (bottom)

Immunoprecipitation of mMSR and sMSR with antibodies against the extracellular or cytoplasmic domains of MSR

BgPMϕ were treated *in vitro* for 48 hours with M-CSF (1000units/ml) and metabolically labeled with Tran^{35}S-label™ for 16 hours. Immunoprecipitation was performed with mAb 2F8 (extracellular domain of MSR) and polyclonal sera raised against the N-terminal cytoplasmic domain of murine MSR. Lysate precipitates were adjusted to represent equivalent total protein concentrations, and samples separated by SDS-PAGE in the presence of β-mercaptoethanol. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed tc film at -70°C. Molecular weight standards in kDa are indicated by arrowheads.


LYSATES
MEDIUM
CONTROL
VIT. D3
DXM
INDOCID
CONTROL
VIT. D3
DXM
INDOCID
200 ▶
97.4 ▶
69 ▶


CELLS
MEDIUM
α-extracell. domain
α-cytopl. domain
α-extracell. domain
α-cytopl. domain
CONTROL
M-CSF
CONTROL
M-CSF
CONTROL
M-CSF
CONTROL
M-CSF
200 ▶
97.4 ▶
69 ▶
46 ▶

most of the extracellular domain of mMSR.

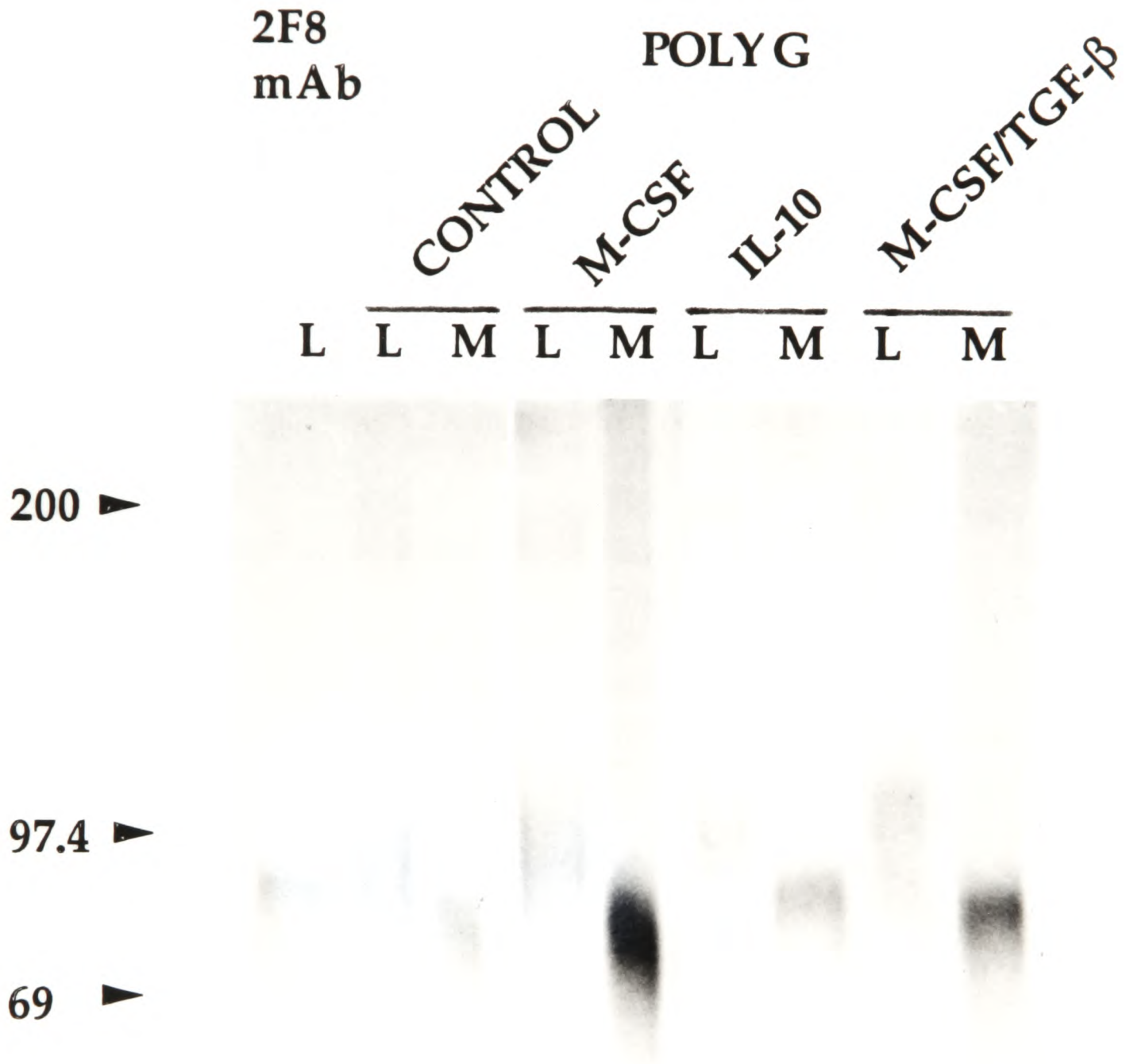
6.3. Bioactivity of soluble MSR

In view of the roles of the MSR as both an endocytic molecule with broad ligand-binding scavenging abilities and an adhesion molecule, the possible functional significance of its soluble form was further investigated. This was first done by attempting to demonstrate the presence of an AcLDL-binding protein in culture supernatants; the approach followed was similar to a study where activated mononuclear cells were described to produce an IL-1β-specific soluble binding protein (Symons *et al.* 1990). A soluble covalent cross-linking procedure was used to characterize the binding protein and its similarities to MSR. After culture supernatants from both cytokine-treated BgPMϕ and human monocytes were incubated with ^{125}I-AcLDL, complexes were cross-linked with disuccinimidyl suberate (DSS) and analyzed by SDS-PAGE. This approach was also followed to determine if any AcLDL-binding moieties, as an indirect indication of functional sMSR, were present in human and/or murine serum under non-stimulated and in the case of murine, LPS-stimulated circumstances. As this did not involve the use of species-specific reagents (such as mAb 2F8), the presence of sMSR in human and murine serum by virtue of its ability to be bound and cross-linked to labeled AcLDL could be assessed. These experiments unfortunately did not work and interpretation of results was unfortunately complicated by non-specific background due to the lipid component of AcLDL. No conclusions could be drawn as to the *in vivo* presence of a soluble AcLDL-binding protein.

The functional nature of sMSR itself was convincingly shown by the ability of immunoprecipitable MSR to bind poly G (a well-characterized ligand) bound to sepharose beads (fig. 6.7). In this assay, BgPMϕ were treated with cytokines and growth factors for 48 hours and metabolically labeled overnight. Both lysates and culture medium were subsequently cleared of cellular debris by centrifugation and pre-cleared overnight at 4°C with protein G-sepharose to diminish non-specific binding. MSR was immunoprecipitated with mAb 2F8 as previously described, washed and the immune complexes boiled to dissociate the MSR antigen from the mAb. Labeled MSR was then incubated with buffer containing Poly G sepharose to determine functional ligand binding; analysis of poly G bound MSR was done by 6% SDS-PAGE under reducing conditions. In the first lane on the left of fig. 6.7, MSR monomer precipitated by mAb 2F8 from untreated cell lysates is depicted for comparative purposes. As expected, poly G sepharose bound MSR

Fig. 6.7 **Soluble MSR in culture supernatants of treated labeled BgPMϕ is functionally active and binds poly G.**

BgPMϕ were treated *in vitro* for 48 hours with M-CSF (1000units/ml), IL-10 (50units/ml) and TGF-β (5ng/ml) and metabolically labeled with Tran^{35}S-label™ for 16 hours. Cell lysates (in 500µl lysis buffer) and culture supernatants (in 1ml labeling medium) were cleared of cells and cellular debris by two successive centrifugation steps at 4°C, one at 13000 rpm for 5 min, and the second at 55000 rpm for 30 min. Samples were pre-cleared overnight at 4°C with protein G-sepharose to diminish non-specific binding, and MSR immunoprecipitated with mAb 2F8 as previously described. Subsequently, samples were washed and the immune complexes boiled to dissociate the MSR antigen from the mAb. Labeled MSR was then incubated with buffer containing Poly G sepharose to determine functional ligand binding; analysis of poly G bound MSR was done by 6% SDS-PAGE under reducing conditions. In the first lane on the left of fig. 6.8, MSR monomer precipitated by mAb 2F8 from untreated cell lysates is depicted for comparative purposes. Lysate precipitates were adjusted to represent equivalent total protein concentrations, and samples separated by SDS-PAGE in the presence of β-mercaptoethanol. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed tc film at -70°C. Molecular weight standards in kDa are indicated by arrowheads.


2F8
mAb
POLY G
CONTROL
M-CSF
IL-10
M-CSF/TGF-β
L L M L M L M L M
200
97.4
69

from the various cell lysates; more interestingly, the smaller soluble form of MSR present in culture medium was also efficiently bound. This confirms the smaller M_r of the cleaved soluble form and suggests retention of functional capability (as least as far as poly G binding is concerned) post-cleavage. The MSR collagenous domain has previously been shown to mediate poly G binding (Acton *et al.* 1993; Doi *et al.* 1993) and this domain is therefore still intact and functional in the soluble MSR form.

PhosphorImager analysis was done in an attempt to quantify poly G-bound soluble MSR expression (table 6.2). From this single experiment it is apparent that similar results were obtained as far as cytokine/growth factor regulation of MSR synthesis is concerned whether poly G binding of cell-associated MSR or mAb 2F8 immunoprecipitation were used (as described in Chapter 3 and 5). M-CSF increased MSR synthesis 2.9-fold and TGF-β decreased this M-CSF mediated increase by 41%. The same trend is seen when soluble MSR expression from treated cells is analyzed. The extent of M-CSF induced upregulation of soluble MSR (5.2-fold) is noteworthy; this may suggest yet another mode of M-CSF action on the MSR pathway where increased surface MSR expression is accompanied by a disproportionate increase in cleaved MSR perhaps in an attempt to downregulate surface expression. This hypothesis needs further investigation; multiple further experiments are needed for reliable quantification and determination of ratios for release of sMSR to mMSR. The TGF-β mediated reduction in M-CSF upregulation of sMSR (52%) was of the same order as that seen with cell-associated mMSR. In these experiments, IL-10 had little effect on MSR synthesis or soluble MSR expression.

Further attempts were made at detecting and quantifying soluble MSR in mouse serum using species-specific mAb 2F8. To determine the effects of Mϕ activation on sMSR expression, mice were given LPS (100ng) intraperitoneally at intervals 2, 4, 8, 12, 24 and 48 hours; sacrificed and serum taken for analysis. This was done by MSR immunoblotting after serum samples had been pre-cleared with protein G, and either immunoprecipitated with mAb 2F8 or incubated with poly G sepharose, followed by SDS-PAGE separation and transfer to nitrocellulose. Due to the laborious and time-consuming nature of the above assays, a dot blot system was also investigated where cytokine and growth factor-treated BgPMϕ culture supernatants and serum samples were analyzed by MSR blotting and visualization by chemiluminescence of a peroxidase-labelled second anti-rat IgG antibody; this was abandoned due to non-specific binding. Neither approach yielded any consistently positive results; emphasising the need for further specific MSR reagents

Table 6.2: Poly G Sepharose binding of immunoprecipitated labeled MSR from lysates and culture medium of M-CSF and TGF-β treated BgPMϕ

Treatment	*Arbitrary PhosphorImager Units (APUs)*	*Percentage Control*
Control - lysate	4317	
- medium	3888	
M-CSF - lysate	12350	286%
- medium	20226	520%
M-CSF/TGF-β - lysate	7337	170%
- medium	9789	252%

BgPMϕ were treated *in vitro* for 48 hours and metabolically labeled overnight. Immunoprecipitable MSR in lysates and culture medium were incubated with poly G Sepharose as decribed in the text and separated by 6% SDS-PAGE under reducing conditions. Quantification of protein bands on dried gels was done by PhosphorImager and results expressed as a ratio of arbitrary APUs. Results are expressed as percentage of control and are derived from a single experiment.

to facilitate the development of sensitive ELISA and/or other solid-phase detection assays for measuring sMSR levels.

6.4 Discussion

In summary, immunochemical evidence presented in this chapter shows the presence of a soluble form of MSR in culture supernatants which lacks the cytoplasmic domain of membrane-bound MSR and is therefore presumed to have been cleaved prior to release. M-CSF increases release of this fragment *in vitro*. Soluble MSR, by binding poly G, is shown to be functionally active.

6.4.1 Physiological significance of soluble MSR

The induced enzymatic cleavage of MSR may have two major functional implications; the *in vivo* regulation of surface expression and the production of functional soluble forms. Most of the molecules cleaved in response to cell stimulation are involved in intercellular interactions, including CD14 (Beekhuizen *et al.* 1991), CD23 (Aubry *et al.* 1992), CD43 (Rosenstein *et al.* 1991), CD44 (Haynes *et al.* 1989), CD106 (Gearing and Newman 1993), L-and E-selectins (Bevilacqua and Nelson

1993) and pro-TGF-α (Anklesaria *et al.* 1990). Thus, their cleavage, accompanied by downregulation of surface expression, may represent a mechanism controlling adhesion/de-adhesion processes during activation and extravasation of leukocytes. Under conditions where cleavage is slow and accompanied by re-expression of the receptor on the cell surface, steady-state surface expression may not be affected. However, if the kinetics of cleavage is faster than the kinetics of receptor re-expression, or if the re-expression is stopped, the receptor may be lost from the cell surface.

For example, the cleavage of L-selectin is initiated by soluble inflammatory factors and appears part of the regulatory mechanism orchestrating the sequence of adhesion events during granulocyte migration to inflamed tissues (Kishimoto *et al.* 1989). Ligand-mediated induction of membrane molecule cleavage may modulate adhesion interactions, and this hypothesis is supported by studies of CD44 expression on lymphocyte homing *in vivo* (Willerford *et al.* 1989). Peripheral blood lymphocytes entering secondary lymphoid organs mostly comprise cells with markedly higher surface expression of CD44, compared with lymphocytes collected from the thoracic duct, which are thought to have exited secondary lymphoid organs. CD44 may be lost from the surface of lymphocytes during their migration through secondary lymphoid organs; and this is accompanied by an increased level of soluble CD44 in the serum (Camp *et al.* 1993). Another example of a membrane molecule whose cleavage regulates certain cellular activities is CD43, a highly sialylated membrane glycoprotein widely expressed on leukocytes. It imparts a high negative charge on the cell surface which is proposed to prevent non-specific intercellular interactions through cell-cell repulsion; and cleavage of CD43 from neutrophils *in vitro* is associated with homotypic adhesion and promotes cell spreading to protein-coated surfaces (Nathan *et al.* 1993).

Apart from the regulation of surface expression, cleavage of membrane molecules may control production of their soluble forms. This may represent a mechanism distinct from alternative splicing of mRNA. Although a number of soluble molecules cleaved from membrane precursors are detectable in various body fluids and have been shown to have physiological activities, for the main however, physiological functions for the soluble forms of membrane molecules remain highly speculative. They have been suggested to function as: i) inhibitors blocking interactions between ligands and their membrane receptors (e.g. soluble TNFR, ICAM-1 or L-selectin); ii) synergistic factors that mediate interactions between ligands and their receptors (e.g. soluble IL-6R,which forms a complex with IL-6 and the gp130 signal-

transducing molecule; or soluble CD14, which mediates interaction of LPS with an unidentified receptor expressed on $CD14^-$ cells); iii) growth factors that act systemically, in contrast to their membrane precursors (e.g. M-CSF, TNF-α, TGF-α); and iv) ligand transporters, as proposed for some of the soluble cytokine receptors (Bazil 1995).

In addition to technical constraints such as reagent availability and limited circulating levels, the inability to detect soluble MSR *in vivo*, either directly or indirectly, may be attributable to other factors. Firstly, circulating MSR may merely represent the free pool that is available for measurement; this may miss a significant proportion that is shed, but unavailable, due to adhesion/binding of circulating or localized serum ligand (e.g. modified LDL, LPS, ECM component). Analogous to this, soluble L-selectin was identified adsorbed to the luminal surface of high endothelium (Watson *et al.* 1990). This has important implications when considering whether circulating adhesion molecules in general have de-adhesive or other biological effects. Secondly, the measurable levels of soluble MSR reflect both synthesis/release and clearance and little is known about the clearance mechanisms or circulating half-lives of these isoforms.

The MSR, in its soluble form, could be implicated in any of the two broad areas of possible physiological significance. Increased induction due to M-CSF stimulation could serve as a negative feedback control mechanism to downregulate surface expression. Circulating soluble forms may function firstly, as ligand scavengers, and due to their broad ligand-binding capacity bind modified lipoproteins, LPS and lipoteichoic acid, potentially transporting these toxic molecules for rapid clearance by sinusoidal Mϕ in the liver and spleen; and secondly, could bind ligands thereby blocking interaction with membrane-bound MSR, e.g. binding modified lipoprotein prior to engagement of MSR on the cell surface and foam cell formation. Elevation in levels of circulating E-selectin seems to indicate endothelial activation or damage as a component of a particular pathology (diabetes mellitus, sepsis, inflammatory and atherosclerotic vascular disease) (Gearing and Newman 1993; Blann *et al.* 1995); whereas cleaved L-selectin could signify leukocyte activation. Similarly, it may be hypothesized that the presence of sMSR is indicative of Mϕ activation and reliable assay systems may enable Mϕ contributions to chronic inflammatory pathological states to be characterized.

6.4.2 Enzymes involved in membrane receptor cleavage

The suggested regulatory role of soluble receptors lends

prominence to their mechanisms of formation and regulation of this process. In principle, several modes of production could be considered: i) alternative splicing of mRNA transcripts producing soluble molecules lacking membrane spanning domains as shown for the sIL-4R and the sIL-7R; ii) posttranslational modification and subsequent secretion; and iii) release from the cell surface.

Membrane proximal cleavage has recently emerged as a specific mechanism to modulate rapidly surface expression of a limited but diverse subset of transmembrane proteins, such as leukocyte membrane proteins CD14, CD16, CD43, CD44, TNF-R and angiotensin-converting enzyme (ACE). This mechanism is however not limited to leukocytes, but appears involved in regulating cell surface expression of proteins on many cell types (Kahn *et al.* 1994). A protease involved in membrane proximal cleavage has not been identified in any system. It is not clear whether these diverse transmembrane proteins are cleaved by one common membrane proximal protease activity or whether multiple related or unrelated enzymes selectively cleave individual receptors.

Characterization of these enzymes thus far has been sporadic. Formal demonstration that a proteolytic mechanism is involved in membrane protein cleavage may require demonstration of its sensitivity to protease inhibitors. Serine protease inhibitors inhibit release of CD14, CD32 and CD44 (Bazil and Strominger 1991; Sarmay *et al.* 1991; Bazil and Strominger 1994); while metalloprotease inhibitors inhibit release of CD16, and downregulation of CD43 appears sensitive to both serine and metalloprotease inhibitors (Bazil 1995). In contrast, the proteolytic mechanism involved in downmodulating L-selectin expression seems distinct because of its lack of sensitivity to a wide range of protease inhibitors (Kahn *et al.* 1994).

The cleavage of TGF-α and *kit* ligand has been mapped to a region rich in apolar residues, such as alanine and valine (Pandiella *et al.* 1992). In contrast, the cleavage region of L-selectin is strikingly devoid of these residues and the actual cleavage site (K↑SFS) occurs after a charged lysine residue (Kahn *et al.* 1994). It may be that positional effects or a protease docking site may be more critical than sequence specificity at the cleavage site (Pandiella *et al.* 1992). The possibility of a common recognition sequence has however been suggested by the presence of the SFS motif in the proximal transmembrane domain of CD16 (Ravetch and Perussia 1989) and a related SFL motif in TNF-R type II and ACE (Soubrier *et al.* 1988; Kohno *et al.* 1990). There is no such obvious motif present in the MSR proximal transmembrane region.

LPS binding protein (LBP) and CD14 are two effector molecules involved in the LPS response. LBP, a 60K serum glycoprotein, functions

as a catalytic transfer protein which delivers LPS to soluble and possibly membrane CD14 after binding to its lipid A moiety (Gegner *et al.* 1995). Membrane CD14, a 55K glycoprotein LPS receptor, has a glycosylphosphatidylinositol (GPI) anchor; its intracellular signalling mechanism is as yet unknown. In addition to responding to LPS, cells internalize LPS (Kitchens *et al.* 1992); and the MSR has been implicated in this clearance. MSR ligands, such as AcLDL competed, although incompletely, for binding and degradation with lipid IV_A, the bioactive precursor of lipid A, in RAW 264.7 and MSR-transfected CHO cells (Hampton *et al.* 1991).

Soluble CD14 was demonstrated to be produced by two different mechanisms; one protease dependent and the other protease independent. The release of sCD14 by limited proteolysis of its membrane form is serine protease inhibitor sensitive; the protease-independent mechanism involves direct secretion of sCD14 which had escaped GPI-anchor attachment (Labeta *et al.* 1993; Durieux *et al.* 1994; Bufler *et al.* 1995). The demonstration of functional sMSR raises the interesting prospect of another co-existent system for binding and clearance of circulating LSP and requires further investigation. A recent study suggests however that internalization is initiated by LBP-LPS complexes first anchoring to mCD14 and that this process occurs independently of the MSR (Gegner *et al.* 1995).

Complement receptor type I (CD35) release by surface cleavage from neutrophils, and HL60 (promyelomonocytic) and U937 (monocytic) cell lines is accelerated by activation by TNF-α and formyl-methionyl-leucyl-phenylalanine (FMLP) (Danielsson *et al.* 1994). These reagents also rapidly increase CR1 surface expression on neutrophils due to mobilization of an intracellular pool. This is similar to the effect M-CSF has on MSR surface expression and the sequence of events may well be an initial increase in cell surface expression followed by proteolytic cleavage. It is impossible, from the current data, to differentiate between accelerated cell surface cleavage and release from a putative intracellular pool of soluble MSR.

Analogous to L-selectin, MSR processing has been difficult to determine because the large, glycosylated extracellular domain of the monomer migrates as a diffuse band by SDS-PAGE. The sensitivity of MSR cleavage to protease inhibitors, such as aprotinin or PMSF (serine proteases); EDTA, leupeptin (metalloproteases) and pepstatin (aspartic proteases) clearly has to be established. Other candidate proteinases expressed by Mϕ which may be involved in this process include gelatinase, elastase, cathepsin G, and N-aminopeptidase (CD13); collagenase (as described in Chapter 4.3) did not show any cleavage effect

on precipitated MSR. The size difference between soluble and intact forms has been estimated at ≈ 10K; which would indicate a transmembrane peptide of ≈ 80-90 amino acids. This would map the putative cleavage site to a short region of the extracellular domain, between the beginning of the transmembrane domain and the membrane proximal part of the spacer domain. To confirm the presence of a ≈ 10K species immunoprecipitates with mAb 2F8 and anti-cytoplasmic polyclonal antiserum will have to be subjected to SDS-PAGE on 10-20% gradient gels.

Further characterization of the transmembrane nature of the ≈ 10K species could involve production of a polyclonal serum against a synthetic peptide (NH_2-LNWEMKNCLVCSRNTSDTSQC-COOH) corresponding to the sequence of this putative membrane-proximal cleavage domain with an additional COOH-terminal cysteine residue. Interestingly, this region is well conserved between mouse, bovine, rabbit and human MSR (Ashkenas *et al.* 1993). To determine the actual cleavage site, the ≈ 10K form would be purified for NH_2-terminal radiochemical sequence analysis after metabolic labeling with ^{35}S-methionine, or other radiolabeled amino acids such as ^{3}H-serine or ^{3}H-phenylalanine; followed by immunoprecipitation, SDS-PAGE, transfer to polyvinylidenefluoride (PVDF) membrane, and excision of the 10K band.

Chapter Seven

The expression and function of MSR transfected into Chinese Hamster Ovary cells

7.1 Introduction

Inhibition of serum-dependent adhesion of Mϕ to tissue culture plastic (TCP) by mAb 2F8 became apparent when divalent cations had been chelated by EDTA. The most likely explanation for this would be that other, divalent cation-dependent adhesion molecules (especially integrins and selectins) contribute to the adhesion of Mϕ to TCP in the presence of serum. In this system, MSR-dependent adhesion becomes limiting only when these other mechanisms have been disabled. The identification of a specific adhesion-promoting ligand(s) for the MSR would enable its contribution to adhesion to be characterized, unless ligands are shared by different receptors which may then be either co-ordinately or combinatorially involved in adhesion. A more direct approach would be to transfect adhesion receptor cDNAs into other non-Mϕ cells and to assess the contribution of a single receptor to adhesion. In the studies described in this chapter the expression and function of MSR cDNAs stably transfected into Chinese Hamster Ovary (CHO) cells were examined. CHO cells are normally adherent to TCP but are easily detachable in the presence of 5mM EDTA.

MAb 2F8 did not detach adherent Mϕ from TCP, even in the presence of EDTA; this suggested two possibilities: i) irreversible ligation of MSR and its adhesion ligand, or ii) polarization of the Mϕ cell membrane into adherent and non-adherent domains as a result of MSR-mediated adhesion, with MSR clustering in the adherent domain, and exogenously applied mAb 2F8 having access only to the MSR-depleted non-adherent domain. This modulation/redistribution of receptors has been demonstrated for phagocytic Mϕ receptors (Rabinovitch *et al.* 1975; Michl *et al.* 1979). Some evidence in support of the second mechanism will be presented in Chapter 9.

It has previously been shown that mAb 2F8 recognizes both the type I and II murine MSR from stably transfected CHO cells in a species-specific fashion (Fraser *et al.* 1993). These cells, stably transfected with either full-length type I or type II murine MSR cDNAs were obtained

from Dr. M. Krieger, Cambridge, USA and Dr. T. Kodama, Tokyo, Japan and are referred to as CHO MSR I and CHO MSR II in the text. Untransfected, wild-type CHO cells were also obtained from the same source, and are referred to as CHO WT. Experiments with MSR CHO transfectants from the Krieger laboratory are described in sections 7.2.1 and 7.2.2; and culture conditions for these cells were as decribed by Ashkenas *et al* (1993). Functional expression of MSR protein by the cells was confirmed by incubation with DiI-labelled AcLDL (Pitas *et al.* 1981) as endocytosis of this ligand is mediated by the MSR (figs. 7.10, 7.11 and 7.14) and does not occur in CHO WT cells. The DiI fluorescent dye accumulates within the cell and is thus readily detected because of its association with the lipid core of the AcLDL particle (and not with the apoprotein component).

In Western blots mAb 2F8 recognizes MSR from stably transfected CHO cell lysates under non-reducing conditions predominantly in its high M_r, trimer form. No binding is detectable when lysates are separated under reducing conditions; this phenomenon has also previously been described for the mAb IgG-D2 against the bovine MSR (Naito *et al.* 1991; Doi *et al.* 1993). In this regard it is noteworthy that binding of the ligand AcLDL to the MSR could previously only be demonstrated with unreduced ligand blots (Kodama *et al* 1988; Via *et al.* 1992; Doi *et al.* 1993; Fraser 1993). Although the ligand-binding domain of the MSR does not contain cysteine residues (Rohrer *et al.* 1990), a role for disulphide bonds in ligand binding has been shown (Kodama *et al* 1988; Via *et al.* 1992). Evidence was provided for two functional classes of disulphide bonds in the MSR; i) the first was extremely sensitive to reducing agents, did not influence AcLDL binding but affected multimerization of MSR monomers and ii) the second was more resistant to reduction, and appeared to contribute to MSR AcLDL binding. This second class of disulfide bonds, whilst not implicated directly in the MSR ligand-binding site, may play a role in maintaining the MSR in the appropriate ligand-binding conformation (Doi *et al.* 1993).

7.2 MSR expression and function in CHO transfectants

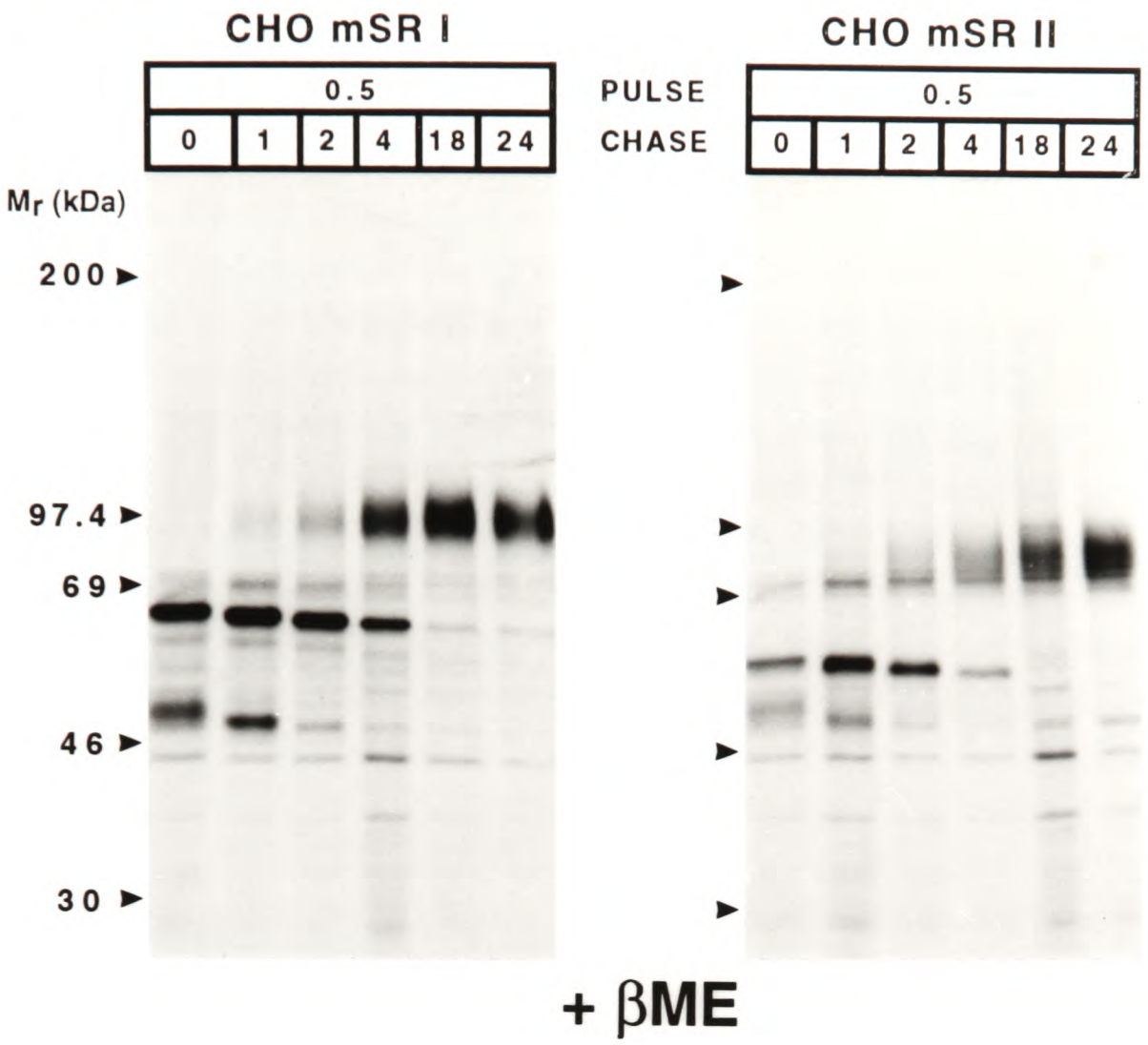
7.2.1 Biosynthesis of MSR in CHO transfectants

In the first instance, the biosynthesis of murine MSR transfected into CHO cells was investigated by pulse-labeling with Tran^{35}S (in association with Dr. I. Fraser) (fig. 7.1) as previously reported (Penman *et al* 1991; Ashkenas *et al* 1993). The differences in M_r of type I and II

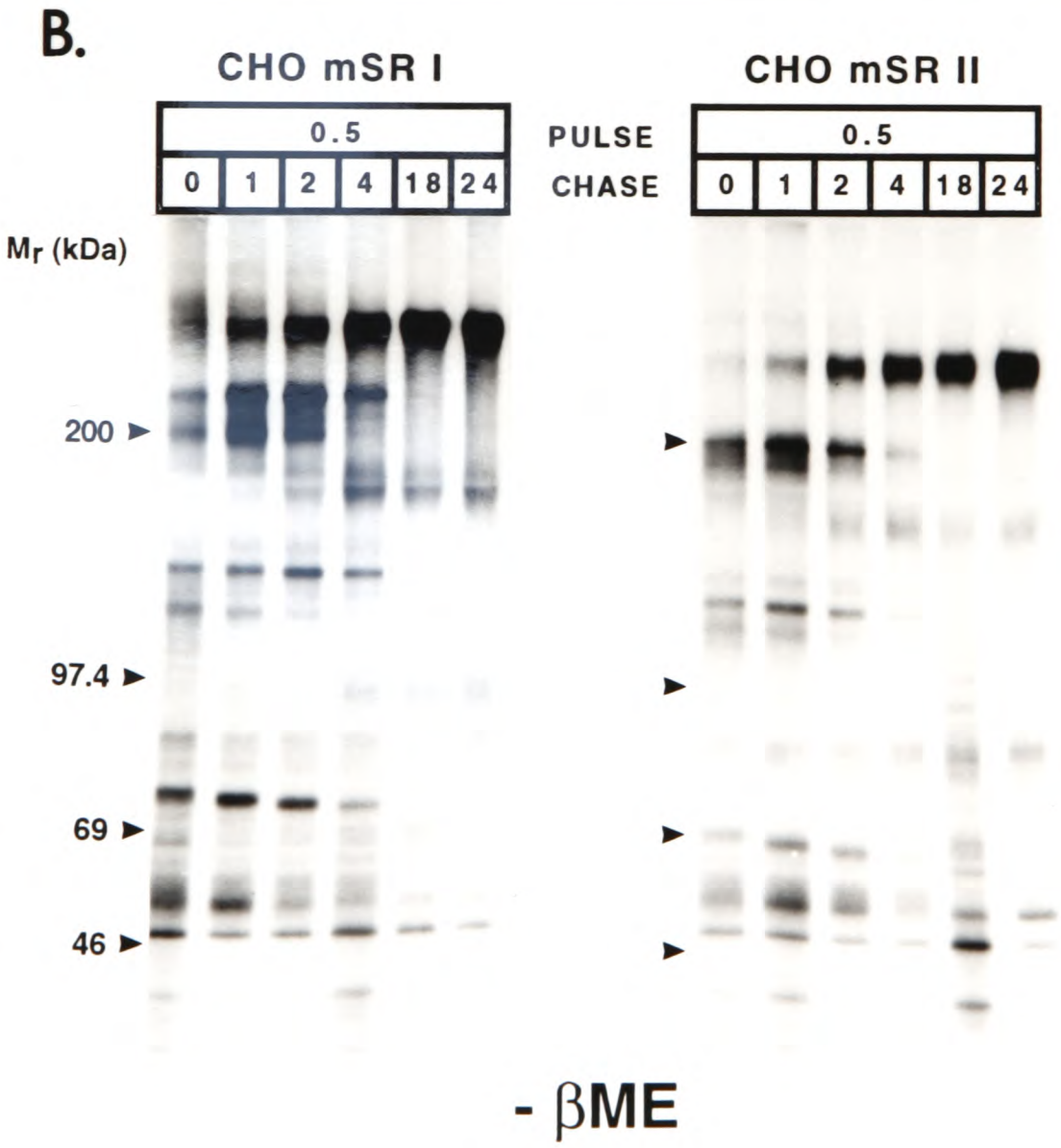
Fig. 7.1 **Biosynthesis of MSR in CHO transfectants**

CHO MSR I and MSR II transfectants were plated in 35mm TCP dishes and grown to confluence. Cells were washed and incubated with methionine-free medium containing 10% dialysed FCS (pulse medium) for a further hour. Pulse medium containing 500 μCi/ml Tran^{35}S-label was then added to the cells for 30 min, following which cells were washed, and the medium replaced with MEM plus 10% FCS. After the indicated chase times, cells were washed, lysed in NP-40 lysis buffer and subjected to immunoprecipitation with mAb 2F8. Precipitates were separated by 5-10% SDS-PAGE in the presence (+βME) **(A)** or absence (-βME) **(B)** of β-mercaptoethanol. Equivalent cell numbers were loaded in each lane. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Pulse and chase times are indicated in hours.

A.


CHO mSR I
CHO mSR II
PULSE
CHASE
0.5
0 1 2 4 18 24
Mr (kDa)
200
97.4
69
46
30
+ βME

B.


CHO mSR I
CHO mSR II
PULSE
CHASE
0.5
0 1 2 4 18 24
Mr (kDa)
200
97.4
69
46
- βME

receptors were readily apparent (for both the precursor and mature products). Samples run under reducing conditions were easiest to interpret due to the multimerization of monomers that occurs during MSR synthesis. Type-specific precursor bands, presumably consisting of core protein plus cotranslationally added high-mannose oligosaccharide chains, had almost disappeared by four hours of chase and the intensity of the mature MSR product increased with time during the chase. Interpretation of the non-reduced precipitates was complicated by the presence of monomer, dimer and trimer, each in precursor, intermediate and mature forms. The type-specific trimer seemed dominant at all time points, suggesting that trimerization occurs early during biosynthesis; but the possibility of artefactual trimerization *in vitro* after lysis cannot be excluded (Penman *et al.* 1991). The kinetics of biosynthesis in transfected CHO cells did not differ qualitatively from those seen with primary BgPMϕ (Ashkenas *et al.* 1993; Fraser 1993). As described below, differences were however detected in intracellular transport of MSR in the different cell types.

7.2.2 Cell surface vs intracellular expression of MSR

The Krieger CHO MSR transfectants, when first obtained, failed to adhere to TCP surfaces in the presence of EDTA (Fraser 1993). This raised doubts as to the identity of the 2F8 antigen as the MSR; but identification by immunoprecipitation from these cells was unequivocal and explanations for this discrepancy between expression and function of MSR were investigated. Flow cytometry of unpermeabilised cells revealed a reduced amount of cell surface MSR on CHO transfectants when compared with RAW 264.7 cells (fig. 7.2). Most transfectants appeared to express little or no cell surface MSR, with only a sub-population of cells expressing surface antigen in the RAW 264 range. While reduced expression at the CHO cell surface resulted in an inability of these cells to sustain divalent cation-independent adhesion, sufficient receptor was present to mediate efficient uptake of DiI-AcLDL and all transfectants were able to mediate AcLDL endocytosis.

To explore this discrepancy between surface and internal expression further, RAW 264.7 and MSR transfected CHO cells were stained with mAb 2F8 in the presence or absence of permeabilising agents such as saponin or triton X-100 and examined by immunofluorescence microscopy (fig. 7.3). In unpermeabilised RAW 264.7 cells, MSR expression was confined to, and evenly distributed on the plasma membrane of the cell. Following permeabilisation, cell surface staining was lost, but a large pool of intracellular antigen was

Fig. 7.2 **Flow cytometry of MSR: RAW 264.7 cells and CHO transfectants**

Cells were grown to confluence in RPMI plus 10% FCS (RAW 264.7) or F-12 Ham Nutrient Medium plus 5% FCS (CHO cells) in 100 mm diameter TCP plates. CHO cells were harvested with 5mM EDTA in PBS, while RAW 264.7 cells required the addition of lidocaine (4 mg/ml) for retrieval. After washing in PBS, cells were resuspended in PBA and processed for flow cytometry as decribed. Staining was with either mAb 2F8 (solid line) or the isotype-matched irrelevant antibody CAMPATH-1G (dashed line). Data were collected in the FL1 photomultiplier of the FACScan and are depicted as log fluorescence intensity (x-axis) versus relative cell number (y-axis). The results shown are representative of two independent experiments.

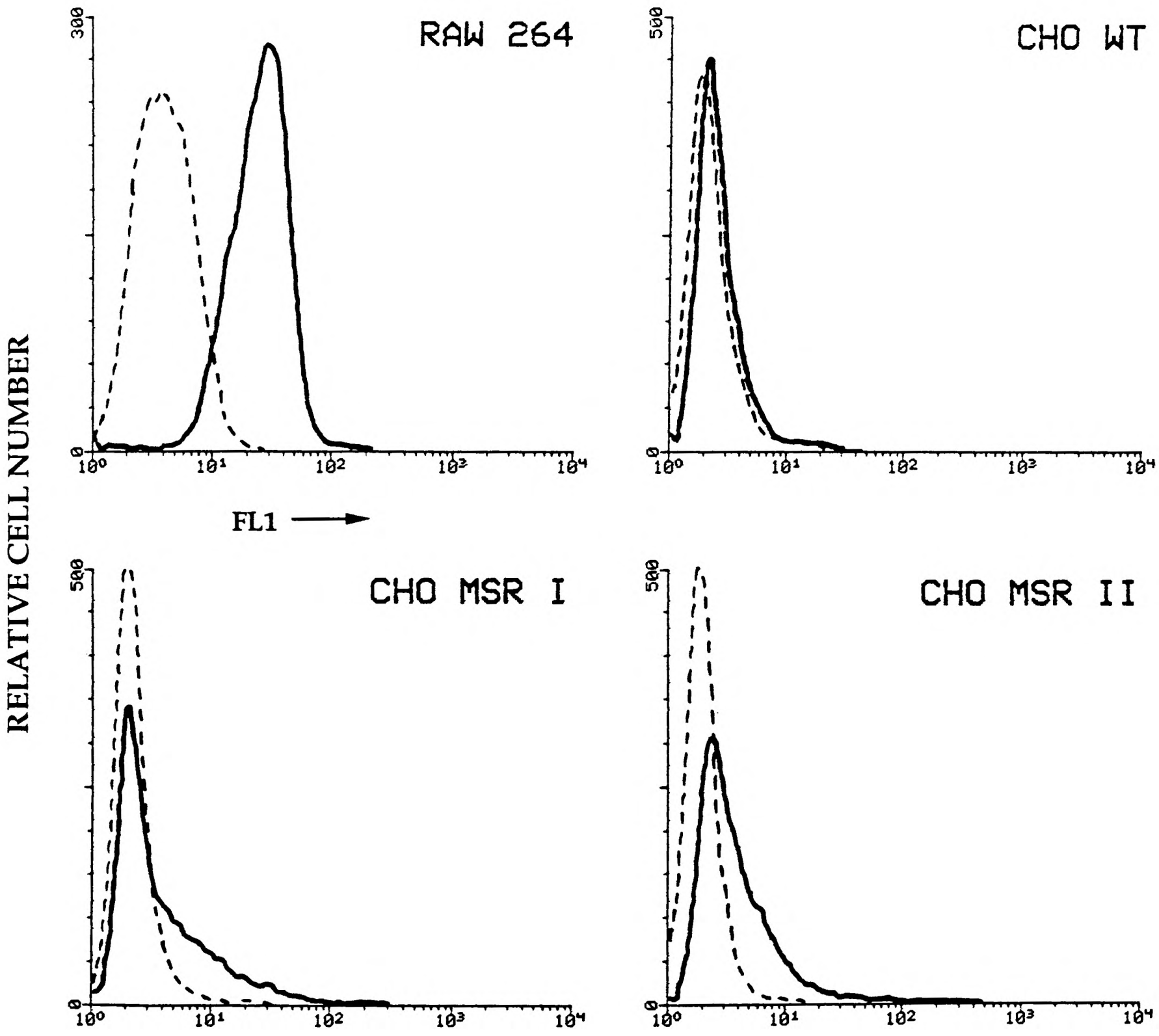

RAW 264
CHO WT
CHO MSR I
CHO MSR II
RELATIVE CELL NUMBER
FL1
300
500
0
10^0
10^1
10^2
10^3
10^4

Fig. 7.3 **Immunofluorescence of MSR: RAW 264.7 cells and CHO transfectants**

Cells were grown on glass coverslips in RPMI plus 10% FCS (RAW 264.7) or F-12 Ham Nutrient Medium plus 5% FCS (CHO). After washing in PBS, cells were fixed in PBS containing 3% (w/v) paraformaldehyde before being processed for staining with mAb 2F8 and FITC-conjugated second antibody. Where indicated, cells were stained and washed in PBS containing 0.1% Triton-X to allow antibody access across the plasma membrane ("permeabilized"). No staining was visible with CAMPATH-1G (not shown). Cells were photographed under oil immersion (objective lens = 100 X) with standard FITC excitation and emission filters. All immunofluorescence exposures were of equivalent duration.

UNPERMEABILISED

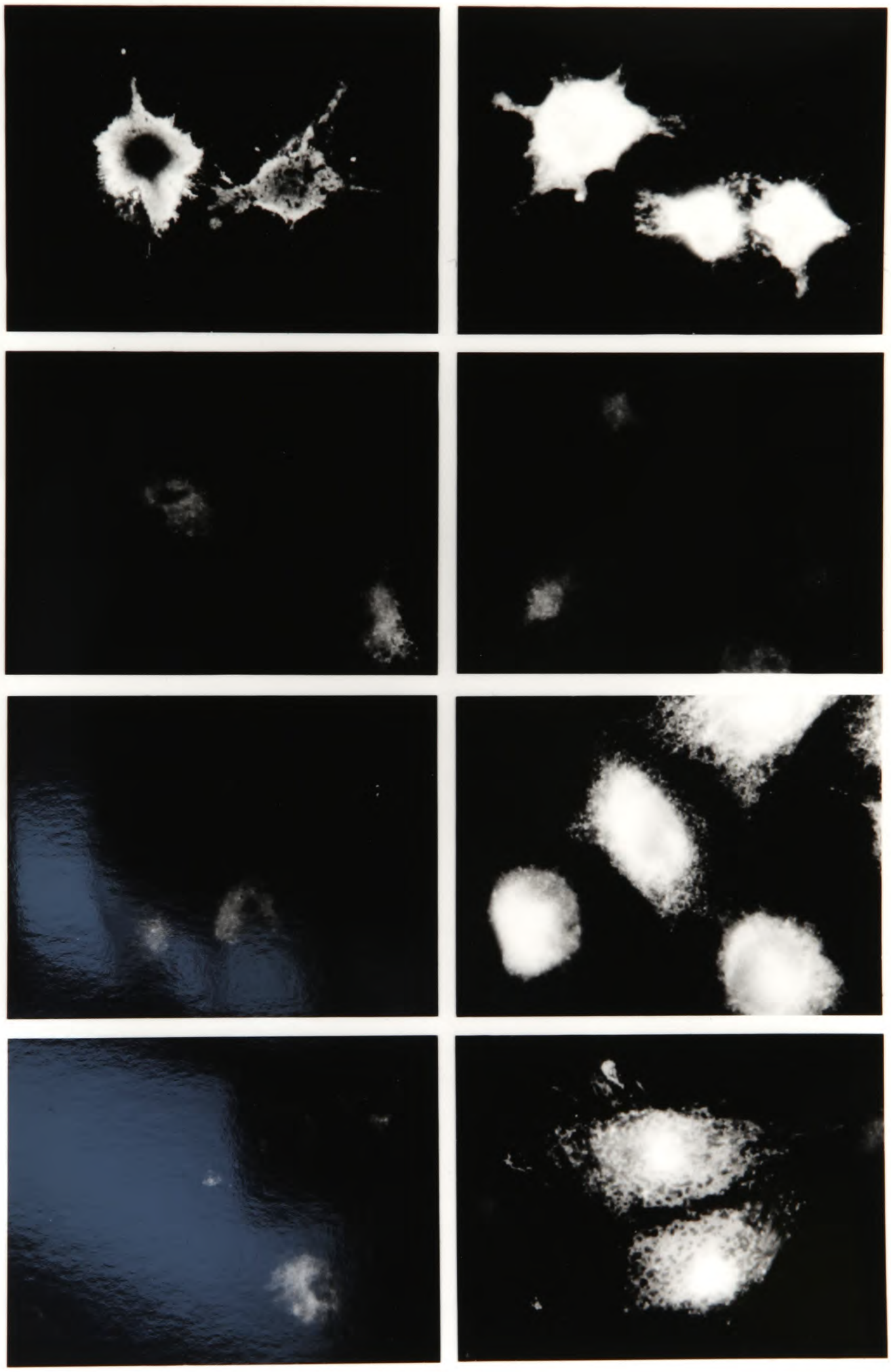
+ TRITON X100

RAW 264

CHO WT

CHO mSR I

CHO mSR II



readily visualised. The extensive, perinuclear pattern of intracellular expression was suggestive of localisation to the ER and Golgi apparatus, and further localisation studies are described in Chapter 9. The intracellular distribution of MSR in RAW 264.7 cells was also noted to be markedly different from that of macrosialin, a Mϕ-restricted late endosomal glycoprotein (Holness *et al.* 1993) (not shown). This RAW 264.7 cell pattern of intracellular distribution of MSR was similar to that seen in primary peritoneal exudate BgPMϕ (not shown), and was therefore not an artefact related to the cell line.

When CHO transfectants were permeabilised and stained with mAb 2F8, considerable intracellular staining was detected (fig. 7.3). Whereas staining at the cell surface in these transfectants was limited to a small sub-population of the cells, permeabilized cells seemed qualitatively to express comparable levels of MSR. Polypeptides that are misfolded, misassembled or unassembled are known to be retained within the ER, and targeted for specific degradation (Hurtley and Helenius 1989). Due to the extensive posttranslational modification and oligomerisation required for MSR biosynthesis, it is feasible that the alien environment of the CHO cells' ER did not promote optimal processing of the murine MSR polypeptide, leading to retention within the ER, and a failure of MSR to continue along the secretory pathway. This argument is countered by the observations from pulse-chase experiments (fig. 7.1) indicating that the approximate half-life, and apparent trimerisation of MSR were no different in transfectants relative to those in primary Mϕ.

An alternative explanation for the failure of MSR to reach the CHO cell surface may lie in the lack of potential accessory transport molecules. Well-described examples of these include: i) the T cell receptor (TCR), in which the αβ heterodimer is not expressed at the cell surface unless associated with the other chains of the TCR complex (Klausner *et al.* 1990), ii) the class II major histocompatibility complex antigen (MHC class II) which requires invariant chain (Ii; CD74) for efficient transport to the cell surface (Viville *et al.* 1993), and iii) the human formyl peptide receptor which requires an additional factor for functional expression when transfected into *Xenopus* oocytes (Murphy and McDermott 1991). Cell type-specific accessory molecules required for functional activity have been described in other systems; for example the FcγRIII (CD16) molecule transduced a signal comparable to that of wild type when transfected into haematopoietic, but not into CHO cell lines (Wirthmueller *et al.* 1992). It will thus be of interest to determine if MSR cDNA transfection into haematopoietic cell lines in general (such as the non-adherent B cell line, A20), or Mϕ-like lines in particular, is

able to confer EDTA-resistant adhesion. It should be noted that immunoprecipitation experiments have never shown any additional bands that could be considered MSR accessory molecules.

7.2.3 Upregulation of MSR expression and function in transfected CHO cells (from Dr. T. Kodama)

To correlate surface expression of MSR with its dual endocytic and adhesion functions, upregulation of the surface expression of type II MSR in transfected CHO cells was attempted; this involved selection and clonal expansion of those CHO transfectants expressing high levels of MSR. CHO MSR II cells used in these experiments were obtained from the laboratory of Dr. T. Kodama, Tokyo and were grown for a prolonged period (3 months) in selection medium (so-called MAC medium), containing 3% (v/v) newborn calf lipoprotein-deficient serum (Sigma), 0.5mg/ml geneticin (G418, Gibco), 250μM **M**evalonate, 3μg protein/ml **A**cLDL, and 40μM **C**ompactin. This medium provides nutritional selection for cells expressing functional scavenger receptors as well as drug selection for transfected cells (Freeman *et al.* 1991; Ashkenas *et al.* 1993).

The effect of the upregulating selection medium on expression levels of CHO MSR II was initially examined by immunoblotting and flow cytometry (in association with Dr. I. Fraser) (figs. 7.4. and 7.5). It is apparent from the immunoblot shown in fig. 7.4 (equal quantities of cell proteins were loaded in each lane) that CHO MSR II cells grown in MAC medium expressed more or less the same amount of mature MSR trimer as RAW 264.7 cells or CHO MSR II cells grown in G418 selection medium alone. Lower M_r bands present in lanes 1 (RAW) and 3 (CHO MSR II upregulated), presumably representing trimer precursor and dimer MSR forms, were however increased in intensity. Flow cytometry showed the increased expression of intracellular MSR in permeabilized CHO MSR II cells following MAC medium selection (fig. 7.5). This was not however associated with marked upregulation of surface MSR expression which confirms the relative inefficiency of MSR transport to the CHO cell surface after synthesis.

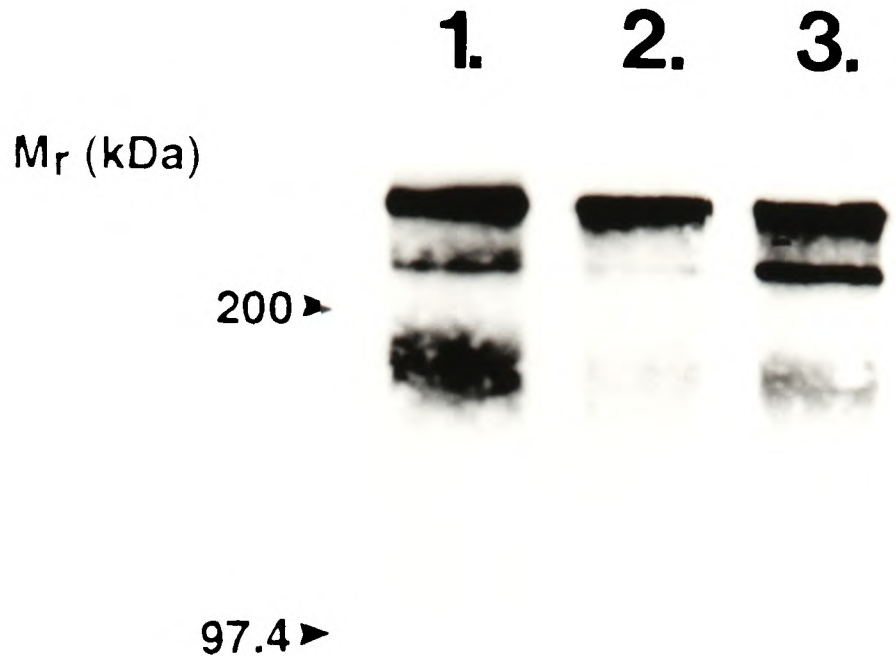
The effect of MAC medium on MSR surface and intracellular distribution was further investigated by biochemically quantifying the fraction of total MSR on the CHO cell surface at steady state (table 7.1 and fig. 7.6). Cells were metabolically labelled overnight (16h) to equilibrium followed by surface biotinylation, on ice, by the membrane-impermeant probe sulfo-NHS-SS-biotin (Harter and Mellman 1992); MSR immunoprecipitation was further done as described in Chapter 2. Total

Fig. 7.4 (top) **Immunoblot of MSR in CHO MSR II and CHO MSR II MAC transfectants (3 month upregulation period)**

RAW 264.7 **(lane 1)** and CHO MSR II cells **(lane 2)** were grown as previously decribed, lysed in lysis buffer containing NP-40 and the lysate protein concentrations determined using the BCA assay (Pierce). In addition, MSR protein levels were also determined in upregulated CHO MSR II cell lysates **(lane 3)**. These CHO MSR II MAC cells had been grown for a prolonged period (3 months) in functional selection medium (so-called MAC medium), containing 3% (v/v) newborn calf lipoprotein-deficient serum (Sigma), 0.5mg/ml geneticin (G418, Gibco), 250μM Mevalonate, 3μg protein/ml AcLDL, and 40μM Compactin. Equal amounts of total cellular protein (20 ug) were subjected to 6% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. Blots were probed with mAb 2F8 and binding detected by peroxidase-conjugated second antibody and chemiluminescence. The top bands in each lane represent mature MSR trimers and lower two trimer precursor and dimer respectively.

Fig. 7.5 (bottom) **Flow cytometry of surface and internal MSR expression in CHO MSR II and MSR II MAC transfectants**

CHO cells were grown to confluence in 35 mm TCP dishes and harvested with 5mM EDTA in PBS. After washing in PBS, cells were resuspended in PBA and processed for flow cytometry by staining with mAb 2F8 as decribed. Internal MSR antigen levels were determined in cells that had been initially permeabilized with 1% (w/v) saponin and all subsequent processing was done in the presence of 0.1% (w/v) saponin. Data were collected in the FL1 photomultiplier of the FACScan and are depicted as log fluorescence intensity (x-axis) versus relative cell number (y-axis). The results shown (CHO WT: dashed line; CHO MSR II: solid line; and CHO MSR II MAC cells: dot-dashed line) are representative of two independent experiments.


1.
2.
3.
Mr (kDa)
200
97.4

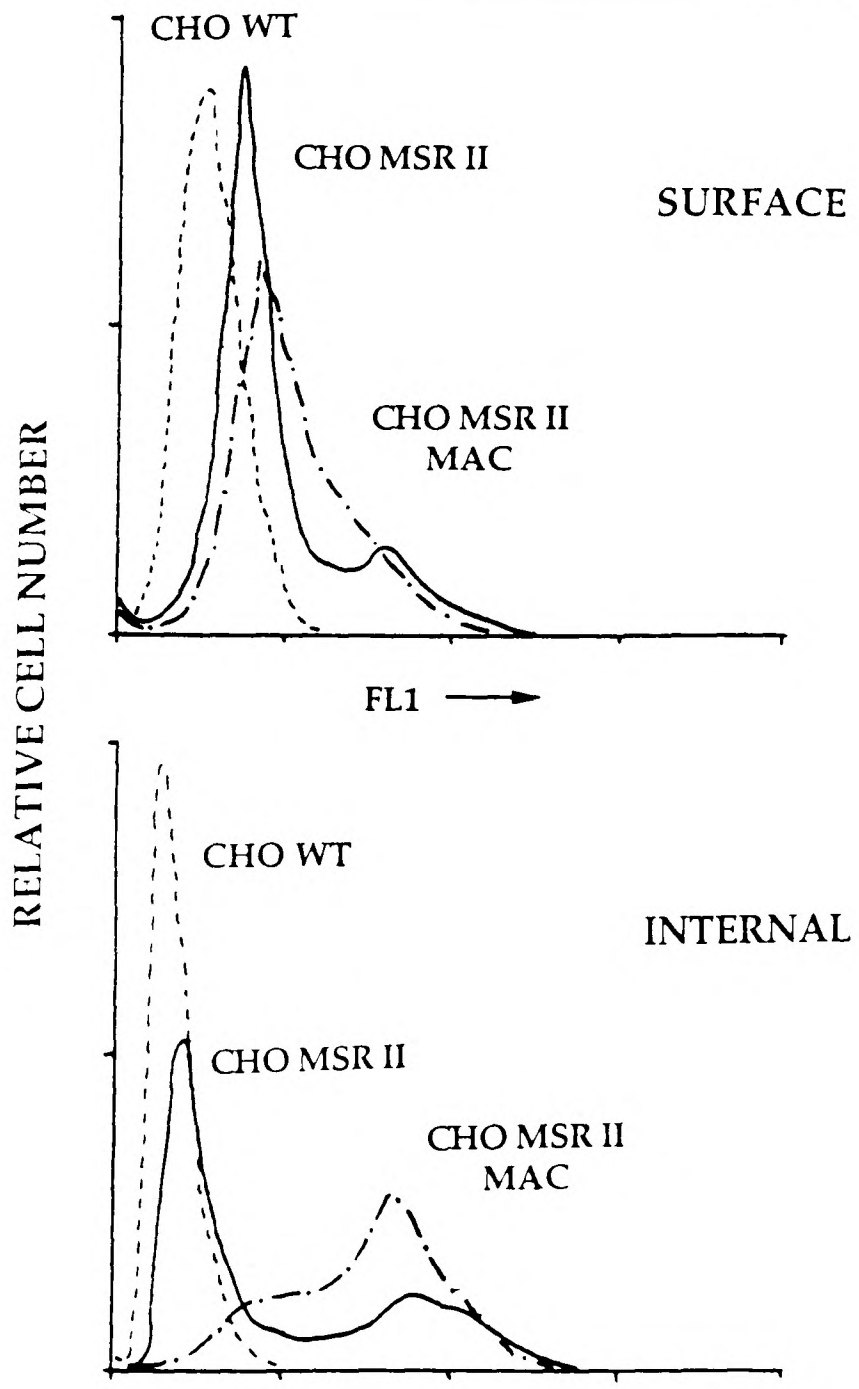
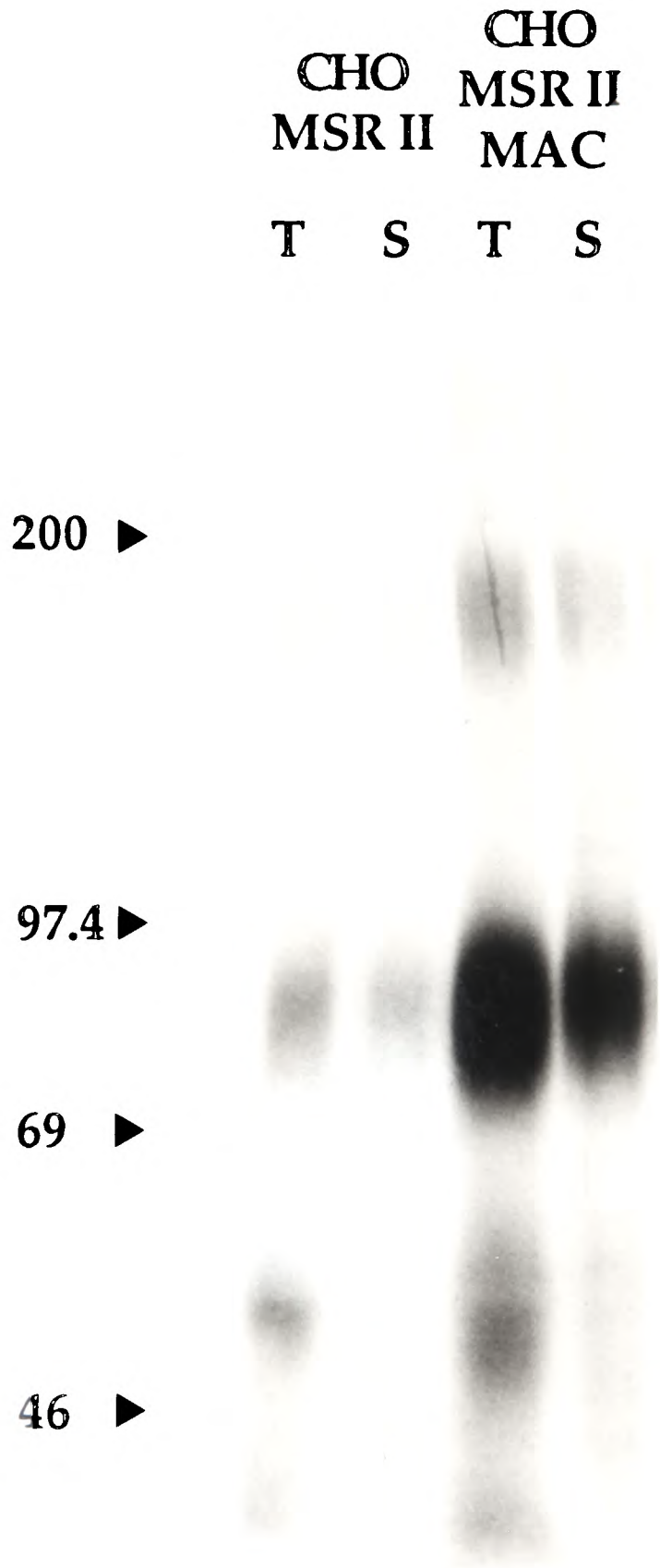

CHO WT
CHO MSR II
SURFACE
CHO MSR II
MAC
RELATIVE CELL NUMBER
FL1
CHO WT
INTERNAL
CHO MSR II
CHO MSR II
MAC

Fig. 7.6 **The effect of MAC selection medium on MSR II protein surface expression in CHO transfectants**

CHO MSR II and CHO MSR II MAC cells were grown to confluence in 35 mm TCP dishes and metabolically labelled with Tran^{35}S-label™ overnight. This was followed by surface biotinylation, on ice, by the membrane-impermeant probe sulfo-NHS-SS-biotin as decribed. After cell lysis, total MSR was precipitated by mAb 2F8 and the resulting immunoprecipitate divided into two equal aliquots. Biotinylated "surface" MSR was precipitated from one aliquot using streptavidin-agarose and "total" MSR was precipitated from the other aliquot by a second round of mAb 2F8 immunoprecipitation. Precipitates were adjusted to represent equivalent total protein concentrations, and separated by SDS-PAGE in the presence of β-mercaptoethanol. MSR precursor and monomer migrate at Mr ≈ 64 and ≈ 90K respectively. Gels were fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Molecular weight standards in kDa are indicated by arrowheads. Total and biotinylated MSR were also quantitated after SDS-PAGE by PhosphorImager analysis of dried gels (table 7.1).


CHO MSR II
CHO MSR II MAC
T
S
T
S
200
97.4
69
46

and biotinylated MSR were quantitated after SDS-PAGE by PhosphorImager analysis of dried gels.

In these experiments incubation in MAC medium increased both surface MSR expression and total MSR protein synthesis 12-fold. MAC selection medium upregulation was however not associated with any redistribution of the cellular pool of MSR from a mainly intracellular compartment (70% of total) to a surface location. The ratio of surface to

Table 7.1 Effect of MAC medium on CHO cell surface distribution of MSR.

Treatment	*Total*	*Surface*
CHO MSR II	6598	1984
% surface:total	30%	
CHO MSR II MAC	78902	24581
% surface:total	31%	

CHO cells stably transfected with MSR type II and cultured in G418 or MAC selection media were metabolically labelled and surface biotinylated using sulfo-NHS-SS-biotin as described below. Quantification of protein bands on dried gels was done by PhosphorImager analysis and results expressed as a ratio of arbitrary PhosphorImager units (APUs). The values are derived from a single experiment.

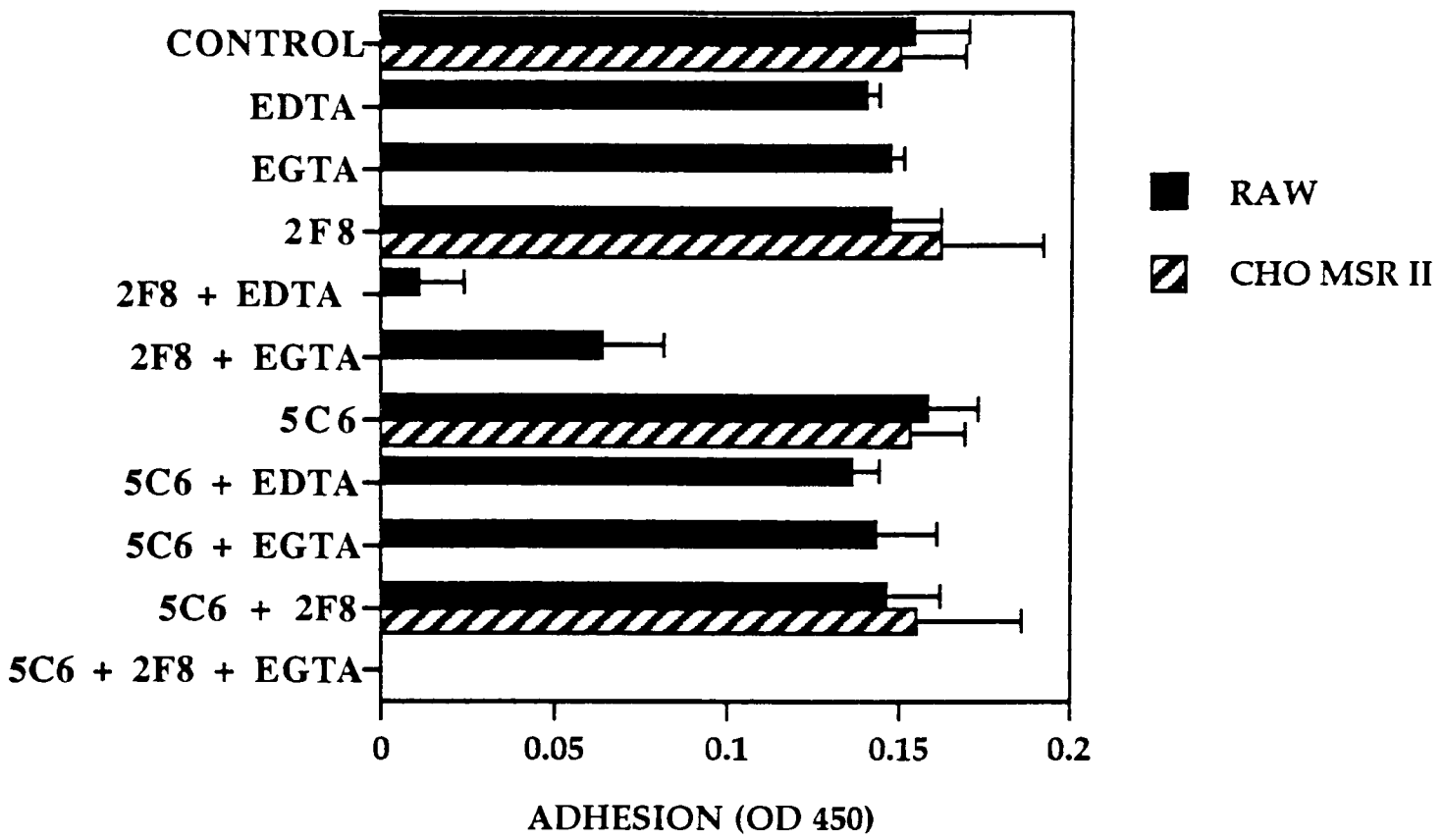
total MSR was indeed remarkably similar to that seen when primary elicited peritoneal cells (BgPMϕ) were metabolically labeled, surface biotinylated and MSR immunoprecipitated (31%) (Chapter 3.2 and de Villiers *et al.* 1994). This result would suggest that, even in CHO transfectants, sufficient mature MSR molecules appear on the plasma membrane to mediate function, e.g. AcLDL endocytosis (fig. 7.14).

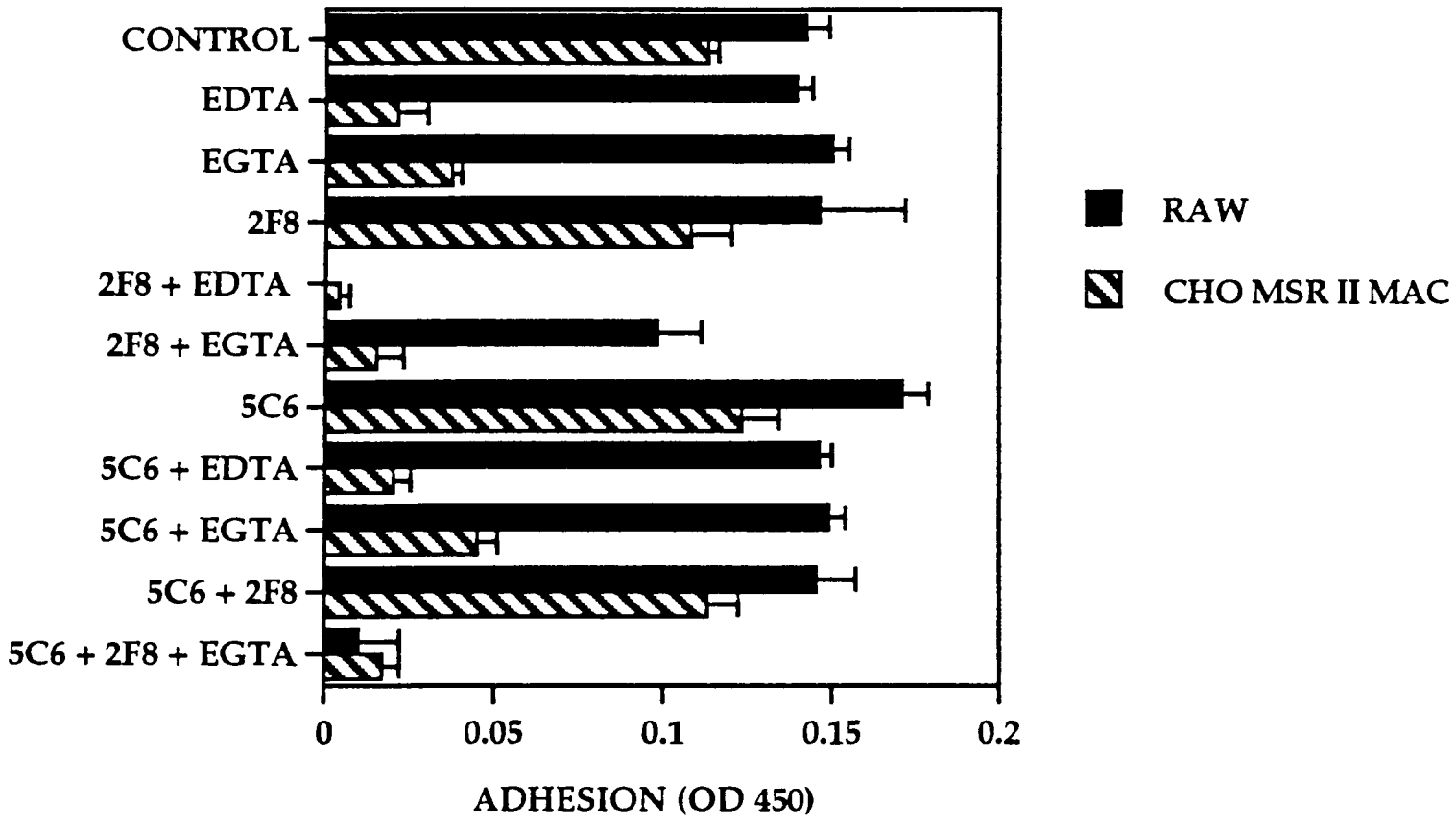
The demonstrated increase in MSR biosynthesis and surface expression in the MAC medium-treated CHO transfectants was presumed to be associated with increased functional activity and the adhesion properties of these cells to TCP were next examined (fig. 7.7 top and bottom). It is apparent from Fig. 7.7 (top) that the Mϕ-like cell line RAW 264.7 show the expected described adhesion characteristics (Fraser *et al.* 1993) (i.e. a considerable EDTA-resistant component which is inhibited by the addition of the mAb 2F8 to MSR). In contrast, EDTA and EGTA completely abolished the adherence of CHO MSR II cells to TCP; this demonstrates both the extreme divalent cation and calcium-sensitive nature of CHO MSR II cell adhesion in this context and the lack of any MSR contribution. Fig. 7.7 (bottom) depicts the adhesion to

Fig. 7.7 **Adhesion of RAW 264.7 cells and CHO transfectants to TCP**

Adhesion assays were performed as described by comparing the TCP adhesion properties of RAW 264.7 and CHO MSR II cells **(top)** and RAW 264.7 and CHO MSR II MAC cells **(bottom)**. Chelators (EDTA and EGTA) were used at a final concentration of 5mM. Purified mAbs (2F8 and 5C6) were used at a final concentration of 2 μg/ml. After staining with Giemsa, plates were washed, dried, and the retained dye solubilised in methanol. Stain was quantified by measuring absorbance at 450nm in an automatic plate reader. Results are derived from a single experiment in triplicate and are representative of three such similar experiments.

ADHESION OF RAW 264.7, CHO MSR II AND CHO MSR II MAC TO TCP





TCP of RAW 264.7 and CHO MSR II cells grown in MAC selection medium. The upregulated CHO cells exhibit a slight increase in EDTA-resistant adhesion to TCP (but much less so than RAW 264.7 cells). This small, but perceptible, increase is mAb 2F8 (MSR)-inhibitable and insensitive to inhibition by mAb 5C6 (CR3) and thus consistent with an upregulation in MSR surface expression in the transfected CHO cells.

The small increase in MSR-dependent adhesion to TCP in MSR CHO transfectants (despite the biochemically apparent increase in surface expression) may be explained by i) the need for co-operativity with other adhesion receptors, cytoskeletal or signalling molecules (which may be absent in CHO cells) in Mϕ and Mϕ-like cell lines for complete MSR-dependent adhesion to be revealed; or ii) transfected MSR on the surface of MAC medium-treated CHO cells may exhibit subtle structural differences due to increased ER retention, misfolding, misassembly, degradation or differences in glycosylation in an alien CHO cell environment; all of which may adversely influence the MSR's functional ability as far as adhesion is concerned.

7.2.4 Further upregulation of MSR expression and function in CHO transfectants.

To extend these studies on the contribution of MSR to adhesion, the expression of type II MSR in stably transfected CHO cells was further upregulated by prolonged (more than 9 months) incubation in MAC selection medium. MSR expression levels were then analyzed by immunoblot analysis (fig. 7.8) and flow cytometry (fig. 7.9). Total MSR protein expression (fig. 7.8) was markedly increased in the MAC medium-treated CHO transfectants compared to the G418-treated CHO MSR II cells; and this increase appeared more impressive than that seen in fig. 7.4 when levels had been assayed after a shorter period of functional upregulation. Flow cytometry analysis of surface and internal (following saponin permeabilization) MSR levels confirmed the upregulation in expression following MAC medium selection (fig. 7.9). In addition, the MAC upregulated CHO MSR II cells appeared a more distinct and homogeneous population compared to earlier results (fig. 7.5). Quantitative assessment of the geometric mean fluorescence units revealed a relatively higher increase in MSR surface expression level (2.6-fold) than internal expression (2-fold) in the MAC-treated CHO MSR II cells; this may indicate a higher functional MSR surface expression than evident previously.

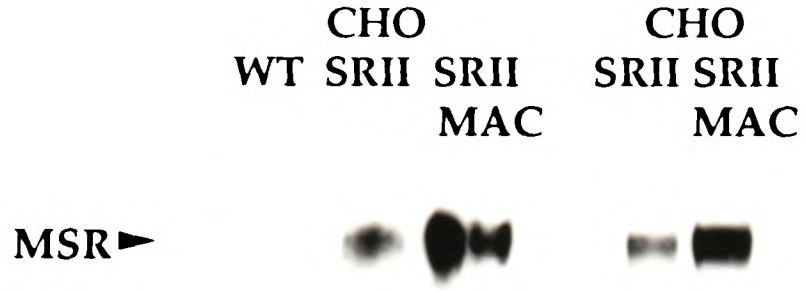
MSR endocytic activity in the CHO transfectants was assayed by their ability to take up DiI-AcLDL (4 μg/ml final concentration). It is

Fig. 7.8 (top) **Immunoblot of MSR in CHO MSR II and CHO MSR II MAC transfectants (9 month upregulation period)**

CHO WT, CHO MSR II and CHO MSR II MAC cells that had been selected in MAC medium for a prolonged period (> 9 months) were grown as previously decribed, lysed in lysis buffer containing NP-40 and the lysate protein concentrations determined using the BCA assay (Pierce). Equal amounts of total cellular protein (20 µg) were subjected to 6% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. Blots were probed with mAb 2F8 and binding detected by peroxidase-conjugated second antibody and chemiluminescence. Results from two different sets of lysates are depicted.

Fig. 7.9 (bottom) **Flow cytometry of surface and internal MSR expression in CHO MSR II and MSR II MAC transfectants**

CHO cells were grown to confluence in 35 mm TCP dishes and harvested with 5mM EDTA in PBS. After washing in PBS, cells were resuspended in PBA and processed for flow cytometry by staining with mAb 2F8 as decribed. Internal MSR antigen levels were determined in cells that had been initially permeabilized with 1% (w/v) saponin and all subsequent processing was done in the presence of 0.1% (w/v) saponin. Data were collected in the FL1 photomultiplier of the FACScan and are depicted as log fluorescence intensity (x-axis) versus relative cell number (y-axis). The results shown are representative of two independent experiments.


CHO
WT SRII SRII
MAC
CHO
SRII SRII
MAC
MSR

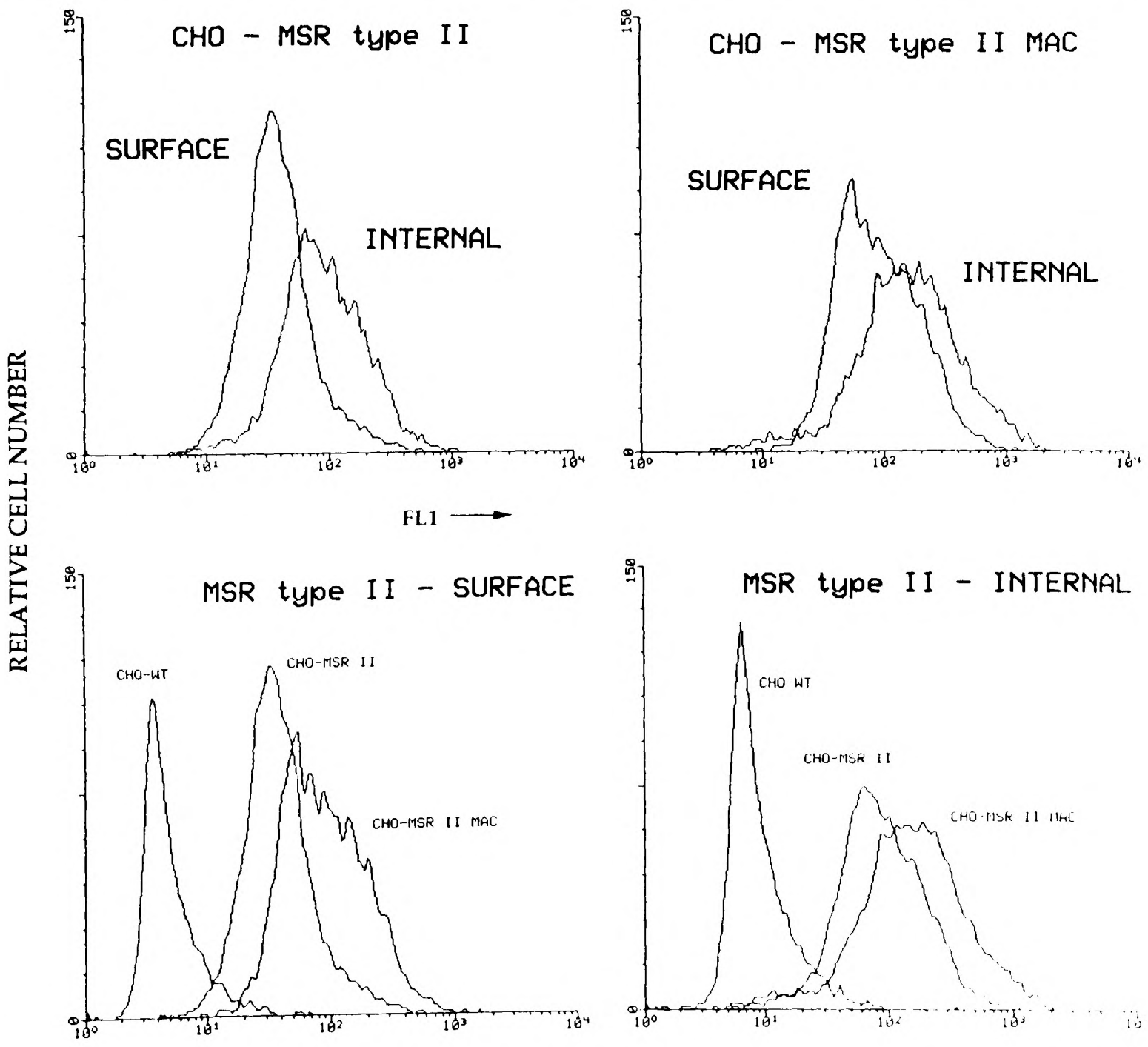
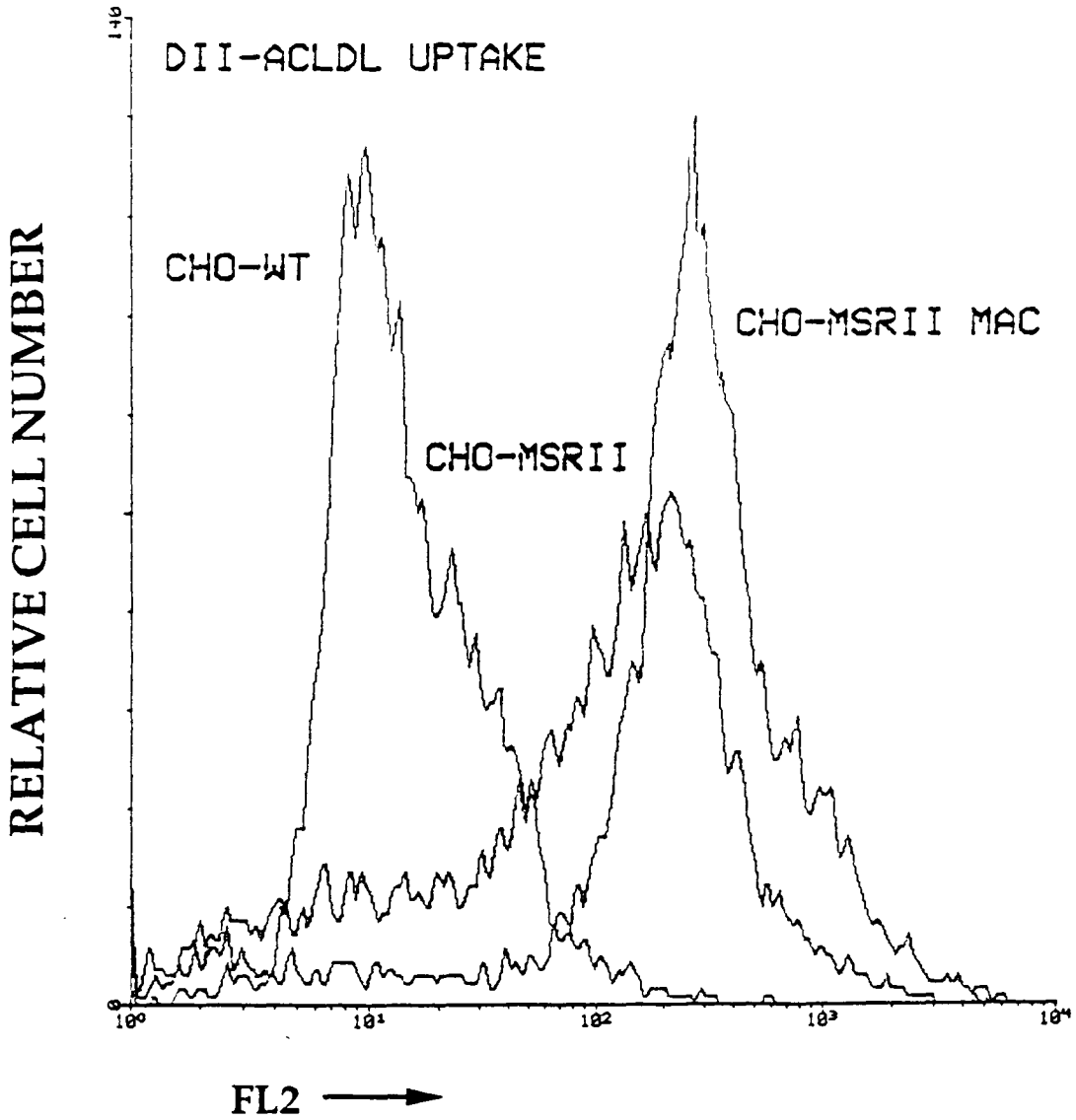
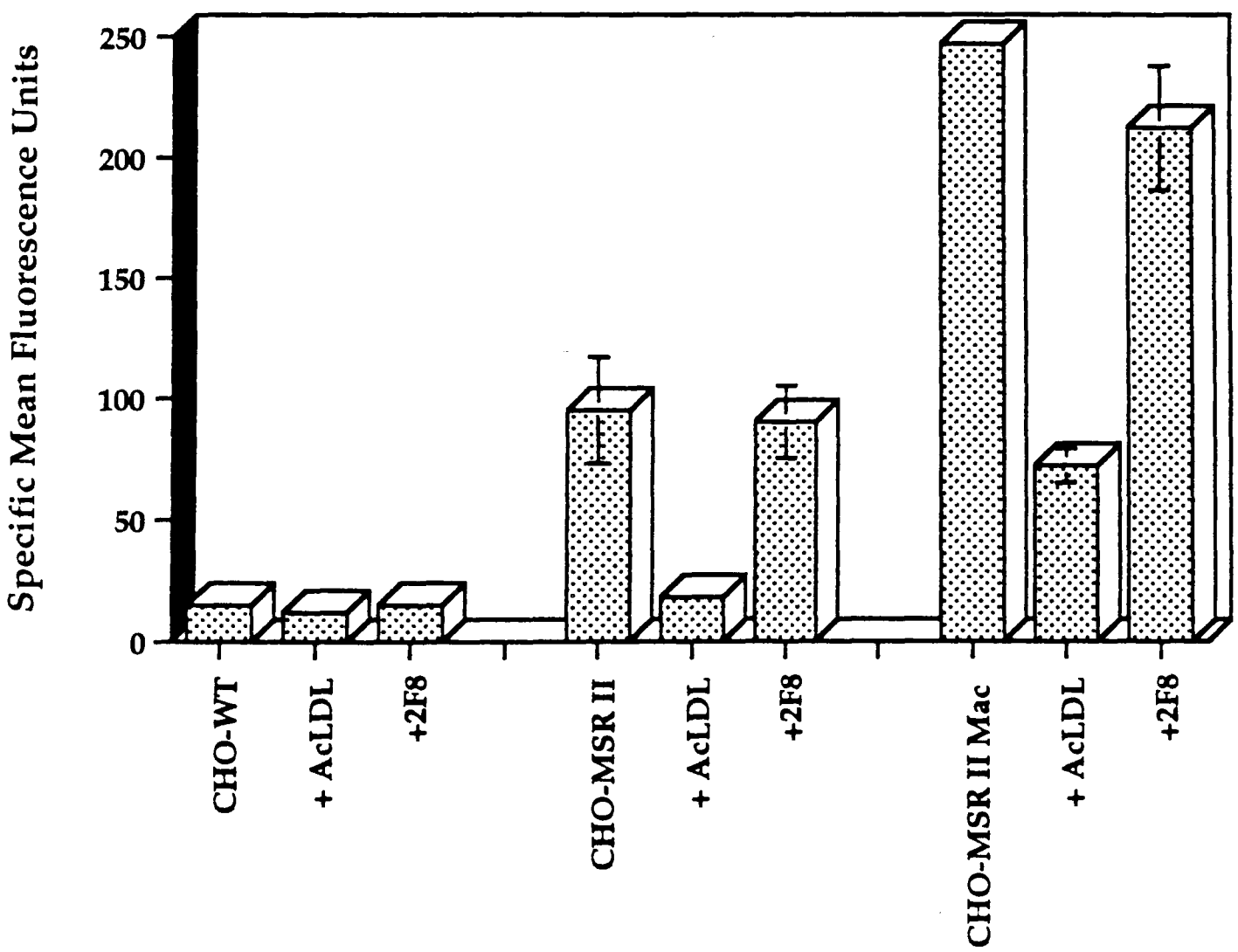

CHO - MSR type II
SURFACE
INTERNAL
CHO - MSR type II MAC
SURFACE
INTERNAL
RELATIVE CELL NUMBER
FL1
MSR type II - SURFACE
CHO-WT
CHO-MSR II
CHO-MSR II MAC
MSR type II - INTERNAL
CHO-WT
CHO-MSR II
CHO-MSR II MAC

Fig. 7.10 (top) and fig. 7.11 (bottom) **The uptake of DiI-AcLDL by CHO MSR transfectants as assessed by flow cytometry**

CHO WT, CHO MSR II and CHO MSR II MAC cells that had been selected in MAC medium for a prolonged period (> 9 months) were grown as previously decribed. Cells were plated in 24 well TCP plates and after washing in PBS, DiI-AcLDL at 4 μg/ml was added. After incubation for 3 hours at 37°C, cells were washed, harvested using 5mM EDTA in PBS, and resuspended in PBA for analysis by flow cytometry (FL2 photomultiplier). Results shown in fig 7.10 (top) are representative of samples done in triplicate from two separate experiments. In a further two experiments depicted in fig 7.11 (bottom) the ability of unlabelled AcLDL (200 μg/ml final concentration) and purified mAb 2F8 (10μg/ml final concentration) to inhibit DiI-AcLDL uptake by the CHO transfectants was assessed. Results show the mean values (two independent experiments in duplicate) of specific fluorescence intensities obtained from subtracting geometric mean autofluorescence from the fluorescence obtained after uptake of DiI-AcLDL.


DII-ACLDL UPTAKE
CHO-WT
CHO-MSRII
CHO-MSRII MAC
RELATIVE CELL NUMBER
FL2


DiI-AcLDL UPTAKE BY CHO
MSR TRANSFECTANTS
Specific Mean Fluorescence Units
250
200
150
100
50
0
CHO-WT
+ AcLDL
+2F8
CHO-MSR II
+ AcLDL
+2F8
CHO-MSR II Mac
+ AcLDL
+2F8

clear from the flow cytometry profiles in fig. 7.10 that the CHO MSR II MAC cells represent a distinct functional population from the CHO WT cells as far as DiI-AcLDL uptake is concerned (specific mean fluorescent uptake 16-fold increased). The CHO MSR II cells were more heterogeneous and a sub-population took up relatively little DiI-AcLDL; resulting in a 6-fold increase in comparison to CHO WT cells. Functional selection pressure from prolonged culture in MAC medium contributed to a further 2.6-fold upregulation of DiI-AcLDL uptake in CHO MSR II MAC cells. This is graphically illustrated in fig. 7.11; in addition a 50-fold excess unlabeled AcLDL (200μg/ml) inhibited DiI-AcLDL uptake specifically (81% and 71% in the CHO MSR II and CHO MSR II MAC cells respectively). Interestingly, mAb 2F8 (at a saturating concentration of 10μg/ml) reproducibly had no inhibitory effect on DiI-AcLDL uptake by the CHO transfectants. This is in contrast to the situation in RAW 264.7 cells where mAb 2F8 was reported to inhibit DiI-AcLDL uptake partially and an isotype-matched antibody (CAMPATH-1G) (to control for RAW 264.7 Fc receptor involvement) had no effect (Fraser *et al.* 1993). This does not exclude the possibility that the partial inhibitory effect of mAb 2F8 on AcLDL uptake requires the presence of Fc receptors (which are absent in CHO cells) in Mϕ and Mϕ-like cell lines. This phenomenon needs to be further investigated.

The contribution of MSR to TCP adhesion in the upregulated CHO MSR II MAC medium-treated cells was also explored (fig, 7.12). Serum-dependent adhesion of these cells to TCP appeared qualitatively more EDTA-resistant than CHO WT or G418-selected CHO MSR II cells. Quantitatively, adhesion to TCP remained predominantly EDTA-sensitive, but the MSR-dependent (EDTA-resistant, mAb 2F8-inhibitable) component seemed to increase slightly from 8% in CHO MSR II transfectants to 22% in CHO MSR II MAC upregulated cells. This increase was however not significant.

7.2.5 Discussion

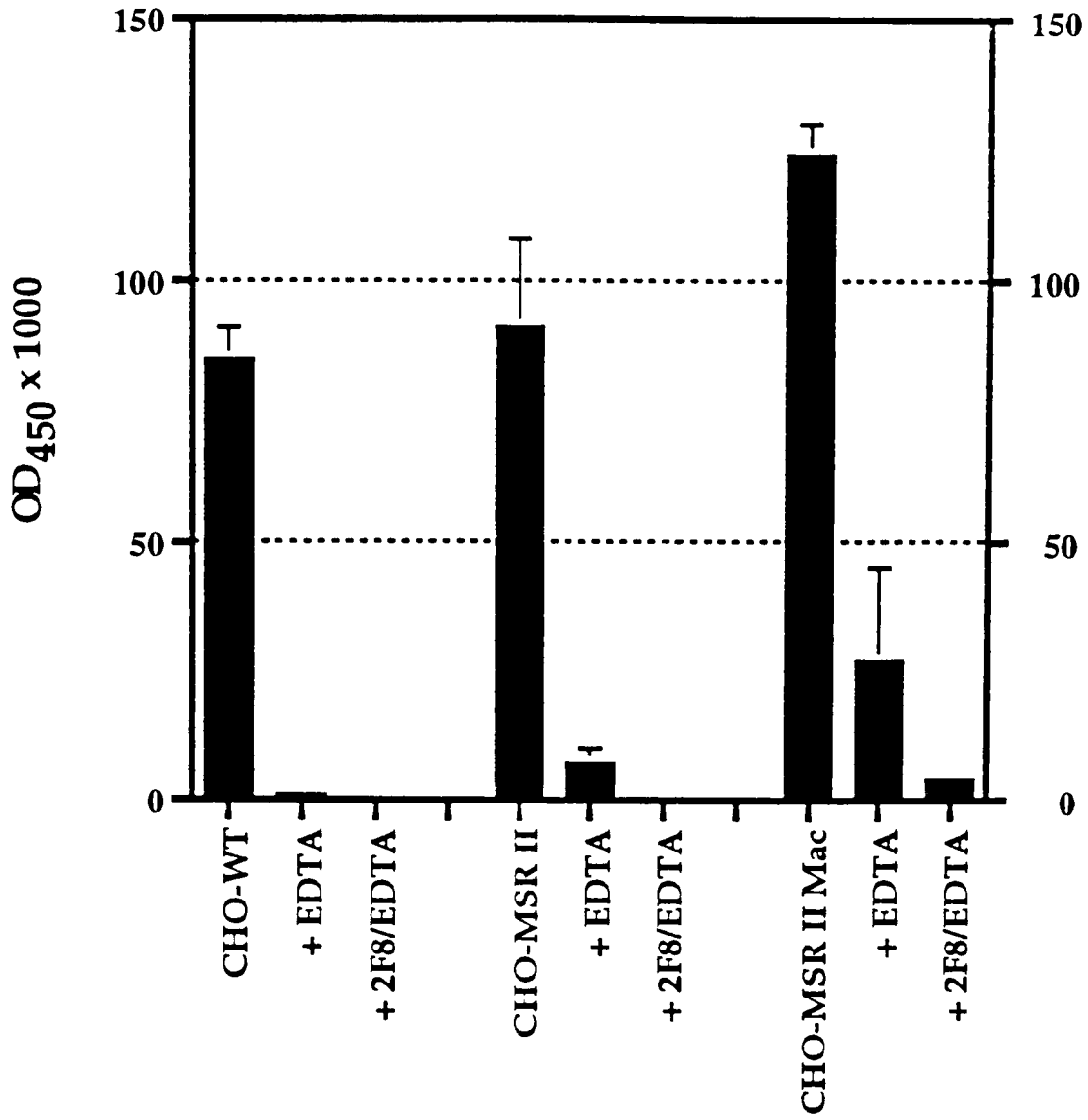
In the studies described the expression of type II MSR cDNAs stably transfected into CHO cells was manipulated in an attempt to characterize the endocytic function and contribution to adhesion of this single receptor. Fluorescent AcLDL uptake was a sensitive and specific marker of MSR transfection even in the presence of low cell surface expression. Uptake was markedly enhanced by culturing transfected cells in a scavenger-specific functional selection medium in which AcLDL remained the only source for cellular cholesterol; this increase is most likely due to selective pressure for high expressors in the CHO .

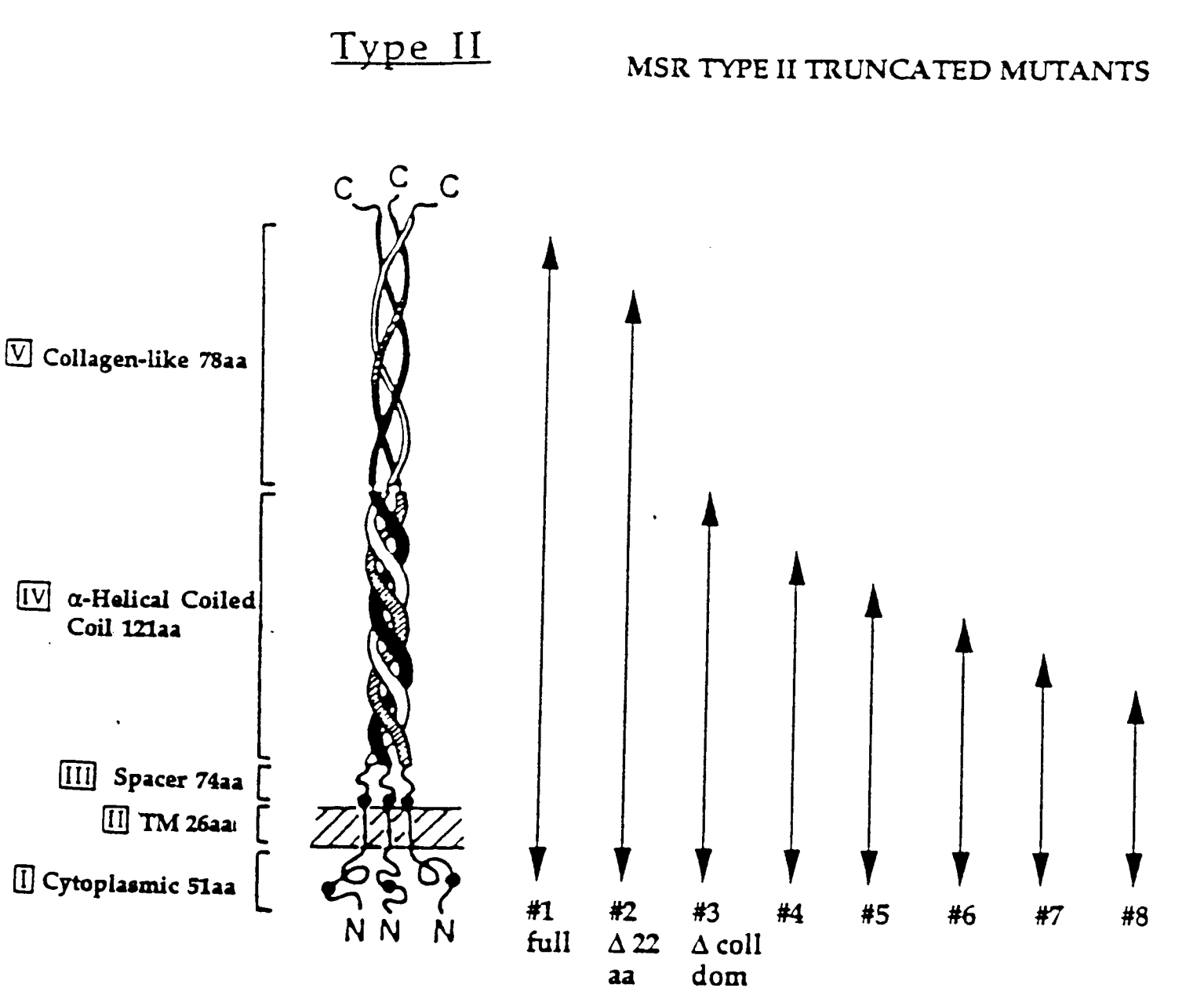
Fig. 7.12 (top) **Adhesion of CHO MSR II and CHO MSR II MAC transfectants to TCP**

Adhesion assays were performed as described by comparing the TCP adhesion properties of CHO MSR II cells and CHO MSR II MAC cells that had been grown in the MAC functional selection medium for > 9 months. EDTA and purified mAb 2F8 were used at final concentrations of 5mM and 2 μg/ml respectively. After staining with Giemsa, plates were washed, dried, and the retained dye solubilised in methanol. Stain was quantified by measuring absorbance at 450nm in an automatic plate reader. Results are derived from a single experiment in triplicate and are representative of two such similar experiments.

Fig. 7.13 (bottom) **Schematic diagram of murine MSR type II truncated mutants stably transfected into CHO cells and used in studies to determine the MSR binding site of mAb 2F8.**

A series of murine MSR truncation mutants stably transfected into CHO cells was obtained from Drs. M. Honda and T. Kodama, Tokyo and characterized. Truncated mutants were constructed in the expression vector pRC/CMV (Invitrogen, San Diego, CA) which contains *HindIII* and *XbaI* restriction sites as well as a neomycin resistance gene. The DNA sequences of all mutant genes were determined by the dideoxy method. Expression vectors containing the various mutants were transfected into CHO cells using the polybrene method. To isolate transfectants expressing the mutants, CHO cells were grown, selected and cloned by dilution plating in F-12 HAM Nutrient Medium supplemented with 5% (v/v) FCS, 100units/ml penicillin, 100μg/ml streptomycin, 2mM glutamine and 300 μg/ml geneticin (G418) for at least 6 weeks.


TCP ADHESION OF CHO MSR TRANSFECTANTS
OD450 x 1000
150
100
50
0
CHO-WT
+ EDTA
+ 2F8/EDTA
CHO-MSR II
+ EDTA
+ 2F8/EDTA
CHO-MSR II Mac
+ EDTA
+ 2F8/EDTA


Type II
MSR TYPE II TRUNCATED MUTANTS
C C C
V Collagen-like 78aa
IV α-Helical Coiled Coil 121aa
III Spacer 74aa
II TM 26aa
I Cytoplasmic 51aa
N N N
#1 full
#2 Δ 22 aa
#3 Δ coll dom
#4
#5
#6
#7
#8
355 aa 325 aa 277 aa 249 aa 226 aa 197 aa 183 aa 167 aa

transfectant population and the loss of negative cells. The MSR contribution to adhesion was more difficult to demonstrate and only became apparent in upregulated transfected cells expressing high levels of surface MSR.

Some possible mechanisms to explain this duality in both function and sensitivity were invoked, and include the possible need for accessory transport molecules to increase functional surface expression and the requirement for additional surface membrane molecules such as Fc receptors or Mϕ-specific cytoskeletal and/or signalling molecules to facilitate or mediate MSR-dependent adhesion. The EDTA-sensitive nature of CHO cell adherence to TCP complicated assessment of the role for transfected MSR in adhesion. In contrast to RAW 264.7 adhesion to TCP, other divalent cation-dependent adhesion molecules (probably integrins) contribute the major component to the adhesion of untransfected CHO WT cells to TCP. In this system, transfected MSR-dependent adhesion would only come into play when these other mechanisms are non-operative and MSR surface expression had been upregulated to a high degree. This upregulation was hampered by the relative inefficiency of CHO transfectants to redistribute MSR from a predominantly intracellular pool to a surface location. The shortcoming of this general approach (as was clearly demonstrated) is that transfected receptors do not always behave in a functional (in this case adhesion-promoting) fashion when expressed in an alien cellular environment. An alternative approach may be to transfect MSR cDNAs into otherwise non-adherent cells (such as the B cell line A20) and then assess its contribution to adhesion. The identification of a specific adhesion-promoting ligand(s) for the MSR could allow its contribution to adhesion to be characterized; but this may be complicated if such ligands are found to be shared by different receptors.

7.3 Localization of the MSR binding site of mAb 2F8: implications for a functional adhesion-promoting site

In order to examine further the molecular basis for the bifunctional endocytic and adhesive nature of MSR, a series of murine MSR truncation mutants stably transfected into CHO cells was obtained from Drs. M. Honda and T. Kodama, Tokyo and characterized (fig. 7.13). These mutants include **#1 CHO-MSR II** expressing the complete MSR type II (350 aa); **#2 CHO-Δ22aa** (325 aa) lacking the C-terminal 22 amino acids containing the putative AcLDL binding site; **#3 CHO-Δcollagen-like domain** (277 aa) lacking the collagen-binding domain; and **#4-#8** a series of deletion mutants in the α-helical coiled-coil domain (249, 226, 197, 183

and 167 aa respectively). Truncated mutants were constructed in the expression vector pRC/CMV (Invitrogen, San Diego, CA) which contains *HindIII* and *XbaI* restriction sites as well as a neomycin resistance gene. The DNA sequences of all mutant genes were determined by the dideoxy method. Expression vectors containing the various mutants were transfected into CHO cells using the polybrene method. To isolate transfectants expressing the mutants, CHO cells were grown, selected and cloned by dilution plating in F-12 Ham Nutrient Medium supplemented with 5% (v/v) FCS, 100units/ml penicillin, 100µg/ml streptomycin, 2mM glutamine and 300 µg/ml geneticin (G418) for at least 6 weeks.

Functional expression of #1 CHO MSR II was shown by its ability to take up DiI-AcLDL (fig. 7.14). None of the other truncation mutants retained this ability and, as has been described for the bovine MSR (Acton *et al* 1993; Doi *et al.* 1993), deletion of the C-terminal 22 amino acids of the collagen-like domain abolished AcLDL uptake; this occurred in spite of expression of the truncated receptor on the cell surface as shown by mAb 2F8 immunostaining. This region contains a conserved (among bovine, human, rabbit and murine MSR) lysine cluster which, based on computational modeling, has been proposed to contribute to a "charged collagen" structure. A positively charged groove in the lysine cluster is suggested to interact specifically with AcLDL and other negatively charged ligands (Doi *et al.* 1993). The EDTA-sensitive nature of CHO WT and non-upregulated CHO MSR II adhesion to TCP was described above; as well as the poor correlation between the extent of an EDTA-resistant component and the level of MSR expression. Analogous to the G418-selected non-upregulated CHO MSR II cells, the various mutants would not be expected to exhibit marked surface expression of truncated MSR; and none of them showed any qualitative increase in EDTA resistance to TCP adhesion. Upregulation of expression to examine relative contributions to adhesion, by using the AcLDL-containing MAC selection medium, is not an option as the mutants lack the AcLDL-binding site. A possible option would be to sort high MSR mutant expressors by mAb 2F8 selction.

To characterize the binding site of mAb 2F8, which in view of its adhesion inhibitory activity may delineate a functional adhesion domain in MSR, the antibody was used to detect truncated MSR protein in the transfected CHO cells. This approach was also necessitated by the caveats described above relating to functional expression levels of truncated MSR transfected into CHO cells. CHO transfectants #1- #3 were metabolically labeled overnight, MSR immunoprecipitated from the lysates, and the precipitates separated by SDS-PAGE under reducing

Fig. 7.14 **Immunofluorescence microscopy of DiI-AcLDL uptake by CHO WT, CHO MSR II and #2 CHO-Δ22aa cells**

CHO WT and CHO MSR II cells as well as #2 CHO-Δ22aa transfectants lacking the MSR C-terminal 22 amino acids containing the putative AcLDL binding site were grown on acid-washed coverslips in 35 mm TCP dishes. After washing in PBS, cells were incubated with DiI-AcLDL at 4 μg/ml for 3 hours at 37°C. The cells were then washed and fixed in PBS containing 3% (w/v) paraformaldehyde. Cells were mounted in Mowiol mountant and allowed to dry overnight in the dark. Cells were photographed under both phase and epifluorescence microscopy (identical fields) using oil immersion (objective lens = 100 x) and standard DiI excitation and emission filters. All exposures were of equivalent duration. None of the other truncation mutants exhibited any DiI-AcLDL uptake under similar experimental conditions (results not shown).

A. CHO WT cells (phase).

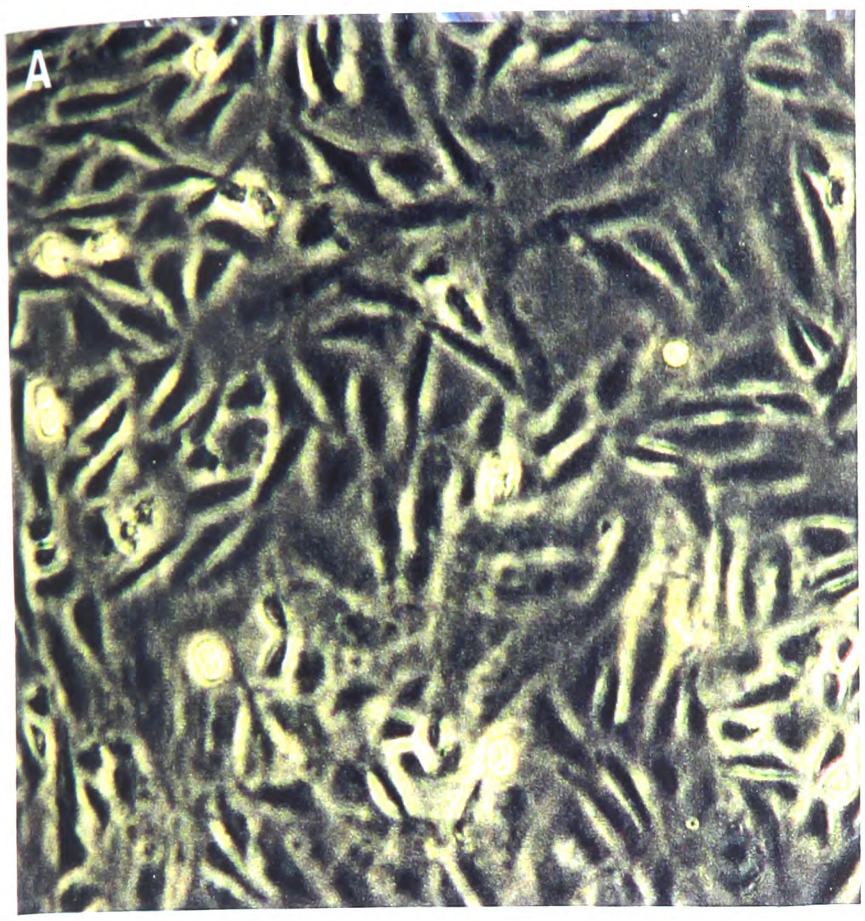
B. CHO WT cells (fluorescence).

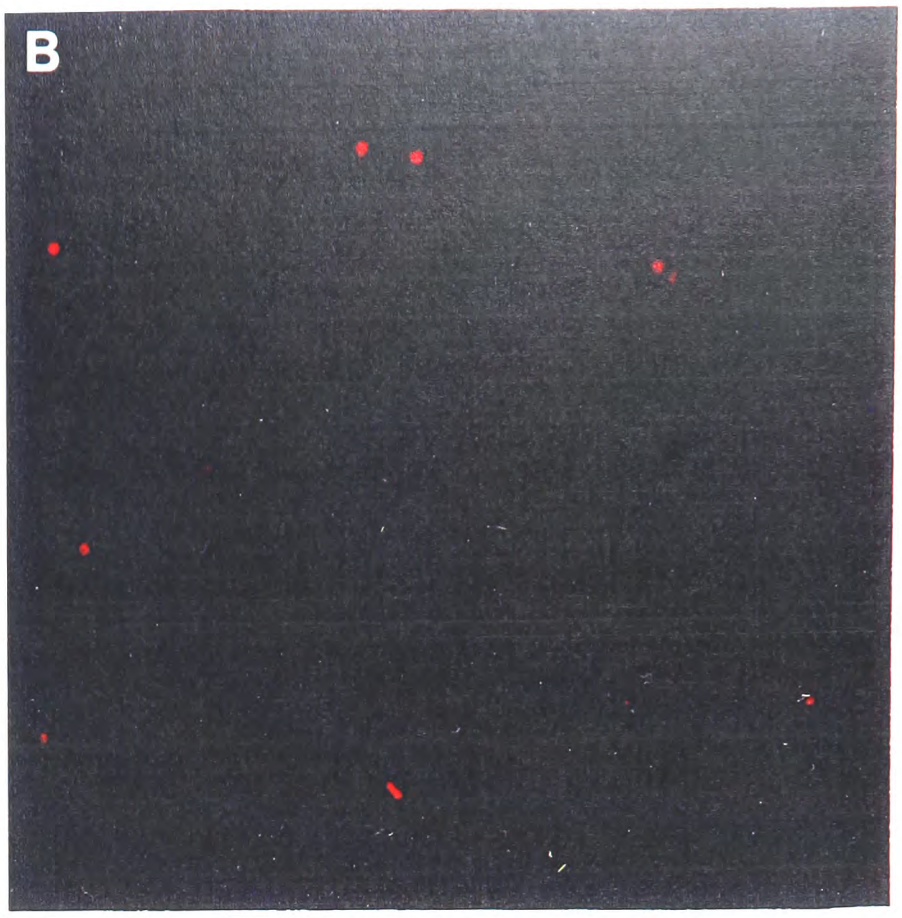
C. CHO MSR II cells (phase).

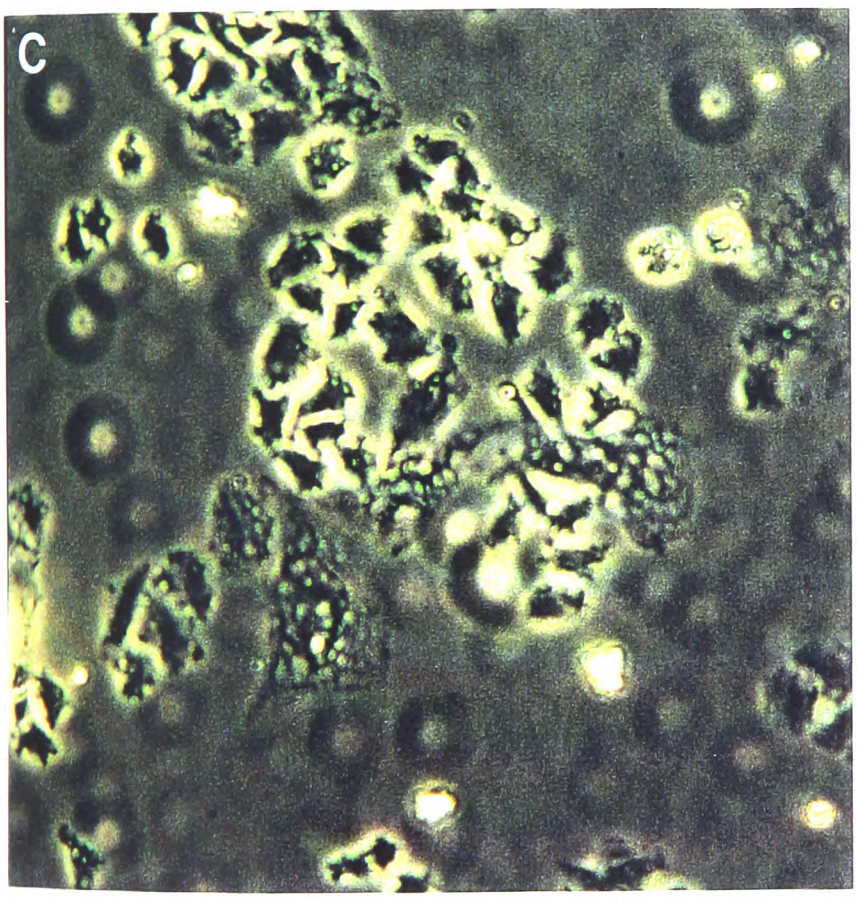
D. CHO MSR II cells (fluorescence).

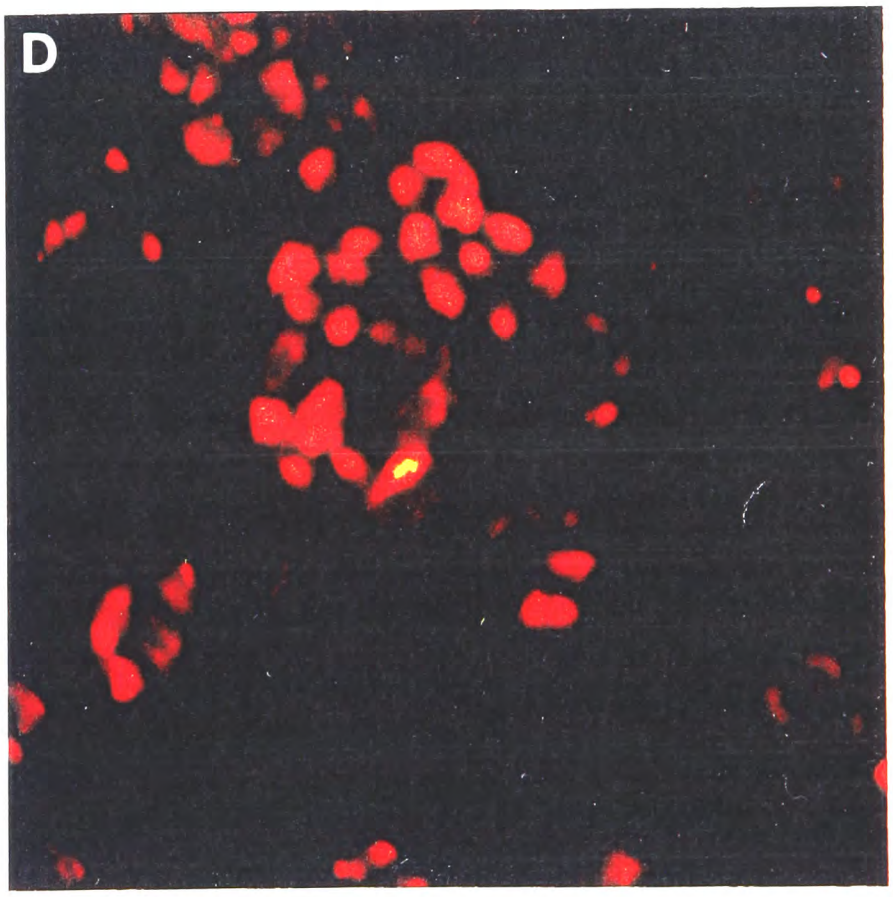
E. #2 CHO-Δ22aa transfectants (phase).

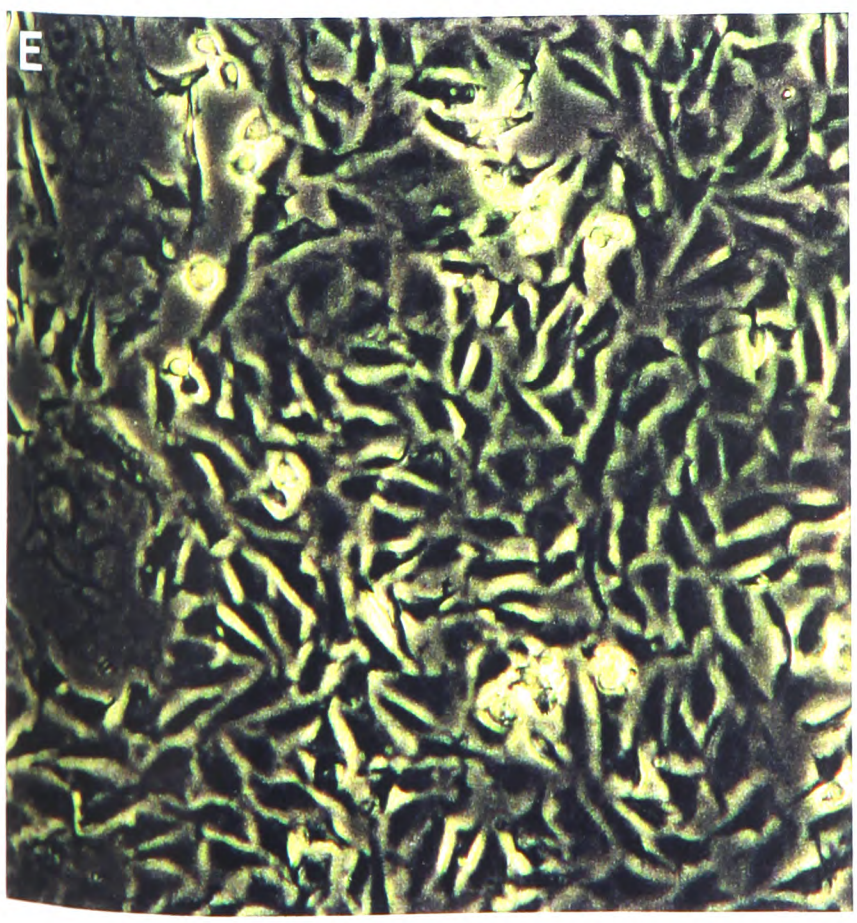
F. #2 CHO-Δ22aa transfectants (fluorescence).


A


B


C


D


E

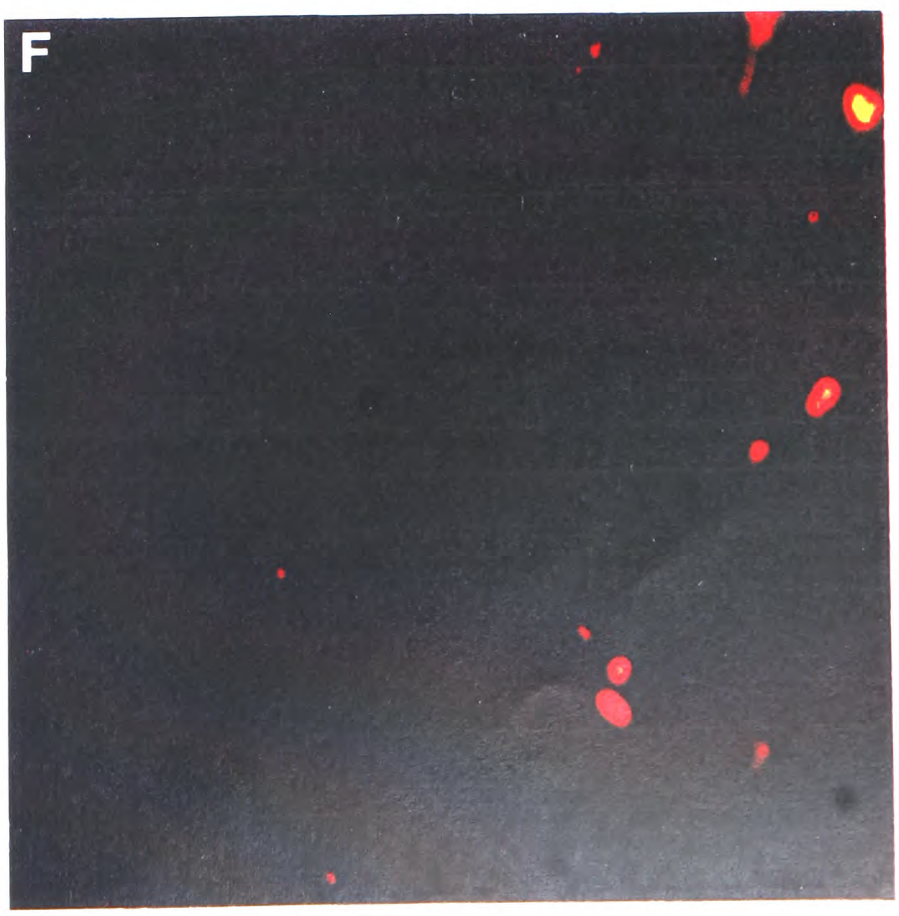
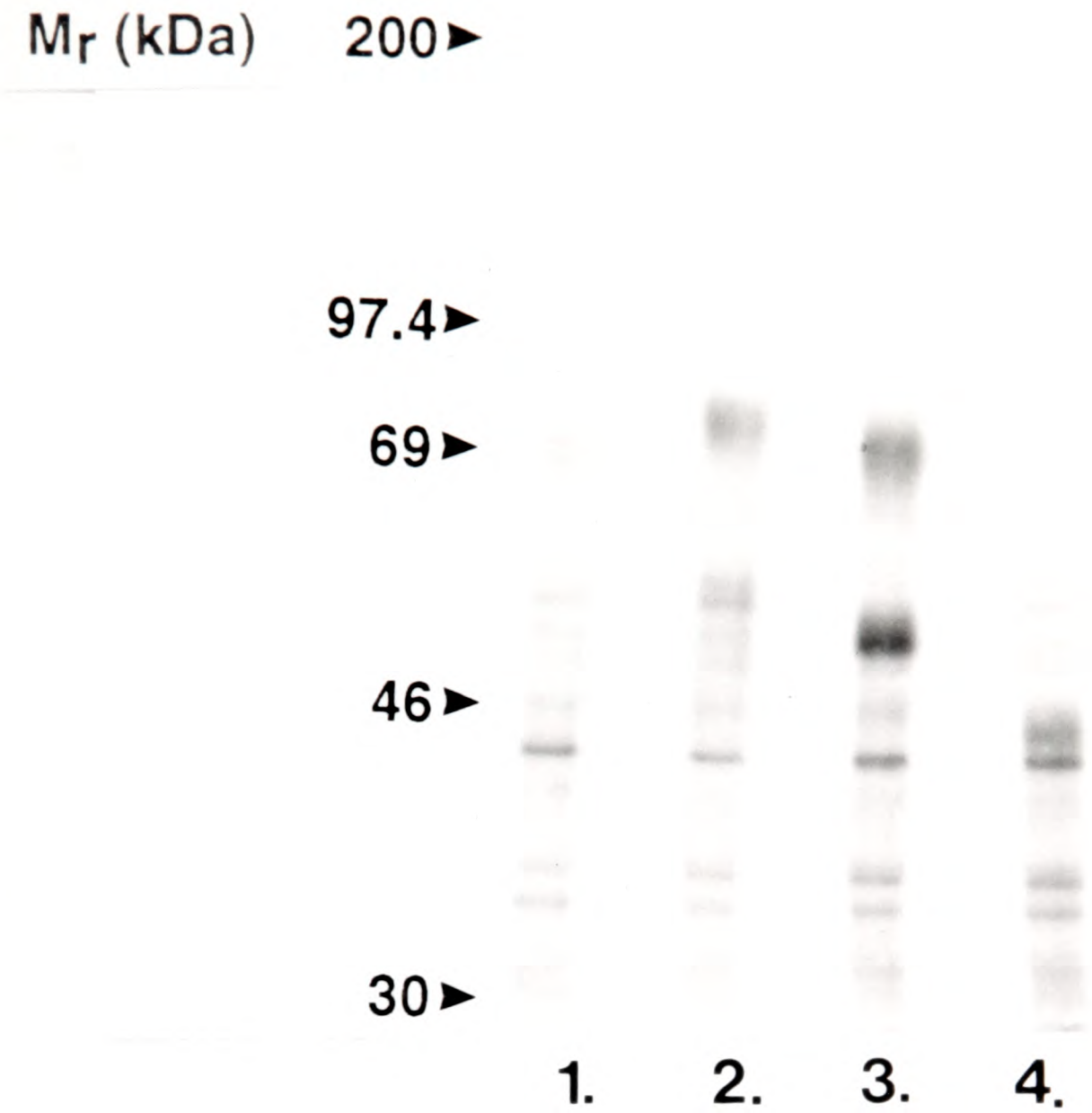

F

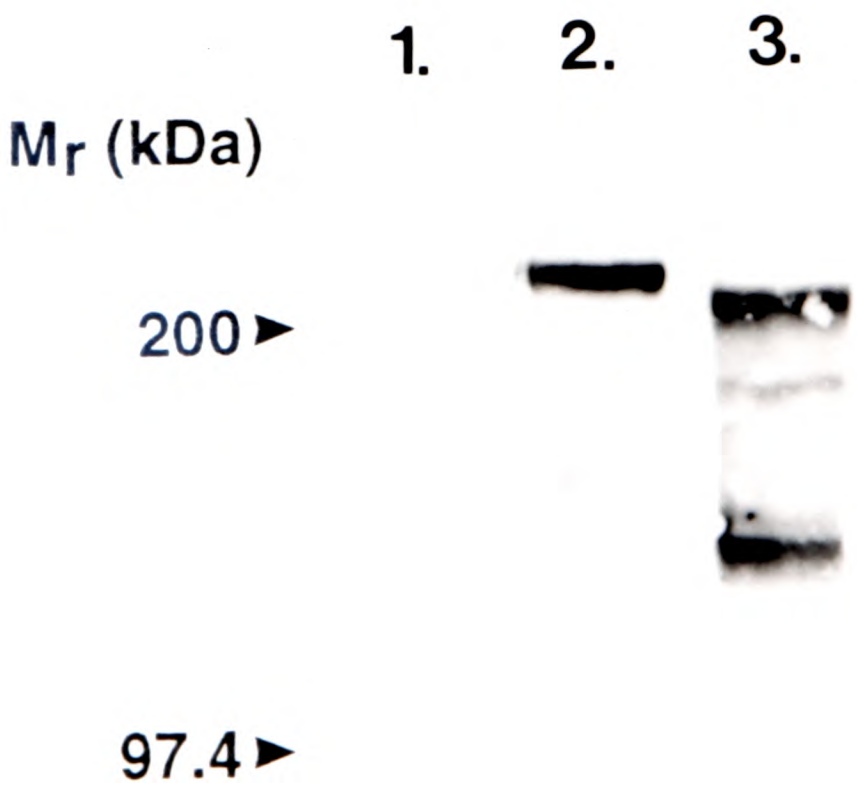
Fig. 7.15 (top) **Immunoprecipitation of MSR from CHO cells transfected with truncated MSR cDNAs**

CHO WT cells **(lane 1)**, and CHO MSR II **(lane 2)**, #2 CHO-Δ22aa **(lane 3)** and #3 CHO-Δcollagen-like domain **(lane 4)** transfectants were grown to confluence in 35 mm TCP dishes. Cells were then metabolically labelled by overnight incubation with 50 μCi/ml Tran^{35}S, lysed and subjected to immunoprecipitation with mAb 2F8 as described. Immunoprecipitates were separated by 5-10% SDS-PAGE (equivalent cell protein per lane) in the presence of β-mercaptoethanol, fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C.

Fig. 7.16 (bottom) **Immunoblot of MSR from CHO cell transfected with MSR truncation mutants**

CHO WT cells **(lane 1)**, and CHO MSR II **(lane 2)**, and #2 CHO-Δ22aa **(lane 3)** transfectants were grown as previously decribed, lysed in lysis buffer containing NP-40 and the lysate protein concentrations determined using the BCA assay (Pierce). Equal amounts of total cellular protein (20 μg) were subjected to 6% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. Blots were probed with mAb 2F8 and binding detected by peroxidase-conjugated second antibody and chemiluminescence. Results are representative of three different sets of lysates.


Mr (kDa)
200►
97.4►
69►
46►
30►
1.
2.
3.
4.


1.
2.
3.
Mr (kDa)
200►
97.4►

conditions (fig. 7.15). Both deletion mutants #2 CHO-Δ22aa (325 aa) (≈69K) and #3 CHO-Δcollagen-like domain (277 aa) (≈46K) (lanes 3 and 4 respectively) were still recognized by mAb 2F8 and the apparent smaller M_r of the shortened MSR monomers correlated with their predicted sizes. These results indicated that the MSR epitope for mAb 2F8 binding resides proximally to the collagen-like domain. Different regions of the MSR may mediate its endocytic (C-terminal 22 amino acids of the collagen-like domain) and adhesion functions; but steric effects have to be considered and without functional data no definite conclusions can yet be drawn.

Immunoblot analysis (under non-reducing conditions) by mAb 2F8 was subsequently performed on the truncated MSR proteins after transfer to nitrocellulose. It is important to note that the antibody recognizes predominantly non-reduced trimeric MSR forms and that the α-helical coiled-coil domain (and not the collagen-like domain) has previously been shown to be necessary for MSR trimerization (Doi *et al.* 1993). This may explain some of the findings shown in figs. 7.16 and 7.17. Prominent bands of the expected size were present in mutants #1-#3 (confirming the biosynthesis result); but faint lower M_r bands appeared (with prolonged exposure) in mutants #5 (226 aa) and #6 (197 aa); but not in #4 (249 aa). This may be artefactual MSR non-specific binding or ascribed to specific conformational requirements for mAb 2F8 binding which truncated mutant #4 may lack after SDS-PAGE. Mutants #7 (183 aa) and #8 (167 aa) reproducibly lacked any discernible mAb 2F8 binding. An approach to this problem may be immunoprecipitation of the various MSR truncation mutants with the anti-cytoplasmic domain polyclonal antiserum (as used in Chapter 6) and mAb 2F8 following short pulse metabolic labeling.

The expressed mutant MSR proteins were further detected by mAb 2F8 immunohistochemical staining (fig. 7.18). In contrast to the immunoblots, mutant #4 revealed prominent staining suggesting the continued presence of the mAb 2F8 recognition site in this mutant. Similar to the immunoblot experiments, mutants #7 (183 aa) and #8 (167 aa) reproducibly did not bind mAb 2F8. Note that mutants #6 (197 aa), #7 (183 aa) and #8 (167 aa) were obtained later and appropriate negative and positive staining controls were performed (using CAMPATH-1G antibody and CHO-WT cells MSR transfectants). These results would, given the above caveats, suggest that mAb 2F8 binds MSR proteins of 197 amino acids and longer and that the epitope recognized by mAb 2F8 is located in the α-helical coiled-coil domain between amino acids 183 (mutant #7) and 197 (mutant #6) (shown diagrammatically in fig. 7.19). No known adhesion-promoting motifs (e.g. RGD etc.) are

Fig. 7.17 **Immunoblot of MSR from CHO cells transfected with MSR truncation mutants**

CHO WT cells and various CHO MSR transfectants (as decribed in the figure legend) were grown as previously decribed, lysed in lysis buffer containing NP-40 and the lysate protein concentrations determined using the BCA assay (Pierce). Equal amounts of total cellular protein (20 µg) were subjected to 6% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose. Blots were probed with mAb 2F8 and binding detected by peroxidase-conjugated second antibody and chemiluminescence. Results are representative of three different sets of lysates.

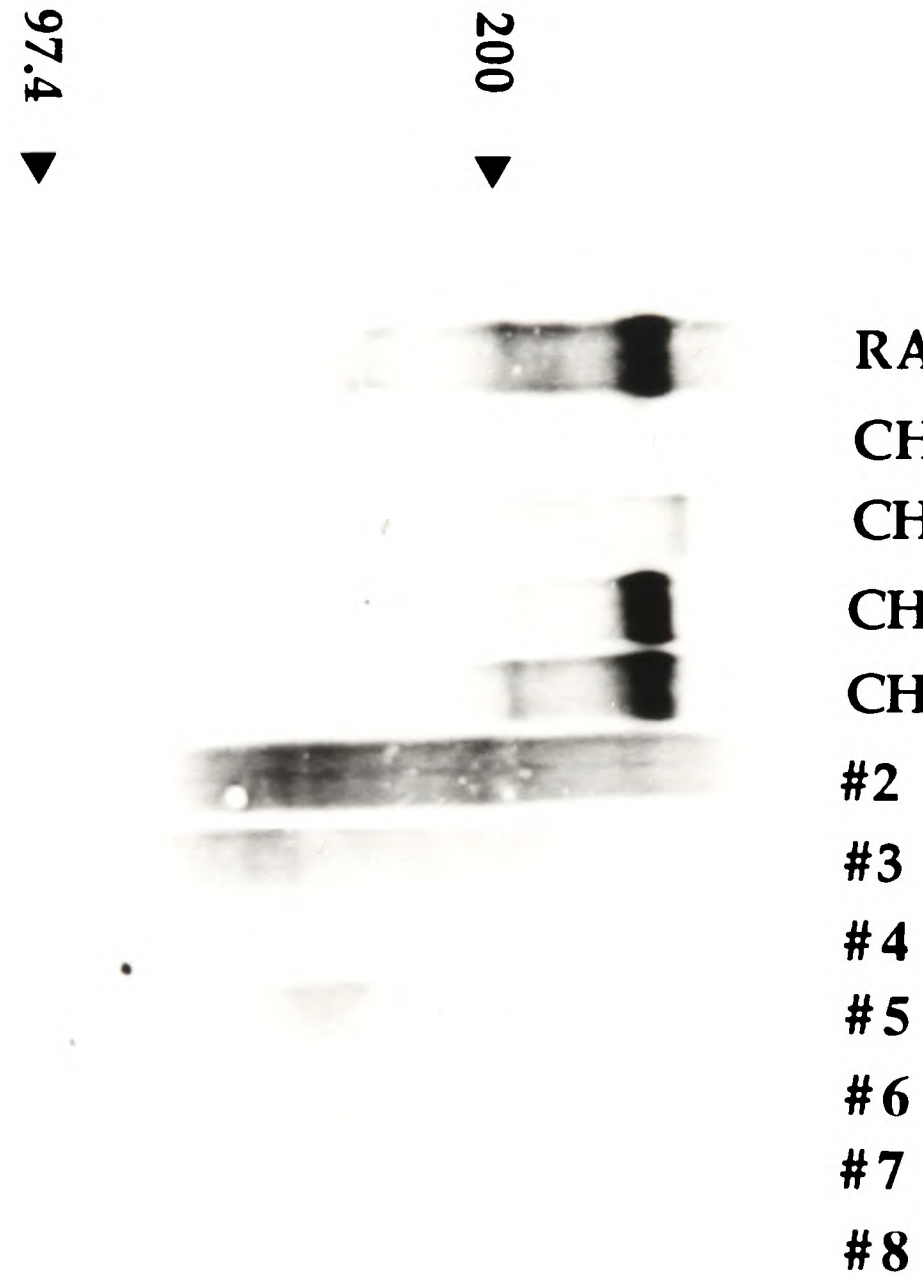
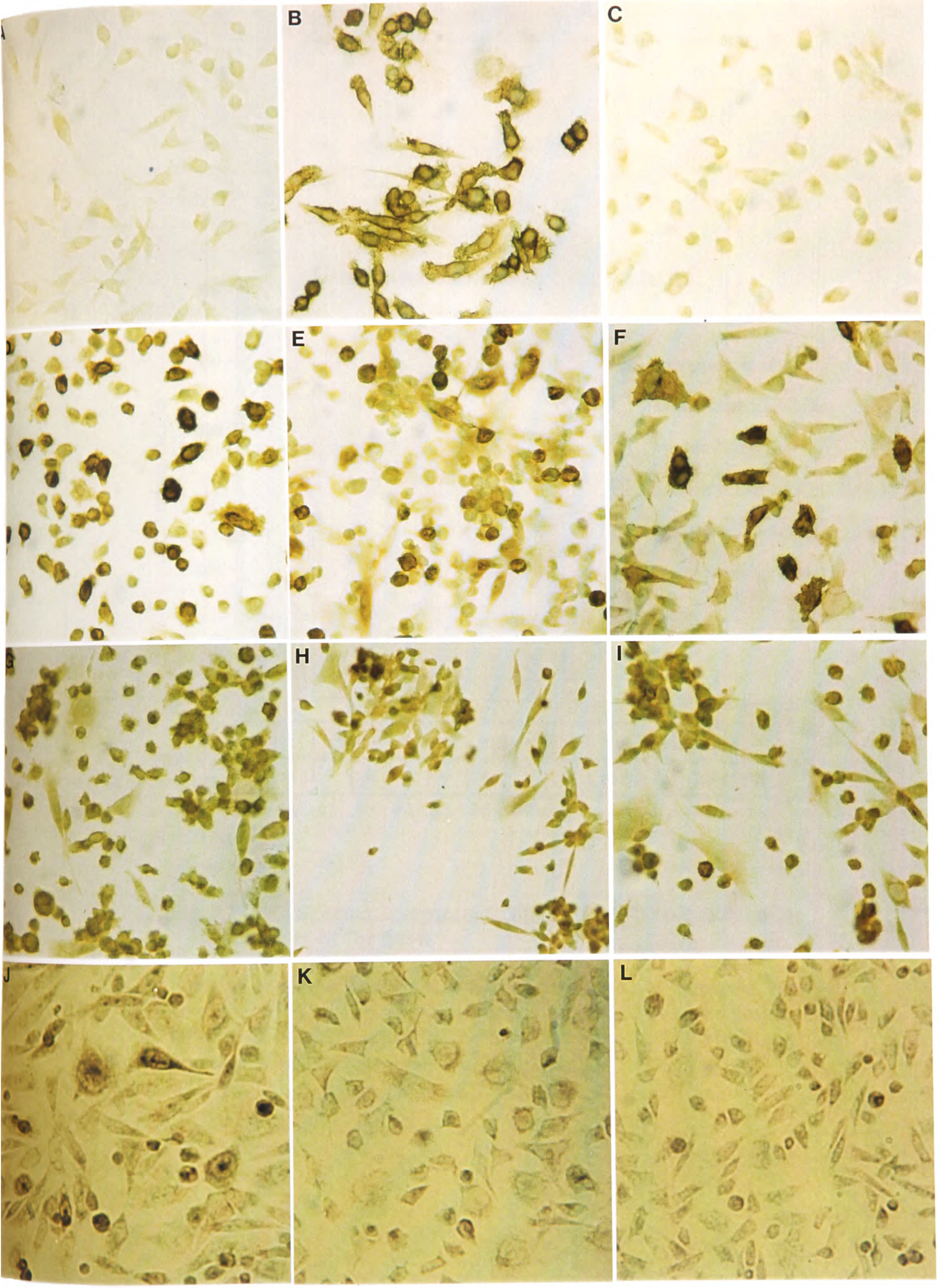

97.4
200
RAW 264.7
CHO WT
CHO MSR I
CHO MSR II
CHO MSR II MAC #2
#3
#4
#5
#6
#7
#8

Fig. 7.18 **Immunocytochemical staining of CHO transfectants by mAb 2F8**

CHO WT cells and various CHO MSR transfectants were cultured in the previously described culture media on 25mm diameter acid-washed glass coverslips in 6 well TCP plates. After washing with PBS, cells were fixed for 30 minutes at room temperature in PBS containing 3% Paraformaldehyde or acetone : methanol (1:1 by volume) for 10 minutes at 4°C. Unreacted groups were quenched for one hour with PBS plus 10% FCS, following which cells were stained immediately, or kept in PBA at 4°C until used. MAbs 2F8 and CAMPATH-1G and PBS as appropriate staining controls were incubated with the cells for one hour at room temperature in the presence of 5% normal goat serum. Antibody binding was detected with the appropriate biotinylated second antibody by the avidin-biotin peroxidase method with diaminobenzidine as the chromogen. Endogenous peroxidase activity was eliminated by incubating the cells for 20 minutes at room temperature in methanol with 0.3% H_2O_2. Counterstaining was with Mayer's haematoxylin. Stained cells were dehydrated and mounted in DePeX mountant. Cells were photographed using bright field microscopy (objective lens = 40x). MAb 2F8 unless otherwise specified.

A.	RAW 264.7 (CAMPATH-1G)	**B.**	RAW 264.7
C.	CHO WT	**D.**	CHO MSR I (454aa)
E.	#1 CHO MSR II (355aa)	**F.**	#2 CHO-Δ22aa (325aa)
G.	#3 CHO-Δcollagen (277aa)	**H.**	#4 CHO MSR (249aa)
I.	#5 CHO MSR (226aa)	**J.**	#6 CHO MSR (197aa)
K.	#7 CHO MSR (183aa)	**L.**	#8 CHO MSR (167aa)


B
C
E
F
G
H
I
J
K
L

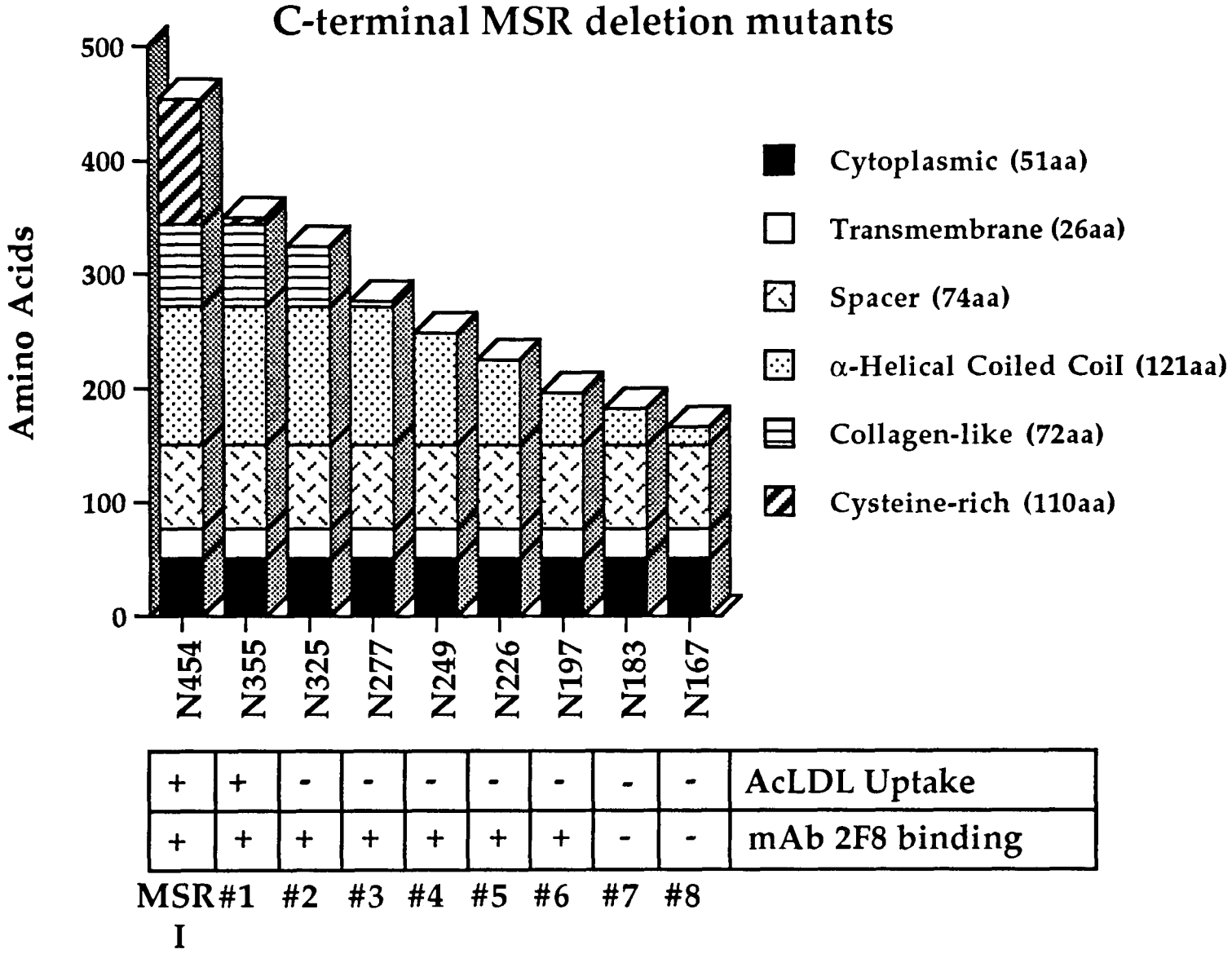


Fig. 7.19 Schematic representation of the C-terminal deletion mutants of the MSR

Each of the five domains is distinguished by a different type of shadowing. The ligand uptake activities determined by fluorescent labeled DiI-AcLDL and MSR recognition by mAb 2F8 binding of the mutants are shown as positive (+) or negative (-) respectively.

present in this region or its flanking areas. Interestingly, the binding site of the non-functional mAb IgG-D2 against the bovine trimeric MSR was previously localized to the α-helical coiled-coil domain and the antibody recognized mutant bovine MSR forms of 221 amino acids and longer (Doi *et al.* 1993). Epitope mapping of the mAb 2F8 does not necessarily identify this site as the adhesion-promoting region in MSR; as alluded to previously the adhesion inhibitory quality of mAb 2F8 is complex and may require specific conformational features in serum adhesion ligand(s) or MSR domains, as well as the co-ordinate or combinatorial involvement of other membrane molecules.

This complexity was demonstrated when the ability of a number of polyanionic inhibitors/ligands for MSR-mediated AcLDL endocytosis and their corresponding negative controls to inhibit MSR-dependent EDTA-resistant adhesion of RAW 264.7 Mϕ-like cells to TCP was investigated (Fraser 1993). Inhibition by known MSR ligands was found to be variable. Poly I inhibited adhesion at very low concentrations; dextran sulfate inhibited adhesion at low, but not higher concentrations, while fucoidan and polyvinyl sulfate did not inhibit adhesion at all. This phenomenon may be explained by a number of potential mechanisms. Non-reciprocal cross-competition between AcLDL and LDL for MSR binding is well described (Sparrow *et al.* 1989; Freeman *et al.* 1991); and the underlying basis for this appears to reside in the specific clustering of basic amino residues in the collagen-like domain of MSR, with different ligands using different basic residue clusters to bind (Doi *et al.* 1993).

Data presented in this chapter suggest that the adhesion-promoting site(s) and AcLDL-binding site in the MSR may be distinct and that multimerization or conformational changes in MSR structure may be required for adhesion to occur. MSR thus seems to be a bifunctional receptor, having both endocytic and adhesive properties (analogous to the β_2-integrin, CR3 (Rosen 1990). Another explanation may be related to the ability of serum components to act as adhesion-promoting ligands (rather than soluble inhibitors of adhesion) in the *in vitro* adhesion assay. Fucoidan and polyvinyl sulfate (and dextran sulfate at higher concentrations) could therefore have a higher affinity for TCP than MSR, thus becoming immobilized on TCP and acting as the main adhesion-promoting ligand in the assay. Possibilities for future investigation may include: i) determining the adhesion-inhibitory abilities of a soluble peptide containing residues 183-197 of the MSR α-helical coiled coil domain as well as antibodies raised against this region; very conceivably though a monomeric peptide may not have any phenotype in view of the MSR's trimeric structure and ii) co-

transfection of types I and II murine MSR into CHO cells and assessing the influence (if any) of heterotrimers on function (adhesion). The differences between CHO cells and Mϕ as far as requirements for efficient MSR transport to the cell surface are concerned (e.g. protein folding, chaperonins, accessory transport molecules and glycosylation capabilities), also need further biochemical and cell biological investigation. Studies describing the morphology of MSR expression at the adhesive interface are presented in Chapter 9.

Chapter Eight

Macrophage phenotype, including MSR expression, in mice deficient in both M-CSF (op) and apolipoprotein E

8.1 Introduction

The arterial fatty streak is an early and reversible precursor to advanced fibroproliferative atherosclerotic lesions (Ross 1993). These early lesions contain mostly monocyte-derived Mϕ that have taken up modified lipoproteins to become lipid-enriched foam cells. In addition to Mϕ foam cells, human lesions also contain T cells and smooth muscle-derived foam cells (Ross 1993), and the essential role of monocyte-derived Mϕ in atherosclerosis remains unproven.

Apolipoprotein E (ApoE)-deficient mice are hypercholesterolemic, and unlike other rodent models of atherosclerosis, develop the disease spontaneously on a low-fat chow diet (Plump *et al.* 1992). The hypercholesterolemia and atherosclerosis in these mice are diet responsive, and are increased by a high-fat Western-type diet (Plump *et al.* 1992). Moreover, these lesions are excellent models for human atherosclerosis as they progress with age from early fatty streaks to complex fibrous lesions with necrotic cores and are found at the same sites of predilection as human lesions (Nakashima *et al.* 1994). Oxidized lipoprotein epitopes are present in the lesions of apoE-deficient mice, and sera from these mice contain high titers of autoantibodies that recognize rabbit oxidized lipoproteins and lesions (Palinski *et al.* 1994). ApoE-deficient mice are therefore an attractive model system in which to evaluate factors that mediate or modulate the pathogenesis of atherosclerosis.

The osteopetrotic (op/op) mouse has a spontaneously derived recessive mutation in the gene encoding M-CSF and the phenotype has been well characterized in many previous studies (Wiktor-Jedrzejczak *et al.* 1990; Witmer-Pack *et al.* 1993). The *op* defect in the M-CSF gene is a frame-shift mutation leading to the complete absence of M-CSF activity in the serum and tissues (Yoshida *et al.* 1990); and partial reversal of the op/op phenotype can be achieved by repeated M-CSF injection (Felix *et al.* 1990). Osteopetrotic mice lack osteoclasts; this results in impaired

bone-remodeling and skeletal deformities, the most severe being a deficiency in tooth eruption so that young op/op mice require a soft diet to survive (Kodama *et al.* 1991). These mice also have decreased body weight and fertility and the females have reduced lactational ability. Haematologically, op/op mice have normal haematocrits and granulocyte counts, but markedly decreased blood monocytes and peritoneal Mϕ (Wiktor-Jedrzejczak *et al.* 1982). The op/op mouse provides an opportunity to examine the contribution of M-CSF to specific Mϕ populations *in vivo*, with particular regard to levels of MSR expression and function.

Immunohistochemical studies have shown that, apart from osteoclasts, other Mϕ populations are also critically dependent on M-CSF *in vivo* (Witmer-Pack *et al.* 1993). These M-CSF dependent populations include peritoneal Mϕ, splenic marginal zone metallophils, and lymph node subcapsular sinus Mϕ. Liver Mϕ (Kupffer cells) are reduced, but still readily identifiable; other Mϕ populations, including those within the thymic cortex, splenic red pulp, lymph node medulla, intestinal lamina propria, lung (alveolar Mϕ) and brain (microglia), as well as dendritic cells, remain present in substantial numbers and thus appear for the most part M-CSF independent.

M-CSF and its effects on Mϕ development and function may play a key role in atherogenesis. M-CSF injections reduce cholesterol levels in rabbits, primates and humans (Motoyoshi and Takaku 1989; Stoudemire and Garnick 1991; Schaub *et al.* 1994). The best characterized *in vivo* role of the MSR has been in atherogenesis through its involvement in foam cell formation. As described in Chapter Three (de Villiers *et al.* 1994) M-CSF potently and selectively increases MSR expression, stability, endocytic and adhesion functions in murine Mϕ *in vitro*. M-CSF has also been shown to reduce atherosclerosis and carrageenan-induced granuloma lipid accumulation in Watanabe hyperlipidemic rabbits (Inoue *et al.* 1992; Schaub *et al.* 1994).

To determine the role of monocyte-derived Mϕ (and M-CSF) in atherogenesis *in vivo*, op/op mice were recently crossed onto the apoE-deficient background by Smith and colleagues (Rockefeller University, New York) (Smith *et al.* 1995). The double mutant mice had an almost three-fold increase in plasma cholesterol compared with apoE-deficient controls. Interestingly, despite this severe hypercholesterolemia, proximal aorta atherosclerosis in these mice was significantly decreased. Atherosclerosis lesion area was affected by both *op* genotype and gender (increased lesions in females); however, confounding variables, such as body weight, plasma cholesterol, and monocyte count (all secondary to the *op* genotype), had no significant additional effects.

To extend the *in vitro* work on growth factor and cytokine regulation of MSR expression and function that has been the subject of this thesis thus far, *in vivo* murine models of atherosclerosis were explored. A collaboration was entered into with Dr. Jonathan Smith of the Rockefeller University, New York, for the supply of organs from apoE-deficient mice (E0; E for apoE followed by the number of wild-type alleles to describe the mouse genotype) as well as mice doubly mutant for the apoE gene and M-CSF genes (hetero- and homozygous) (op1/E0; op0/E0) (op2, op1 or op0; op for osteopetrotic followed by the number of wild-type alleles). These animals were fed a high-fat (42% of calories from fat) Western-type diet and sacrificed at 16 weeks of age. The following issues were addressed:

i) Mϕ subpopulations in the op2/E0 and op0/E0 double mutant crosses were characterized by immunohistochemistry of Mϕ membrane molecules (with special emphasis on MSR expression) (the mAbs used are described in table 8.1). In addition, the effects of exposure to a 16-week high-fat Western-type diet could also be determined.

Table 8.1. Monoclonal antibodies used in this study

Name	Specificity	Isotype	Reference
F4/80	Macrophage	Rat IgG2b	Austyn and Gordon 1981
FA-11	Macrosialin (CD68) on Mϕ	Rat IgG2a	Smith and Koch 1987
5C6	CD11b subunit of CR3	Rat IgG2b	Rosen and Gordon 1987
2F8	MSR (SR-AI and II)	Rat IgG2b	Fraser *et al.* 1993
3D6	Sialoadhesin on Mϕ	Rat IgG2a	van den Berg 1992
8D2	CD44	Rat IgG2b	Doyle, unpublished
2.4G2	FcγRII (CD32)	Rat IgG2b	Unkeless 1979
TIB120	MHC class II, IA/IE	Rat IgG2b	Bhattacharya *et al.* 1981
NLDC145	Interdigitating (dendritic cells)	Rat IgG2a	Kraal *et al.* 1986
GK 1.5	CD4	Rat IgG2b	Dialynas *et al.* 1983
53.6.72	CD8	Rat IgG2a	Ledbetter and Herzenberg 1979

ii) Mϕ subpopulations present in atherosclerotic lesions were

characterized by phenotype analysis of Mϕ membrane molecules; which may elucidate Mϕ functional heterogeneity in these murine models of atherogenesis. Using a rabbit anti-mouse Mϕ polyclonal antiserum for immunohistochemistry, Smith and colleagues reported prominent and homogeneous Mϕ staining in the small raised foam cell lesions from both op2/E0 and op0/E0 mice (Smith *et al.* 1995). Mϕ staining of the more advanced lesions in the op2/E0 mice was less homogeneous perhaps reflecting the presence of SMCs and necrotic regions. This pan-Mϕ staining would not be expected to reveal any Mϕ functional heterogeneity in lesions. Interestingly, the reduced monocyte number in the op0/E0 mice was shown statistically not to affect lesion formation independently (Smith *et al.* 1995), and the crucial factor may rather be the anti-atherogenic functional modulation of the remaining Mϕ. A hypothesis could be that diminished MSR expression on the remaining Mϕ contributes to decreased foam cell formation and atherosclerosis in the doubly mutant mice.

iii) Investigation of the possible role of MSR in Mϕ adhesion in atherogenesis by frozen section adhesion assay. The Stamper-Woodruff lymphocyte adhesion assay was modified to investigate RAW 264.7 (Mϕ-like cell line) and TPMϕ adhesion to tissue sections; MSR-dependent (EDTA-resistant, mAb 2F8-inhibitable) adhesion has previously been demonstrated by this approach (Hughes *et al.* 1995). The exact role of MSR in adhesion *in vivo* could be determined by performing adhesion assays of this nature on the atherosclerotic lesions and other organs in the op2/E0 and op0/E0 mice and preliminary studies in this regard are described.

8.2 Results

8.2.1 Characterization of Mϕ phenotypes in atherosclerotic lesions in op2/E0, op1/E0 and op0/E0 mice

Every 16-week old op1/E0 and op2/E0 mouse analyzed had atheromatous lesions in the aortic root, situated on the valve cusps and in the areas between valve cusps. These lesions comprised a spectrum of both simple raised foam cell type and larger, more developed fibroproliferative plaques. In fig. 8.1A and B immunohistochemistry from representative advanced lesions (as shown by oil red O staining) in an op2/E0 mouse, and in fig. 8.1C an op1/E0 animal, is depicted. Recruited Mϕ within the lesions display prominent expression of the Mϕ-specific late endosomal membrane glycoprotein, macrosialin (Rabinowitz and Gordon 1991). This mostly intracellular membrane

Fig. 8.1 **Mϕ phenotype analysis in atherosclerotic lesions in op2/E0 and op1/E0 mice**

Op2/E0 animals fed an atherogenic diet for 16 weeks, were sacrificed and saline perfused prior to embedding in OCT, freezing and sectioning, as described in chapter 2.

A. A representative advanced lesion from an op2/E0 animal was stained with oil red O, FA-11, 2F8, 5C6, 2.4G2, TIB120, 3D6 and F4/80. 100x. Mϕ phenotypic heterogeneity is present and tabulated in table 8.2.

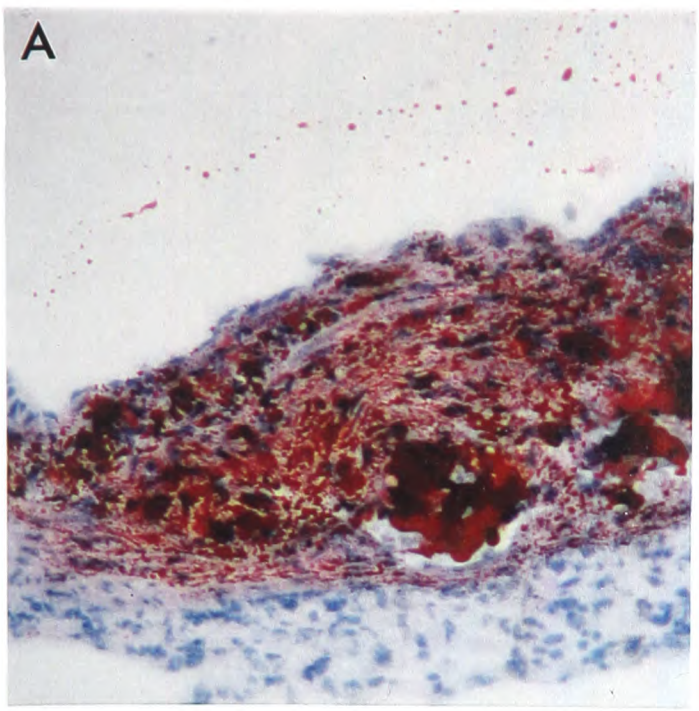
A.	Oil red O	B.	FA-11
C.	2F8	D.	5C6
E.	2.4G2	F.	TIB120
G.	3D6	H.	F4/80

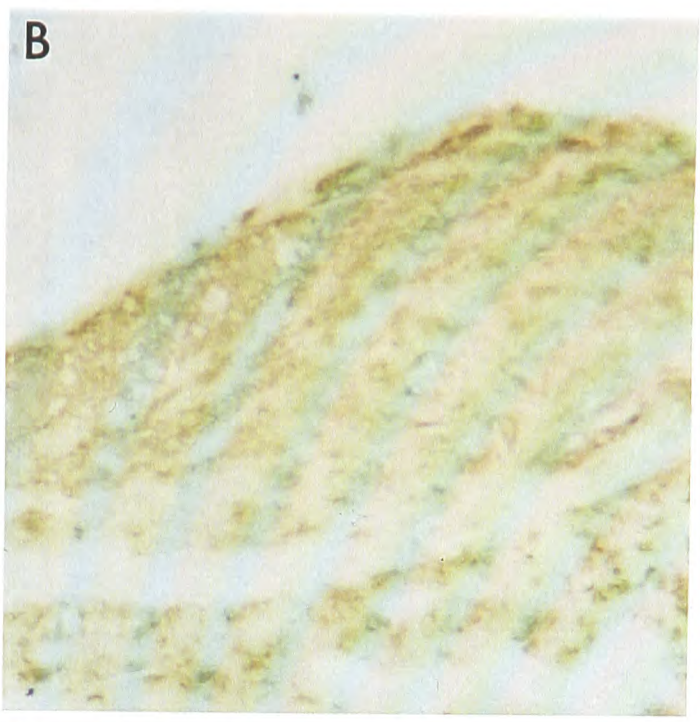
B. Staining from another op2/E0 advanced lesion is depicted (FA-11, 5C6, F4/80 and TIB120) emphasising Mϕ phenotypic heterogeneity within the base, shoulders and necrotic core of lesions. 300x

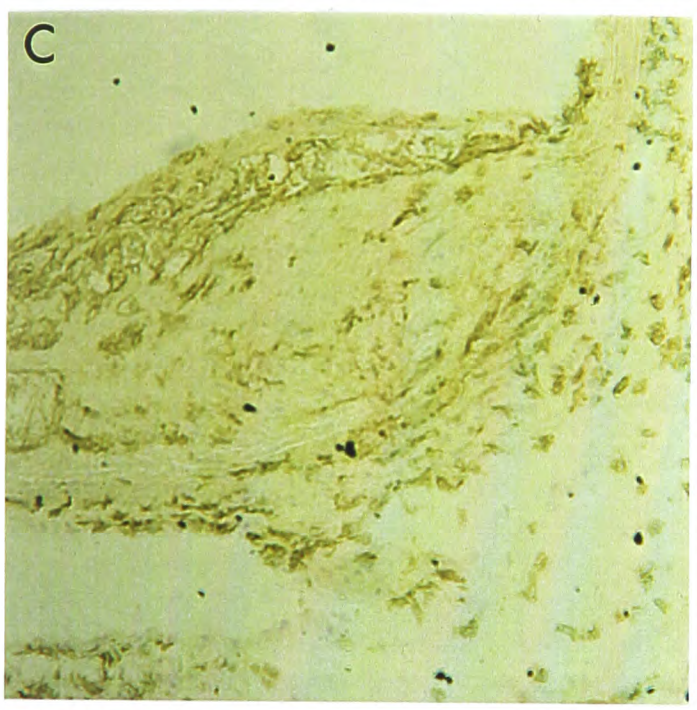
A.	FA-11	B.	5C6
C.	F4/80	D.	TIB120

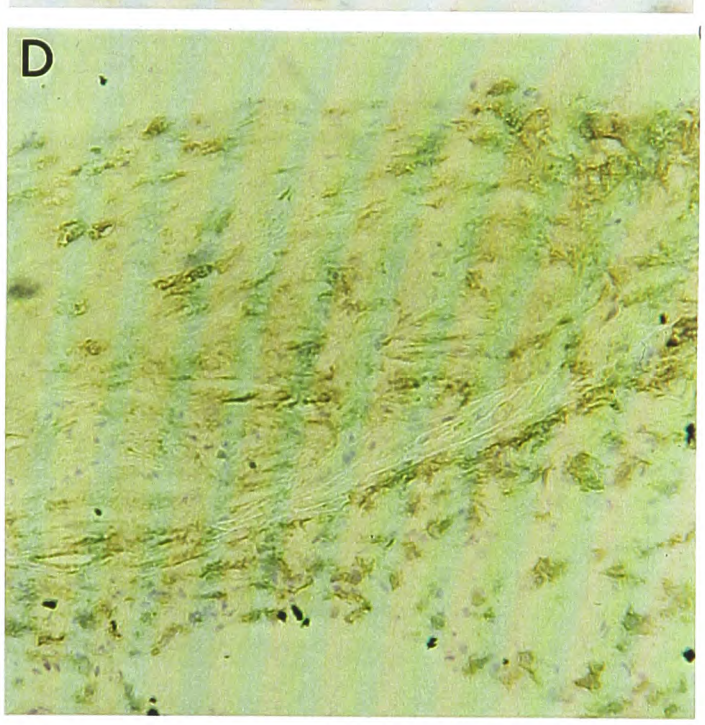
C. An advanced lesion from an op1/E0 animal stained with FA-11, 2F8 and 5C6. The absence of one normal M-CSF allele did not affect Mϕ phenotype markedly. 100x

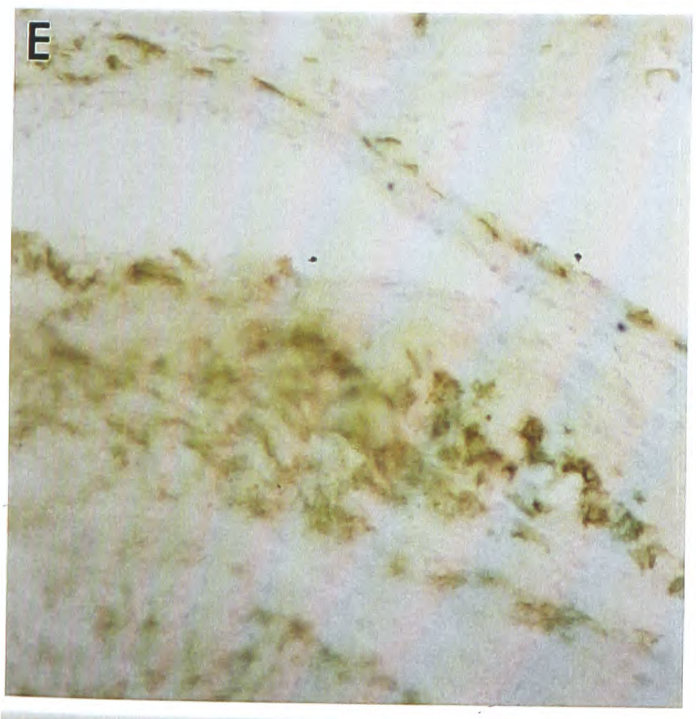
A.

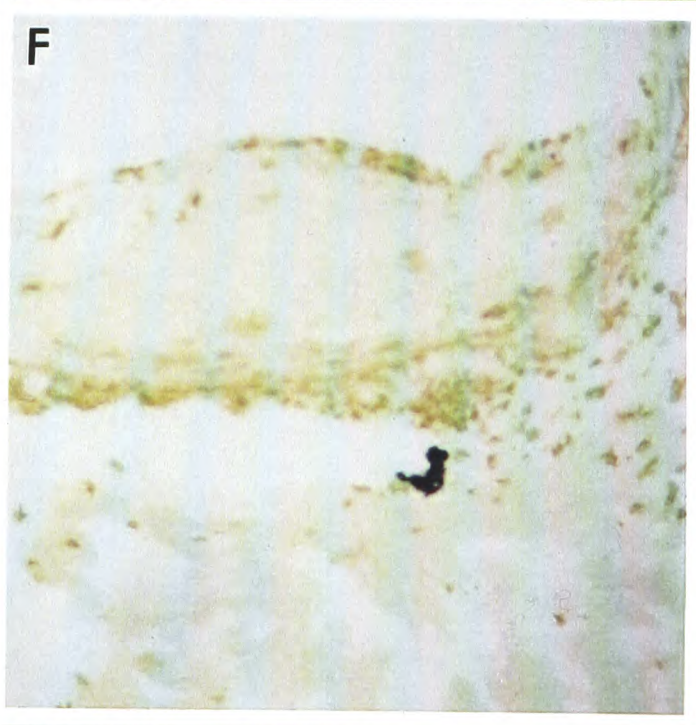


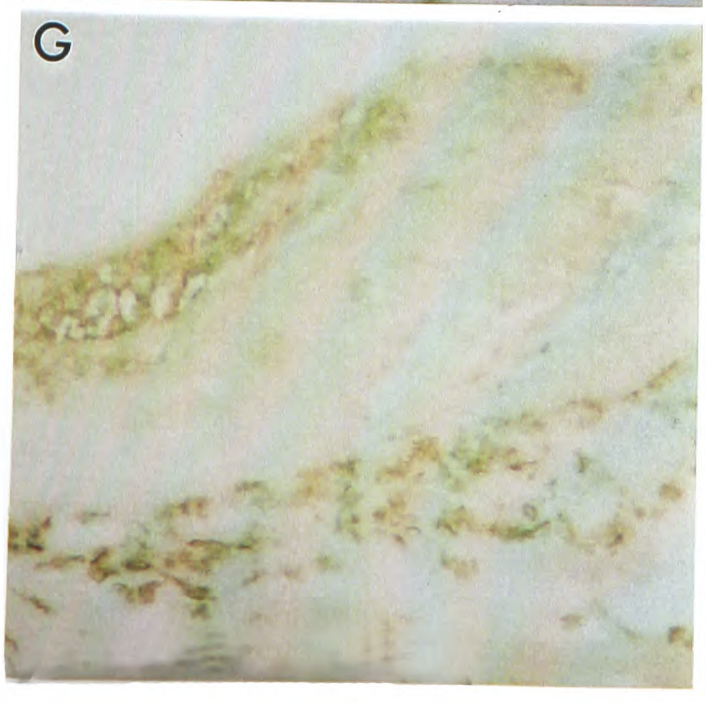


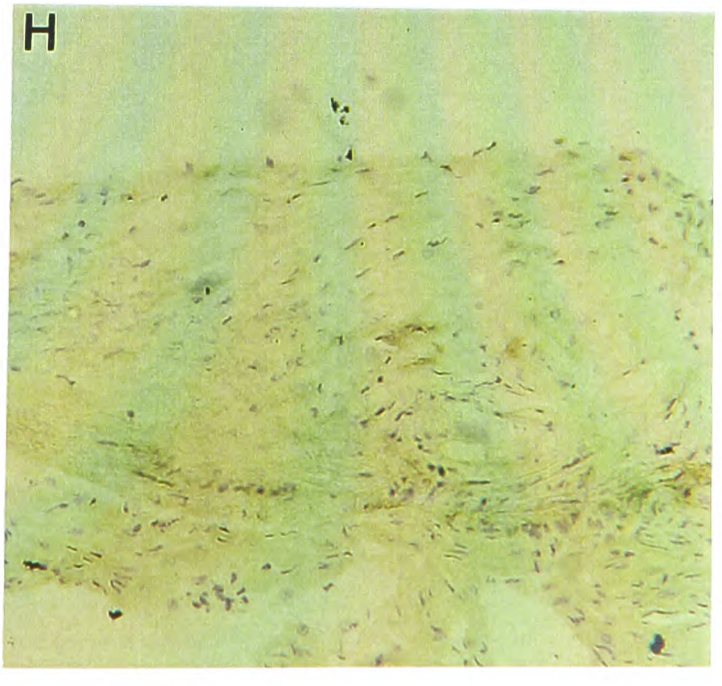




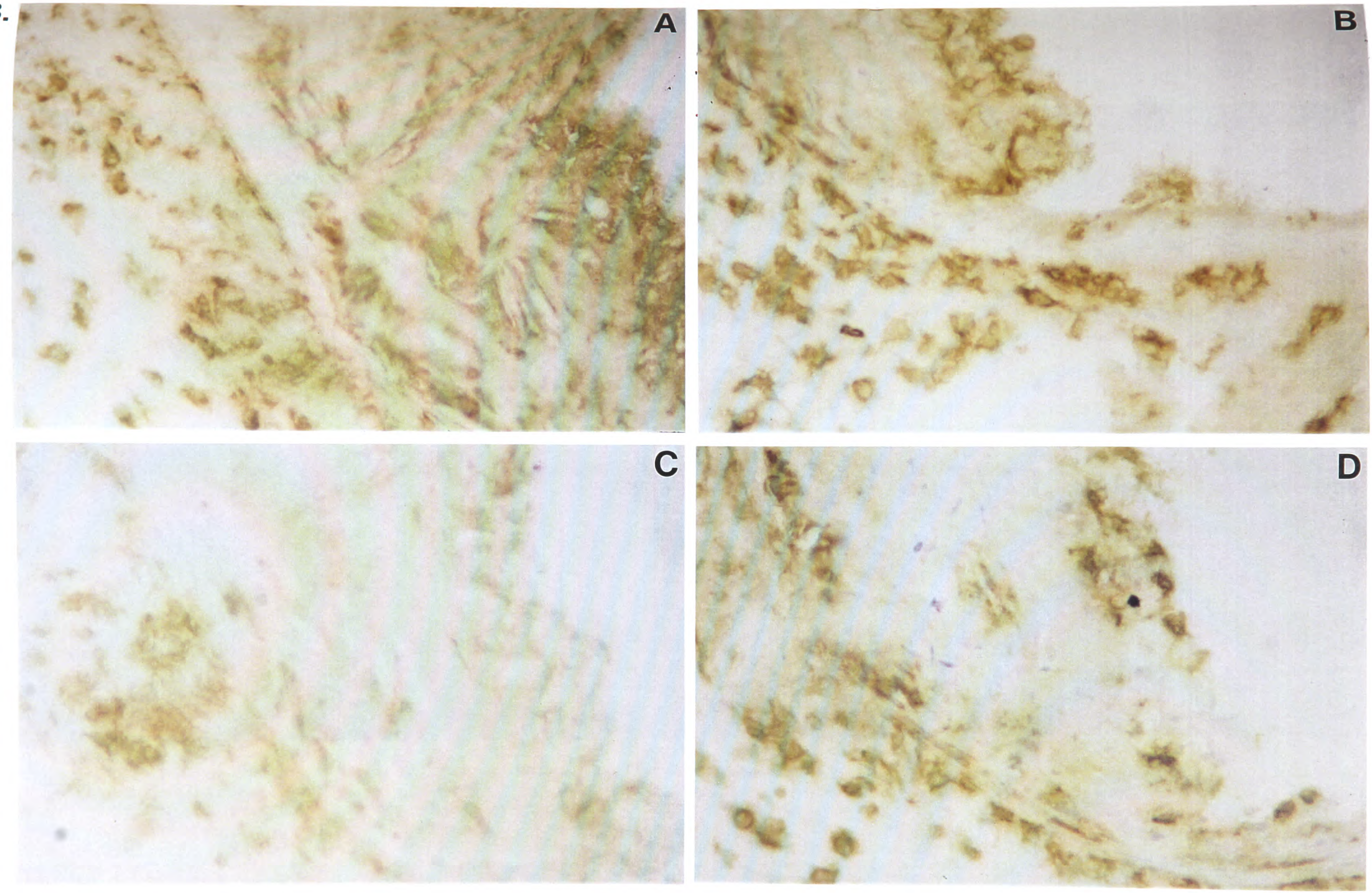




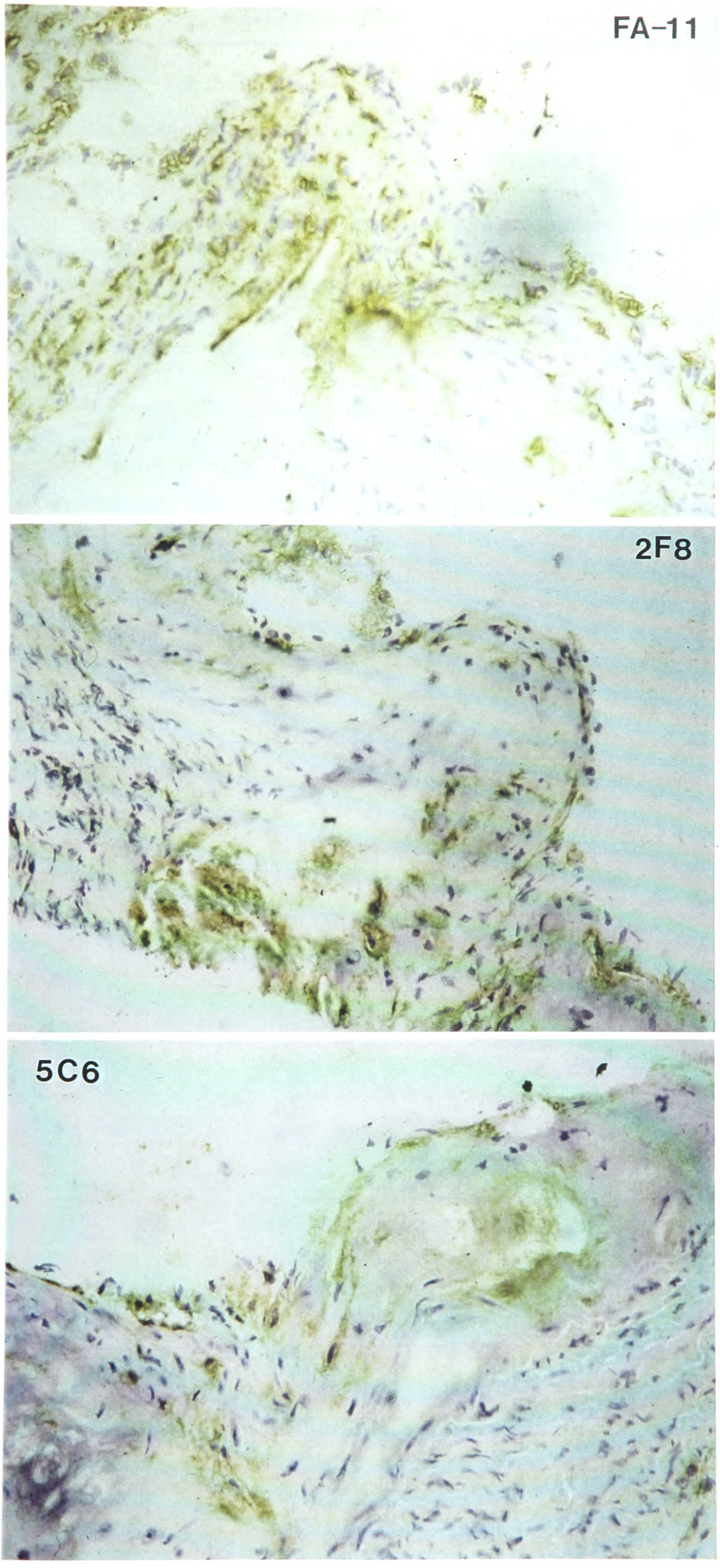




B.



C.



molecule (homologous to human CD68 (Holness and Simmons 1993)) is present throughout the lesion, including within its base, shoulders and necrotic core.

CR3 and MSR, both examples of Mϕ membrane molecules which have endocytic and adhesive functions, are also prominently expressed in the lesion. CR3 is generally considered a marker of recently recruited Mϕ and neutrophils and PMNs were conspicuous by their absence in lesions. MSR expression accompanies monocyte to Mϕ differentiation, and different functional subsets of lesional Mϕ may thus be characterized by these markers. Interestingly, in view of its envisaged role in foam cell formation, marked MSR staining was present on foam cell membranes in a smaller immature lesion in an op1/E0 animal (fig. 8.2). CD44, another adhesion molecule possibly involved in Mϕ recruitment and retention within the atheromatous micro-environment, is homogeneously displayed in the lesion and immediate surrounding areas (not shown). Staining with an irrelevant isotype control antibody, CAMPATH-1G, was negative. This fairly non-specific staining pattern is, probably for the main part, due not to Mϕ, but accounted for by extracellular matrix (hyaluronate) binding and the increased expression of CD44 on most activated leukocytes (T and B cells, monocytes and granulocytes), and its well reported presence on epithelial cells, fibroblasts and skeletal muscle (Stamenkovic *et al* 1989).

Mϕ in the atherosclerotic lesions also express FcγRII (CD32); this molecule may be functionally relevant in IgG-mediated phagocytosis as oxidized lipoprotein epitopes are present in the lesions of E0 mice, and sera from these mice contain high titers of autoantibodies to oxidized lipoproteins (Palinski *et al.* 1994). The prominent MHC class II staining indicates the presence mainly of activated lesional Mϕ; as dendritic cells (NLDC145) and T lymphocytes (CD4 and CD8) (not shown) were virtually absent. It is unclear whether upregulation of MHC class II expression on these Mϕ results by IFN-γ-dependent- or -independent mechanisms. In contrast to these markers of a recruited Mϕ population, F4/80 and sialoadhesion expression levels in the lesions were unimpressive and consistent with their status as resident and stromal Mϕ markers respectively (Gordon *et al.* 1992). Lesions in op1/E0 animals (fig. 8.1C) showed a similar Mϕ presence and phenotype as op2/E0 mice; and a gene dosage effect of M-CSF deficiency could not be demonstrated. The distribution of Mϕ membrane molecules within op2/E0 atherosclerotic lesions is tabulated below (table 8.2) with + to +++ indicating the graded presence and - the absence of antigen expression.

Fig. 8.2. **MSR presence in an atherosclerotic lesion from an op1/E0 animal**

Prominent MSR expression, by 2F8 staining, was present on foam cell membranes in an earlier atheromatous lesion from an op1/E0 animal. 300x

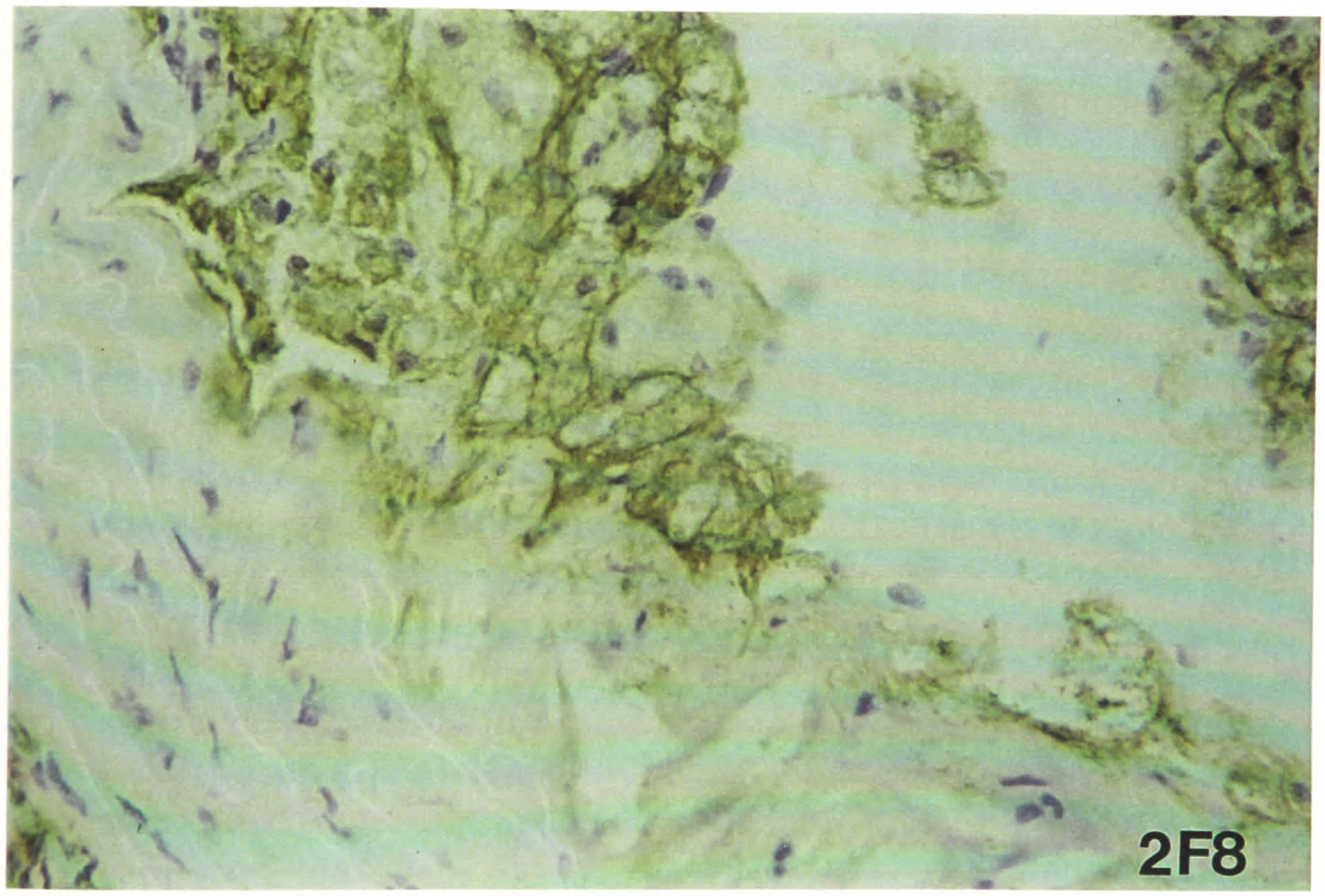

2F8

Table 8.2 Distribution of Mϕ membrane molecules in op2/E0 atheromatous lesions

Location	FA-11	5C6	2F8	2.4G2	8D2	TIB120	F4/80	3D6
Base	++	+++	++	++	+++	++	+	++
Shoulder	++	+++	++	++	+++	++	+/-	+
Necrotic core	++	+	+	++	+++	-	+/-	-
Fibrous cap	++	+	++	-	+++	+	-	+/-

The modulation in E0 atherogenesis brought about the op/op mutation was readily apparent (fig. 8.3). Oil red O staining revealed that M-CSF deficiency resulted in fewer and smaller lesions in op0/E0 mice; these were of mostly early foam cell type that had not progressed to more advanced stages and were primarily found on valve cusps. This agrees with the findings reported by (Smith *et al.* 1995). The reduction in atherosclerotic lesions was paralleled by an overall reduction in Mϕ expressing the molecules so prominently displayed by lesional Mϕ in op2/E0 mice. It was not possible to ascertain whether MSR expression was disproportionately reduced in op0/E0 lesional Mϕ in comparison to macrosialin, CR3, FcRII, MHC class II or CD44 levels of expression. M-CSF deficiency in op0/E0 animals did not have a selective effect on functional heterogeneity in Mϕ within atherosclerotic lesions, but rather reflected the functional heterogeneity among Mϕ populations systemically; and the recruited lesional Mϕ population in op2/E0 mice thus appeared predominantly M-CSF dependent. It is noteworthy that some Mϕ (representing an M-CSF independent population of cells expressing low levels of the markers mentioned above) still remained as an integral component of those smaller lesions present in op0/E0 mice.

All the lesions present in op0/E0 animals were at the aortic root associated with the aortic valve cusps. Until recently, the only mouse model available for studies of atherogenesis was the atherogenic diet-susceptible C57BL/6 mouse (Paigen *et al.* 1990) which develops foam cell lesions at a similar location; little is known about Mϕ phenotypic heterogeneity in this model. Apart from extensive Mϕ non-specific CD44 expression, few cells stained positive for Mϕ in the aorta (not shown). The FcγRII molecule appeared to be a sensitive marker for subendothelial Mϕ (fig. 8.4) in the vascular tree of op2/E0 mice. This is in addition to its prominent staining of lesional Mϕ and it may represent an early immunohistochemical marker for foam cell formation. Few FcγRII-positive cells were present in the subendothelium of op0/E0 mice indicating the M-CSF dependency of this population in general.

Fig. 8.3 **Mϕ phenotype analysis in atherosclerotic lesions in op0/E0 mice**

Op0/E0 animals fed an atherogenic diet for 16 weeks, were sacrificed and saline perfused prior to embedding in OCT, freezing and sectioning, as described in chapter 2. Lesions were fewer and less advanced than op2/E0 animals and located exclusively on valves and valve cusps.

A. An early foam cell type lesion from an op0/E0 animal is shown, stained with oil red O, FA-11, 2F8, 5C6, TIB120, 2.4G2 and 8D2. 100x.

A.	Oil red O	B.	FA-11
C.	2F8	D.	5C6
E.	TIB120	F.	2.4G2
G.	8D2		

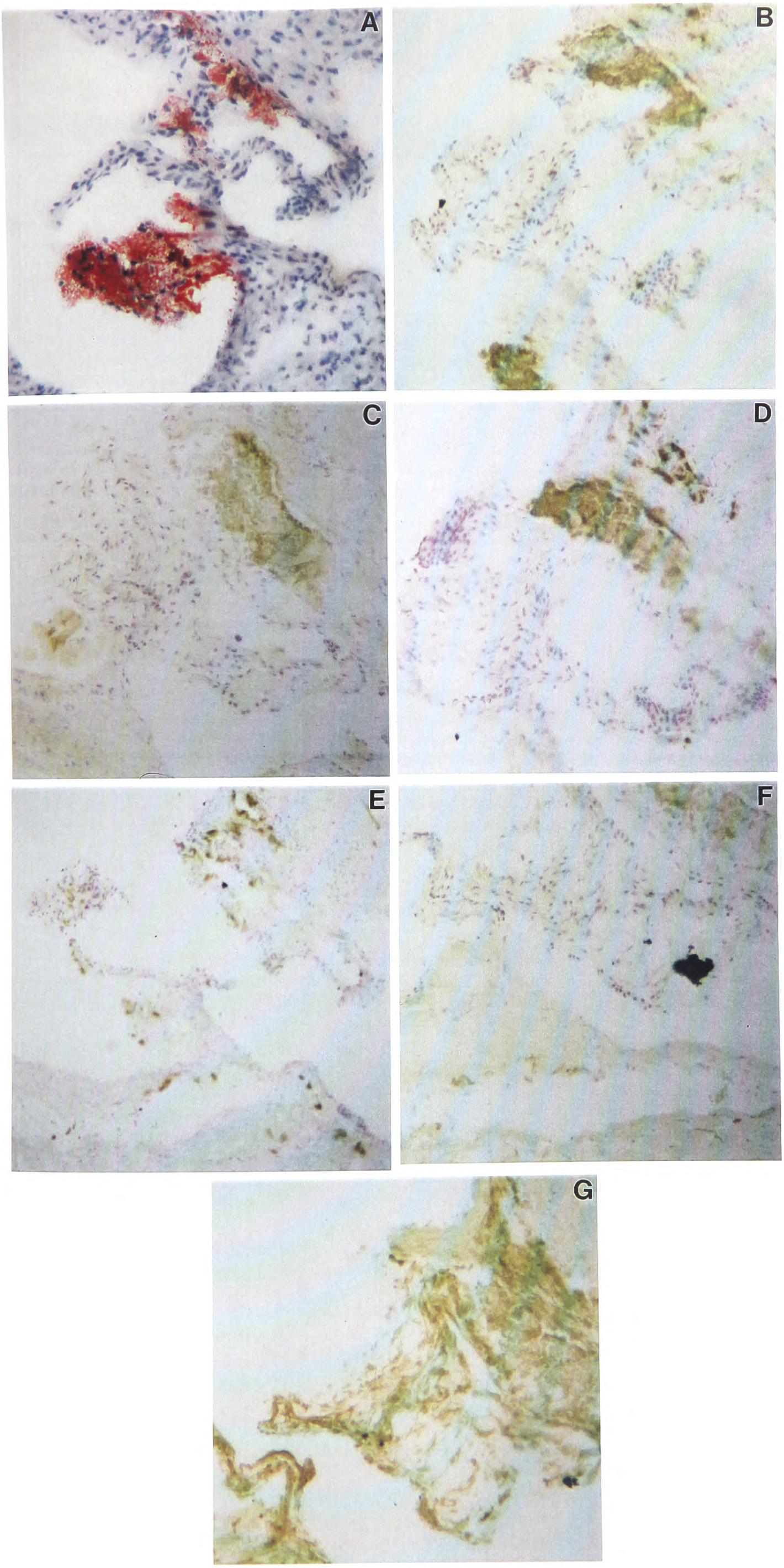
B. Staining from an earlier op0/E0 lesion is depicted (oil red O, FA-11, 5C6, 2F8,TIB120 and 8D2) showing Mϕ presence within these early lesions. 100x

A.	Oil red O	B.	FA-11
C.	2F8	D.	5C6
E.	TIB120	F.	8D2

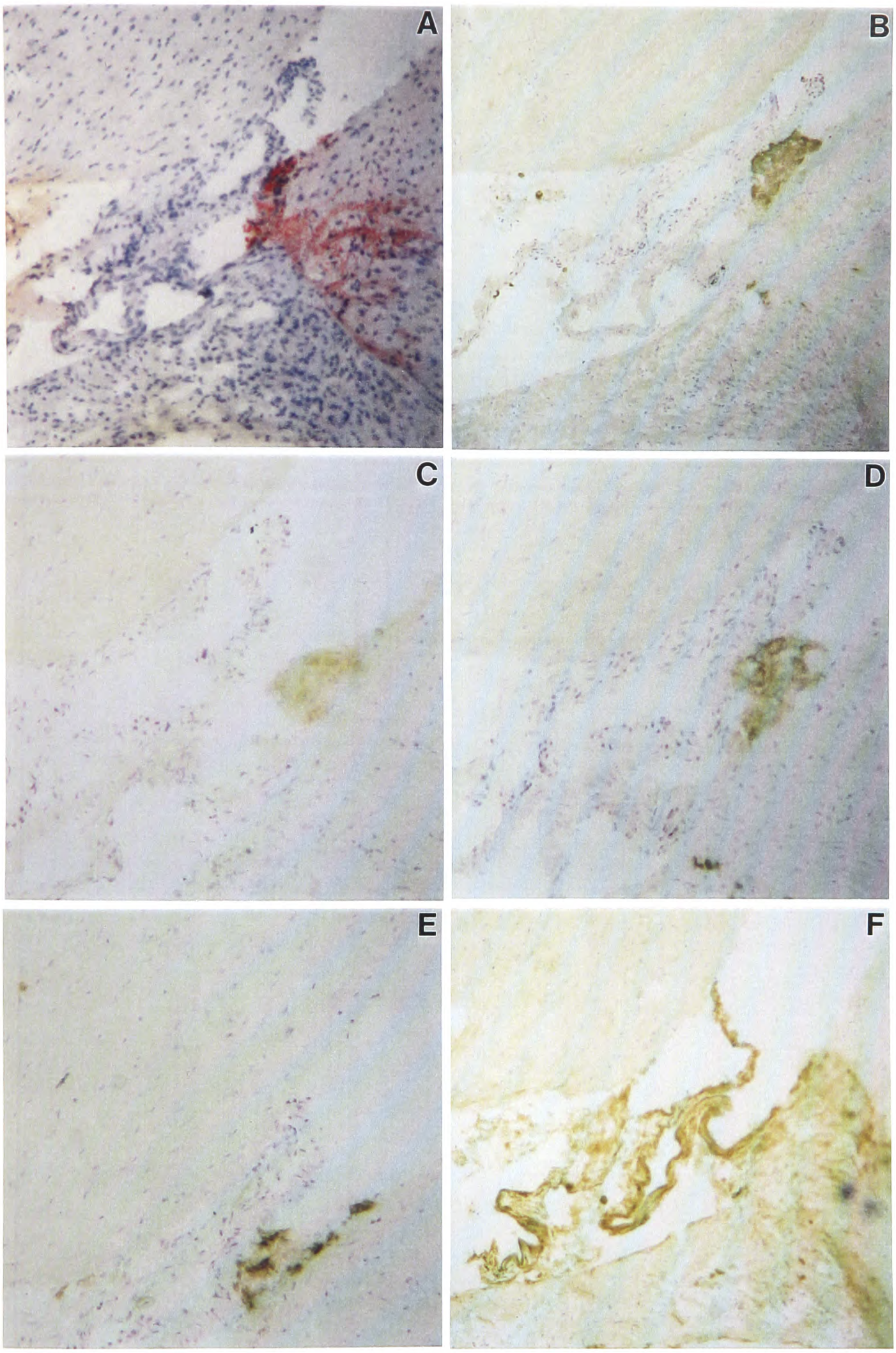
C. Another example of an early lesion from an op0/E0 animal stained with oil red O, FA-11, 5C6, 2F8, 2.4G2 and TIB120. 100x

A.	Oil red O	B.	FA-11
C.	2F8	D.	5C6
E.	TIB120	F.	8D2

A.



B.



C.

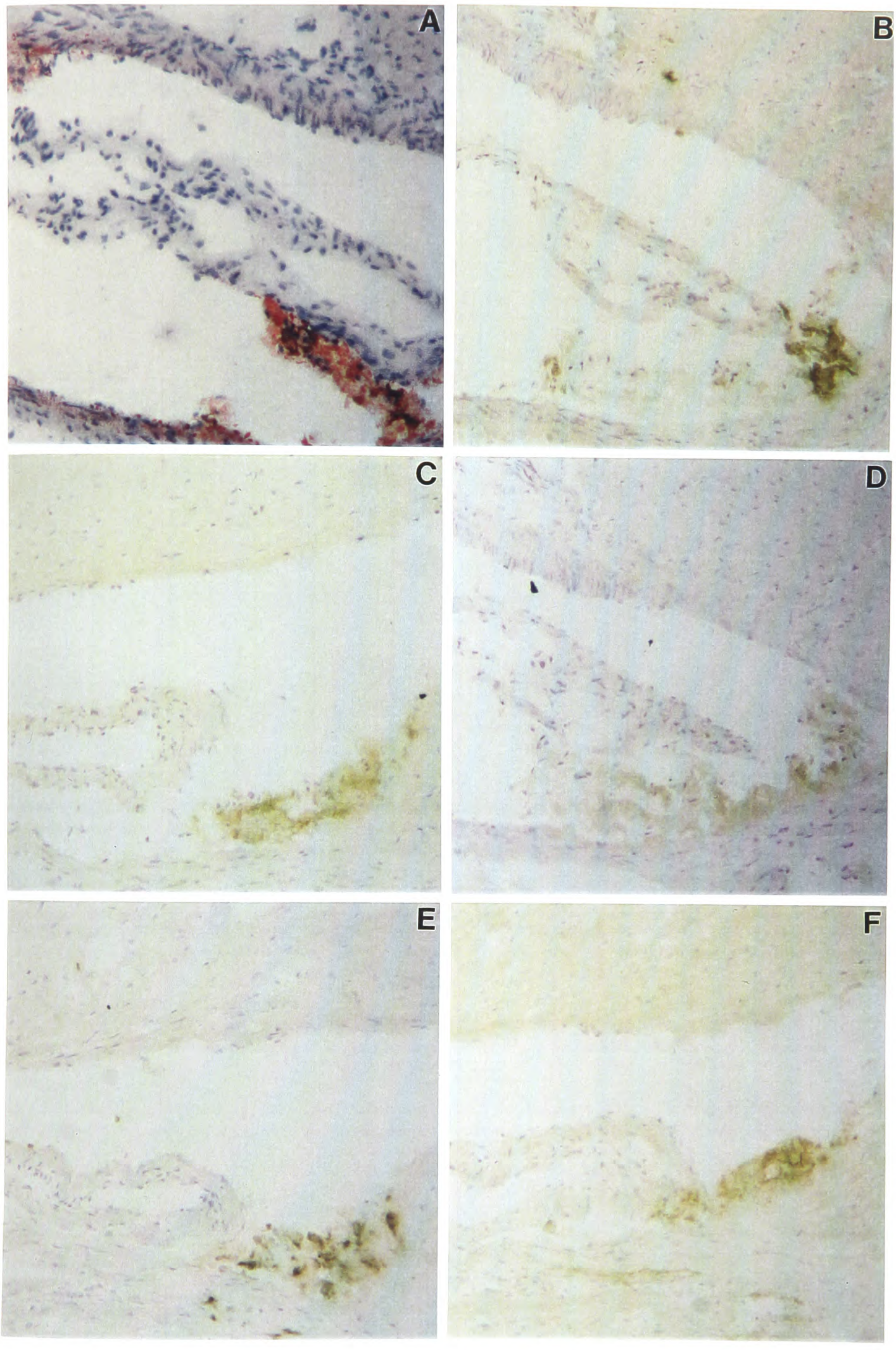


Fig. 8.4 **FcγRII expression in vascular tree of op2/E0 mice**

Subendothelial Mϕ in the vascular tree of op2/E0 mice stained prominently with 2.4G2. 100 x. This was reduced in op0/E0 animals (not shown) indicating the M-CSF dependent nature of this Mϕ population.

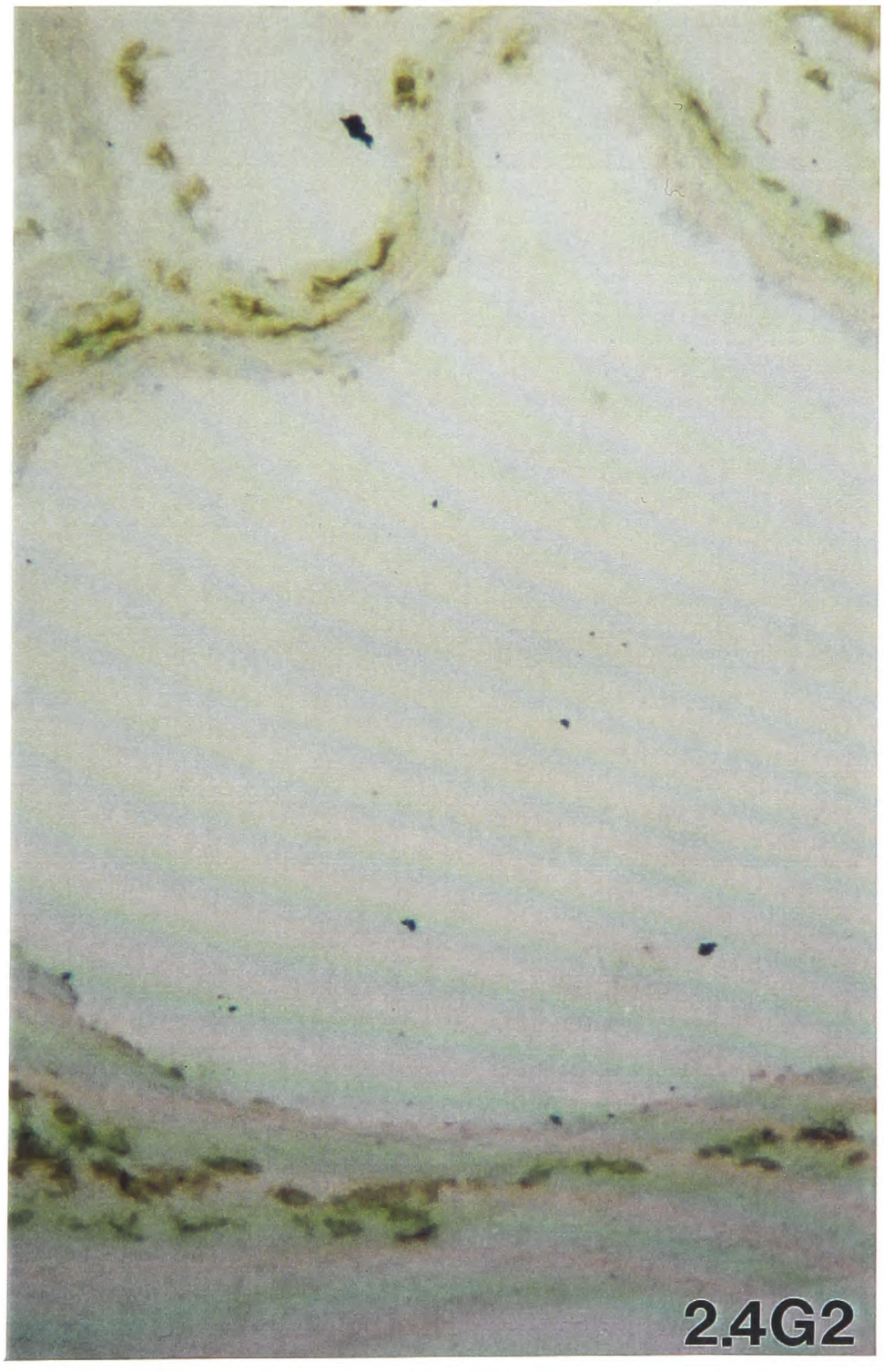
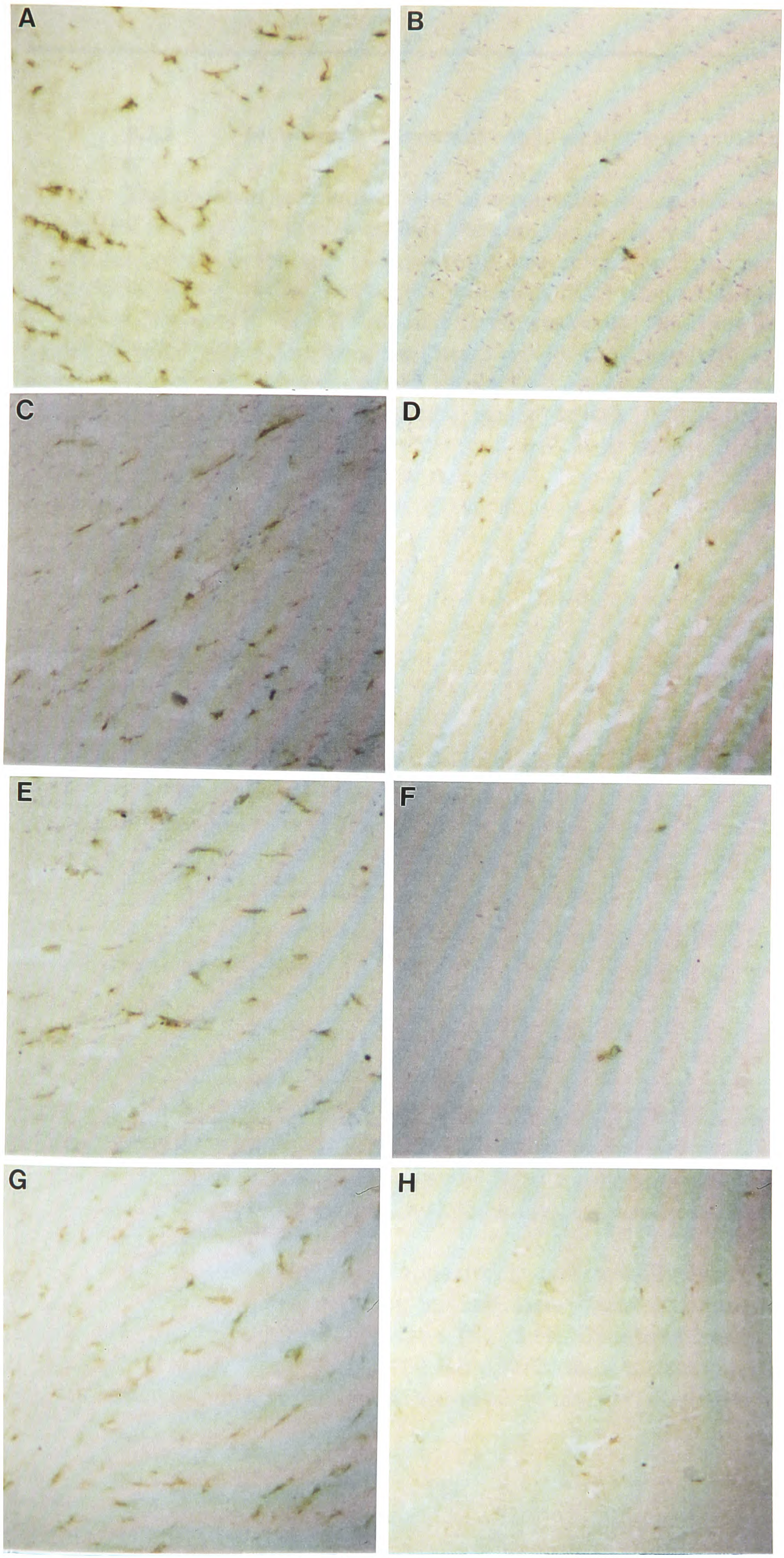

2.4G2

Fig. 8.5 **Characterization of myocardial interstitial Mϕ in op2/E0 and op0/E0 mice**

Myocardial tissues from op2/E0 and op0/E0 animals fed an atherogenic diet for 16 weeks, were stained with FA-11, 8D2, 2F8 and TIB120. 100x The M-CSF dependent nature of this Mϕ population is apparent; as indicated by reduction in op0/E0 mice.

op2/E0		**op0/E0**	
A.	FA-11	B.	FA-11
C.	8D2	D.	8D2
E.	2F8	F.	2F8
G.	TIB120	H.	TIB120


A
B
C
D
E
F
G
H

8.2.2 Myocardial interstitial Mϕ in op2/E0 and op0/E0 mice

The phenotypic characteristics of myocardial interstitial Mϕ in the op2/E0 and op0/E0 mouse models are depicted in fig. 8.5. Numerous Mϕ are present in the op2/E0 myocardial interstitium as is evident from immunostaining of the Mϕ-specific molecules macrosialin and MSR, as well as CD44, MHC class II, CR3, and FcγRII molecules (last two antigens not shown). These Mϕ have the characteristics of a recruited, and not resident population, as suggested by the low expression of F4/80 and sialoadhesin (not shown). Myocardial interstitial Mϕ are less prominent in C57BL/6 mice on a normal chow diet and seem mostly resident in nature (Hughes 1994). Extensive phenotyping has not previously been performed, and as op2/E2 C57BL/6 mice were not included in this study, the effects of a high-fat diet on Mϕ phenotype could not be ascertained. The recruitment of interstitial Mϕ into the myocardium of atherosclerosis-prone Western-type diet fed op2/E0 mice may be secondary to myocardial mucle injury, although no areas of focal necrosis or fibrosis due to myocardial infarction could be detected. Skeletal muscle injury has been reported to be associated with a Mϕ influx of similar recruited phenotype.

In contrast, expression of Mϕ membrane molecules, including MSR, was markedly reduced in op0/E0 animals (fig. 8.5). This would suggest a decrease in the interstitial myocardial Mϕ population as a whole in op0/E0 mice, and also indicates the M-CSF dependent nature of this population.

8.2.3 Mϕ populations in non-cardiovascular organs of op2/E0 and op0/E0 mice

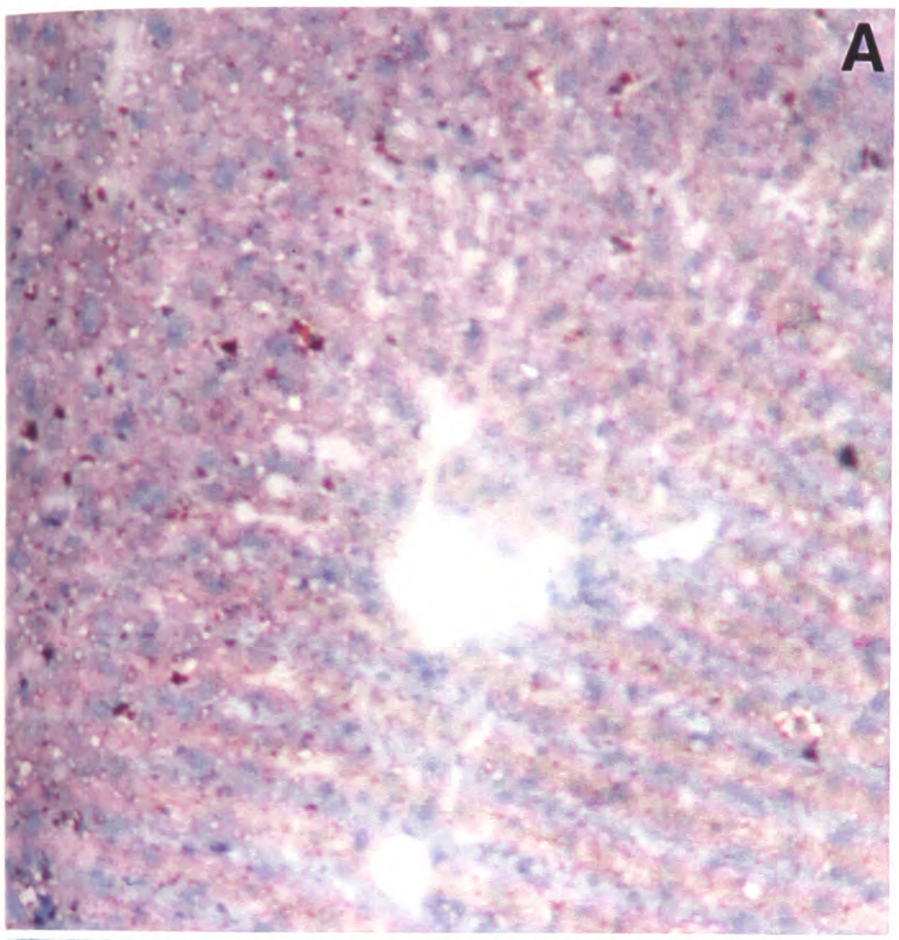
Hypercholesterolemia was present in both op2/E0 and op0/E0 mice fed a high-fat Western-type diet (1.25% cholesterol, 15% fat) (Smith *et al.* 1995). Cholesterol levels were increased 6-fold in the op2/E0 and more markedly, 14-fold in the op0/E0 animals compared to reported levels in normal C57BL/6 and 129 mice fed a similar diet (Zhang *et al.* 1994). To determine relative levels of lipid accumulation in various op2/E0 and op0/E0 organs, oil red O staining of tissue sections was performed (fig. 8.6).

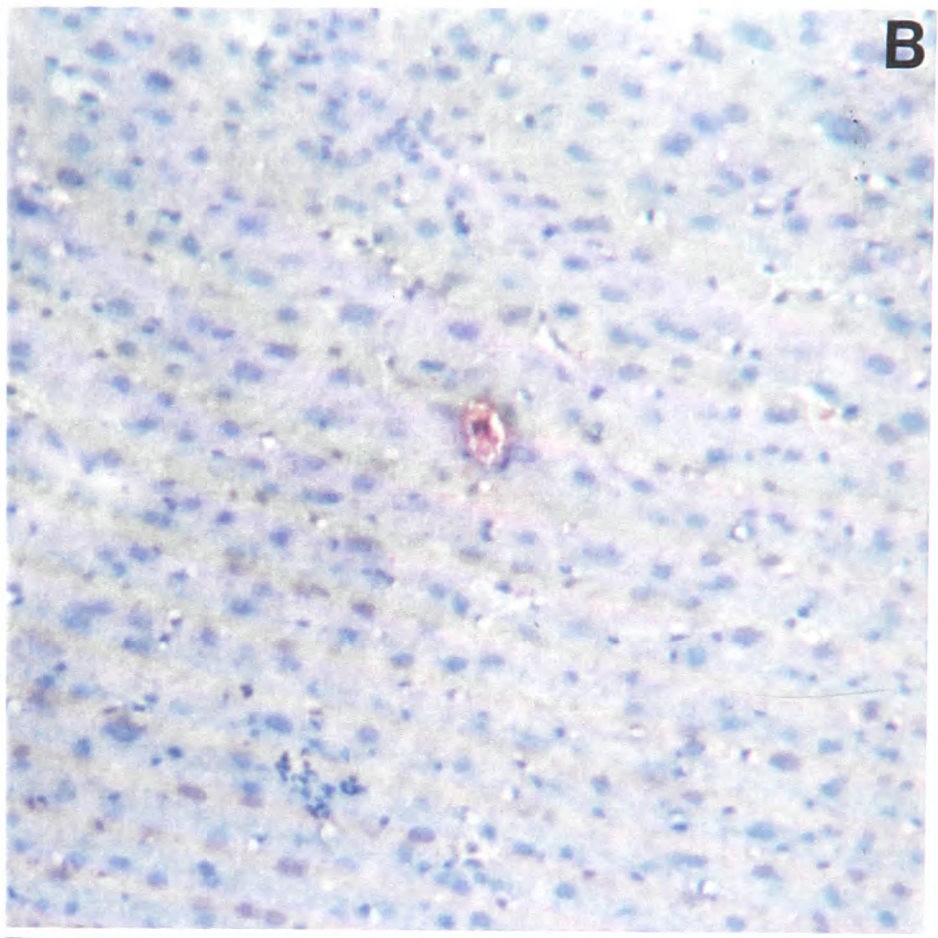
The **liver** in op2/E0 mice appeared macroscopically enlarged and fatty and this was confirmed by marked foamy lipid accumulation in both Kupffer cells and hepatocytes (fig. 8.6). Immunohistochemical analysis of hepatic Mϕ from op2/E0 and op0/E0 mice is shown in fig. 8.7. The Kupffer cell population expressed macrosialin and CD44

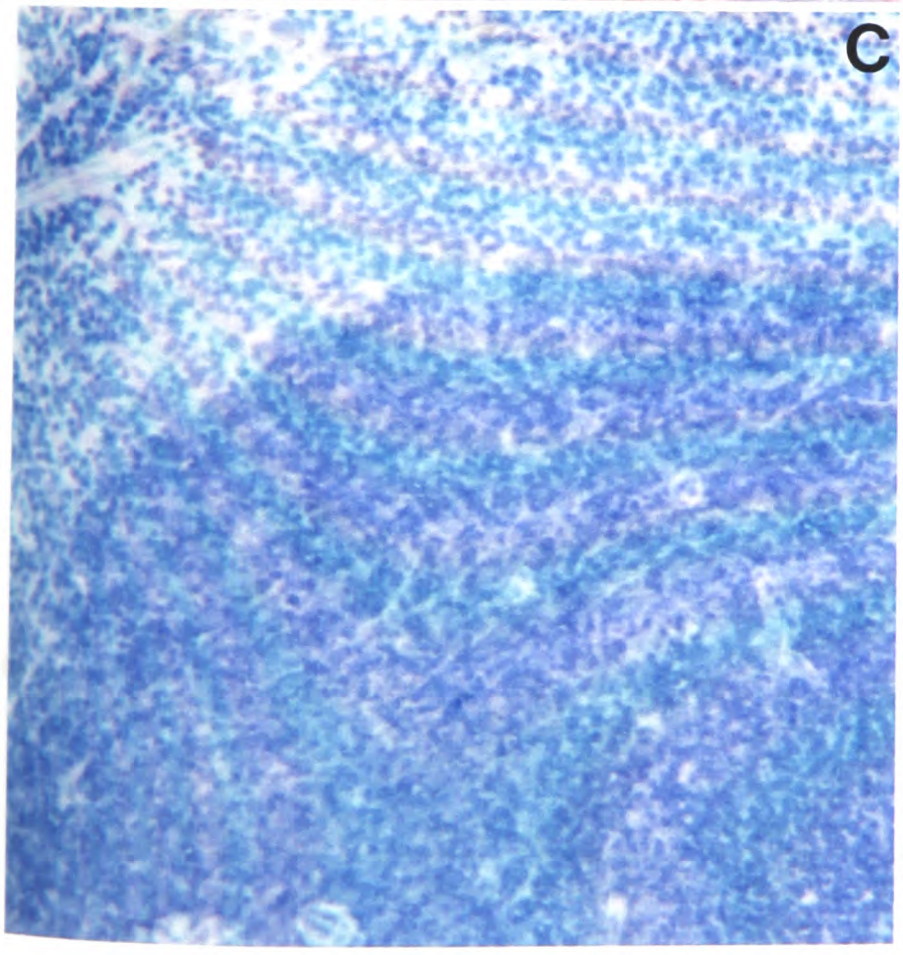
Fig. 8.6 **Lipid accumulation in non-cardiovascular organs of op2/E0 and op0/E0 mice fed an atherogenic diet**

Liver and spleen sections from op2/E0 and op0/E0 animals fed an atherogenic diet for 16 weeks, were stained with oil red O. 100x Both sets of animals were hypercholesterolemic; op2/E0 mice had 6-fold raised plasma cholesterol levels and op0/E0 mice showed a 14-fold increase. In op2/E0 animals, both Kupffer cells and hepatocytes accumulated lipid, while only Kupffer cells (reduced in number) in op0/E0 mice stained oil red O-positive. The reverse applied in spleen sections from these animals; with increased lipid uptake by op0/E0 red pulp Mϕ, but not op2/E0 spleens.

A. liver: op2/E0 B. liver: op0/E0
C. spleen: op2/E0 D. spleen: op0/E0


A


B


C

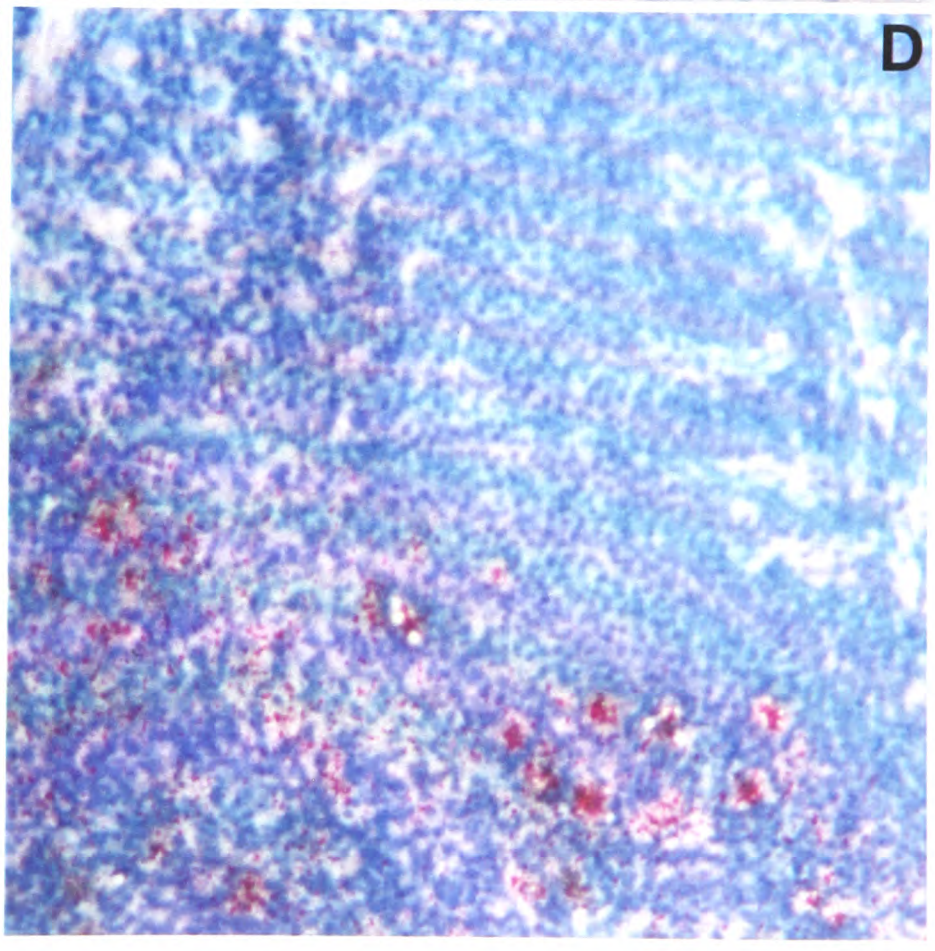

D

Fig. 8.7 **Immunohistochemistry of livers from op2/E0 and op0/E0 mice fed an atherogenic diet**

Liver tissues were obtained from op2/E0 and op0/E0 animals fed an atherogenic diet for 16 weeks, and stained with FA-11, 8D2, and 2F8. 100x FA-11 and 8D2 stained only Kupffer cells, and both the reduction in absolute numbers and foamy nature of op0/E0 Kupffer cells are clearly apparent. MSR was present on Kupffer cells and sinusoidal endothelium in op2/E0 mice; in op0/E0 mice, expression was maintained by sinusoidal ECs, but not Kupffer cells.

op2/E0		**op0/E0**	
A.	FA-11	B.	FA-11
C.	2F8	D.	2F8
E.	8D2	F.	8D2

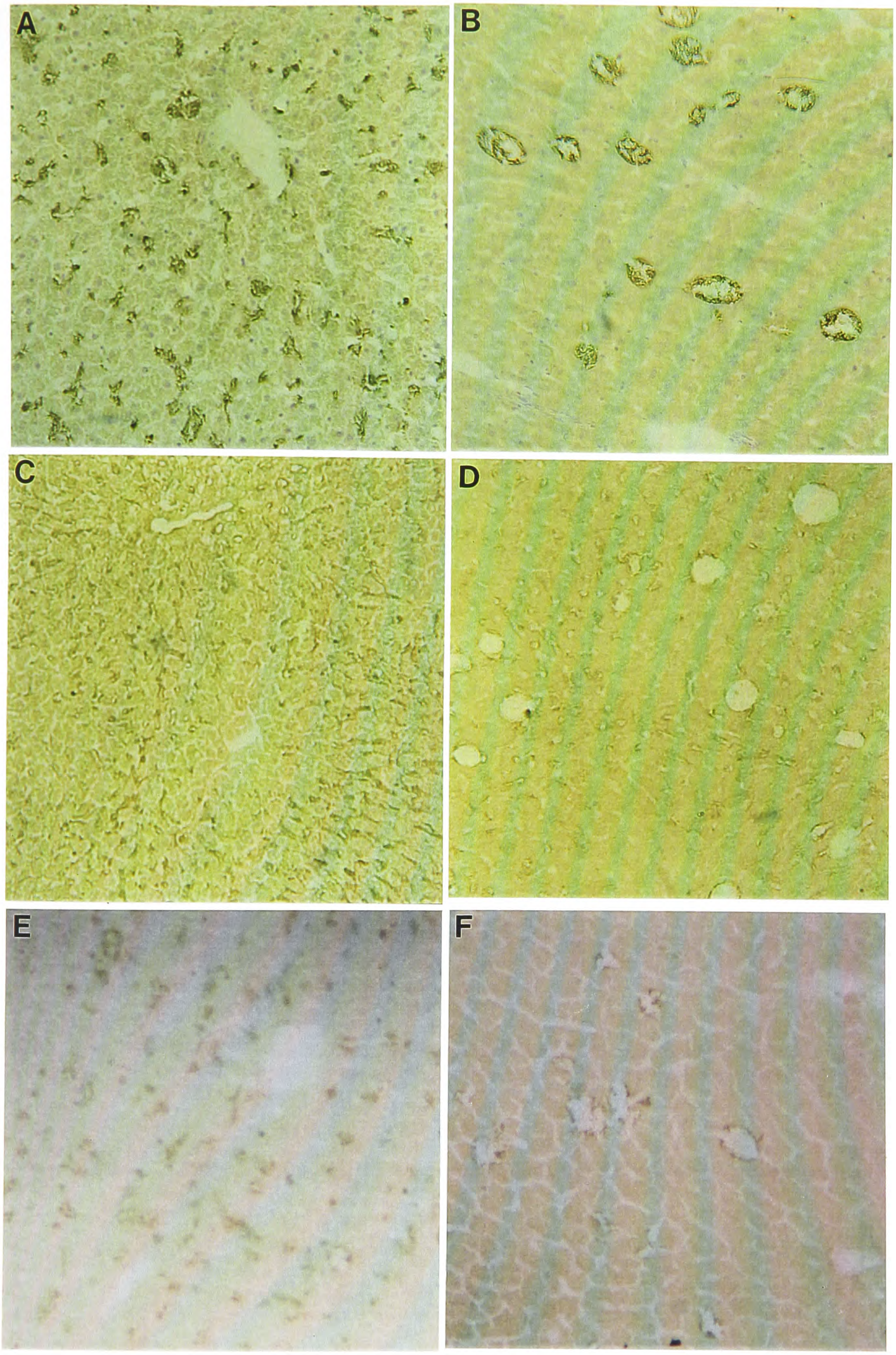
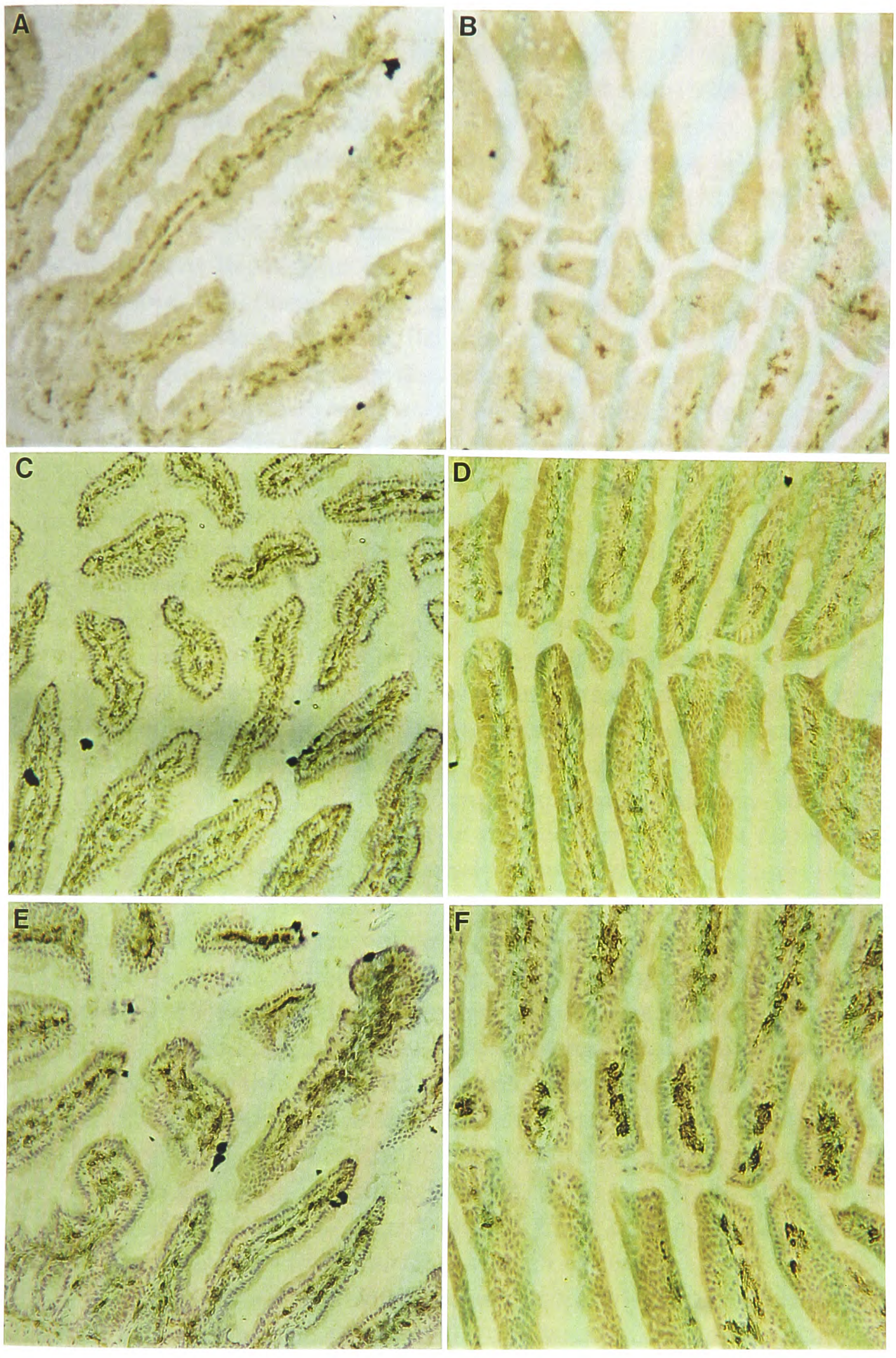

A
B
C
D
E
F

Fig. 8.8 **Characterization of intestinal lamina propria Mϕ in op2/E0 and op0/E0 mice**

Small intestinal tissues were obtained from op2/E0 and op0/E0 animals fed an atherogenic diet for 16 weeks, and lamina propria Mϕ were stained with FA-11, 5C6, and 2F8. 100x This Mϕ population seemed for the most part, M-CSF independent. MSR staining however appeared reduced in op0/E0 lamina propria Mϕ compared to op2/E0 animals.

op2/E0		**op0/E0**	
A.	FA-11	B.	FA-11
C.	2F8	D.	2F8
E.	5C6	F.	5C6


A
B
C
D
E
F

prominently. Interestingly, both MSR and FcγRII molecules were abundantly expressed by Kupffer cells and sinusoidal endothelium, and Kupffer, but not endothelial cells appeared foamy. MSR staining of sinusoidal endothelium was more apparent than previously described (Hughes *et al.* 1995) and this would be consistent with the increased level of hepatic MSR expression shown in tissue blots in Chapter 4. The prominent expression of MSR by sinusoidal endothelial cells (and its possible functional relevance in clearing atherogenic lipoproteins) contrasts markedly with its complete absence from endothelial cells elsewhere and especially the myocardial endothelium. The phenotypic status of Kupffer cells as a mature resident Mϕ population was confirmed by the lack of CD11b expression (not shown) and low levels of sialoadhesin staining. Less F4/80 antigen than expected was present (usually well expressed on Kupffer cells (Gordon *et al.* 1992)); which may reflect downregulation by the atherogenic diet or technical reasons. Prominent F4/80 staining of splenic red pulp Mϕ argues against the latter.

In contrast, the livers from op0/E0 mice appeared non-fatty despite the extremely high levels of plasma cholesterol. Hepatocytes showed little evidence of lipid accumulation, but Kupffer cells (reduced in number) were still oil red O-positive and thus capable of lipid uptake (fig. 8.6). The M-CSF dependent reduction in Kupffer cells was further demonstrated by the decrease in macrosialin and CD44 staining (fig. 8.7), and foamy Mϕ were sparsely grouped throughout the hepatic architecture. The reduction in Kupffer cells (although substantial numbers were still present) was paralleled by decreased MSR and FcγRII expression on Kupffer cells in op0/E0 liver sections. Interestingly, MSR and FcγRII remained well preserved on the sinusoidal EC population; these cells did not, however, appear foamy. That M-CSF does not appear to influence the expression of MSR on sinusoidal EC *in vivo* is not altogether surprising as they do not express the M-CSF receptor (*c-fms*) (Sherr 1990); the functional significance of sinusoidal EC expression remains unclear. Hepatic MSR expression in op0/E0 animals, although reduced, could not be demonstrated to be disproportionately decreased in comparison to the other Kupffer cell markers.

In the **small intestine** lamina propria Mϕ from op2/E0 or op0/E0 did not accumulate lipid (by oil red O staining) or appear foamy (fig. 8.8). This differentiated Mϕ population appeared M-CSF independent and both op2/E0 and op0/E0 animals expressed the antigens FA-11, CD11b (fig. 8.8), 2.4G2, F4/80 and 3D6 (not shown) abundantly; and the Mϕ population size in op0/E0 mice did not appear reduced. Interestingly, as is apparent from fig. 8.8, MSR expression in op0/E0 mice did seem

decreased in this Mϕ population compared to op2/E0 controls.

The **spleens** from op2/E0 mice showed no lipid accumulation (fig. 8.6) and splenic Mϕ also did not appear fatty. In contrast, in op0/E0 mice, the red pulp stained positive for oil red O and red pulp Mϕ (depicted by macrosialin (fig. 8.9), F4/80 antigen, and CD44 (not shown) (Gordon *et al.* 1992; Gordon *et al.* 1992)) appeared foamy, possibly signifying some selective pressure for increased lipid uptake in this Mϕ subpopulation. Some white pulp Mϕ, stained with mAb FA-11, also had a lipid-engorged appearance. In general, as tabulated below (table 8.3), op2/E0 splenic Mϕ displayed the expected range and distribution of antigen markers. MSR expression, in particular, was prominent in the marginal zone and less obvious in the white pulp.

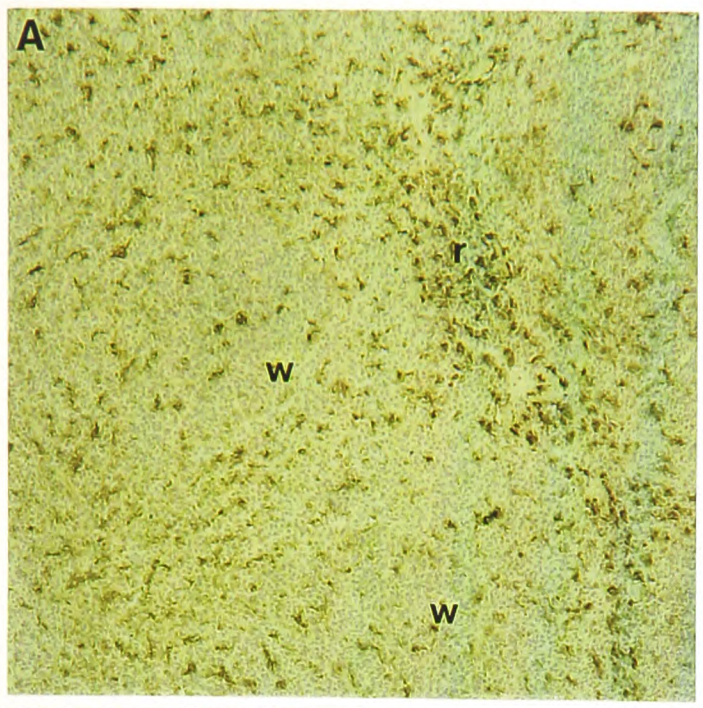
Numerous phagocytes were present in the splenic red pulp of op0/E0 mice (stained with F4/80 and FA-11) (fig. 8.9). The amount of red pulp also appeared increased; this would fit with the need for extramedullary haematopoiesis as a result of reduction in bone marrow Mϕ in op0 mice (Felix *et al.* 1990). Splenic marginal zone metallophil Mϕ are profoundly reduced in op0 mice (Witmer-Pack *et al.* 1993). Sialoadhesin staining was absent in the spleens of op0/E0 mice confirming the op0 status of the doubly mutant animals (fig. 8.9). The CD11b molecule, found on the same splenic Mϕ populations as sialoadhesin, was also absent from the same regions; neutrophils in the red pulp stained prominently. Differences in MSR staining in op0/E0 mice compared to op2/E0 mice were similar to that described before (Hughes 1994). MSR expression in op0/E0 mice was absent on the M-CSF dependent marginal zone metallophil population. The combined absence of 5C6, 3D6 and 2F8 staining would seem to indicate loss of a specific Mϕ subpopulation rather than selective decreases in antigens. Interestingly, loss of those cells in op0 mice which normally express high levels of MSR would be consistent with an anchoring role for MSR at particular locations within tissues.

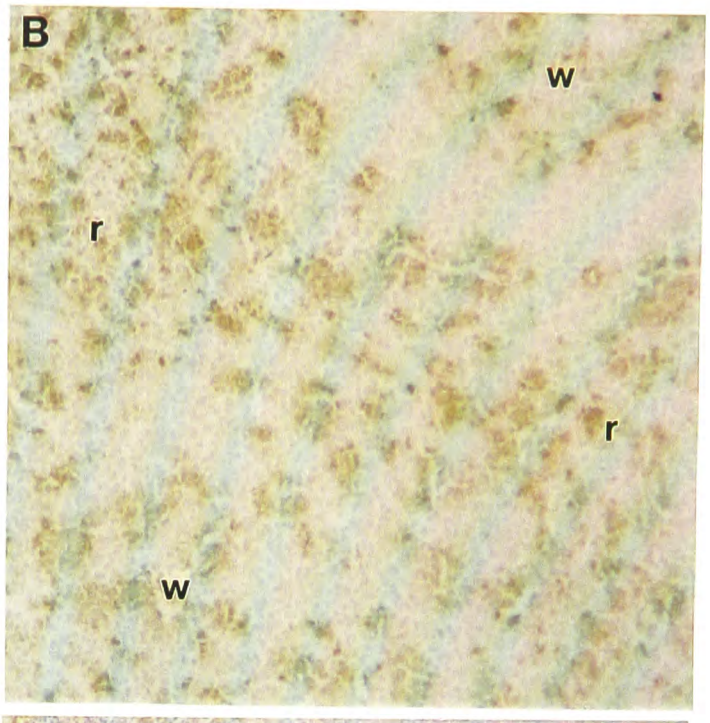
Alveolar Mϕ in the **lung** have previously been reported as an M-CSF independent population in op0 mice (Witmer-Pack *et al.* 1993) although the numbers appeared slightly reduced relative to op2 controls. No lipid accumulation could be detected in the lungs of op2/E0 and op0/E0 mice and alveolar Mϕ were readily detected by a variety of Mϕ markers, including MSR (fig. 8.10). Saline perfusion may explain the prominent CR3 staining (probably due to neutrophils) in both op2/E0 and op0/E0 animals; because alveolar Mϕ, unless activated, usually lack CR3 (Gordon *et al.* 1992). Interestingly, the dendritic cell marker, NLDC145, was prominent in op0/E0 mice (although alveolar Mϕ may have contributed (Kraal *et al.* 1986)); confirming the M-CSF independent

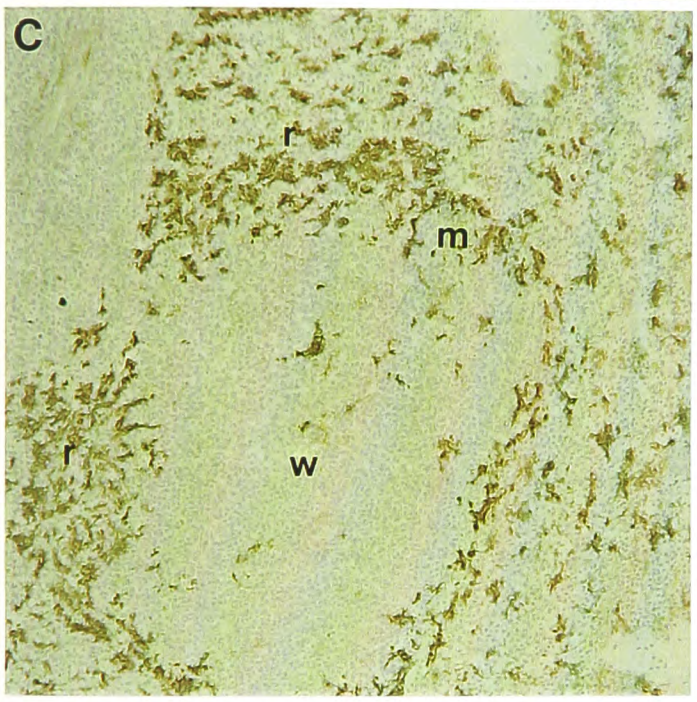
Fig. 8.9 **Macrophage phenotype in spleens from op2/E0 and op0/E0 mice**

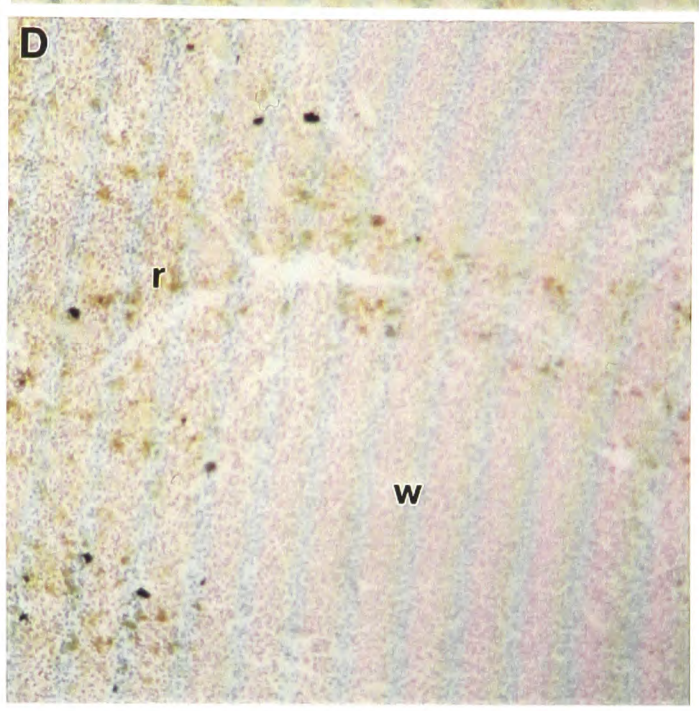
Spleens were obtained from op2/E0 and op0/E0 animals fed an atherogenic diet for 16 weeks, and stained with FA-11, 5C6, and 3D6. 100x w = white pulp; r = red pulp; m = marginal zone. FA-11-positive Mϕ in op0/E0 mice appear lipid-engorged and foamy, whilst the osteopetrotic phenotype of the op0/E0 animals was confirmed by loss of the marginal zone Mϕ subpopulation as indicated by the absence of 5C6 and 3D6 staining. Neutrophils in the red pulp are 5C6-positive. MSR expression (not shown) was similarly absent from the op0/E0 marginal zone.

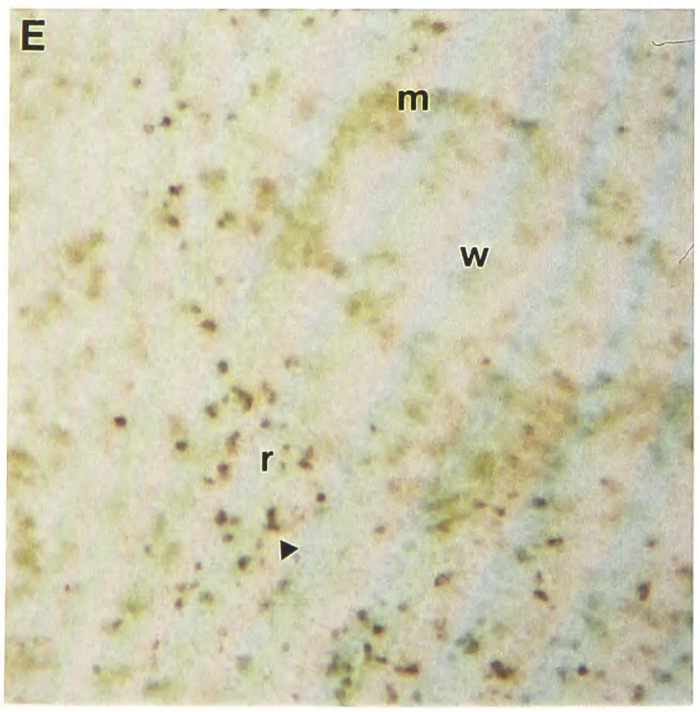
op2/E0		**op0/E0**	
A.	FA-11	B.	FA-11
C.	3D6	D.	3D6
E.	5C6	F.	5C6


A
r
w
w


B
w
r
r
w


C
r
m
r
w


D
r
w


E
m
w
r

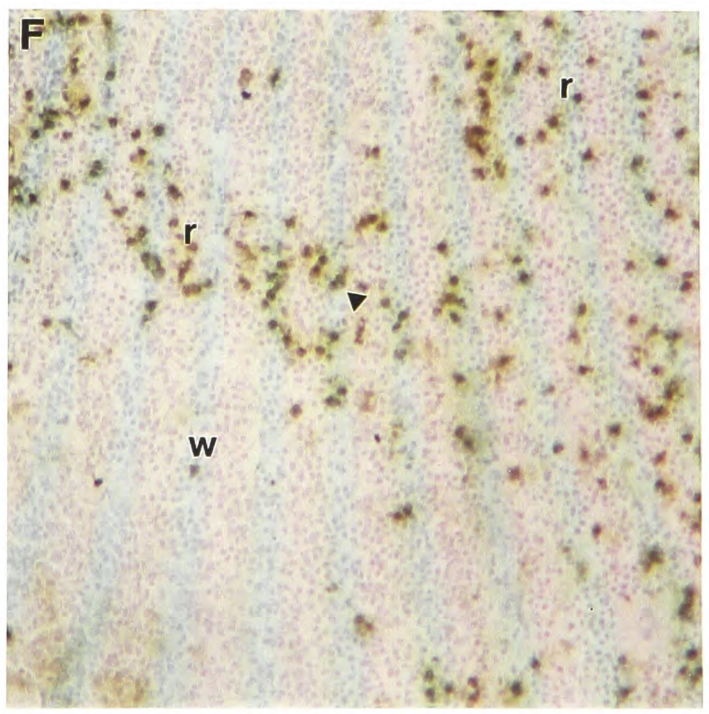

F
r
r
w

Fig. 8.10 **Immunohistochemistry of pulmonary tissues from op2/E0 and op0/E0 mice**

Lungs were obtained from op2/E0 and op0/E0 animals fed an atherogenic diet for 16 weeks, and sections were stained with FA-11, 5C6, 2F8 and NLDC145. 100x The alveolar Mϕ population (FA-11 and 2F8) seemed for the most part, M-CSF independent. The prominent CR3 expression may be explained by the presence of neutrophils, as saline perfusion and not PLP perfusion-fixation, was used in tissue preparation. The marked NLDC145 expression would suggest that op0/E0 alveolar Mϕ, characterized by a dendritic cell marker, represent a M-CSF independent population.

op2/E0		**op0/E0**	
A.	FA-11	B.	FA-11
C.	5C6	D.	5C6
E.	2F8	F.	2F8
		G.	NLDC145

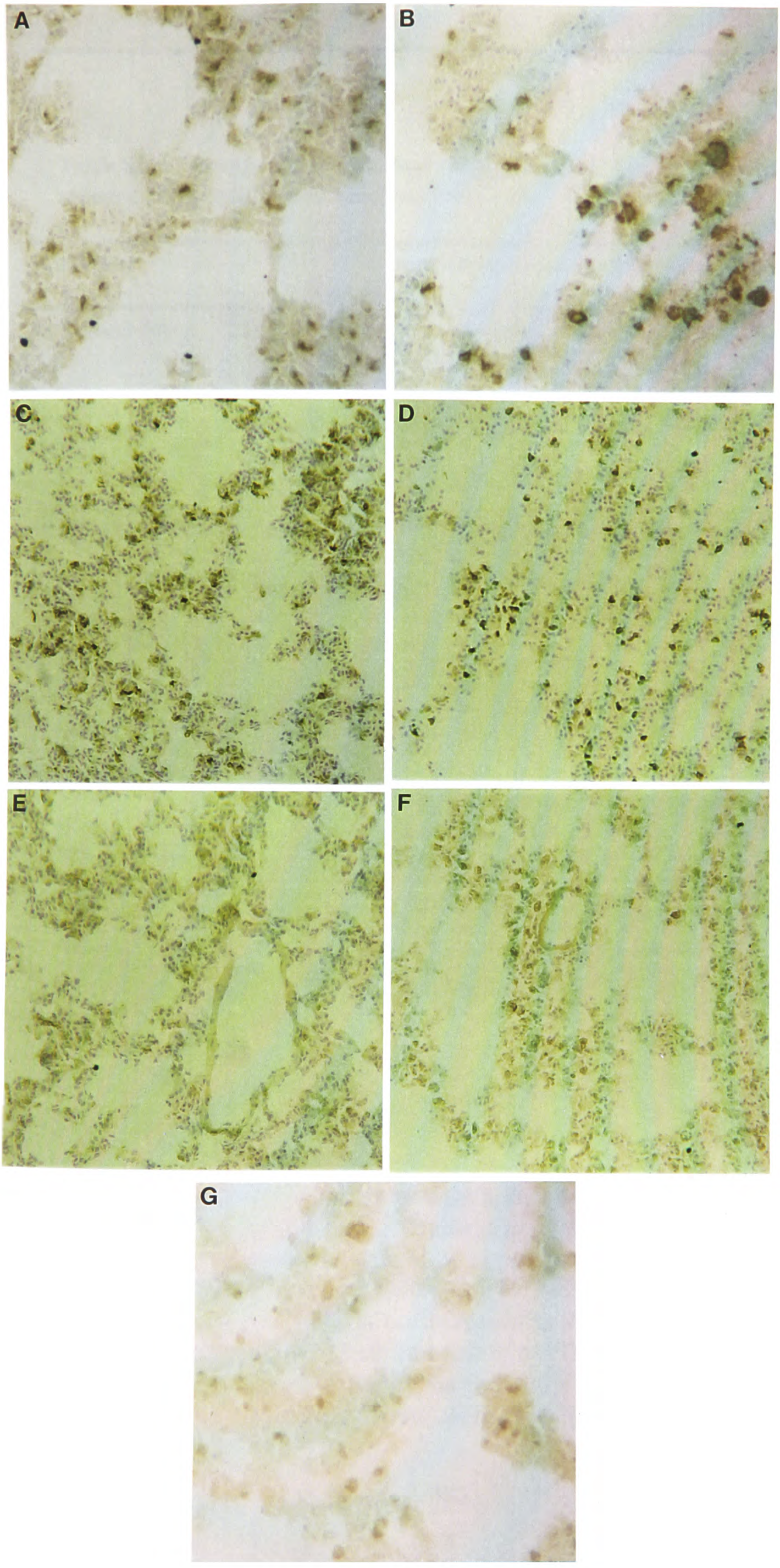

A
B
C
D
E
F
G

Table 8.3 Immunohistochemical detection of Mϕ populations in spleens from op2/E0 and op0/E0 mice

Cell type	Antigen expression and comments	
	op2/E0	op0/E0
Red pulp Mϕ	FA-11 +++, F4/80 ++, 5C6 (Mϕ and PMNs) +++, 8D2 +++, 3D6+	FA-11 +++, F4/80 ++, 5C6 (Mϕ and PMNs) +++, 8D2 +++ Oil red O +, foamy
White pulp Mϕ	FA-11++, 2F8+, TIB120+	FA-11++, 2F8-, TIB120+ Foamy cells
Marginal zone Mϕ	3D6 ++, 5C6++, 2F8++	3D6 -, 5C6-, 2F8-

nature of this cell population (Witmer-Pack *et al.* 1993).

8.3 Discussion

The immunohistochemical findings presented in this Chapter can be summarized as follows:

i) Op0/E0 develop fewer and smaller atherosclerotic lesions than op2/E0 mice. Mϕ in op0/E0 lesions are reduced and constitute a predominantly recruited and M-CSF dependent population. Phenotypic heterogeneity is shown by differences in marker expression by lesional Mϕ in different parts of the lesion (table 8.2) and by an overall reduction in F4/80 and sialoadhesin levels. MSR expression is still present, although reduced in proportion to the decrease in Mϕ numbers; suggesting that M-CSF is not an absolute requirement for its expression *in vivo*. Myocardial interstitial Mϕ are a similarly recruited and M-CSF dependent population and in the vascular tree, FcγRII expression proved a good marker for the detection of subendothelial Mϕ in op2/E0 mice.

ii) Prominent lipid accumulation in op2/E0 Kupffer cells and hepatocytes contrasts markedly with the low level of hepatic lipid uptake by Kupffer Mϕ in op0/E0 animals. MSR is abundantly expressed on both Kupffer cells and sinusoidal ECs in op2/E0 mice. Op0/E0 Kupffer cells are partially M-CSF dependent as shown by a non-selective decrease of Mϕ markers; and MSR expression on these cells is not disproportionately reduced. Sinusoidal EC MSR expression in op0/E0 remains preserved. There is no increased or differential lipid uptake apparent in the lung and small intestine of op2/E0 and op0/E0 mice. In the spleen, lipid accumulates in the red pulp Mϕ of op0/E0, but not op2/E0 mice, indicating some selective pressure on this Mϕ

subpopulation. Splenic marginal zone Mϕ, including those cells normally expressing the highest levels of MSR, are absent in op0/E0 animals, thereby confirming their true op0 status. M-CSF independent populations thus express MSR in the apparent absence of M-CSF (op0/E0); therefore, although M-CSF upregulates MSR expression on Mϕ *in vitro*, M-CSF is not required for MSR expression on Mϕ and sinusoidal endothelium.

Peritoneal Mϕ are very M-CSF dependent and op0 mice have few and sometimes no peritoneal cavity phagocytes (Witmer-Pack *et al.* 1993). Functional MSR expression in op0 thioglycollate-elicited peritoneal Mϕ was determined by assessing MSR-dependent adhesion to TCP (Hughes 1994). In these experiments op0 TPMϕ lacked any EDTA-resistant component to adhesion suggesting the absence of any functional MSR. The immunohistochemical expression of MSR on op0 peritoneal Mϕ was similarly reduced or undetectable; this would be compatible with an *in vivo* role for M-CSF in the induction or maintenance of MSR expression.

In association with Dr. D. Hughes the possible role of MSR in Mϕ adhesion in atherogenesis was investigated by frozen section adhesion assay. The Stamper-Woodruff lymphocyte adhesion assay was modified to investigate RAW 264.7 (Mϕ-like cell line) and TPMϕ adhesion to tissue sections; MSR-dependent (EDTA-resistant, mAb 2F8-inhibitable) adhesion has previously been demonstrated by this approach (Hughes *et al.* 1995). To elucidate the exact role of MSR in adhesion *in vivo*, adhesion assays of this nature were performed on the atherosclerotic lesions and other organs in the op2E0 and op0E0 mice. These studies are ongoing but preliminary results are encouraging in that the ability of mAb 2F8 to block the EDTA-resistant adhesion of Mϕ correlated with MSR expression in some op2/E0 tissues (results not shown).

Apart from intestinal lamina propria Mϕ, a convincing disproportionate reduction in MSR levels could not be shown in op0/E0 animals. A possible explanation may be their age (16 weeks) at time of sacrifice. MSR is expressed at low levels in normal mice aged three to eight weeks; at this age op0 animals completely lack MSR expression (Hughes 1994). An M-CSF gene dosage effect was also detectable when examining MSR expression in spleen, liver, thymus and lung in 3 week old normal (op2), op1 and op0 mice. MSR expression was reduced in op1 mice compared to op2 controls and was virtually absent in op0 animals. However, from eleven weeks MSR expression was present in both normal and op0 mice; and it has been suggested that some

reconstitution of op0 mice might occur after eight weeks (Begg *et al.* 1993; Nilsson and Bertoncello 1994). The lack of a selective reduction in MSR expression and M-CSF gene dosage on lesional and other Mϕ populations in op1/E0 and op0/E1 mice may therefore not be surprising. The general M-CSF requirement of monocyte-derived Mϕ for survival, differentiation and proliferation should also be considered (Stanley 1994).

An unexpected finding from the study of Smith and colleagues (Smith *et al.* 1995) was the significant inverse correlation between plasma cholesterol levels and lesion area in the op2/E0, op1/E0 and op0/E0 populations; implicating M-CSF gene dosage as the underlying cause for both phenomena. Op0/E0 mice thus had significantly less atherosclerosis than their op1/E0 and op2/E0 littermates, despite markedly raised cholesterol levels averaging more than 34 mmol/l. They concluded that M-CSF, by either altering the number or functional status of monocyte-derived Mϕ, therefore affects susceptibility to atherosclerosis profoundly. The level of MSR expression and function *in vivo* may be such a property affected by M-CSF, and this was not excluded as a functionally relevant possibility by the current findings. Other factors which should be considered include i) the effects of M-CSF, produced locally in atherosclerotic lesions by ECs, SMCs and Mϕ, on Mϕ proliferation within the lesions (Clinton *et al.* 1992); and ii) the increased expression of LDL-receptor related protein (LRP) by M-CSF (Hussaini *et al.* 1990). Both of the above may contribute to the positive gene dosage effect of M-CSF on β-VLDL uptake by Mϕ (from normal and E0 mice), and affect lesion growth and plasma cholesterol levels.

Mϕ within atherosclerotic lesions express considerable heterogeneity; and may represent functionally diverse subsets of cells as far as adherence, lipid endocytosis, secretion of growth and migration factors and roles in coagulation and fibrinolysis are concerned. In addition, replication of Mϕ occurs more frequently in advanced atherosclerotic lesions than commonly appreciated and many Mϕ in lesions are not "terminally" differentiated cells (Rosenfeld and Ross 1990). Lesional Mϕ foam cell functional heterogeneity may be influenced by i) the duration of residence within the lesion; ii) the local micro-environment of stimulatory and inhibitory growth factor and cytokine signals; and iii) regional location within the lesion. For example, Mϕ at the shoulder of lesions seem to express stromelysin activity selectively (Henney *et al.* 1991); whilst within the necrotic core of advanced plaques Mϕ cell death may be either programmed (apoptosis) (Mitchinson *et al.* 1995) or result from toxic accumulation of modified lipoprotein derivatives and nitric oxide, or local depletion of M-CSF

required for survival (Libby and Clinton 1993).

Mϕ are generally thought to be activated by modified lipoproteins (Quinn *et al.* 1987; Barath *et al.* 1990; Frostregard *et al.* 1992; Stiko-Rahm *et al.* 1992), but some studies also suggest anti-inflammatory effects (Hamilton *et al.* 1990). Such confusion probably reflects the variability of experimental preparations, and the differences between most Mϕ studied *in vitro* and lesional foam cells (which are themselves heterogeneous in function). Many Mϕ gene products present in atherosclerotic lesions *in vivo* (table 1.2) have known *in vitro* effects on Mϕ activation, proliferation, or lipid metabolism. A simplistic approach has to be cautioned against, however, as it is difficult to predict any specific functional role for lesional Mϕ subsets; and in most cases there is little evidence that gene expression by lesional Mϕ *in vivo* causes changes in cellular behaviour or has an impact on the initiation or progression of atherosclerosis.

Given these caveats, the current study depicts a recruited M-CSF dependent lesional Mϕ population in op2/E0 mice which parallels lesion development. The pan-Mϕ molecule, macrosialin, proved an excellent marker for the presence of intralesional Mϕ. Its ubiquitous presence and localization as a late endosomal membrane protein, may suggest a role in Mϕ lipid loading; and recent work is consistent with its involvement in oxidized lipoprotein uptake (Ramprasad *et al.* 1995). The prominent lesional expression of FcγRII on Mϕ was also interesting as, in addition to IgG phagocytosis, a scavenging role including the uptake of oxidized lipoprotein, has been proposed for this molecule (Stanton *et al.* 1992). CR3-positive cells within the lesions were all Mϕ, and not neutrophils, indicating monocyte-specific recruitment and rapid turnover within a chronic inflammatory focus. The C-C chemokine, MCP-1, is thought to have an important role in intimal monocyte recruitment in atherogenesis (discussed in Chapter 5.3). The preserved presence of CR3-positive staining in op0/E0 mice could argue against a M-CSF requirement for MCP-1 expression *in vivo*.

A recent study demonstrated the phenotypic heterogeneity of Mϕ within human atherosclerotic lesions (van der Wal *et al.* 1992). Antibodies to clusters of differentiation (CD) antigens were used to show that Mϕ within different types of lesions or in different locations within the same lesion differed phenotypically by their varying expression of combinations of marker antigens. Almost all the Mϕ within lesions from the aorta, coronary and carotid arteries expressed CD68 and the HLA-DR antigen; which agrees with the findings in op2/E0 and op0/E0 mice. The other results are tabulated below; it is clear from fig. 8.1 that CR3 expression in the murine advanced lesion is not only confined to

the deeper layers, but also occurs in the superficial layers and necrotic core.

Mϕ in different lesion types	CR3	CD14	CD36
1) Fatty streaks	+	-	+
2) Diffuse intimal thickening	-	+	-
3) Advanced lesions			
- superficial layers	-	+	-
- deeper layers	+	+	+/-
- necrotic core	-	-	+

(van der Wal *et al.* 1992)

Mϕ within atherosclerotic lesions may also be heterogeneous with regard to expression of MSR isoform (type I or II). Although mAb 2F8 recognizes both types of murine MSR, data shown in Chapters 4 (tissue blots) and 7 (CHO transfectants) would suggest that type II MSR is the predominant murine form expressed *in vivo.* Tissue blotting of MSR expressed in op2/E0 atherosclerotic lesions (and comparison to the M_r of MSR types I and II transfected into CHO cells) could be attempted to determine type specificity. The functional importance of the SRCR domain in type I MSR is still unknown, but subtle differences in ligand-binding properties between type I and II isoforms have been described (Ashkenas *et al.* 1993). Selective MSR type I upregulation (at the mRNA level) has recently been suggested to account for the increased MSR activity observed during human monocyte to Mϕ differentation (Geng *et al.* 1994); this increased expression was also maintained after foam cell transformation *in vitro.* This contrasts with *in vivo* results where no differential expression of human MSR isoforms could be detected in Mϕ from various organs and atherosclerotic foam cells immunostained with polyclonal sera generated against type-specific synthetic peptides (Naito *et al.* 1992).

An intriguing finding in the current study was the lipid accumulation in the livers of op2/E0, but not op0/E0 mice, despite a comparable 4-fold increase in plasma cholesterol. This may be consistent with a crucial M-CSF dependent role for Kupffer cells in lipoprotein uptake, transfer to hepatocytes and biliary excretion of cholesterol. In contrast, M-CSF independent splenic Mϕ in op0/E0 animals remained capable of lipid uptake (more so than op2/E0 mice). Though severely hypercholesterolemic, op0/E0 mice are protected against atherosclerosis and exhibit both fewer Mϕ and low MSR expression on remnant M-CSF independent Mϕ populations. The importance of M-CSF in maintaining cholesterol homeostasis and in atherogenesis is clearly illustrated by this model; the direct involvement of MSR however remains unproven.

A recent study used genetically engineered mice which overexpress hepatic MSR to investigate the functional role of these site-specific receptors in clearing potentially atherogenic lipoproteins (Wolle *et al.* 1995). The mouse transferrin promoter was used to target expression of the bovine MSR type I to the murine liver. Overexpression of hepatic MSR enhanced cholesterol flux and transgenic MSR mice on an atherogenic diet accumulated less apoB containing lipoprotein cholesterol and secreted more biliary cholesterol as bile acids. This increased removal of modified lipoproteins would be consistent with a protective anti-atherogenic role for MSR.

The explanation for the seeming paradox in the atherogenic effects of low and high MSR expression may be the tissue location of expression and resulting cholesterol flux. In addition, the balance or competition that most likely exists *in vivo* between arterial subendothelial MSR and liver Kupffer and sinusoidal endothelial SR for removal of modified lipoproteins may prove of critical importance. Thus, when hepatic MSR is overexpressed, modified lipoproteins would be less likely to bind MSR present on Mϕ in the aortic subendothelium (anti-atherogenic scenario). ApoE-deficient mice, in contrast, develop hypercholesterolemia due to decreased hepatic clearance of atherogenic lipoproteins, thereby increasing access of modified lipoproteins to subendothelial Mϕ; this shift in balance leads to increased MSR binding, enhanced arterial lipid deposition and foam cell formation (pro-atherogenic scenario). In op0/E0 mice hepatic uptake and clearance of atherogenic lipoproteins is inefficient due to reduced Kupffer cell numbers and MSR, resulting in severe hypercholesterolemia. The M-CSF dependent reduction in vascular tree Mϕ numbers (subendothelial Mϕ and myocardial interstital Mϕ) and MSR expression is of such an extent that arterial modified lipoprotein uptake is limited and severe atherosclerosis fails to develop.

This scenario would be consistent with several studies that have demonstrated M-CSF-induced decreases in plasma cholesterol in normal rabbits and hypercholesterolemic LDL-receptor-defective Watanabe heritable hyperlipidemic (WHHL) rabbits, non-human primates, normo- and hypercholesterolemic humans, as well as patients with homozygous familial hypercholesterolemia (FH) (Motoyoshi and Takaku 1989; Stoudemire and Garnick 1991; Schaub *et al.* 1994; Sherman *et al.* 1995). M-CSF also decreased foam cell development in Mϕ-rich carrageenan-induced granulomas in WHHL rabbits and decreased progression and enhanced regression of atherosclerotic lesions in WHHL and cholesterol-fed New Zealand White (NZW) rabbits (Inoue *et al.* 1992; Schaub *et al.* 1994; Schaub *et al.* 1995). The cholesterol-lowering

effects of M-CSF have been ascribed to enhanced biliary cholesterol excretion (Schaub *et al.* 1995), the upregulation of apoE secretion by Mϕ (Clinton *et al.* 1992), suggesting a protective role by enhancing Mϕ cholesterol efflux and reverse cholesterol transport, and increased expression of additional receptors important in cholesterol clearance, such as the apoE-binding protein, LDL-receptor-related protein (LRP), in Mϕ (Hussaini *et al.* 1990; Watanabe *et al.* 1994).

The question whether monocyte-derived Mϕ (and MSR) are required for atherogenesis (although suggestive) could not be answered definitively, because op0/E0 mice still have substantial remaining M-CSF independent Mϕ sub-populations which express low levels of MSR. GM-CSF also stimulates the production and function of Mϕ (Caroccilo *et al.* 1987) and might be expected to maintain the M-CSF independent Mϕ populations in op0 mice. A recent study showed that mice lacking both M-CSF and GM-CSF have coexistent osteopetrosis and severe lung disease (alveolar proteinosis) (Lieschke *et al.* 1994). These double mutant mice still have circulating monocytes and phagocytically active Mϕ at levels comparable to op0 animals; this would indicate that, in addition to M-CSF and GM-CSF, other factors influence Mϕ production and function *in vivo*. A further cross of these animals with E0 mice to study the Mϕ role in atherogenesis would not therefore be expected to be informative (apart from technical difficulties due to their reduced survival). Another approach may be to administer M-CSF to op2/E0 op0/E0 mice fed an atherogenic diet to determine the progression or regression of atherosclerotic plaques.

Although the majority of apoE in plasma is of hepatic origin, apoE is synthesized by a variety of cell types, including Mϕ (Basu *et al.* 1981; Basu *et al.* 1982). Indirect evidence for the important role played by Mϕ in atherogenesis was provided by the recent demonstration that atherosclerosis in irradiated apoE-deficient mice can be prevented by bone marrow transplantation from mice with the normal apoE gene; implying that apoE synthesized by bone marrow-derived Mϕ (which contribute ≈10% of total apoE) corrected the hyperlipidemia and atherosclerosis caused by apoE deficiency (Boisvert *et al.* 1995; Linton *et al.* 1995).

MSR-deficient mice may prove a valuable research tool in determining the exact contribution of the MSR to atherogenesis. These mice have been genetically engineered by Kodama's group in Japan and a breeding colony has been established at the Dunn School of Pathology. Possible approaches to answer the question of MSR's pro- or anti-atherogenic role may include assessing the development of atherosclerotic lesions in i) 129 MSR-deficient mice bred on a

atherosclerosis-susceptible C57BL/6 background and fed a high-fat Western-type diet and ii) doubly mutant apoE- and MSR-deficient mice subjected to normal and high-fat diets. These studies are currently in progress.

Chapter Nine

MSR Cell Biology: Morphological Studies of Expression

9.1 Introduction

In animal cells receptor-mediated endocytosis via clathrin-coated pits and vesicles provides an efficient pathway for taking up specific macromolecules from the extracellular fluid (Brown and Goldstein 1986). The early endosomal compartment acts as a main sorting station in the endocytic pathway. The fates of the various receptor proteins (and their ligands) vary according to their specific types and four possible pathways have been described: i) the LDL receptor dissociates from its ligand in the early endosome and recycles to the cell surface, whilst the ligand is destined for lysosomal degradation; ii) the transferrin receptor remains bound to transferrin and recycles back to the plasma membrane as a receptor-transferrin complex; iii) epidermal growth factor (EGF) does not recycle but ends up in the lysosome and is degraded with its ligand and iv) in the process of transcytosis, receptors on polarized epithelial cell surfaces transfer specific macromolecules (e.g. maternal IgG) to a different domain of the plasma membrane.

The unregulated intracellular lipid deposition in Mϕ that results in foam cell formation is a pivotal process in atherogenesis; and MSR-mediated endocytosis of modified lipoproteins is a major pathway that may modulate this process (Krieger *et al.* 1993). The MSR pathway (fig. 9.1) resembles the type described for the LDL receptor above; and includes initial synthesis and processing as an integral membrane protein in the endoplasmic reticulum (ER). Further post-translational modification such as N-linked glycosylation (spacer and α-helical coiled-coil domains), hydroxylation of prolines and lysines (collagenous domain) and trimerization occurs in the ER (Krieger and Herz 1994). Trimers are subsequently transported to the Golgi apparatus and *trans*-Golgi network (TGN) for additional processing and sorting to the cell surface. Receptor-mediated endocytosis of its multiple ligands follows high-affinity receptor-ligand binding, and invagination of cell-surface coated pits containing the receptor-ligand complex results in the formation of coated endocytic vesicles. These are converted to endosomes where the low pH in the endosomal lumen induces

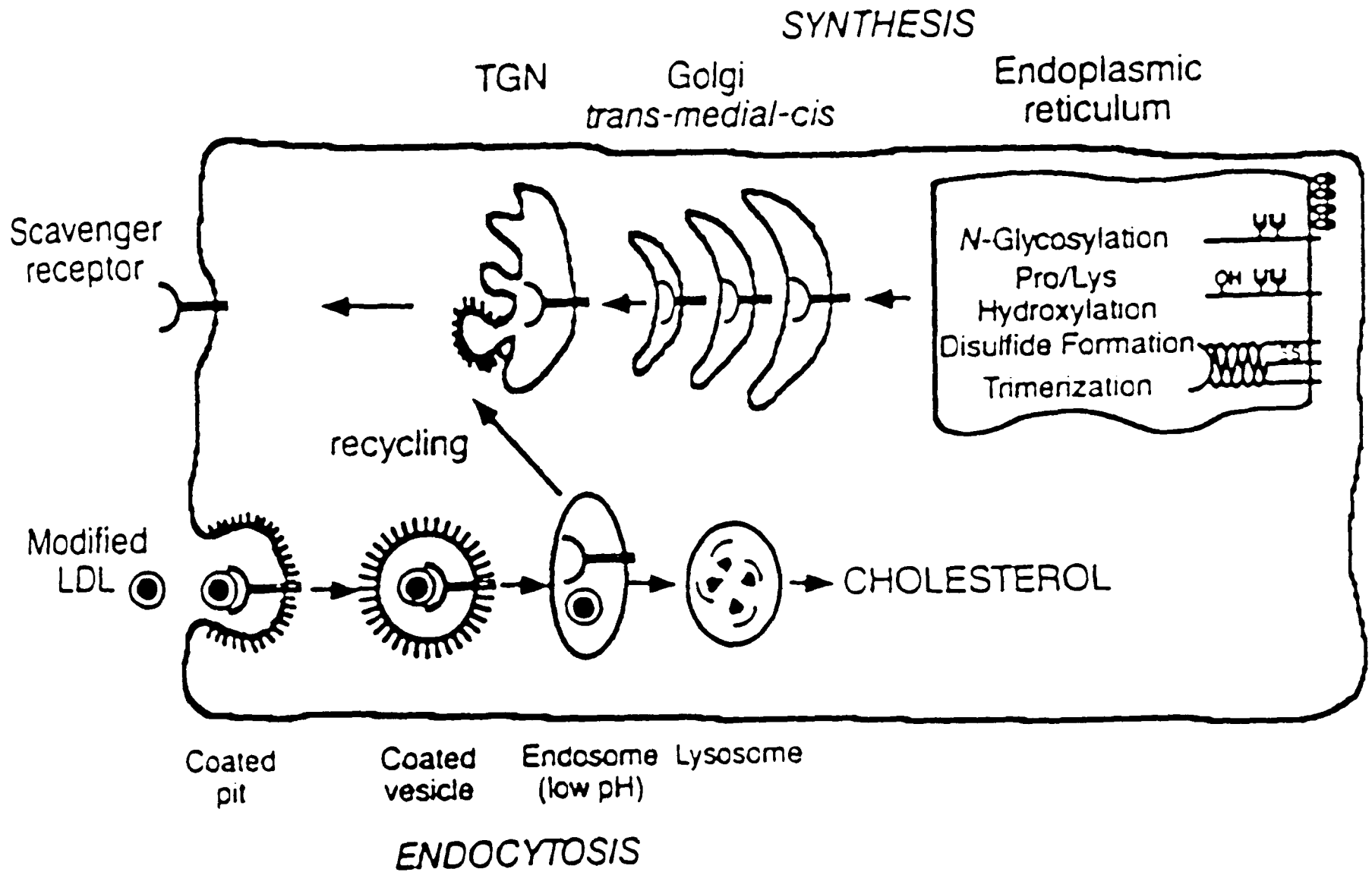


Fig. 9.1 **Diagrammatic representation of the MSR cellular pathway.**

The steps in the pathway are discussed in the text. (adapted from Krieger, 1992).

receptor-ligand dissociation, with consequent recycling of the receptor to the cell surface through the *trans*-Golgi apparatus and lysosomal digestion of the ligand (Mori *et al.* 1994). Endocytosis of modified lipoprotein results in cholesterol release and conversion to cholesteryl ester droplets in the cytoplasm (foam cell formation).

Domains and amino acid residues of critical importance for the endocytic function of bovine MSR have recently been identified. The ligand-binding domain for AcLDL was determined by using truncated and site-directed mutants of the protein (Doi *et al.* 1993). The collagen-like domain was characterized as being important in the binding of modified lipoproteins, and Lys337 was critical for mediating this interaction. The functional importance of the α-helical coiled-coil region was defined by substituting two histidines (His168 and His260) by site-directed mutagenesis. Substitution had no effect on MSR binding of AcLDL, but intracellular degradation of the AcLDL protein moiety was greatly diminished after uptake by His260-modified receptors due to an inability of the ligand to dissociate from the receptor at acidic pH (Doi *et al.* 1994). Immunoelectron microscopy showed the cells containing the His260 mutant MSR were unable to release gold-labeled AcLDL in endosomes. This suggests that protonation of His260 in the acidic endosomal compartment results in allosterical ligand dissociation from the collagenous domain.

In addition to endocytosis, MSR also plays a role in Mϕ adhesion (cell-cell or cell-extracellular matrix) (Fraser *et al.* 1993; El Khoury *et al.* 1994). To understand the underlying molecular basis for the dual functional nature of MSR, a series of murine MSR truncation mutants stably transfected into CHO cells was characterized and the results described in Chapter 7. The epitope recognized by mAb 2F8, which in view of its adhesion inhibitory activity may delineate a functional MSR adhesion domain, is located in the α-helical coiled-coil domain between amino acids 183 and 197. Epitope mapping however, does not necessarily identify this site as the adhesion-promoting region in MSR; as the adhesion inhibitory quality of mAb 2F8 is complex and may require specific conformational features in serum adhesion ligand(s) or MSR domains, as well as the co-ordinate or combinatorial involvement of other membrane molecules.

To characterize the adhesion function of MSR further, studies on the morphology of MSR expression and distribution were performed on BgPMϕ and MSR transfected CHO cells adhered to TCP under various culture conditions. M-CSF treated BgPMϕ and CHO MSR type II transfectants grown in MAC medium were of special interest due to the high levels of MSR expressed in these cells. To characterize the

adherent cell surface, imaging techniques that provide better resolution than conventional light microscopy were required. The techniques used included confocal laser scanning microscopy and electron microscopy (including immunogold conjugates); and the collaboration of Dr. M. Hollinshead is gratefully acknowledged in these experiments.

9.2 The morphology of MSR-mediated adhesion in M-CSF treated BgPMϕ

Bio-Gel-elicited Mϕ were adhered on glass cover slips and cultured in RPMI 1640 plus 10% FCS and recombinant human M-CSF (1000 units/ml) for 48 hours. The cells were fixed with 4% PFA in PBS for 10 min, and 8% PFA for 50 min at room temperature. Indirect immunofluorescence (with rhodamine-conjugated goat anti-rat $F(ab')_2$ fragments as secondary antibody) was performed using confocal laser scanning microscopy with special attention to the adhesive cell surface immediately adjacent to the glass. Surface staining (fig. 2A) revealed a prominent rim of mAb 2F8-positive fluorescence with central clear areas. After Triton-X 100 permeabilization, substantial MSR-specific fluorescence with a fairly homogeneous fenestrated appearance at the Mϕ base (contact area) was uncovered (fig. 2B). This MSR-positive "structure" revealed by permeabilization was a consistent morphological finding (fig. 3A). Untreated BgPMϕ did not show such prominent basal MSR fluorescence (fig. 3B), suggesting that its appearance is either dependent on M-CSF effects on Mϕ spreading or on M-CSF mediated upregulation of MSR cellular expression.

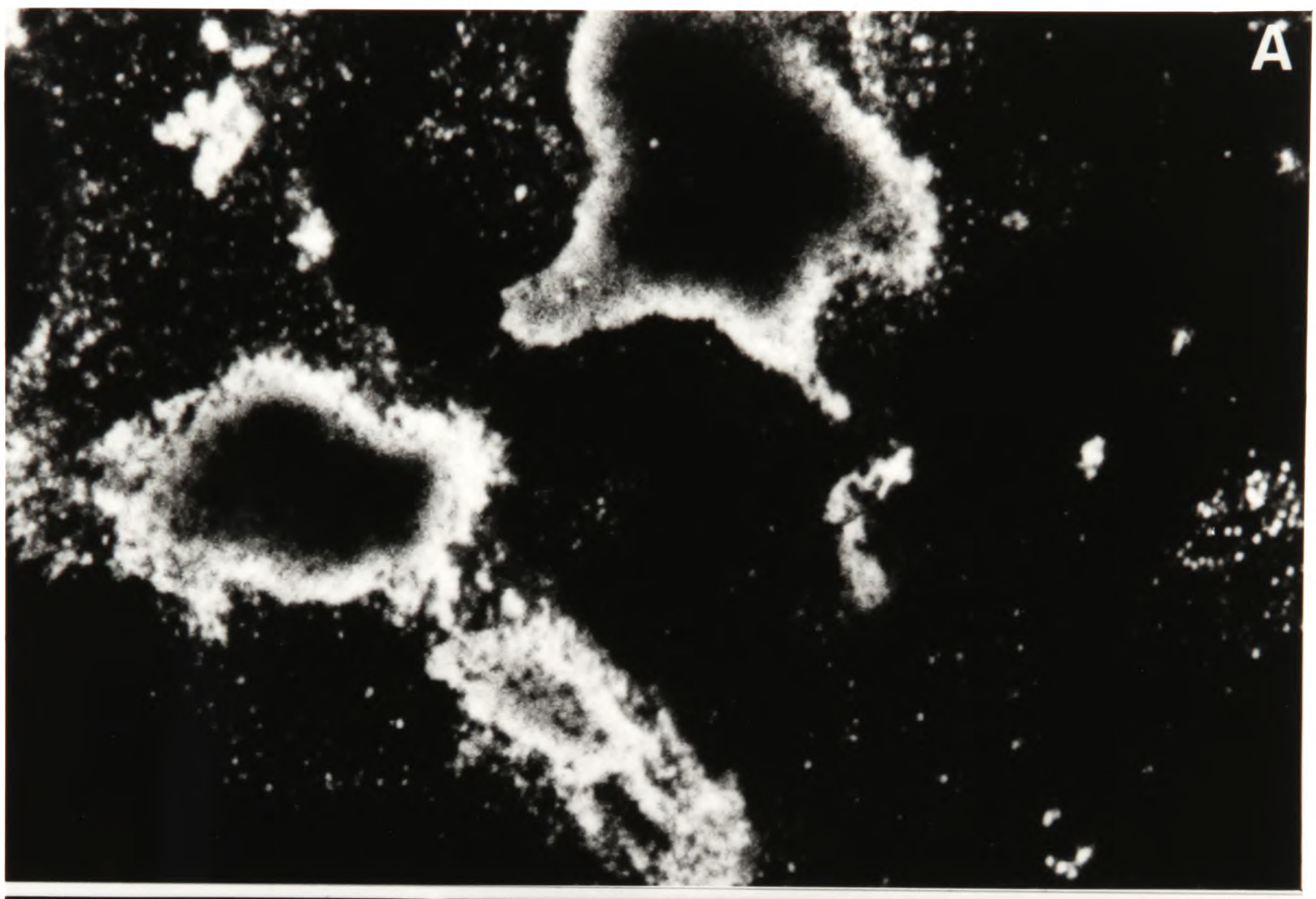
The appearance was reminiscent of studies which showed that phagocytosing Mϕ (Fc receptors) exclude proteins from zones of contact with IgG opsonized targets ("black hole" experiments) (Wright and Silverstein 1984). Mϕ form a protein-tight seal at the periphery of their contact with ligand-coated surfaces creating a closed compartment between cell and target and HRP is excluded from this zone. This closed compartment could conceivably become available for MSR fluorescence subsequent to permeabilization. The origin of the bright MSR staining was however unclear; possibilities were i) artefactual collection of primary antibody in subcellular exclusion zone, ii) a substantial intracellular MSR pool adjacent to the adherent cell surface, or iii) directed redistribution and concentration of MSR in the plasma membrane towards contact zones, representing a polarization process. The first possibility seems unlikely, as identical MSR fluorescence patterns were apparent when mAb 2F8 was added to M-CSF treated BgPMϕ for 60 min prior to fixation, permeabilization and addition of

Fig. 9.2 **MSR immunofluorescence confocal laser scanning microscopy of M-CSF treated BgPMϕ**

BgPMϕ were adhered on glass cover slips and cultured in RPMI 1640 plus 10% FCS and recombinant human M-CSF (1000 units/ml) for 48 hours. The cells were fixed with 4% PFA in PBS for 10 min, and 8% PFA for 50 min at room temperature. Indirect immunofluorescence (with rhodamine-conjugated goat anti-rat $F(ab')_2$ fragments as secondary antibody) was performed with mAb 2F8 (10 µg/ml) in the presence or absence of Triton-X 100 (permeabilization). CLSM was performed as described in chapter 2.14 with special attention to the adhesive cell surface immediately adjacent to the glass. 150x

A. Surface. Note the prominent rim of mAb 2F8-positive fluorescence with central clear areas.

B. Permeabilized. Substantial MSR-specific fluorescence with a homogeneous fenestrated appearance becomes apparent.


A

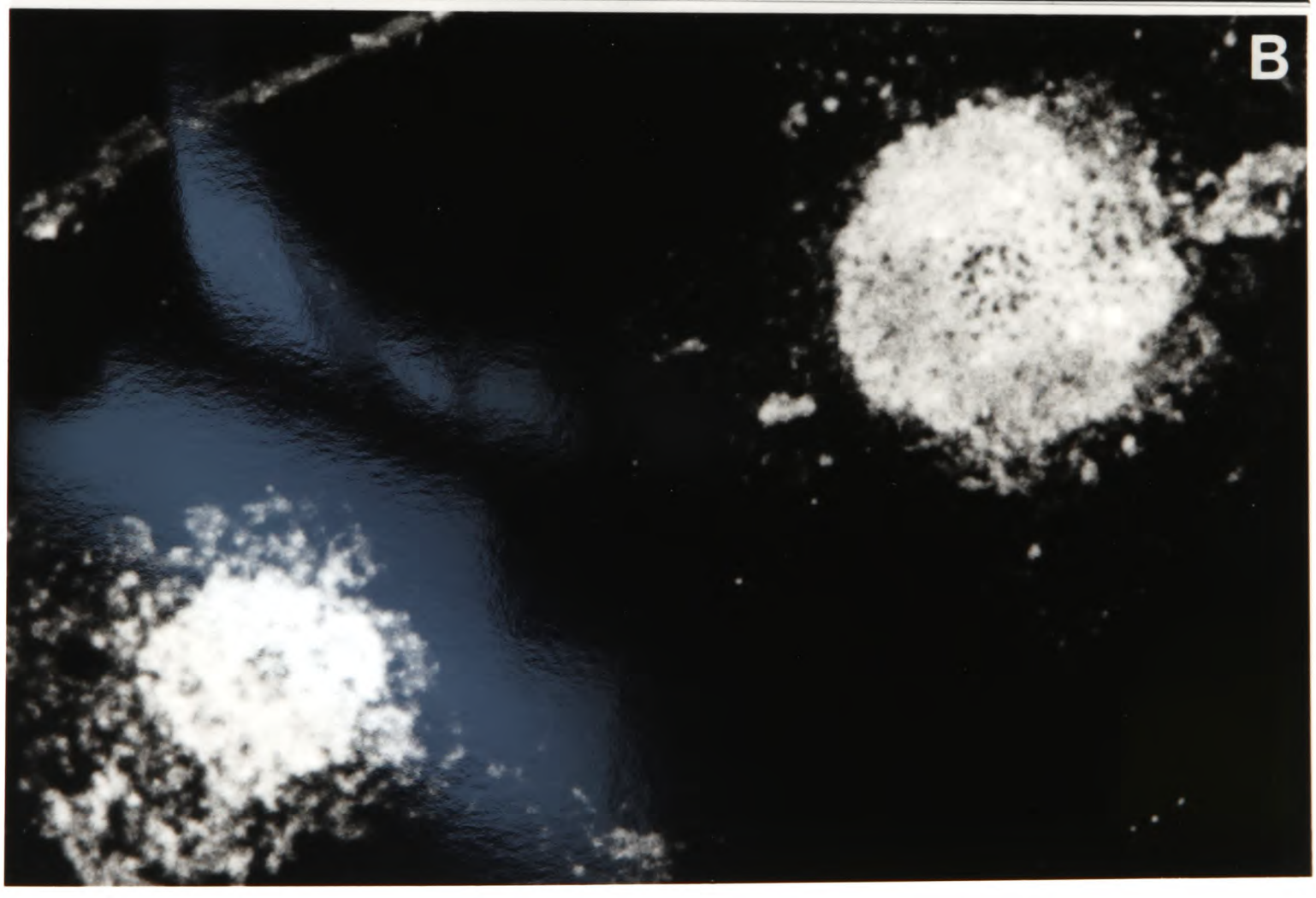
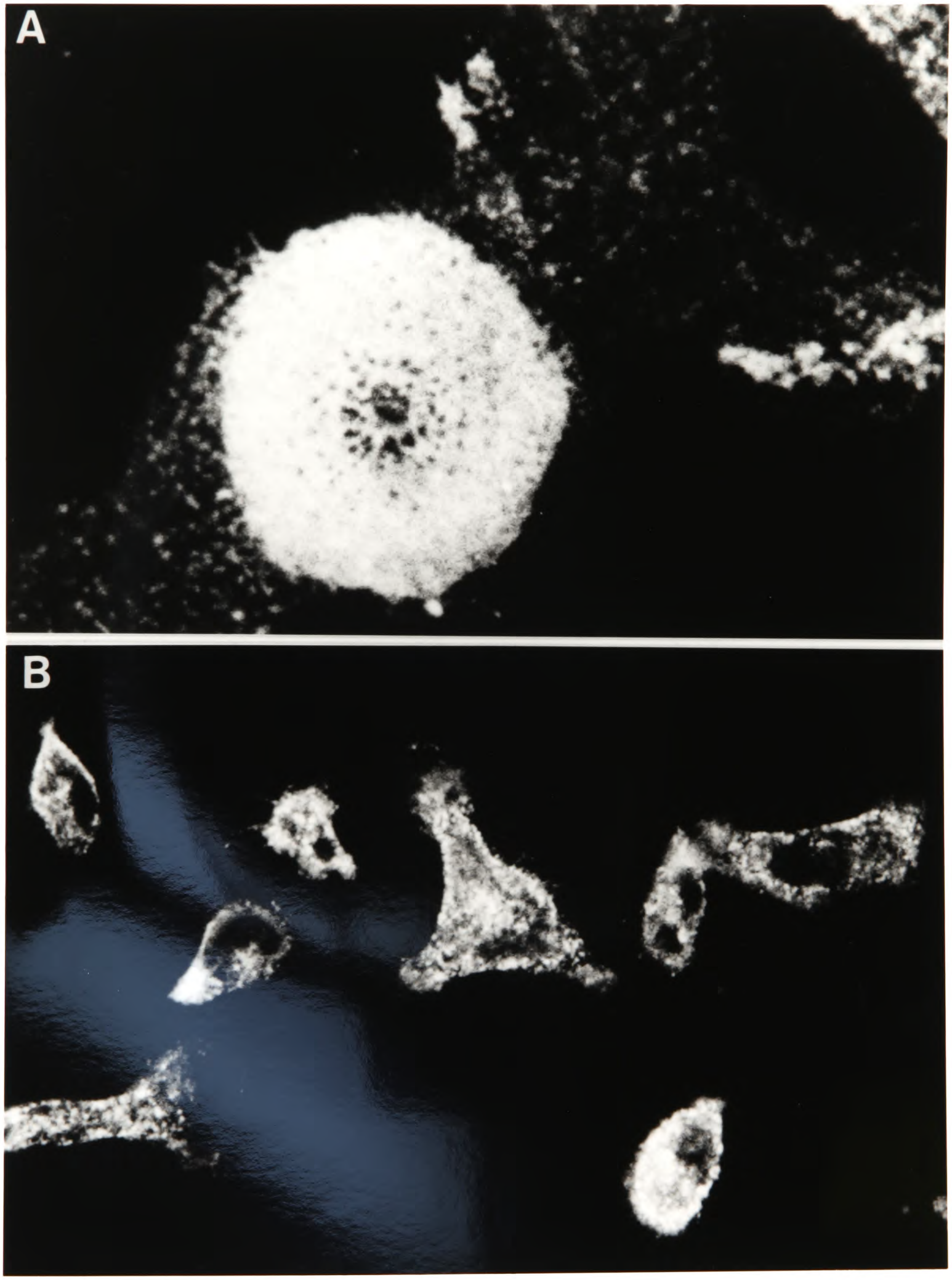

B

Fig. 9.3 **MSR immunofluorescence confocal laser scanning microscopy of control and M-CSF treated BgPMϕ**

BgPMϕ were cultured and processed (permeabilized) as in fig. 9.2.

A. M-CSF treated BgPMϕ. The MSR-positive "structure" revealed by permeabilization is shown to be a a consistent morphological finding. 150x

B. Untreated BgPMϕ. In the same optical plane, at the adhesive surface, there is no evidence of basal accumulation of MSR-positive fluorescence. 60x


A
B

secondary antibody (not shown).

The phenomenon however appeared peculiar to MSR, as staining for other Mϕ-specific membrane markers, such as CR3, F4/80 antigen (not shown) and macrosialin in M-CSF-treated BgPMϕ did not show a similar fluorescence pattern by confocal microscopy (fig. 9.4A and B). Macrosialin is a Mϕ-specific late endosomal membrane glycoprotein and localized to the perinuclear region. Secondary antibody alone, and the isotype-matched irrelevant antibody CAMPATH-1G acted as negative controls and showed no fluorescence (not shown). The distribution of other Mϕ non-specific molecules, such as the transferrin receptor and a marker for a 38K protein of the *trans*-Golgi network (TGN 38 antibody), were as expected and remained unaffected by M-CSF treatment (fig. 9.4C and D). Interestingly, the MSR-specific staining pattern was absent when M-CSF treated BgPMϕ were cultured in a proprietary serum-free medium (OptiMEM), suggesting a serum-dependent process (not shown). In a time course study, the phenomenon could only be detected after 24 hours, and not 12 hours, of M-CSF treatment in serum-containing medium. This may indicate the period required for upregulation of MSR expression and redistribution to the surface to be sufficiently detectable.

Computer-assisted modification of confocal microscopy images enabled the distribution of CR3 and MSR molecules in adherent M-CSF treated BgPMϕ to be studied in vertical sections (fig. 9.5). Both molecules have dual endocytic and adhesive functional roles; but their cellular distribution *in vitro* differed markedly. CR3 was predominantly localized on the non-adhesive Mϕ surface, whilst a substantial proportion of MSR was present at the adhesive interface and had a fenestrated appearance. To assess the effect of an endocytic stimulus on MSR distribution, M-CSF treated BgPMϕ were exposed to 4 μg/ml DiI-AcLDL for 3 hours (fig. 9.6). DiI-AcLDL uptake was prominent and showed a speckled appearance in the endosomal/lysosomal perinuclear region, as was readily apparent from a vertical section. Double staining of MSR showed no redistributive effect from the cell base as a result of AcLDL endocytosis (not shown); implying that the capacity of the receptor to fulfil both functional roles was not saturated. Interestingly, AcLDL ligand fluorescence was widely distributed intracellularly, and the adhesive region was not excluded. It may thus be argued that MSR molecules at the contact zone are not exclusively involved in adhesion, but, when unoccupied, may also stochastically bind and endocytose ligand.

MSR-deficient mice had recently been obtained from Dr. T. Kodama (Tokyo, Japan) and a breeding colony established at the Dunn

Fig. 9.4 **Immunofluorescence confocal laser scanning microscopy of M-CSF treated BgPMϕ to assess the distribution of other antigens**

BgPMϕ were cultured and processed (permeabilized) as in fig. 9.2 and the basal, adhesive surface of the cell examined by CLSM. The prominent basal fluorescence detected with mAb 2F8 appears specific to MSR.

A. CR3 (mAb 5C6). 60x

B. Macrosialin (mAb FA-11). Note the typical late endosomal perinuclear distribution pattern of this Mϕ-specific membrane molecule. 60x

C. Transferrin receptor. 120x

D. 38K protein of the *trans*-Golgi network. 120x

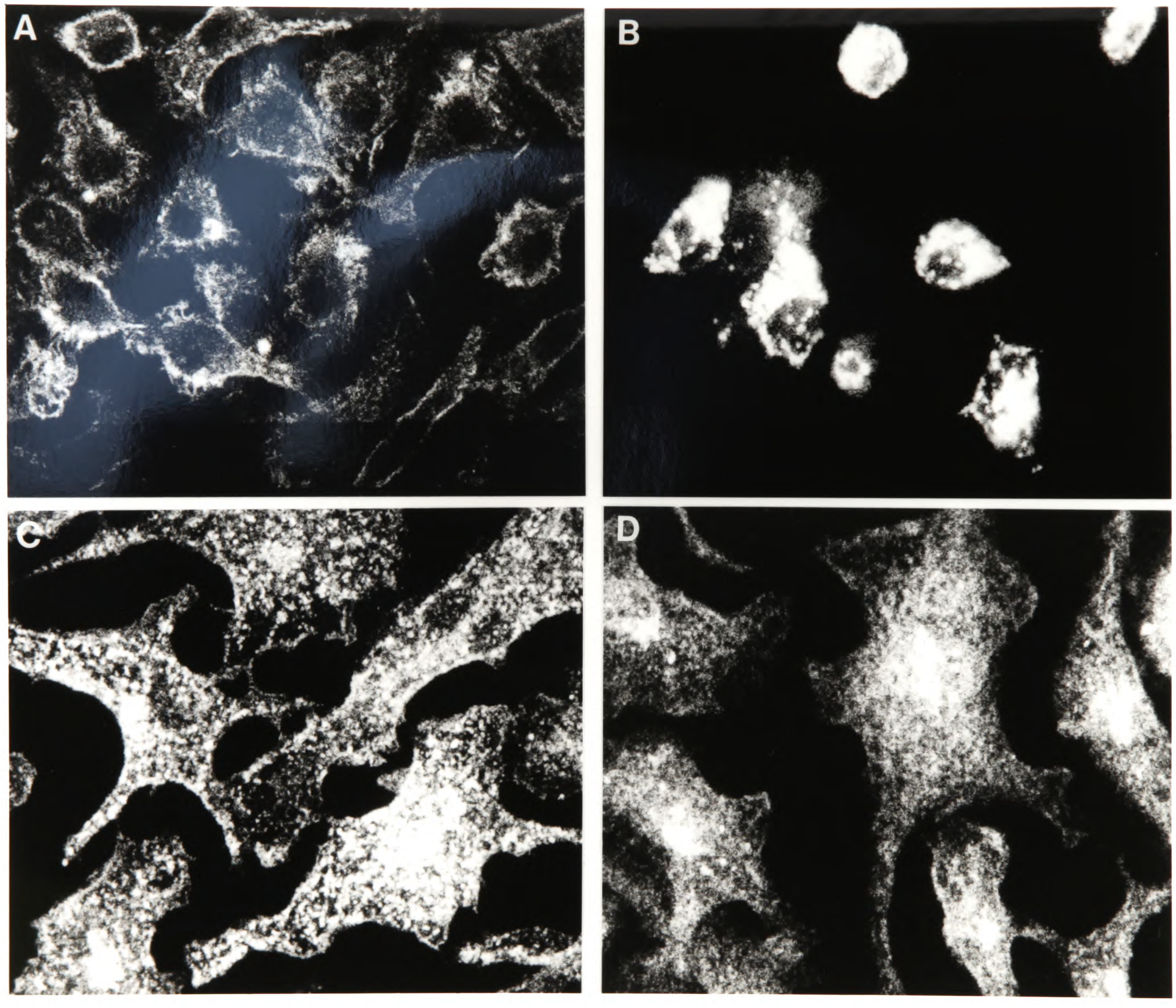
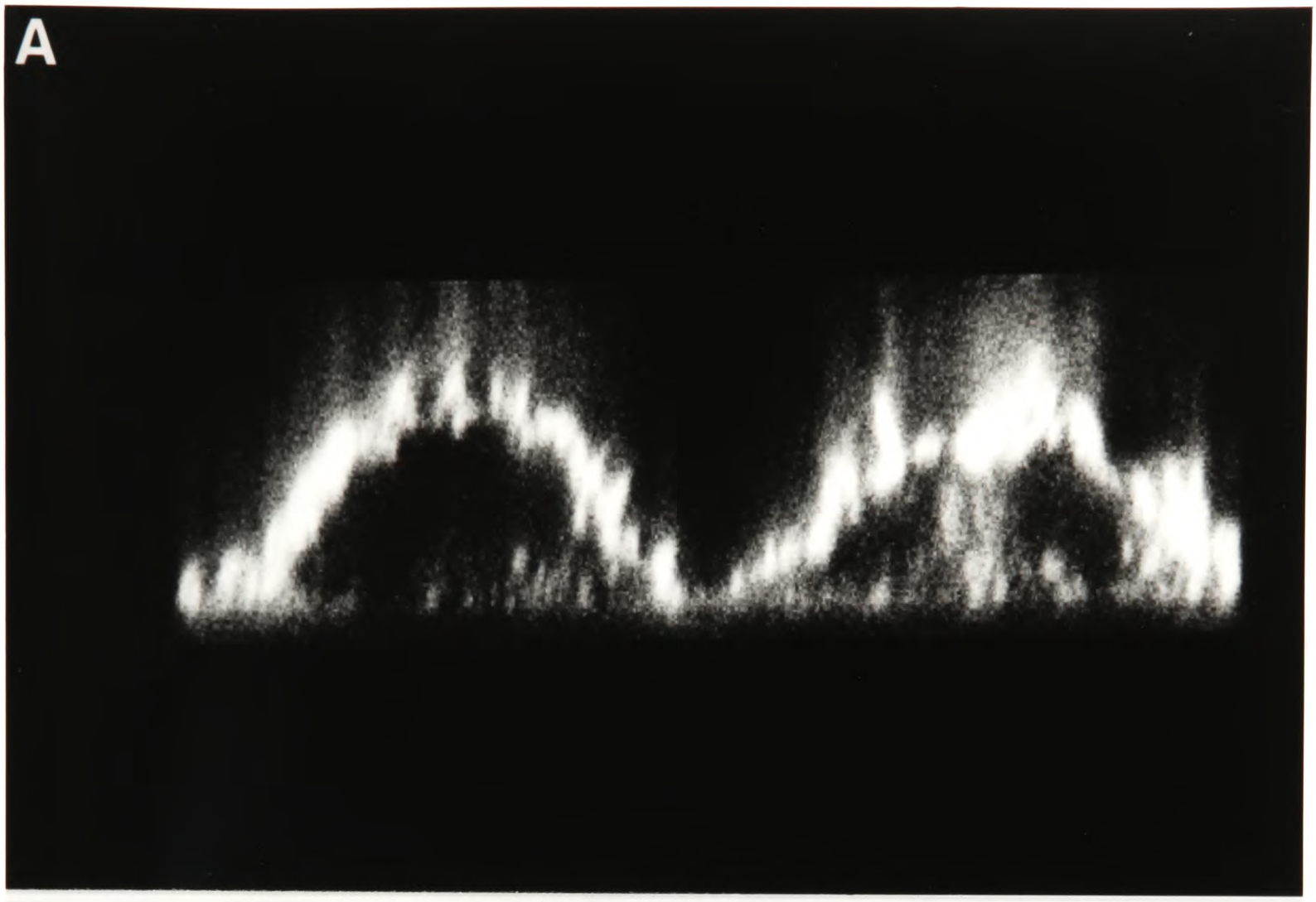

A
B
C
D

Fig. 9.5 **Immunofluorescence confocal laser scanning microscopy of M-CSF treated BgPMϕ to assess the distribution of MSR and CR3 in vertical sections**

BgPMϕ were cultured and processed (permeabilized) as in fig. 9.2 and the basal, adhesive surface of the cell examined by CLSM. Computer-assisted modification of confocal microscopy images enabled the distribution of CR3 **(A)** (150x) and MSR **(B)** (120x) molecules in adherent M-CSF treated BgPMϕ to be studied in vertical sections. Fluorescence scattering seen in fig. 9.5A is due to technical reasons, so-called "point spread function". Both molecules have dual endocytic and adhesive functional roles; but their cellular distribution *in vitro* differed markedly. CR3 was predominantly localized on the non-adhesive Mϕ surface, whilst a substantial proportion of MSR was present at the adhesive interface and had a fenestrated appearance.


A

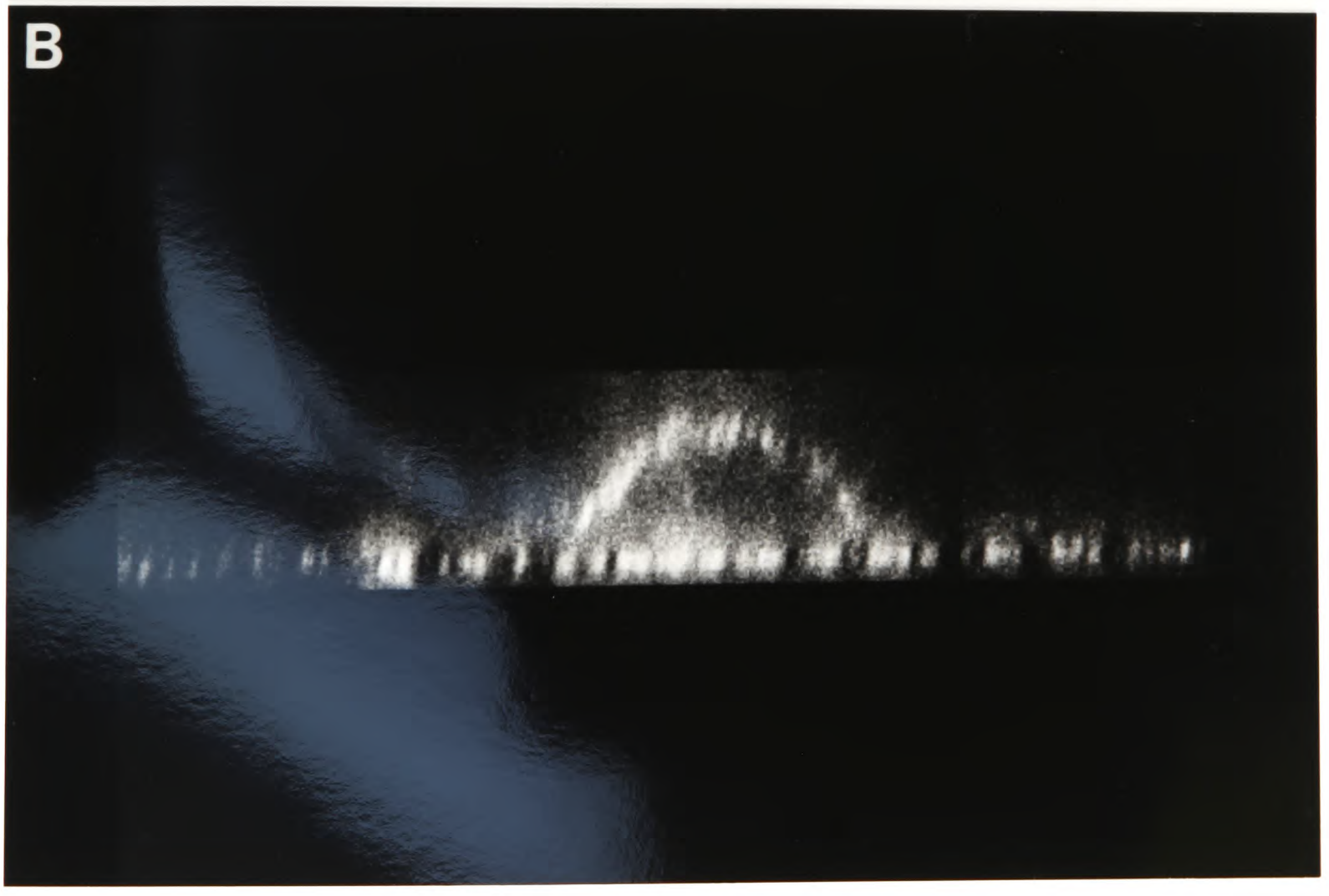
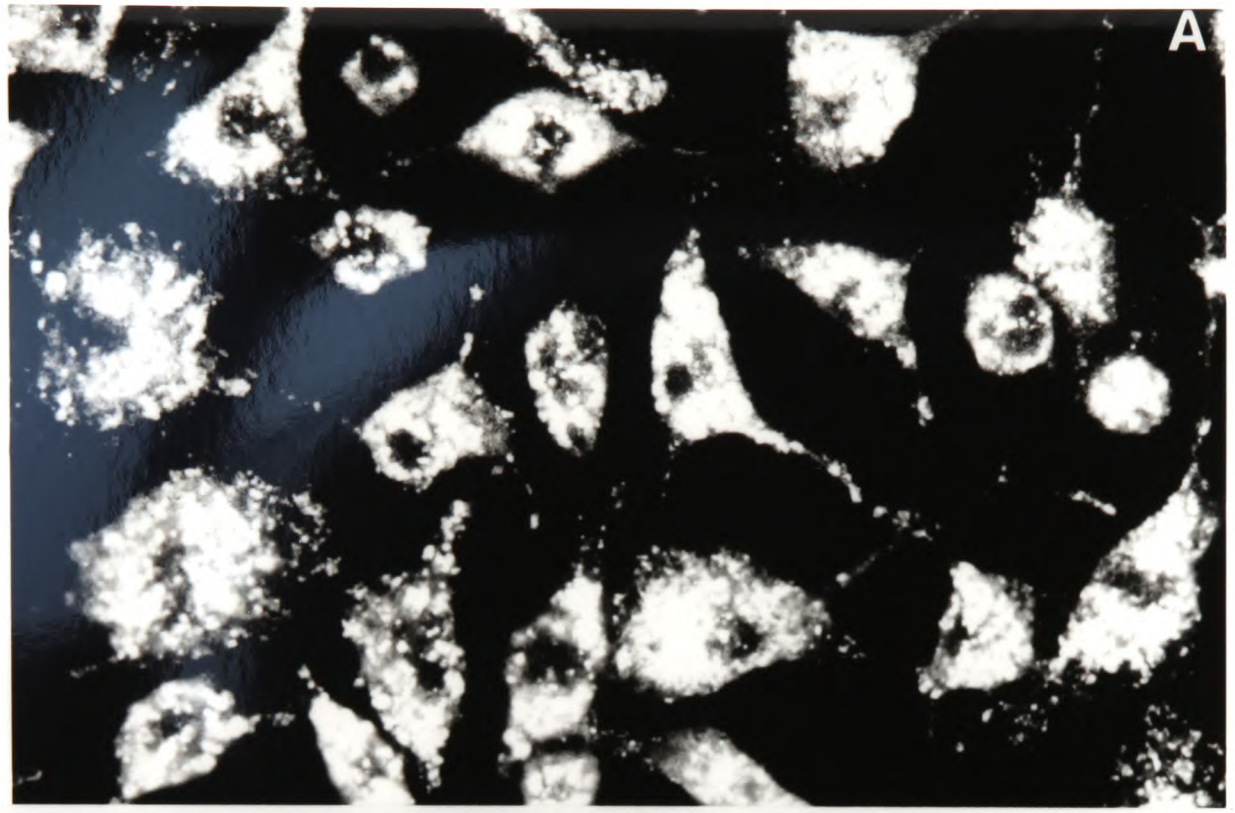

B

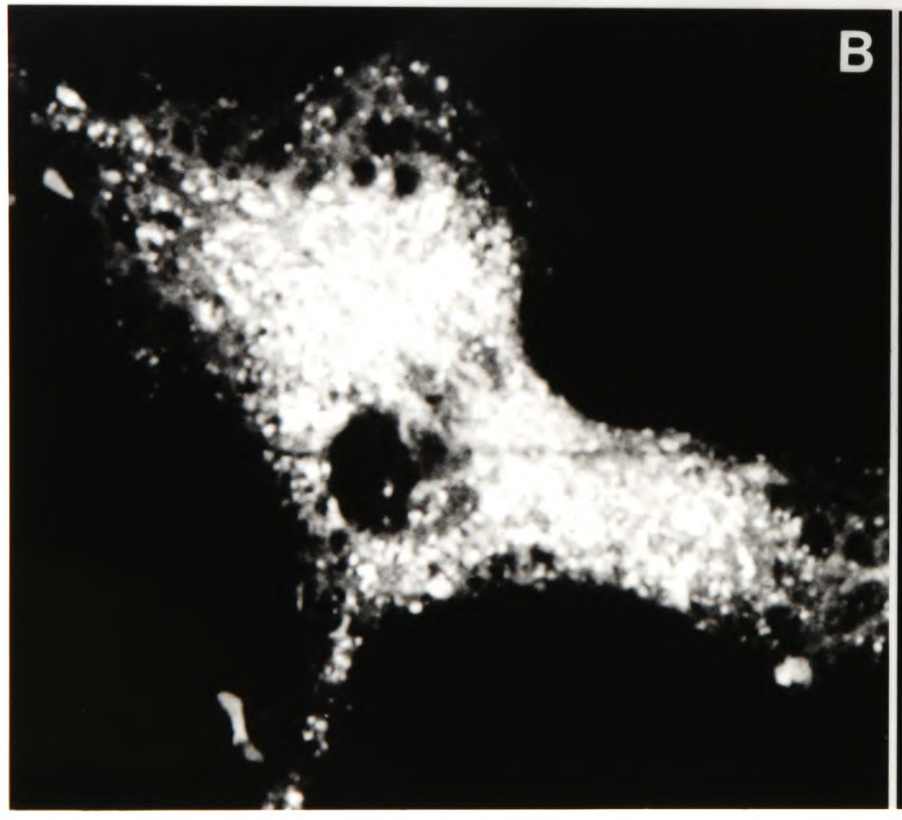
Fig. 9.6 **The cellular distribution of DiI-AcLDL uptake in M-CSF treated BgPMϕ**

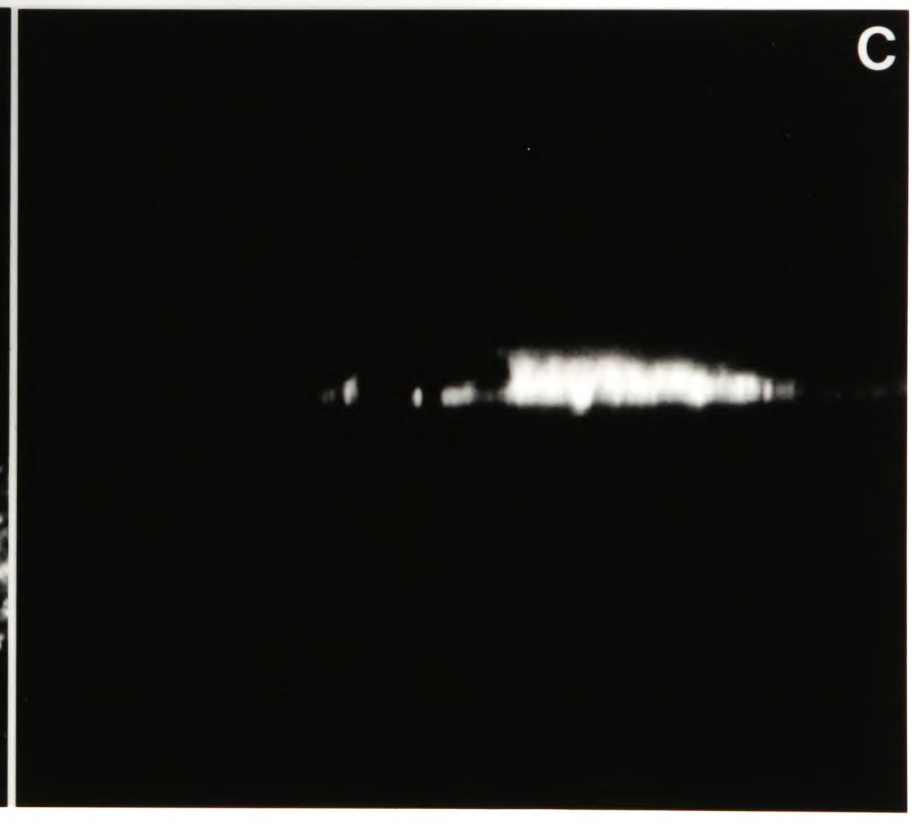
BgPMϕ were cultured and processed (permeabilized) as described and exposed to 4 μg/ml DiI-AcLDL for 3 hours before washes and fixation. Ligand distribution was then examined by CLSM.

A. DiI-AcLDL uptake. Note the prominent AcLDL uptake by Mϕ concentrated in a speckled fashion in the endosomal/lysosomal perinuclear region. 60x

B and C. DiI-AcLDL uptake was examined in a single cell to determine distribution in a vertical section **(C.)**. The vertical section image was computed from fluorescence obtained from the cell in **(B)**; note the clear horizontal line in panel B through the nucleus; this represents photobleaching. 180x


A


B


C

School of Pathology. BgPMϕ from these animals were studied as to their adhesive ability *in vitro* and preliminary results are described (fig. 9.7). The MSR-deficient status of the animals was confirmed by the absence of mAb 2F8 precipitable MSR protein from metabolically labeled BgPMϕ lysates from two different MSR-deficient mice. MSR staining could also not be detected by indirect immunofluorescence microscopy. Interestingly, MSR-deficient BgPMϕ retained their spreading ability on TCP following M-CSF treatment; and this effect is therefore not a MSR-dependent phenomenon. This is not surprising as M-CSF has also been described to increase β_1 integrin expression in BMDMϕ and their calcium-dependent attachment to fibronectin (Shima *et al.* 1995).

Electron microscopy was subsequently done with 9nm immuno-gold conjugates to determine MSR distribution in M-CSF treated BgPMϕ (fig. 9.8). These studies were done on cells in a steady state, adherent to TCP, and did not include an added ligand stimulus or temperature shift. MSR was readily apparent on the cell membrane, and in coated pits, coated vesicles and tubular endosomal network adjacent to the Golgi apparatus. The findings were characteristic of a receptor thought to be involved in receptor-mediated endocytosis. AcLDL has been shown to be internalized through coated pits and to follow a lysosome-directed pathway through endosomes (Traber *et al.* 1983; Mommaas-Kienhuis *et al.* 1985; Henson *et al.* 1989). The MSR distribution in murine peritoneal Mϕ is consistent with a previous study, in which a mAb to bovine MSR was used to demonstrate the presence of MSR on the cell membrane and in coated pits of alveolar Mϕ (Naito *et al.* 1991; Mori *et al.* 1994). After a temperature shift to 37°C, MSR could be detected in endosomal vesicles and vacuoles and subsequent disappearance of MSR immunoreactivity from lysosomes suggested a receptor recycling pathway.

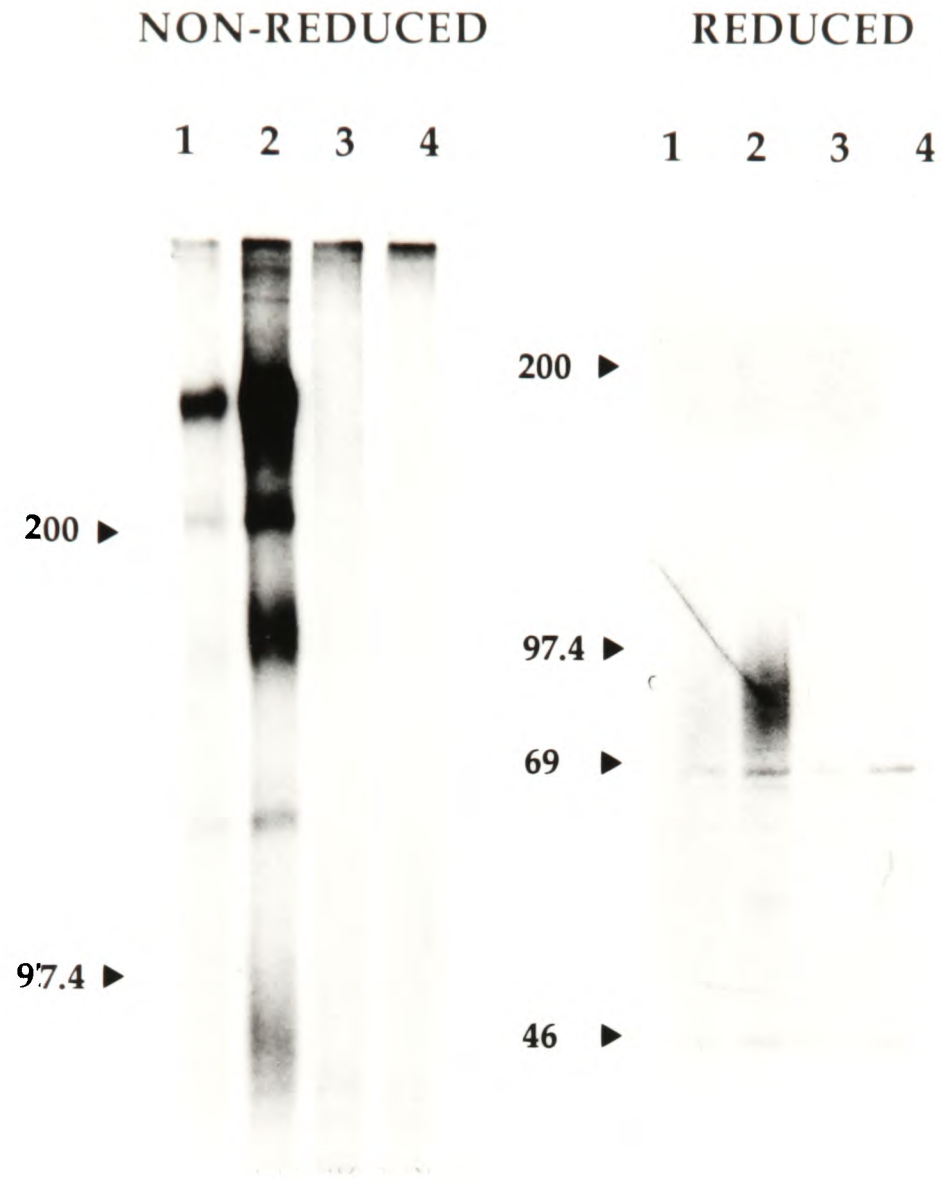
Further electron microscopy (without MSR immuno-gold labeling) to examine the Mϕ adhesive surface revealed an elaborate fenestrated cisternal network immediately adjacent to the plasma membrane (fig 9.9). Mϕ are known to reorganize their cellular membranes rapidly during Fc receptor-mediated frustrated phagocytosis of immune complex-coated surfaces (Bainton *et al.* 1989). As they spread, anastomosing tubules and large, clear basal vacuoles form, which are occasionally contiguous with the adherent surface. It is unclear whether the phenomenon shown in fig. 9.9 is identical to that which occurs during frustrated phagocytosis; or whether it may be associated with the brightly fluorescent MSR-positive "structures" seen at the adhesive surface after Mϕ permeabilization. Immuno-gold labeling studies to address this question and to localize the MSR specifically at this interface are currently under way.

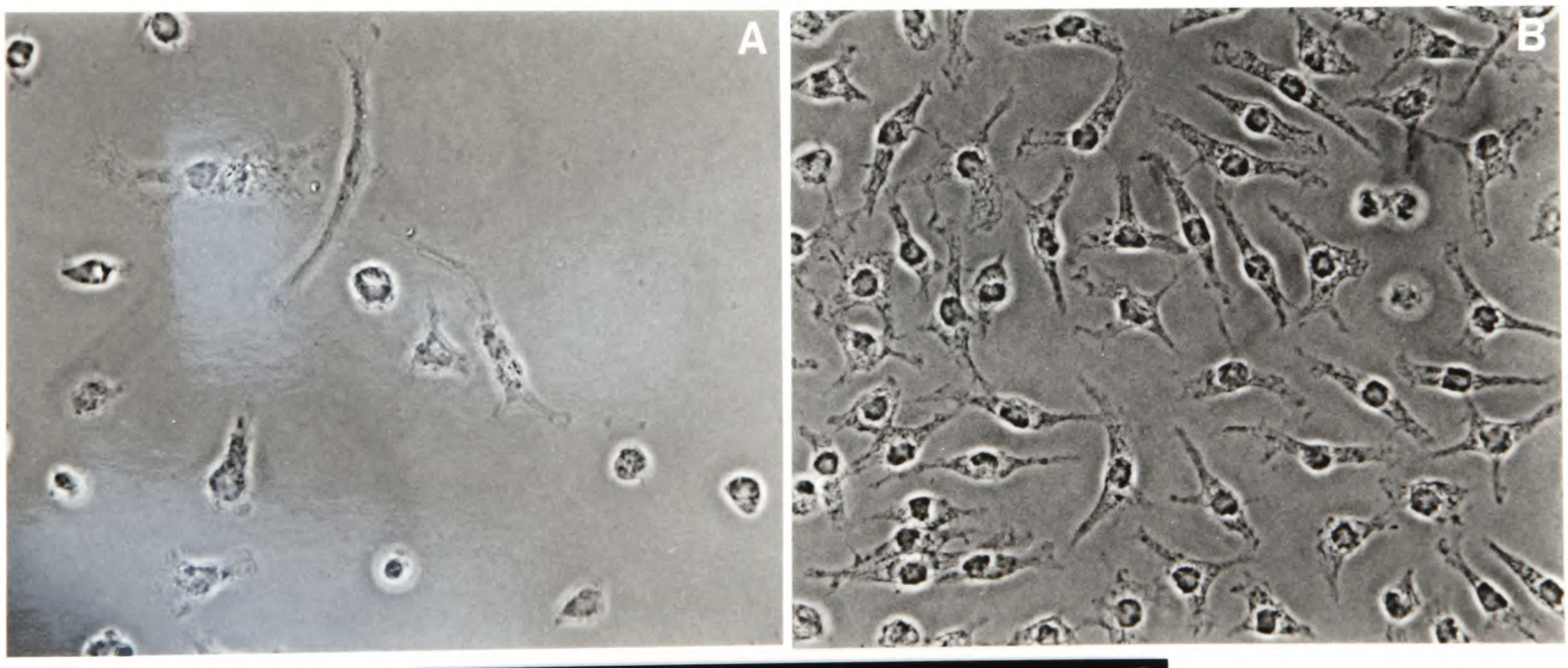
Fig. 9.7 MSR immunoprecipitation and immunofluorescence in BgPMϕ from MSR-deficient mice.

Day five BgPMϕ from MSR-deficient mice were harvested and treated *in vitro* with M-CSF (1000units/ml) for 48 hours.

Immunoprecipitation (top). Cells were then metabolically labelled by overnight incubation with 50 μCi/ml Tran^{35}S, lysed and subjected to immunoprecipitation with mAb 2F8 as described. Immunoprecipitates were separated by 5-10% SDS-PAGE (equivalent cell protein per lane) in the presence of β-mercaptoethanol, fixed, impregnated with EN^{3}HANCE, dried and exposed to film at -70°C. Note the absence of any immunoprecipitable reduced or non-reduced MSR in BgPMϕ from two different MSR-deficient mice (lanes 3 and 4).

Phase contrast and immunofluorescence microscopy (bottom). Cells were cultured in the presence **(B.)** or absence **(A.)** of M-CSF and examined by phase contrast microscopy. 60x. Note the increase in spreaded adherent cells after M-CSF treatment. Untreated MSR-deficient BgPMϕ were rounded and seemed to adhere poorly to TCP. Independent studies have confirmed MSR-deficient TPM lack any EDTA-resistant component (MSR contribution) to their adhesion to TCP (R. Haworth). No immunofluorescence could be detected by mAb 2F8 staining in MSR-deficient BgPMϕ **(C.)**.


NON-REDUCED
REDUCED
1 2 3 4
1 2 3 4
200 ▶
97.4 ▶
200 ▶
97.4 ▶
69 ▶
46 ▶


A
B

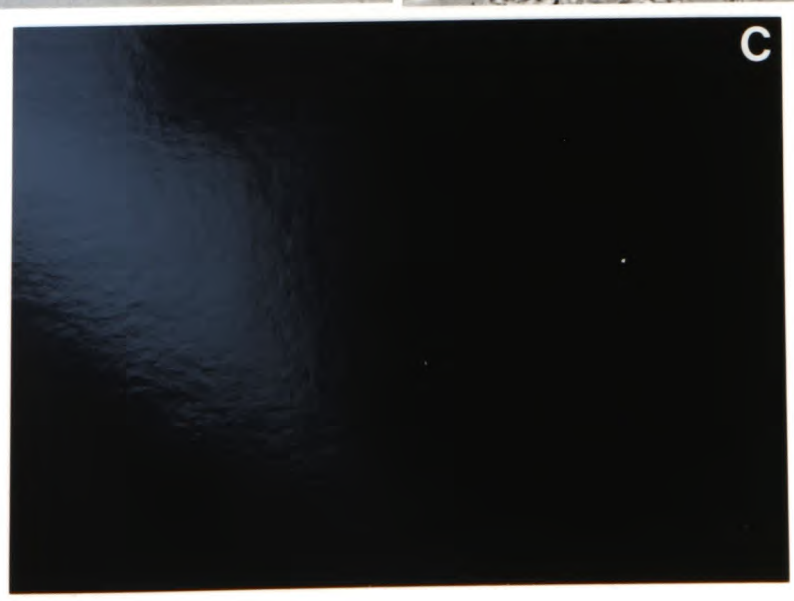
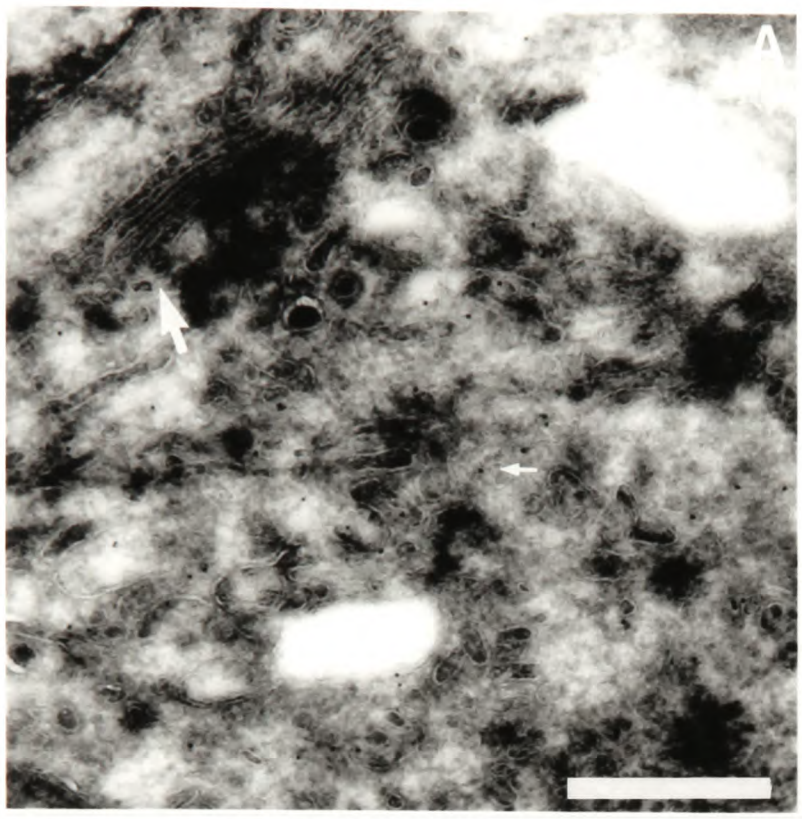
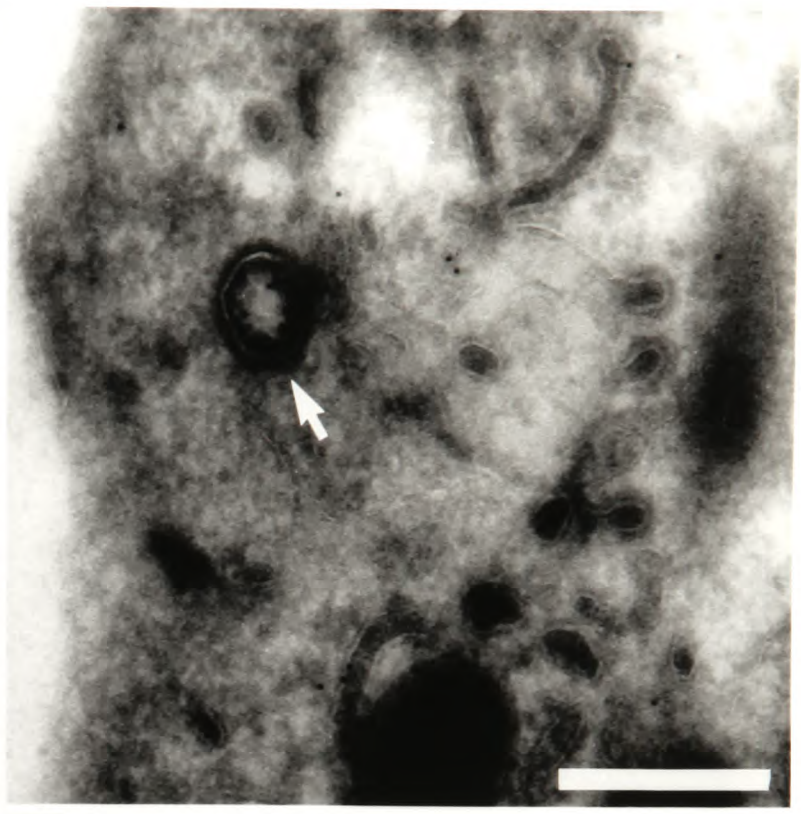
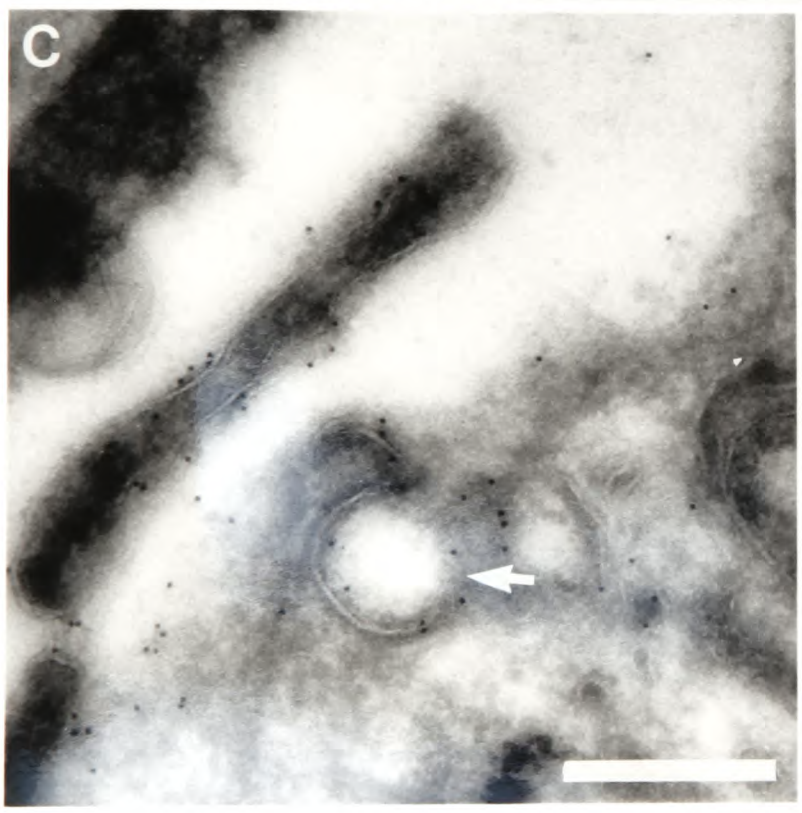

C

Fig. 9.8 **MSR immunogold electron microscopy of M-CSF treated BgPMϕ**

BgPMϕ were cultured with M-CSF (1000units/ml) for 48 hours and processed for electron microscopy as described in section 2.14, using 9 nM immunogold conjugates. Note the prominent presence of MSR on the cell surface, coated pits, coated vesicles (large arrows in **B.** and **C.**) and tubular endosomal network (small arrow) next to the Golgi stack (large arrow) in **A.** Bars: A, B, and C, 200nM; D, 100nM.


A




C

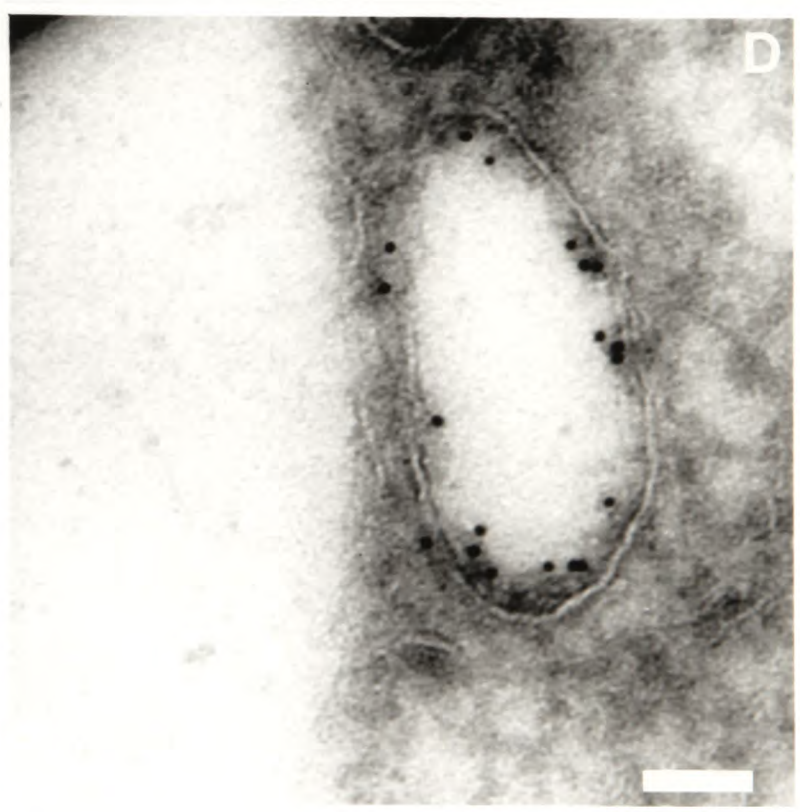
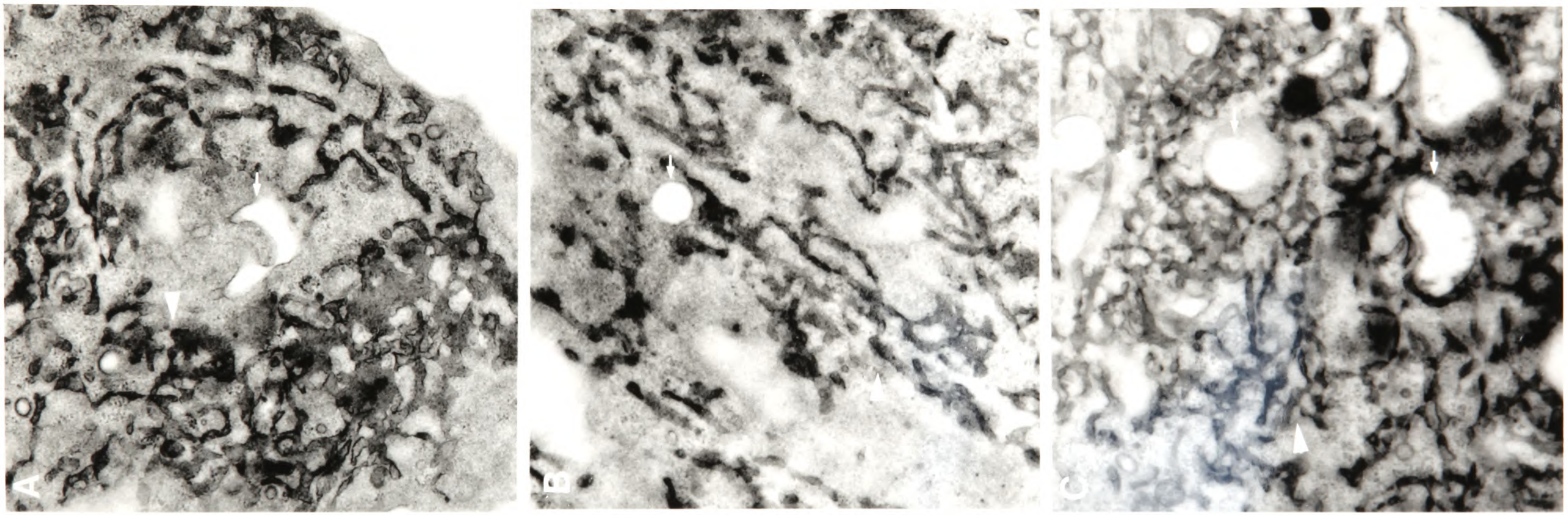

D

Fig. 9.9 **Ultrastructure of M-CSF treated BgPMϕ as determined by electron microscopy**

BgPMϕ were cultured with M-CSF (1000units/ml) for 48 hours and processed for electron microscopy as described in section 2.14 with special emphasis on the basal, adhesive surface. Note the elaborate fenestrated cisternal network immediately adjacent to the plasma membrane. Tubular network shown by arrowheads and the extracellular areas by small arrows. 4000x.


A
B
C

To determine the possible association between cytoskeletal elements and MSR at the adhesive surface and whether these elements were required for its presence, M-CSF treated BgPMϕ were treated with various cytoskeletal perturbants. Nocodazole disrupts microtubules (by inhibiting monomer addition) and causes the loss of normal positioning and morphology of organelles including the Golgi apparatus, mitochondria and ER (Terasaki *et al.* 1986). Treatment also however rounded up adherent BgPMϕ and affected cell morphology to such an extent that the MSR-microtubule association could not be explored (not shown). The morphological expression of MSR, and the effects of cytoskeletal perturbants on its distribution, were examined further in CHO cells stably transfected with murine MSR type II, in which MSR expression had been upregulated by selection in MAC medium as discussed in Chapter 7.

9.3 Morphology of MSR expression in CHO MSR II MAC cells

CHO MSR II MAC cells were cultured overnight on glass cover slips in F-12 Ham's, 5% FCS and G418 selection medium prior to manipulation and fixation as described previously. Indirect immunofluorescence was performed as described using confocal laser scanning microscopy with special attention to the adhesive cell surface immediately adjacent to the glass. The MSR fluorescence pattern, both on staining the cell surface and after permeabilization, was remarkably similar to that seen with M-CSF treated BgPMϕ (fig. 9.10). The fenestrated appearance of MSR expression near the adhesion surface was especially prominent on higher magnification, and resembled cellular stress microfilaments in appearance. These findings indicate i) that MSR is associated with the cellular contact zone, even when expressed in an alien cellular environment and ii) that the MSR-positive "structure" is not directly M-CSF dependent, but requires high levels of MSR expression to become apparent. Vertical sections of CHO MSR II MAC cells also showed prominent fenestrated MSR staining at the cell-glass interface (fig. 9.10), suggesting its involvement in adhesion.

Efficient intracellular transport of MSR to the cell surface was previously suggested only to occur in Mϕ (Naito *et al.* 1991). This was based on the demonstration that bovine MSR transiently transfected into COS or human erythroleukemic (HEL) cells accumulated in the nuclear envelope, ER and Golgi apparatus, but not on the cell membrane. MSR expression on the cell surface only became apparent after transfected HEL cells were transformed into Mϕ-like cells by phorbol ester treatment. The presence of a substantial intracellular MSR

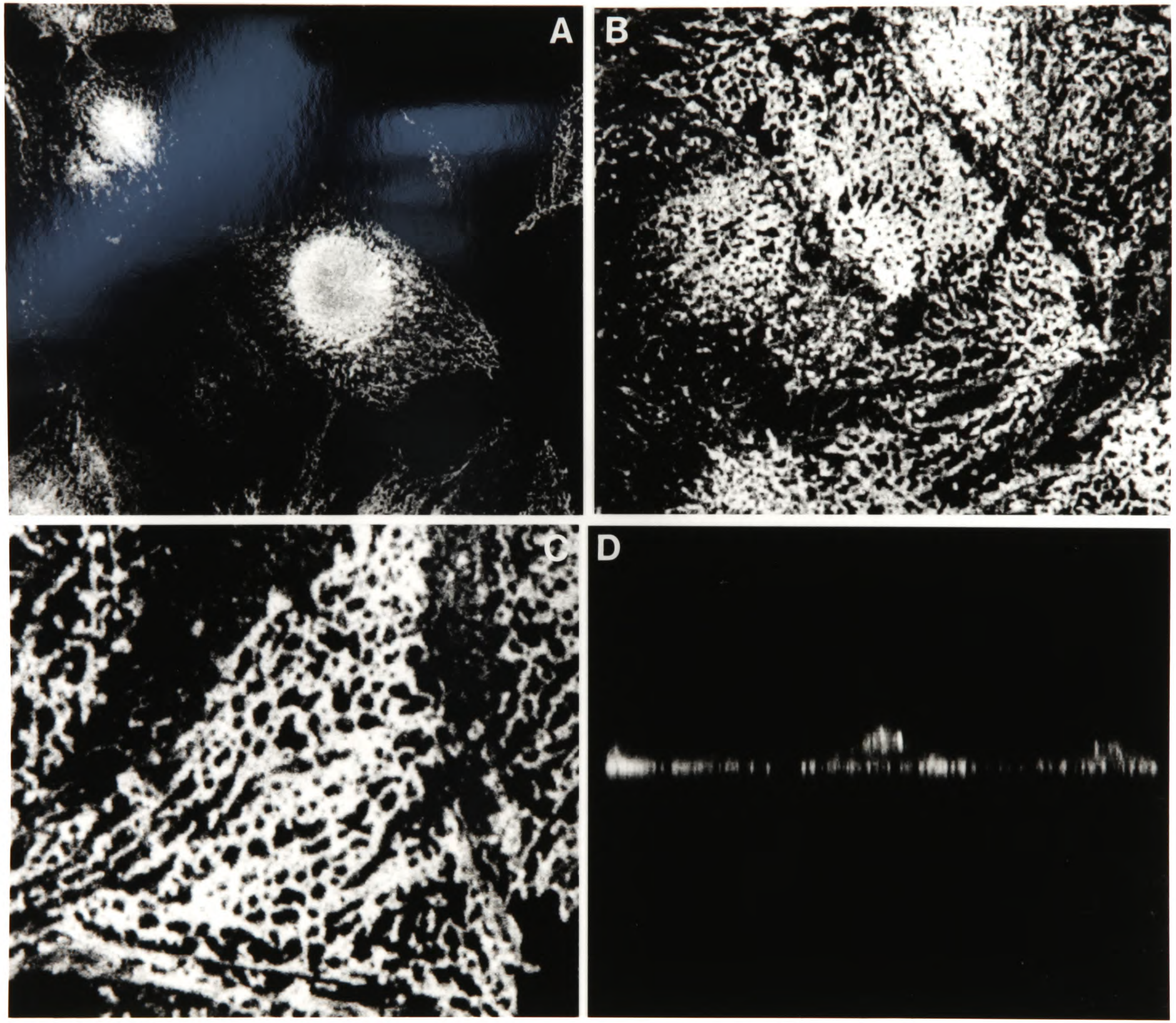
Fig. 9.10 **MSR immunofluorescence confocal laser scanning microscopy of CHO MSR II MAC cells**

CHO MSR II MAC cells were cultured as described in the text and processed (permeabilized) as in fig. 9.2. The basal, adhesive surfaces of the cells were examined by CLSM to determine MSR distribution.

A. 120x

B. 180x and **C.** 240x. Higher magnification show the fenestrated appearance of MSR fluorescence at the adhesion surface which seem to run concurrently with stress fibres.

D. Vertical section. 120x Note the fenestrated MSR appearance basally.


A
B
C
D

pool in CHO MSR II MAC cells, and the requirement for prolonged incubation with MAC selection medium prior to significant upregulation of surface MSR expression (as shown in Chapter 7), would be consistent with these findings and the existence of a Mϕ-specific transport mechanism.

The presence of a prominent intracellular MSR pool was demonstrated when CHO MSR II MAC cells were treated with brefeldin A (BFA) (10 μg/ml) (fig. 9.11). BFA is used as a specific reversible inhibitor of membrane transport and dysregulates membrane traffic throughout the central vacuolar system. In BFA-treated cells protein secretion is blocked at an early pre-Golgi compartment step, thereby resulting in retention of secretory and membrane proteins in the ER (Klausner *et al.* 1992). The effects of BFA are thought to be due to inhibition of regulatory coat protein binding (especially the 110K protein/β-COP and ADP-ribosylation factor (ARF)) to the Golgi apparatus (Rothman 1994). A major morphological effect of BFA is the rapid induction of tubules in the Golgi apparatus; but this has also been observed in peripheral organelles such as endosomes, lysosomes, and the TGN (Tooze and Hollinshead 1991; Klausner *et al.* 1992). Membrane tubulation of the Golgi apparatus due to BFA could clearly be observed by TGN 38 immunofluorescence (fig.9.11) and was distinct from the pattern shown by an antibody to the ER marker, protein disulfide isomerase (PDI). Prominent perinuclear MSR staining was observed at a more apical optical plane and in vertical sections following BFA treatment (fig. 9.11); this would be consistent with redistribution of intracellular MSR to the ER subsequent to collapse of the Golgi apparatus and tubular endosomal network. A substantial proportion of MSR however remained associated with the basal adhesive surface.

The cytoskeletal perturbants nocodazole and cytochalasin D were subsequently used to examine the possible association of microtubules and microfilaments to the MSR-positive "structure" at the adhesive surface. CHO WT and CHO MSR II MAC cells were incubated with 10 μg/ml nocodazole for 10 and 60 min. Disruption of microtubules occurred in both cell types, resulting in punctate tubulin fluorescence at the centrosome or microtubule organizing centre (MTOC). The intensity and distribution of MSR staining at the contact zone remained unchanged (fig. 9.12). Similar experiments were performed with cytochalasin D (1μM final concentration) followed by rhodamine phalloidin staining to assess the role of actin microfilaments (fig. 9.12). Cytochalasin D clearly interfered with actin microfilament morphology, but the MSR fluorescence pattern was unaffected. The distribution of talin, part of the actin-binding complex, was similarly unchanged (not

Fig. 9.11 **The effects of brefeldin A on MSR distribution in CHO MSR II MAC cells**

CHO MSR II MAC cells were cultured as described in the text and processed (permeabilized) as in fig. 9.10. Prior to fixation and examination by CLSM, brefeldin A (10 μg/ml) was added to the medium for 10 to 30 min.

A. and B. A substantial proportion of MSR still remains at the adhesive interface **(A.)**, but prominent perinuclear MSR fluorescence becomes apparent after brefeldin A treatment (visible at a more apical optical plane in **B.**). 60x

C. Vertical section confirms redistribution of MSR to the perinuclear region, consistent with brefeldin A-induced collapse of the Golgi apparatus and tubular endosomal network. 90x

D. Membrane tubulation post-brefeldin A treatment as shown by TGN38 immunofluorescence. 120x

E. Protein disulfide isomerase fluorescence is not affected by brefeldin A treatment. 120x

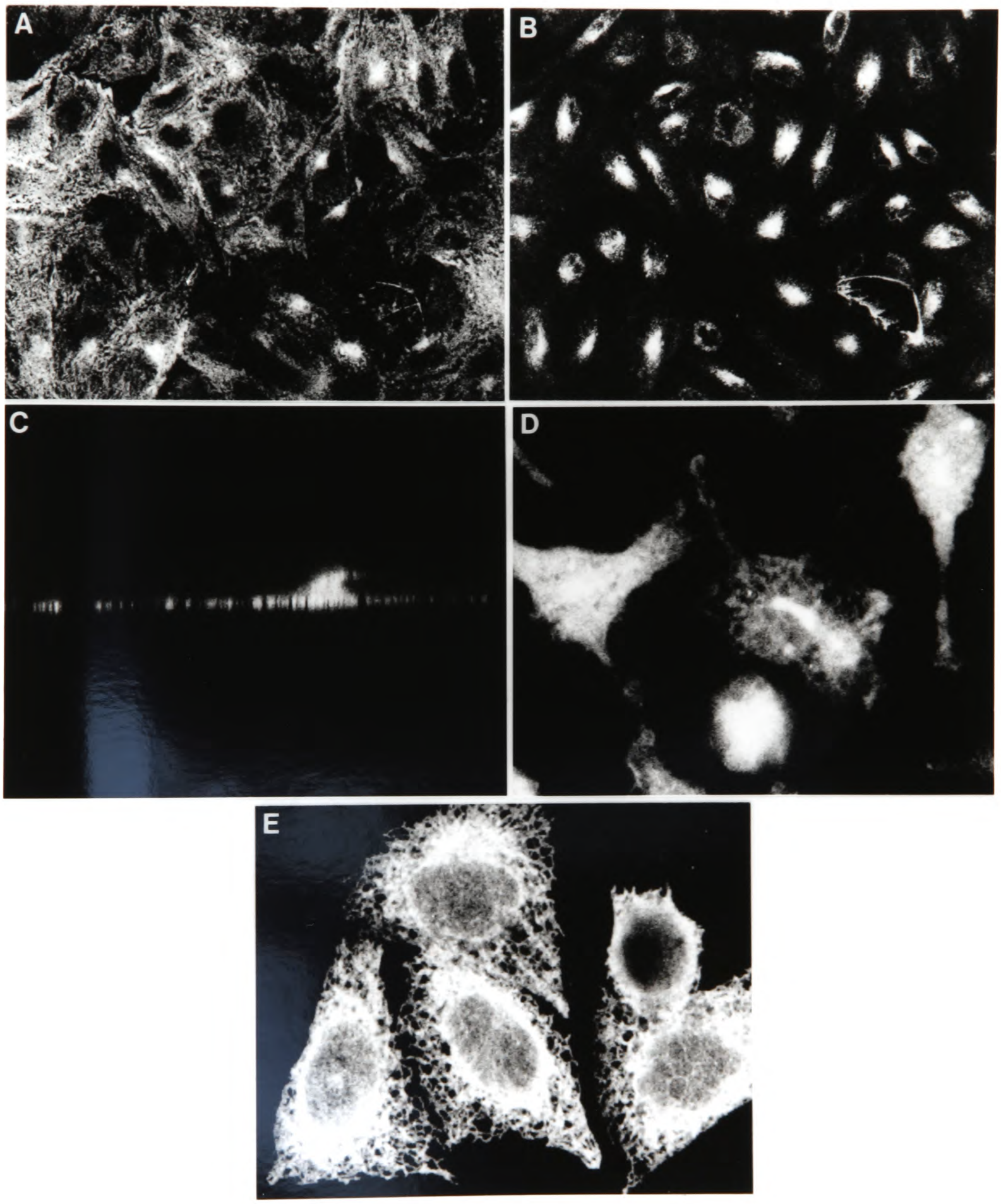

A
B
C
D
E

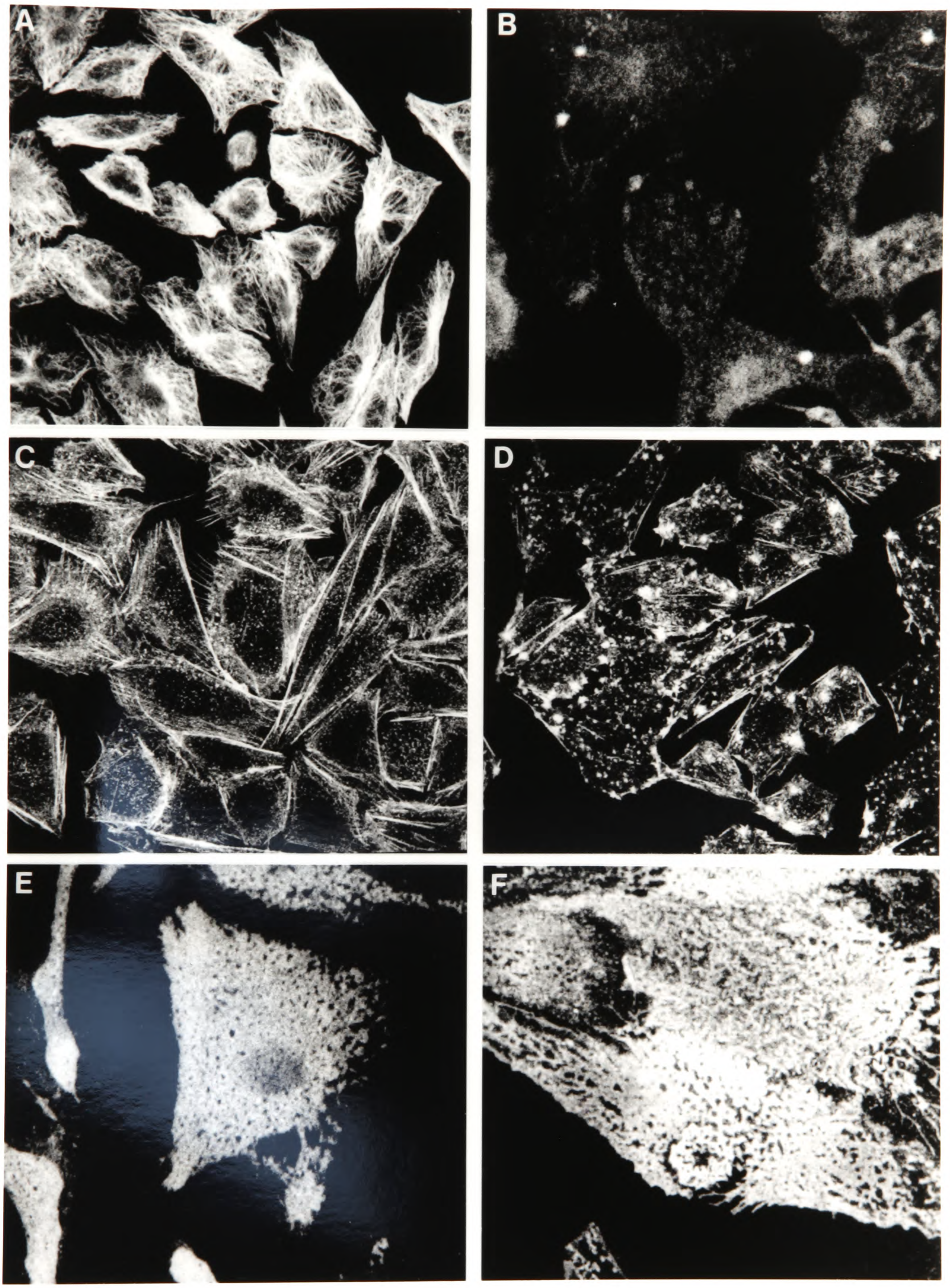
Fig. 9.12 **The effects of nocodazole and cytochalasin D on MSR distribution in CHO MSR II MAC cells**

CHO MSR II MAC cells were cultured as described in the text and processed (permeabilized) as in fig. 9.10. Prior to fixation and examination by CLSM, nocodazole (10 μg/ml) and cytochalasin D (1μM) were added to the medium for 10 to 60 min.

A. and B. Tubulin. Nocodazole disrupted the microtubular network in these cells (**A**. 60x) resulting in punctate tubulin fluorescence at the MTOC (**B**. 120x).

C. and D. Actin microfilaments. Cytochalasin D had a similar disruptive effect on microfilaments as shown by rhodamine phalloidin staining (**D**.). 90x.

E. and F. The MSR fluorescence pattern showed no change after any of the treatments (this set following cytochalasin D treatment). 180x


A
B
C
D
E
F

shown). These results would suggest that MSR distribution at the adhesive basal cell surface is microtubule- and microfilament-independent.

Intermediate filaments (IFs) represent the third major class of cytoskeletal elements, and are thought to maintain the mechanical integrity of cells in tissue. Their precise functions have remained elusive and recent studies have suggested that vimentin-type intermediate filaments may also play a role in post-lysosomal intracellular cholesterol transport (Evans 1994). Regular vimentin IF structures have been described to surround membrane-free lipid droplets in adipose cells, cholesterol-loaded Mϕ, and foam cells in atherosclerotic lesions. Electron microscopy of adipocytes showed regularly spaced arrays of vimentin filaments at the surface of lipid droplets adjacent to ER cisterna (Franke *et al* 1987) Human adrenal tumour cell lines that lack any detectable vimentin IF network have a striking defect in their capacity to esterify lipoprotein-derived cholesterol compared with cells containing vimentin IFs (Sarria *et al.* 1992). These studies remain correlative, and it is unclear how these apolar structures may be involved in cholesterol transport. The possible association or co-localization of vimentin with MSR will be examined by anti-vimentin immunofluorescence. The lack of compounds that specifically affect the vimentin IF component of the cytoskeleton has prevented its potential role to be distinguished from that of the other cytoskeletal elements.

The epitope recognized by mAb 2F8 is located in the α-helical coiled-coil domain between amino acids 183 and 197 (shown in Chapter 7) and may represent a functional MSR adhesion domain. Interestingly, the MSR truncation mutant #6 (length 197 aa) failed to show a similar MSR immunofluorescence pattern to that of CHO MSR II MAC cells and primary M-CSF treated BgPMϕ (fig. 9.13). This may indicate i) that certain MSR structural regions are required (such as presence of α-helical coiled-coil domain and trimerization) for concentration to occur at the cell contact zone or ii) that low transfection efficiency and expression levels in CHO cells prevented redistribution or detection of MSR towards the cell base.

The contact area of CHO MSR II MAC cells was further investigated by electron microscopy (without MSR-specific gold-labeling) (fig. 9.14). Cell morphology appeared broadly similar to that seen in M-CSF treated BgPMϕ. A prominent fenestrated cisternal network was present close to the adhesive surface and also extended centrally to include tubular structures (probably endosomes) adjacent to the centrosome (the region in which MSR localized after BFA treatment (fig. 9.11)). The vesiculotubular network also appeared associated with stress

Fig. 9.13 **MSR immunofluorescence in CHO MSR mutant #6 compared with CHO MSR II MAC cells**

CHO MSR II MAC and CHO cells stably transfected with truncated MSR mutant #6 (as described in Chapter 7) were cultured as described in the text and processed (permeabilized) as in fig. 9.10, and the adhesive surface analyzed by CLSM.

A. CHO MSR II MAC cells.

B. CHO MSR mutant #6. Note the absence of prominent MSR fluorescence.


A

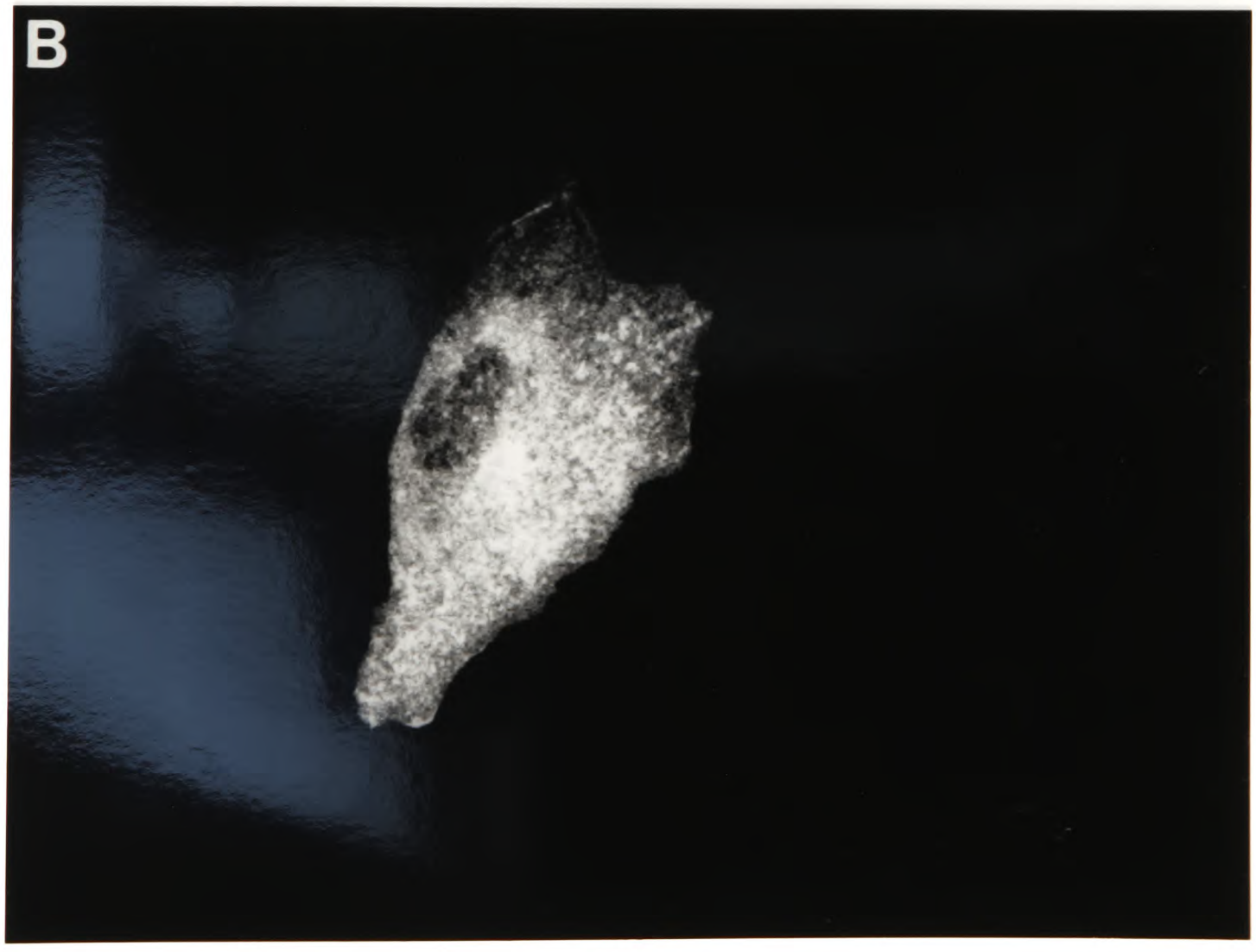

B

Fig. 9.14 **Ultrastructure of CHO MSR II MAC cells as determined by electron microscopy**

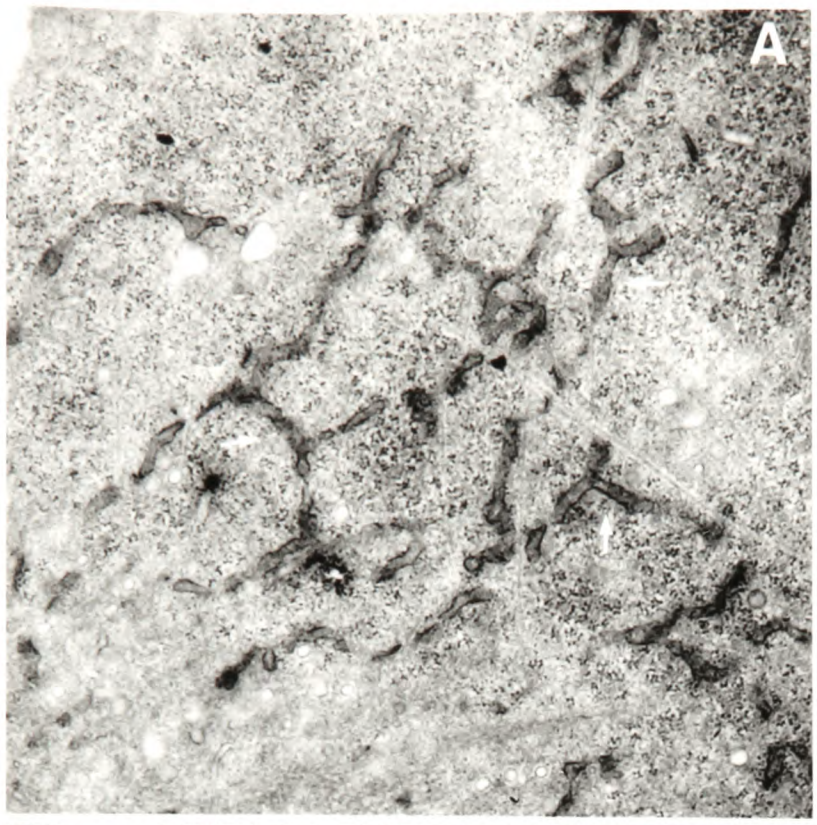
CHO MSR II MAC were processed for electron microscopy as described in section 2.14 with special emphasis on the basal, adhesive surface. Note the elaborate fenestrated cisternal network (similar to the appearance in BgPMϕ in fig. 9.9) immediately adjacent to the plasma membrane.

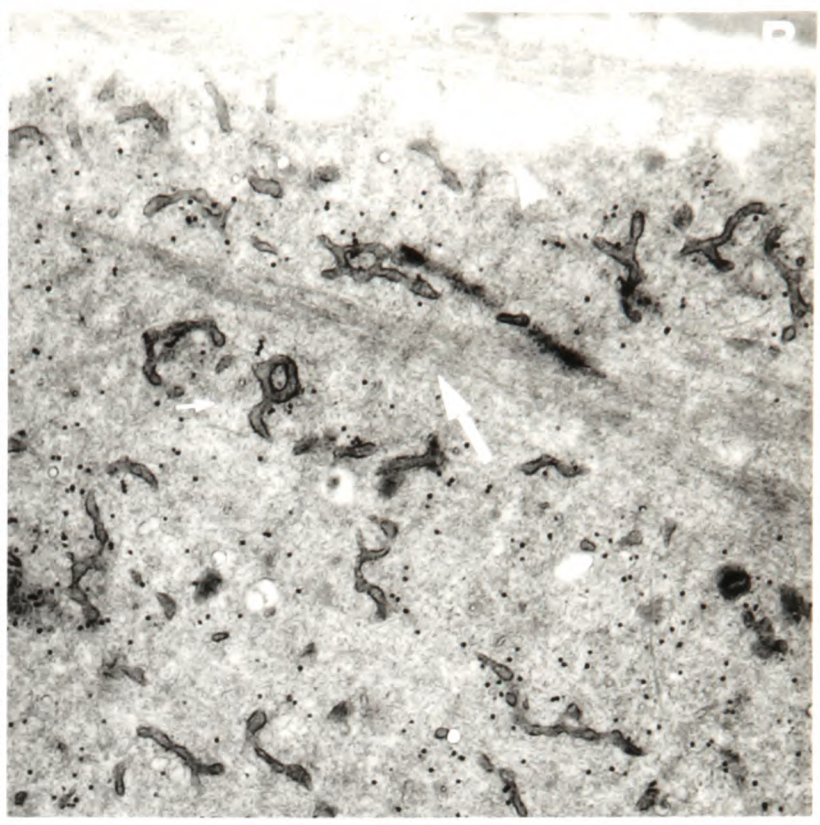
A. and B. Small arrows depict tubular fenestrated network. Arrowhead shows extracellular region and large arrow highlights actin bundles at the dge of the plasma membrane. 2560x

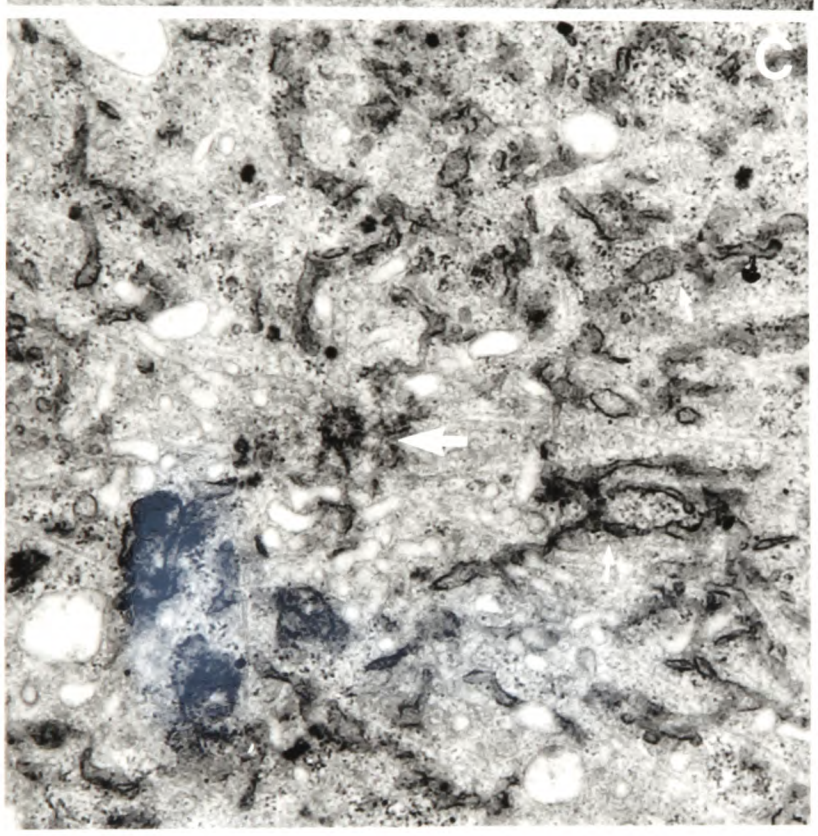
C. Tubular network shown by small arrow and centriole by large arrow. 4000x.

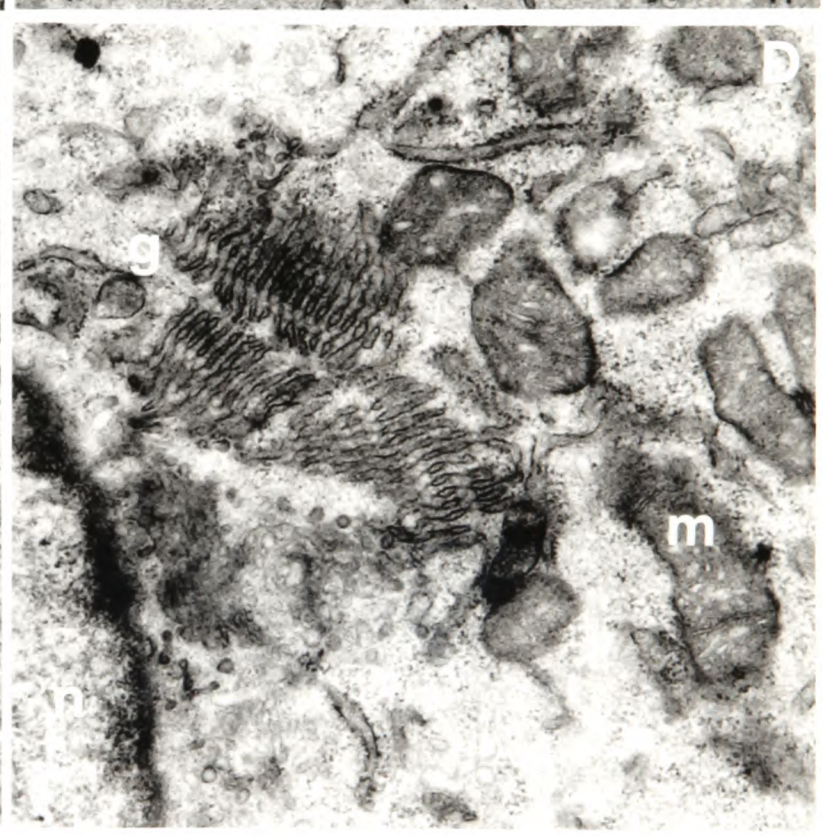
D. Note prominent Golgi stack (g) and mitochondria (m). 4000x

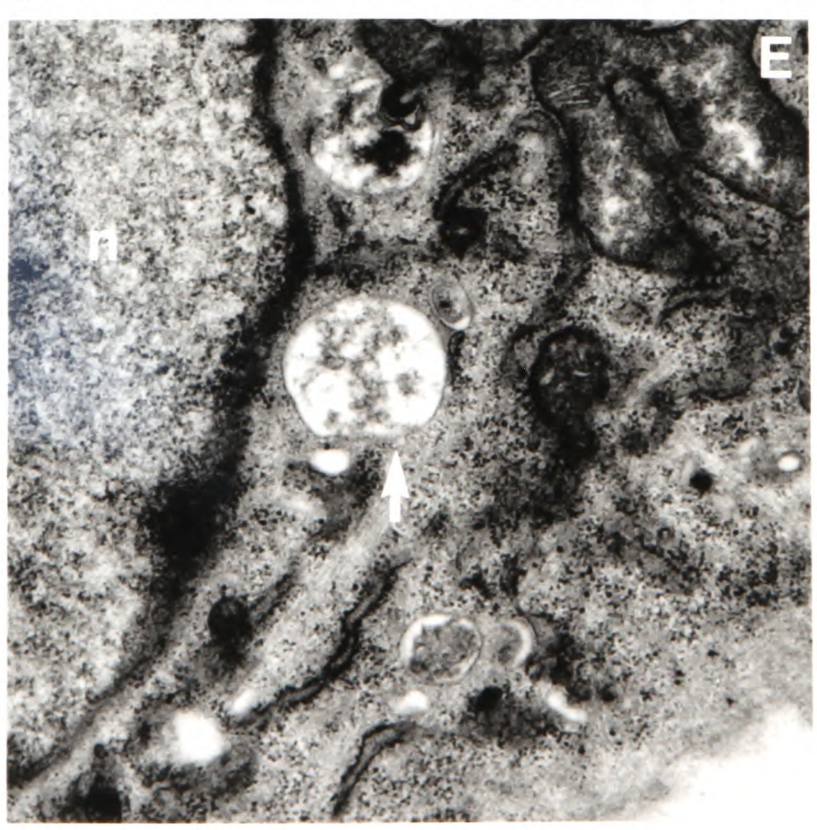
E. Note rough ER and lysosome (arrow) adjacent to nucleus (n). 4000x


A


B


C
n


D
g
m
n


E
n

actin bundles which stretched to the edge of the plasma membrane.

To determine the relationship between the tubules close to the adherent surface, the plasma membrane and the area immediately extracellular, CHO MSR II MAC cells were incubated with HRP as a fluid phase marker, for 2 min and 60 min at room temperature prior to fixation (fig. 9.15). The cells were not permeabilized and the adherent surface was then examined by electron microscopy. At the 2 min time point, the most obvious features were HRP bathing the cell exterior and the presence of many round HRP-dense areas which could represent either plasma membrane invaginations or early pinocytic vesicles. The important observation was that HRP was not excluded from the zone between the cell and the substrate. The "black hole" visualized with surface staining was thus not due to the formation of a protein-tight seal to the adherent surface that exclude soluble proteins. An alternative explanation may be that the "black hole" phenomenon in M-CSF treated BgPMϕ and CHO cells represents a fixation artefact due to tight mAb 2F8-impermeable cross-linking; subsequent to Triton-X 100 permeabilization, the previously inaccessible area becomes available for immunofluorescence.

After 60 min HRP incubation, the marker was absent from the extracellular space and had largely been transferred to the late endosomal/lysosomal compartments as expected (fig. 9.15). Very little HRP is visible in the cytoplasm immediately adjacent to the adherent surface; stress actin bundles were also prominent in this area. The electron microscopic image of the cellular contact zone (fig. 9.15E) was computer-modified to depict extracellular areas as black, and the plasma membrane/cytoplasm as grey (fig. 9.15F). The resulting fenestrated "spotted leopard" appearance was very similar to that of MSR-specific fluorescence shown by confocal microscopy at the contact areas of CHO MSR II MAC cells (fig. 9.15D). This would be consistent with (as shown diagrammatically in fig. 9.16) the black extracellular or non-fluorescent areas representing invaginations of the plasma membrane and the grey or white MSR-positive areas depicting contact zones with the substratum. In this model, Mϕ membrane remodeling following adhesion is suggested to result in polarized redistribution of MSR towards cellular contact zones.

9.4 Discussion

In this chapter, morphological evidence is presented in support of a role for the MSR in adhesion *in vitro* in primary Mϕ and transfected CHO cells; this may also be an important function of the MSR *in vivo*.

Fig. 9.15 **Ultrastructural analysis of the adhesive surface in CHO MSR II MAC cells as using horseradish peroxidase as a fluid phase marker.**

CHO MSR II MAC were incubated with HRP as a fluid phase marker for 2 min (**A. and B.**) and 60 min (**C, E. and F.**) at room temeperature prior to fixation and processing for electron microscopy as described in section 2.14.

A. and B. At 2 min, HRP is still clearly present at the cell exterior (arrows) and in plasma membrane invaginations or pinocytotic vesicles. 4000x

C. and E. At 60 min, very little HRP is present in the cytoplasm immediately adjacent to the adherent surface. HRP-dense collection bottom left of **C.** represents technical artefact. Note the prominent stress actin bundles in this area (arrow). C. 800x and E. 1600x

D. MSR immunofluorescence of CHO MSR II MAC cells shown for comparison. 120x.

E. and F. The electron microscopic image of the cellular contact zone (**E.**) (1600x) was computer-modified to depict extracellular areas (arrowheads) as black, and the plasma membrane/cytoplasm as grey (**F.**). The resulting fenestrated "spotted leopard" appearance was very similar to that of MSR-specific fluorescence at the contact areas of CHO MSR II MAC cells (**D.**).

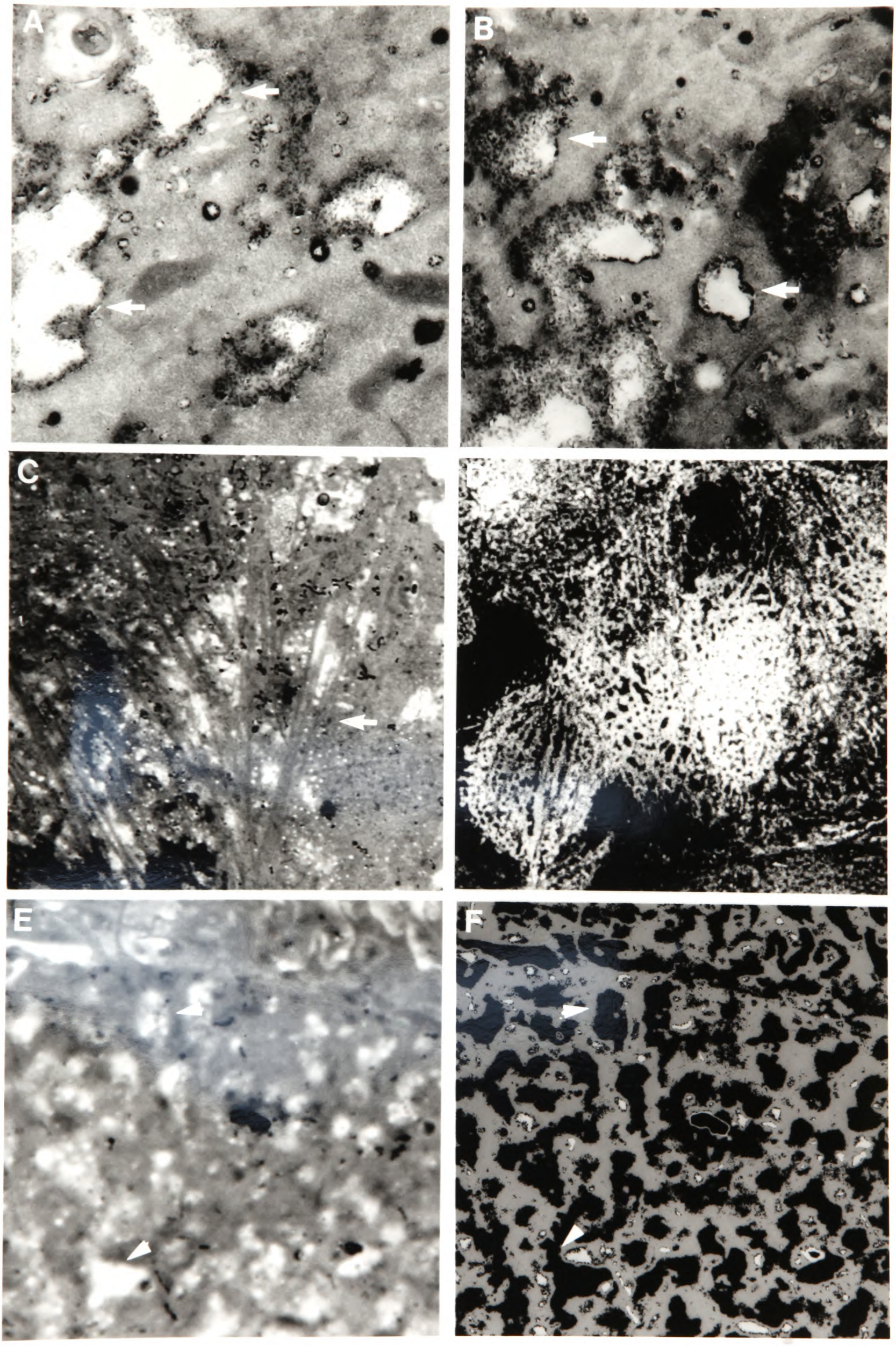

A
B
C
E
F

Fig. 9.16 **Diagrammatic representation of MSR adhesion to substratum.**

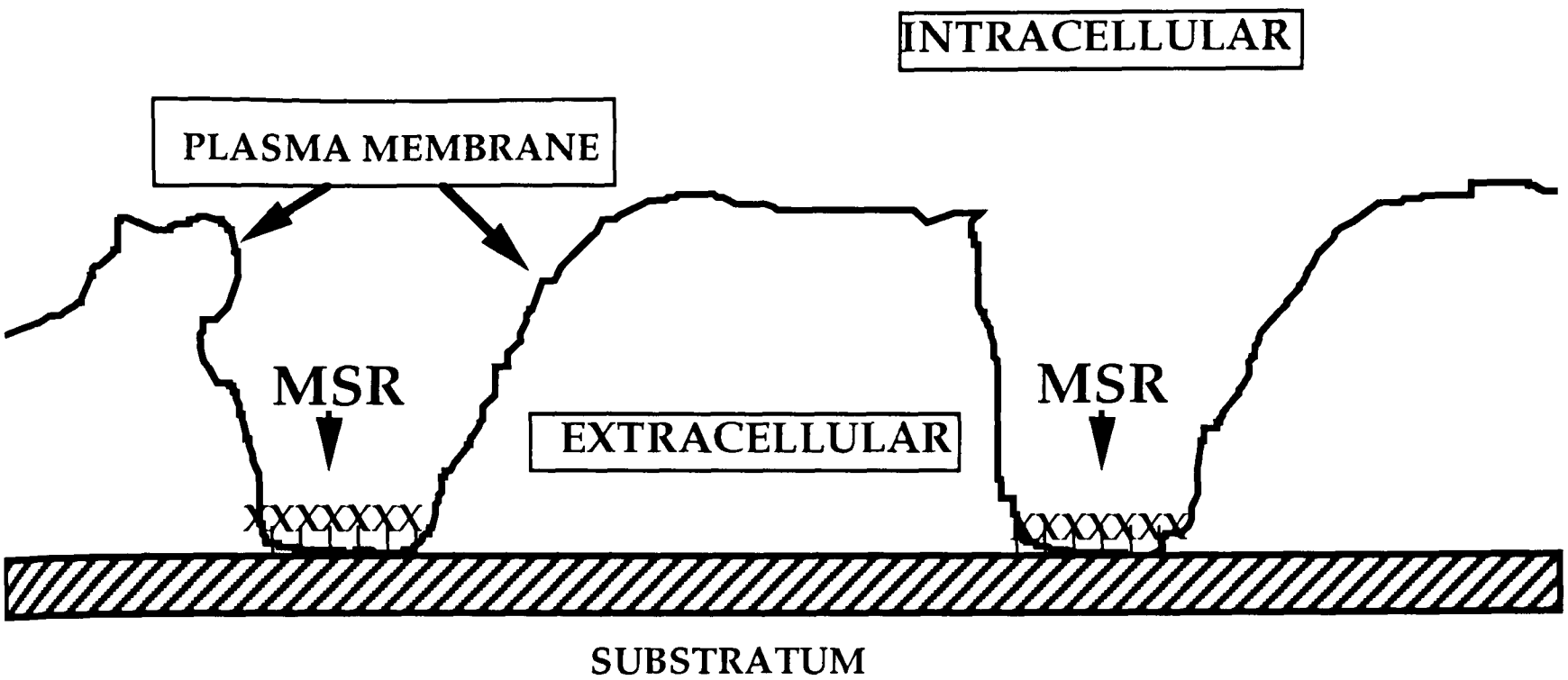
A. Side on view (high power) of basal surface of cell (Mϕ or CHO MSR II MAC) adhering to substratum. MSR (XXX) redistribute to contact areas with ligand(s) on adhesive surface and these areas are brightly MSR-positive. Plasma membrane not in contact with substratum is relatively devoid of MSR.

B. The area shown in **A.** is viewed from above. Brightly fluorescent MSR concentrate at cellular contact areas, which appear homogeneous due to resolution of CLSM; the higher resolution of EM reveals further substructures consistent with PM invaginations.

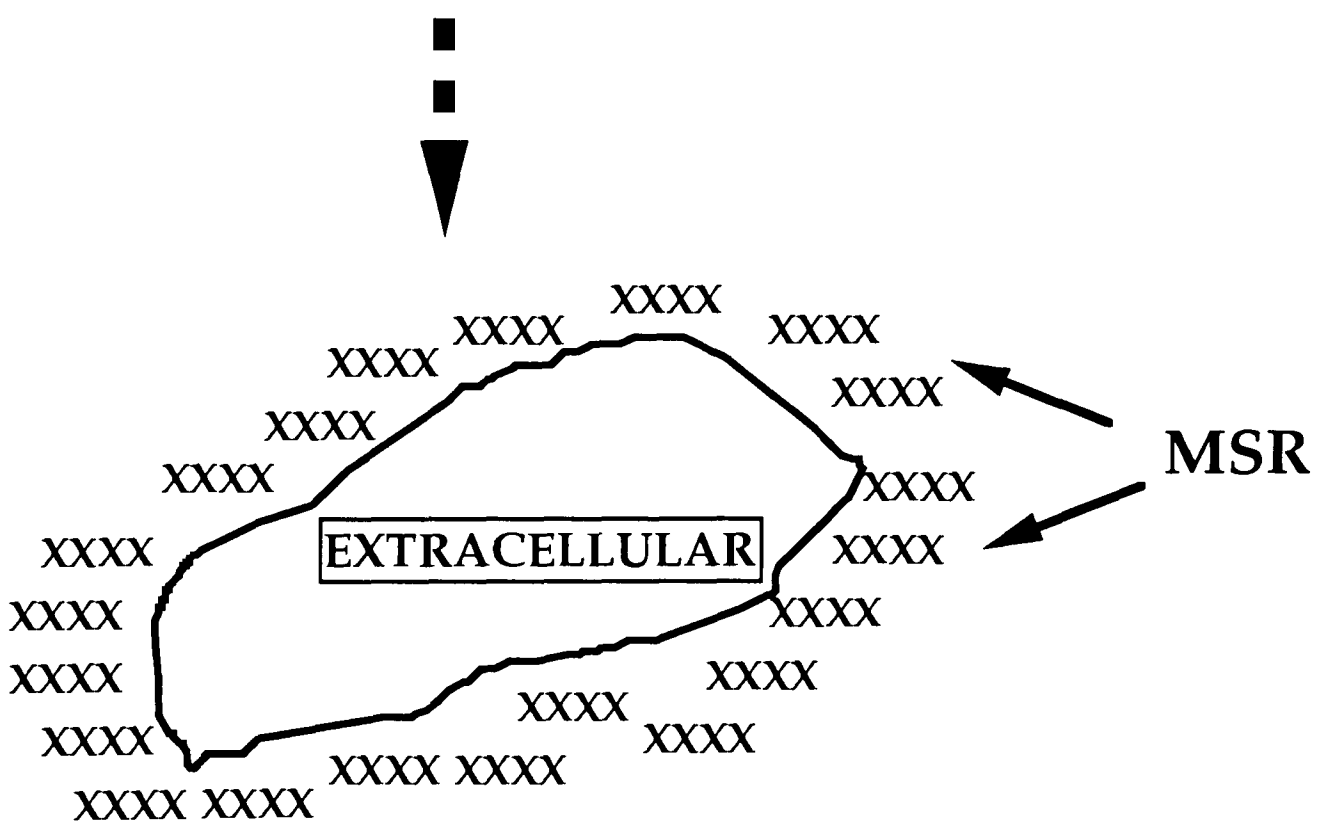
C. At lower magnification the "leopard spot" appearance of MSR staining at the adhesive interface becomes apparent. Contact areas are MSR-positive whilst PM invaginations or extracellular areas are negative.

FIG. 9.16

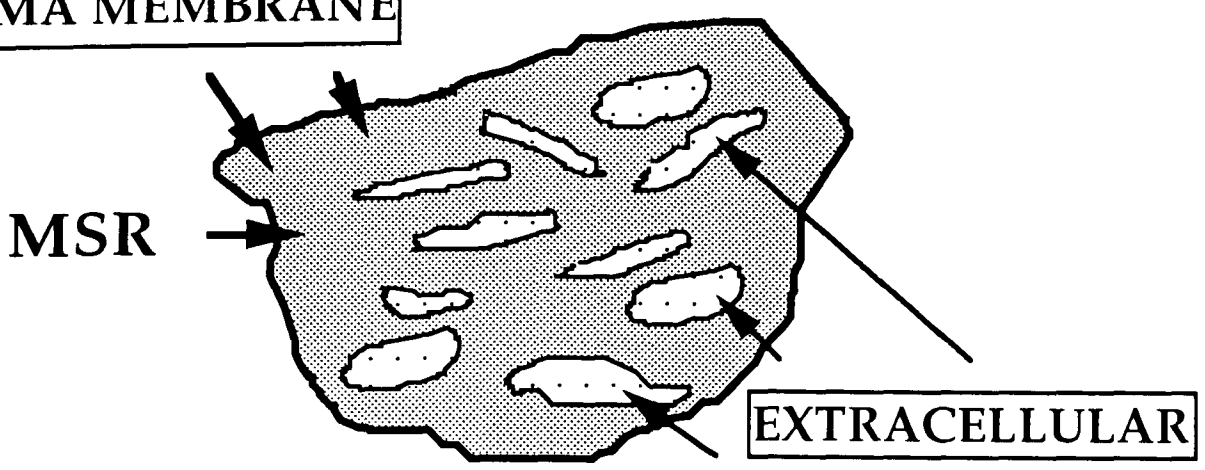
A.


INTRACELLULAR
PLASMA MEMBRANE
MSR
MSR
EXTRACELLULAR
SUBSTRATUM

B.


MSR
EXTRACELLULAR
INTRACELLULAR
INTRACELLULAR
PLASMA MEMBRANE

C.


MSR
EXTRACELLULAR

MSR is prominently expressed at the adherent surface and preliminary data suggest its distribution may be restricted to cellular contact areas with the substratum. This distribution is not affected by disruption of microtubules or microfilaments and the ability of the cell to endocytose AcLDL did not seem qualitatively impaired.

The suggested polarized redistribution of the MSR towards contact zones could also have other functional implications for cholesterol metabolism in the subcellular space. AcLDL and cholesterol crystals have been reported to be sequestered by Mϕ in endocytic compartments which remain connected to the extracellular space (Kruth *et al.* 1995); providing a lipid reservoir prior to further processing. When Mϕ spread on immobile immune complexes, repositioning and reorganization of Golgi membranes occur and exocytosis of secretory granules containing apoE is directed towards the adherent surface and basal vacuoles (Bainton *et al.* 1989; Werb *et al.* 1989).

The MSR has not been demonstrated to be specifically (by immuno-gold labeling) associated with the fenestrations and cellular contact zones and these experiments are in progress. Other morphological approaches which may prove useful in characterizing the contribution of MSR of adhesion include i) ultrastructural studies of adherent Mϕ from MSR-deficient mice, the various CHO MSR truncation mutants and, for comparison, CHO WT cells; ii) immunofluorescent localization of vimentin to explore the possible association with MSR; iii) characterization of the effect on MSR distribution following Mϕ adherence to a MSR ligand (e.g. M-BSA) coated surface and iv) exploring MSR distribution in MSR-upregulated (M-CSF treated BgPMϕ) non-adherent cells to determine effects on MSR polarization.

Chapter Ten

Conclusions and Future Directions

10.1 Summary of main findings

The Mϕ foam cell in the atherosclerotic plaque micro-environment is subject to a constant barrage of stimuli from cytokines and growth factors secreted by SMCs, ECs, platelets and lesional Mϕ themselves. Work presented in this thesis examined the effects of some of these soluble factors on a Mϕ membrane molecule, the MSR, considered pivotal in foam cell formation and atherogenesis, and was based on the following hypothesis:

i) that Mϕ use the MSR as an additional adhesion molecule for their recruitment and retention within lipoprotein-rich atherosclerotic plaques;

ii) that regulation of MSR expression and activity may represent an important determinant of the extent of atherogenesis and

iii) that therapeutic modulation of MSR activity may influence the rate and extent of atherosclerosis development.

M-CSF was shown to enhance MSR expression in murine elicited peritoneal Mϕ markedly and selectively by a number of mechanisms. MSR functional activity (including MSR-dependent adhesion) is likewise increased. The documented presence of M-CSF in the atheromatous lesion and its induced secretion by minimally modified LDL suggest a plausible mechanism whereby the MSR could contribute to the recruitment of Mϕ into the arterial wall and, at the post-endothelial stage, anchor Mϕ at specific locations, thus favouring foam cell formation.

The modulation by T lymphocyte products of MSR expression and activity *in vitro* was subsequently explored. Th1 (IFN-γ) and Th2 (chiefly IL-4) lymphokines display divergent effects with IL-4 upregulating and IFN-γ either maintaining or downregulating MSR status. An intriguing finding was cytokine-specific mediated micro-heterogeneity in MSR structure; IL-4 effected changes in N-linked glycosylation, specifically sialylation. MSR-mediated AcLDL uptake in IL-4 treated primary Mϕ appeared more resistant to inhibition by various MSR ligands, especially lipoteichoic acid. This phenomenon may be

related to structural differences in MSR N-linked glycosylation following IL-4 treatment. Interestingly, MSR structural heterogeneity was also shown *in vivo* in immunologically active locations, although this could not be linked to specific cytokine actions. To determine this, MSR expression in organs from IFN-γ or IL-4 deficient mice may be examined by tissue immunoblots.

The classical Mϕ deactivators, IL-10 and TGF-β, were demonstrated to affect MSR expression and function differently. In contrast to IL-10, TGF-β functions as a potent dose-dependent inhibitor of MSR upregulation in M-CSF stimulated BgPMϕ. Endogenously produced TGF-β inhibits MSR function in BgPMϕ, the Mϕ-like cell line RAW 264.7 and foam cells *in vitro*. In this experimental system, TGF-β proved as potent an inhibitor of MSR function as M-CSF is a stimulator. Active TGF-β inhibits vascular SMC proliferation. Decreased levels of active TGF-β (due to Lp(a) inhibition of plasmin generation) result in vascular SMC proliferation *in vitro* and are associated wth advanced atherosclerosis *in vivo* (Grainger *et al.* 1993; Grainger *et al.* 1994; Grainger *et al.* 1995). The lack of active TGF-β may, in addition, be considered pro-atherogenic because of an increase in foam cell formation due to the unopposed upregulation of MSR activity by M-CSF. Of the C-C chemokines and other soluble factors examined, only MCP-1 reproducibly enhanced BgPMϕ MSR-dependent adhesion without affecting MSR expression levels.

Immunochemical evidence was presented for the presence in BgPMϕ culture supernatants of a cleaved soluble form of MSR which lacks the cytoplasmic domain. Release of the fragment is regulatable, and the theme of M-CSF as a potent MSR stimulator was reinforced when it increased soluble MSR release *in vitro*. Soluble MSR was shown to be functionally active. The physiological significance of this phenomenon is unclear, but if present *in vivo*, can be envisaged to serve a circulating scavenging anti-inflammatory role. Definition of its role in human physiology and pathophysiology requires the development of specific human MSR reagents which are lacking at present.

In studies with murine MSR cDNAs transfected into CHO cells, a MSR-dependent contribution to CHO cell adhesion to TCP was difficult to ascertain. This may be due to impaired surface expression of MSR in a non-Mϕ alien cellular environment, as high levels of MSR protein were present intracellularly. Following upregulation of MSR surface expression by prolonged culture in scavenger receptor-specific selection medium, a modest MSR-dependent contribution to adhesion became apparent. This may indicate the requirement of MSR-mediated

adhesion for additional Mϕ-specific surface membrane molecules (Fc receptors), or cytoskeletal and signalling events to become fully operative.

To determine a possible adhesion-promoting region in the MSR, the binding site of mAb 2F8 was mapped using a series of MSR truncation mutants, and localized to residues 183 to 197 in the proximal α-helical coiled-coil domain. No obvious known adhesion motifs are present in this region, but the data would suggest distinct adhesion-promoting and AcLDL-binding sites in the receptor. This would be consistent with its bifunctional nature, but does not exclude multimerization or conformational changes in the MSR as adhesion-promoting requirements.

Morphological evidence, obtained by confocal and electron microscopy, is presented for an adhesion role for the MSR in primary Mϕ and transfected CHO cells. MSR expression is prominently directed to the adherent surface and its distribution may be restricted to cellular contact areas with the substratum. The distribution of MSR at the adherent surface depends on high levels of MSR expression, and requires prior M-CSF exposure in primary Mϕ and upregulation in MAC medium in type II MSR transfected CHO cells. The basal surface accumulation of MSR is also resistant to microfilament and microtubule disruption and did not qualitatively impair AcLDL endocytosis. Apart from its putative mechanical role in anchoring the Mϕ to an adhesive surface (cell or extracellular matrix), the MSR may help in establishing a functional extracellular compartment at the adhesive interface. A possible beneficial physiological consequence of this secluded micro-environment would be in tissue repair and remodeling due to localized apoE secretion, extracellular acidification (analogous to osteoclasts) and prevention of access to protease inhibitors.

Organs and atherosclerotic lesions from op2/E0, op1/E0 and op0/E0 mice were examined by immunohistochemistry to determine the effects of M-CSF on Mϕ phenotype (including MSR expression) and lesion development *in vivo*. Though severely hypercholesterolemic, op0/E0 mice are protected against atherosclerosis and exhibit fewer Mϕ and low MSR expression on remaining M-CSF independent populations. An intriguing finding is prominent lipid accumulation in the livers of op2/E0, but not op0/E0 mice, despite a comparable 4-fold increase in plasma cholesterol. This may be consistent with a crucial M-CSF dependent role for Kupffer cells in lipoprotein uptake, transfer to hepatocytes and biliary excretion of cholesterol. The importance of M-CSF in maintaining cholesterol homeostasis and in atherogenesis is clearly illustrated by this model; the direct involvement of MSR

however remains unproven.

10.2 Does the MSR play a role in Mϕ adhesion in vivo?

To determine the biological significance of the MSR *in vivo*, MSR gene knock-out animals were engineered using embryonic stem cell recombinant technology (Mansour *et al.* 1988; Capecchi 1989). A colony of these MSR-deficient mice was established at the Dunn School of Pathology, Oxford and preliminary results (Honda *et al.* 1995; Takahashi *et al.* 1995) indicate them to be healthy and fertile. MSR could not be detected in any organs or tissues from homozygous MSR -/- mice by mAb 2F8 immunohistochemistry or immunoelectron microscopy. The degradation of AcLDL and OxLDL by peritoneal Mϕ from MSR-deficient animals was decreased by 75% and 70% respectively. In addition, MSR-deficient peritoneal Mϕ appeared rounded, exhibited poor glass adherence and also showed decreased uptake of AGEs, surfactant and CD14-independent lipopolysaccharide. The adhesion of MSR-deficient peritoneal Mϕ to TCP lacks an EDTA-resistant (MSR-dependent) component (R. Haworth and S. Gordon). Hepatic granulomas following Zymocel injection were larger and more irregular than in heterozygotes or normal mice. All these findings would support an important role for the MSR in Mϕ adhesion as well as in the uptake of negatively charged macromolecules *in vivo*.

10.3 Identification of the MSR adhesion-promoting ligand

MSR-mediated adhesion of murine Mϕ *in vitro* depends critically on the presence of serum coating the artificial substratum. The nature of the adhesion-promoting component in murine and human serum will be of considerable interest due to its physiological relevance. A methodical approach to its isolation is currently in progress in this laboratory (Hughes 1994), and both FCS and human serum have been fractionated to identify ligand-containing fractions, from which relevant proteins could be purified and characterized by protein sequencing or the generation of polyclonal antisera. Alternative approaches include the addition of isolated candidate ligands to serum free medium or removing them by affinity chromatography, and assessing the effects on Mϕ adhesion *in vitro*. Another strategy may be the use of soluble MSR proteins (Resnick *et al.* 1993), or MSR-Fc chimeras (Watson *et al.* 1990); this may prove problematical because of the doubtful ability of MSR monomers to engage ligand (Via *et al.* 1985). Preliminary studies have excluded lipoproteins as important adhesion ligands in normal sera and

other candidate molecules currently investigated are the acute phase response protein serum amyloid P (SAP), surfactant proteins A and D (Kuroki and Voelkers 1994), and proteoglycan-LDL (PG-LDL) complexes such as PG-M-CSF-LDL (Suzu *et al.* 1994). It is also important to bear in mind that multiple serum ligands may exist for MSR adhesion, either in isolation or complexed, and that the tissue ligand(s) responsible for MSR-compatible adhesion in frozen section adhesion assays may again be different.

10.4 Characterization of Mϕ phenotypes in atherosclerotic lesions in humans and in mouse models of atherosclerosis

As is evident from the immunohistochemical studies involving mice deficient in apoE and M-CSF, the development of genetically engineered mouse models have validated their use as models for human atherosclerosis. The solution to the conundrum of the MSR's pro- or anti-atherogenic role could involve either subjecting MSR-deficient mice to an atherogenic diet or crossing them with apoE-deficient animals; the results from these studies are eagerly awaited. Another atherosclerosis model in which Mϕ phenotypic heterogeneity may be illuminating, is the apo(a) transgenic mouse. Atherosclerotic lesions in these mice show reduced levels of active TGF-β (Grainger *et al.* 1994); and MSR expression on lesional Mϕ may, based on the *in vitro* data, be increased. In the dynamic micro-environment of the atheromatous plaque, an intimate relationship appears to exist between the MSR, its regulation by M-CSF and TGF-β, and the Mϕ secretion of apoE; the perturbation of any of these factors may affect the extent of foam cell formation and atherogenesis.

The relevance of the MSR as a functional adhesion molecule for Mϕ in these lesions and models may be investigated using a modified Stamper-Woodruff lymphocyte adhesion assay. EDTA-resistant Mϕ adhesion to frozen tissue sections have, in this *ex vivo* analysis, implicated MSR (Hughes *et al.* 1995). Adhesion assays of vascular tree atherosclerotic lesions (as well as other organs) in the above described mouse models of atherosclerosis will be informative.

The analysis of Mϕ heterogeneity in human atherosclerotic lesions has been limited thus far. Investigation of an adhesion role for MSR in humans has been hampered by lack of specific reagents; polyclonal antisera against type-specific peptides have yielded unimpressive results. The development of specific anti-human MSR reagents is a major future objective and will require raising neutralizing mAbs against human MSR, targeting its divalent cation-independent

adhesion function as a screening strategy. The following approaches may be used:

i) Immunization with expressed MSR (type I and type II) protein. Production of MSR type-specific proteins may be engineered using an expression system for soluble protein expression. Purified protein may then be used as immunizing agent, and to screen for mAbs, accompanied by functional assays.

ii) Immunization with primary human monocyte/Mϕ and monocytoid cell lines expressing MSR activity and mRNA. Human blood monocytes differentiated to Mϕ after culture in human serum may be used as immunogen. Similarly, human Mϕ-like cell lines, including PMA-treated THP-1 cells, HL60, U937 and Mono Mac1 and 6 cells could be assessed as to their levels of MSR activity (AcLDL endocytosis and divalent cation-independent adhesion) and suitability for use as immunizing agents.

iii) DNA vaccination (Tang *et al.* 1992; Fynan *et al.* 1993). Using this novel approach, mRNA from PMA-treated THP-1 cells (which express abundant MSR) can be reverse transcribed yielding total cellular cDNA. The extracellular portion of the human MSR (type I and type II) can be PCR amplified using specific primers, subcloned and the functional expression of MSR protein confirmed by fluorescent DiI-AcLDL uptake in transfected COS cells. Rats can subsequently be immunized intramuscularly with MSR DNA and fusions done using Y3 myeloma cells. Functional screens may include inhibition of divalent cation-independent adhesion and DiI-AcLDL uptake.

Specific mAbs would enable characterization of human MSR on monocytes, culture-differentiated Mϕ and *in situ* analysis of Mϕ in normal and diseased tissues, including atherosclerotic lesions.

10.5 Signalling through the MSR relevant to atherogenesis.

A signalling role for the MSR in Mϕ activation may be important in Mϕ biology and atherogenesis. The major difficulty in interpreting published studies with ligands lies in the control for the MSR-specificity of the post-ligation/-internalization response. Modified LDL treatment of murine Mϕ causes secretion of arachidonic acid and reactive oxygen metabolites (Hartung *et al.* 1986; Hamilton *et al.* 1987), and induction of Mϕ urokinase-type plasminogen activator secretion; this response is protein kinase C-dependent (Falcone 1988; Falcone *et al.* 1991; Falcone *et al.* 1993). IL-1, neutral proteases, and early response proteins are secreted by Mϕ in response to MSR ligation, and MHC class II, TNF α, and PDGF are downregulated (Johnson *et al.* 1987; Hamilton *et al.* 1990; Malden *et*

al. 1991; Palkama 1991). A recent study suggests ligation of the MSR by OxLDL suppresses TNF-α gene expression by modulation of NFκB activation (Shackelford *et al.* 1995).

Data from my work (not shown) highlight some of the difficulties alluded to above. Cross-linking of murine MSR by intact mAb 2F8 increased IL-1 mRNA levels as measured by semi-quantitative RT-PCR assay. Involvement of FcγR-mediated signalling was controlled for by using an isotype matched mAb; this approach yielded inconsistent results. Subsequent efforts to generate functional $F(ab')_2$ fragments from mAb 2F8 were unsuccessful (N. Platt). These pilot experiments should be extended, but specific human and murine anti-MSR reagents need to be used, probably in solid-phase cross-linking experiments, to analyze the effects of MSR ligation and dissect second messengers and intracellular signalling pathways.

Characterization of Mϕ phenotypes in murine and human atherosclerotic lesions and analysis of an adhesion function for the MSR could shed light on the mechanisms underlying Mϕ recruitment, retention and involvement in atherogenesis. The development of specific mAbs, of possibly neutralizing and inhibiting functional nature, to the human MSR might serve as effective tools to dissect its role in human atherogenesis. These studies, together with increasing information as to the control of MSR function by cytokines and growth factors, could give rise to new therapeutic approaches to minimize the development of atherosclerosis.

References

Abrams, J.M., Lux, A., Steller, H. and Krieger, M. (1992). "Macrophages in *Drosophila* embryos and L2 cells exhibit scavenger receptor-mediated endocytosis." *Proc. Natl. Acad. Sci. USA* **89** : 10375-10379.

Acton, S., Resnick, D., Freeman, M., Ekkel, Y., Ashkenas, J. and Krieger, M. (1993). "The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificities for polyanionic ligands." *J. Biol. Chem.* **268** : 3530-3537.

Acton, S.L., Scherer, P.E., Lodish, H.F. and Krieger, M. (1994). "Expression cloning of SR-B1, a CD36-related class B scavenger receptor." *J. Biol. Chem.* **269** : 21003-21009.

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H., Krieger, M. (1996). "Identification of scavenger receptor SR-B1 as a high density lipoprotein receptor." *Science* **271**: 518-520.

Adams, J.L. and Czuprynski, C.J. (1994). "Mycobacterial cell wall components induce the production of TNF-α, IL-1, and IL-6 by bovine monocytes and the murine macrophage cell line RAW 264.7." *Microb. Pathol.* **16** : 401-411.

Aftring, R.P. and Freeman, M.W. (1995). "Structure of the murine macrophage scavenger receptor gene and evaluation of sequences that regulate expression in the macrophage cell line, $P388D_1$." *J. Lipid Res.* **36** : 1305-1314.

Akeson, A.L., Schroeder, K., Woods, C., Schmidt, C.J. and Jones, W.D. (1991). "Suppression of interleukin-1 beta and LDL scavenger receptor expression in macrophages by a selective protein kinase C inhibitor." *J. Lipid Res.* **32** : 1699-1707.

Amento, E.P., Ehsani, N., Palmer, H. and Libby, P. (1991). "Cytokines positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells." *Arteriosclerosis* **11** : 1223-1230.

Anderson, D.C. and Springer, T.A. (1987). "Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1 and p150,95 glycoproteins." *Ann. Rev. Med.* **38** : 175-194.

Anklesaria, P., Teixido, J., Laiho, M., Pierce, J.H., Greenberger, J.S. and Massague, J. (1990). "Cell-cell adhesion mediated by binding of membrane-anchored transforming growth factor α to epidermal growth factor receptors promotes cell proliferation." *Proc. Natl. Acad. Sci. USA* **87** : 3289-3293.

Aqel, N.M., Ball, R.Y., Waldmann, H. and Mitchinson, M.J. (1985). "Identification of macrophages and smooth muscle cells in human atherosclerosis using monoclonal antibodies." *J. Pathol.* **146** : 197-204.

Arai, H., Kita, T., Yokode, M., Naramiya, S. and Kawai, C. (1989). "Multiple receptors for modified LDL in mouse peritoneal macrophages: Different uptake mechanisms for acetylated and oxidized LDL." *Biochem Biophys Res Commun* **159** : 1375-1382.

Araki, N., Higashi, T., Mori, T., Shibayama, R., Kawabe, Y., Kodama, T., Takahashi, K., Shichiri, M. and Horiuchi, S. (1995). "Macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the Maillard reaction." *Eur. J. Biochem.* **230** : 408-415.

Aruffo, A., Melnick, M.B., Linsley, P.S. and Seed, B. (1991). "The lymphocyte glycoprotein CD6 contains a repeated domain structure characteristic of a new family of cell surface and secreted proteins." *J. Exp. Med.* **174** : 949-952.

Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M. and Krieger, M. (1993). "Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors." *J. Lipid Res.* **34** : 983-1000.

Assoian, R.K., Fleurdelys, B.E., Stevenson, H.C., Miller, P.J., Madtes, D.K., Raines, E.W., Ross, R. and Sporn, M.B. (1987). "Expression and secretion of type β transforming growth factor by activated human macrophages." *Proc. Natl. Acad. Sci. USA* **84** : 6020-6024.

Aubry, J.-P., Pochon, S., Graber, P., Jansen, K.V. and Bonnefoy, J.-

Y. (1992). "CD21 is a ligand for CD23 and regulates IgE production." *Nature* **358** : 505-507.

Austyn, J.M. and Gordon, S. (1981). "F4/80, a monoclonal antibody directed specifically against the mouse macrophage." *Eur. J. Immunol.* **11** : 805-815.

Aviram, M. (1989). "Low-density lipoprotein and scavenger receptor activities are modulated by secretory products derived from cells of the arterial wall." *Metabolism* **38** : 445-449.

Aviram, M. (1989). "Platelet secretory products enhance LDL receptor activity and inhibit scavenger receptor activity in human monocyte derived macrophages." *Metabolism* **38** : 425-430.

Bainton, D.F., Takemura, R., Stenberg, P.E. and Werb, Z. (1989). "Rapid fragmentation and reorganization of Golgi membranes during frustrated phagocytosis of immobile immune complexes by macrophages." *Am. J. Pathol.* **134** : 15-26.

Baird, A., Moremede, P. and Bohlen, P. (1985). "Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor." *Biochem. Biophy. Res. Comm.* **1263** : 358-364.

Barath, P., Fishbein, M.C., Cao, J., Berenson, J., Helfant, R.H. and Forrester, J.S. (1990). "Tumor necrosis factor gene expression in human vascular intimal smooth muscle cells detected by *in situ* hybridization." *Am. J. Pathol.* **137** : 503-509.

Basu, S.K., Brown, M.S., Ho, Y.K., Havel, R.J. and Goldstein, J.L. (1981). "Mouse macrophages synthesize and secrete a protein resembling apolipoprotein E." *Proc. Natl. Acad. Sci. USA* **78** : 7545-7549.

Basu, S.K., Ho, Y.K., Brown, M.S., Bilheimer, D.W., Anderson, R.G.W. and Goldstein, J.L. (1982). "Biochemical and genetic studies of the apoprotein E secreted by mouse macrophages and human monocytes." *J. Biol. Chem.* **257** : 9799-9795.

Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen-Pope, D.F. and Ross, R. (1990). "TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine loop." *Cell* **63** : 515-524.

Bazil, V. and Strominger, J.L. (1991). "Shedding as a mechanism of downmodulation of CD14 on stimulated human monocytes." *J. Immunol.* **147** : 1567-1574.

Bazil, V. and Horejsi, V. (1992). "Shedding of the CD44 adhesion molecule from leukocytes induced by anti-CD44 monoclonal antibody simulating the effect of a natural receptor ligand." *J. Immunol* **149** : 747-753.

Bazil, V. and Strominger, J.L. (1994). "Metalloprotease and serine-protease are involved in cleavage of CD43, CD44, and CD16 from stimulated human granulocytes - induction of cleavage of L-selectin via CD16." *J. Immunol.* **152** : 1314-1322.

Bazil, V. (1995). "Physiological enzymatic cleavage of leukocyte membrane molecules." *Immunol. Today* **16** : 135-140.

Becker, S., Warren, M.K. and Haskill, S. (1987). "Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures." *J. Immunol.* **139** : 3703-3709.

Beekhuizen, H., Blokland, I., Carsel van Tilburg, A.J., Koning, F. and van Furth, R. (1991). "CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells." *J. Immunol.* **147** : 3761-3767.

Beekhuizen, H., Verdegaal, E.M.E., Blokland, I. and van Furth, R. (1992). "Contribution of ICAM-1 and VCAM-1 to the morphological changes in monocytes bound to human venous endothelial cells stimulated with recombinant interleukin-4 (rIL-4) or rIL-1α." *Immunology* **77** : 469-472.

Begg, S.K., Radley, J.M., Pollard, J.W., Chisholm, O.T., Stanley, E.R. and Bertoncello, I. (1993). "Delayed hematopoietic development in osteopetrotic (*op/op*) mice." *J. Exp. Med.* **177** : 237-242.

Beppu, M., Hayashi, T., Hasegawa, T. and Kikugawa, K. (1995). "Recognition of sialosaccharide chains of glycophorin on damaged erythrocytes by macrophage scavenger receptors." *Bioch. Biophys. Acta* **1268** : 9-19.

Berliner, J.A., Navab, M., Fogelman, A., Frank, J.S., Demer, L.L., Edwards, P.A., Watson, A.D. and Lusis, A.J. (1995). "Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics." *Circulation* **91** : 2488-2496.

Bevilacqua, M.P. (1993). "Endothelial-leukocyte adhesion molecules." *Annu. Rev. Immunol.* **11** : 767-804.

Bevilacqua, M.P. and Nelson, R.M. (1993). "Selectins." *J. Clin. Invest.* **91** : 379-387.

Bickel, P.E. and Freeman, M.W. (1992). "Rabbit aortic smooth muscle cells express inducible macrophage scavenger receptor messenger RNA that is absent from endothelial cells." *J. Clin. Invest.* **90** : 1450-1457.

Bjorkerud, S. (1991). "Effects of transforming growth factor β1 on human arterial smooth muscle cells *in vitro*." *Arteriosclerosis* **11** : 892-902.

Blann, A.D., McCollum, C.N., Steiner, M. and Jayson, M.I.V. (1995). "Circulating adhesion molecules in inflammatory and atherosclerotic vascular disease." *Immunol. Today* **16** : 251-252.

Bogdan, C. and Nathan, C. (1993). "Modulation of macrophage function by transforming growth factor beta, interleukin-4, and interleukin-10." *Ann N Y Acad Sci* **685** : 713-39.

Bogdan, C., Vodovotz, Y. and Nathan, C. (1991). "Macrophage deactivation by interleukin 10." *J. Exp. Med.* **174** : 1549-1555.

Boisvert, W.A., Spangenberg, J. and Curtiss, L.K. (1995). "Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation." *J. Clin. Invest.* **96** : 1118-1124.

Bottalico, L.A., Wager, R.E., Agellon, L.B., Assoian, R.K. and Tabas, I. (1991). "Transforming growth factor-beta 1 inhibits scavenger receptor activity in THP-1 human macrophages." *J. Biol. Chem.* **266** : 22866-22871.

Bouhours, D. and Bouhours, J.F. (1988). "Tissue specific

expression of GM3 (NeuGs) and GD3 (NeuGc) in epithelial cells of the small intestine of strains of inbred rats. Absence of NeuGc in intestine and presence of kidney gangliosides in Brown Norway and spontaneously hypertensive rats." *J. Biol. Chem.* **263** : 15540-15545.

Brogi, E., Winkles, J., Underwood, R., Clinton, S., Alberts, G. and Libby, P. (1994). "Distinct patterns of expression of fibroblast growth factors and their receptors in human atheroma and non-atherosclerotic arteries: association of acidic FGF with plaque microvessels and macrophages." *J. Clin. Invest.* :

Brown, D.L., Phillips, D.R., Damsky, C.H. and Charo, I.F. (1989). "Synthesis and expression of the fibroblast fibronectin receptor in human monocytes." *J. Clin. Invest.* **84** : 366-370.

Brown, K.D., Zurawski, S.M., Mosmann, T.R. and Zurawski, G. (1989). "A family of small inducible proteins secreted by leukocytes are members of a new superfamily that includes leukocyte and fibroblast-derived inflammatory agents, growth factors, and indicators of various activation processes." *J. Immunol.* **142** : 679-687.

Brown, M.S. and Goldstein, J.L. (1983). "Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis." *Ann. Rev. Biochem.* **52** : 223-261.

Brown, M.S. and Goldstein, J.L. (1986). "A receptor-mediated pathway for cholesterol homeostasis." *Science* **232** : 34-47.

Brown, M.S., Herz, J., Kowal, R.C. and Goldstein, J.L. (1991). "The low density lipoprotein receptor-related protein: double agent or decoy?" *Curr. Opin. Lipidol.* **2** : 65-72.

Brungs, M., Radmark, O., Samuelsson, B. and Steinhilber, D. (1995). "Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor β and 1,25-dihydroxyvitamin D_3." *Proc. Natl. Acad. Sci. USA* **92** : 107-111.

Bufler, P., Stiegler, G., Schuchmann, M., Hess, S., Kruger, C., Stelter, F., Eckerskorn, C., Schutt, C. and Engelmann, H. (1995). "Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants." *Eur. J. Immunol.* **25** : 604-610.

Camp, R.L., Scheynius, A., Johansson, C. and Pure, E. (1993). "CD44 is necessary for optimal allergic responses but is not required for normal leukocyte extravasation." *J. Exp. Med.* **178** : 497-507.

Campbell, E.J., Cury, J.D., Shapiro, S.D., Goldberg, G.I. and Welgus, H.G. (1991). "Neutral proteinases of human mononuclear phagocytes. Cellular differentiation markedly alters cell phenotypes for serine proteinases, metalloproteinases, and tissue inhibitor of metalloproteinases." *J. Immunol.* **146** : 1286-1293.

Capecchi, M.R. (1989). "Altering the genome by homologous recombination." *Science* **244** : 1288-1292.

Carew, T.E., Schwenke, D.C. and Steinberg, D. (1987). "Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe Heritable Hyperlipidemic rabbit." *Proc. Natl. Acad. Sci. USA* **84** : 7725-7729.

Carlos, T., Kovach, N., Schwartz, B., Rosa, M., Newman, B., Wayner, E., Benjamin, C., Osborn, L., Lobb, R. and Harlan, J. (1991). "Human monocytes bind to two, cytokine-induced adhesive ligands on cultured human endothelial cells: Endothelial-leukocyte adhesion molecule-1 and Vascular cell adhesion molecule-1." *Blood* **77** : 2266-2271.

Carlos, T.M. and Harlan, J.M. (1994). "Leukocyte-endothelial adhesion molecules." *Blood* **84** : 2068-2101.

Caroccilo, D., Shirsat, N., Wong, G.G., Lange, B., Clark, S. and Romera, G. (1987). "Recombinant human macrophage colony stimulating factor (M-CSF) requires subliminal concentrations of granulocyte macrophage (GM) CSF for optimal stimulation of human macrophage colony formation *in vitro*." *J. Exp. Med.* **166** : 1851-1860.

Charo, I.F. (1992). "Monocyte-endothelial cell interactions." *Curr. Opin. Lipidol.* **3** : 335-343.

Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J. and Coughlin, S.R. (1994). "Molecular cloning and functional expression of two monocyte chemoattractant protein-1 receptors reveals alternative

splicing of the carboxyl-terminal tails." *Proc. Natl. Acad. Sci. USA* **91** : 2752-2756.

Chen, W.J., Goldstein, J.L. and Brown, M.S. (1990). "NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor." *J. Biol. Chem.* **265** : 3116-3123.

Cherwinski, H.M., Schumacher, J.H., Brown, K.D. and Mosmann, T.R. (1987). "Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies." *J. Exp. Med.* **166** : 1229-1244.

Clinton, S.K., Fleet, J.C., Loppnow, H., Salomon, R.N., Clark, B.D., Cannon, J.G. and al, e. (1991). "Interleukin-1 gene expression in rabbit vascular tissue *in vivo*." *Am. J. Pathol.* **138** : 1005-1014.

Clinton, S.K., Underwood, R., Hayes, L., Sherman, M.L., Kufe, D.W. and Libby, P. (1992). "Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis." *Am. J. Pathol.* **140** : 301-316.

Cohen, C. and Parry, D.A.D. (1990). "Alpha-helical coiled coils and bundles: how to design an alpha-helical protein." *Proteins* **7** : 1-15.

Collawn, J.F., Stangel, M., Kuhn, L.A., Esekogwu, V., Jing, S.Q., Trowbridge, I.S. and Tainer, J.A. (1990). "Transferrin internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis." *Cell* **63** : 1061-1072.

Collin, M., Montaner, L.J., Herbein, G. and Gordon, G. (1995). *HIV infection of macrophages*. IRL. 63-75.

Collins, T. (1992). *J. Vasc. Surg.* **15** : 923-924.

Cook, D.N., Beck, M.A., Coffman, T.M., Kirby, S.L., Sheridan, J.F., Pragnell, I.B., Smithies, O. (1995). "Requirement of MIP-1α for an inflammatory response to viral infection." *Science* **269** : 1583-1585.

Corcoran, M.I., Stetler-Stevenson, W.G., Brown, P.D. and Wahl, L.M. (1992). "Interleukin 4 inhibition of prostaglandin E2 synthesis

blocks interstitial collagenase and 92-kDa type IV collagenase/gelatinase production by human monocytes." *J. Biol. Chem.* **267** : 515-519.

Corfield, A.P., Wagner, S.A., Paaskeva, C., Clamp, J.R., Durdey, P., Reuter, G. and Schauer, R. (1992). "Loss of sialic acids O-acetylation in human colorectal cancer cells." *Biochemal Society Transactions* **20** : 94S.

Crocker, P.R., Kelm, S., Dubois, C., Martin, B., McWilliam, A.S., Shotton, D.M., Paulson, J.C. and Gordon, S. (1991). "Purification and properties of sialoadhesin, a sialic acid-binding receptor of murine tissue macrophages." *EMBO J.* **10** : 1661-1669.

Crocker, P.R., Mucklow, S., Bouckson, V., McWilliam, A., Willis, A.C., Gordon, S., Milon, G., Kelm, S. and Bradfield, P. (1994). "Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains." *EMBO J.* **13** : 4490-4503.

Cushing, S.D., Berliner, J.A., Valente, A.J., Territo, M.C., Navab, M., Parhami, F., Gerrity, R., Schwartz, C.J. and Fogelman, A.M. (1990). "Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells." *Proc. Natl. Acad. Sci. USA* **87** : 5134-5138.

Cushing, S.D. and Fogelman, A.M. (1992). "Monocytes may amplify their recruitment into inflammatory lesions by inducing monocyte chemoattractant protein." *Arterioscler. Thromb.* **12** : 78-82.

Cybulsky, M.I. and Gimbrone, M.A.J. (1991). "Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis." *Science* **251** : 788-791.

D'Andrea, A., Ma, X., Aste-Amezaga, M., Paganin, C. and Trinchieri, G. (1995). "Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production." *J Exp Med* **181** : 537-46.

Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A and Stewart TA (1993). "Multiple defects of immune cell function in mice with disrupted interferon-γ genes." *Science* **259** : 1739-1742.

Dangott, L.J., Jordan, J.E., Bellet, R.A. and Garbers, D.L. (1989). "Cloning of the mRNA for the protein that crosslinks to the egg peptide speract." *Proc. Natl. Acad. Sci. USA* **86** : 2128-2132.

Danielsson, C., Pascual, M., French, L., Steiger, G. and Schifferli, J.-A. (1994). "Soluble complement receptor type 1 (CD35) is released from leukocytes by surface cleavage." *Eur. J. Immunol.* **24** : 2725-2731.

Das, S.K., Stanley, E.R., Guilbert, L.J. and Forman, L.W. (1981). "Human colony-stimulating factor (CSF-1) radioimmunoassay: resolution of three subclasses of human colony-stimulating factor." *Blood* **58** : 630-641.

de Bono, D. (1994). "Significance of raised plasma concentrations of tissue type plasminogen activator and plasminogen activator inhibitor in patients at risk from ischaemic heart disease." *Br. Heart J.* **71** : 504-507.

De Nichilo, M.O. and Burns, G.F. (1993). "Granulocyte-macrophage and macrophage colony-stimulating factors differentially regulate αv integrin expression on cultured human macrophages." *Proc. Natl. Acad. Sci. USA* **90** : 2517-2521.

de Rijke, Y.B. and van Berkel, T.J. (1994). "Rat liver Kupffer and endothelial cells express different binding proteins for modified low density lipoproteins. Kupffer cells express a 95-kDa membrane protein as a specific binding site for oxidized low density lipoproteins." *J. Biol. Chem.* **269** : 824-827.

de Villiers, W.J.S., Fraser, I.P. and Gordon, S. (1994). "Cytokine and growth factor regulation of macrophage scavenger receptor expression and function." *Immunology Letters* **43** : 73-79.

de Villiers, W.J.S., Fraser, I.P., Hughes, D.A., Doyle, A.G. and S., G. (1994). "Macrophage-colony-stimulating factor selectively enhances macrophage scavenger receptor expression and function." *J. Exp. Med.* **180** : 705-709.

de Villiers, W.J.S., Fraser, I.P., Hughes, D.A. and Gordon, S. (1995). *Macrophage adhesion in atherosclerosis: a role for the scavenger receptor?* Atherosclerosis X, Montreal, Elsevier Science B.V.

de Waal Malefyt R, Abrams J, Bennett B, Figdor CG and de Vries JE (1991). "Interleukin 10 inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes." *J Exp Med* **174** : 1209-1220.

De Waal Malefyt, R., Figdor, C.G., Huijbens, R., Mohan-Peterson, S., Bennett, B., Culpepper, J., Dang, W., Zurawski, G. and De Vries, J.E. (1993). "Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. Comparison with IL-4 and modulation by IFN-γ or IL-10." *J Immunol* **151** : 6370-6381.

de Whalley, C.V. and Riches, D.W.H. (1991). "Influence of the cytocidal macrophage phenotype on the degradation of acetylated low density lipoproteins: dual regulation of scavenger receptor activity and of intracellular degradation of endocytosed ligand." *Exp. Cell Res.* **192** : 460-468.

Dejager, S., Mietus-Snyder, M., Friera, A. and Pitas, R.E. (1993). "Dominant negative mutations of the scavenger receptor. Native receptor inactivation by expression of truncated variants." *J. Clin. Invest.* **92** : 894-902.

Dejager, S., Mietus-Synder, M. and Pitas, R.E. (1993). "Oxidized low density lipoproteins bind to the scavenger receptor expressed by rabbit smooth muscle cells and macrophages." *Arterioscler. Thromb.* **13** : 371-378.

Demaria, S., Schwab, R., Gottesman, S.R.S. and Bushkin, Y. (1994). "Soluble β2-microglobulin-free Class I heavy chains are released from the surface of activated and leukemia cells by a metalloprotease." *J. Biol. Chem.* **269** : 6689-6694.

Diamond, M.S., Staunton, D.E., de Fougerolles, A.R., Stacker, S.A., Garcia-Aguilar, J., Hibbs, M.L. and Springer, T.A. (1990). "ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18)." *J. Cell Biol.* **111** : 3129-3139.

Doi, T., Higashino, K.-i., Kurihara, Y., Wada, Y., Miyazaki, T., Nakamura, H., Uesugi, S., Imanishi, T., Kawabe, Y., Itakura, H., Yazaki, Y., Matsumoto, A. and Kodama, T. (1993). "Charged collagen structure mediates the recognition of negatively charged macromolecules by macrophage scavenger receptors." *J. Biol. Chem.* **268** : 2126-2133.

Doi, T., Kurasawa, M., Higashino, K.-i., Imanishi, T., Mori, T., Naito, M., Takahashi, K., Kawabe, Y., Wada, Y., Matsumoto, A. and Kodama, T. (1994). "The histidine interruption of an alpha-helical coiled-coil domain allosterically mediated a pH-dependent ligand dissociation from macrophage scavenger receptors." *J. Biol. Chem.* **269** : 25598-25604.

Doyle, A.G., Herbein, G., Montaner, L.J., Minty, A.J., Caput, D., Ferrara, P. and Gordon, S. (1994). "Interleukin-13 alters the activation state of murine macrophages *in vitro*: comparison with interleukin-4 and interferon-γ." *Eur. J. Immunol.* **24** : 1441-1445.

Dunne, D.W., Resnick, D., Greenberg, J., Krieger, M. and Joiner, K.A. (1994). "The type I macrophage scavenger receptor binds to Gram-positive bacteria and recognizes lipoteichoic acid." *Proc. Natl. Acad. Sci. USA* **91** : 1863-1867.

Durieux, J.-J., Vita, N., Popescu, O., Guette, F., Calzada-Wack, J., Munker, R., Schmidt, R.E., Lupker, J., Ferrara, P., Ziegler-Heitbrock, H.W.L. and Labeta, M.O. (1994). "The two soluble forms of the lipopolysaccharide receptor, CD14: characterization and release by normal human monocytes." *Eur. J. Immunol.* **9** :

Dustin, M.L. and Springer, T.A. (1991). "Role of lymphocyte adhesion receptors in transient interactions and cell locomotion." *Ann. Rev. Immunol.* **9** : 27-66.

Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987). "Protein serine/threonine kinases." *Ann. Rev. Biochem.* **56** : 567-613.

El Khoury, J., Thomas, C.A., Loike, J.D., Hickman, S.E., Cao, L. and Silverstein, S.C. (1994). "Macrophages adhere to glucose-modified basement membrane collagen IV via their scavenger receptors." *J. Biol. Chem.* **269** : 10197-10200.

Elbein, A.D. (1987). "Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains." *Ann. Rev. Biochem.* **56** : 497-534.

Elford, P.R., Felix, R., Cecchini, M., Trechsel, U. and Fleisch, H. (1987). "Murine osteoblastlike cells and the osteogenic cell MC3T3-E1 release a macrophage colony-stimulating activity in culture." *Calcif. Tissue Int.* **41** : 151-156.

Elliott, M.J., Vadas, M.A., Cleland, L.G., Gamble, J.R. and Lopez, A.F. (1990). "IL-3 and Granulocyte-Macrophage Colony-Stimulating Factor stimulate two distinct phases of adhesion in human monocytes." *J. Immunol.* **145** : 167-176.

Elomaa, O., Kangas, M., Sahlberg, C., Tuukkanen, J., Sormunen, R., Liakka, A., Thesleff, I., Kraal, G. and Tryggvason, K. (1995). "Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages." *Cell* **80** : 603-609.

Emi, M., Asaoka, H., Matsumoto, A., Itakura, H., Kurihara, Y., Wada, Y., Kanamori, H., Yazaki, Y., Takahashi, E.-i., Lepert, M., Lalouel, J.-M., Kodama, T. and Mukai, T. (1993). "Structure, organization, and chromosomal mapping of the human macrophage scavenger receptor gene." *J. Biol. Chem.* **268** : 2120-2125.

Endemann, G., Stanton, L.W., Madden, K.S., Bryant, C.M., White, R.T. and Protter, A.A. (1993). "CD36 is a receptor for oxidized low density lipoprotein." *J. Biol. Chem.* **268** : 11811-11816.

Etzioni, A., Frydman, M., Pollack, S., Avidor, I., Phillips, M.L., Paulson, J.C. and Gershoni-Baruch, R. (1992). "Recurrent severe infections caused by a novel leukocyte adhesion deficiency." *New Engl. J. Med.* **327** : 1789-1792.

Evans, R.M. (1994). "Intermediate filaments and lipoprotein cholesterol." *Trends Cell Biology* **4** : 149-151.

Faggiotto, A., Ross, R. and Harker, L. (1984). "Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation." *Arteriosclerosis* **4** : 323-340.

Falcone, D.J. (1988). "Heparin stimulation of plasminogen activator secretion by macrophage-like cell line RAW 264.7: role of the scavenger receptor." *J. Cell. Physiol.* **140** : 219-226.

Falcone, D.J., McCaffrey, T.A., Haimovitz-Friedman, A., Vergilio, J.-A. and Nicholson, A.C. (1993). "Macrophage and foam cell release of matrix-bound growth factors." *J. Biol. Chem.* **268** : 1191-11958.

Falcone, D.J., McCaffrey, T.A. and Vergilio, J.-A. (1991).

"Stimulation of macrophage urokinase expression by polyanions is protein kinase C-dependent and requires protein and RNA synthesis." *J. Biol. Chem.* **266** : 22726-22732.

Fan, K., Ruan, Q., Sensenbrenner, L. and Chen, B. (1992). "Transforming growth factor B1 bifunctionally regulates murine macrophage proliferation." *Blood* **79** : 1679-1685.

Faruqi, R., de la Motte, C. and DiCorleto, P.E. (1994). "α-Tocopherol inhibits agonist-induced monocytic cell adhesion to cultured human endothelial cells." *J. Clin. Invest.* **94** : 592-600.

Faulkner, L., Aruoma, O.I., Brickell, P.M., Davies, M.J., Halliwell, B., Woolf, N. and Katz, D.R. (1993). "Effects of the synthetic anti-oxidant, probucol, on the U937 monoblastoid cell line." *Atherosclerosis* **99** : 1-13.

Fauve, R.M., Jusforgues, H. and Hevin, B. (1983). "Maintenance of granuloma macrophages in serum-free medium." *J. Immunol. Methods* **64** : 345-351.

Felix, R., Cecchini, M.G. and Fleisch, H. (1990). "M-CSF restores *in vivo* bone resorption in the op/op mouse." *Endocrinology* **127** : 2592-2594.

Felix, R., Cecchini, M.G., Hofstetter, W., Elferel, P.C., Sutzer, A. and Fleisch, H. (1990). "Impairment of macrophage colony stimulating factor production and lack of resident bone marrow macrophages in the osteopetrotic op/op mouse." *J. Bone Mineral Res.* **5** : 781-789.

Ferns, G., Raines, E.W., Sprugel, K., Motani, A., Reidy, M. and Ross, R. (1991). "Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF." *Science* **253** : 1129-1132.

Ferns, G.A.A., Forster, L., Stewart-Lee, A., Konneh, M., Nourooz-Zadeh, J. and Anggard, E.E. (1992). "Probucol inhibits neointimal thickening and macrophage accumulation after balloon injury in the cholesterol-fed rabbit." *Proc. Natl. Acad. Sci. USA* **89** : 11312-11316.

Fischer, D.G., Tal, N., Novick, D., Barak, S. and Rubinstein, M. (1993). "An antiviral soluble form of the LDL receptor induced by interferon." *Science* **262** : 250-253.

Fitch, F.W., McKisic, M.D., Lancki, D.W. and Gajewski, T.F. (1993). "Differential regulation of murine T lymphocyte subsets." *Annu Rev. Immunol.* **11** : 29-48.

Floros, J., Steinbrink, R., Jacobs, K., Phelps, D., Kriz, R., Recny, M., Sultzman, L., Jones, S., Taeusch, H.W., Frank, H.A. and Fritsch, E.F. (1986). "Isolation and characterisation of cDNA clones for the 35-kD pulmonary surfactant-associated protein." *J. Biol. Chem.* **261** : 9029-9033.

Fogelman, A.M., Haberland, M.E., Seager, J., Hokom, M. and Edwards, P.A. (1981). "Factors regulating the activities of the low density lipoprotein receptor and the scavenger receptor on human monocyte-macrophages." *J. Lipid Res.* **22** : 1131-1141.

Fogelman, A.M., Seager, J., Haberland, M.E., Hokom, M., Tanaka, R. and Edwards, P.A. (1982). "Lymphocyte-conditioned medium protects human monocyte-macrophages from cholesteryl ester accumulation." *Proc. Natl. Acad. Sci. USA* **79** : 922-926.

Fong, L.G., Fong, T.A.T. and Cooper, A.D. (1990). "Inhibition of mouse macrophage degradation of acetyl-low density lipoprotein by Interferon-γ." *J. Biol. Chem.* **265** : 11751-11760.

Fowler, S., Shio, H. and Haley, N.J. (1979). "Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations." *Lab. Invest.* **41** : 372-378.

Franek, K.J., Wolcot, F.R., Chervenak, R. (1994). " Reliable method for the simultaneous detection of cytoplasmic and surface CD3ε expression by murine lymphoid cells." *Cytometry* 17: 224-236.

Franke, W.W., Hergt, M., Grundt, C. (1987). " Rearrangement of the vimentin cytoskeleton during adipose conversion: formation of an intermediate filament cage around lipid droplets." *Cell.* **49** : 131-141.

Fraser, I. and Gordon, S. (1993). "An overview of receptors of MPS cells." *Blood Cell Biochem.* **5** : 1-27.

Fraser, I., Hughes, D. and Gordon, S. (1993). "Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor." *Nature* **364** : 343-346.

Fraser, I.P. (1993). Macrophage haemagglutinins and scavenger receptors: their roles in adhesion and endocytosis. University of Oxford.

Freeman, M., Ashkenas, J., Rees, D.J.D., Kingsley, D.M., Copeland, N.G., Jenkins, N.A. and Krieger, M. (1990). "An ancient, highly conserved family of cysteine-rich protein domains revealed by cloning type I and type II murine macrophage scavenger receptors." *Proc. Natl. Acad. Sci. USA* **87** : 8810-8814.

Freeman, M., Ekkel, Y., Rohrer, L., Penman, M., Freedman, N.J., Chisolm, G.M. and Krieger, M. (1991). "Expression of type I and type II bovine scavenger receptors in Chinese hamster ovary cells: lipid droplet accumulation and nonreciprocal cross competition by acetylated and oxidized low density lipoprotein." *Proc. Natl. Acad. Sci. USA* **88** : 4931-4935.

Freeman, M.W. (1994). "Macrophage scavenger receptors." *Curr. Opin. Lipidol.* **5** : 143-148.

Fridman, W.H., Teillaud, J.-L., Bouchard, C. *et al.* (1993). " Soluble Fcγ receptors." *J. Leukocyte Biol.* **54** : 504-512.

Friedman, J., Trahey, M. and Weissman, I. (1993). "Cloning and characterization of cyclophilin C-associated protein: A candidate natural cellular ligand for cyclophilin C." *Proc. Natl. Acad. Sci. USA* **90** : 6815-6819.

Frostregard, J., Wu, R., Giscombe, R., Holm, G., Lefvert, A.K. and Nilsson, J. (1992). "Induction of T-cell activation by oxidized low density lipoprotein." *Arteriosclerosis Thromb.* **12** : 461-467.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C. and Robinson, H.L. (1993). "DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations." *Proc. Natl. Acad. Sci. USA* **90** : 11478-11482.

Galis, Z.S., Sukhova, G.K., Kranzhofer, R., Clark, S. and Libby, P. (1995). "Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases." *Proc. Natl. Acad. Sci. USA* **92** : 402-406.

Garg, U.C. and Hassid, A. (1989). "Nitric oxide-generating vasodilators and 8-romo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells." *J. Clin. Invest.* **83** : 1774-1777.

Gay, C.G. and Winkles, J.A. (1991). "Interleukin-1 regulates heparin-binding growth factor 2 gene expression in vascular smooth muscle cells." *Proc. Natl. Acad. Sci. USA* **88** : 296-300.

Gearing, A.J.H. and Newman, W. (1993). "Circulating adhesion molecules in disease." *Immunology Today* **14** : 506-512.

Gegner, J.A., Ulevitch, R.J. and Tobias, P.S. (1995). "Lipopolysaccharide (LPS) signal transduction and clearance. Dual roles for LPS binding protein and membrane CD14." *J. Biol. Chem.* **270** : 5320-5325.

Geng, Y.-j. and Hansson, G.K. (1992). "Interferon-γ inhibits scavenger receptor expression and foam cell formation in human monocyte-derived macrophages." *J. Clin. Invest.* **89** : 1322-1330.

Geng, Y.-J., Kodama, T. and Hansson, G.K. (1994). "Differential espression of scavenger receptor isoforms during monocyte-macrophage differentiation and foam cell formation." *Arterioscler. Thromb.* **14** : 798-806.

Geng, Y.-J. and Hansson, G.K. (1995). "High endothelial cells of postcapillary venules express the scavenger receptor in human peripheral lymph nodes." *Scand. J. Immunol.* **42** : 289-296.

Gerrity, R.G., Goss, J.A. and Soby, L. (1985). "Control of monocyte recruitment by chemotactic factor(s) in lesion-prone areas of swine aorta." *Arteriosclerosis* **5** : 55-66.

Gierasch, L.M. (1989). "Signal sequences." *Biochem.* **28** : 923-930.

Gipson, I.K., Spurr-Michaud, S., Tisdale, A., Elwell, J. and Stepp, M.A. (1993). "Redistribution of the hemidesmosome components α6β4 integrin and bullous pemphigoid antigens during epithelial wound healing." *Exp. Cell Res.* **207** : 86-98.

Giry, C., Giroux, L.-M., Roy, M., Davignon, J. and Minnich, A.

(1995). "Characterization of inherited scavenger receptor overexpression and abnormal macrophage phenotype in a normolipidemic subject with planar xanthomas." *J. Lipid Res.* : in press.

Gisselbrecht, S., Sola, B., Fichelson, S., Bordereaux, D., Tambourin, P., Mattei, M.-G., Simon, D. and Guenet, J.-L. (1989). "The murine M-CSF gene is localized on chromosome 3." *Blood* **73** : 1742-1746.

Goldberger, G., Bruns, G.A.P., Rits, M., Edge, M.D. and Kwiatkowski, D.J. (1987). "Human complement factor I: analysis of cDNA-derived primary structure and assignment of its gene to chromosome 4." *J. Biol. Chem.* **262** : 10065-10071.

Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979). "Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein producing massive cholesterol deposition." *Proc Natl Acad Sci USA* **76** : 333-337.

Gong, Q. and Pitas, R.E. (1995). "Synergistic effects of growth factors on the regulation of smooth muscle cell scavenger receptor activity." *J. Biol. Chem.* **270** : 21672-21678.

Gordon, D., Reidy, M.A., Benditt, E.P. and Schwartz, S.M. (1990). "Cell proliferation in human coronary arteries." *Proc. Natl. Acad. Sci. USA* **87** : 4600-4604.

Gordon, S., da Silva, R., McWilliam, A. and Stein, M. (1992). *Macrophage membrane glycoproteins and inflammation.* Leukocyte adhesion. Basic and clinical aspects. Elsevier Science Publishers.

Gordon, S., Lawson, L., Rabinowitz, S., Crocker, P.R., Morris, L. and Perry, V.H. (1992). "Antigen markers of macrophage differentiation in murine tissues." *Curr. Topics Microbiol. Immunol.* **181** : 1-37.

Gordon, S., Clarke, S., Greaves, D. and Doyle, A. (1995). "Molecular immunobiology of macrophages: recent progress." *Curr. Opin. Immunol.* **7** : 24-33.

Gougos, A. and Letarte, M. (1990). "Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells." *J. Biol. Chem.* **265** : 8361-8364.

Goyert, S.M., Ferrero, E., Rettig, W.J., Yenamandra, A.K., Obata, F. and Le Beau, M.M. (1988). "The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors." *Science* **239** : 497-500.

Grainger, D.J., Kirschenlohr, H.L., Metcalfe, J.C., Weissberg, P.L., Wade, D.P. and Lawn, R.M. (1993). "Proliferation of human smooth muscle cells promoted by lipoprotein(a)." *Science* **260** : 1655-1658.

Grainger, D.J., Kemp, P.R., Liu, A.C., Lawn, R.M. and Metcalfe, J.C. (1994). "Activation of transforming growth factor-β is inhibited in transgenic apolipoprotein(a) mice." *Nature* **370** : 460-462.

Grainger, D.J., Kemp, P.R., Metcalfe, J.C., Liu, A.C., Lawn, R.M., Williams, N.R., Grace, A.A., Schofield, P.M. and Chauhan, A. (1995). "The serum concentration of active transforming growth factor-β is severely depressed in advanced atherosclerosis." *Nature Medicine* **1** : 74-79.

Griffiths, G., McDowall, A.W., Back, R., Dubochet, J. (1984). "On the preparation of cryosections for immunochemistry." *J. Ultrastruct. Res.* **89**: 65-78.

Haak-Frendscho, M., Wynn, T.A., Czuprynski, C.J. and Paulnock, D. (1990). "Transforming growth factor-β1 inhibits activation of macrophage cell line RAW 264.7 for cell killing." *Clin. Exp. Immunol.* **82** : 404-410.

Hale, G., Cobbold, S.P., Waldmann, H., Easter, G., Matejtschuk, P. and Coombs, R.R. (1987). "Isolation of low-frequency class-switch variants from rat hybrid myelomas." *J. Immunol. Met.* **103** : 59-67.

Hamilton, J.A., Vairo, G., Knight, K.R. and Cocks, B.G. (1991). "Activation and proliferation signals in murine macrophages. Biochemical signals controlling the regulation of macrophage urokinase-type plasminogen activator activity by colony-stimulating factors and other agents." *Blood* **77** : 616-627.

Hamilton, T.A., Gainey, P.V. and Adams, D.O. (1987). "Maleylated-BSA suppresses Ifn-γ–mediated Ia expression in murine peritoneal macrophages." *J. Immunol.* **138** : 4063-4068.

Hamilton, T.A., Guoping, M. and Chisolm, G.M. (1990). "Oxidized low density lipoprotein suppresses the expression of tumor necrosis factor-α mRNA in stimulated murine peritoneal macrophages." *J. Immunol.* **144** : 2343-2350.

Hampton, R.Y., Golenbock, D.T., Penman, M., Krieger, M. and Raetz, C.R.H. (1991). "Recognition and plasma clearance of endotoxin by scavenger receptors." *Nature* **352** : 342- 344.

Hanamura, T., Motoyoshi, K., Yoshida, K., Saito, M., Miura, Y., Kawashima, T., Nishida, M. and Takaku, F. (1988). "Quantitation and identification of human monocyte colony-stimulating factor in human serum by enzyme-linked immunosorbent assay." *Blood* **72** : 886-892.

Hansson, G.K., Holm, J. and Jonasson, L. (1989). "Detection of activated T lymphocytes in the human atherosclerotic plaque." *Am. J. Pathol.* **135** : 169-175.

Hara, H., Tanishita, H., Yokoyama, S., Tajima, S. and Yamamoto, A. (1987). "Induction of acetylated low density lipoprotein receptor and suppression of low density lipoprotein receptor on the cells of human monocytic leukemia cell line (THP-1 cell)." *Biochem. Biophys. Res. Comm.* **146** : 802-808.

Hara, S., Nagano, Y., Sasada, M. and Kita, T. (1992). "Probucol pretreatment enhances the chemotaxis of mouse peritoneal macrophages." *Arterioscler. Thromb.* **12** : 593-600.

Harrison, D., Phillips, J.H. and Lanier, L.L. (1991). "Involvement of a metalloprotease in spontaneous and phorbol ester-induced release of natural-killer cell-associated Fcγ-RIII (CD16-II)." *J. Immunol.* **147** : 3459-3465.

Harter, C. and Mellman, I. (1992). "Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane." *J Cell Biology* **117** : 311-325.

Hartung, H.P., Kladetzky, R.G., Melnik, B. and Hennerici, M. (1986). "Stimulation of the scavenger receptor on monocytes-macrophages evokes release of arachidonic acid metabolites and reduced oxygen species." *Lab. Invest.* **55** : 209-216.

Hausmann, E.H., Hao, S.Y., Pace, J.L. and Parmely, M.J. (1994). "Transforming growth factor-β1 and γ- interferon provide opposing signals to lipopolysaccharide-activated mouse macrophages." *Infect. Immun.* **62** : 3625-3632.

Haynes, B.F., Telen, M.J., Hale, L.P. and Denning, S.M. (1989). "CD44 - a molecule involved in leukocyte adherence and T cell-activation." *Immunol. Today* **10** : 423-428.

Hemler, M.E. (1990). "VLA proteins in the integrin family: structures, functions, and their role on leukocytes." *Ann. Rev. Immunol.* **8** : 365-400.

Henney, A.M., Wakeley, P.R., Davies, M.J., Foster, K., Hembry, R., Murphy, G. and Humphries, S. (1991). "Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization." *Proc. Natl. Acad. Sci. USA* **88** : 8154-8158.

Henson, D.A., St. Clair, R.W. and Lewis, J.C. (1989). "Morphological characterization of β-VLDL and acetylated-LDL binding and internalization by cultured pigeon monocytes." *Exp. Molec. Pathol.* **51** : 243-263.

Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C. and Klagsbrun, M. (1991). "A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF." *Science* **251** : 936-939.

Hirsch, L.J. and Mazzone, T. (1986). "Dexamethasone modulates lipoprotein metabolism in cultured human monocyte-derived macrophages. Stimulation of scavenger receptor activity." *J. Clin. Invest.* **77** : 485-490.

Hoffman, R.A., Kung, P.C., Hansen, W.P. and Goldstein, G. (1980). "Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood." *Proc. Natl. Acad. Sci. USA* **77** : 4914-4917.

Hogg, N. (1992). "The role of integrins in macrophage biology." *Pathobiol.* **60(S1)** : 14.

Hogg, N. and Landis, R.C. (1993). "Adhesion molecules in cell interactions." *Curr. Opin. Immunol.* **5** : 383-390.

Holers, V.M., Ruff, T.G., Parks, D.L., McDonald, J.A., Ballard, L.L. and Brown, E.J. (1989). "Molecular cloning of a murine fibronectin receptor and its expression during inflammation." *J. Exp. Med.* **169** : 1589-1605.

Holness, C.L., da Silva, R.P., Fawcett, J., Gordon, S. and Simmons, D.L. (1993). "Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family." *J. Biol. Chem.* **268** : 9661-9666.

Holness, C.L. and Simmons, D.L. (1993). "Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins." *Blood* **81** : 1607-1613.

Honda, M., Wada, Y., Suzuki, H., Jishage, K., Kawabe, Y., Shimokawa, T., Kurihara, Y., Kurihara, H., Asaoka, H., Matsumoto, A., Itakura, H., Yazaki, Y., Naito, M., Mori, T., Takahashi, K., Nakamura, H., Matsushita, M., Emi, M., Doi, T. and Kodama, T. (1995). *Physiological role of macrophage scavenger receptors.* Atherosclerosis X, Montreal, Elsevier Science B.V.

Horowitz, M.C. (1993). "Cytokines and estrogen in bone: anti-osteoporotic effects." *Science* **260** : 626-627.

Horvai, A., Palinski, W., Wu, H., Moulton, K.S., Kalla, K. and Glass, C.K. (1995). "Scavenger receptor A gene regulatory elements target gene expression to macrophages and to foam cells of atherosclerotic lesions." *Proc. Natl. Acad. Sci. USA* **92** : 5391-5395.

Hsieh C-S, Macatonia SE, Tripp CS, Wolf SF, O'Garra A and Murphy KM (1993). "Development of T_H1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages." *Science* **260** : 547-549.

Hsu, S.M., Raine, L. and Fanger, H. (1981). "The use of avidin-biotin complex (ABC) in immunoperoxidase techniques; a comparison between ABC and unlabelled antibody (PAP) procedures." *J. Histochem. Cytochem.* **29** : 577-587.

Huang, S., Hendriks, W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilek, J., Zinkernagel, R.M. and Aguet, M. (1993). "Immune responses in mice that lack the interferon-γ receptor." *Science* **259** : 1742-1745.

Hughes, D.A. (1994). Murine macrophage molecules: characterization and function. Oxford University.

Hughes, D.A., Fraser, I.P. and Gordon, S. (1995). "Murine macrophage scavenger receptor: *in vivo* expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs." *Eur. J. Immunol.* **25** : 466-473.

Hume, D.A., Pavli, P., Donahue, R.E. and Fidler, I.J. (1988). "The effect of human recombinant macrophage colony-stimulating factor (CSF-1) on the murine mononuclear phagocyte system in vivo." *J. Immunol.* **141** : 3405-3409.

Hurtley, S.M. and Helenius, A. (1989). "Protein oligomerization in the endoplasmic reticulum." *Ann. Rev. Cell Biol.* **5** : 277-307.

Hussaini, I.M., Srikumar, K., Quesenberry, P.J. and Gonias, S.L. (1990). "Colony-stimulating factor-1 modulates alpha 2-macroglobulin receptor expression in murine bone marrow macrophages." *J. Biol. Chem.* **265** : 19441-19446.

Hynes, R.O. (1992). "Integrins: versatility, modulation, and signalling in cell adhesion." *Cell* **69** : 11-25.

Inaba, T., Gotoda, T., Shimano, H., Shimada, M., Harada, K., Kozaki, K., Watanabe, Y., Hoh, E., Motoyoshi, K., Yazaki, Y. and Yamada, N. (1992). "Platelet-derived growth factor induces *c-fms* and scavenger receptor genes in vascular smooth muscle cells." *J. Biol. Chem.* **267** : 13107-13112.

Inaba, T., Shimano, H., Gotoda, T., Harada, K., Shimada, M., Kawamura, M., Yazaki, Y. and Yamada, N. (1993). "Macrophage colony-stimulating factor regulates both activities of neutral and acidic cholesteryl ester hydrolases in human monocyte-derived macrophages." *J. Clin. Invest.* **92** : 750-757.

Inaba, T., Shimano, H., Gotoda, T., Harada, K., Shimada, M., Ohsuga, J., Watanabe, Y., Kawamura, M., Yazaki, Y. and Yamada, N. (1993). "Expression of platelet-derived growth factor β receptor on human monocyte-derived macrophages and effects of platelet-derived growth factor BB dimer on the cellular function." *J. Biol. Chem.* **268** : 24353-24360.

Inoue, I., Inaba, T., Motoyoshi, K., Harada, K., Shimano, H., Kawamura, M., Gotoda, T., Oka, T., Shiomi, M., Watanabe, Y., Tsukuda, T., Yazaki, Y., Takaku, F. and Yamada, N. (1992). "Macrophage colony stimulating factor prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits." *Atherosclerosis* **93** : 245-254.

Ishibashi, S., Inaba, T., Shimano, H., Harada, K., Inoue, I., Mokuno, H., Mori, N., Gotoda, T., Takaku, F. and Yamada, N. (1990). "Monocyte colony-stimulating factor enhances uptake and degradation of acetylated low density lipoproteins and cholesterol esterification in human monocyte-derived macrophages." *J. Biol. Chem.* **265** : 14109-14117.

Janeway, C.A.J. (1992). "The immune system evolved to discriminate infectious nonself from noninfectious self." *Immunol. Today* **13** : 11-16.

Johnson, P.A., Jansen, M.M., Somers, S.D., Adams, D.O. and Hamilton, T.A. (1987). "Maleyl-BSA and fucoidan induce expression of a set of early proteins in murine mononuclear phagocytes." *J. Immunol.* **138** : 1551-1558.

Johnson, R.B.J., Godzik, C.A. and Cohn, Z.A. (1978). "Increased superoxide anion production by immunologically activated and chemically elicited macrophages." *J. Exp. Med.* **148** : 115-127.

Jonasson, L., Holm, J., Skalli, O., Bondjers, G. and Hansson, G.K. (1986). "Regional accumulation of T cells, macrophages, and smooth muscle cels in the human atherosclerotic plaque." *Arteriosclerosis* **6** : 131-138.

Jones, N.H., Clabby, M.L., Dialynas, D.P., Huang, H.-J.S., Herzenberg, L.A. and Strominger, J.L. (1986). "Isolation of complementary DNA clones encoding the human lymphocyte glycoprotein T1/Leu-1." *Nature* **323** : 346-349.

Joris, T., Nunnari, J.J., Krolikowski, F.J. and Majno, G. (1983). "Studies on the pathogenesis of atherosclerosis. I. Adhesion and emigration of mononuclear cells in the aorta of hypercholesterolemic rats." *Am. J. Pathol.* **113** : 341-358.

Joseph-Silverstein, J., Moscatelli, D. and Rifkin, D.B. (1988). "The identification of bFGF in adherent elicited murine peritoneal macrophages." *J. Immunol. Methods* **110** : 183-192.

Jouni, Z.E. and McNamara, D.J. (1991). "Lipoprotein receptors of human macrophages. Effects of differentiation with tetramyristic phorbol acetate and 1,25-dihydroxyvitamin D_3." *Arterioscler. Thromb.* **11** : 995-1006.

Jungi, T.W., Brcic, M., Eperon, S. and Albrecht, S. (1994). "Transforming growth factor-β and IL-10, but not IL-4, down-regulate procoagulant activity and tissue factor expression in human monocyte-derived macrophages." *Thromb. Res.* **76** : 463-474.

Kahn, J., Ingraham, R.H., Shirley, F., Migaki, G.I. and Kishimoto, T.K. (1994). "Membrane proximal cleavage of L-selectin: identification of the cleavage site and a 6-kD transmembrane peptide fragment of L-selectin." *J. Cell Biol.* **125** : 461-470.

Kasama, T., Strieter, R.M., Standiford, T.J., Burdick, M.D. and Kunkel, S.L. (1993). "Expression and regulation of human-derived macrophage inflammatory protein 1α." *J. Exp. Med.* **178** : 63-72.

Kawasaki, E.S. and Ladner, M.B. (1990). *Molecular biology of macrophage colony-stimulating factor. Colony-stimulating factors. Molecular and cellular biology.* New York and Basel, Marcel Dekker. 155-176.

Kishimoto, T.K., Hollander, N., Roberts, T.M., Anderson, D.C. and Springer, T.A. (1987). "Heterogeneous mutations in the beta subunit common to the LFA-1, Mac-1, and p150,95 glycoproteins cause leukocyte adhesion deficiency." *Cell* **50** : 193-202.

Kishimoto, T.K., Jutila, M.A., Berg, E.L. and Butcher, E.C. (1989). "Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors." *Science* **245** : 1238-1241.

Kita, T., Nagano, Y., Yokode, M., Ishi, K., Kume, N., Ooshima, A., Yoshida, H. and Kawai, C. (1987). "Probucol prevents the progression of atherosclerosis in the Watanabe Heritable Hyperlipidemic rabbit, an animal model for familial hypercholesterolemia." *Proc. Natl. Acad. Sci. USA* **84** : 5928-5931.

Kitazawa, S., Ross, F.P., McHugh, K. and Teitelbaum, S.L. (1995). "Interleukin-4 induces expression of the integrin $\alpha_v\beta_3$ via transactivation of the β_3 gene." *J. Biol. Chem.* **270** : 4115-4120.

Kitchens, R.L., Ulevitch, R.J. and Munford, R.S. (1992). "Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway." *J. Exp. Med.* **176** : 485-494.

Klausner, R.D., Lippincott-Schwartz, J. and Bonifacino, J.S. (1990). "The T cell antigen receptor: insights into organelle biology." *Ann. Rev, Cell Biol.* **6** : 403-431.

Klausner, R.D., Donaldson, J.G. and Lippincott-Schwartz, J. (1992). "Brefeldin A: Insights into the control of membrane traffic and organelle structure." *J. Cell Biol.* **116** : 1071-1080.

Klurfeld, D.M. (1985). "Identification of foam cells in human atherosclerotic lesions as macrophages using monoclonal antibodies." *Arch Pathol Lab Med* **109** : 445-449.

Kobzik, L. (1995). "Lung macrophage uptake of unopsonized environmental particulate. Role of scavenger-type receptors." *J. Immunol.* **155** : 367-376.

Kodama, H., Nose, M., Niida, S. and Yamasaki, A. (1991). "Essential role of macrophage colony-stimulating factor in the osteoclast differentiation supported by stromal cells." *J. Exp. Med.* **173** : 1291-1294.

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. and Krieger, M. (1990). "Type I macrophage scavenger receptor contains α-helical and collagen-like coiled coils." *Nature* **343** : 531-535.

Kodama, T., Reddy, P., Kishimoto, C. and Krieger, M. (1988). "Purification and characterization of a bovine acetyl low density lipoprotein receptor." *Proc. Natl. Acad. Sci. USA* **85** : 9238-9242.

Kohno, T., Brewer, M.T., Baker, S.L., Schwartz, P.E., King, M.W., Hale, K.K., Squires, C.H., Thompson, R.C. and Vannice, J.L. (1990). "A second tumor necrosis factor receptor gene product can shed a naturally occuring tumor necrosis factor inhibitor." *Proc. Natl. Acad. Sci. USA* **87** :

8331-8335.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H. and Kohler, G. (1993). "Disruption of the murine IL-4 gene blocks Th2 cytokine responses." *Nature* **362** : 245-248.

Koren, H.S., Handwerger, B.S. and Wunderlich, J.R. (1975). "Identification of macrophage-like characteristics in a cultured murine tumor line." *J. Immunol.* **114** : 894-897.

Koths, K., Taylor, E., Halenbeck, R., Casipit, C. and Wang, A. (1993). "Cloning and characterization of a human Mac-2-binding protein, a new member of the superfamily defined by the macrophage scavenger receptor cysteine-rich domain." *J. Biol. Chem.* **268** : 14245-14249.

Kowala, M.C., Mazzucco, C.E., Hartl, K.S., Seiler, S.M., Warr, G.A., Abid, S. and Grove, R.I. (1993). "Prostacyclin agonists reduce early atherosclerosis in hyperlipidemic hamsters. Octimibate and BMY 42393 suppress monocyte chemotaxis, macrophage cholesteryl ester accumulation, scavenger receptor activity, and tumor necrosis factor production." *Arterioscler. Thromb.* **13** : 435-444.

Kraal, G., Breel, M., Janse, M. and Bruin, G. (1986). "Langerhans cells, veiled cells and interdigitating cells in the mouse recognized by a monoclonal antibody." *J. Exp. Med.* **163** : 981-997.

Krieger, M. (1992). "Molecular flypaper and atherosclerosis: structure of the macrophage scavenger receptor." *Trends Biochem Sci* **17** : 141-146.

Krieger, M., Acton, S., Ashkenas, J., Pearson, A., Penman, M. and Resnick, D. (1993). "Molecular flypaper, host defense, and atherosclerosis." *J. Biol. Chem.* **268** : 4569-4572.

Krieger, M. and Herz, J. (1994). "Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP)." *Annu. Rev. Biochem.* **63** : 601-637.

Kriegler, M., Perez, C., DeFay, K., I., A. and Lu, S.D. (1988). "A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF." *Cell* **53** : 45-53.

Kruth, H.S., Skarlatos, S.I., Lilly, K., Chang, J. and Ifrim, I. (1995). "Sequestration of acetylated LDL and cholesterol crystals by human monocyte-derived macrophages." *J. Cell Biology* **129** : 133-145.

Kulkarni, A.B., Huh, C.H., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M. and Karlsson, S. (1993). "Transforming growth factor-β1 null mutation in mice causes excessive inflammatory response and early death." *Proc. Natl. Acad. Sci. USA* **91** : 770-774.

Kume, N., Arai, H., Kawai, C. and Kita, T. (1991). "Receptors for modified low-density lipoproteins on human endothelial cells: different recognition for acetylated low-density lipoprotein and oxidized low-density lipoprotein." *Biochim. Biophys. Acta* **1091** : 63.

Kume, N., Cybulsky, M.I. and Gimbrone, M.A.J. (1992). "Lysophophatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells." *J. Clin. Invest.* **90** : 1138-1144.

Kume, N. and Gimbrone, M.A.J. (1994). "Lysophophatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells." *J. Clin. Invest.* **93** : 907-911.

Kume, S., Takeya, M., Mori, T., Araki, N., Suzuki, H., Horiuchi, S., Kodama, T., Miyauchi, Y. and Takahashi, K. (1995). "Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific antibody." *Am. J. Pathol.* **147** : 654-667.

Kurihara, Y., Matsumoto, A., Itakura, H. and Kodama, T. (1991). "Macrophage scavenger receptors." *Curr. Opin. Lipidol.* **2** : 295-300.

Kuroki, Y. and Voelkers, D.R. (1994). "Pulmonary surfactant proteins." *J. Biol. Chem.* **269** : 25943-25946.

Labeta, M.O., Durieux, J.-J., Fernandez, N., Herrmann, R. and Ferrara, P. (1993). "Release from a human monocyte-like cell line of two different soluble forms of the lipopolysaccharide receptor, CD14." *Eur. J. Immunol.* **23** : 2144-2151.

Lachmann, P.J. (1967). "Conglutinin and immunoconglutinins." *Adv. Immunol.* **6** : 479-527.

Lacraz, S., Nicod, L., Galve-de-Rochemontiex, B., Baumberger, C., Dayer, J.M. and Welgus, H.M. (1992). "Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4." *J. Clin. Invest.* **90** : 382-388.

Laemmli, U.K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature* **227** : 680-681.

Larsen, E., Palabrica, T., Sajer, S., Gilbert, G.E., Wagner, D.D., Furie, B.C. and Furie, B. (1990). "PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15)." *Cell* **63** : 467-474.

Lasky, L.A. (1992). "Selectins: interpreters of cell-specific carbohydrate information during inflammation." *Science* **258** : 964-969.

Lastres, P., Almendro, N., Bellon, T., Lopez-Guerrero, J.A., Eritja, R. and Bernabeu, C. (1994). "Functional regulation of platelet/endothelial adhesion molecule-1 by TGF-β1 in promonocytic U-937 cells." *J. Immunol.* **153** : 4206.

Lastres, P., Bellon, T., Cabanas, C., Sanchez-Madrid, F., Acevedo, A., Gougos, A., Letarte, M. and Bernabeu, C. (1992). "Regulated expression on human macrophages of endoglin, an Arg-Gly-Asp-containing surface antigen." *Eur. J. Immunol.* **22** : 393-397.

Lau, W., Devery, J.M. and Geczy, C.L. (1995). "A chemotactic S100 peptide enhances scavenger receptor and Mac-1 expression and cholesteryl ester accumulation in murine peritoneal macrophages *in vivo*." *J. Clin. Invest.* **95** : 1957-1965.

Lawrence, M.B. and Springer, T.A. (1991). "Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins." *Cell* **65** : 859-873.

Lee, W.K., Bell, J., Kilpatrick, E., Hayes, M., Lindop, G.B. and Dominiczak, M.H. (1993). "Collagen-linked fluorescence in human atherosclerotic plaques." *Atherosclerosis* **98** : 219-227.

Leeuwenberg, J.F.M., Jeunhomme, T.M.A.A. and Buurman, W.A. (1994). "Slow release of soluble TNF receptors by monocytes *in vitro*." *J. Immunol.* **152** : 4036-4043.

Lendon, C.L., Davies, M.J., Born, G.V. and Richardson, P.D. (1991). "Atherosclerotic plaque caps are locally weakened when macrophage density is increased." *Atherosclerosis* **87** : 87-90.

Letellier, M., Nakajima, T., Pulido-Cejudo, G., Hofstetter, H. and Delespesse, G. (1990). "Mechanisms of formation of human IgE-binding factors (soluble CD23). III. Evidence for a receptor (FcεRII)-associated proteolytic activity." *J. Exp. Med.* **172** : 693-700.

Li, H., Cybulsky, M.I., Gimbrone, M.A.J. and Libby, P. (1993). "An atherogenic diet rapidly induces VCAM-1, a cytokine regulatable mononuclear leukocyte adhesion molecule, in rabbit endothelium." *Arteriosclerosis Thromb* **13** : 197-204.

Li, H., Freeman, M.W. and Libby, P. (1995). "Regulation of smooth muscle scavenger receptor expression in vivo by atherogenic diets and in vitro by cytokines." *J. Clin. Invest.* **95** : 122-133.

Liao, F., Andalibi, A., deBeer, F.C., Fogelman, A.M. and Lusis, A.J. (1993). "Genetic control of inflammatory gene induction and NF-κB-like transcription factor activation in response to an atherogenic diet in mice." *J. Clin. Invest.* **91** : 2572-2579.

Liao, F., Berliner, J.A., Mehrabian, M., Navab, M., Demer, L.L., Lusis, A.J. and Fogelman, A.M. (1991). "Minimally modified low density lipoprotein is biologically active *in vivo* in mice." *J. Clin. Invest.* **87** : 2253-2257.

Liau, G. and Chan, L.M. (1989). "Regulation of extracellular matrix RNA levels in cultured smooth muscle cells. Relationship to cellular quiescence." *J. Biol. Chem.* **264** : 1-6.

Libby, P., Warner, S.J.C. and Friedman, G.B. (1988). "Interleukin-1: A mitogen for human vascular smooth muscle cells that induces the release of growth-inhibitory prostanoids." *J. Clin. Invest.* **88** : 487-498.

Libby, P. and Clinton, S.K. (1993). "The role of macrophages in atherogenesis." *Curr. Opinion Lipid.* **4** : 355-363.

Lieschke, G.J., Stanley, E., Grail, D., Hodgson, G., Sinickas, V., Gall, J.A.M., Sinclair, R.A. and Dunn, A.R. (1994). "Mice lacking both macrophage- and granulocyte-macrophage colony-stimulating factor have macrophages and coexistent osteopetrosis and severe lung disease." *Blood* **84** : 27-35.

Linton, M.F., Atkinson, J.B. and Fazio, S. (1995). "Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation." *Science* **267** : 1034-1037.

Lundgren, C.H., Sawa, H., Sobel, B.E. and Fujii, S. (1994). "Modulation of expression of monocyte/macrophage plasminogen activator activity and its implications for attenuation of vasculopathy." *Circulation* **90** : 1927-1934.

Luoma, J., Hiltunen, T., Sarkioja, T., Moestrup, S.K., Gliemann, J., Kodama, T., Nikkari, T. and Yla-Herttuala, S. (1994). "Expression of alpha2-macroglobulin receptor/low density lipoprotein-related protein and scavenger receptor in human atherosclerotic lesions." *J. Clin. Invest.* **93** : 2014-2021.

Lupu, F., Bergonzelli, G.E., Heim, D.A., Cousin, E., Genton, C.Y., Bachmann, F. and Kruithof, E.K. (1993). "Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries." *Arterioscler. Thromb.* **13** : 1090-1100.

Lyons, C.R., Orloff, G.J. and Cunningham, J.M. (1992). "Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line." *J. Biol. Chem.* **267** : 6370-6374.

Madtes, D.K., Raines, E.W., Sakariassen, K.S., Assoian, R.K., Sporn, M.B., Bell, G.I. and Ross, R. (1988). "Induction of transforming growth factor-α in activated human alveolar macrophages." *Cell* **53** : 285-293.

Makgoba, M.W., Sanders, M.E., Luce, G.E.G., Dustin, M.L., Springer, T.A., Clark, E.D., Mannoni, P. and Shaw, S. (1988). "ICAM-1, a ligand for LFA-1 dependent adhesion of B, T and myeloid cells." *Nature* **331** : 86-88.

Malden, L.T., Chait, A., Raines, E.W. and Ross, R. (1991). "The

influence of oxidatively modified low density lipoproteins on expression of platelet-derived growth factor by human monocyte-derived macrophages." *J. Biol. Chem.* **266** : 13901-13907.

Mansour, S.L., Thomas, K.R. and Cappecchi, M.R. (1988). "Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes." *Nature* **336** : 348-352.

Martinet, Y., Bitterman, P.B., Mornex, J.F., Grotendorst, G.R., Martin, G.R. and Crystal, R.G. (1986). "Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity." *Nature* **319** : 158-160.

Massague, J. (1990). "The transforming growth factor-β family." *Annu. Rev. Cell Biol.* **6** : 597-641.

Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asaoka, H., Hayakawa, I., Kanamori, H., Aburatani, H., Takaku, F., Suzuki, H., Kobari, Y., Miyai, T., Takahashi, K., Cohen, E.H., Wydro, R., Housman, D.E. and Kodama, T. (1990). "Human macrophage scavenger receptors: primary structure, expression, and localization in atherosclerotic lesions." *Proc Natl Acad Sci USA* **87** : 9133-9137.

Mattana, J., Margiloff, L. and Singhal, P.C. (1995). "Oxidation of mesangial matrix using a mixed function oxidase system augments adhesion of macrophages: possible role of macrophage scavenger receptors." *Biochem. Biophys. Res. Comm.* **212** : 63-69.

Mayadas, T.N., Johnson, R.C., Rayburn, H., Hynes, R.O. and Wagner, D.D. (1993). "Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice." *Cell* **74** : 541-554.

McKnight, A.J., Macfarlane, A.J., Dri, P., Turley, L., Willis, A.C., Gordon, S. (1996). "Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family." *J. Biol Chem.* **271**: 486-489.

McGrath, J.A., Gatalica, B., Christiano, A.M., Li, K., Owaribe, K., McMillan, J.R., Eady, R.A.J. and Uitto, J. (1995). "Mutations in the 180-kD bullous pemphigoid antigen (BPAG2), a hemidesmosomal

transmembrane collagen (COL17A1), in generalized atrophic benign epidermolysis bullosa." *Nature Genetics* **11** : 83-86.

Medof, M.E., Walter, E.I., Rutgers, J.L., Knowles, D.M. and Nussenzweig, V. (1987). "Identification of the complement decay accelerating factor on epithelium and glandular cells and in body fluids." *J. Exp. Med.* **165** : 848-864.

Meek, R.L., Eriksen, N. and Benditt, E.P. (1992). "Murine serum amyloid A_3 is a high density apolipoprotein and is secreted by macrophages." *Proc. Natl. Acad. Sci. USA* **89** : 7949-7952.

Melkko, J., Hellevik, T., Risteli, L., Risteli, J. and Smedsrod, B. (1994). "Clearance of NH_2-terminal propeptides of types I and III procollagen is a physiological function of the scavenger receptor in liver endothelial cells." *J. Exp. Med.* **179** : 405-412.

Mellman, I., Fuchs, R. and Helenius, A. (1986). "Acidification of the endocytic and exocytic pathways." *Ann. Rev. Biochem.* **55** : 663-700.

Michl, J., Pieczonka, M.M., Unkeless, J.C. and Silverstein, S.C. (1979). "Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages." *J. Exp. Med.* **150** : 607-621.

Minty, A. (1995). *Structure, function and mechanism of action of interleukin-13. Human cytokines: Handbook for basic and clinical research.* Oxford, Oxford University Press. in press.

Minty, A., Chalon, P., Derocq, J.-M., Dumont, X., Guillemot, J.-C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shire, D., Ferrara, P. and Caput, D. (1993). "Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses." *Nature* **362** : 248-250.

Mitchinson, M.J., Reid, V.C., Hardwick, S.J., Clare, K., Carpenter, K.L.H. and Marchant, C.E. (1995). *LDL-induced macrophage toxicicty.* Atherosclerosis X, Montreal, Elsevier Science B.V.

Moestrup, S.K., Kaltoft, K., Petersen, C.M., Pedersen, S., Gliemann, J. and Christensen, E.I. (1990). "Immunocytochemical identification of the human alpha 2-macroglobulin receptor in monocytes and

fibroblasts: monoclonal antibodies define the receptor as a monocyte differentiation antigen." *Exp. Cell Res.* **190** : 195-302.

Mommaas-Kienhuis, A.M., van der Schroeff, J.G., Wijsman, M.C., Daems, W.T. and Vermeer, B.J. (1985). "Conjugates of colloidal gold with native and acetylated low density lipoproteins for ultrastructural investigations on receptor-mediated endocytosis by cultured human monocyte-derived macrophages." *Histochemistry* **83** : 29-35.

Moore, K.L., Stults, N.L., Diaz, S., Smith, D.F., Cummings, R.D., Varki, A. and McEver, R.R. (1992). "Identification of a specific glycoprotein ligand for P-Selectin (CD62) on myeloid cells." *J. Cell Biol.* **118** : 445-456.

Moore, K.W., O'Garra, A., de Waal Malefyt, R., Vieira, P. and Mosmann, T.R. (1993). "Interleukin-10." *Annu Rev Immunol* **11** : 165-190.

Moore, R.N., Larsen, H.S., Horohov, D.W. and Rouse, B.T. (1984). "Endogenous regulation of macrophage proliferative expansion by conony-stimulating factor-induced interferon." *Science.* **223** : 178-180.

Moore, R.N., Oppenheim, J.J., Farrar, J.J., Carter, C.S., Waheed, A. and Shadduck, P.K. (1980). "Production of lymphocyte-activating factor (interleukin-1) by macrophages activated with colony stimulating factors." *J. Immunol.* **125** : 1302-1305.

Morganelli, P.M., Rogers, R.A., Kitzmiller, T.J. and Bergeron, A. (1995). "Enhanced metabolism of LDL aggregates mediated by specific monocyte IgG Fc receptors." *J. Lipid Res.* **36** : 714-724.

Mori, T., Takahashi, K., Naito, M., Kodama, T., Hakamata, H., Sakai, M., Miyazaki, A., Horiuchi, S. and Ando, M. (1994). "Endocytic pathway of scavenger receptors via trans-Golgi system in bovine alveolar macrophages." *Laboratory Investigation* **71** : 409-416.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L. (1986). "Two types of murine helper T cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins." *J. Immunol.* **136** : 2348-2357.

Motoyoshi, K. and Takaku, F. (1989). "Serum cholesterol-lowering

activity of human monocytic colony-stimulating factor." *Lancet* **ii** : 326-327.

Moulton, K.S., Wu, H., Barnett, J., Parthasarathy, S. and Glass, C.K. (1992). "Regulated expression of the human acetylated low density lipoprotein receptor gene and isolation of promoter sequences." *Proc. Natl. Acad. Sci. USA* **89** : 8102-8106.

Moyer, C.F., Sajuthi, D., Tulli, H. and Williams, J.K. (1991). "Synthesis of IL-1 alpha and IL-1 beta by arterial cells in atherosclerosis." *Am. J. Pathol.* **138** : 951-960.

Mui, A.L.-F., Kay, R.J., Humphries, R.K. and Krystal, G. (1992). "Ligand-induced phosphorylation of the murine interleukin 3 receptor signals its cleavage." *Proc. Natl. Acad. Sci. USA* **89** : 10812-10816.

Mullberg, J., Schooltink, H., Stoyan, T. *et al.* (1993). "The soluble interleukin-6 receptor is generated by shedding." *Eur. J. Immunol.* **23** : 473-480.

Muller, W.A., Weigl, S.A., Deng, X.H. and Phillips, D.M. (1993). "PECAM-1 is required for transendothelial migration of leukocytes." *J. Exp. Med.* **178** : 449-460.

Murphy, P.M. (1994). "The molecular biology of leukocyte chemoattractant receptors." *Annu. Rev. Immunol.* **12** : 593-633.

Murphy, P.M. and McDermott, D. (1991). "Functional expression of the human formy peptide receptor in *Xenopus* oocytes requires a complementary human factor." *J. Biol. Chem.* **266** : 12560-12567.

Nabel, E.G., Shum, L., Pompili, V.J., Yang, Z.Y., San, H., Shu, H.B., Liptay, S., Gold, L., Gordon, D., Derynck, R., Nabel, G.J. (1993). "Direct transfer of the TGF-β gene into arteries stimulates fibrocellular hyperplasia." *Proc. Natl. Acad. Sci. USA* **90** : 10759-10763.

Nagelkerke, J.F., Barto, K.P. and van Berkel, T.J.C. (1983). "*In vivo* and *in vitro* uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer, and parenchymal cells." *J. Biol. Chem.* **258** : 12221.

Naito, M., Kodama, T., Matsumoto, A., Doi, T. and Takahashi, K.

(1991). "Tissue distribution, intracellular localization, and *in vitro* expression of bovine macrophage scavenger receptors." *Am. J. Pathol.* **139** : 1411-1423.

Naito, M., Suzuki, H., Mori, T., Matsumoto, A., Kodama, T. and Takahashi, K. (1992). "Coexpression of type I and type II human macrophage scavenger receptors in macrophages of various organs and foam cells in atherosclerotic lesions." *Am. J. Pathol.* **141** : 591-599.

Nakashima, Y., Plump, A.S., Raines, E.W., Breslow, J.L. and Ross, R. (1994). "ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree." *Arterioscler. Thromb.* **14** : 133-140.

Nassar, G.M., Morrow, J.D., Roberts, L.J., Lakkis, F.G. and Badr, K.F. (1994). "Induction of 15-lipoxygenase by interleukin-13 in human blood monocytes." *J. Biol. Chem.* **269** : 27631-27634.

Nathan, C. and Sporn, M. (1991). "Cytokines in context." *J. Cell Biol.* **113** : 981-986.

Nathan, C., Xie, Q., Halbwachs-Mecarelli, L. and Jin, W.W. (1993). "Albumin inhibits neutrophil spreading and hydrogen peroxide release by blocking the shedding of CD43 (sialophorin, leukosialin)." *J. Cell Biol.* **122** : 243-256.

Navab, M., Hama, S.Y., Nguyen, T.B. and Fogelman, A.M. (1994). "Monocyte adhesion and transmigration in atherosclerosis." *Coronary Artery Disease* **5** : 198-204.

Neish, A.S., Williams, A.J., Palmer, H.J., Whitley, M.Z. and Collins, T. (1992). "Functional analysis of the human vascular cell adhesion molecule 1 promoter." *J. Exp. Med.* **176** : 1583-1593.

Nelken, N., Coughlin, S., Gordon, D. and Wilcox, J. (1991). "Monocyte chemoattractant protein-1 in human atheromatous plaques." *J. Clin. Invest.* **88** : 1121-1127.

Neote, K., DiGregorio, D., Mak, J.Y., Horuk, R. and Schall, T.J. (1993). "Molecular cloning, functional expression and signaling characteristics of a C-C chemokine receptor." *Cell* **72** : 415-425.

Newman, P.J., Berndt, M.C., Gorski, J., White, G.C., Lyman, S., Paddock, C. and Muller, W.A. (1990). "PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily." *Science* **247** : 1219-1222.

Nikol, S., Isner, J.M., Pickering, J.G., Kearney, M., Leclerc, G. and Weir, L. (1992). "Expression of transforming growth factor-beta 1 is increased in human vascular restenosis lesions." *J Clin Invest* **90** : 1582-92.

Nilsson, S.K. and Bertoncello, I. (1994). "Age-related changes in extramedullary hematopoiesis in the spleen of normal and perturbed osteopetrotic (*op/op*) mice." *Exp. Hematology* **22** : 377-383.

Nimer, S.D., Champlin, R.E. and Golde, D.W. (1988). "Serum cholesterol-lowering activity of granulocyte-macrophage colony-stimulating factor." *JAMA* **260** : 3297-3300.

Nishikawa, K., Arai, H. and Inoue, K. (1990). "Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phopspholipids by mouse peritoneal macrophages." *J. Biol. Chem.* **265** : 5226-5231.

Nozaki, S., Kashiwagi, H., Yamashita, S., Nakagawa, T., Kostner, B., Tomiyama, Y., Nakata, A., Ishigami, M., Miyagawa, J., Kameda-Takemura, K., Kurata, Y. and Matsuzawa, Y. (1995). "Reduced uptake of oxidized low density lipoproteins in monocyte-derived macrophages from CD36-deficient subjects." *J. Clin. Invest.* **96**: 1859-1865.

O'Brien, K., Allen, M., McDonald, T., Chait, A., Harlan, J., Fishbein, D., McCarty, J., Ferguson, M., Hudkins, K., Benjamin, C., Lobb, R. and Alpers, C. (1993). "Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques: implications for the mode of progression of advanced coronary atherosclerosis." *J. Clin. Invest.* **92** : 945-951.

O'Brien, K.D., Gordon, D., Deeb, S., Ferguson, M. and Chait, A. (1992). "Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques." *J. Clin. Invest.* **89** : 1544-1550.

Ohno, M., Cooke, J.P., Dzau, V.J. and Gibbons, G.H. (1995). "Fluid

shear stress induces endothelial transforming growth factor beta-1 transcription and production." *J. Clin. Invest.* **95** : 1363-1369.

Ottnad, E., Parthasarathy, S., Sambrano, G.R., Ramprasad, M.P., Quehenberger, O., Kondratenko, N., Green, S. and Steinberg, D. (1995). "A macrophage receptor for oxidized low density lipoprotein distinct from the receptor for acetyl low density lipoprotein: partial purification and role in recognition of oxidatively damaged cells." *Proc. Natl. Acad. Sci. USA* **92** : 1391-1395.

Owens, G.K., Geisterfer, A.A., Yang, Y.W. and Komoriya, A. (1988). "Transforming growth factor-b-induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells." *J. Cell Biol.* **107** : 771-780.

Pace, J., Hayman, M.J. and Galan, J.E. (1993). "Signal transduction and invasion of epithelial cells by S. typhimurium." *Cell* **72** : 505-514.

Paigen, B., Ishida, B.Y., Verstuyft, J., Winters, R.B. and Albee, D. (1990). "Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice." *Arteriosclerosis* **10** : 316-323.

Palinski, W., Ord, V.A., Plump, A.S., Breslow, J.L., Steinberg, D. and Witztum, J.L. (1994). "Apo E-deficient mice are a model of lipoprotein oxidation. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum." *Arterioscler. Thromb.* **14** : 605-616.

Palkama, T. (1991). "Induction of interleukin-1 production of ligands binding to the scavenger receptor in human monocytes and the THP-1 cell line." *Immunology* **74** : 432-438.

Pandiella, A. and Massague, J. (1991). "Cleavage of the membrane precursor for transforming growth factor alpha is a regulated process." *Proc. Natl. Acad. Sci. USA* **88** : 1726-1730.

Pandiella, A., Bosenberg, M.W., Huang, E.J., Besmer, P. and Massague, J. (1992). "Cleavage of membrane-anchored growth factors involves distinct protease activities regulated through common mechanisms." *J. Biol. Chem.* **267** : 24028-24033.

Pantazis, P., Kharbanda, S., Scott Goustin, A., Galanopoulos, T.

and Kufe, D. (1991). "Coexpression of the genes for platelet-derived growth factor B-chain receptor and macrophage colony-stimulating factor 1 receptor during monocytic differentiation." *Proc. Natl. Acad. Sci. USA* **88** : 2481-2485.

Parker, T.S., Levine, D.M., Donnelly, T.M., Stoudemire, J.B., and Garnick, M.B. (1990). "Recombinant human macrophage colony-stimulating factor lowers LDL and raises HDL in WHHL rabbits." *Circulation* **82 (suppl III)** : III-233.

Pearson, A., Lux, A. and Krieger, M. (1995). "Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*." *Proc. Natl. Acad. Sci. USA* **92** : 4056-4060.

Pearson, A.M., Rich, A. and Krieger, M. (1993). "Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices." *J. Biol. Chem.* **268** : 3546-3554.

Penman, M., Lux, A., Freedman, N.J., Rohrer, L., Ekkel, Y., Mckinstry, H., Resnick, D. and Krieger, M. (1991). "The type I and type II bovine scavenger receptors expressed in Chinese Hamster Ovary cells are trimeric proteins with collagenous triple helical domains comprising noncovalently associated monomers and Cys^{83}-disulfide-linked dimers." *J. Biol. Chem.* **266** : 23985-23993.

Pitas, R.E. (1990). "Expression of the acetyl low density lipoprotein receptor by rabbit fibroblasts and smooth muscle cells: up-regulation by phorbol esters." *J. Biol. Chem.* **265** : 12722-12727.

Pitas, R.E., Innerarity, T.L., Weinstein, J.N. and Mahley, R.W. (1981). "Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages *in vitro* by fluorescence microscopy." *Arteriosclerosis* **1** : 177-185.

Plump, A.S., Smith, J.D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J.G., Rubin, E.M. and Breslow, J.L. (1992). "Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells." *Cell* **71** : 343-353.

Polacek, D., Lal, R., Volin, M.V. and Davies, P.F. (1993). "Gap junctional communication between vascular cells. Induction of

connexin43 messenger RNA in macrophage foam cells of atherosclerotic lesions." *Am. J. Pathol.* **1426** : 593-606.

Pollard, J.W., Bartocci, A., Arceci, R., Orlofsky, A., Ladner, M.B. and Stanley, E.R. (1987). "Apparent role of the macrophage growth factor, CSF-1, in placental development." *Nature* **330** : 484-486.

Poole, J.C.F. and Florey, H.W. (1958). "Changes in the endothelium of the aorta and the behavior of macrophages in experimental atheroma of rabbits." *J. Path. Bact.* **75** : 245-253.

Price, L.K.H., Choi, H.U., Rosenberg, L. and Stanley, E.R. (1992). "The predominant form of secreted colony stimulating factor-1 is a proteoglycan." *J. Biol. Chem.* **267** : 2190-2199.

Printseva, O.Y., Peclo, M.M. and Gown, A.M. (1992). "Various cell types in human atherosclerotic lesions express ICAM-1. Further immunocytochemical and immunochemical studies employing monoclonal antibody 10F3." *Am. J. Pathol.* **140** : 889-896.

Quinn, M.T., Parthasarathy, S., Fong, L.G. and Steinberg, D. (1987). "Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis." *Proc. Natl. Acad. Sci. USA* **84** : 2995-2998.

Rabinovitch, M. and de Stefano, M.J. (1976). "Cell shape changes induced by cationic anesthetics." *J. Exp. Med.* **143** : 290-304.

Rabinovitch, M., Manijas, R.E. and Nussenzweig, V. (1975). "Selective phagocytic paralysis induced by immune complexes." *J. Exp. Med.* **142** : 827-838.

Rabinowitz, S. and Gordon, S. (1991). "Macrosialin, a macrophage-restricted membrane sialoprotein differentially glycosylated in response to inflammatory stimuli." *J. Exp. Med.* **174** : 827-836.

Rabinowitz, S., Horstmann, H., Gordon, S. and Griffiths, G. (1992). "Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages." *J. Cell Biol.* **116** : 95-112.

Rabinowitz, S.S. (1988). Macrophage membrane glycoproteins

defined by wheat germ agglutinin. Oxford.

Radoff, S., Cerami, A. and Vlassara, H. (1990). "Isolation of surface binding protein specific for advanced glycosylation end products from mouse macrophage-derived cell line RAW 264.7." *Diabetes* **39** : 1510-1518.

Raines, E.W., Dower, S.K. and Ross, R. (1989). "Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA." *Science* **243** : 393-396.

Rajavashisth, T.B., Andalibi, A., Territo, M.C., Berliner, J.A., Navab, M., Fogelman, A.M. and Lusis, A.J. (1990). "Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins." *Nature* **344** : 254-257.

Ralph, P., Prichard, J. and Cohn, M. (1975). "Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity." *J. Immunol.* **114** : 898-905.

Ralph, P., Warren, M., Lee, M., Csejty, J., Weaver, J., Broxmeyer, H., Williams, D., Stanley, E. and Kawasaki, E. (1986). "Inducible production of human macrophage growth factor, CSF-1." *Blood* **68** : 633-689.

Ramprasad, M.P., Fischer, W., Witztum, J.L., Sambrano, G.R., Quehenberger, O. and Steinberg, D. (1995). "The 94- to 97-kDa mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of human CD68." *Proc. Natl. Acad. Sci. USA* **92** : 9580-9584.

Rankin, S.M., Parthasarathy, S. and Steinberg, D. (1991). "Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages." *J. Lipid Res.* **32** : 449-456.

Raschke, W.C., Baird, S., Ralph, P. and Nakoinz, I. (1978). "Functional macrophage cell lines transformed by Abelson Leukemia Virus." *Cell* **15** : 261-267.

Ravetch, J.V. and Perussia, B. (1989). "Alternate membrane forms

of FcγRIII (CD16) on human natural killer cells and neutrophils." *J. Exp. Med.* **170** : 481-497.

Reddy, S.T., Gilbert, R.S., Xie, W., Luner, S. and Herschman, H.R. (1994). "TGF-β1 inhibits both endotoxin-induced prostaglandin synthesis and expression of the TIS10/prostaglandin synthase 2 gene in murine macrophages." *J. Leukoc. Biol.* **55** : 192-200.

Reddy, V.Y., Zhang, Q.-Y. and Weiss, S.J. (1995). "Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages." *Proc. Natl. Acad. Sci. USA* **92** :

Resnick, D., Freedman, N., Xu, S. and Krieger, M. (1993). "Secreted extracellular domains of macrophage scavenger receptors form elongated trimers which specifically bind crocidolite asbestos." *J. Biol. Chem.* **268** : 3538-3545.

Resnick, D., Pearson, A. and Krieger, M. (1994). "The SRCR superfamily: a family reminiscent of the Ig superfamily." *TIBS* **19** : 5-8.

Richardson, M., Hadcock, S.J., DeReske, M. and Cybulsky, M.I. (1994). "Increased expression *in vivo* of VCAM-1 and E-selectin by the aortic endothelium of normolipidemic and hyperlipemic diabetic rabbits." *Arterioscler. Thromb.* **14** : 760-769.

Rigotti, A., Acton, S.L. and Krieger, M. (1995). "The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids." *J. Biol. Chem.* **270** : 16221-16224.

Roebuck, K.A., Rahman, A., Lakshminarayanan, V., Janakidevi, K. and Malik, A.B. (1995). "H_2O_2 and tumor necrosis factor-α activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter." *J. Biol. Chem.* **270** : 18966-18974.

Rohrer, L., Freeman, M., Kodama, T., Penman, M. and Krieger, M. (1990). "Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II." *Nature* **343** : 570-572.

Rollins, B.J. and Pober, J.S. (1991). "Interleukin-4 induces the synthesis and secretion of MCP-1/JE by human endothelial cells." *Am. J.*

Pathol. **138** : 1315-1319.

Rollins, B.J., Yoshimura, T., Leonard, E.J. and Pober, J.S. (1990). "Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE." *Am. J. Pathol.* **136** : 1229-1233.

Rosen, H. (1990). "Role of CR3 in induced myelomonocytic recruitment: insights from in vivo monoclonal antibody studies in the mouse." *J. Leuk. Biol.* **48** : 465-469.

Rosen, H. and Gordon, S. (1987). "Monoclonal antibody to the murine type 3 complement receptor inhibits adhesion of myelomonocytic cells in vitro and inflammatory cell recruitment in vivo." *J. Exp. Med.* **166** : 1685-1701.

Rosenfeld, M.E., Tsukuda, T., Gown, A.M. and Ross, R. (1987). "Fatty streak initiation in Watanabe heritable hyperlipemic and comparably hypercholesterolemic fat-fed rabbits." *Arteriosclerosis* **7** : 9-23.

Rosenfeld, M.E. and Ross, R. (1990). "Macrophage and smooth muscle cell proliferation in atherosclerotic lesions of WHHL and comparably hypercholesterolemic fat-fed rabbits." *Arteriosclerosis* **10** : 680-687.

Rosenfeld, M.E., Yla-Herttuala, S., Lipton, B.A., Ord, V.A., Witztum, J.L. and Steinberg, D. (1992). "Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans." *Am. J. Path.* **140** : 291-300.

Rosenfeld, M.E., Butler, S., Ord, V.A., Lipton, B.A., Dyer, C.A., Curtiss, L.K., Palinski, W. and Witztum, J.L. (1993). "Abundant expression of apoprotein E by macrophages in human and rabbit atherosclerotic lesions." *Arterioscler. Thromb.* **13** : 1382-1389.

Rosenstein, Y., Park, J.K., Hahn, W.C., Rosen, F.S., Bierer, B.E. and Burakoff, S.J. (1991). "CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1." *Nature* **354** : 233-235.

Ross, R., Masuda, J., Raines, E.W., Gown, A.M., Katsuda, S., Sasahara, M. and al, e. (1990). "Localization of PDGF-B protein in macrophages in all phases of atherogenesis." *Science* **248** : 1009-1012.

Ross, R. (1993). "The pathogenesis of atherosclerosis; a perspective for the 1990s." *Nature* **362** : 801-809.

Rothman, J.E. (1994). "Mechanisms of intracellular transport." *Nature* **372** : 55-63.

Salomon, R.N., Underwood, R., Doyle, M.V., Wang, A. and Libby, P. (1992). "Increased apolipoprotein E and *c-fms* expression without elevated interleukin 1 or 6 mRNA levels indicates selective activation of macrophage functions in advanced human atheroma." *Proc. Natl. Acad. Sci. USA* **89** : 2814-2818.

Salonen, J.T., Yla-Herttuala, S., Yamamoto, R., Butlar, S., Korpela, H., Salonen, R., Nyyssonen, K., Palinski, W. and Witztum, J.L. (1992). "Autoantibody against oxidised LDL and progression of carotid atherosclerosis." *Lancet* **339** : 883-889.

Sambrano, G.R., Parthasarathy, S. and Steinberg, D. (1994). "Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low density lipoprotein." *Proc. Natl. Acad. Sci. USA* **91** : 3265-3269.

Sambrano, G.R. and Steinberg, D. (1995). "Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine." *Proc. Natl. Acad. Sci. USA* **92** : 1396-1400.

Sarmay, G., Rozsnyay, Z., Szabo, I., Biro, A. and Gergely, J. (1991). "Modulation of type II Fcγ receptor expression on activated human B lymphocytes." *Eur. J. Immunol.* **21** : 541-549.

Sarria, A.J., Panini, S.R. and Evans, R.M. (1992). "A functional role for vimentin intermediate filaments in the metabolism of lipoprotein-derived cholesterol in human SW-13." *J. Biol. Chem.* **267** : 19455-19463.

Sastry, K., Zahedi, K., Lelias, J.M., Whitehead, A. and Ezekowitz, R.A.B. (1991). "Molecular characterization of the mouse mannose-binding proteins." *J. Immunol.* **147** : 692-697.

Sastry, K. and Ezekowitz, R.A. (1993). "Collectins: pattern

recognition molecules involved in first line host defense." *Curr. Opin. Immunol.* **5** : 59-66.

Schall, T.J. (1995). *The chemokines. The Cytokine Handbook.* New York, Academic Press. 3 rd. ed.

Schaub, R.G., Bree, M.P., Hayes, L.L., Rudd, M.A., Rabbani, L., Loscalzo, J. and Clinton, S.K. (1994). "Recombinant human macrophage colony-stimulating factor reduces plasma cholesterol and carrageenan granuloma foam cell-formation in Watanabe heritable hyperlipidemic rabbits." *Arterioscler. Thromb.* **14** : 70-76.

Schaub, R.G., Donnelly, L.H., Parker, T.S., Clinton, S.K. and Garnick, M.B. (1995). *The protective role of the macrophage in atherogenesis: insight from using M-CSF.* Atherosclerosis X, Montreal, Elsevier Science.

Schauer, R. (1991). "Biosynthesis and function of N- and O-linked substituted sialic acids." *Glycobiology* **1** : 449-452.

Schleiffenbaum, B., Spertini, O. and Tedder, T.F. (1992). "Soluble L-selectin is present in human plasma at high levels and retains functional activity." *J. Cell Biol.* **119** : 229-238.

Schmid, K., Hediger, M.A., Brossmer, R. *et al.* (1992). "Amino acid sequences of human plasma galactoglycoprotein: identity with the extracellular region of CD43 (sialophorin)." *Proc. Natl. Acad. Sci. USA* **89** : 663-667.

Schmidt, L., Pozzati, R., Li, H., Wei, M.-H., Vocke, C., Linehan, W.M. and Tory, K. (1994). "A microsatellite, D8S602, adjacent to the MSR gene." *Human Molecular Genetics* **3** : 67.

Schneider, C., Owen, M.J., Banville, D. and Williams, J.G. (1984). "Primary structure of human transferrin receptor deduced from the mRNA sequence." *Nature* **311** : 675-678.

Schnitzer, J.E., Sung, A., Horvat, R. and Bravo, J. (1992). "Preferential interaction of albumin-binding proteins, gp30 and gp18, with conformationally modified albumins." *J. Biol. Chem.* **267** : 24544-24553.

Schwartz, B.S. and Bradshaw, J.D. (1992). "Regulation of plasminogen activator inhibitor mRNA levels in lipopolysaccharide-stimulated human monocytes. Correlation with production of the protein." *J. Biol. Chem.* **267** : 7089-7094.

Schwartz, C.J. and Valente, A.J. (1995). *The monocyte-macrophage in atherosclerosis: historical background and overview.* Atherosclerosis X, Montreal, Elsevier Science B.V.

Seifert, P.S. and Hansson, G.K. (1989). "Complement receptors and regulatory proteins in human atherosclerotic lesions." *Arteriosclerosis* **9** : 802-811.

Seifert, P.S., Roth, I., Scmiedt, W., Oelert, H., Okada, N., Okada, H. and Bhakdi, S. (1992). "CD59 (homologous restriction factor 20), a plasma membrane protein that protects against complement C5b-9 attack, in human atherosclerotic lesions." *Atherosclerosis* **96** : 135-145.

Shackelford, R.E., Misra, U.K., Florine-Casteel, K., Thai, S.-F., Pizzo, S.V. and Adams, D.O. (1995). "Oxidized low density lipoprotein suppresses activation of NFκB in macrophages via a pertussis toxin-sensitive signaling mechanism." *J. Biol. Chem.* **270** : 3475-3478.

Sherman, M.L., Stein, E.A., Isaacsohn, J., Lees, R.S. and Garnick, M.B. (1995). *Role of recombinant human macrophage colony-stimulating factor (rhM-CSF) in cholesterol metabolism and atherosclerosis.* Atherosclerosis X, Montreal, Elsevier Science.

Sherr, C.J. (1990). "Colony-stimulating factor-1 receptor." *Blood* **75** : 1-12.

Sherr, C.J., Borzillo, G.V., Kato, J.-Y., Shurtleff, S.A., Downing, J.R. and Roussel, M.F. (1991). "Regulation of cell growth and differentiation by the colony-stimulating factor 1 receptor." *Sem. Hematol.* **28** : 143-151.

Shih, Y.J., Baynes, R.D., Hudson, B.G., Flowers, C.H., Skikne, B.S., Cook, J.D. (1990). "Serum transferrin receptor is a truncated form of tissue receptor." *J. Biol. Chem.* **265** : 19077-19081.

Shima, M., Teitelbaum, S.L., Holers, V.M., Ruzicka, C., Osmack, P. and Ross, F.P. (1995). "Macrophage-colony-stimulating factor regulates expression of the integrins α4β1 and α5β1 by murine bone marrow

macrophages." *Proc. Natl. Acad. Sci. USA* **92** : 5179-5183.

Shimada, M., Inaba, T., Shimano, H., Gotoda, T., Watanabe, Y., Yamamoto, K., Motoyoshi, K., Yazaki, Y. and Yamada, N. (1992). "Platelet-derived growth factor BB-dimer suppresses the expression of macrophage colony stimulating-factor inhuman vascular smooth muscle cells." *J. Biol. Chem.* **267** : 15455-15458.

Shimano, H., Yamada, N., Ishibashi, S., Harada, A., Matsumoto, N., Mori, T., Inaba, K., Motoyoshi, H., Itakura, H. and Takaku, F. (1990). "Human monocyte colony-stimulating factor enhances the clearance of lipoproteins containing apolipoproteins B-100 via both low density lipoprotein receptor-dependent and -independent pathways in rabbits." *J. Biol. Chem.* **265** : 12869-12875.

Shimano, H., Yamada, N., Katsuki, M., Shimada, M., Gotoda, T., Harada, K., Murase, T., Fukazawa, C., Takaku, F. and Yazaki, Y. (1992). "Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia." *Proc. Natl. Acad. Sci. USA* **89** : 1750-1754.

Shimano, H., Ohsuga, J., Shimada, M., Namba, Y., Gotoda, T., Harada, K., Katsuki, M., Yazaki, Y. and Yamada, N. (1995). "Inhibition of diet-induced atheroma formation in transgenic mice expressing apolipoprotein E in the arterial wall." *J. Clin. Invest.* **95** : 469-476.

Shimokado, K., Raines, E.W., Madtes, D.K., Barrett, T.B., Benditt, E.P. and Ross, R. (1985). "A significant part of macrophage-derived growth factor consists of at least two forms of PDGF." *Cell* **43** : 277-286.

Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N. and Doetschman, T. (1992). "Targeted disruption of the mouse transforming growth factor- β1 gene results in multifocal inflammatory disease." *Nature* **359** : 693-699.

Shyy, Y.-J., Wickham, L.L., Hagan, J.P., Hsieh, H.-J., Hu, Y.-L., Telian, S.H., Valente, A.J., Sung, K.-L.P. and Chien, S. (1993). "Human monocyte colony-stimulating factor stimulates the gene expression of monocyte chemotactic protein-1 and increases the adhesion of monocytes to endothelial monolayers." *J. Clin. Invest.* **92** : 1745-1751.

Smith, J.D., Trogan, E., Ginsberg, M., Grigaux, C., Tian, J. and Miyata, M. (1995). "Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (*op*) and apolipoprotein E." *Proc. Natl. Acad. Sci. USA* **92** : 8264-8268.

Smith, M.J. and Koch, G.L.E. (1987). "Differential expresson of murine macrophage surface glycoprotein antigens in intracellular membranes." *J. Cell Sci.* **87** : 113-119.

Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G. and Corvol, P. (1988). "Two putative active centers in human angiotensin I-converting enzyme revealed by molecular cloning." *Proc. Natl. Acad. Sci. USA* **85** : 9386-9390.

Sparrow, C.P., Parthasarathy, S. and Steinberg, D. (1989). "A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein." *J. Biol. Chem.* **264** : 2599-2604.

Spertini, O., Luscinskas, F.W., Kansas, G.S., Munro, J.M., Griffin, J.D., Gimbrone, M.A. and Tedder, T.F. (1991). "Leukocyte adhesion molecule-1 (LAM-1; L-Selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion." *J. Immunol.* **147** : 2565-2573.

Springer, T.A. (1990). "Adhesion receptors of the immune system." *Nature* **346** : 425-434.

Stamenkovic, I., Amiot, M., Pesando, J.M., Seed, B. (1989). "A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family." *Cell* **56** : 1057-1062.

Stanley, E.R. (1994). *Colony Stimulating Factor-1 (Macrophage Colony Stimulating Factor). The Cytokine Handbook*. San Diego, Academic Press. 387-418.

Stanley, E.R. and Heard, P.M. (1977). "Factors regulating macrophage production and growth. Purification and some properties of the colony stimulating factor from medium conditioned by mouse L-cells." *J Biol Chem* **252** : 4305-4312.

Stanton, L.W., White, R.T., Bryant, C.M., Protter, A.A. and Endemann, G. (1992). "A macrophage Fc receptor for IgG is also a

receptor for oxidized low density lipoprotein." *J. Biol. Chem.* **267** : 22446-22451.

Starkey, P.M., Turley, L. and Gordon, S. (1987). "The mouse macrophage-specific glycoprotein defined by monoclonal antibody F4/80: characterization, biosynthesis and demonstration of a rat analogue." *Immunology* **60** : 117-122.

Stein, J. and Rettenmier, C.W. (1991). "Proteolytic processing of a plasma membrane-bound precursor to human macrophage colony-stimulating factor (CSF-1) is accelerated by phorbol ester." *Oncogene* **6** : 601-605.

Stein, M., Keshav, S., Harris, N. and Gordon, S. (1992). "Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation." *J. Exp. Med.* **176** : 287-292.

Stein, M.L. and Gordon, S. (1991). "Regulation of tumour necrosis factor release by murine peritoneal macrophages: role of cell stimulation and specific phagocytic plasma membrane receptors." *Eur. J. Immunol.* **21** : 431-437.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989). "Beyond cholesterol - modifications of low-density lipoprotein that increase its atherogenicity." *New Engl. J. Med.* **320** : 915-924.

Steinberg, D. (1995). *The oxidative modification hypothesis of atherogenesis: strengths and weaknesses.* Atherosclerosis X, Montreal, Elsevier Science B.V.

Steinbrecher, U.P., Lougheed, M., Kwan, W.-C. and Dirks, M. (1989). "Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation." *J. Biol. Chem.* **264** : 15216-152223.

Stemme, S., Faber, B., Holm, J., Wiklund, O., Witztum, J.L. and Hansson, G.K. (1995). "T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein." *Proc. Natl. Acad. Sci. USA* **92** : 3893-3897.

Stemme, S. and Hansson, G.K. (1994). "Immune mechanisms in atherosclerosis." *Coronary Artery Disease* **5** : 216-222.

Stiko-Rahm, A., Hultgardh-Nilsson, A., Regnstrom, J., Hamsten, A. and Nilsson, J. (1992). "Native and oxidized LDL enhances production of PDGF AA and the surface expression of PDGF receptors in cultured human smooth muscle cells." *Arteriosclerosis Thromb.* **12** : 1099-1109.

Stockinger, H., Gadd, S.J., Eher, R., Majdic, O., Schreiber, W., Kasinrerk, W., Strass, B., Schnabl, E. and Knapp, W. (1990). "Molecular characterization and functional analysis of the leukocyte surface protein CD31." *J. Immunol.* **145** : 3889-3897.

Stopeck, A.T., Nicholson, A.C., Mancini, F.P. and Hajjar, D.P. (1993). "Cytokine regulation of low density lipoprotein receptor gene transcription in HepG2 cells." *J. Biol. Chem.* **268** : 17489-17494.

Stoudemire, J.B. and Garnick, M.B. (1991). "Effects of recombinant macrophage colony-stimulating factor on plasma cholesterol levels." *Blood* **77** : 750-755.

Strassmann, G., Cole, M.D. and Newman, W. (1988). "Regulation of colony-stimulating factor 1-dependent macrophage precursor proliferation by type β transforming growth factor." *J. Immunol* **140** : 2645-2651.

Suzu, S., Inaba, T., Yanai, N., Kawashima, T., Yamada, N., Oka, T., Machinami, R., Ohtsuki, T., Kimura, F., Kondo, S., Torikata, C., Nagata, N. and Motoyoshi, K. (1994). "Proteoglycan form of macrophage colony-stimulating factor binds low density lipoprotein." *J. Clin. Invest.* **94** : 1637-1641.

Symons, J.A., Eastgate, J.A. and Duff, G.W. (1990). "A soluble binding protein specific for interleukin-1β is produced by activated mononuclear cells." *Cytokine* **2** : 190-198.

Takahashi, K., Naito, M., Kodama, T., Suzuki, H., Mori, T. and Matsumoto, A. (1992). "Expression of macrophage scavenger receptors in various human tissues and atherosclerotic lesions." *Clin. Biochem.* **25** : 365-368.

Takahashi, K., Takeya, M., Kodama, T., Suzuki, H., Gordon, S., de

Villiers, W.J.S. and Horiuchi, S. (1995). *Biological and pathological features of scavenger receptor-deficient mice.* The macrophage: basic and clinical aspects. Ninth annual conference of the European macrophage study group., Amsterdam,

Takeya, M., Yoshimura, T., Leonard, E.J. and Takahashi, K. (1993). "Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal antibody." *Hum. Pathol.* **24** : 534-539.

Tanaka, Y., Adams, D.H., Hubscher, S., Hirano, H., Siebenlist, U. and Shaw, S. (1993). "Proteoglycan-immobilized MIP-1β induces adhesion of T cells." *Nature* **361** : 79-82.

Tang, D., DeVit, M. and Johnston, S.A. (1992). "Genetic immunization is a simple method for eliciting an immune response." *Nature* **356** : 152-154.

Terasaki, M., Chen, L.B. and Fujiwara, K. (1986). "Microtubules and the endoplasmic reticulum are highly interdependent structures." *J. Cell Biology* **103** : 1557-1568.

Thiel, S. and Reid, K.B.M. (1989). "Structures and function associated with the group of mammalian lectins containing collagen-like sequences." *FEBS Lett.* **250** : 78-84.

Thompson, C.B. (1995). "Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differnetiation?" *Cell* **81** : 979-982.

Tipping, P.G. and Hancock, W.W. (1993). "Production of tumor necrosis factor and interleukin-1 by macrophages from human atheromatous plaques." *Am. J. Pathol.* **142** : 1721-1728.

Tokuyasu, K.T. (1980). "Immunocytochemistry in ultra-thin frozen sections." *Histochem. J.* **12**: 381-403.

Tooze, J. and Hollinshead, M. (1991). "Tubular early endosomal networks in AtT20 and other cells." *J. Cell Biology* **115** : 635-654.

Towbin, H., Staehelin, T. and Gordon, J. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." *Proc. Natl. Acad. Sci. USA* **76** : 4350-

4354.

Traber, M.G., Kallman, B. and Kayden, H.J. (1983). "Localization of the binding sites of native and acetylated low-density lipoprotein (LDL) in human monocyte-derived macrophages." *Exp. Cell Res.* **148** : 281-292.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980). "Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)." *Int. J. Cancer* **26** : 171-176.

Tsukuda, T., Rosenfeld, M., Ross, R. and Gown, A.M. (1986). "Immunocytochemical analysis of cellular components in lesions of atherosclerosis in the Watanabe and fat-fed rabbit using monoclonal antibodies." *Arteriosclerosis* **6** : 601-613.

Tyrell, D., James, P., Rao, N., Foxall, C., Abbas, S., Dasgupta, F., Nashed, M., Hasegawa, A., Kiso, M. and Ada, D. (1991). "Structural requirements for the carbohydrate ligand of E-selectin." *Proc. Natl. Acad. Sci. USA* **88** : 10372-10376.

Unkeless, J.C. (1979). "Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors." *J. Exp. Med.* **150** : 580-596.

Utermann, G. (1989). "The mysteries of lipoprotein (a)." *Science* **246** : 904-910.

Valente, A.J., Rozek, M.M., Abramova, M. and Schwartz, C.J. (1995). *Monocyte-macrophage recruitment to the arterial intima: a role for MCP-1 and its receptor. Atherosclerosis X.* Amsterdam, Elsevier. 586-592.

Van Berkel, T.J.C., De Rijke, Y.B. and Kruijt, J.K. (1991). "Different fate *in vivo* of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats." *J. Biol. Chem.* **266** : 2282-2289.

van den Berg, T.K., Breve, J.J., Damoiseaux, J.G., Dopp, E.A., Kelm, S., Crocker, P.R., Dijkstra, C.D. and Kraal, G. (1992). "Sialoadhesin on macrophages: its identification as a lymphocyte adhesion molecule." *J. Exp. Med.* **176** : 647-655.

van den Eijnden, D.H. and Joziasse, D.H. (1993). "Enzymes

associated with glycosylation." *Current Opinion in Structural Biology* **3** : 711-721.

van der Wal, A.C., Das, P.K., Tigges, A.J. and Becker, A.E. (1992). "Macrophage differentiation in atherosclerosis. An in situ immunohistochemical analysis in humans." *Am. J. Pathol.* **141** : 161-168.

Van Lenten, B.J. and Fogelman, A.M. (1992). "Lipopolysaccharide-induced inhibition of scavenger receptor expression in human monocyte-macrophages is mediated through tumor necrosis factor-alpha." *J. Immunol.* **148** : 112-116.

Van Lenten, B.J., Fogelman, A.M., Seager, J., Ribi, E., Haberland, M.E. and Edwards, P.A. (1985). "Bacterial endotoxin selectively prevents the expression of scavenger-receptor activity on human monocyte-macrophages." *J. Immunol.* **134** : 3718-3721.

Via, D.P., Dresel, H.A., Cheng, S.-L. and Gotto, A.M.J. (1985). "Murine macrophage tumors are a source of a 260 000-Dalton acetyl-low density lipoprotein receptor." *J. Biol. Chem.* **260** : 7379-7386.

Via, D.P., Kempner, E.S., Pons, L., Fanslow, A.E., Vignale, S., Smith, L.C., Gotto, A.M., Jr. and Dresel, H.A. (1992). "Mouse macrophage receptor for acetylated low density lipoprotein: Demonstration of a fully functional subunit in the membrane and with purified receptor." *Proc. Natl. Acad. Sci. USA* **89** : 6780-6784.

Viville, S., Neefjes, J., Lotteau, V., Dierich, A., Lemeur, M., Ploegh, H., Benoist, C. and Mathis, D. (1993). "Mice lacking the MHC Class II-associated invariant chain." *Cell* **72** : 635-648.

Vlassara, H., Brownlee, M. and Cerami, A. (1986). "Novel macrophage receptor for glucose-modified proteins is distinct from previously described scavenger receptors." *J. Exp. Med.* **164** : 1301-1309.

Vlassara, H., Fuh, H., Makita, Z., Krungkrai, S., Cerami, A. and Bucala, R. (1992). "Exogenous advanced glycosylation end products induce complex vascular dysfunction in normal animals: a model for diabetic and aging complications." *Proc. Natl. Acad. Sci. USA* **89** : 12043-12047.

Wahl, S.M. (1994). "Transforming growth factor β: the good, the

bad, and the ugly." *J. Exp. Med.* **180** : 1587-1590.

Wahl, S.M., Allen, J.B., Wong, H.L., Dougherty, S.F. and Ellingsworth, L.R. (1990). "Antagonistic and agonistic effects of transforming growth factor-β and IL-1 in rheumatoid synovium." *J. Immunol.* **145** : 2514-2519.

Waldmann, T.A. (1991). "The interleukin-2 receptor." *J. Biol. Chem.* **266** : 2681-2684.

Warren, M.K. and Ralph, P. (1986). "Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity." *J. Immunol.* **137** : 2281-2285.

Watanabe, Y., Inaba, T., Shimano, H., Gotoda, T., Yamamoto, K., Mokuno, H., Sato, H., Yazaki, Y. and Yamada, N. (1994). "Induction of LDL receptor-related protein during the differentiation of monocyte-macrophages: possible involvement in the atherosclerotic process." *Arterioscler. Thromb.* **14** : 1000-1006.

Watson, K.E., Bostrom, K., Ravindranath, R., Lam, T., Norton, B. and Demer, L.L. (1994). "TGF-β1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify." *J. Clin. Invest.* **93** : 2106-2113.

Watson, S.R., Imai, Y., Fennie, C., Geoffroy, J.S., Rosen, S.D. and Lasky, L.A. (1990). "A homing receptor-IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules." *J. Cell Biol.* **110** : 2221-2229.

Wayner, E.A., Garcia-Pardo, A., Humphries, M.J., McDonald, J.A. and Carter, W.G. (1989). "Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin." *J. Cell Biol.* **109** : 1321-1330.

Welgus, H.G., Campbell, E.J., Cury, J.D., Eisen, A.Z., Senior, R.M., Wilhelm, S.M. and Goldberg, G.I. (1990). "Neutral metalloproteinases produced by human mononuclear phagoctes. Enzyme profile, regulation, and expression during cellular development." *J. Clin. Invest.* **86** : 1496-1502.

Welgus, H.G., Senior, R.M., Parks, W.C., Kahn, A.J., Ley, T.J.,

Shapiro, S.D. and Campbell, E.J. (1992). "Neutral proteinases expression by human mononuclear phagoctes: a prominent role of cellular differentiation." *Matrix* **1** : 363-367.

Werb, Z., Takemura, R., Stenberg, P.E. and Bainton, D.F. (1989). "Directed exocytosis of secretory granules containing apolipoprotein E to the adherent surface and basal vacuoles of macrophage spreading on immobile immune complexes." *Am. J. Pathol.* **134** : 661-670.

Whitney, G.S., Starling, G.C., Bowen, M.A., Modrell, B., Siadak, A.W. and Aruffo, A. (1995). "The membrane-proximal scavenger receptor cysteine-rich domain of CD6 contains the activated leukocyte cell adhesion molecule binding site." *J. Biol. Chem.* **270** : 18187-18190.

Wick, G., Kleindienst, R., Dietrich, H. and Xu, Q. (1992). "Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock protein 65." *Trends Food Sci. Technol.* **3** : 114-119.

Wijngaard, P.L., Metzelaar, M.B., MacHugh, N.D., Morrison, W.I. and Clevers, H.C. (1992). "Molecular characterization of the WC1 antigen expressed specifically on bovine CD4-CD8- Gamma Delta T lymphocytes." *J. Immunol.* **149** : 3273-3277.

Wiktor-Jedrzejczak, W., Ahmed, A., Szczylik, C. and Skelly, R.R. (1982). "Hematologic characterization of congenital osteopetrosis in op/op mouse - Possible mechanism for abnormal macrophage differentiation." *J. Exp. Med.* **156** : 1516-1527.

Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A.W., Ahmed-Ansari, A., Sell, K.W., Pollard, J.W. and Stanley, E.R. (1990). "Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse." *Proc. Natl. Acad. Sci. USA* **87** : 4828-4832.

Wilcox, J.N., Smith, K.M., Schwartz, S.M. and Gordon, D. (1989). "Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque." *Proc. Natl. Acad. Sci. USA* **86** : 2839-2843.

Willerford, D.M., Hoffman, P.A. and Gallatin, W.M. (1989). "Expression of lymphocyte adhesion receptors for high endothelium in primates. Anatomoc partitioning and linkage to activation." *J. Immunol.* **142** : 3416-3422.

Williams, A.F. and Barclay, A.N. (1988). "The immunoglobulin superfamily-domains for cell surface recognition." *Ann. Rev. Immunol.* **6** : 381-405.

Wilson, A.C., Schaub, R.G., Goldstein, R.C. and Kuo, P.T. (1990). "Suppression of aortic atherosclerosis in cholesterol-fed rabbits by purified rabbit interferon." *Arteriosclerosis* **10** : 208-214.

Wing, E.J., Waheed, A., Shadduck, R.K., Nagle, L.S. and Stephenson, K. (1982). "Effect of colony stimulating factor on murine macrophages. Induction of antitumor activity." *J. Clin. Invest.* **69** : 270-276.

Wirthmueller, U., Kurosaki, T., Murakami, M.S. and Ravetch, J.V. (1992). "Signal transduction by FcγRIII (CD16) is mediated through the gamma chain." *J. Exp. Med.* **175** : 1381-1390.

Wissler, R.W. *et al.* (1994). "New insights into the pathogenesis of atherosclerosis as revealed by PDAY." *Atherosclerosis* **108**: S3-S20.

Witmer-Pack, M.D., Hughes, D.A., Schuler, G., Lawson, L., McWilliam, A., Inaba, K., Steinman, R.M. and Gordon, S. (1993). "Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse." *J. Cell Sci.* **104** : 1021-1029.

Witte, O.N. (1990). "Steel locus defines new multipotent growth factor." *Cell* **63** : 5-6.

Wolle, S., Via, D.P., Chan, L., Cornicelli, J.A. and Bisgaier, C.L. (1995). "Hepatic overexpression of bovine scavenger receptor type I in transgenic mice prevents diet-induced hyperlbetalipoproteinemia." *J. Clin. Invest.* **96** : 260-272.

Wright, S.D. and Silverstein, S.C. (1984). "Phagocytosing macrophages exclude proteins from the zones of contact with opsonized targets." *Nature* **309** : 359-361.

Wright, S.D., Tobias, P.S., Ulevitch, R.J. and Ramos, R.A. (1989). "Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages." *J. Exp. Med.* **170** : 1231-1241.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J. and Mathison, J.C. (1990). "CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein." *Science* **249** : 1431-1433.

Xie, B., Dong, Z. and Fidler, I.J. (1994). "Regulatory mechanisms for the expression of type IV collagenases/gelatinases in murine macrophages." *J. Immunol.* **152** : 3637-3644.

Xu, Q., Dietrich, H. and Steiner, H.J. (1992). "Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock-protein-65." *Arterioscler. Thromb.* **12** : 789-799.

Yamada, N., Inoue, M., Harada, K., Watanabe, Y., Shimano, H., Gotoda, T., Shimada, M., Kohzaki, K., Tsukuda, T., Shiomi, M., Watanabe, Y. and Yazaki, Y. (1992). "Apolipoprotein E prevents the progression of atherosclerosis in Watanabe Heritable Hyperlipidemic Rabbits." *J. Clin. Invest.* **89** : 706-711.

Yeung, Y.G., Jubinsky, P.T., Sengupta, A., Yeung, D.C.-Y. and Stanley, E.R. (1987). "Purification of the colony-stimulating factor 1 receptor and demonstration of its tyrosine kinase activity." *Proc. Natl. Acad. Sci. USA* **84** : 1268-1271.

Yla-Herttuala, S., Rosenfeld, M.E., Parthasarathy, S., Glass, C.K., Sigal, E., Witztum, J.L. and Steinberg, D. (1990). "Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions." *Proc. Natl. Acad. Sci. USA* **87** : 6959-6963.

Yla-Herttuala, S., Rosenfeld, M.E., Parthasarathy, S., Sigal, E., Sarkioja, T., Witztum, J.L. and Steinberg, D. (1991). "Gene expression in macrophage-rich human atherosclerotic lesions: 15-lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts." *J Clin Invest* **87** : 1146-1152.

Yla-Herttuala, S., Lipton, B.A., Rosenfeld, M.E., Goldberg, I.J., Steinberg, D. and Witztum, J.L. (1991). "Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions." *Proc. Natl. Acad. Sci. USA* **88** : 10143-10147.

Yla-Herttuala, S., Lipton, B.A., Rosenfeld, M.E., Sarkioja, T., Yoshimura, T., Leonard, E.J., Witztum, J.L. and Steinberg, D. (1991). "Expression of monocyte chemoattractant protein-1 in macrophage-rich areas of human and rabbit atherosclerotic lesions." *Proc. Natl. Acad. Sci. USA* **88** : 5252-5256.

Yla-Herttuala, S., Luoma, J., Viita, H., Hiltunen, T., Sisto, T. and Nikkari, T. (1995). "Transfer of 15-lipoxygenase into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein." *J. Clin. Invest.* **95** : 2692-2698.

Yoshida, H., Hayashi, S.-I., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L.D. and Nishikawa, S.-I. (1990). "The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene." *Nature* **345** : 442-444.

Zhang, H., Yang, Y. and Steinbrecher, U.P. (1993). "Structural requirements for the binding of modified proteins to the scavenger receptor of macrophages." *J. Biol. Chem.* **268** : 5535-5542.

Zhang, S.H., Reddick, R.L., Piedrahita, J.A. and Maeda, N. (1992). "Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E." *Science* **258** : 468-471.

Zhang, S.H., Reddick, R.L., Burkey, B. and Maeda, N. (1994). "Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption." *J. Clin. Invest.* **94** : 937-945.

Zuckerman, S.H., Evans, G.F. and O'Neal, L. (1992). "Cytokine regulation of macrophage apo E secretion: opposing effects of GM-CSF and TGF-β." *Atherosclerosis* **96** : 203-214.

Zurawski, G. and De Vries, J.E. (1994). "Interleukin-13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells." *Immunol Today* **15** : 19-26.