

The role of HLA-B27 in the pathogenesis of spondyloarthritis

A thesis submitted for the degree of D.Phil

University of Oxford

Trinity Term 2011



Kirsty McHugh

New College

Medical Research Council (MRC) Human Immunology Unit
Weatherall Institute of Molecular Medicine
John Radcliffe Hospital
Oxford

Supervisor: Professor Paul Bowness

The role of HLA-B27 in the pathogenesis of spondyloarthritis

Abstract

The Human Leukocyte Antigen (HLA)-B27 is a Major Histocompatibility Complex (MHC) class I antigen that is strongly associated with development of a group of closely related arthritic diseases, collectively known as the spondyloarthropathies (SpA). However, the mechanism by which HLA-B27 confers this susceptibility is unclear. Studies have shown that HLA-B27 heavy chains can form classical heterotrimers associated with peptide and β_2 -microglobulin (B27HT), and also non-classical heavy chain homodimers (B27₂). B27₂ assemble intracellularly during maturation and are also expressed at the cell surface following endosomal recycling of B27HT. A pathogenic role for B27₂ has been proposed in two of the current theories of pathogenesis: the B27 homodimer theory and the B27 misfolding and UPR theory. Yet, determinations of the extent, distribution, and triggers of B27₂ expression, as well as the functional consequences of its receptor interactions in AS pathogenesis, have been hampered by the lack of a specific detection reagent. Therefore, to investigate the role of B27₂ in AS, we generated a novel antibody to B27₂ – HD6 – using phage display technology, which binds to *in vitro* refolded B27₂ but not B27HT complexes by ELISA. This thesis provides evidence that HD6-reactive molecules, which include B27₂, are expressed at the cell surface in both cell lines and in the context of a disease setting. Recognition is B27-specific and strongly correlated with the magnitude of B27 expression, which could account for the lack of staining in some cell subsets. Moreover, staining was comparable in cell lines expressing the disease-associated B*27:05 and the less disease-associated subtype B*27:09. In addition, I have shown cells expressing physiologic levels of B27, including EBV-transformed BCLs and AS patient PBMCs, are capable of expressing the HD6 epitope upon low pH treatment. Interestingly, these ‘acid-inducible HD6’ molecules were absent from cells lacking a functional PLC. Finally, I have shown that HD6-reactive molecules can derive from pre-existing folding B27 molecules at the cell surface, which may be inhibited by the addition of exogenous B27-binding peptides. These findings are consistent with a mechanism of pathogenesis involving the surface expression and recognition of B27₂ and/or other aberrantly folded forms of B27, as proposed in the homodimer theory. HD6 will be a powerful tool to address the potential pathogenic role of B27₂ in SpA and may additionally have therapeutic potential.

Declaration

I declare that the work presented in this D.Phil thesis entitled “The role of HLA-B27 in the pathogenesis of spondyloarthritis” is entirely my own work, except for where the contributions of my collaborators have been clearly acknowledged. No part of my thesis has been submitted for any degree or other qualification in this University or elsewhere.

Trinity Term 2011

Kirsty McHugh

MRC Human Immunology Unit

Weatherall Institute of Molecular Medicine

Acknowledgements

This research was undertaken at the Weatherall Institute of Molecular Medicine and supported by a doctoral studentship from the Medical Research Council, for which I am very grateful. I am especially indebted to Professor Paul Bowness for his invaluable supervision and guidance throughout the duration of the project. The continuous support and encouragement he provided is something for which I am immensely grateful, and the freedom he afforded me, both with respect to the development of the project and in regard to the conferences he supported me in attending, was hugely beneficial to not only the thesis itself but to my development as a research scientist in general.

I would also like to thank the members of the Bowness and Ogg groups, past and present, for providing such a friendly and supportive environment in which to work. This undoubtedly made carrying out my research a far more rewarding experience. In particular, special thanks go to Simon Kollnberger, Jackie Shaw, Joanna Giles, Anna Ridley, Liye Chen, Isabel Wong and Laura Crack. As most who engage in it would attest, scientific research can at times be frustrating and difficult, yet the friendship, helpful advice, and enthusiasm for science offered by these lab members made those exasperating moments more bearable.

I also very much appreciate the kind efforts of several members of staff at the WIMM and of our collaborators, whose contributions of reagents as well as helpful discussion were essential for developing this project. In particular, I wish to thank Kati DiGleria, Becky Horsfall and Alastair Waugh for supplying the peptides and several of the antibodies; Simon Milling and colleagues for provision of numerous rat samples; Antony Antoniou and colleagues for generating several of the cell lines analysed; and Christoph Renner and colleagues for the continuous supply of the purified HD6 antibody.

Having been both an undergraduate and graduate student at New College, and lived within the College grounds for the majority of this time, I would like to express my gratitude to all the members of New College staff who helped me during that time. My particular thanks go to my tutor, Professor George Ratcliffe, whose initial guidance was instrumental in both my undergraduate education and in my decision to embark upon graduate studies.

I am truly fortunate to have such great friends and family. I feel privileged to have shared my PhD journey at the WIMM with two of my closest friends and fellow scientists, Abigail Culshaw and Joanna Giles, and am grateful for their friendship. This thesis would certainly not have existed without the enormous emotional and moral support of Gideon Elford. Throughout my time in Oxford, his unwavering confidence in me was the motivation needed whenever difficult times were faced. His deep understanding and unfaltering patience during this long process will not be forgotten.

Finally, to my parents, the debt of gratitude that I owe is immeasurable; for raising me in a loving environment, supporting me in all my endeavours, and exemplifying a work ethic that I have aspired to follow. Without the foundation they have provided me, nothing I have done would have been possible. It is my dad's example in particular that encouraged my interest in rheumatology research, and continues to inspire. This thesis is therefore dedicated to Linda and Neil McHugh, to whom I owe so much.

Contents

Abstract	ii
Declaration	iii
Acknowledgements	iv
Contents	vi
List of Tables	xii
List of Figures	xiii
List of Abbreviations	xvii
Chapter One : Introduction	1
1.1 Spondyloarthropathies	1
1.1.1 Overview of the Spondyloarthropathies	1
1.1.2 Ankylosing Spondylitis	1
1.1.3 Diagnosis	5
1.1.4 Treatment	8
1.2 MHC class I antigen processing and presentation	14
1.2.1 The functions of MHC class I molecules	14
1.2.2 The structure of MHC class I molecules	18
1.2.3 The structure of HLA-B27	21
1.2.4 Overview of the MHC class I folding pathway	25
1.2.5 The folding of HLA-B27	29
1.2.6 Overview of MHC class I antigen processing	30
1.3 Genetic Factors and AS	33

1.3.1 Susceptibility loci within the MHC region	35
A. B27 and subtypes	37
B. peptide repertoires and B27 subtypes	41
C. non-B27 genes within the MHC	43
1.3.2 Susceptibility loci outside of the MHC region	44
A. ERAP1	45
B. IL23R	47
1.4 HLA-B27 and SpA pathogenesis	50
1.4.1 Animal Models of SpA	50
A. HLA-B27 transgenic mice	51
B. Spontaneous inflammatory arthritis (SA) mouse model	53
C. ANKENT mouse model	56
D. DBA/1 mouse model	57
E. HLA-B27 transgenic rats	58
1.4.2 Theories of pathogenesis	64
A. Arthritogenic peptide theory	64
B. HLA-B27 misfolding and UPR theory	66
C. HLA-B27 homodimer theory	70
Project Objectives	76
Chapter Two : Materials and Methods	78
2.1.1 General cell culture	78
2.1.2 Adherent cell culture	78
2.1.3 Cell lines	79
2.1.4 Generation of lentivirus and lentiviral transduction of cells	81

2.1.5 Human peripheral blood mononuclear cell (PBMC)	82
samples and ethical approval	
2.1.6 Preparation of peripheral blood mononuclear cells	
(PBMCS)	83
2.1.7 Cryopreservation of cells	83
2.1.8 Antibodies	84
2.1.9 FACS staining and analysis	86
2.1.10 Intracellular staining	86
2.1.11 Generation of B27-restricted CD8+ T cell lines	87
2.1.12 Tetramer staining	87
2.1.13 Generation of HLA-B27 tetramers	88
2.1.14 Synthetic peptides	89
2.1.15 Exogenous peptide addition to cells	89
2.1.16 Low pH iso-osmotic treatment of cells	91
2.1.17 Papain treatment of cells	91
2.1.18 CD107a degranulation assay	91
2.1.19 IFN- γ ELISpot	92
Chapter Three : HLA-B27 expression in cell lines	94
3.1 Introduction	94
3.2 Analysis of HD6 epitope expression in human B cell lines	97
3.2.1 HD6 stains transfected B cell lines expressing HLA-B27	97
3.2.2 The HD6 epitope is not expressed on EBV-transformed B	
cell lines under standard conditions	101
3.2.3 HD6 staining of B cell lines transduced with HLA-	

expressing lentiviruses	104
3.3 Analysis of HD6 epitope expression in non-B cell HLA-B27	
expressing cell lines	110
3.3.1 HD6 does not stain non-B cell lines expressing HLA-B27	110
3.4 Preliminary studies suggest HD6 may additionally	
recognise an intracellular epitope not confined to HLA-B27	119
3.5 Discussion	123
Chapter Four : Characterisation of the HLA-B27	
epitope recognised by HD6	135
4.1 Introduction	135
4.2 The effect of exogenous peptide addition on HLA-B27	
expression	136
4.2.1 Addition of exogenous HLA-B27-binding peptides	
reduces HD6 staining of HLA-B27-expressing lymphoblastoid	
B cell lines	136
4.2.2 Peptide addition abrogates HD6 reactivity for up to 24h	136
4.2.1 Addition of exogenous HLA-B27-binding peptides	
reduces HD6 staining of HLA-B27-expressing lymphoblastoid	
B cell lines	137
4.2.3 Exogenous peptide addition reduces HD6 staining in	
HeLa cells lentivirally-transduced to express HLA-B27	138
4.2.1 Addition of exogenous HLA-B27-binding peptides	
reduces HD6 staining of HLA-B27-expressing lymphoblastoid	
B cell lines	136

4.3 The effect of iso-osmotic low pH treatment of cells on HD6 reactivity	146
4.3.1 Low pH treatment of transfected .221 and C1R BCLs increases HD6 reactivity	146
4.3.2 HD6 staining of BCLs expressing HLA-B27 is induced by low pH treatment	147
4.3.3 Acid-induced HD6 staining is independent of HLA-B27 Subtype polymorphisms in the F pocket	147
4.3.4 Acid-induced HD6 staining is dependent on surface expression levels of HLA-B27	151
4.3.5 The unpaired cysteine residues at positions 67, 308 and 325 of HLA-B27 are not necessary for acid-induced HD6 reactivity	152
4.3.6 Acid-induced HD6 staining requires key components of the peptide complex, tapasin and TAP, but does not require calnexin	158
4.4 The use of papain to investigate the formation of HD6-reactive molecules	160
4.4.1 HD6-reactive B27 surface molecules are susceptible to digestion by papain in the absence of cysteine	161
4.4.2 Cell surface HD6-reactivity partially recovers in the presence of BFA after treatment with papain alone but not cysteine-activate papain	162
4.4.3 The absence of functional tapasin in .220B27 cells	

influences the recovery of surface HLA-B27 molecules	
following papain treatment and protein transport inhibition	165
4.5 Preliminary Epitope Mapping of the HD6 epitope	170
4.6 Discussion	173
Chapter Five : HD6 recognition of HLA-B27 in an	
animal model of SpA and in SpA patients	195
5.1 Introduction	195
5.2 Expression of HD6-reactive HLA-B27 molecules in an	
HLA-B27 transgenic rat model of Spondyloarthritis	197
5.2 Expression of HD6-reactive HLA-B27 molecules in an	
HLA-B27 transgenic rat model of Spondyloarthritis	197
5.2.1 HD6 binds to HLA-B27 transgenic rat cell subsets	197
5.2.2 HD6 binding to HLA-B27 transgenic rat cell subsets is	202
dependent on HLA-B27 expression levels	
5.2.3 HD6 stains cells from HLA-B27 but not HLA-B7	207
transgenic rats	
5.3 The effect of B27 stability on HD6 staining in transgenic	
rats	212
5.3.1 HD6 staining is reduced by addition of exogenous HLA-	
B27 binding peptides	212
5.3.2 HD6 staining of B27 TG rat splenocytes is increased by	
low pH treatment	212
5.4 HD6 epitope expression on peripheral blood mononuclear	
cells from patients with Ankylosing Spondylitis	218

5.4.1 HD6 staining of AS patient PBMCs	218
5.4.2 HD6 staining is induced in AS patient PBMCs upon low pH treatment	219
5.5 Discussion	222
Concluding remarks and future perspectives	232
Appendices	242
References	250

List of Tables

Table 1.1 Prevalence of HLA-B27 in SpA	3
Table 1.2 Clinical symptoms of HLA-B27 associated SpA	4
Table 1.3 Modified New York Criteria for diagnosis of AS	6
Table 1.4 ASAS classification criteria for axial SpA	7
Table 1.5 Common HLA-B27 subtype frequencies (%) in different global populations	37
Table 1.6 Common HLA-B27 subtype frequencies (%) in different global populations	39
Table 1.7 MHC and non-MHC genes associated with AS	49
Table 1.8 The first HLA transgenic rat lines	63
Table 2.1 Cell Culture Media	79
Table 2.2 Cell Lines	80
Table 2.3 Antibodies	84
Table 2.4 Synthetic Peptides	90
Table 4.1 Overlapping peptides used in epitope mapping experiments	170

List of Figures

Chapter One

Figure 1.1 The structure of an MHC class I molecule	20
Figure 1.2 Key amino acid residues in HLA-B27	23
Figure 1.3 Proposed model of the HLA-B27 HC homodimer (B27 ₂) structure	24
Figure 1.4 Positions of the polymorphic residues in HLA-B27	38
Figure 1.5 Disease progression in HLA-B27 transgenic rats	62
Figure 1.6 HLA-B27 homodimer Theory	74
Figure 1.7. HLA-B27 heterotrimer and homodimer interactions with innate immune receptors	75

Chapter Three

Figure 3.1 Antibody Epitopes	95
Figure 3.2 Cartoon schematic of the generation of B27 ₂ -specific monoclonal antibodies	96
Figure 3.3 721.220 and 721.221 transfected B cell lines expressing HLA-B27 stain with HD6	99
Figure 3.4 HD6 stains transfected C1R cells expressing HLA-B27 but not other HLA alleles	102
Figure 3.5 HD6 does not stain EBV-transformed B cell lines that express HLA-B27	103
Figure 3.6 Schematic representation of the HLA-B27 Lentivirus constructs used	104
Figure 3.7 Lentivirus transduction of .220 and .221 cell lines and HD6 epitope expression	106
Figure 3.8 HD6 staining of LV-transduced cells expressing HLA-B*27:05 or HLA-B*27:09	107
Figure 3.9 The presence of a C-terminal HA tag abrogates HD6 epitope expression	109
Figure 3.10 The HD6 epitope is not detectable in several non-B cell lines	111
Figure 3.11 HD6 epitope is not expressed by KG-1 cell lines	

transfected with HLA-B27 before or even after PMA-induced maturation	112
Figure 3.12 HD6 does not stain the C58 rat thymoma cell line transfected with HLA-B27	114
Figure 3.13 HD6 staining of LV-transduced HeLa cells	115
Figure 3.14 HD6 staining of HeLa cells expressing HLA-B27 SCT molecules	117
Figure 3.15 HeLa cells expressing B27 molecules as SCTs under an eIF1 α promoter can activate a specific CTL response	120
Figure 3.16 HeLa cells expressing B27 molecules as SCTs under a CMV promoter can activate a specific CTL response	121
Figure 3.17 Intracellular staining of cell lines with HD6	122
Chapter Four	
Figure 4.1 Overnight addition of B27-binding peptides reduces HD6 binding to HLA-B27-expressing B cell lines	139
Figure 4.2 Overnight incubation of .220B27 cells with the B27-binding peptides recognised by the peptide-dependent B27-specific MARB4 antibody reduces HD6 staining	140
Figure 4.3 Peptide addition to .220B27 cells abrogates HD6 binding for up to 24h	141
Figure 4.4 Peptide addition to .220B27TPN cells abrogates HD6 binding for up to 24h	142
Figure 4.5 Peptide addition to C1RB27 cells abrogates HD6 binding for up to 24h	143
Figure 4.6 Recovery of HD6 staining is delayed by the presence of peptide in the media	144
Figure 4.7 Incubation of HLA-B27-expressing HeLa cells with peptide abrogates HD6 reactivity	145
Figure 4.8 HD6 staining is increased in .221 and C1R BCLs expressing HLA-B27 but not control HLA alleles after acid treatment	148
Figure 4.9 Low pH treatment of EBV-transformed BCLs induces	

HD6 epitope expression in cells expressing HLA-B27 but not control alleles	149
Figure 4.10 Acid-induced HD6 reactivity is independent of HLA-B27 subtype polymorphism at position 116 in the F pocket	150
Figure 4.11 Acid-induced HD6 correlates with surface expression levels of HLA-B27 in the rat C58 cell line	154
Figure 4.12 HD6 staining can be induced in C58 cells expressing HLA-B27 by low temperature treatment, is augmented upon low pH treatment, and does not require cysteine residues at positions 67, 308, and 325	155
Figure 4.13 Acid-induced HD6 staining is dependent on tapasin and not inhibited by a C-terminal HA tag	157
Figure 4.14 Acid treatment of HLA-expressing TAP-deficient but not calnexin-deficient cells induces HD6 epitope expression	159
Figure 4.15 HD6-reactive molecules are susceptible to papain stripping in the absence of cysteine	163
Figure 4.16 Recovery of HD6 and HC-10 surface staining is inhibited by BFA only after treatment of cells with cysteine-activated papain	164
Figure 4.17 Recovery after treatment with activated-papain and BFA reveals a cohort of HD6- and HC-10-reactive molecules in .220B27 cells not present in .220B27TPN cells	168
Figure 4.18 Re-expression of cell surface HD6 reactivity following stripping with activated papain requires egress of newly synthesised proteins from the ER	169
Figure 4.19 Mapping of the HD6 epitope	172
Figure 4.20 Proposed model for the formation of steady-state HD6-reactive B27 molecules	193
Figure 4.21 Proposed model for the formation of 'Acid-inducible HD6' molecules at the cell surface	194

Chapter Five

Figure 5.1 HLA-B27 transgenic rat splenocytes have an increased

relative percentage of cells in the granulocyte gate	199
Figure 5.2 HD6 stains splenocytes from an adult disease-prone HLA-B27 transgenic rat but not from an age-matched WT rat	200
Figure 5.3 HD6 stains lymphocyte and monocyte but not granulocyte cell subsets in splenocytes from an adult disease-prone HLA-B27 transgenic rat	201
Figure 5.4 Cell surface expression of HLA-B27 on rat splenocytes is increased with age in disease-prone HLA-B27 transgenic rats	203
Figure 5.5 HD6 staining of splenocyte subsets from disease-prone HLA-B27 transgenic rats is increased with age	204
Figure 5.6 HD6 staining of cell subsets is increased with age in disease-prone HLA-B27 transgenic rats	208
Figure 5.7 HD6 stains cell surface molecules on splenocytes from young HLA-B27 TG rats but not HLA-B7 TG rats	209
Figure 5.8 HD6 recognises cell surface molecules on splenocytes from HLA-B27 but not HLA-B7 TG rats	210
Figure 5.9 HD6 staining of adult HLA-B27 transgenic rat splenocytes is reduced upon addition of a B27-binding peptide but not a control peptide	211
Figure 5.10 Low pH treatment of splenocytes from a young HLA-B27 transgenic rat induces HD6 staining	214
Figure 5.11 Low pH treatment of splenocytes from an older HLA-B27 rat increases and/or induces HD6 staining	215
Figure 5.12 Low pH treatment of splenocyte from a young HLA-B7 transgenic rat increases surface free heavy chains but does not induce HD6 staining	215
Figure 5.13 A comparison of the effect of acid treatment on HLA-B27 and HLA-B7 transgenic rat splenocytes	217
Figure 5.14 HD6 does not bind to peripheral blood mononuclear cells from B27+ AS patients under standard conditions.	220
Figure 5.15 HD6 staining is induced by low pH treatment on PBMCs from B27+ AS patients but not controls	221

List of Abbreviations

AAU	Acute anterior uveitis
ANKENT	Ankylosing enthesopathy
APC	Antigen presenting cell
AS	Ankylosing spondylitis
ASAS	Assessment in AS
ATF6	Activating transcription factor 6
β2m	Beta ₂ -microglobulin
B7	Human leukocyte antigen-B7
B27	Human leukocyte antigen-B27
B27₂	B27 homodimer
BASDAI	Bath AS disease activity index
BASFI	Bath AS functional index
BASMI	Bath AS metrological index
BFA	Brefeldin A
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
CHOP	C/EBP homologous protein
CIA	Collagen-induced arthritis
CI	MHC class II molecule
CMV	Cytomegalovirus
Cnx	Calnexin
CRP	C-reactive protein
Crt	Calreticulin
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMARDs	Disease modifying anti-rheumatic drugs
DMSO	Dimethyl sulphoxide
dTGM	Double transgenic mice

DTT	Dithiothreitol
EA	Enteropathic arthritis
EBV	Epstein barr virus
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
EOR	ER overload response
ER	Endoplasmic reticulum
ERAAP	ER associated aminopeptidase (<i>murine</i>)
ERAD	ER-associated degradation
ERAP	ER aminopeptidase (<i>human</i>)
ERGIC	ER-Golgi intermediate compartment
ESR	Erythrocyte sedimentation rate
Fab	Fragment antigen binding
FACS	Fluorescent activated cell sorting
FasL	Fas ligand
FBC	Full blood count
FCS	Fetal calf serum
FPLC	Fast performance liquid chromatography
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GI	Gastrointestinal
GWAS	Genome-wide association study
HA	Haemagglutinin
HC	Heavy chain
HLA	Human leukocyte antigen
HPLC	High pressure liquid chromatography
HSP	Heat shock protein
HT	Heterotrimer

IBD	Inflammatory bowel disease
IBP	Inflammatory back pain
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin-like transcript
IP	Immunoprecipitation
IRAK	IL-1-receptor activation kinase
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer immunoglobulin-like receptor
KO	Knockout
LB	Luria bertani
LILR	Leukocyte immunoglobulin-like receptor
LPS	Lipopolysaccharide
LV	Lentivirus
MHC	Major histocompatibility complex
MHCI	MHC class I
NK	Natural killer
NSAIDs	Non-steroidal anti-inflammatory drugs
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
PE	Phycoerythrin
PERK	Pancreatic ER kinase
PIR	Paired immunoglobulin-like receptor
PLC	Peptide loading complex
PMA	Phorbol myristate acetate
pMHCI	Peptide-loaded MHC class I molecule

PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
ReA	Reactive arthritis
RCT	Randomised control trial
SCT	Single chain trimer
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sTGM	Single transgenic mice
SJ	Sacroiliac joint
SpA	Spondyloarthopathy/Spondyloarthritis
SNP	Single nucleotide polymorphism
SPR	Surface plasmon resonance
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TG	Transgenic
Th	T helper
TNF	Tumour necrosis factor
TNFR	TNF receptor
TPN	Tapasin
UPR	Unfolded protein response
uSpA	Undifferentiated SpA
XBP1	X box binding protein 1

Chapter One: Introduction

1.1 Spondyloarthropathies

1.1.1 Overview of the Spondyloarthropathies

The spondyloarthropathies (SpA) are a group of closely related chronic inflammatory diseases, typically affecting the axial skeleton, that share certain clinical features, underlying pathology and a strong genetic association with the human leukocyte antigen HLA-B27 (reviewed in (Calin, 1998)) (Table 1.1). They can be subdivided into 5 main adult subtypes, based on their clinical manifestations: ankylosing spondylitis (AS); reactive arthritis (ReA); enteropathic arthritis (EA); psoriatic arthritis (PsA); and undifferentiated Spondyloarthritis (uSpA). They can also be distinguished from other common inflammatory arthritides, such as rheumatoid arthritis (RA), by their hallmark characteristics of enthesitis and sacroiliitis (McGonagle et al., 1998), and the absence of rheumatoid factor (Table 1.2). Other symptoms include peripheral oligoarthritis, acute anterior uveitis (AAU), and sub-type defining features such as psoriasis and colitis (Martin et al., 2002; Orchard et al., 2000; Suarez-Almazor and Russell, 1990). AAU is the most common extra-articular feature, occurring in up to 25% of AS patients. Together, SpA are the leading cause of inflammatory arthritis, with the major subtype, AS, affecting approximately 200,000 people in the UK.

1.1.2 Ankylosing Spondylitis

Ankylosing Spondylitis (AS) is a chronic, progressive rheumatic disease that affects between 0.1 - 0.5% of the UK population (Brown et al., 1996). It is more common in males than females, with a bias of approximately 2:1, and usually develops in early adulthood,

with onset of symptoms typically beginning around 26 years of age (Feldtkeller et al., 2003). Inflammation typically affects two major sites: the entheses, which are sites where ligaments and tendons attach to bone; and the sacroiliac joints (SJ), which are where the base of the spine meets the pelvis. Disease primarily affects the spine, however peripheral joint involvement and associated disorders at other sites, including uveitis, psoriasis and inflammatory bowel disease, are common (Table 1.2). Typically, AS patients experience inflammatory back pain (IBP) as the earliest symptom, which come and goes in fluctuating periods of intensity. This IBP is distinct from mechanical back pain; it is characterised as persistent back pain that worsens with periods of immobility, such as during the night, but improves with light exercise. It is associated with morning spinal stiffness and is often accompanied by alternating buttock pain.

Persistence of disease then leads to progressive joint and spinal inflammation (spondylitis), which can result in extensive structural changes throughout the spine, mainly as a result of new bone formation. This includes the ossification of spinal ligaments and the appearance of bony outgrowths known as syndesmophytes, which are a typical radiographic feature of AS. In the most severe cases, this ultimately results in complete fusion of the vertebrae (ankylosis), which causes curvature of the spine, often referred to as bamboo spine, limited spinal mobility, and gives the individual a characteristic posture and gait (reviewed in (Braun and Sieper, 2007)).

Although no longer considered life threatening, the functional restrictions that AS places on individuals, alongside the debilitating pain experienced and the eventual progression to irreversible spinal damage, emphasises the importance for development of preventative

and symptom-alleviating therapies. This requires an improved understanding of the molecular events, genetic factors and environmental triggers that predispose and initiate disease pathogenesis. Yet, despite intense investigation, the fundamental mechanism(s) by which disease arises remain ill defined.

SpA disease group	HLA-B27 Frequency (%)
Ankylosing Spondylitis (AS)	96
Reactive Arthritis (ReA)	30-70
Psoriatic Spondylitis (PsA)	40-50
Enteropathic Arthritis (EA)	33-75
Undifferentiated Spondyloarthritis (uSpA)	70

Table 1.1 Prevalence of HLA-B27 in SpA Adapted from (McMichael and Bowness, 2002)

Clinical symptoms	SpA disease subgroup			
	AS	ReA	PsA	EA
Sacroiliitis or spondylitis	100%	<50%	~20%	~10%
Peripheral arthritis	25%	90%	95%	90%
Gastrointestinal inflammation	Common, usually asymptomatic	Common, often symptomatic	Uncommon	All
Skin and nail involvement	Rare	Common	Almost always	Uncommon
Genitourinary involvement	Uncommon	Common	Uncommon	Rare
Eye involvement	10-20%	Common	Occasional	Occasional
Cardiac involvement	<5%	<5%	Rare	Rare

Table 1.2 Clinical symptoms of HLA-B27 associated SpA (Adapted from (Hammer et al., 1990))

1.1.3 Diagnosis

The diagnosis of AS is difficult, and recognising it at an early stage of disease is one of the major barriers to its successful treatment. Aside from the difficulties of distinguishing between inflammatory back pain and mechanical back pain, few clinical tests are available to aid the process. With no reliable disease markers, blood tests look for indicators of inflammation, such as the full blood count (FBC), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, along with the presence of HLA-B27, which, given its occurrence in healthy individuals, cannot be used as a definitive indicator alone. However, the absence of a raised ESR or CRP is common in AS patients and does not rule out inflammation.

Thus, until recently, diagnosis has been based on the modified New York criteria, a set of guidelines that depend upon the presence of both clinical features and radiographic features (Table 1.3) (van der Linden et al., 1984). Yet, the structural changes identified on conventional radiographs can often take up to 10 years to develop, meaning that this criteria excludes the possibility of detecting disease early, before axial progression. The advent of MRI scanning, which can detect both acute inflammation and chronic damage within the spine and sacroiliac joints, has provided a means for the better detection of pre-radiographic SpA. This has led to the recent development of new diagnostic guidelines, the ASAS criteria, which incorporate the use of MRI evidence (Table 1.4) (Rudwaleit et al., 2009a; Rudwaleit et al., 2009b). By enabling the identification of patients with early AS, patients may have access to more effective treatment sooner in the disease course, preventing further damage. This is particularly relevant given that some of the anti-TNF studies have reported improved outcomes with early treatment (Rudwaleit et al., 2004).

Once diagnosed, AS disease activity and function are assessed using a combination of indices, some of which involve a patient's self-assessment of symptom severity and function, and all of which produce a score out of 10: the Bath Ankylosing Spondylitis Function Index (BASFI), which assesses functional limitation; the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), a measure for assessing signs of disease activity including fatigue, axial pain, peripheral pain, enthesitis and morning stiffness; the Bath Ankylosing Spondylitis Disease Metrology Index (BASMI), which combines various aspects of spinal mobility to give an indication of the limitation of movement as a result of a patient's AS. For example, a BASDAI score higher than 4 is deemed indicative of active disease. These index scores are also helpful in monitoring the efficacy of treatment strategies: the BASFI is one of 4 domains (physical function, spinal pain, patient global assessment, and inflammation) that comprise the ASAS response criteria, which was constructed as a measure to assess the treatment response in AS trials, whereas the BASDAI is also often used in clinical trials.

Clinical criteria	Radiological criterion
1) Low back pain and stiffness for more than 3 months which improves with exercise, but is not relieved by rest 2) Limitation of motion of the lumbar spine in both the sagittal and frontal planes 3) Limitation of chest expansion relative to normal values corrected for age and sex	1) Sacroiliitis grade ≥ 2 bilaterally or sacroiliitis grade 3-4 unilaterally
<u>Definite AS</u> is present if the radiological criterion is associated with at least 1 clinical criterion <u>Probable AS</u> is present if either 3 clinical criteria are present or the radiologic criterion is present without any symptoms satisfying the clinical criteria	

Table 1.3 Modified New York criteria for diagnosis of AS (van der Linden et al., 1984)

Sacroiliitis on imaging* + ≥1 SpA feature**	OR	HLA-B27 + ≥2 other SpA features**
* sacroiliitis on imaging: - active (acute) inflammation on MRI highly suggesting of sacroiliitis associated with SpA Or - definite radiographic sacroiliitis according to modified NY criteria **SpA features: - inflammatory back pain - arthritis - enthesitis (heel) - uveitis - dactylitis - psoriasis - Crohn's disease/ulcerative colitis - Good response to NSAIDs - Family history of SpA - HLA-B27 - Elevated CRP		

Table 1.4 ASAS classification criteria for axial SpA (in patients with back pain for more than 3 months and age at onset less than 45 years) (Sieper et al., 2009)

1.1.4 Treatment

With no current therapies available that directly target bone ankylosis, and, until recently, no effective disease modifying anti-rheumatic drugs (DMARDs) for AS, the standard treatment is aimed at reducing inflammation and maintaining patient function. Non-steroidal anti-inflammatory drugs (NSAIDs), including those traditionally used (ibuprofen, diclofenac and naproxen) and the more recently developed COX-2 specific inhibitors (celecoxib (Celebrex)), are prescribed in combination with specifically tailored exercise programmes. The efficacy of this treatment strategy is supported by the findings of a number of randomised control trials (RCTs). For example, those assessing the efficacy of physiotherapy for AS have reported improved function in the short term when patients undergo home exercise regimes (Fernandez-de-Las-Penas et al., 2006). Similarly, the efficacy of NSAIDs at significantly improving inflammatory symptoms has been widely reported, with the majority of patients reporting rapid pain relief. Whether there are additional benefits of this therapeutic strategy in the long term, such as a potential influence on the bone remodelling processes or an improved disease prognosis, remains under investigation. Notably, one long-term study, carried out over a 2-year period, has suggested that continuous NSAID treatment may delay radiographic disease progression (Wanders et al., 2005). Yet, continuous use of NSAIDs is problematic, and associated with an increased risk of adverse side effects, including gastrointestinal bleeding. Furthermore, a proportion of patients remain refractory to NSAIDs. Thus, the concept of NSAID treatment alone being a viable long-term therapeutic option for most patients is flawed.

Traditional disease-modifying anti-rheumatic drugs (DMARDs), such as sulfasalazine, leflunomide, and methotrexate, have proved very effective at suppressing disease activity

and progression in RA but, unfortunately, not in AS. In contrast, the last decade has seen the advent of new promising biological therapies for the treatment of both inflammatory arthritides. These “biologics”, designed to interfere with the inflammatory process, have been directed at the action of the proinflammatory cytokines TNF α , IL-1, IL-6 and, most recently, IL-17 and IL-23. First trialled in RA patients, the rationale to extend their application to AS has come from the success of anti-TNF therapy in the treatment of Crohn’s disease, an inflammatory bowel disease that shares several clinical features with SpA, and the findings of elevated levels of TNF α in the serum and at the sacroiliac joints of AS patients.

Three current anti-TNF α therapies have been approved for AS treatment: the monoclonal antibodies infliximab (Remicade) and adalimumab (Humira), and the soluble receptor etanercept (Enbrel). Two further anti-TNF α drugs that have been passed by NICE for use in RA, golimumab (Simponi) and certolizumab pegol (Cimzia), are in ongoing clinical trials and awaiting approval for use in AS. All of these agents target the action of TNF α , blocking the ensuing signalling cascade. TNF α is a pro-inflammatory cytokine primarily produced by activated monocytes and macrophages but may also be produced by neutrophils, activated lymphocytes (B and T), natural killer (NK) cells, endothelial cells, fibroblasts, osteoclasts and mast cells. It is expressed as a membrane-bound protein that can be cleaved into a soluble form by the proteolytic activity of the TNF α converting enzyme (TACE, also known as ADAM17). It plays a central role both in host defense against viral and bacterial infection and in anti-tumor immunity. Through binding to two distinct cell surface receptors, TNFRI and TNFRII, TNF α induces the expression of adhesion molecules on endothelial cells, including intercellular adhesion molecule-1

(ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, allowing the recruitment of leukocytes into regions of inflammation. It also triggers the release of several other pro-inflammatory interleukins, including IL-1, IL6, and IL-8, resulting in the potent activation of neutrophils. In addition, TNF α acts on the local environment of a joint: it induces the proliferation of fibroblast-like synoviocytes (FLS), stimulates the production of tissue destructive enzymes and mediators, including collagenase and prostaglandin E2 (PGE2), while inhibiting production of proteoglycans, and, through its regulation of osteoclast differentiation and function, it can stimulate osteoclastogenesis, resulting in bone resorption (Dayer et al., 1986; Gitter et al., 1989; Saklatvala, 1986).

Animal models of arthritis also highlight the role of TNF α in inflammation (reviewed in (Li and Schwarz, 2003)): mice transgenic for TNF α develop spontaneous and progressive polyarthritis, whereas TNF α knockout mice are more resistant to the induction of serum-transfer arthritis. Moreover, treatment with anti-TNF α antibodies alleviates disease symptoms and reduces histologic features of joint damage in several current mouse models of arthritis, including adjuvant-induced (AIA), collagen-induced (CIA), and serum-transfer arthritis (STA).

Initial human clinical trials were set up with each anti-TNF α agent in order to compare their efficacy to a placebo arm and have demonstrated their efficacy at significantly improving various clinical and functional parameters (Davis et al., 2003; Inman et al., 2008; van der Heijde et al., 2006). For example, in one RCT, 80% of patients receiving etanercept achieved ASAS20 at 4 months compared with 30% of placebo-treated patients. Furthermore, in a 6-month extension, an ASAS20 was achieved in 95% of the etanercept-

treated patients (Gorman et al., 2002). In an infliximab trial, 53% of patients receiving the anti-TNF treatment, compared with 9% in the control arm, experienced regression of disease activity of at least 50%, as well as significant improvement in function and quality of life (Braun et al., 2002). With clear improvements demonstrated, ongoing trials now investigate their use in combination with other drugs, and whether their efficacy can be improved if used earlier in disease. One recent paper identifies young age and short disease duration as predictors for good clinical responses to TNF therapy (Rudwaleit et al., 2008). Whether TNF therapy reduces or prevents skeletal damage remains unclear.

TNF blockade is not without limitations. Whilst the majority of patients receiving treatment respond well, there remains a subset that is refractory. Furthermore, relapse is common when treatment is discontinued. Adverse side effects include a risk of allergy, particularly in the case of infliximab, which is administered as an IV infusion and injection site reactions are common. However, the most significant side effect is the increased risk of infection that comes with inhibiting TNF α signalling. In particular, the reactivation of tuberculosis (TB) is a recognised risk, and so patients are screened for both active and inactive TB infection prior to treatment. Finally, the cost of the anti-TNF drugs has meant that NICE guidelines must be met before this treatment can even be considered, and so these biologicals are rarely used as a first-line therapy.

The success of TNF therapy in the treatment of inflammatory arthritis has led to the development of biologics targeting other cytokine pathways, including the pro-inflammatory cytokines IL-1 and IL-6 as well two key cytokines involved in Th17 cell biology: IL-17 and IL-23. In the case of the former, there is increasing evidence that these

cytokines can inhibit bone formation and induce bone resorption in a similar way to TNF α (reviewed in (Schett, 2008)). For example, bone erosions do not develop after induction of arthritis in IL-6 knockout mice (Wong et al., 2006). One anti-IL6R antibody, Tocilizumab (RoActemra) is currently available for the treatment of RA, having shown benefits in reducing joint inflammation and damage, and has entered trials for AS, whilst another antibody targeting the IL-6 receptor, SAR153191 (REGN88) awaits approval (<http://clinicaltrials.gov>). For targeting the IL-1 pathway, an IL-1 receptor antagonist, anakinra, and an IL-1 β neutralising antibody, canakinumab, have been developed and trialled for treatment of inflammatory arthritis. Yet, results for these reagents thus far have not been as convincing as the anti-TNF drugs, and further trials on their effectiveness in AS are needed. Their value may lie in the treatment of patients that do not respond first to TNF α antagonists.

Th17 cells are a T cell subset implicated in a number of human autoimmune diseases including AS, which has made them a potential therapeutic target. Their differentiation is promoted by the cytokine IL-23, and they secrete IL-17, a pro-inflammatory cytokine that can behave in a similar manner to TNF α ; inducing secretion of further proinflammatory cytokines, stimulating osteoclast formation and bone resorption, and recruiting neutrophils and monocytes to sites of inflammation. Two neutralising antibodies that target the major isoform of IL-17, IL-17A, have entered phase II/III development, AIN457 (secukinumab) and LY2439821. In addition, monoclonal antibodies under evaluation in ongoing trials include those designed to target the p40 subunit of IL-23, a subunit shared with IL-12, or the IL-23 specific p19 subunit (<http://clinicaltrials.gov>).

Whether these therapies will match or even supersede the efficacy of TNF α drugs awaits analysis, however the risk of complications and increased infection remains. There is therefore a need for future drug development that can be more directed at specific disease pathogenic mechanisms, without interfering with normal innate and adaptive immune responses. Thus, an improved understanding of the molecular mechanisms underlying SpA will enable the identification of better therapeutic targets.

1.2 Major Histocompatibility Complex class I antigen presentation

HLA-B27 is a Major Histocompatibility Complex (MHC) class I molecule and, as such, its principle function is to present cell-derived peptides to CD8⁺ cytotoxic T cells (CTL) as part of the adaptive immune response (reviewed in (Bjorkman and Parham, 1990)). MHC class I molecules also play a critical role in regulating the innate response, through their ability to interact with receptors on NK cells. The following section first outlines the role of MHC class I molecules in the immune system. This is followed by detailed descriptions of the structure and assembly of MHC class I molecules, along with their antigen processing and presentation pathways, with particular emphasis on how these relate to HLA-B27 and its unusual features that set it apart from other MHC class I molecules.

1.2.1 The functions of MHC Class I molecules in innate and adaptive immunity

MHC class I proteins are expressed on the surface of virtually all nucleated cells as part of a surveillance system that allows the immune system to detect and respond to abnormal cell activity. This is achieved through the inherent ability of cells to process endogenous proteins and present them on the surface, in the context of MHC molecules, as cell-derived peptides. Under abnormal conditions, such as infection or cancerous growth, these cell-derived peptides will include foreign peptides, which, when presented at the surface, target the cell for CD8⁺ CTL-mediated lysis. Naïve CD8⁺ T cells acquire their cytotoxic capabilities as a result of the interaction of these peptide-MHCI complexes with the T cell receptor (TCR), a heterodimeric protein expressed on the surface of CTLs. This interaction is stabilised by additional contacts between the MHCI and the CD8 co-receptor present on the CTL. Signal transduction is then achieved through the close association of the TCR with the CD3 complex. Thus, appropriate stimulation of naïve CD8⁺ T cell produces

mature effector CTLs capable of lysing target cells that bear the cognate pMHC I molecules.

CTL-mediated cytotoxicity is carried out by several lytic mechanisms. Of these, the most well known involve either the exocytosis of pre-formed vesicles containing lytic proteins or the receptor-ligand binding of members of the Fas family. In the case of the former, some CTLs have cytoplasmic granules containing perforin, a pore-forming protein that forms channels in the target cell membrane, and granzymes, a group of serine proteases that can induce target cell apoptosis through the cleavage and concomitant activation of caspase precursors. Upon target recognition, CTL activation releases these lytic proteins via granule exocytosis in the direction of the target cell to induce cell death. Alternatively, target cell apoptosis can be induced via the Fas ligand (FasL), which is expressed on the surface of activated CTLs. FasL binds to Fas molecules on the surface of target cells, which also leads to the activation of a caspase cascade within the target cell and culminates in death by apoptosis. Thus, MHC class I molecules are a major component of the adaptive immune system.

MHC class I molecules are also important in the initiation and regulation of the innate response. This is through their ability to interact with receptors displayed on a variety of cell types including natural killer (NK) cells. NK cells are phenotypically defined by the surface expression of CD56 and absence of CD3. Although additional roles in transplantation and pregnancy have been established, (Goss et al., 1985; Moffett-King, 2002), they are involved primarily in the control and clearance of malignant and virus-infected cells. This is achieved through their natural cytotoxic activity, which allows them

to directly kill target cells utilising mechanisms similar to those employed by CTLs. In addition, activated NK cells secrete cytokine and chemokines, including IFN γ and TNF α , thereby serving as regulatory cells during inflammation, with the ability to influence the adaptive immune response that follows. Unsurprisingly, therefore, impaired NK cell activity is associated with several autoimmune diseases including multiple sclerosis, systemic lupus erythematosus and type 1 diabetes mellitus (reviewed in (Baxter and Smyth, 2002; Flodstrom et al., 2002)).

Activation of NK cells is mediated by two sets of receptors: activating receptors and inhibitory receptors (Lanier, 1998). The inhibitory receptors predominantly recognise MHC class I ligands, whereas the activatory receptors include both MHC class I-like molecules and unrelated proteins, with many ligands still to be determined. Following ligand recognition, it is the integration of signals resulting from the triggering of these receptors that dictates whether the NK cell becomes activated for effector function. In the presence of normal MHCI expression, inhibitory signalling protects healthy cells from NK cell mediated lysis, overriding any activatory signalling that occurs. However, this NK cell tolerance can be overcome in situations of infection or tumorigenesis. For example, if MHCI expression is downregulated on target cells, which is a common strategy employed by viruses to escape CTL recognition, NK cell inhibition is relieved and activation ensues. This forms the basis of “missing-self” recognition, which was first discovered by Karre *et al.* in transplantation experiments involving mice and tumor cell lines that had been selected for deficient MHCI expression (Ljunggren and Karre, 1990). The molecular basis for missing-self recognition was subsequently provided by the identification of the first inhibitory receptor to recognise MHC class I (Karlhofer et al., 1992). Alternatively,

activatory signalling can also override inhibition if further activatory receptor ligands are induced on abnormal cells. This process has been termed ‘induced self’ recognition.

Until recently, it has been generally accepted that NK cell responsiveness is also determined by a process known as NK cell licensing. Although not fully understood, this process stipulates that a requirement for NK cell licensing is that the NK cell must express at least one inhibitory receptor capable of binding self-MHC. This was put forward to explain the observed hyporesponsiveness of i) NK cells from MHC class I deficient mice and ii) of naturally occurring NK cell subsets that lack any known inhibitory receptor for self MHC class I (Kim et al., 2005). It postulates that during maturation, the ligation of inhibitory receptors, prior to target cell encounter, allows NK cells to become functionally mature and licensed, without which they become defective or anergic. However, very recent evidence from the Lanier group challenges this view and proposes a previously unidentified role for unlicensed NK cells in the efficient killing of some abnormal targets (Orr et al., 2010).

Despite the uncertainties surrounding the requirements for NK cell activation, the central role of MHC class I molecules in this process seems certain. Therefore, MHC class I molecules play a critical role in regulating both the innate and adaptive arms of the immune response through their ability to interact with receptors on NK cells and CTLs respectively.

1.2.2 The structure of MHC class I molecules

MHC class I molecules comprise two polypeptide chains; a long α chain, also called the heavy chain (HC), and a short noncovalently associated invariant chain, β_2 -microglobulin (β_2m). In humans, the HCs are encoded on chromosome 6 within the MHC region, whereas the β_2m gene is located outside this region on chromosome 15. The HC is a 45 kDa glycosylated polypeptide of about 350 amino acids, with one N-linked oligosaccharide at positions N86. It comprises an extracellular region that is folded into three separate domains, which are linked to a 70 amino acid C-terminal transmembrane and cytoplasmic portion. β_2m is a 12 kDa polypeptide with no transmembrane region, encoded outside of the MHC locus. Upon association, the HC: β_2m heterodimer is able to bind a peptide to form a heterotrimeric complex (HT) that presents the peptide antigen on the surface of antigen-presenting cells (APCs) for recognition by the T cell receptor (TCR) of CD8⁺ cytotoxic T cells (CTLs).

The first three-dimensional structure of a class I molecule was determined for HLA-A2 (Bjorkman et al., 1987a, b; Saper et al., 1991). This has been followed by the determination of the structure a number of other class I molecules, including HLA-B27, some of which have been solved with a number of different peptide ligands (www.pdb.org). These X-ray crystallographic studies have revealed that class I molecules share a very similar overall structure, with the most variable region located in the $\alpha 1$ and $\alpha 2$ domains, which together form the peptide binding groove. From these studies, the detailed structure features of class I molecules have emerged. The heavy chain itself is composed of 3 α domains: $\alpha 1$ and $\alpha 2$ form the peptide binding groove; the $\alpha 3$ domain associates with β_2m and sits below. The peptide binding groove comprises two anti-

parallel α -helices, one from each domain, that form the walls and a β -pleated sheet forming the base of a groove containing several pockets (designated A-F) to accommodate amino acid side chains (Madden et al., 1991). The groove is approximately 30Å long, 12Å wide and 11Å deep in the middle and can accommodate peptides of 8-10 amino acids long in an extended conformation, with defined C- and N-terminal orientation (Figure 1.1). The $\alpha 3$ and β_2m domains are highly conserved and fold into an immunoglobulin-like (Ig-like) β -sandwich structure. There are extensive non-covalent interactions between the β_2m and the underside of the peptide-binding groove, and some further contacts with the $\alpha 3$ domain. The HC structure is stabilised by two intra-molecular disulphide bonds: one in the $\alpha 2$ domain (Cys 101-Cys164), and one in the hydrophobic core of the $\alpha 3$ domain (Cys203-Cys259), whereas β_2m has a single disulfide bond (Cys25-Cys80).

As mentioned, the heavy chain also comprises one N-linked glycan, at position N86 in the $\alpha 1$ domain. The precise biological role of this glycan is still unclear. There is some evidence suggesting that the inhibition of glycosylation in cells compromises CTL recognition without affecting MHC class I cell surface expression (Hart, 1982; Pimlott and Miller, 1986; Ploegh et al., 1981). Yet, experiments involving murine MHC class I molecules, which have a further two N-linked glycans (N176 in $\alpha 2$ domain and N256 in $\alpha 3$ domain) report no role for glycans in antigen recognition (Goldstein and Mescher, 1986; Miyazaki et al., 1986). Instead, these studies have suggested that the biological role of these N-linked oligosaccharides may be in the efficiency of the intracellular folding and transport pathways for MHC class I, which are discussed in a following section.

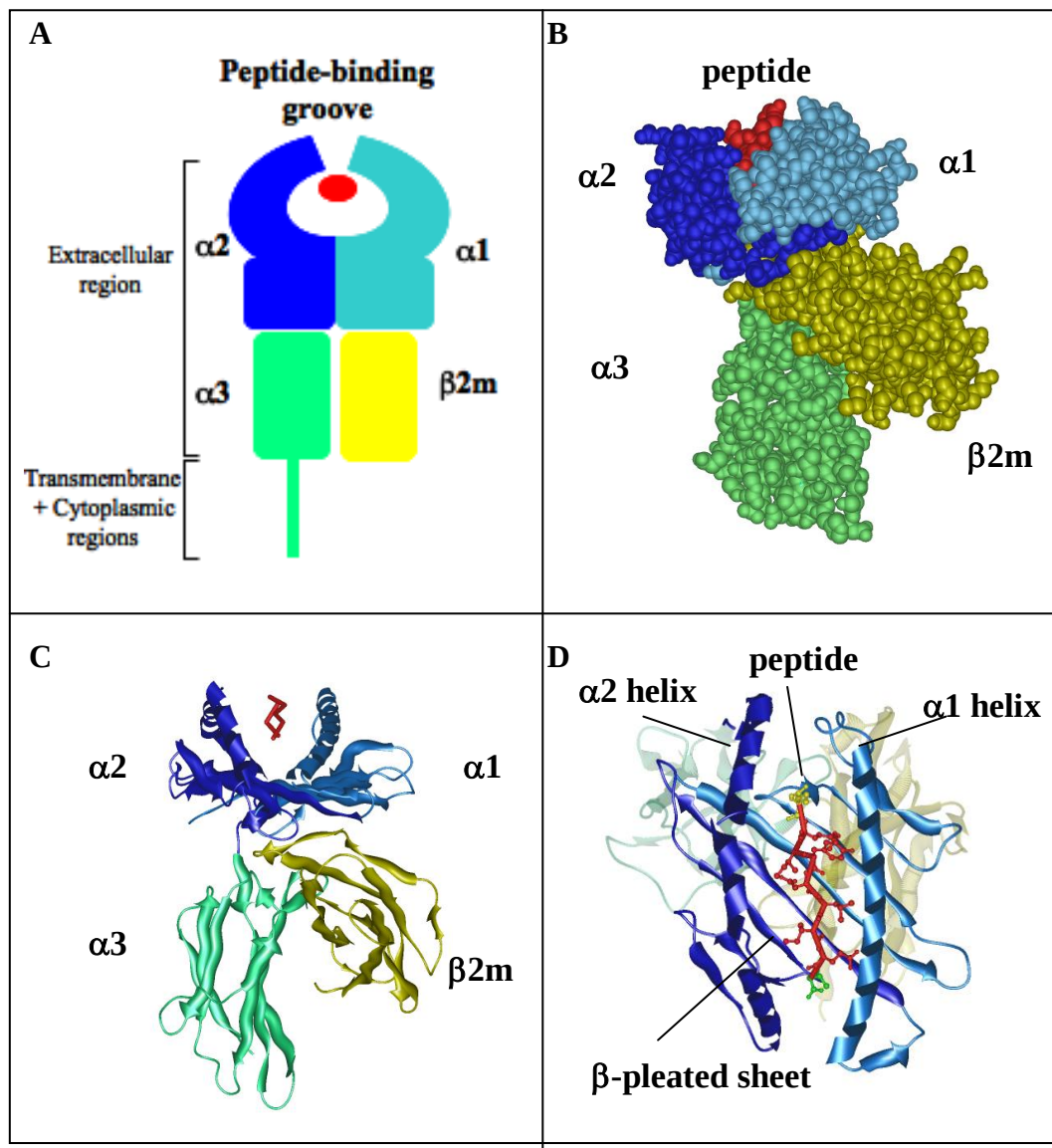


Figure 1.1 The structure of an MHC class I molecule. (A) Cartoon schematic of the extracellular and intracellular portions of an MHC class I molecule. The three α domains (light blue, dark blue, and green), comprising the heavy chain, non-covalently associate with β_2 -microglobulin (yellow) and peptide (red) to form a heterotrimeric complex. Panels (B-D) show the crystal structure of the extracellular portion of HLA-B27 (Protein Data Bank ID 2BSR). (B) Space-fill model and (C) Ribbon model of the structure of HLA-B27 with bound peptide (side view). (D) Top view of peptide binding groove. Peptide sits within the peptide binding groove, which is formed by 2 α -helices and 8 anti-parallel β sheets. Amino acid residues at the N- and C-termini are shown (yellow and green respectively).

1.2.3 The structure of HLA-B27

In the case of HLA-B27, since the first crystal structure was published by Madden *et al.* (Madden *et al.*, 1991), a number of other structures have been solved, including several with different peptide ligands. These show that the overall structure is largely similar to other HLA class I molecules. Yet, they have also revealed some distinguishing features within its peptide binding groove. Specifically, the B pocket, which accommodates the 2nd residue of the peptide (p2), contains a combination of key amino acids (H9, T24, and E45) that confer a strong preference for an arginine at p2 (Figure 1.2). This is reflected in sequencing studies that reveal most B27 endogenous peptides to have arginine as the 2nd residue (Colbert *et al.*, 1993; Jardetzky *et al.*, 1991; Stodulkova *et al.*, 2006).

In addition, unlike most other class I molecules, B27 possesses an unpaired cysteine residue at position 67 (Cys67), also in the B pocket (Figure 1.2). This residue is highly thiol-reactive, in part owing to the close proximity of a neighbouring lysine residue (L70) (Whelan and Archer, 1993), and has been implicated in the formation of β_2m -free, disulphide-linked B27 HC homodimers (B27₂) (Allen *et al.*, 1999). A proposed structure for the B27 homodimer has been modelled on the basis of the known structure of the extracellular region of HLA-B27 (Figure 1.3) (Bowness *et al.*, 1999; McMichael and Bowness, 2002). Here, exposed C67 residues of two B27 HC react to form a disulfide bond that holds the dimer together, stabilised by hydrogen bonding between residues 36-43 across the dimer interface. This model proposes that disulfide bond formation results in partial unfolding of the $\alpha 1$ helix at residues 62-67. Both peptide binding grooves retain their ability to bind peptide, although the peptide repertoire may be altered as a result of the conformational changes. However, the orientation of the dimer prevents TCR binding

to either peptide binding groove in the conventional way. This would suggest that any role for homodimers in disease pathogenesis is likely to not involve classical T cell recognition of pMHCI.

C67 is not a unique feature to HLA-B27, with five other known B alleles possessing this free cysteine: HLA-B14, -B15, -B38, -B39, and -B73. Of these, only B73 also has the nearby K70 residue. In addition, three have been linked to AS disease susceptibility independent of HLA-B27: -B14, -B38, and -B39. Yet, very few studies have addressed homodimer formation of these other HLA-B alleles; one published report has demonstrated that HLA-B14 forms homodimers in a transfected B cell line (Merino et al., 2008). It is therefore worth investigating the capacity of these other alleles to form homodimers, which will not only help determine whether C67 alone or with K70 is sufficient for homodimer formation but may also provide further support for the involvement of homodimers in AS pathogenesis. However, although the idea of homodimers being the common factor shared by disease-associated HLA-B alleles is attractive, at least two exceptions would need to be made. Firstly, HLA-B52 and HLA-B*40:01 have also been linked to AS yet do not possess C67, and so in these cases disease association would warrant an alternative explanation. Secondly, as discussed later, not all HLA-B27 subtypes are disease-associated despite the presence of the C67 residue, which also needs to be taken into account.

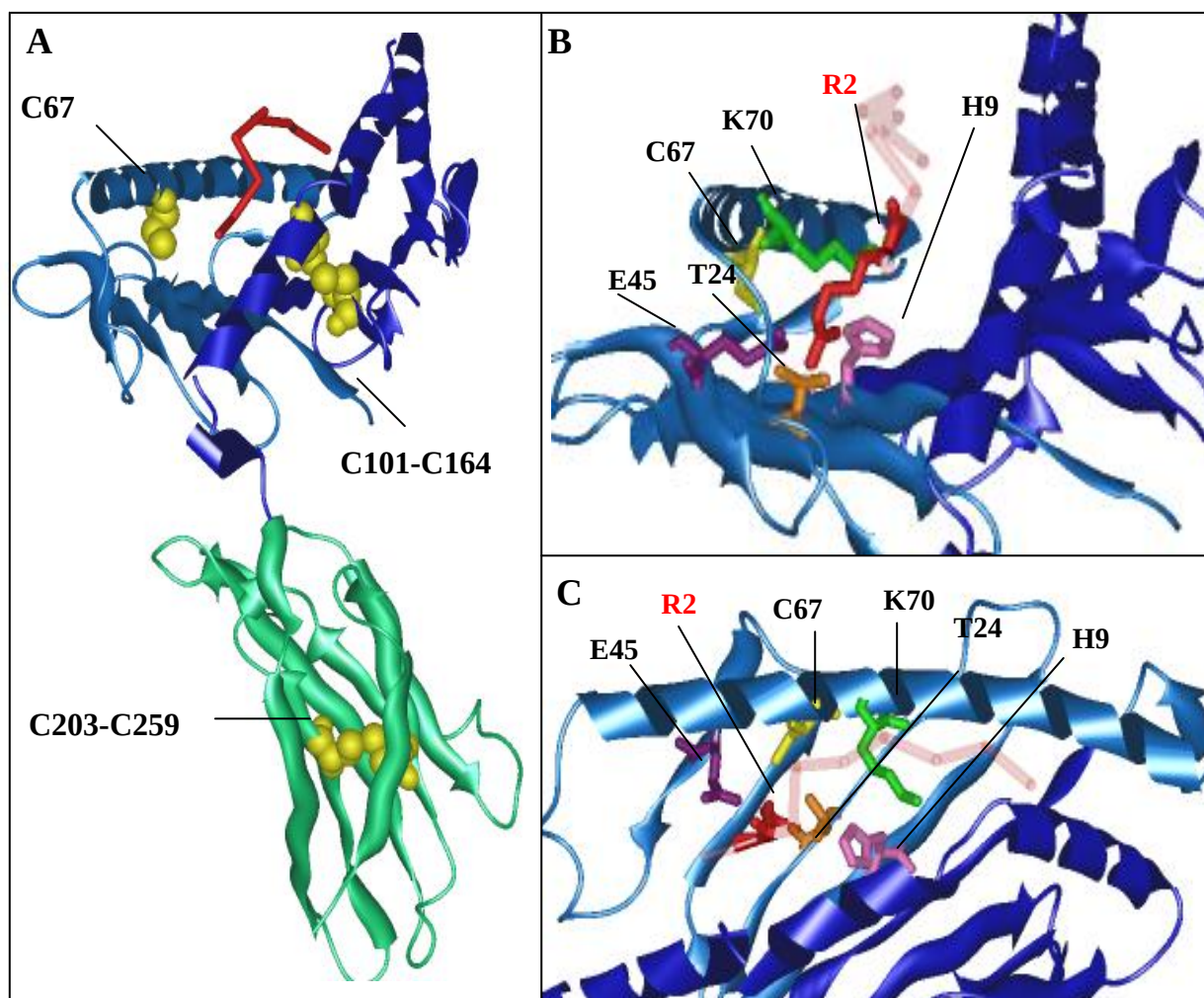


Figure 1.2 Key amino acid residues in HLA-B27. (A) Ribbon diagram of HLA-B27 highlighting the five cysteine residues in yellow spheres. Not shown are the two further cysteine residues in the cytoplasmic tail region (C308 and C325). Side view (B) and top-down view (C) of key residues in the peptide binding groove of HLA-B27: H9 (pink); T24 (purple); E45 (orange); C67 (yellow); K70 (green). Arginine at position 2 of the peptide (R2) is shown in red. Amino acid residues are depicted in wire style and all diagrams modelled using iMol software (Protein Data Bank ID 2BSR).

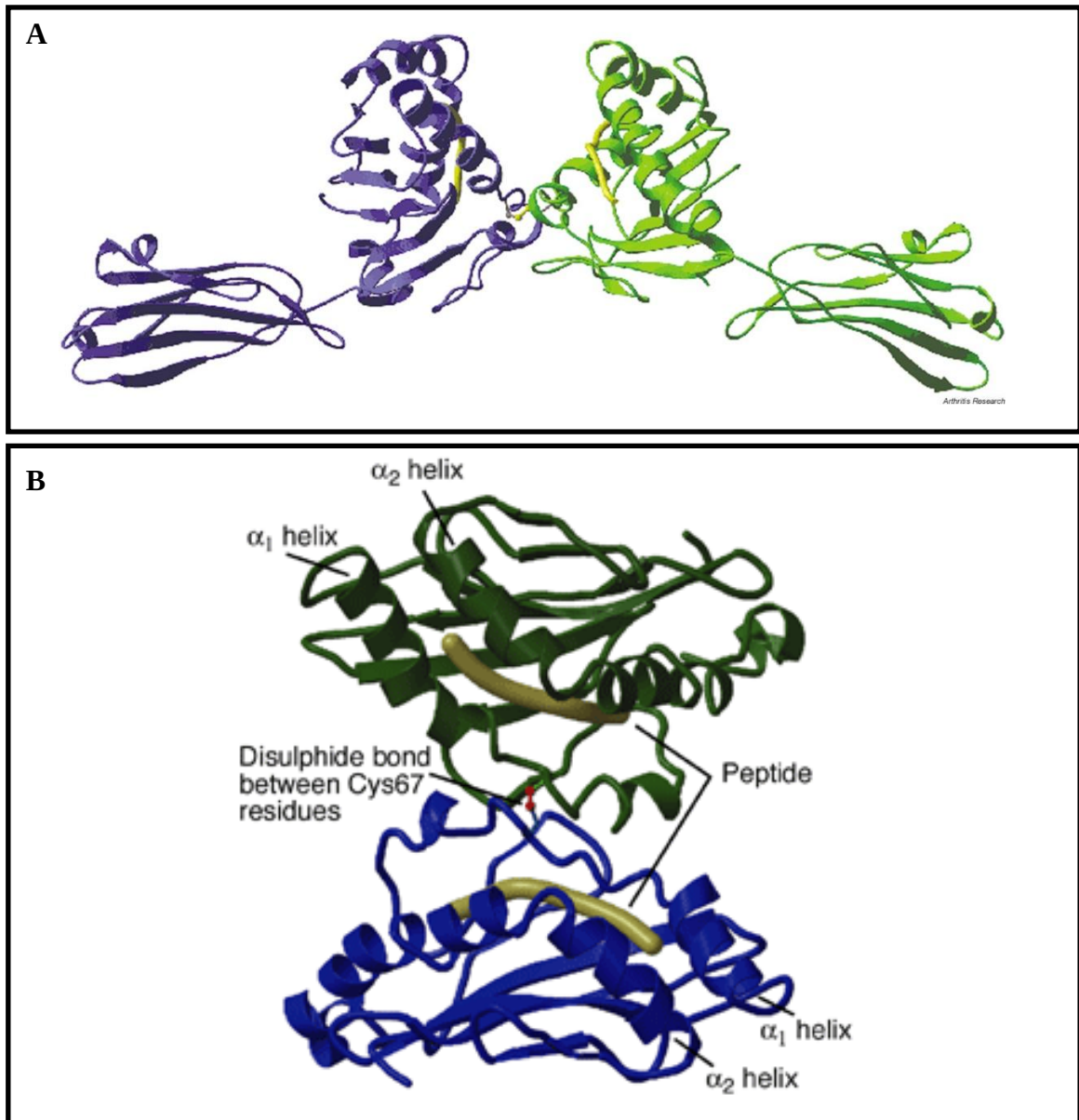


Figure 1.3 Proposed model of the HLA-B27 HC homodimer (B27₂) structure taken from Bowness *et al.* (Bowness *et al.*, 1999; McMichael and Bowness, 2002). Ribbon diagram representation of two HLA-B27 HC, shown in green and purple (A) or blue (B), which are joined by a C67-C67 disulfide bond in the centre (yellow in (A) or red in (B)). The model is orientated so that (A) is a side view with the cell surface at the bottom, whereas (B) is a top view looking down towards the cell surface. Putative peptides bound in the peptide binding grooves are shown as yellow tubes.

A third uncommon property of HLA-B27, related to the structure of its peptide binding groove, is its ability to bind and present peptides longer than the typical nonamer peptide. Peptide elution studies have revealed that the binding groove can accommodate peptides up to 33 amino acids long (Merino et al., 2008; Urban et al., 1994), whilst presentation and CTL recognition of longer peptides have been demonstrated in several reports (Huet et al., 1990; Nixon et al., 1988). A possible consequence for HLA-B27 molecules, as a result of binding to these longer peptides, is that their folded conformation may be more relaxed than if bound to a nonamer peptide. This may have important implications for HLA-B27 homodimer formation, if these more relaxed conformers represent a pool of molecules more prone to dimerisation.

Further atypical features of HLA-B27 relate to its assembly in the ER and egression to the cell surface. These features will be discussed in the following section after an outline of the MHC class I folding pathway.

1.2.4 Overview of the MHC class I folding pathway

The assembly of MHC class I molecules is a highly orchestrated process involving a number of chaperones that culminates in the surface expression of stably-loaded heterotrimeric class I complexes (pMHCI) (reviewed in (Van Hateren et al., 2010)). It begins in the endoplasmic reticulum (ER) where newly synthesised HC are picked up by calnexin (Cnx), a membrane-bound lectin chaperone. Cnx is associated with a thiol oxidoreductase, ERp57, a member of the protein disulfide isomerase (PDI) family. This Cnx:ERp57 heterodimer is believed to promote HC folding and disulfide bond formation and subsequent association with β_2m to form the MHCI heterodimer. Upon β_2m binding,

Cnx is displaced by its soluble homologue calreticulin (Crt) and the resulting HC:β₂m:Crt complex is then incorporated within the peptide loading complex (PLC) via the association with tapasin. Upon full assembly, the PLC comprises mainly of HC:β₂m, Crt, tapasin, ERp57 and the transporter associated with antigen processing (TAP) protein. Other components also reported to be present include protein disulfide isomerase (PDI), a further thiol oxidoreductase, and Bap31, a cargo receptor for ER export of transmembrane proteins. Once incorporated into this dynamic multimolecular complex, the MHCI heterodimer acquires peptide cargo to form heterotrimeric pMHCI molecules.

Tapasin is a key component of the PLC. Like Cnx, it also associates with ERp57, with recent studies demonstrating that it exists predominantly as a tapasin:ERp57 disulfide-linked heterodimer. It serves two important functions in PLC assembly. Firstly, it interacts with the TAP protein, a heterodimer comprising TAP1 and TAP2 units. TAP, a member of the ATP-binding cassette family of transporters, is the main source of peptide for most MHCI molecules; it catalyses the transfer of cytosolic peptides into the ER lumen for MHCI peptide loading. Thus, tapasin bridges the HC:β₂m heterodimer with the TAP peptide transporter, allowing the MHCI to acquire peptide cargo and form heterotrimeric pMHCI molecules. Secondly, tapasin promotes the binding of high affinity peptides to MHCI molecules in preference to those with a lower affinity (Williams et al., 2002). This optimisation of the peptide repertoire allows for MHCI molecules to be loaded with peptides with slow dissociation rates, which extends their life-span at the surface and increases the chance of relevant TCR recognition. Once stable pMHCI molecules have formed, they dissociate from the PLC and egress to the cell surface. This transport is thought to be a receptor-mediated process, involving the cargo receptor Bap31 and COP-II

coated vesicles, and occurs via the Golgi, where final N-linked glycosylation events take place. Once at the surface, MHCI are then continually endocytosed and recycled via the endosomal compartments to the cell surface. This process is thought to be dependent on conserved motifs within the cytoplasmic tail of the HC (Capps et al., 1989); in particular, for HLA-A and HLA-B molecules, a key tyrosine residue at position 320 is involved (Le Gall et al., 1998).

In order to ensure that only correctly folded and optimally loaded pMHCI molecules reach the cell surface, a number of quality control mechanisms are in place both within the ER and within the Golgi (post-ER quality control). Incompletely folded proteins are retained in the ER until they fold correctly. However, misfolded proteins that cannot be salvaged are sorted and targeted for degradation by ER-associated degradation (ERAD) (reviewed in (Vembar and Brodsky, 2008)). This consists of four main stages: (1) recognition of misfolded protein, (2) ubiquitination, (3) translocation back into the cytoplasm (retrotranslocation) and (4) proteasomal degradation. The components of ERAD and how this ER quality control system works are still being characterised, with several ERAD pathways identified so far. For MHCI molecules, initial recognition is thought to involve molecular chaperones such as Cnx, Crt, Grp94, BiP and other 70 kDa heat shock protein (Hsp70) family members, although how this discriminates between misfolded and correctly folded proteins is largely unknown. Once identified, misfolded MHCI molecules are ubiquitinated via the ubiquitination pathway, involving a series of enzymes, E1-E3, of which the E3 ubiquitin ligase enzyme confers substrate specificity. Ubiquitinated proteins are then transported back into the cytosol via the retrotranslocation machinery. This process employs a multi-molecular complex, termed the retrotranslocon. Its composition is

determined by which ERAD pathway is involved, but includes an E3 enzyme and associated scaffold proteins, a channel-forming protein, and a p97 ATPase complex, which couples ATP hydrolysis to the translocation process (Lilley and Ploegh, 2004). Further characterisation of this complex has been made possible through the study of two HCMV-encoded proteins, US2 and US11, which down-regulate MHCI expression on host cells to evade immune detection. Both proteins bind directly to MHCI heavy chains to target them for degradation, but they employ distinct retrotranslocation complexes: US2 targets HC to a complex comprising the signal peptide peptidase and the E3 ligase TRC8; in contrast, US11 targets HC to a complex containing the E3 ligase Hrd1 and its binding partner Sel1, and the channel-forming protein Derlin-1, among other subunits. Moreover, a recent publication has demonstrated the importance of the Hrd1 pathway for degradation of non- β 2m bound MHCI HC under normal cell conditions (Burr et al., 2011). Thus, multiple ERAD pathways exist that may be used in MHCI degradation when misfolding events occur. Once in the cytosol, the misfolded protein is further ubiquitinated and eventually destroyed by the 26S proteasome.

Until recently, the ER was considered the only checkpoint for MHCI molecules to pass through, wherein tapasin and the PLC operated to ensure that only optimally loaded MHCI complexes could exit from the ER. However, studies have since shown that sub-optimally loaded MHCI complexes can be efficiently exported from the ER, irrespective of peptide affinity, and enter the ER-Golgi intermediate compartment (ERGIC) (Garstka et al., 2007). Nevertheless, the fact that only stable pMHCI molecules reach the cell surface is evidence for the existence of a second quality control checkpoint within the post-ER compartment, operating as an ER retrieval mechanism whereby misfolded MHCI in the post-ER

compartments are sent back to the ER to either refold correctly or undergo ERAD. This mechanism is thought to also involve components of the PLC, specifically tapasin and calreticulin, which have been detected in post-ER compartments and which possess the C-terminal ER retrieval sequences KKxx (Paulsson et al., 2006) and KDEL (Paulsson et al., 2002) respectively. Upon binding to or continued association with misfolded MHCI in the cis-Golgi, or by some alternative mechanism, tapasin and Crt may be recognised by receptors, such as the KDEL receptor, through their terminal retrieval sequences, which direct chaperone-MHCI complexes back to the ER in COP-1 coated vesicles. The importance of the KDEL motif in this process has been shown in mutation studies, whereby Crt lacking the KDEL sequence fails to retrieve sub-optimally loaded pMHCI complexes and transient MHCI expression at the cell surface results (Howe et al., 2009). Whether additional components of the PLC, in particular ERp57, which can associate with both tapasin and calreticulin and therefore may affect the role of these two chaperones in MHCI binding, contribute to this retrieval mechanism remains to be identified (Paulsson et al., 2006; Paulsson et al., 2002). Furthermore, how sub-optimal molecules are distinguished from others by these recycling chaperones remains unknown.

1.2.5 The folding of HLA-B27

The folding of HLA-B27 exhibits two atypical features, which may contribute to its pathogenicity. Firstly, the folding of its heavy chain appears to be slower than many other class I molecules (Antoniou et al., 2004; Dangoria et al., 2002; Mear et al., 1999; Tran et al., 2004). As a consequence, it has a tendency to misfold following synthesis in the ER, even in the presence of a normal supply of peptide and β_2m . This is thought to be due to the specific B pocket residues of B27, as mutating these residues to those found in HLA-

A2 reduces the accumulation of aberrantly folded HC in the ER (Mear et al., 1999). Secondly, HLA-B27 is less dependent upon tapasin in comparison to other class I molecules for surface expression (Peh et al., 1998). This results in the egress from the ER of HLA-B27 complexes loaded with more weakly bound peptides, which can dissociate at the surface. Consistent with this, Benjamin *et al* have reported the presence of stable 'empty' forms of B27 on the surface of B cells that are peptide-receptive and so are able to bind extracellular peptides not normally accessible to the class I processing pathway (Benjamin et al., 1991). Empty surface MHCI molecules can also be seen in cell lines expressing other class I alleles but requires low-temperature induction (Ljunggren et al., 1990). Consequently, it is possible that the 'empty' B27 molecules are a result of suboptimal peptide loading in the ER, owing to the reduced tapasin dependency, which leads to the formation of unstable heterotrimers at the cell surface from which peptide can more easily dissociate. Without peptide, β_2m will also dissociate, leaving a supply of free HC at the surface that are capable of forming disulphide-linked heavy chain multimers.

1.2.6 Overview of MHC class I Antigen Processing Pathway

The peptides that are presented by MHCI molecules come from at least two distinct intracellular pools generated by the proteolysis of intracellular proteins. The first involves the classical antigen processing pathway and is a major source of MHCI peptides. Peptides of lengths 3-30 amino acids are generated by proteasomal degradation of cytosolic proteins, which are targeted for destruction both randomly and due to damage or surplus requirements of cellular proteins. This will also include any bacterial or viral proteins present as a result of any infection. Alternatively, a second pool of intracellular peptides is generated from the degradation of defective ribosomal products (DRiPs) (Yewdell et al.,

1996). These peptides may be a result of translational errors (protein truncations or amino acid substitutions), post-translational errors (misfolding, erroneous covalent modifications, or inappropriate chaperone association), or RNA processing errors (splicing or RNA synthesis errors). Several papers have highlighted the significance of DRiP generated antigens, with two demonstrating that active protein synthesis is required for generation of TAP substrates (Reits et al., 2000; Schubert et al., 2000). Furthermore, the advantage of generating peptides from DRiPs is that their quick generation allows for very fast presentation of antigenic peptides almost immediately after synthesis, and so enables rapid T cell recognition of acute viral infection.

Irrespective of the source of antigen, peptide generation depends upon the proteolytic activity of the proteasome, the importance of which can be seen in inhibition experiments, whereby blocking proteasomal activity results in a poor peptide supply to MHCI and reduced MHCI antigen presentation (Rock et al., 1994). The 26S proteasome is a multiple subunit complex constitutively expressed in all cells, comprising a 20S catalytic core that associates with two 19S regulator complexes that enable substrate binding. With three active sites that differ in their cleavage specificity, the proteasome is able to cleave after almost every amino acid residue, which is important in the generation of the vast array of peptide antigens for MHCI presentation. Furthermore, in response to IFN γ induction, alternative subunits are incorporated into the proteasome to generate what is known as the immunoproteasome. This involves replacing the 3 catalytic subunits with the subunits LMP2, MECL1 and LMP7, and the inclusion of a further IFN γ -induced subunit, the proteasome activator PA28. The resulting complex has altered cleavage site specificities with a preference that generates the correct C-terminal anchor residues of an MHCI

epitope and in many cases generates N-terminally extended peptides to facilitate TAP transport.

MHCI epitopes are usually 8-11 amino acids long, yet the majority of peptides generated by the proteasome are either too short (~60%) to bind to MHCI, and are lost from the pool, or are extended peptides (~20%) that require further trimming (Cascio et al., 2001; Kisselev et al., 1999). A number of cytosolic aminopeptidases, including tripeptidyl peptidase II (TPP II), leucine aminopeptidase (LAP), thimet oligopeptidase (TOP), bleomycin hydrolase and puromycin sensitive aminopeptidase (PSA) function to trim the N-terminus to a near-optimal peptide length, although they can also completely degrade peptides (reviewed in (Herberts et al., 2003)). It is estimated that more than 99% of intracellular peptides are in fact destroyed before translocation into the ER (Reits et al., 2003). Those that are then transported into the ER via TAP include some N-terminally extended peptides, which are trimmed within the ER by the ER resident aminopeptidase ERAP1 for successful MHCI binding. This enzyme cleaves peptides to a length of about 8-9 amino acids (York et al., 2002), although the mechanism by which this is achieved has been debated. ERAP1 function is further discussed later. Notably, the importance of retaining N-terminal extensions on certain peptides prior to TAP transport can be seen for the transport of proline-containing epitopes. Several MHCI alleles preferably bind peptides containing a proline residue at P2 or P3 as an anchor, yet these peptides are inefficiently transported by TAP. Thus, N-terminal extensions facilitate the TAP transport of these epitope precursors, while ERAP1 cleavage generates the final epitope. Moreover, studies of the mouse ortholog enzyme, ERAAP, have shown the effect these ER-trimming events can have on the surface expression of class I molecules, wherein the generation of ERAAP

knockout mice or the use of RNA interference to reduce ERAAP expression altered the normal peptide repertoire, markedly decreased MHCI on the surface and diminished CD8+ T cell responses (Hammer et al., 2006; Hammer et al., 2007; Serwold et al., 2002).

As mentioned, transport of cytosolic peptides into the ER is catalysed by TAP. TAP binds peptide with a length of 8-16 residues with equal affinity, although translocation of shorter peptides up to 12 residues is most efficient. It also exhibits a preference for sequences containing hydrophobic or basic C-terminal residues, a feature shared with the MHCI peptide binding groove. The essential role of TAP in the delivery of antigenic peptides into the ER can be seen in deletion studies and in the TAP-defective T2 cell line, whereby class I surface expression is drastically reduced.

Components of the MHC class I antigen processing pathway have been implicated in AS pathogenesis. In a study employing two-dimensional electrophoresis and quantitative mass spectrometry analysis, monocytes from AS patients had upregulated expression of proteins belonging to the Ubiquitin Proteasome Pathway (UPP), including the proteasomal activator complex PA28 (Wright et al., 2009). Furthermore, this subunit was shown to increase the generation of HLA-B27 antigenic epitopes *in vitro*. In addition, an association of ERAAP1 with AS has been convincingly demonstrated in a number of genetic association studies, which is discussed below.

1.3 Genetic Factors and AS

HLA-B27 was identified as a risk factor for AS in 1973; the first association of an HLA allele with inflammatory disease to be discovered (Brewerton et al., 1973b). From studies

with monozygotic and dizygotic twins, it has been estimated that approximately 90% of susceptibility to AS is genetically linked, yet only a third of this risk can be attributed to B27 (Brown et al., 1997b; Jarvinen, 1995; Pedersen et al., 2008). B27⁺ relatives of AS patients have a 5-16 times greater risk of developing AS compared with other B27⁺ individuals, suggesting the presence of shared familial risk factors other than B27 (Calin et al., 1983; van der Linden et al., 1983). The recurrence risk for AS among monozygotic twins and first-degree relatives are 63% and 8.2% respectively, compared with only 0.1% in the general population (Brown et al., 2000). Moreover, the fact that disease can occur in non-B27⁺ individuals and that the majority of B27⁺ individuals are healthy indicates the multifactorial nature of the disease. Other genetic and environmental factors therefore play a significant role in the development and pathogenesis of disease (Brown, 2008; Penttinen et al., 2004). This is particularly the case in ReA, where disease onset is strongly correlated with certain microbial infections (reviewed in (Girschick et al., 2008)).

While the strongest association remains with HLA-B27, linkage studies combined with recent advances in the genetics of AS have identified several other genes and genetic regions, including both MHC and non-MHC loci, that are involved in the disease. Most notable have been the findings from two key Genome-Wide Association Studies (GWAS): the 2007 Wellcome Trust Case Consortium & The Australo-Anglo-American Spondylitis Consortium (WTCC-TASC) study, which included 1000 AS patients and 1500 healthy individuals and surveyed 14,436 coding single nucleotide polymorphisms (SNPs); and the subsequent ‘TASC’ study, which was the first GWAS to be carried out specifically for AS and compared 2053 European AS cases with 5140 controls. These are the first reports to demonstrate strong associations with *ERAP1* and *IL23R* (Burton et al., 2007; Reveille et

al., 2010). These ‘definite’ associations (genome-wide significance, replicated) will be discussed below, along with the HLA-B27 subtype associations, and the ‘suggestive’ associations of other HLA-B alleles and non-MHC genes that have yet to reach genome-wide significance.

1.3.1 Susceptibility loci within the MHC region

1.3.1A HLA-B27 and its subtypes

The association of HLA-B27 with AS is one of the strongest known associations between an HLA molecule and disease, with 96% of patients carrying at least one HLA-B27 allele compared with only 9% of healthy people (Brewerton et al., 1973b). Significant association, albeit to a lesser extent, is also seen for the other members of the SpA family (Brewerton et al., 1973a, 1974a, b). Furthermore, although B27 homozygosity does not affect clinical manifestations, it triples the risk of disease occurrence (Jaakkola et al., 2006; Kim et al., 2009) .

HLA-B has been identified as the most polymorphic gene in the human genome (Mungall et al., 2003), with over 2000 named alleles, of which approximately 1600 encode MHC class I proteins (<http://hla.alleles.org>). This extensive polymorphism is further exemplified by the fact that over 60 subtypes for HLA-B27 have been identified thus far. Yet, an assessment of their putative associations with AS has only been possible for the more prevalent subtypes, as the majority are either too rare or too recent for analyses.

The major subtypes include B*27:02, B*27:03, B*27:04, B*27:05, B*27:06, B*27:07, and B*27:09. These differ from each other at only a few amino acids, which are mainly within

the $\alpha 1$ and $\alpha 2$ domains that form the peptide binding groove (Table 1.5 and Figure 1.4). However, they differ markedly in their global distribution profiles, with different subtypes predominating in different populations (Table 1.6). B*27:05 is the most widespread subtype, and has been the focus of most research into B27 and SpA. It is thought to be the common ancestor from which the rest of the subtypes have evolved. It is further subdivided into B*27:05:02-B*27:05:13 alleles as a result of silent single nucleotide substitutions, with B*27:05:02 being the most prevalent, and is the predominant disease-associated subtype in Caucasian populations.

Other common disease-associated subtypes include B*27:02 and B*27:04; the former being restricted to Caucasian populations, and prevailing in Jewish communities, while the latter is the most common subtype among the Asian populations. These distinct distributions over diverse ethnic background makes it difficult to directly compare their strength of associations with AS. However, recent studies within the same ethnic groups and environments have helped establish a hierarchy whereby the most strongly associated subtype appears to be B*27:04, with B*27:05 and B*27:02 similarly, albeit slightly less strongly associated (Liu et al., 2006).

	Amino acid residue number										
Subtype	α 1 domain					α 2 domain					α 3
	59	77	80	81	97	113	114	116	131	152	211
B*27:05	Y	D	T	L	N	Y	H	D	S	V	A
B*27:02	-	N	-	A	-	-	-	-	-	-	-
B*27:03	H	-	-	-	-	-	-	-	-	-	-
B*27:04	-	S	-	-	-	-	-	-	-	E	G
B*27:06	-	S	-	-	-	-	D	Y	-	E	G
B*27:07	-	-	-	-	S	H	N	Y	R	-	-
B*27:09	-	-	-	-	-	-	-	H	-	-	-

Table 1.5 Amino acid substitutions in most the common HLA-B27 subtypes (Sequence information from <http://hla.alleles.org/>)

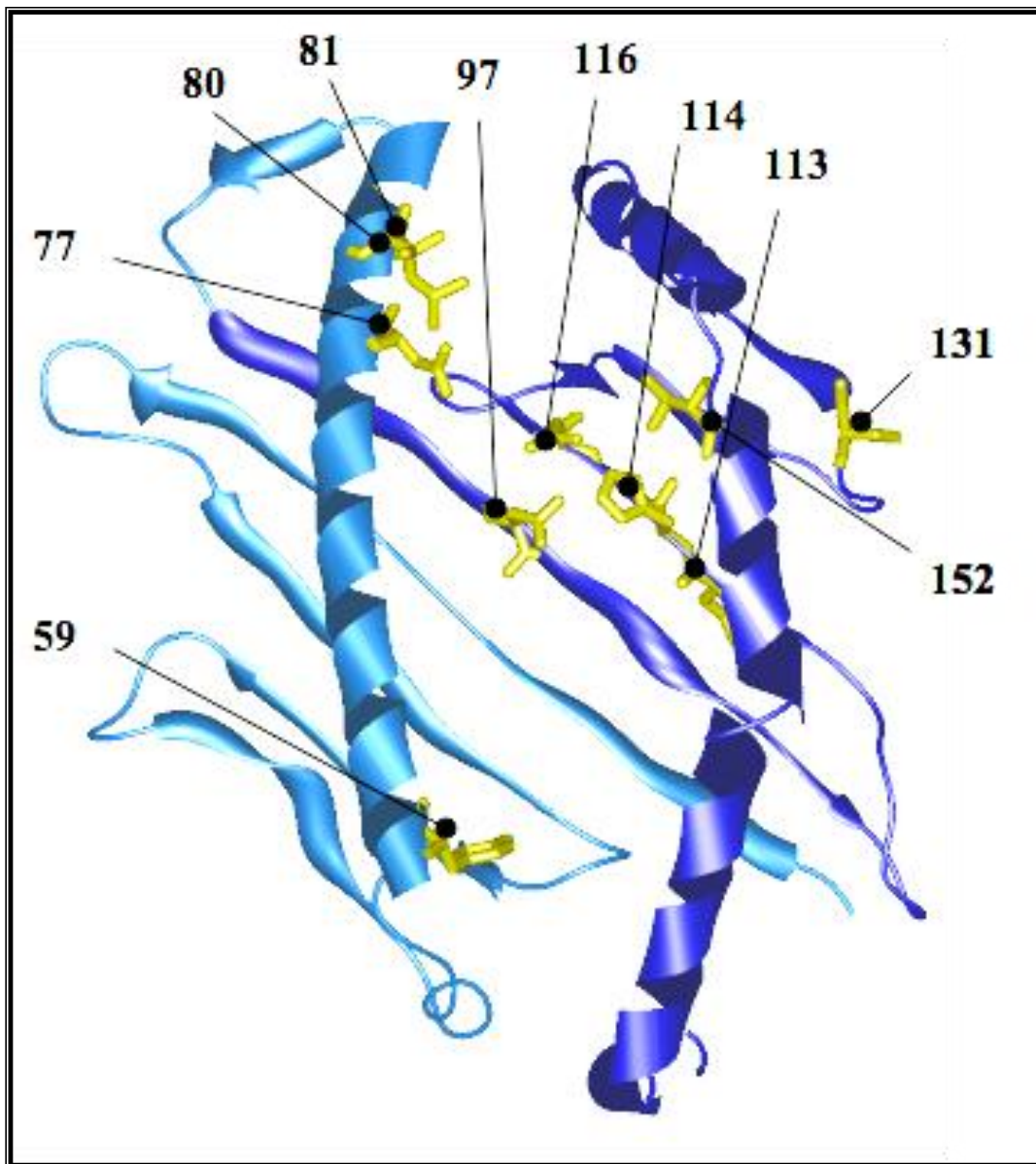


Figure 1.4 Positions of the polymorphic residues in HLA-B27. Polymorphic positions within the HLA-B*27:05 peptide binding groove shown in yellow and labelled.

Population	HLA-B27 subtype						
	02	03	04	05	06	07	09
Northern Europe	10	-	-	90	-	-	-
Southern Europe	20	-	-	80	-	-	-
Azores	-	7	-	86	-	7	-
Italy	30	-	-	65	-	2	3
Sardinia	3	-	-	77	-	-	20
Greece	34	8	-	50	-	8	-
Cyprus (Greece)	52	-	-	32	-	17	-
Northern India	-	-	33	61	-	6	-
China	2	-	66	31	-	2	-
Japan	-	-	82	18	-	-	-
Taiwan	0.5	3	87	4	7	2	-
Thailand	-	-	42	5	53	-	-
Native Indonesians	-	-	6	6	89	-	-
North Africa	50	-	-	50	-	-	-
West Africa	-	32	-	68	-	-	-
Brazil	10	6	-	80	-	-	-

Table 1.6 Common HLA-B27 subtype frequencies (%) in different global populations. (Adapted from (Reveille and Maganti, 2009))

Not all subtypes are disease-associated; B*27:06 and B*27:09 have been shown to be less associated with AS than their subtype-associated counterparts. Studies have demonstrated that B*27:06, common in Thai and Indonesian populations, has a similar frequency to B*27:04 in the healthy B27+ control populations, yet the AS patient cohort comprised almost entirely of B*27:04 (Lopez-Larrea et al., 1995; Nasution et al., 1997). Similarly, for B*27:09, which is found almost exclusively in the Sardinian population, studies have found a distinct lack of association of this allele with AS. The lack of reported cases of these subtypes in AS patients initially led to suggestions they may be protective for disease. However, patients carrying these alleles have since been identified. For B*27:06, 4 out of 676 AS patients have been reported ($p = 2.6 \times 10^{-11}$), whereas at least six B*27:09 SpA cases have been reported, two of which had AS (Cauli et al., 2007; Olivieri et al., 2000). It is noteworthy, however, that both of these AS cases could possibly be explained by the presence of alternative risk factors: one carried the HLA-B*14:03 allele, which, as mentioned previously, has been found to be associated with AS in a Togolese population; the other had ulcerative colitis. Thus, although not definitive, B*27:06 and B*27:09 show a much lower predisposition to AS, compared with the disease-associated subtypes.

Although definitive associations of B*27:03 and B*27:07 with AS have been shown, there have been studies in certain populations providing noticeable exceptions to this. In particular, there is a lack of AS in the Gambian population, despite the prevalence of the B*27:05 and B*27:03 alleles (Brown et al., 1997a), and similarly, a disease association of the B*27:07 allele in a Greek Cypriot population has not been demonstrated (Varnavidou-Nicolaidou et al., 2004). This emphasises the importance of other genetic or environmental factors in distinct populations that may play a role in AS susceptibility.

1.3.1B Peptide repertoires of B27 subtypes

The differential associations of the HLA-B27 subtypes with AS within the same population suggests that key amino acids may determine the pathogenicity of the class I molecule (D'Amato et al., 1995; Lopez-Larrea et al., 1995; Nasution et al., 1997). An alternative possibility is that a non-B27 gene is responsible; however, this would require the candidate susceptibility gene to occur in disequilibrium exclusively with the disease-associated subtypes, and numerous studies have failed to provide evidence for such a linkage.

B*27:09 differs from B*27:05 by just one amino acid residue, D116H, whereas B*27:06 differs from B*27:04 at two positions, H114D and D116Y (Figure 1.4 and Table 1.5). These two positions constitute the F pocket of the peptide-binding groove, which interacts with the side chain of the peptide C-terminus. Interestingly, they also determine the dependence on the accessory protein, tapasin, for surface expression of class I molecules. A number of studies, therefore, have addressed how subtype polymorphism affects both the repertoire of bound peptides and degree of shared T cell reactivity. B*27:05 and B*27:09 share 79% and 88%, respectively, of their peptide repertoires, while the repertoires of B*27:04 and B*27:06 differ by 10-12% (Ramos et al., 2002; Sesma et al., 2002). In both cases, the sequence differences are largely confined to the C-terminus, presumably because of the changes in the F pockets. In addition, another study comparing peptides eluted from B*27:02, B*27:05 and B*27:09, found the less-associated B*27:09 to have a more restricted specificity; with a strict preference for hydrophobic residues at the peptide C-terminus (Fiorillo et al., 1997; Marti et al., 2001). In contrast, the highly conserved B pocket, at least among these common subtypes, suggests the residues within

this region, which anchors the N terminus, are unlikely to play a role in pathogenesis. Thus, polymorphism in B27 influences the nature of the C-terminal amino acids of bound peptides.

Subtype polymorphism also impacts on T cell recognition, as evidenced in several studies investigating the cross-reactivity of allospecific CTLs. For example, B*27:05 and B*27:09 are recognised by 80% and 90%, respectively, of the allospecific CTLs generated against the two molecules (Montserrat et al., 2003). Several studies focusing on B*27:05 and B*27:09 have identified peptide epitopes that elicit distinct subtype dependent T cell responses (Fiorillo et al., 2000; Fiorillo and Sorrentino, 2009). In particular, a self peptide derived from vasoactive intestinal peptide receptor 1 (pVIPR, RRKWRRWHL), despite binding to both alleles, only evoked CTL responses in B*27:05 individuals. Interestingly, subsequent crystallographic studies have revealed this epitope to bind to B*27:05 in two different conformations, only one of which is seen for B*27:09 (Fiorillo et al., 2005). This dual conformation has also been seen for a second B27 epitope, derived from the glucagon receptor (pGR, RRRWHRWRL), although in this case both conformations are seen and elicit CTL responses for both subtypes (Hulsmeyer et al., 2004; Nurzia et al., 2010; Ruckert et al., 2006). Whether this phenomena is a unique feature of B27 or if other class I molecules can also display peptides in dual conformations is uncertain.

Thus, differences in both the peptide repertoires bound by the B27 subtypes and their differential CTL recognition profiles may explain their differential disease associations and be significant in disease pathogenesis. However, studies thus far have failed to identify

peptide epitopes or indeed B27-restricted autoreactive CTLs shared among to the disease-associated subtypes and not the others.

1.3.1C Non-B27 genes within the MHC

The classical Major Histocompatibility Complex (MHC) is located on the short arm of chromosome 6 and spanning about 3.6 Mb. It contains 224 known genes, of which at least 128 are functional, and can be divided into 3 major regions: class I, class III and class II regions. An extended MHC (xMHC) region also includes the extended class I sub-region and the extended class II sub-region, and encompasses a total of 7.6 Mb. The class I region contains the genes encoding the α chain of the classic MHC class I molecules: HLA-A, -B and -C and pseudogenes from HLA-E to HLA-L. In the class II region are the genes encoding for HLA-DP, -DQ, -DR, -DM and -DO along with several genes for other proteins involved in antigen presentation pathways, including -LMP and TAP genes. Between these two regions lies the HLA class III region, containing many genes with various functions, some of which also relating to the immune system such as complement proteins (C4A, C4B, and C2), tumor necrosis factor (TNF) and heat shock protein 70 (HSP70) genes.

The strong linkage between the MHC region and AS was first demonstrated by Rubin *et al.* through linkage analyses of 15 multiple-case AS families (Rubin et al., 1994), and further genetic epidemiology studies have since indicated that between 40 - 50% of the whole genetic risk may be contributed by genes in this region. Although most of the association of this locus can be attributed to HLA-B27, several other HLA-B alleles have been linked, albeit not to the same extent, including HLA-B60, -B14, -B15, -B38, and -

B39, as well as other MHC genes including the class II gene HLA-DR1 (Brown et al., 1998). For example, HLA-B60 has been shown to increase the risk by 3-fold in both B27+ and B27- individuals (Brown et al., 1996). Furthermore, Brown and colleagues have now demonstrated that this association is with the split antigen HLA-B*40:01 and not HLA-B*40:02 (Brown et al., 2009). In this same study, they also report associations with HLA-B38 and HLA-B52, noting, however, that confirming these associations requires further linkage disequilibrium studies (reviewed in (Thomas and Brown., 2010)).

Importantly, for HLA-B27, the increasing evidence from multiple well-powered GWAS studies, alongside the strong association of AS with the different HLA-B27 subtypes and the fact that AS occurs in widely different ethnic groups, has largely ruled out the likelihood of a ‘linked-gene’ accounting for its disease susceptibility. Moreover, the development of HLA-B27 transgenic rodent models, discussed in a later section, supports the direct involvement of B27 with disease pathogenesis. However, it is harder to exclude this possibility for the other B alleles, especially given the extensive linkage disequilibrium within the MHC region. Yet, at least for HLA-B60, the association has been widely replicated in several studies and in ethnically diverse populations (Brown et al., 1996; Wei et al., 2004).

1.3.2 Susceptibility loci outside the MHC region

While the MHC region accounts for part of the genetic risk for AS, and the association of HLA-B27 remains the strongest predisposing factor, GWAS studies have found significant associations with non-MHC genes. In particular, *ERAP1* and *IL23R* were shown to be

responsible for 26% and 9% of the population-attributable risk in AS, respectively. Other genes not described here are reviewed in Reveille *et al.* (Reveille, 2011) (Table 1.7).

1.3.2A ERAP1

ERAP1 is a multi-functional enzyme with two proposed functions in immunity, both of which could potentially explain its association with AS. In humans, it can associate with ERAP2 to form heterodimeric complexes, although the importance of this for enzyme function is uncertain. As an aminopeptidase, its best characterised role is in the trimming of N-terminally extended peptides in the ER for MHC class I antigen presentation (Saric *et al.*, 2002; York *et al.*, 2002). ERAP1 shows a strong preference for substrates with large hydrophobic residues at the N and C-termini, and does not cleave before or after proline residues. In addition to this substrate specificity, it also exhibits a length specificity, preferentially processing peptides of 9-16 residues down to 8-9 residues, the optimal length for presentation by MHCI molecules. This observation led to the proposal that it acts as a 'molecular ruler' (Chang *et al.*, 2005), a theory which has gained further support from the recent determination of crystal structures of ERAP1 in open and closed conformations (Kochan *et al.*, 2011; Nguyen *et al.*, 2011). These studies have revealed key features of the enzyme, including a large interior compartment and a shallow groove extending from the active site that could accommodate long peptides, which have provided the molecular basis for a possible mechanism of peptide processing that allows for the enzymes broad substrate specificity and length dependency. Further functional studies of its mouse ortholog, ERAAP, have shown the enzyme influences both the peptide repertoire of MHC class I molecules and their expression levels; cells from ERAAP-deficient mice exhibit markedly reduced surface MHCI and lack a subset of peptide epitopes, findings also confirmed in

human cell lines (Hammer et al., 2006; Kanaseki et al., 2006; Serwold et al., 2002). Interestingly, a novel set of pMHCI molecules also appear in ERAAP-deficient mice that are not present in WT, indicating that both the destruction and generation of epitopes contributes to the altered peptide repertoire in the absence of ERAAP activity.

ERAP1 is also suggested to be involved in the ectodomain shedding of the cell surface receptors for IL-1, IL-6, and TNF α ; three prominent pro-inflammatory cytokines in inflammatory disease. Evidence for this came from several reports, whereby ERAP1 or its mouse ortholog ERAAP were either over-expressed or knocked down using RNA interference, and receptor levels both on the surface and in the supernatant were monitored. A decrease in ERAP1 activity correlated with a decrease in cytokine receptor shedding (Cui et al., 2002; Cui et al., 2003a, b). However, as yet, there is no *in vivo* data to support these *in vitro* findings nor any confirmation of this data by a second group, and the underlying mechanism is poorly defined.

The mechanism by which ERAP1 is involved in AS pathogenesis may involve either of these biological functions. However, findings from two recent GWAS support a more likely role for the antigen processing activity of ERAP in disease susceptibility: Strange *et al* report a strong interaction between ERAP1 and HLA-C in psoriasis, while Harvey *et al* demonstrate a SNP association in ERAP1 with reduced risk of AS only in the context of HLA-B27 (Harvey et al., 2009; Strange et al., 2010). *In vitro* peptide trimming assays with disease-associated ERAP mutants have reported mixed results on how these mutations affect ERAP function. Nevertheless, in relation to AS disease susceptibility, a mechanism is proposed whereby a change in ERAP activity, as a result of polymorphism, alters the

peptide repertoire which can result in i) the presentation of novel ‘arthritogenic’ peptides, ii) more unstable HLA-B27 complexes in the ER that misfold and activate of the UPR, or iii) more unstable HLA-B27 complexes that leave the ER and dissociate at the cell surface to form HLA-B27 homodimers. These theories of AS pathogenesis are discussed in more detail later, but all have a capacity for a role for ERAP in disease.

1.3.1B IL23R

The association of *IL23R* with AS was first demonstrated in the WTCCC-TASC study, and has since been replicated in a number of different populations, including Portuguese, Spanish and Canadians, with the primary associated SNP identified as rs11209026 (Karaderi et al., 2009; Rahman et al., 2008; Rueda et al., 2008). This SNP results in an R381Q substitution in IL23R and is protective for AS, as well as IBD and psoriasis. Interestingly, however, no association of *IL23R* is seen in the east Asian Han Chinese and Korean populations, where the rs11209026 SNP has been shown not to be polymorphic (Clavin et al., 2008; Sung et al., 2009).

IL23R is a cytokine receptor expressed mainly on activated/memory T cells, T cell clones and NK cells, although low levels of expression have also been detected on dendritic cells, monocytes and macrophages. The IL-23/IL23R signalling pathway is essential for the terminal differentiation and maintenance of the pro-inflammatory CD4 T cell subset, Th17. In humans, Th17 cells are reported to develop from naive CD4⁺ CD161⁺ T cells upon activation by IL-6 and IL-1 β , which induce expression of ROR γ t and upregulate IL-23R, thus enabling cells to respond to IL-23. IL-23R signalling via a STAT3-associated pathway then promotes the production of IL-17 from activated Th17 cells, promoting their

expansion and stimulating prolonged IL-17 upregulation (reviewed in (Layh-Schmitt and Colbert, 2008)).

The identification of *IL23R* as a gene involved in AS susceptibility was the first convincing evidence of a role for Th17 cells in AS pathogenesis. Additional studies with mouse models have also implicated Th17 cells in the pathology of several other autoimmune diseases. For example, mice lacking IL-23 are resistant to experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease and collagen induced arthritis. Th17 CD4⁺ T cells and their products have also been associated with human diseases including MS, Crohn's disease, Psoriasis and RA. For SpA, elevated levels of IL-17 as well as increased numbers of circulating Th17 cells have been reported in the peripheral blood and/or the synovial fluid of AS patients, while a correlation between IL-17 levels and a marker of bone resorption, tartrate-resistant alkaline phosphatase (TRAP), has also been demonstrated (Jandus et al., 2008; Shen et al., 2009; Singh et al., 2007; Wang et al., 2009; Wendling et al., 2007). Furthermore, IL-23 has also recently been shown to be over-expressed in intestinal biopsies of AS patients, although IL-17 levels were normal (Ciccia et al., 2009). Finally, genetic associations with other components of the IL-23 pathway have also been identified, including the *STAT3*, *IL12B*, *CARD9*, and *PTGER4* genes (reviewed in (Brown, 2011)).

Gene	Chromosome	Protein Function
Genes definitely associated with AS		
<i>HLA-B27</i>	6p21.3	Presents endogenously processed antigens to T cells
<i>HLA-B60</i> (<i>B*4001</i>)	6p21.3	Presents endogenously processed antigens to T cells
<i>ERAP1</i>	5q15	Trims intracellular peptides to the optimal length for loading onto MHCI molecules
<i>IL23-R</i>	1p31.3	Promotes the differentiation of CD4 T cells into Th17 cells
<i>Kinesin family 21B</i> (<i>KIF21B</i>)	1q31	A kinesin motor protein used to transport essential cellular components along axonal and dendritic microtubules
<i>IL-1R2</i>	2q11-12	Acts as a decoy receptor, interfering with the binding to IL-1 to IL-1R1
<i>Anthraxin receptor 2</i> (<i>ANTXR2</i>)	4q21	Binds to collagen IV and laminin, possibly involved in extracellular matrix adhesion
<i>STAT3</i>	17q21	A cytoplasmic transcription that that is activated by IL-5 IL-6, IL-11 among others
Genes replicated but not consistently associated with AS		
<i>CYP2D6</i>	22q13.1	Involved in the metabolism of xenobiotics, which are reported promoters of inflammation via T cells
<i>IL-1 genes</i> (primarily <i>IL-1A</i>)	2q12.1	Important modulators of the Th1 response
<i>ANKH</i>	5p15.2	Exports inorganic pyrophosphate from intracellular to extracellular compartments. Regulates tissue calcification
<i>TLR4</i>	9q32-33	A pattern recognition receptor in innate immunity for lipopolysaccharide
<i>Killer Ig-like receptors</i> (<i>KIR</i>)	19q13.4	Regulates activation of NK cells via recognition of MHCI molecules on target cells
Genes associated with AS in one study but failed replication		
<i>TFGB1</i>	19q13.1	Mediates inflammation, fibrosis and bone remodelling
<i>CD14</i>	5q31.1	Receptor on monocytes important in apoptosis, binds lipopolysaccharide
<i>TNAP</i>	1p36.1	A phosphoethanolamine and pyridoxal-5'-PO4 acting-ectophosphatase. Degrades PPI
Genes implicated but did not achieve genome-wide significance		
<i>TNFR1</i>	12p13	A receptor for TNF α that can activate NK κ B, mediate apoptosis and function as a regulator of inflammation
<i>TRADD</i>	16q22	An adaptor molecule that interacts with TNFR1 and mediates apoptosis and NF κ B activation
<i>CARD9</i>	9q34	A component of the signalling pathway downstream of the NOD family of pattern recognition receptors
<i>TNFSF15</i>	9q32	A cytokine of the TNF ligand family that can bind to the DR3 receptor and induce Th17 cell proliferation

Table 1.7 MHC and non-MHC genes associated with AS (Adapted from Reveille *et al.* (Reveille, 2011))

Thus, increasing evidence from patients, experimental models of SpA, and genetic association studies are consistent with the hypothesis that Th17 cells play a significant role in AS pathogenesis.

1.4 HLA-B27 and SpA pathogenesis

Despite the wealth of research that has been carried out since the association between HLA-B27 and AS was first described, there continues to be considerable debate over its role in pathogenesis. It had been suggested that HLA-B27 may simply be a marker for a closely linked gene that was the real factor contributing to disease. However, increasing evidence from animal models, further linkage analysis studies, and, most recently, the convincing findings of two genome-wide association studies provide strong support for B27 involvement. The immunological functions of HLA-B27 have been the basis for several theories put forward to explain the involvement of B27 in disease. These include those proposing B27 to act in its natural capacity as an antigen presenting molecule, and others that consider several unique properties of B27 to be implicated in its mechanism of pathogenesis, independent of its usual immune function. The forthcoming sections begin with an overview of the animal models used to investigate SpA pathogenesis; in particular, the various HLA-B27 transgenic models that have been derived in order to help elucidate the relationship between B27 and disease. This is followed by a discussion of the three main theories under investigation that have been put forward to explain this relationship.

1.4.1 Animal models of SpA pathogenesis

Traditional models of induced or spontaneous arthritis in mice can be used as models for human inflammatory arthritis, particularly RA. These include the collagen-induced arthritis

(CIA) model, the antigen-induced arthritis (AIA) model, the K/BxN serum transfer model, and SKG mice. However, the lack of axial disease or other SpA-like features in these animals rule them out as suitable models for SpA. Instead, the last two decades has seen the generation of several potential animal models for SpA pathogenesis that can be classified into three major groups: (1) HLA-B27 transgenic mice, including the Spontaneous Inflammatory Arthritis (SA) murine model, (2) spontaneous ankylosing enthesopathies, including the ‘ANKENT’ and DBA/1 murine models, and (3) HLA-B27 transgenic rats. In addition, recent studies with the human TNF α transgenic mouse model have shown these mice to also develop sacroiliitis (Redlich et al., 2004). Previously studied as a model of RA, these mice have been used to examine the effects of anti-TNF therapy on inflammation and bone remodelling (Hayer et al., 2010; Uderhardt et al., 2010), and appears to be a useful tool in the study of sacroiliac joint ankylosis. Two further mouse models also show some SpA-like symptoms: Proteoglycan-induced arthritis model, where mice develop sacroiliitis after repeated immunisation with aggrecan; and the *Ank* mouse, in which mice with mutations in the *ank* gene develop murine progressive ankylosis (MPA). However these models appear to have little relevance for studying the pathogenesis of human SpA, and therefore focus here remains on those models relating to HLA-B27 and its role in pathogenesis.

1.4.1A HLA-B27 transgenic mice

Several groups have generated HLA-B27 transgenic mice (B27 TGM) expressing either the B*27:02 or B*27:05 disease-associated B27 subtype, and with or without human β 2m (Kievits et al., 1987; Krimpenfort et al., 1987; Taurog et al., 1988; Weiss et al., 1990). Initial studies were focused on delineating the role of HLA-B27 in anti-viral responses but

did not report the presence of any pathological features that may relate to human SpA. However, they established several key points. Firstly, the co-expression of human $\beta 2m$ in B27 TGM enhances HLA-B27 surface expression, the degree to which appears dependent on the B27 transgene copy number: HLA-B27 single transgenic mice (sTGM) with high copy number (~ 10) can more efficiently express B27 than mice with a lower copy number ($\sim 1-5$) in the absence of human $\beta 2m$ (Weiss et al., 1990). This may explain why earlier reports had disagreed on the importance of human $\beta 2m$ for physiological expression of HLA-B27, as in these studies B27 transgene copy number had not been determined. It is also worth noting here that the antibody commonly employed to detect correctly folded class I molecules, W6/32, recognises human MHC class I molecules (HLA-A, -B, and -C) only when associated with human, primate or bovine $\beta 2m$ and not murine $\beta 2m$. Therefore, the lack of W6/32 reactivity reported by Krimpenfort *et al.* as evidence for the absence of HLA-B27 expression in sTGM, cannot exclude the possibility of HLA-B27:murine $\beta 2m$ heterodimer expression. In these circumstances, the HLA-B27 specific antibody ME-1 used in Kievits *et al.*, which binds a determinant on the HLA-B27 heavy chain when it is correctly folded, is a more useful tool. That said, several reports find HLA-B27 less readily associates with mouse $\beta 2m$ than other class I molecules (Hochman et al., 1988; Rein et al., 1987). Secondly, these studies demonstrate that HLA-B27 molecules are fully functional in TGM as shown by CTL recognition and induction, antibody binding and induction, skin graft rejection and viral antigen presentation. Moreover, despite the clear quantitative difference in expression of HLA-B27 in sTGM compared with dTGM, the absence of human $\beta 2m$ in sTGM has no effect on the ability of B27 to function. Thirdly, the level of HLA-B27 expression in TGM is dependent on the host H-2 haplotype; mice homozygous for the majority of H-2 haplotypes exhibit intermediate to high HLA-B27 expression,

however, H2-q or H2-d mice fail to express B27 despite similar DNA and RNA levels. Interestingly, these latter haplotypes encode for three rather than two endogenous class I molecules (K, D and L). One possible implication of this is that, contrary to previous thinking, the supply of β 2m for MHC class I folding is limited and upon introduction of MHC class I transgenes competition ensues whereby expression is determined by the availability of and affinity for mouse β 2m. Lastly, two studies have shown that, when compared with their non-transgenic littermates, HLA-B27 TGM are more susceptible to infection with two different strains of *Yersinia enterocolitica*, one of the proposed arthritogenic bacteria species (Ismail et al., 1994; Nickerson et al., 1990). Although no SpA-like symptoms were seen in these infected B27 mice, this data suggests a role for B27 in impairing the immune response to infection by certain bacteria, which may contribute to their arthritogenic nature in human SpA.

It has been suggested that the lack of arthritis-like manifestations in these transgenic mice reflects the low susceptibility of mice to arthritis in general. Nonetheless, two groups have reported SpA-like pathology in transgenic mice with at least some specificity for HLA-B27. These are the Spontaneous Inflammatory Arthritis (SA) mouse model and the ANKENT mouse model.

1.4.1B Spontaneous inflammatory arthritis (SA) mouse model

Spontaneous arthritis has been reported in HLA-B*27:05 TGM lacking endogenous murine β 2m (Khare et al., 1995). Disease was dependent on the transfer of animals from a specific-pathogen free (SPF) environment into conventional housing, and symptoms presented within 2-4 weeks of transfer. These symptoms began with redness and swelling

of a single toe in a hind paw, and progressed to swelling, deformity and, in severe cases, ankylosis of both hind paws. Front paws were rarely affected, and nail changes were seen primarily in male mice. Moreover, this spontaneous arthritis developed more frequently and more severely in male mice compared with female mice, paralleling the sex bias observed in AS. Histological changes included increased synovial cell and mononuclear cell proliferation, and erosion of cartilage and subchondral bone. Taken together, the potential contribution from an infectious agent, the clinical features and the progressive nature of this mouse disease share similarities with human SpA. With regard to the potential disease mechanism, these authors use this model to investigate the possible involvement of two alternative forms of HLA-B27 in disease pathogenesis: 1) HLA-B27 free heavy chains and 2) HLA-B27 derived peptides. The lack of endogenous murine β_2m ($\mu\beta_2m$) in these $B27^+/\mu\beta_2m^0$ TGM resulted in no detectable surface expression of HLA-B27 on either splenocytes or PBMCs. However, upon Con-A stimulation, low level expression of B27 HC could be detected in splenocytes that could be recognised by HLA-B27-restricted CTLs. Negligible numbers of CD8 T cells but a normal repertoire of CD4 T cells were also observed. Importantly, no spontaneous joint and nail changes developed in littermates expressing mouse β_2m . In order for HLA-B27 expression to resemble that in human disease, human β_2m was introduced to make HLA-B27⁺/hu β_2m^+ / $\mu\beta_2m^0$ mice (Khare et al., 1996). In these animals, found to exhibit the same spontaneous arthritis, higher surface expression of B27 was detected and the CD8 T cell count was restored. Furthermore, the PBLs from these mice express HLA-B27 HC without the need for Con-A stimulation. Remarkably, treatment of $B27^+/\mu\beta_2m^+/mo\beta_2m^0$ mice with an antibody against free heavy chains, HC10, delayed the onset of arthritis and lowered the disease incidence, strongly implicating B27 HC in pathogenesis. In contrast,

this effect was not seen when treated with the control antibodies ME1, recognising correctly folded HLA-B27, or 3F212, an isotype matched anti-H2-A^b. It is also worth mentioning that these studies also report a mild and transient arthritis in ~10-15% of B27 negative control animals and hu β_2m^+ /mo β_2m^0 mice expressing the -Cw3 or -B7 transgene.

The idea that B27-derived peptides may be the causative agent in disease was put forward on the basis of sequence analysis studies that show several regions of HLA-B27 to contain peptide motifs shared with disease-implicated bacteria, along with the presence of CD4⁺ T cells in diseased joints, suggesting that, in disease, MHC class II may present these peptides to CD4 T cells. However, using this mouse model, Khare *et al.* have presented several lines of evidence against the involvement of MHC class II molecules. Firstly, mice deficient for MHC class II (B27⁺/hu β_2m^+ /mo β_2m^0 /Ab⁰) were found to be equally susceptible to disease (Khare et al., 1998). Secondly, generation of a chimeric MHC molecule containing the peptide binding domain of HLA-B27 ($\alpha1\alpha2$) and the $\alpha3$ domain of H-2K^d demonstrated the importance of the antigen presentation capacity of HLA-B27 for disease (Khare et al., 1999). Thirdly, as mentioned, treatment of B27⁺/hu β_2m^+ /mo β_2m^0 mice with the MHC class II antibody 3F12 had no effect on disease, altogether suggesting that pathogenesis is unlikely to involve presentation by class II molecules. Interestingly, despite this apparent independence from MHCII, further data indicates a role for CD4⁺ T cells in this model, as CD4-deficiency renders the TGM mice almost completely resistant to arthritis.

Thus, in this mouse model of spontaneous inflammatory arthritis, β_2m is necessary for surface expression of HLA-B27. Surface complexes may then dissociate to leave free

heavy chains that participate in disease pathogenesis, possibly through the presentation of exogenous peptides to CD8 T cells, or through their dimerisation to resemble class II molecules, enabling them to present exogenous peptides to potentially autoreactive CD4 T cells. In the presence of mouse β_2m , however, B27 forms more stable complexes that do not dissociate thereby preventing disease occurrence.

Significantly, however, are the reported failings of at least three groups to independently confirm this model of arthritis in β_2m deficient B27 TGM (Taurog, 1998). Moreover, findings by Kingsbury and colleagues are in direct disagreement, demonstrating that β_2m deficiency alone is sufficient to cause spontaneous arthritis and is not affected by transgenic B27 expression (Kingsbury et al., 2000). This study also highlights the role of the non-MHC genetic background, with spontaneous arthritis varying considerably between different strains of β_2m^0 mice with the same H-2 haplotype.

1.4.1C ANKENT model

Ankylosing enthesopathy (ANKENT) is a progressive enthesitis that occurs spontaneously at a low frequency in laboratory mice. It was discovered upon close examination of B27 transgenic and non-transgenic control mice and has distinct features to those in SA, including its phenotype, pathology and incidence susceptibility (Weinreich et al., 1995). In particular, disease progresses from mild joint swelling and inflammation in the hind paws to cartilage proliferation and ossification mainly at the entheses, a prominent feature also of SpA. However, no other pathology is observed, including any spinal abnormalities or extra-articular manifestations such as uveitis or the nail changes seen in SA, and gut inflammation. Males are almost exclusively affected, with castration experiments

suggesting that the testis is critically important for disease development. In addition to sex and age, both genetic and environmental risk factors have also been identified. It occurs more often in mice with the C57Bl background and with particular H-2 haplotypes, and its prevalence is significantly higher in HLA-B27 TGM. However, in contrast to SA, B27 is only a risk factor when co-expressed with murine β_2m ; ANKENT frequency in β_1m -deficient mice is drastically reduced irrespective of the presence of HLA-B27 with and without co-expression of human β_2m (Weinreich et al., 1997). Germ-free conditions protect animals from ANKENT, with one study implicating anaerobic intestinal bacteria as important triggering organisms (Rehakova et al., 2000; Sinkorova et al., 2008). This model appears to exclude a critical role for high surface expression of B27 molecules, perhaps suggesting that, under some circumstances, certain mouse class I molecules can perform the same pathological function as B27.

1.4.1D DBA/1 mouse model

Ankylosing enthesitis also develops spontaneously in group-housed DBA/1 mice, with distinct pathology from ANKENT. These mice develop polyarthrititis and toe stiffness from an age of 4 months. The initial description characterised this condition as similar to CIA, however closer examination of the histologic features revealed it is primarily an enthesopathy that can lead to ankylosis (Corthay et al., 2000). Moreover, unlike CIA, this spontaneous arthritis did not require T cells, which leads the authors to suggest that the bone remodelling processes of the joints in human SpA may also be T cell independent.

1.4.1E HLA-B27 transgenic rats

The very first transgenic rats were generated by Hammer *et al.* by introducing the B*27:05 and hu β_2 m transgenes into the inbred LEW and F344 rat strains (Hammer et al., 1990). Several lines were developed that demonstrated integration of both these transgenes. Of these transgenic rat lines, four LEW lines and one F344 line expressed both HLA-B27 and hu β_2 m at the cell surface, and immunologic function of surface HLA-B27 molecules was confirmed by their ability to allogeneically prime CTLs in skin graft transfer experiments. Notably, the two lines with the highest B27 transgene copy number developed spontaneous inflammatory disease bearing a strong resemblance to human SpA, with symptoms including prominent intestinal, joint, skin, and male genital inflammatory lesions. Furthermore, disease also presented when these rat lines were hemizygous for the B27 locus. In contrast, in a line bearing only an intermediate copy number, disease only manifested in B27 homozygous animals. Moreover, lines with fewer transgenes remained healthy, even when homozygous. These transgenic rat lines and those subsequently generated are detailed in Table 1.8. Importantly, only B27 transgenic rats were associated with this chronic and persistent multisystem inflammatory disease: control HLA-B7 transgenic rats were healthy, and HLA-Cw6 transgenic rats exhibited only transient mild diarrhoea and paw swelling. These control lines had intermediate levels of transgenes comparable to the 21-3 line. (Taurog et al., 1993) (Table 1.8). This implies that disease susceptibility correlates with gene expression and supports the idea that SpA is B27-specific. In agreement with this, mRNA and protein expression levels, both intracellularly and at the surface, were shown to correlate with disease susceptibility; disease-prone rats had consistently higher expression than resistant lines. In contrast, endogenous levels of the rat RT1 class I molecules did not increase in parallel with HLA-B27, indicating the

high level of B27 expression in disease is not simply a consequence of disease, but that, conversely, disease occurrence is the result of high levels of B27 expression. Interestingly, In the human disease, one group have reported statistically higher levels of B27 expression on PBMC from patients with AS compared with healthy B27+ subjects (Cauli et al., 2002). Confirmation of this data requires a larger number of patients, but this preliminary study provides evidence for a higher level B27 surface expression as being a further susceptibility factor in human SpA.

In addition to HLA-B27, other genetic factors affect disease susceptibility in rats. Backcrossing the 33-3 locus onto the rat PVG strain had little effect on disease phenotype. However, backcrossing onto the DA strain completely attenuated disease, indicating the presence of protective allele(s) within the DA strain (Taurog and Hammer, 1996). Intriguingly, this strain specificity is reversed in CIA, where F344 rats remain disease free.

Further characterisation of these transgenic rat lines identified several additional features relating to the disease pathogenesis. Firstly, the important role of environmental factors was demonstrated when the 21-4H and 33-3 disease-prone animals were re-derived under germ-free conditions (Rath et al., 1996; Taurog et al., 1994). These animals lacked any signs of intestinal inflammation or peripheral joint damage unless they were re-introduced to a conventional housing unit, wherein they reverted to the inflammatory phenotype. Interestingly, germ-free animals still presented with skin and male genital lesions suggesting that these symptoms are unaffected by the normal bacterial flora and a result of an alternative pathogenic mechanism. Further reconstitution experiments introducing known bacteria species to the germ-free rats identified enteric bacteria, in particular

Bacteriodes vulgatus, as key mediators of the intestinal inflammation in this model (Rath et al., 1996; Rath et al., 1999). Whether particular bacteria were important for arthritis in these germ-free rats was less clear. Interestingly, however, susceptibility to arthritis but not gut inflammation was markedly reduced in B27 transgenic rats co-expressing a transgene for a high affinity HLA-B27 peptide (Taurog et al., 1999; Zhou et al., 1998) (*note erratum* in (Zhou, 2000)). Here, mini-gene constructs were generated containing either the NP1 transgene, which targets the high affinity B27 influenza NP peptide (SRYWAIRTR) to the ER, or the NP2 transgene, which contains an altered peptide sequence that cannot bind to B27 (SLRWAIRTR). These transgenes were introduced to the disease prone transgenic rats and offspring were observed for disease symptoms. A ~50% reduction in arthritis prevalence was seen only in the NP1⁺B27⁺ rats. Together, these data suggest that distinct mechanisms for the involvement of B27 in gut and joint pathology may exist. Consistent with this theory, disease-prone B27 transgenic rats expressing a mutant B27 molecule C67S developed predictable intestinal inflammation but with a lower prevalence of arthritis. Whether this is a result of the mutation itself, or simply due to lower B27 expression these animals is unclear, as they express only a few more copies of the B27 and huβ₂m transgenes than the disease-resistant lines (Table 1.8). In addition, introducing extra huβ₂m to the disease-prone rat line 21-3 prevented gut pathology but exacerbated arthritic disease. These rats more closely resembled human SpA than the previously derived disease-prone lines.

Studies have also addressed the cellular basis of disease pathogenesis in these rats. Cell transfer experiments demonstrated bone marrow stem cells from disease-prone donors were capable of reproducing disease in both transgenic and non-transgenic healthy

recipients (Breban et al., 1993). Furthermore, transfer of spleen and lymph node cells alone could not induce the disease phenotype. These and additional control experiments suggested a role in disease induction for a BM-derived cell other than a lymphocyte such as an antigen-presenting cell (APC). In this regard, several groups have reported a defective DC function preceding disease onset and as a consequence of high levels of B27 expression in the B27 transgenic rats (Dhaenens et al., 2009; Fert et al., 2008; Hacquard-Bouder et al., 2007; Hacquard-Bouder et al., 2004; Stagg et al., 1995). Therefore, defective DCs could be an important factor in disease development in the transgenic animals. However, a critical role for T cells was also indicated in further experiments involving the generation of athymic (rnu/rnu) rats (Breban et al., 1996). Here, nude rats bearing the 33-3 transgene locus were shown to remain healthy compared with their euthymic transgenic littermates. However, T cell transfer from euthymic to athymic transgenic animals was sufficient to induce the disease phenotype in recipients, albeit with reduced joint symptoms (Breban et al., 1993). Furthermore, transfer of CD4⁺ T cells was more efficient at disease inducing gut inflammation compared with CD8⁺ T cell transfer. Two additional studies confirmed that conventional CD8 $\alpha\beta$ ⁺T cells are not important for the full disease phenotype, including arthritis. In disease-prone lines, CD8 $\alpha\beta$ T cell depletion experiments combined with thymectomy failed to suppress either colitis or arthritis (May et al., 2003). Yet, a reduction in arthritis severity was observed when all CD8⁺ cells were depleted, consistent with the possibility of a role of a CD8⁺ cell in disease. Most convincingly, the recent introduction of a null mutation in the CD8 α gene in disease-prone rats demonstrated the lack of CD8 did not prevent development of the disease phenotype (Taurog et al., 2009). Consistent with this, B27-restricted T cells have also been difficult to find in rats.

Together, this supports the lack of involvement of CD8, and thereby conventional B27 peptide presentation, in the rat model of Spondyloarthritis.

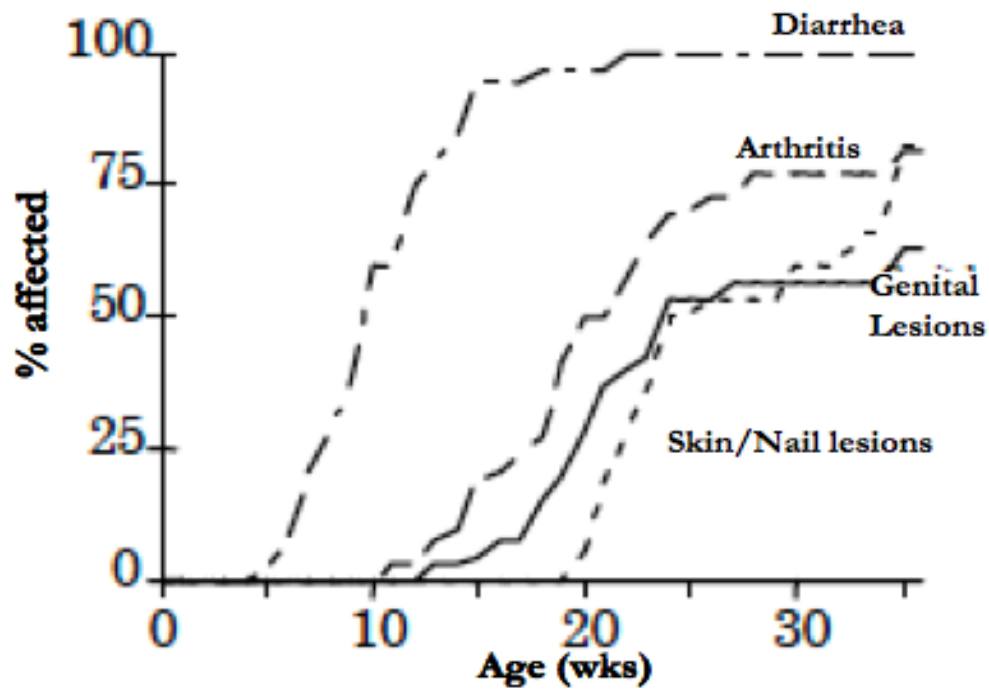


Figure 1.5 Disease progression in HLA-B27 Transgenic Rats (adapted from (Taurog et al., 1993)). Cumulative incidence of clinically evident disease manifestations in the male 33-3 transgenic rat line. Nearly all rats develop diarrhea and intestinal inflammation as the earliest symptom. Within several weeks, most rats develop peripheral arthritis, with skin and nail changes appearing late and more variably.

HLA transgene	Rat line	Original strain	HC gene copy number	huβ ₂ m gene copy number	Disease	Reference
B*27:05	21-4H	LEW	150	90	Yes	(Hammer et al., 1990)
B*27:05	33-3	F344	55	66	Yes	(Hammer et al., 1990)
B*27:05	21-3	LEW	20	15	Only in homozygotes	(Hammer et al., 1990)
B*27:05	21-4L	LEW	6	6	No	(Hammer et al., 1990)
B*27:05	25-6	LEW	7	7	No	(Hammer et al., 1990)
B*07:02	120-4	LEW	26	5	No	(Taurog et al., 1999)
Cw*06:01	201-3	F344	53	21	Mild diarrhoea *	(Taurog et al., 1999)
B*27:05 C67S	133-1	LEW	12	4	Reduced arthritis *	(Taurog et al., 1999)

Table 1.8 HLA transgenic rat lines and arthritis development (adapted from (Taurog et al., 1999)). Gene copy numbers are shown for hemizygote animals. *Disease phenotype only visible in homozygous animals.

1.4.2 Theories of SpA pathogenesis

The findings of these investigations using transgenic animal models of SpA have been used as evidence to support or refute theories put forward to explain the role of B27 in disease pathogenesis. This is discussed in the following, where an outline of the three leading theories is presented: 1) the arthritogenic peptide theory, 2) the misfolding and stress response theory, and 3) the B27₂ homodimer theory.

1.4.2A Arthritogenic peptide theory

The ‘arthritogenic peptide’ theory is one of the first and most extensively studied hypotheses for the involvement of B27 in disease (Benjamin and Parham, 1990). It proposes that disease results from the ability of B27 to bind and present unique antigenic peptides that provoke a B27-restricted CTL response. It is suggested that peptides shared among the disease-triggering pathogens could exhibit molecular mimicry to these ‘arthritogenic’ auto-antigens. As a consequence, upon infection, an activated CTL might overcome tolerance against the auto-antigen leading to a B27-specific, CTL-mediated autoimmune response. This theory is favoured over the previous molecular mimicry model that had postulated cross-reactivity of a humoral response to bacteria and B27 (Chen et al., 1987; Oldstone, 1987), particularly as it can explain both the tissue specificity of disease (joint-specific peptides) and the T-cell dependency seen in animal models (Breban et al., 1996; Breban et al., 1993). It also provides possible reasons as to why most B27⁺ individuals fail to develop SpA: natural polymorphisms in the ‘arthritogenic peptide’ may abrogate binding to B27; individuals require the presence of a triggering infection to initiate disease; variation in tolerance induction in individuals may predispose certain individuals. Furthermore, this may also explain the disease HLA-B27 subtype dependence,

as the less disease-associated alleles may not be able to present the arthritogenic peptide. Additional support for this model was presented in T cell-studies in which B27-restricted CTLs that could recognise both bacterially infected and uninfected target cells were detected in joints and peripheral blood of patients with ReA and AS (Hermann et al., 1993). Also, *Yersinia*- and *Chlamydia*-derived peptides have been detected in synovial fluid of ReA patients, although corresponding auto-antigens for cross-reaction with autoreactive T cells have not been found (Appel et al., 2004; Kuon et al., 2001; Ugrinovic et al., 1997). It is this latter issue that has been a leading caveat to the arthritogenic peptide theory; the inability to identify suitable arthritogenic peptide candidates and to demonstrate CTLs specific for these B27-restricted epitopes. It is also confounded by the findings in transgenic rats that, although T cell dependent, disease appears not to require CD8⁺ T cells (May et al., 2003). Together, this has made it increasingly difficult to accept that B27-restricted CTL-mediated cross-reactivity underlies disease pathogenesis. However, these findings have not ruled out an alternative role for arthritogenic peptides in unconventional recognition of B27 by CD4⁺ T cells (Boyle et al., 2001), a possibility discussed further in the B27₂ homodimer theory section.

An increasing number of studies have implicated certain properties of B27 in disease pathogenesis, relating to its unique biochemistry and cell biology. In particular, as described in the above, the discovery that a spontaneous arthritis phenotype in HLA-B27 transgenic mice develops in the absence of endogenous β_2m indicates that normal cell surface B27 expression is not required for disease and suggests a direct pathogenic role for the β_2m -free heavy chains (Khare et al., 1996; Khare et al., 1995).

1.4.2B HLA-B27 misfolding and UPR hypothesis

The B27 misfolding hypothesis proposes that disease is a result of the accumulation of aberrantly folded HLA-B27 in the ER, resulting in the production of an inflammatory response (Colbert, 2000; Mear et al., 1999). The build up of misfolded proteins can signal a stress response via 2 known pathways: (1) the unfolded protein response (UPR) and (2) the ER overload response (EOR) (reviewed in (Pahl, 1999)). These mechanisms provide stringent quality control measures to ensure that improperly folded proteins are destroyed and that normal ER function is maintained.

In the UPR, triggering activates a series of signalling pathways culminating in the induction of ER-resident chaperones to facilitate protein folding, translational arrest, and the upregulation of ER-associated degradation (ERAD). The key regulator in this pathway is the ER chaperone immunoglobulin-heavy-chain binding protein (BiP), which interacts with the main effector molecules of the UPR: activating transcription factor 6 (ATF6), Inositol-requiring transmembrane kinase/ endonuclease 1 (IRE1) and pancreatic ER kinase (PERK). This sequesters them in the ER and keeps them in an inactive state (Bertolotti et al., 2000). In conditions of ER stress, however, these molecules are released from BiP association. How stress is sensed and how BiP dissociation is triggered remains unclear; one possibility is that BiP itself becomes sequestered through its preferential interaction with increasing levels of unfolded proteins. In any case, BiP dissociation allows the activation of these effectors and ensuing downstream signalling events. For ATF6, this involves its translocation from the ER to the Golgi, where it is processed to release a cytosolic fragment that acts as a transcription factor, binds to ER-stress response elements and induces the expression of UPR associated genes. This includes the genes for several

protein folding chaperones, components of ERAD pathways, and the transcription factors CCAAT/enhancer binding protein homologous protein (CHOP) and X-box binding protein 1 (XBP1). IRE1 and PERK are both ER resident kinases and are often referred to as ER stress transducers. In resting cells they interact with BiP via ER-luminal domain to form IRE1-BiP and PERK-BiP heterodimeric complexes. BiP dissociation results in their oligomerisation, autophosphorylation and activation of effector functions. Phosphorylation of IRE1 activates its endoribonuclease domain, enabling it to splice the mRNA of *XBP1* to generate the active transcription factor XBP1s. Unspliced *XBP1* mRNA encodes XBP1u, a short-lived protein that is rapidly degraded and lacks transcriptional activity. XBP1s translocates into the nucleus and, similar to ATF6, upregulates transcription of a number of UPR associated genes. In addition, its kinase activity may also activate the JNK signalling pathway, culminating in autophagy (Ogata et al., 2006) and/or apoptosis. In contrast, activated PERK phosphorylates the translation-initiation factor eIF2 α , resulting in attenuation of most mRNA translation to alleviate ER stress. However, phosphorylated eIF2 α can also increase expression of certain mRNAs, including the transcription factor ATF4. This in turn increases expression of some UPR targets including CHOP, which acts to repress the anti-apoptotic factor Bcl-2 and induce cell apoptosis. Importantly, these UPR pathways are able to distinguish between acute and chronic ER stress so that apoptosis induction only occurs in cells that fail to recover (reviewed in (Todd et al., 2008)).

The EOR is a response to excessive membrane protein trafficking within the ER. Unlike the UPR, it is a poorly understood pathway, with many signalling components yet to be identified remain. It involves the activation of the transcription factor NF κ B, which in turn can stimulate the synthesis of pro-inflammatory cytokines such as TNF α , IL-1 and IL-6 in

certain cell types (Pahl and Baeuerle, 1997). Recent data suggest that the EOR can activate apoptosis via caspase-3 and caspase-12 activity, and may involve the depletion of ER Ca^{2+} levels (Kuang et al., 2005).

Therefore, in the case of HLA-B27, the misfolding theory postulates that accumulation of B27 HC in the ER provides potent and chronic stimulation of these ER-stress response pathways culminating in the release of pro-inflammatory cytokines that contribute to disease pathogenesis. Initial support for this model came from transgenic animal studies, whereby the disease-prone HLA-B27 transgenic rats were shown to have an accumulation of misfolded B27 HC in both their splenocytes and macrophages (Turner et al., 2005). Another study subsequently found this misfolded B27 more prone to binding to the ER chaperone protein BiP (Tran et al., 2004), a binding event not seen in the HLA-B7 rats, and seemingly independent of the C67 residue. Indeed, the interaction of BiP with B27 HC had previously been alluded to in experiments with B27-transfected human cell lines, and subsequently confirmed by Antoniou *et al.* ((Antoniou et al., 2004; Dangoria et al., 2002)). Furthermore, evidence of UPR activation in the B27 transgenic rats has been presented in several studies. In the first paper, upregulated mRNA levels of BiP, CHOP and XBP-1, as well as an increase in XBP-1 splicing were observed in BM macrophages from B27 rats but not B7 rats. Moreover, these increases were only seen in the B27 rats exhibiting the disease phenotype (10 weeks old), as the younger, premorbid rats (4 weeks) showed no such signs of a UPR. Yet, a UPR could be induced in these premorbid animals upon $\text{IFN}\gamma$ treatment, which also increased B27 HC transcript levels, suggesting B27 expression level as key in inducing the UPR. A second study also demonstrated that the upregulation of B27 mRNA in BM macrophages was accompanied by HC accumulation and preceded UPR activation,

the magnitude of which correlated with the degree of B27 upregulation (Turner et al., 2007). Interestingly, this appeared cell-type specific, as these findings did not extend to HLA-B27 expressing splenocytes from the same animals, which showed little signs of UPR activation. This is consistent with the findings in transgenic animals which predict an important role for a non-lymphocyte BM-derived cell that has high B27 expression. In this regard, signs of an activated UPR have also been reported in the adherent fraction of cells taken from AS patients (Gu et al., 2002).

However, more recent evidence in transgenic rat studies presents a serious challenge to the UPR theory, particularly as the accumulation of misfolded HC could be reversed by over-expression of human β_2m without alleviating the disease-phenotype (Tran et al., 2006). In this study, misfolding and UPR activation was significantly reduced, yet the double transgenic animals developed disease resembling human SpA to a greater degree than the previously described transgenic lines (Taurog et al., 1993). It is possible that misfolding provides one of several signals necessary for the activation of an inflammatory response, and it has been postulated that the misfolding may trigger or in fact augment an innate immune response (Colbert, 2000). On this point, it has been shown in the U937 human macrophage line that cells expressing B27, but not A2, have enhanced NF κ B activation and TNF α production after stimulation LPS (Penttinen et al., 2002). It may be that the misfolding sufficiently lowers the threshold for activation by other stimuli, such as LPS. This also offers a plausible explanation for the link between infection and SpA.

1.4.2C B27₂ homodimer theory

The B27₂ homodimer theory proposes that disease pathogenesis is a result of the immune recognition of disulphide-linked B27 homodimers (B27₂) (Figure 1.6). A trigger, such as bacterial infection, hypoxia or a stress-related signal, promotes the formation of cell surface B27₂ from unstable B27 precursors, which are then recognised by innate immunoregulatory receptors on leukocytes including NK cells, T cells, and other APCs. This results in aberrant signalling in these cells and a shift toward a pro-inflammatory response. Although little is known about the postulated trigger, an increasing number of studies have provided evidence for the both the presence of B27₂ at the surface and their altered interactions with leukocyte receptors.

Soluble recombinant B27 HC have been shown to refold and form disulfide-linked homodimers, dependent on the C67 residue in the α 1 domain (Allen et al., 1999). These homodimers do not contain β ₂m and adopt a different conformation to the heterotrimeric B27 molecules (Figure 1.3). Although they have been shown to be peptide receptive, whether they bind and present peptide *in vivo* is unknown. Three mouse monoclonal antibodies have been used in the characterisation of these homodimers in flow cytometry, immunoprecipitation, and immunoblotting experiments; HC-10, ME-1 and W6/32. HC-10 recognises a number of unfolded HLA-A, -B and -C heavy chains not associated with β ₂m and also reacts with homodimers. In contrast, ME-1 recognises correctly folded HLA-B molecules, while W6/32 recognises an epitope comprising residues from both β ₂m and the α 2 helix of folded HLA-A, -B and -C alleles, although it may also recognise a subset of B27 homodimers (Ellis et al., 1982; Stam et al., 1986). Analysis with a combination of these antibodies has enabled relative proportions of correctly folded and unfolded B27 HC

to be determined. In addition, by immunoblotting with HC-10 under reducing and non-reducing conditions, a 90kD band has been shown to reduce to a 45kD band correlating with B27 HC dimers and monomers respectively, consistent with a role for the C67 disulphide bond in stabilising the molecule. In this way, cell-surface homodimers have been detected in B27-transfected cell lines, AS patient PBMCs, and in leukocytes from B27-transgenic rats (Bird et al., 2003; Kollnberger et al., 2002; Kollnberger et al., 2004; Lan et al., 2004; Raine et al., 2006; Tsai et al., 2002).

Cell surface expressed B27₂ are proposed to form during endosomal recycling (Bird et al., 2003). This is a distinct pool of molecules from those found in the ER that fail to egress to the surface and are postulated to trigger a UPR. Surface homodimers are thought to form as a result of the dissociation of unstable trimeric complexes at the cell surface, a premise made more plausible by their propensity to bind suboptimal peptides in the ER. Upon loss of peptide and β_2m , surface free heavy chains undergo recycling into endosomal vesicles. Evidence for this was provided from experiments that combined the surface cleavage of HLA molecules with the inhibition of egress of newly synthesised HLA molecules (Bird et al., 2003). These demonstrated the formation of new surface B27₂ from pre-existing B27 heterotrimers cycling through the endosomal compartment. Furthermore, inhibition of endosomal acidification prevented the re-acquisition of surface homodimers following cleavage treatment, as did the addition of optimal B27 peptides, which appeared to stabilise B27 molecules at the surface.

As previously outlined, in addition to the TCR, MHCI molecules are also recognised by immunoregulatory receptors in the killer immunoglobulin-like receptor (KIR) and

leukocyte immunoglobulin-like receptor (LILR) families, which are expressed on many types of leukocytes, including NK cells, NKT cells, monocytes, macrophages, DCs and T cells (Brown et al., 2004; Colonna et al., 1999; Lanier, 1998). Both families comprise both inhibitory and stimulatory forms of receptors and they play important roles in immune homeostasis, including promotion of T cell survival, differentiation of DCs and macrophages, and the induction of regulatory T cells (Chang et al., 2002; Colonna et al., 1997; Colonna et al., 1998; Perez-Villar et al., 1997; Ristich et al., 2005; Ugolini et al., 2001). The demonstration of cell surface expression of B27₂ led to the proposal that these immunoregulatory leukocyte receptors might specifically recognise B27₂, which could modulate the immune response towards inflammatory disease. Consistent with this, B27₂ and B27 have demonstrated distinct patterns of binding to some of these immune receptors (Figure 1.7). Tetrameric complexes of recombinant B27₂ or B27 have been used in FACS investigations to assess their binding to a number of transfectants expressing these receptors. In particular, transfectants expressing KIR3DL1 or LILRB2 were recognised by both B27₂ and B27, whereas those expressing LILRB1 were only recognised by B27, and those expressing KIR3DL2 were only recognised by B27₂ (Figure 1.7) (Allen et al., 1999; Kollnberger et al., 2002). Furthermore B27₂ but not B27 binds to rodent paired immunoglobulin-like receptors (PIR), which are related to LILR (Kollnberger et al., 2004).

How the interaction of B27₂ with immunoregulatory receptors leads to AS remains undetermined, however given the role that these receptors play in maintaining immune homeostasis it is plausible that ligation by aberrant forms of B27 may upset this balance in favour of a pro-inflammatory response. One possibility is that the interaction of B27₂ with NK cells and T cells expressing KIR3DL2 results in altered cell signalling to promote the

survival of pro-inflammatory cell subsets that participate in and exacerbate a pro-inflammatory response. Consistent with this, studies have demonstrated an increased number of peripheral blood and synovial fluid NK cells and CD4⁺ T cells expressing KIR3DL2 from AS patients compared to controls (Bowness et al., 2011; Chan et al., 2005). Interestingly, these NK and T cell populations had an activated phenotype, expressing intracellular perforin and high levels of CD38 or high levels of CD38 and low levels of CD62L, respectively, suggesting these cells to have previous experience of activation, and, for the NK cells, to have enhanced killing ability. Importantly, co-culture experiments of these KIR3DL2 cell subsets with B27₂ expressing APCs have illustrated the functional significance of KIR3DL2 ligation by B27₂. KIR3DL2⁺ NK cells were protected from apoptosis in the presence of B27₂-expressing APCs, whereas KIR3DL2⁺ CD4⁺ T cells demonstrated enhanced survival and proliferation. Intriguingly, the KIR3DL2⁺ CD4⁺ T cells produced IL-17 after B27₂ stimulation, and these IL-17-producing KIR3DL2⁺ CD4⁺ T cells were enriched in the peripheral blood and synovial fluid of SpA patients. Together this data supports the hypothesis that B27₂ promotes the survival of KIR3DL2⁺ T and NK cells that go on to participate in the inflammatory response. Whether similar responses are seen as a result of LILRB2 interactions with B27₂ remains to be seen. However, given the expression of this receptor is restricted to cells of the myeloid lineage, it suggests that B27₂:LILRB2 interactions could affect the functioning of APCs themselves. As mentioned previously, defective APC function has been reported in B27 transgenic rat studies, with DCs shown to form defective interactions with T cells. Yet, without any evidence to support this so far, a role for B27₂:LILRB2 interactions in DC dysfunction remains highly speculative.

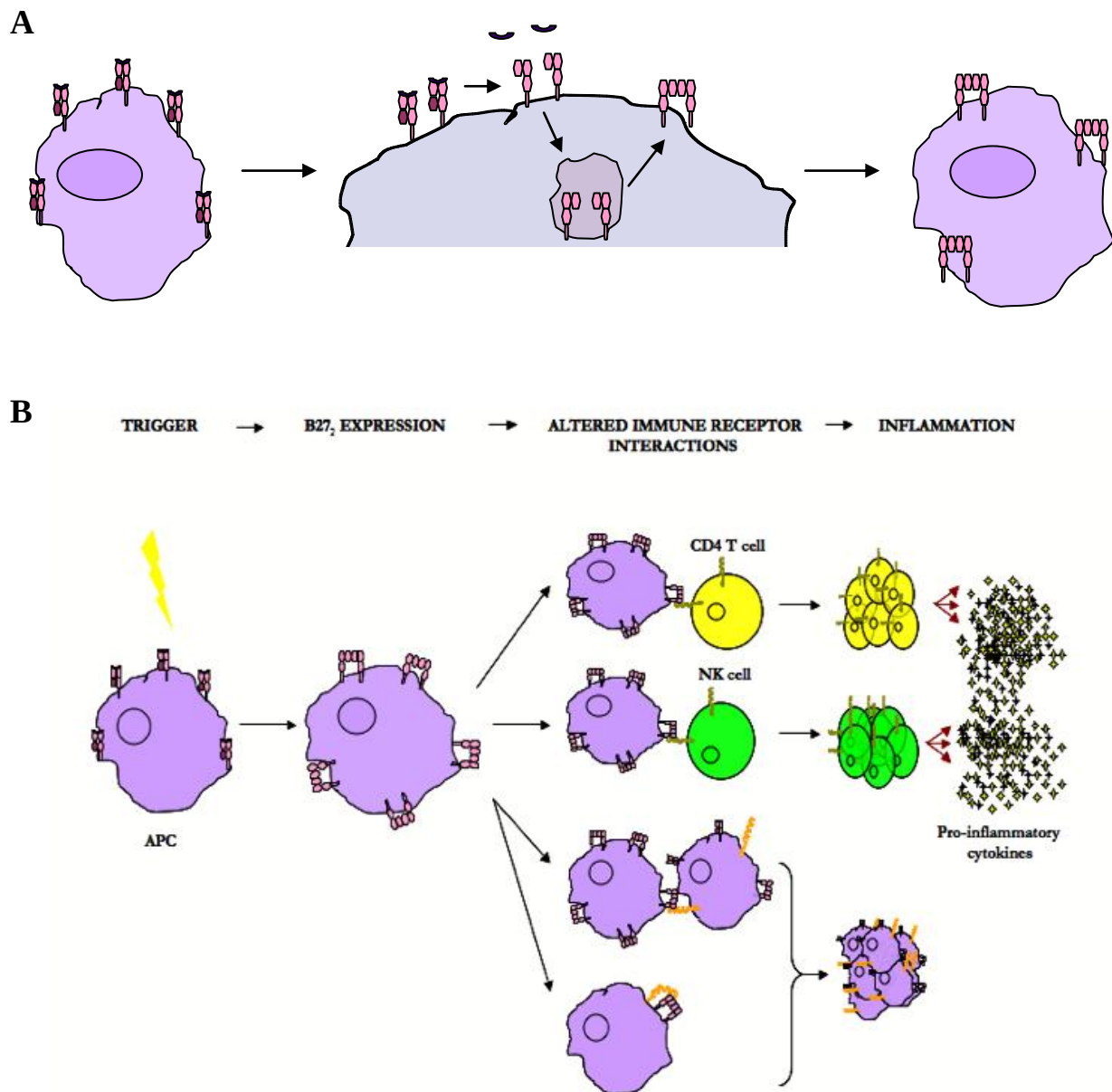


Figure 1.6 HLA-B27 homodimer theory. (A) Cartoon diagram of the formation of B27₂ at the surface following dissociation of unstable heterotrimeric complexes (B) Cartoon diagram of the 4 proposed stages of the homodimer theory.

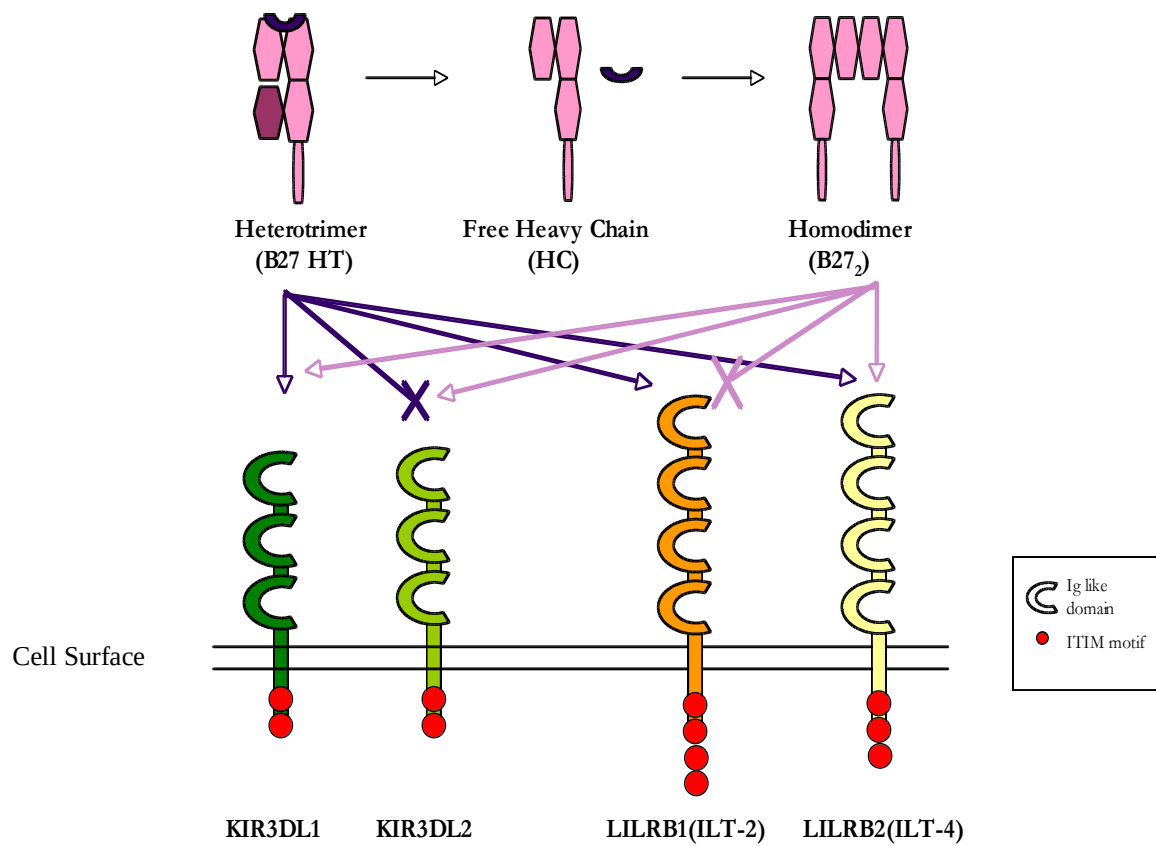


Figure 1.7 HLA-B27 heterotrimers and homodimer interactions with innate immune receptors of the LILR and KIR family. HLA-B27 heterotrimers bind to KIR3DL1, LILRB1 and LILRB2, whereas HLA-B27 homodimers bind to KIR3DL1, KIR3DL2 and LILRB2.

Project Objectives

Despite nearly four decades of research, the mechanism underlying the strong association of HLA-B27 with SpA remains unclear. The normal immunologic function of HLA-B27 is to present peptides for CD8⁺ T cell recognition, yet there is little evidence to indicate that this underlies disease pathogenesis. Moreover, the fact that disease develops in HLA-B27 transgenic rats in the absence of CD8 T cells highlights the involvement of an alternative role for B27 independent of its function as a classical MHC class I molecule. Recent studies have focused on two theories of pathogenesis, both based upon unusual biochemical properties of B27: the B27₂ homodimer theory and B27 misfolding and unfolded protein response theory. The work described herein is based upon the B27₂ homodimer theory, with the principle aim being to investigate the surface expression of aberrant forms of HLA-B27, such as B27₂, by means of a novel and potentially B27₂-specific monoclonal antibody, HD6. Importantly, however, this may also have relevance for the B27 misfolding theory, given that ER-localised B27₂ may contribute to the UPR posited in this theory.

The specific objectives of my research are outlined at the start of each chapter. Briefly, these include:

- I) Determining the binding specificity of HD6 in human cell lines,
- II) Characterising the nature of the HD6 epitope and role of peptide in its formation, and
- III) Determining the expression of HD6-reactive molecules in the context of a disease setting.

Overall, this thesis endeavours to provide further insight into the expression of aberrant forms of HLA-B27, and in doing so, provide solid evidence supporting a role for B27₂ in disease pathogenesis, as proposed in the homodimer theory. An improved understanding of the expression of these molecules, both in cell lines and in the more physiological setting provided by the B27 TG rat model of disease and SpA patients themselves, will hopefully provide further rationale for the development of targeted therapies aimed at these potentially pathogenic molecules, such as the HD6 antibody we have developed. Moreover, establishing whether HD6-reactive aberrant forms of B27 are present in the B27 transgenic rats will open up the prospect of testing HD6 itself as a treatment in these rats, prior to human trials. As HC-10 has been used to treat and ameliorate disease in the HLA-B27-transgenic β_2m -deficient mouse model, thereby implicating a role for free heavy chains in disease pathogenesis, successful treatment of HLA-B27 transgenic rats with HD6 will provide compelling evidence for the role of aberrant forms of B27, such as B27₂, in pathogenesis.

Chapter Two: Materials and methods

2.1.1 General cell culture

Cells were cultured in sterile tissue culture wells or flasks at 37°C with 5% CO₂ in R10 media (see Table 2.1), unless stated otherwise, and split twice weekly. Regular testing for mycoplasma infection was carried out, thanks to Alistair Waugh and Rebecca Horsfall. All disposable plastic ware obtained from Sigma or BD FalconTM (BD Biosciences).

2.1.2 Adherent cell culture

To split adherent HeLa cells, media was aspirated and cells were washed with warmed trypsin-EDTA (CC-5012, Lonza) to remove any remaining media that could otherwise block trypsinisation. 2-3ml of trypsin was then added to flasks or wells for 5-10 min at 37°C with 5% CO₂ until all adherent cells were suspended in the trypsin, applying gentle tapping if necessary. The trypsin was diluted with 5 times its volume of warm D10. Cells were then centrifuged (1500rpm, 5min), and resuspended in warm D10 (see Table 2.1) for splitting into flasks or wells as appropriate. Cells were split on reaching confluence (approx. every 4-5 days). Trypsin treatment unpredictably altered the surface staining of cells with the mAbs HC-10 and HD6 (see Appendix II), therefore removal of adherent HeLa cells immediately prior to mAb staining involved a 3-5 min incubation with a solution of 4mg/ml lidocaine with 5mM EDTA at 37°C with CO₂. Cells were then washed twice in PBS with 10% FCS for subsequent mAb staining.

Table 2.1 Cell Culture Media

R0	RPMI-1640 media (R8758, Sigma, UK), 2mM L-glutamine (BE17-605E) and 50U/ml penicillin/streptomycin (DE17-603E, Lonza Group Ltd, Switzerland)
R10	R0, 10% sterile filtered heat-inactivated fetal calf serum (FCS) (F9665, Sigma, UK)
R10-G418	R10, 0.5mg/ml Geneticin® (G418 sulfate) (11811, Gibco, UK)
R10H	R0, 10% sterile filtered heat inactivated human AB serum (H4522 Sigma, UK)
R10H*	R10H, recombinant IL-2 (200U/ml) (Proleukin, Pharmacy)
D10	Dulbecco's Modified Eagle Medium (DMEM) (D5786, Sigma, UK) 2mM L-glutamine, 50U/ml penicillin/streptomycin, 10% FCS
ID20	Iscove's Modified Dulbecco's Medium (IMDM) (21056-023, Invitrogen, UK), 20% FCS, 50U/ml penicillin/streptomycin
ID20-G418	ID20, 1mg/ml Geneticin®

2.1.3 Cell lines

The cell lines used in this thesis are listed in Table 2.2. Cells were either transfected or lentivirally-transduced to express HLA-B27 or HLA control alleles as indicated throughout. .220, .221 and their lentivirally-transduced counterpart cells, generated together with Jacqueline Shaw and Simon Kollberger, were maintained in R10 medium. Transfected .220 and .221 cells, obtained from Simon Kollnberger and described previously (Bird et al., 2003; Peh et al., 1998), were maintained in R10-G418 medium. C58, CEM, CEM.NKR and lentivirally-transduced versions of these cell lines, received

from Antony Antoniou and Simon Powis and described previously (Fussell et al., 2008; Santos et al., 2006), were maintained in R10 medium. Transfected versions of these cells were also received and maintained in R10-G418 medium. T2, U937, RBL and lentivirally-transduced U937 and RBL cell lines were maintained in R10 medium. Transfected T2 and U937 cell lines were maintained in R10-G418 medium. HeLa cells and lentivirally-transduced HeLa cells, received from Keith Gould and Antony Antoniou, and HEK 293T cells were maintained in D10 medium. KG-1 cells were maintained in ID20 medium, and transfected KG-1 cells were maintained in ID20-G418 media. Baf3 cells and transfected Baf3 cells, described previously in (Kollnberger et al., 2002), were maintained in R10 media. EBV-transformed B cell lines, received from Tim Powell and Louise Jones, were maintained in R10 medium.

Table 2.2 Cell Lines

Cell Line	Information and Source
721.220 (.220)	A human B-LCL derived by mutagenesis in which HLA-A and -B are deleted, low levels of a non-functional mutant tapasin are expressed, and HLA-Cw1 remains intact on one chromosome (Shimizu and DeMars, 1989)
721.221 (.221)	A human B-LCL derived by mutagenesis in which HLA-A, -B, and -C are absent, but functional tapasin is retained (Shimizu and DeMars, 1989)
HM72.C1R (C1R)	A human B-LCL lacking HLA-A and expressing low levels of HLA-B35 (Zemmour et al., 1992)
T2	A TAP-deficient hybrid of the .174 and CEMR.3 cell lines. The cells synthesize but do not express HLA-B5 (DeMars et al., 1984; Salter et al., 1985) (ATCC Number CRL-1992)

CEM	A human T-LCL cell line that expresses no HLA class II antigens, little total HLA class I antigen and no HLA-B (Howell et al., 1985) (ATCC Number CCL-119)
CEM.NKR (NKR)	A CEM-derived T-LCL cell line deficient in calnexin expression (Howell et al., 1985)
HeLa	A human epithelial carcinoma cell line (ATCC Number CCL-2)
C58	A rat thymoma cell line ATCC Number TIB-236)
U937	A human leukemic monocytic lymphoma cell line that serves as an in vitro model for monocyte/macrophage differentiation (ATCC Number CRL-1593.2)
KG-1	A human monocytic-line cell line that spontaneously differentiates to granulocyte and macrophage-like cells (ATCC Number CCL-246)
HEK 293T (293T)	A highly transfectable derivative of the human HEK 293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted. (ATCC Number CRL-11268)
RBL	Rat basophilic cell line (ATCC Number CRL-1378)
Baf3 (Baf)	An immortalised murine bone marrow-derived pro-B-cell line

2.1.4 Generation of Lentivirus and lentiviral transduction of cell lines

All lentivirally-transduced cell lines generated in this thesis were produced using the supernatant from cultured HEK 293T cells that had been transiently transfected to produce lentivirus particles as described below. The lentiviral plasmid pHR-SIN-BX-IRES-Em was used to generate constructs with and without co-expression of eGFP for (i) untagged HLA-

B*27:05 (05), (ii) untagged HLA-B*27:09 (09), (iii) 05 with a C-terminal HA tag, and (iv) 09 with a C-terminal HA tag.

To make lentivirus particles, 293T cells in D10 media were cotransfected at a ratio of 1:1:1 with 3 different plasmids obtained from Demin Li: 1) a lentiviral plasmid (pHR-SIN-BX-IRES-Em); 2) an HIV-based packaging plasmid (pCMVR8.91); 3) an HIV-based envelope plasmid (pMD-G). The pHR-SIN-BX-IRES-Em plasmid contains the transgene, which is flanked by the HIV-1 derived cis-acting sequences necessary for packaging, reverse transcription, and integration (a CMV promoter and the Woodchuck post-transcriptional regulatory element (WPRE)), and contains an eGFP gene. The pCMVR8.91 and pMD-G plasmids encode gag/pol (with *tat*, *rev*, and virulence genes deleted) and the G protein of VSV (VSV-G), respectively. The resulting disabled particles are able to infect most mammalian cells (Zufferey et al., 1997). Fugene 6 transfection reagent (11815091001, Roche) was prepared as per manufacturer's instructions and 1 µg of each vector was added in a 1.5ml sterile screw-cap tube to 150 µl transfection mix. After 30 min incubation at RT, 100 µl was added to 293T cells (~70% confluent) in 6 well plates with 1ml D10 media and cells were incubated at 37°C for 3 days. Supernatant was aspirated and centrifuged (3,000rpm, 15min), and 250-500 µl used to infect 2×10^5 target cells in 24-well plates. After 5 days, successful lentiviral transduction was verified by either checking for GFP expression where appropriate or by staining with MHC class I-specific antibodies.

2.1.5 Human peripheral blood mononuclear cell (PBMC) samples and ethics approval

20 ml peripheral blood samples were obtained from AS patients attending the Nuffield Orthopaedic Centre, Oxford. All AS patients were HLA-B*27:05 positive, as determined

by previous DNA typing. For healthy controls, 25-50 ml of peripheral blood was obtained from willing laboratory volunteers known to be HLA-B*27:05 negative, also determined by previous DNA typing. Samples were obtained with informed consent from all donors and with appropriate ethical permission (CO REC 06/Q1606/139).

2.1.6 Preparation of peripheral blood mononuclear cells (PBMCs)

Venous blood was collected in 50ml sterile polypropene falcon tubes (352070, Becton Dickinson, USA) containing 100µl heparin (≥ 1 U/ml, FP1077, Wockhardt, UK) and diluted with equal volume of R0 media, then layered onto 15ml Ficoll-Hypaque medium (1114547 LymphoprepTM, Axis-Shield PoC AS, Norway) for density gradient centrifugation. The blood was then centrifuged (2000rpm, 25 min) without a brake and the PBMC layer at the interface between the Ficoll-Hypaque and the plasma was aspirated carefully into a new tube and washed twice in R0 (1500 rpm, 5 min). An aliquot was then taken and counted in a 1:1 dilution with 0.4% trypan blue solution (T8154, Sigma-Aldrich, UK) using Hycor Kova Glasstic counting chamber slides (87144, Hycor Biomedical Inc., USA). The cells were resuspended in an appropriate volume of R10 media to achieve the desired concentration.

2.1.7 Cryopreservation of cells

Cells were centrifuged at 1500 rpm for 5 min and the pellet was resuspended in cold freezing media (filter sterilised solution of heat-inactivated FCS supplemented with 10% sterile dimethyl sulphoxide (D2650, Sigma-Aldrich, UK)). 1ml aliquots were transferred into chilled screw-cap freezing vials that were then placed into a 1°C cryo-container (5100, Nalgene, USA) and stored at -80°C or in liquid nitrogen dewars for long term storage.

Frozen cells were removed from storage and immediately placed in a 37°C water bath for thawing, washed twice by centrifugation at 1500 rpm for 5 min in warm R10, then resuspended in appropriate media.

2.1.8 Antibodies

The antibodies used in this thesis, with the exception of HD6, are listed. The monoclonal antibodies generated in house were thanks to Alastair Waugh, Rebecca Horsfall and Alison Han, and were purified from hybridoma supernatants by protein A-Sepharose affinity column as described previously (Ey et al 1978). The generation of the HD6 antibody, carried out in collaboration with Christoph Renner *et al.* using phage display technology, is detailed in Appendix III. 2µg in 50µl R10 of HD6 was used to stain 2×10^5 cells, followed by 0.5µl of the secondary conjugated goat anti-mouse antibody (see Table 2.3).

Table 2.3 Antibodies

Antibody	Further details and use in FACS	Supplier
<i>Unlabelled primary mouse monoclonal antibodies – used 0.5µg/2x10⁵ cells (in 50ul volume)</i>		
HC-10 (IgG2a)	Reacts with β_2 m-free heavy chains of HLA-B, -C, and some –A alleles (Stam et al., 1986)	Made in house (1.4-4mg/ml)
ME-1 (IgG1)	Reacts with MHC class I heavy chains associated with β_2 m (Barnstable et al., 1978)	Made in house (1.4-1.8mg/ml)
W6/32 (IgG2a)	Recognises HLA-B27, -B7, -B42, -B67, -B73 and Bw22 (Ellis et al., 1982)	Made in house (1.0-1.8mg/ml)
BBM.1 (IgG2a)	Recognises free and HLA-associated β_2 m (Brodsky et al., 1979)	Made in house (1.2-1.4mg/ml)
BB7-2 (IgG2a)	Recognises HLA-A2 (Parham and Brodsky, 1981; Wong et al., 2006)	Made in house (0.7-1.5mg/ml)
Anti-ILT4 (IgG2a)	Recognises LILRB2	R&D Systems (287219)

IgG1 (Isotype control)	N/A	BD Pharmingen™ (554121)
IgG2a (Isotype control)	N/A	BD Pharmingen™ (554126)
<i>Secondary conjugated antibodies – used 0.5ul/2x10⁵ cells (in 50ul volume)</i>		
Anti-mouse PE	Goat anti-mouse immunoglobulins	Dako (R0480)
Anti-mouse APC	Goat anti-mouse immunoglobulins	BD Pharmingen™ (550826)
<i>Conjugated Primary Anti-human antibodies (lineage markers) – staining in 50ul volume</i>		
CD3-APC (UCHT1)	Used 1:50 dilution	BD Pharmingen™ (555355)
CD8-APC (clone RPA-T8)	Used 1:50 dilution	BD Pharmingen™ (561953)
CD4-APC (clone RPA-T4)	Used 1:50 dilution	BD Pharmingen™ (555349)
CD14-PB (clone HCD14)	Used 1:50 dilution	Biolegend (325615)
CD11b-APC (clone D12)	Used 1:50 dilution	BD Pharmingen™ (333143)
CD11c-APC (clone B-ly6)	Used 1:50 dilution	BD Pharmingen™ (560895)
CD107-PE (clone H4A3)	Used 1:50 dilution	BD Pharmingen™ (555801)
IgG1-PE (Isotype control)	Used 1:50 dilution	BD Pharmingen™ (551436)
<i>Conjugated Primary Anti-rat antibodies (lineage markers – staining in 50ul volume)</i>		
CD4-FITC (clone OX38)	Used 1:200 dilution	BD Pharmingen™ (554843)
CD8-PerCP (clone OX8)	Used 1:200 dilution	BD Pharmingen™ (558824)
CD11b-A488 (clone OX42)	Used 1:200 dilution	Serotec (MCA275A488)
MHC class II- PerCP (clone OX6)	Used 1:800 dilution	BD Pharmingen™ (557016)
CD45R-FITC (clone HIS24)	Used 1:200 dilution	BD Pharmingen™ (554880)

2.1.9 FACS staining and analysis

Cells were added to 96-well plates at $0.5-2 \times 10^5/\text{ml}$ and washed with cold PBS before addition of primary antibodies against surface markers at appropriate concentrations in 50 μl R10 (see Table 2.2). Cells were incubated on ice for 30 min in dark, washed three times in FACS wash buffer (PBS 1% FCS) and then, where necessary, incubated with secondary antibodies for a further 30 min on ice in dark (see Table 2.2). Cells were washed again twice in FACS wash buffer and then fixed by addition of 200 μl /well Fix buffer (FACS wash buffer with 1.5% formaldehyde (47608, Sigma, UK)). To exclude dead cells, a violet LIVE/DEAD® stain was included by adding 4 μl /well of a 1:100 dilution of the violet fluorescent reactive dye reconstituted as per the manufacturer's instructions (L34955, Invitrogen, UK). For human PBMCs and rat splenocytes, prior to antibody staining, cells were pre-incubated for 15 min on ice with 100 μl blocking buffer (R10 supplemented with 10% Rabbit Serum (R9133, Sigma, UK)) to minimise background staining. Subsequent staining steps were then also carried out in 50 μl blocking buffer. Fixed cells were transferred to FACS tubes (352063, BD) containing 300 μl Fix buffer, read on a 9-colour CyAn ADP flow cytometer (Dako, Denmark) and analysed using FlowJo software (Tree Star Inc).

2.1.10 Intracellular staining

Intracellular staining of cell lines was carried out using the BD Cytofix/Cytoperm™ reagents according to the manufacturer's instructions (554714, BD Biosciences, UK).

2.1.11 Generation of B27-restricted CD8⁺ T cell lines

The generation of B27-restricted CD8⁺ T cell lines is detailed in Appendix I. Briefly, PBMCs from a B27⁺ individual were prepared and 5×10^6 cells were pelleted in two tubes and resuspended in 100 μ l of concentrated 100 μ M sterile peptide solution for 1-2h at 37°C. Cells were then diluted in R10 to 1×10^6 /ml and plated at 2.5×10^6 cells/well in 24 well plates (Corning Inc., USA). On day 3, IL-2 was added (200 U/ml) and cells were maintained in R10H*. Peptide-specific CD8⁺ T cells were sorted as follows: 2×10^6 cells were stained with 5 μ g of appropriate HLA-B27 tetramer and 10 μ l of CD8-APC (BD PharmingenTM), then washed and transferred into sterile polystyrene FACs tubes containing 0.3ml PBS 1% FCS. CD8⁺Tetramer⁺ T cells were collected into tubes containing warmed R10H, thanks to Craig Waugh, then washed twice in PBS and transferred to 24 well plates in 2ml feeder mix. Cells were cultured for 3 weeks in R10H* before restimulation with feeder mix. Feeder mix was generated in collaboration with Abigail Culshaw and Yanchun Peng, and contained irradiated allogeneic PBMCs and 50 μ g/ml PHA. For further information, see Appendix I.

2.1.12 Tetramer staining

For MHC class I tetramer staining a standard and well established protocol was followed (Kollnberger et al., 2004). Briefly, cells were centrifuged at 1500 rpm for 5 min, the supernatant removed, and remaining cells resuspended in residual volume. 5 μ g of PE-conjugated tetramer was then added in 50 μ l R10 and the cells were incubated at 37°C for 20-30 min in dark before washing with FACS wash buffer. Antibodies to cell surface markers were then added for a further 15-20 min in the dark (CD8-APC or CD3-APC) before final washing steps with sterile wash buffer. Cells were next either sorted or fixed in

Fix buffer for FACs analysis. Samples were read on a CyAn ADP flow cytometer and analysed using FlowJo software (Tree Star Inc).

2.1.13 Generation of HLA-B27 tetramers

The generation of HLA-B27 tetramers is detailed in Appendix I. Inclusion bodies for HLA-B*27:05 and β_2 -microglobulin (β_2 -m) were provided by Jacqueline Shaw, having been prepared from plasmids encoding the extracellular domain of HLA-B*27:05 with a C-terminal biotinylation recognition site and His Tag (pLM1-HLA-B27, “B27BH”) or β_2 -microglobulin (β_2 m) (pHN1- β_2 m). These were used to transform recA-BL21 (DE3) pLys(Gold) (Stratagene, Amsterdam, Netherlands) following the Invitrogen protocol. Protein was purified from inclusion bodies and solubilised in 15ml urea (8M). Standard heterotrimeric complexes were then refolded by adding β_2 m (30mg), the required peptide in molar excess (3mg/refold) and B27BH (40mg) to 500ml refolding buffer (100mM Tris, 400mM L-arginine, 2mM EDTA, 5mM reduced glutathione, 0.5mM oxidised glutathione, 0.1mM PMSF in 1L of H₂O) at 4°C. The B27BH was added in several aliquots over time to result in a higher yield of refolded complex. Refolding mixture was kept continuously stirring at 4°C for 48-72h. The refolded protein was centrifuged to remove protein precipitates and then concentrated to a volume of 5-10ml using an ultrafiltration membrane with 10kD cut-off (Millipore, UK) at 4°C and 50psi pressure using a pressurised stirred cell (Amicon, Millipore). Buffer exchange into 10mM Tris pH8 was performed using PD10 columns (GE Healthcare, UK) following the manufacturer’s instructions. The protein was biotinylated using BirA enzyme (10 μ M final concentration) and Biomixes A and B (containing 100mM ATP, pH7 and biotin) (Avidity, Aurora) to facilitate the incorporation of biotin at the enzyme recognition site in the C-terminus of B27BH.

Refolded biotinylated peptide-HLA-B27 complexes were purified by Fast liquid protein chromatography (FPLC) using the AKTA purification system with an sd75 superdex gel filtration column (GE Healthcare) and filter-sterilised gel filtration buffer (20mM Tris pH8, 150mM NaCl). The peak corresponding to the heterotrimeric complex (elution volume 160-175ml) was harvested, concentrated to ~1ml and protease inhibitors were added (leupeptin (1µg/ml) and pepstatin (1ug/ml)) before aliquots of 50µg were frozen and stored at -80°C. Composition was confirmed by nonreducing and reducing SDS-PAGE. Tetramers were conjugated by pulsing 100-200µg protein with Extravidin®-R-phycoerythrin (PE) conjugate (E4011, Sigma, UK) at a 4:1 molar ratio of biotinylated protein:extravidin, then stored at 4°C in the dark. (See Appendix I for elution profiles).

2.1.14 Synthetic peptides

Peptides were synthesised in the in-house facility on an automated peptide synthesiser (396 MPS, Advanced Chemtech) by conventional solid phase Fmoc chemistry, then analysed for purity of >90% by reverse phase high performance liquid chromatography (HPLC), thanks to Dr Kati di Gleria and Zhanru Yu. Peptides were dissolved in PBS or DMSO to 10mg/ml and stored at -20°C. 1mg/ml peptide stock solutions (diluted in RPMI) were filter sterilised with low protein binding 0.2 µm Millex-GV4 disposable microfilter units (SLGP033RS, Millipore, USA). Peptides used are shown in Table 2.4.

2.1.15 Exogenous peptide addition to cells

HLA-B27-binding peptides or control peptides binding to either HLA-A2 or HLA-B7, as described in Table 2.4, were generated and made up to 1mg/ml in RPMI-1640, filter-sterilised, and then added to $0.5-1 \times 10^6$ cells/ml at a final concentration of 50-100µM.

Peptide-pulsed cells were then incubated at 37°C with 5% CO₂ for indicated time periods before washing in FACS wash followed by staining with mAbs.

Table 2.4 Synthetic Peptides

Peptide Sequence	Peptide Name and Natural Source
<i>Non HLA-B27 restricted peptides</i>	
GILGFVFTL	HLA-A2-restricted peptide (A2 peptide 1) influenza matrix protein, residues 58-66
SLYNTVATL	HLA-A2-restricted peptide (A2 peptide 2) HIV-1 gag, residues 77-85
RPMTYKAAV	HLA-B7-restricted peptide (B7 peptide) HIV-1 nef, residues 77-85
<i>HLA-B27 restricted peptides</i>	
SRYWAIRTR	“FLU peptide” Influenza nucleoprotein (NP), residues 383-391
RRIYDLIEL	“EBV peptide” EBNA-3C, residues 258-266
RRYQKSTEL	“RRY peptide” Human histone (H3.3), residues 52-60
KRWIILGLNK	“GAG peptide (L variant)” HIV-1 gag, residues 263-272
KRWIIMGLNK	“GAG peptide (M variant)” HIV-1 gag, residues 263-272

2.1.16 Low pH iso-osmotic treatment of cells

0.5-5x10⁶ of washed cells were gently resuspended in 0.3 ml of a low pH filter-sterilised glycine buffer (PBS with 1% BSA (A9418, Sigma, UK) and 300mM glycine (410225, Sigma, UK), pH 2.4) for 2 min at room temperature. Cell suspensions were then neutralised by adding 25ml RPMI 1640, washed twice with wash buffer, then resuspended in wash buffer for mAb staining and FACS analysis.

2.1.17 Papain treatment of cells

Papain from *Carica papaya* (10108014001, Roche, UK) was left as unactivated (P) or activated (P*) with 10mM L-cysteine (C7352, Sigma, UK) in warmed R10 for 20 min, before being used to treat cells at 3-4mg/ml for 45 min at 37°C. Cells were then washed twice with wash buffer (PBS 1% FCS) and re-incubated in R10 at 37°C for the indicated recovery time periods. Where specified, cells were pre-incubated with 10µg/ml brefeldin A (BFA) (B7651, Sigma, UK) for 45 min prior to papain treatment before recovering in R10 supplemented with 10µg/ml BFA. In addition, where indicated, cells were also pre-incubated with the inhibitors of endosomal acidification bafilomycin A1 (0.01µM final concentration) (B1793, Sigma, UK) or chloroquine (100µM final concentration) (C6628, Sigma, UK).

2.1.18 CD107a degranulation assay

50µl of CTLs at a concentration of 5 x 10⁵/ml were added to a 96-well plate along with 2µl/well of anti-CD107a PE-conjugated antibody and incubated at 4°C for 30 min. Appropriate numbers of target cells were aliquoted into 15 ml falcon tubes, spun down (1500 rpm, 5 min), then resuspended in 50µl of a filter-sterilised 1mg/ml peptide solution

(FLU or EBV peptide) for 1h at 37°C, 5% CO₂. These peptide-pulsed target cells were next washed twice in R10 and then added to the CTLs at an E:T ratio of 1:1 or 0.5:1 for a further 1h at 37°C, 5% CO₂. 10µl of Brefeldin A at a concentration of 100µg/ml (final concentration 10µg/ml) was added to each well before a further 3h incubation at 37°C, 5% CO₂. Cells were then spun down and stained with 2µl/well anti-CD8 APC-conjugated antibody followed by 20 min incubation on ice. Cells were washed twice in FACS wash buffer and resuspended in 300µl Fix buffer. Samples were read on a CyAn ADP flow cytometer and analysed using FlowJo software (Tree Star Inc).

2.1.19 IFN- γ ELISpot

The IFN- γ ELISpot was carried out using ELISpot kit supplied by Mabtech (3420-2A). The hIFN γ capture antibody was diluted with PBS to 15µg/ml. Ninety six-well multi-screen IP-plates (MAIPS4510, Millipore, USA) were pre-coated with 50 µl/well 35% ethanol for 1-3 min, then rinsed with distilled water. The wells were then coated with 50 µl/well of the diluted capture antibody, sealed with parafilm and stored at 4°C overnight. Plates were washed six times with R0 and subsequently blocked with pre-warmed R10 for 1-2 h at 37°C, 5% CO₂. PBMC or T cell lines were added to the plates in R10H at 1 x 10⁵ and 4 x 10⁴ cells/well respectively. Phytohaemagglutinin (PHA) (08E2701, BioStat, UK) was used as a positive control at 2 µg/ml per well and R0 alone was used as a negative control. All conditions were set up in triplicate wells and the mean number of spot forming units was used in analysis. The plate was incubated overnight at 37°C with 5% CO₂. After incubation, the plate was washed 6 times with 200µl of PBS-0.05% Tween (4370829, BDH chemicals, UK) per well. IFN- γ detection antibody was diluted in PBS to a concentration of 1 µg/ml and 100 µl of the diluted antibody was added to each well and

incubated for 2-4h at room temperature. The washing step was repeated and then 100 μ l/well of diluted streptavidin (1 μ g/ml) was added and the plates were incubated at room temperature for a further 1-2 h. The washing step was repeated for a final time and 100 μ l of developing buffer, as per manufacturer's instructions, (170-6432, Biorad, USA) was added per well, and the plate incubated for 10-20 min at room temperature until spots appeared. Plates were then washed with tap water and inverted on paper towels to dry. Once dry, plates were analysed using AID ELISpot Software (version 4.0).

Chapter Three: HLA-B27 expression in cell lines

3.1 Introduction

HLA-B27 forms both heterotrimers associated with peptide and β_2m (B27HT) and heavy chain homodimers (B27₂). A number of monoclonal antibodies are available for the recognition of B27HT, including the conformation-specific antibody ME-1 that recognises an epitope in the heavy chain of β_2m -associated HLA-B7, -B27, -Bw22 and -Bw42 molecules (Figure 3.1). In contrast, there have been no reagents specific for the recognition of B27₂. Instead, detection has relied heavily upon the heavy chain-specific antibody HC-10. This antibody recognises an epitope in the heavy chain of all HLA-B and -C alleles and some -A alleles; the PXXWDR motif (Ellis et al., 1982; Stam et al., 1986). HC-10 recognition of B27₂ has been demonstrated by several groups using western blots to probe lysates of various HLA-B27-expressing cell lines. In one study, further analysis of the ~80 kD dimer band by 2D isoelectric focusing verified it comprises two HLA-B27 heavy chains that reduce to a single monomer upon addition of reducing agent. This indicates a probable covalent association of the heavy chains through the unpaired cysteines at position 67, although alternative cysteines may also stabilise homodimeric species (Capps and Zuniga, 1993; Powis et al., 2009). Yet, the major caveat in using HC-10 for B27₂ detection is its lack of specificity for HLA-B27; not only does it recognise other class I heavy chains but it also recognises several heavy chain species, including monomers and multimers. This heavily restricts its use in flow cytometry experiments to monitor B27₂ surface expression, as background HC-10 staining, present on most cells, makes it impossible to definitively attribute any staining to B27₂ molecules. In particular, although previous studies have demonstrated increased HC-10 epitope expression on peripheral

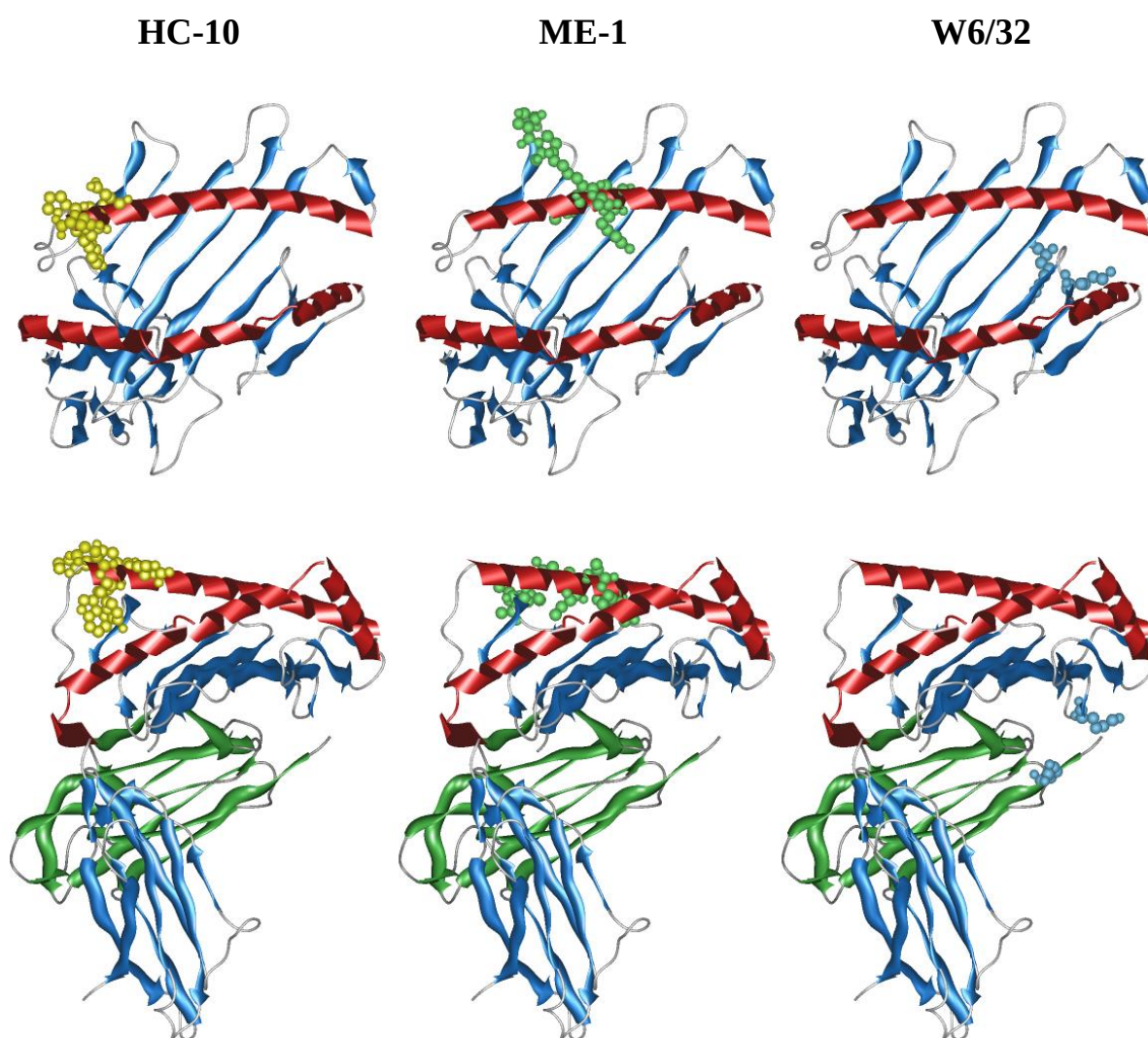


Figure 3.1 Antibody Epitopes (based on 2BSR structure (Stewart-Jones et al., 2005)). Ribbon diagrams of HLA-B27 depicting the amino acid residues comprising the monoclonal antibody epitopes for HC10 (yellow), ME1 (green) and W6-32 (blue) as space-filled spheres. Top panel shows the top view of the peptide binding groove, with a side view of the molecule below. HC-10 recognises residues 57-61 in the heavy chain, the PXXWDR motif. ME-1 is predicted to recognise residues 67-71 of the α 1 helix and residues 41-43 of the connecting loop region. W6-32 epitope includes residues K121 in α 2 helix of HC and R3 in β _{2m}.

blood monocytes of AS patients compared with controls, the exact nature of these molecules recognised remains unknown (Raine et al., 2006; Tsai et al., 2002).

Thus, determination of the extent, distribution and triggers of B27₂ expression in AS patients, and the functional consequences of its receptor interactions in SpA pathogenesis have been hampered by the lack of a specific detection reagent. Therefore, to address this issue, in collaboration with Renner *et al*, our lab sought to generate monoclonal antibodies that specifically recognise B27₂ but not B27HT or other HLA heterotrimeric complexes. Using phage display technology, biotinylated recombinant B27₂ complexes for positive selection and B27 heterotrimeric complexes for negative selection, candidate Fab antibodies were identified and converted into chimeric antibodies comprising the selected human Fab₂ and murine IgG1 Fc (see **Appendix III** for further details). One of these candidate B27₂-specific antibodies, HD6, was selected for further characterisation and its specificity for B27₂ but not B27HT refolded complexes were demonstrated by ELISA (Figure 3.2).

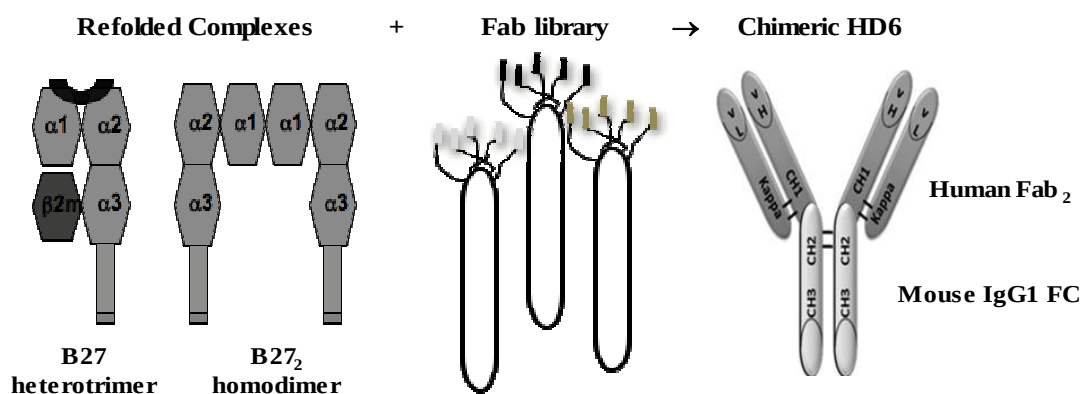


Figure 3.2 Cartoon schematic of the generation of B27₂-specific monoclonal antibodies by phage display. B27HT and B27₂ refolded complexes were used to select phage particles displaying Fab molecules specific for B27₂ complexes (positive selection step) but not B27HT complexes (negative selection step). Selected phages were then used to generate chimeric monoclonal antibodies comprising a human Fab₂ and a mouse IgG1 Fc portion. See Appendix III for more details on the phage display method.

Thus, the aim of this chapter is to further characterise the binding specificity of the candidate B27₂-specific antibody, HD6, by determining its reactivity with HLA-B27-positive cell lines by flow cytometry. I first investigate HD6 surface expression in transfected B cell lines that have previously been shown to contain HC-10 reactive dimers. Following this, HD6 reactivity in a range of other HLA-B27 expressing cell lines is determined, and its dependency on B27 surface expression levels is examined. Whether HD6 expression is dependent on HLA-B27 subtype is also addressed. Unless otherwise stated, HLA-B27 indicates the disease-associated subtype HLA-B*27:05.

3.2 HD6 epitope expression in human B cell lines

3.2.1 HD6 stains transfected B cell lines expressing HLA-B27

721.220 (.220) and 721.221 (.221) are mutagenised derivatives of the human B lymphoblastoid cell line (LCL) 721, which lack normal endogenous class I expression, and therefore offer the opportunity to characterise the behaviour of introduced class I molecules. Both cell lines are a result of γ -ray mutagenesis of a parent mutant line 721.184, which carries a single copy of the MHC region with deletions of the HLA-A and -B loci, followed by negative selection with W6/32 for class I null mutants. .221 cells are devoid of all class I molecule expression as a result of mutation of the remaining HLA-Cw1 locus, diminishing HLA-Cw1 transcription to levels undetectable by Northern blot analysis. Conversely, .220 cells retain the single copy of HLA-Cw1, but surface expression is reduced to low levels as a result of an inability to express functional tapasin. Transfection of class I genes into .221 cells, restores surface expression to levels comparable to other B-LCLs, indicating intact and functional antigen presentation machinery (Shimizu and DeMars, 1989). In contrast, surface expression of class I molecules in .220 cells is only

seen for some alleles, including HLA-B27, indicating a relative independence of these alleles from tapasin (Peh et al., 1998).

Using surface iodination and HC-10 immunoprecipitation, Lucy Bird previously showed that heavy chain homodimers and multimers are expressed at the surface of .220 stably transfected with HLA-B27 (Bird et al., 2003). Moreover, restoring tapasin function in these cells substantially reduced the HC-10 reactive homodimers. Therefore, to assess whether these complexes are recognised by HD6, .220 cells transfected with HLA-B27 or control HLA alleles were stained with HD6 and control antibodies. HD6 stained .220-B27 at consistently high levels (black histograms, Figure 3.3A). No significant binding to untransfected 220 cells or those transfected with the control alleles –B7, -A1, -B8, -B35, and –B44 was observed. Furthermore, additional transfection of human tapasin (TPN), which reduces HC-10 epitope expression, also reduced HD6 epitope expression (Figure 3.3B, left). In line with previously findings, these cells exhibited an increase in correctly folded ME-1-reactive B27 molecules and a reduction in unfolded HC-10-reactive heavy chains compared with .220-B27, supporting a role for partial dependence on tapasin for expression of stable surface molecules. All control cell lines positively stained with W6/32 and HC-10, indicating the presence of both HLA heterotrimers and free heavy chains respectively, although these levels were variable, reflecting the different levels of transfection of the HLA alleles. Interestingly, HD6 reactivity in 220-B27 cells expressing the mutant C67S appears abrogated (Figure 3.3B, right). However, the ME-1 expression in these cells is markedly reduced compared with HLA-B27, and therefore the importance of the C67 residue for HD6 epitope expression is difficult to determine (the role of C67 is further addressed later).

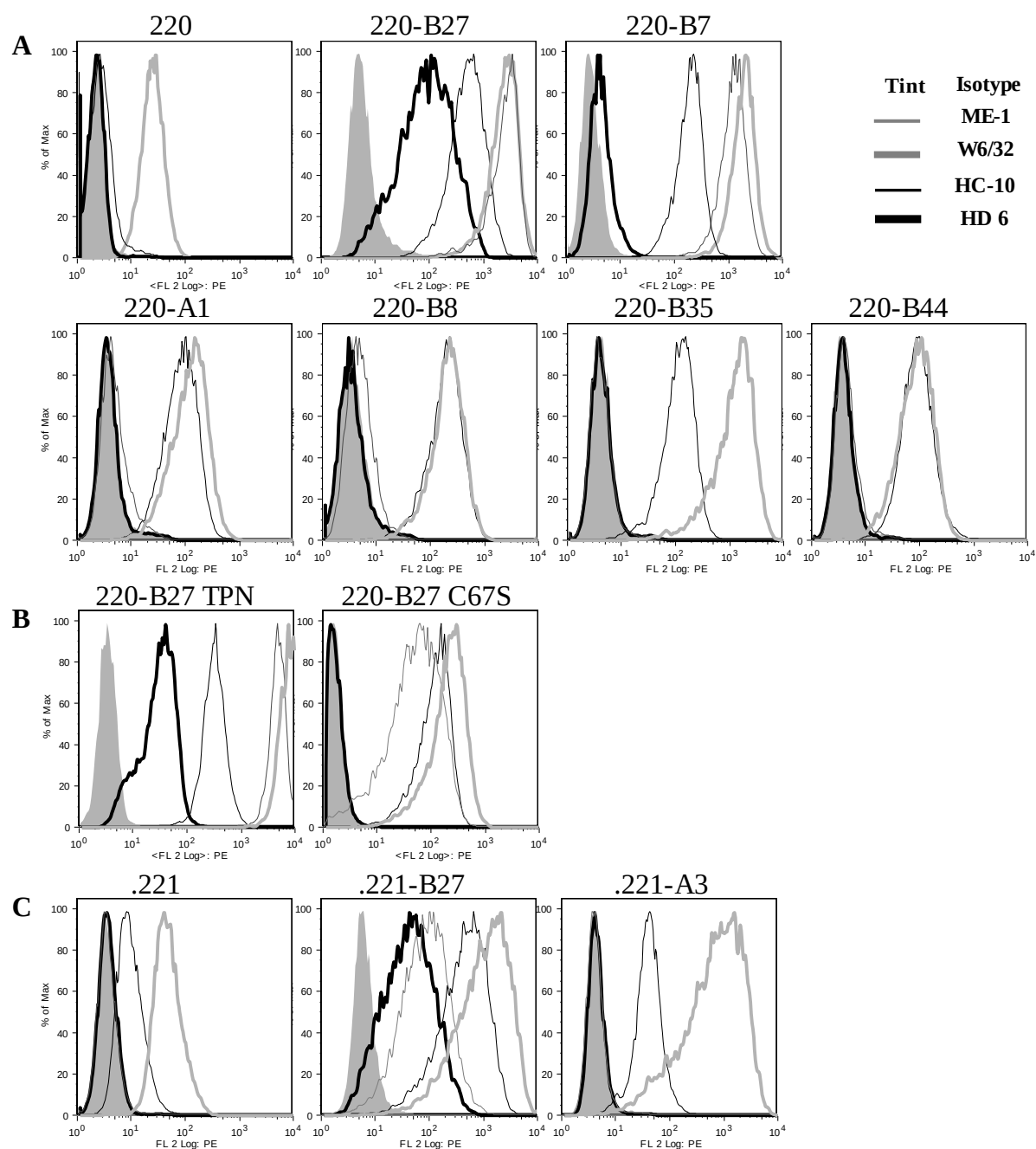


Figure 3.3 721.220 and 721.221 transfected B cell lines expressing HLA-B27 stain with HD6. (A) .220 cells transfected with -B27, -B7, -A1, -B8, -B35, or -B44 HLA molecules, (B) 220-B27 cells expressing additional human tapasin (TPN) or expressing the C67S mutant B27, and (C) .221 cells transfected with -B27 or -A3 were surface-stained using HD6 (thick black histogram) and controls as indicated. Results are representative of at least n=3 experiments.

Similarly, HD6 stained the .221 cell line when transfected with HLA-B27 but not with a control HLA-A3 allele (Figure 3.3C).

The HMy2.C1R (C1R) B-LCL has also been used by several groups to address B27₂ expression (Bird et al., 2003; Dangoria et al., 2002). This line is HLA-A negative but expresses low levels of HLA-B35, allowing HD6 expression to be assessed in the context of an intact class I assembly pathway and in the presence of endogenous HLA molecules, albeit not the full complement. Staining of these cells confirmed HD6 reactivity in the context of HLA-B27 expression but not HLA-A2 expression (Figure 3.4A).

Increased HD6 reactivity in the absence of functional tapasin suggests that epitope formation is favoured under conditions of less optimal peptide loading. Yet, Dangoria *et al.* have previously reported that HC-10 reactive dimers are significantly reduced in the TAP-deficient T2 cell line, indicating in this case at least a partial requirement of peptide loading for B27₂ formation. However, whether fewer B27₂ molecules were expressed at the surface of T2-B27 cells was not determined. Therefore, to further investigate the role of the peptide loading complex in B27₂ surface expression, the TAP-deficient T2 cells transfected with HLA-B27 (T2B27) were examined for HD6 epitope expression. This mutant human cell line is derived from a hybrid of the B-LCL .174 and the T-LCL CEM cell lines, and expresses HLA-A2 and HLA-B51 mRNA but fails to express normal surface levels of these molecules due to lack of a functional TAP heterodimer. Unlike the previously described transfected B-LCL lines, HD6 staining was not observed for T2B27 cells (2nd row (37°C), Figure 3.4B). To determine whether this was a consequence of reduced HLA-B27 surface expression, further staining was carried out at 26°C, which has

been shown to promote surface expression of class I molecules in the absence of TAP (Ljunggren et al., 1990), and to induce HC-10-reactive dimer formation (Antoniou et al., 2004). Lowering the temperature consistently increased ME-1, W6/32 and HC-10 staining in T2B27 transfected cells (1st row (26°C), Figure 3.4B). Interestingly, however, variable results were obtained for HD6 staining. As shown in Figure 3.4B, little change was observed for the first T2B27 cells tested, which was reproduced in two further experiments. However, for a second batch of T2B27 cells, lowering the temperature resulted in detectable HD6 staining (figure 3.4C), which was also reproducible in further experiments with these cells. One notable difference between these two batches of T2B27 cells was the higher ME-1 staining in the 2nd batch, both before and after incubation at 26°C (MFI values of 170 vs 200 at 37°C, and 650 vs 1190 at 26°C).

3.2.2 The HD6 epitope is not expressed on EBV-transformed B cell lines under standard conditions

Having demonstrated HD6 expression in the aforementioned HLA-B27 transfected B cell lines, I next investigated HD6 reactivity in EBV-transformed B cell lines that have endogenous HLA-B27 expression alongside the normal milieu of HLA molecules (BCL1-BCL4). In addition, this analysis also included the Jesthom BCL, known to be homozygous for HLA-B27, an AS patient derived B27+ cell line, and a B27- BCL (BCL5). The table in Figure 3.5B indicates the HLA alleles in these lines where known. Yet, despite high HLA-B27 expression, as indicated by ME-1 staining (thin grey histogram), and the presence of unfolded HC-10 reactive surface molecules (thin black histogram), these cell lines did not show significant HD6 staining compared with the isotype control (black histogram and grey filled histogram respectively) (Figure 3.5A).

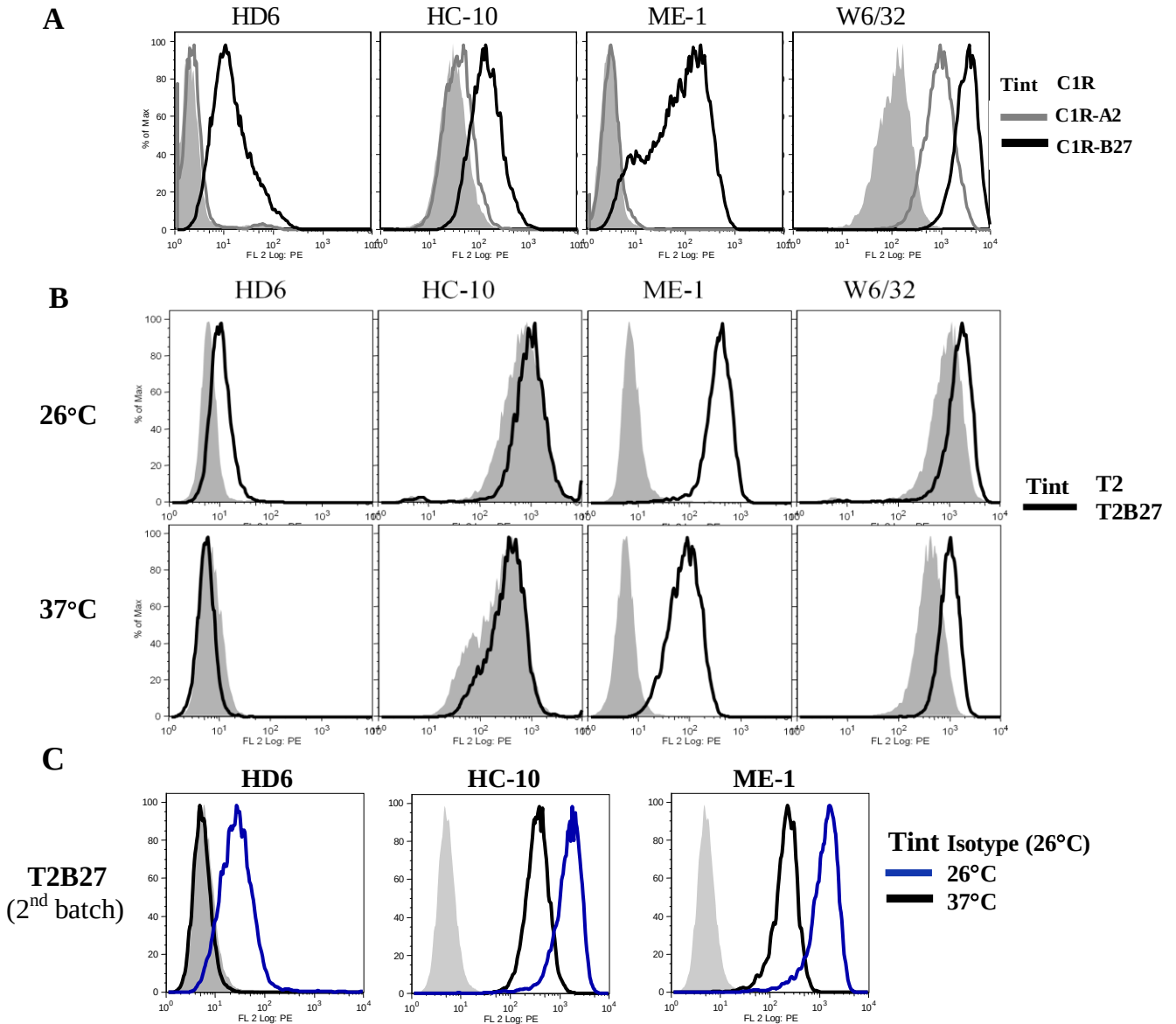


Figure 3.4 HD6 stains transfected C1R cells expressing HLA-B27 but not other HLA allele. (A) Untransfected C1R cells (tinted histograms) and C1R cells transfected with –A2 (grey histograms) or –B27 (black histograms) HLA molecules were stained with HD6, HC-10 ME-1 and W6/32 monoclonal antibodies shown. (B) Untransfected T2 cells (tinted histogram), or those transfected with —B27 (black histograms) were incubated overnight at 26C or 37C as shown and then stained with antibodies as in (A). Results are representative of n=3 experiments for the T2 cells and n=5 for the C1R cells. (C) Staining of a second T2B27 cell line with higher HLA-B27 surface expression after overnight incubation at 37°C (black histograms) or 26°C (blue histograms). Results are representative of n=3 for this cell line.

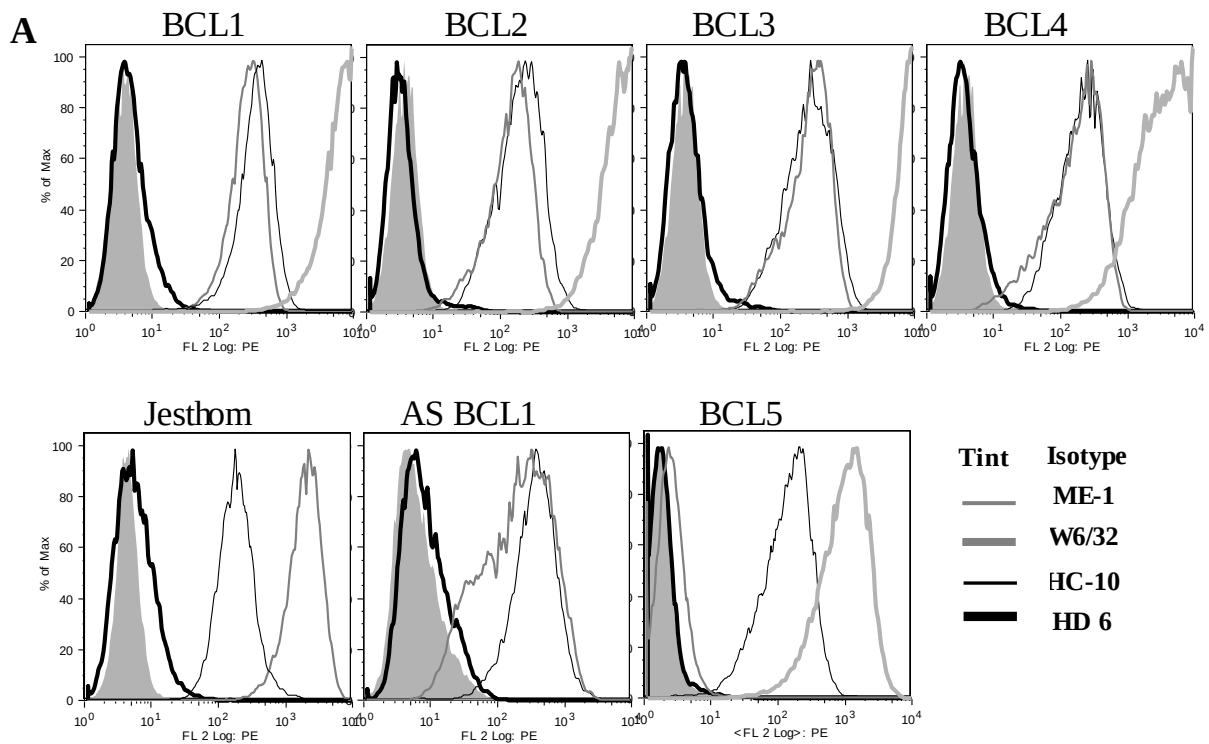


Figure 3.5 HD6 does not stain EBV-transformed B cell lines that express HLA-B27. (A) EBV-transformed B cell lines that were previously generated from four HLA-B27+ non-AS donors (BCL1-BCL4), one B27+ AS patient (AS BCL1), and one B27- non-AS donor (BCL5), and the B27+ Jesthom BCL were obtained and stained with the monoclonal antibodies HD6 (thick black histogram), HC-10 (thin black histogram), ME-1 (thin grey histogram), W6/32 (thick grey histogram) and IgG1 isotype control (tinted histogram). Where possible, HLA typing information was obtained and shown in (B).

3.2.3 HD6 staining of B cell lines transduced with HLA-expressing lentiviruses

To further investigate whether HLA-B27 expression levels affect HD6 epitope expression, our group have generated lentivirus (LV) constructs that allow for different levels of HLA-B27 expression after transduction with different amounts of lentivirus. The first construct, generated by Simon Kollnberger, includes an HA-tag at the C-terminus of the B27 molecule and allows for the co-expression of GFP (HA GFP). Three further constructs were generated by Dr. Kollnberger and Jacqueline Shaw as follows: (i) B27 without the HA-tag but with GFP expression (GFP); (ii) B27 with the HA tag only (HA); and (iii) B27 without the HA tag and with no GFP expression (no tag or NT). A schematic diagram of the lentivirus construct is depicted below (Figure 3.6). Furthermore, they generated these constructs for both the disease-associated subtype HLA-B*27:05 (05) and for the less disease-associated subtype HLA-B*27:09 (09), which allows for a comparison between the two.

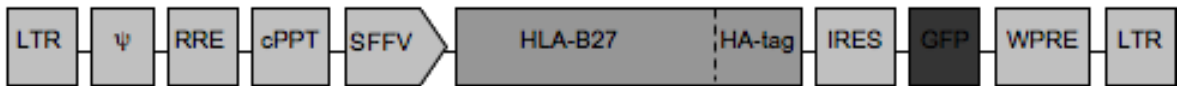


Figure 3.6 Schematic representation of the HLA-B27 lentivirus constructs used. HLA-B*27:05 or HLA-B*27:09 cDNA with (HA) or without (no tag or NT) a C-terminal HA tag was incorporated before the IRES region between known restriction sites. In the GFP and HA GFP constructs, DNA encoding for the GFP protein was included downstream of the IRES site.

I used these constructs to generate lentivirus in 293T cells, the supernatant of which was subsequently used to transduce a number of cell lines, including the .220 and .221 cell lines. Using the same volume of high titre virus, similar levels of surface expression of HLA-B*27:05 were obtained in .220 cells for all four lentiviruses (Figure 3.7A). This

confirmed that the addition of the HA tag at the C terminus of HLA-B27 does not inhibit the expression of correctly folded surface molecules. In addition, GFP expression levels in both the .220 and .221 cell lines confirms the high efficiency of lentiviral transduction using the GFP (left) or HA GFP (right) lentiviruses, as 82-97% of cells acquired GFP expression (Figure 3.7B).

The untagged GFP-expressing cells (GFP) were next used to determine whether HD6 expression was dependent on HLA-B27 levels. By plotting GFP vs. HD6 expression (black events) overlaid with the isotype control (grey events), the correlation of HD6 expression with GFP levels can be seen for both 05 (left) and 09 (right) and in both .220 (top) and .221 (bottom) transduced cell lines: HD6 expression is only detected in cells with relatively high GFP expression (Figure 3.7C). In addition, this is the first data to demonstrate that HD6 reactivity is not exclusive to the HLA-B*27:05 subtype, but also detects HLA-B*27:09 molecules.

These lentivirus-transduced cell lines were then further investigated. Firstly, to confirm the apparent dependence of HD6 expression on high HLA-B27 expression, I transduced .220 and .221 cell lines with either an intermediate (LV1) or high (LV2) titre HLA-B*27:05 NT lentivirus supernatant (Figure 3.8). HD6 expression (black histogram) was seen in the cells transduced with LV2 (right), but not with LV1 (left) (Figure 3.8A). A comparison of the MFI values of ME-1 and W6/32 expression in these cell lines confirmed the higher surface expression of HLA-B27 molecules resulting from transduction with LV2 (white bars) compared with LV1 (black bars).

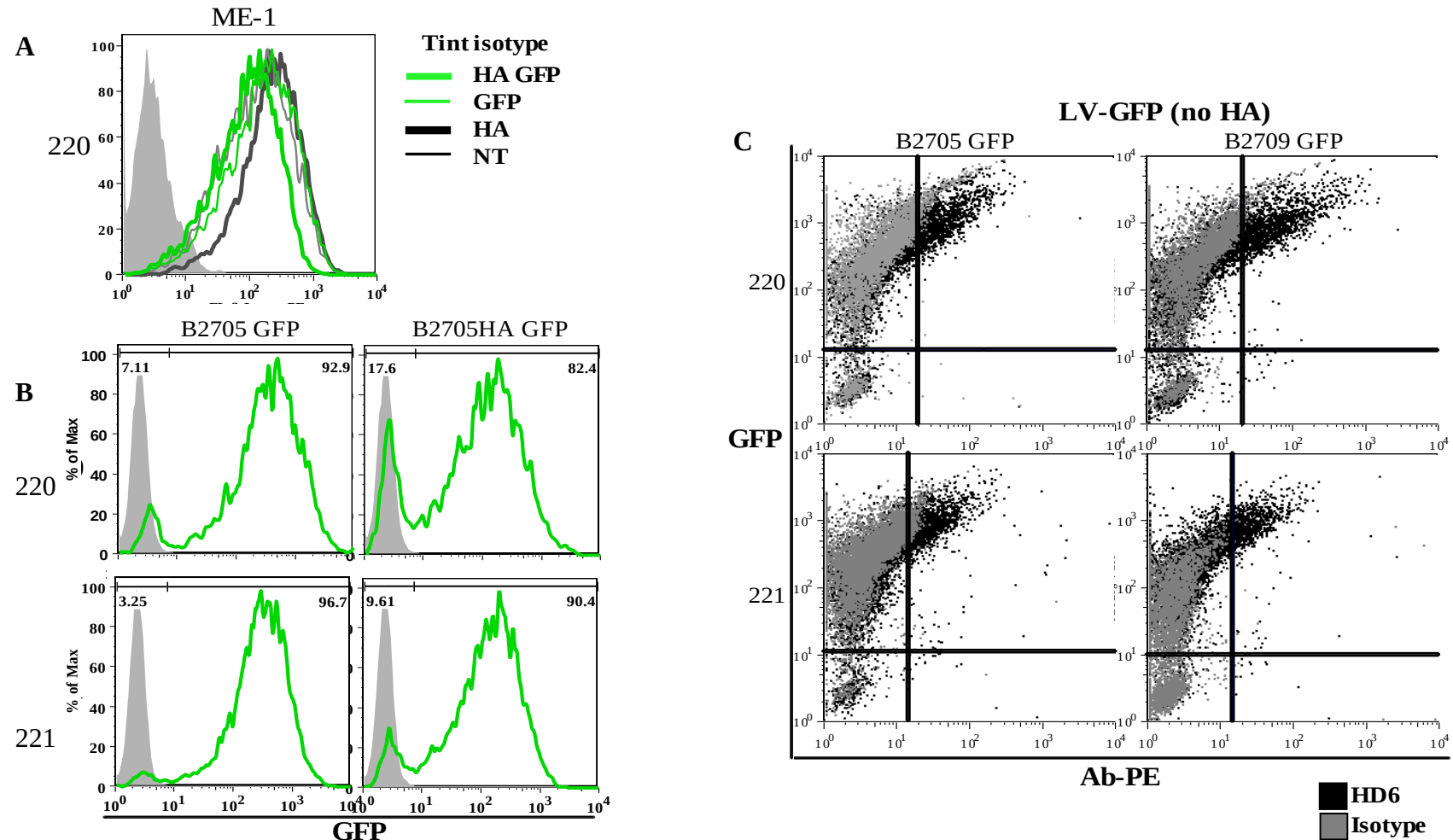


Figure 3.7 Lentivirus transduction of .220 and .221 cell lines and HD6 epitope expression. (A) ME-1 surface expression of .220 cells transduced with lentiviruses to express non-tagged HLA-B27 (NT, thin grey histogram) , HA-tagged HLA-B27 (HA, thick grey histogram), non-tagged HLA-B27 and GFP (GFP, thin green histogram), or HA-tagged HLA-B27 and GFP (HA GFP, thick green histogram). (B) Comparison of the GFP expression in .220 and .221 cells transduced with the GFP or HA GFP lentiviruses. IgG1 isotype control is shown as tinted histogram. Percentage of GFP+ cells shown in top right corner. (C) 220 and 221 cells transduced with B*27:05 (left) or B*27:09 (right) GFP lentiviruses were stained with HD6 (black events) or isotype (grey events) and GFP expression plotted against antibody staining. Results are representative of at least n=2 experiments.

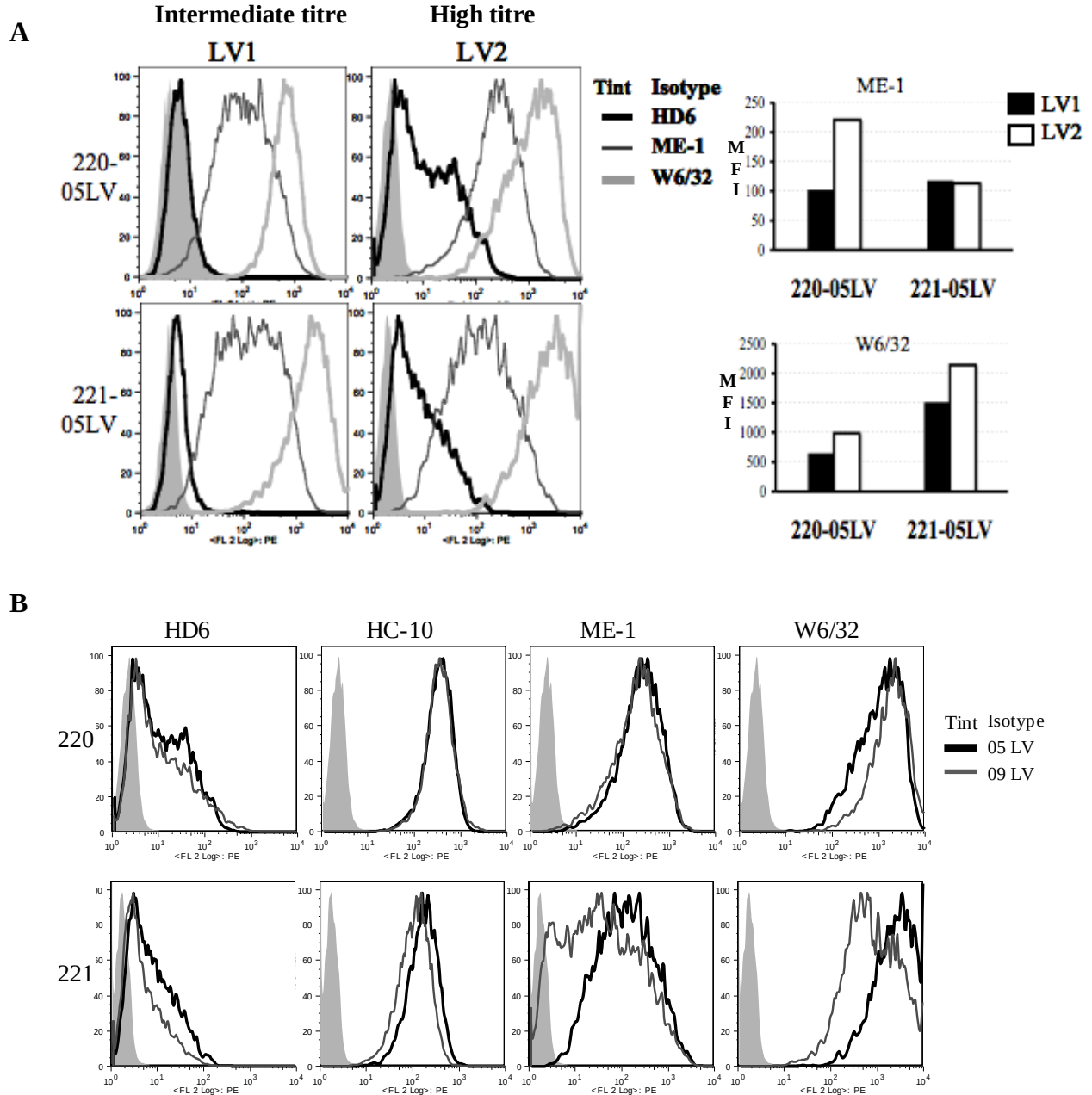


Figure 3.8 HD6 staining of LV-transduced cells expressing HLA-B*27:05 or HLA-B*27:09 (A) .220 and .221 cells were transduced with intermediate (LV1) or high (LV2) titre HLA-B*27:05 untagged lentivirus and analysed for surface expression with HD6 (black histogram), ME-1 (thin grey histogram) and W6/32 (thick grey histogram). MFI values for ME-1 and W6/32 are compared in the bar graphs on the right, with black bars representing LV1 and white bars representing LV2. (B) .220 and .221 cells were transduced with untagged (NT) HLA-B*27:05 (05 LV, black histograms) or HLA-B*27:09 (09 LV, grey histograms) lentiviruses and analysed for expression with the monoclonal antibodies HD6, HC-10, ME-1 and W6/32. Isotype control is shown as tinted histograms. Data is representative of n=2 independent experiments.

To confirm that HD6 reactivity is not restricted to the disease-associated HLA-B*27:05 subtype, expression was analysed in both the .220 and .221 cells transduced with the untagged 05 or 09 NT lentiviruses (05 LV and 09 LV) (Figure 3.8B). As with HC-10 and ME-1, a similar level of HD6 expression was observed for the both subtypes.

Interestingly, when comparing all four lentivirus constructs (GFP, HA GFP, HA, and NT) for both the 05 (Figure 3.9A, top row) and the 09 (bottom row) subtypes, HD6 reactivity was seen for the GFP (thick green histogram) and untagged (thick grey histogram) lentiviruses as previously shown but not for the HA tagged constructs (thin green and thin grey histograms for GFP HA and HA, respectively). This difference was also seen in the .221 cells, whereby a population of untagged B27 molecules (blue) but not HA tagged B27 molecules (red) stained with HD6 (Figure 3.9B, top row). Importantly, this lack of HD6 expression appeared not to be as a result of lower ME-1 expression (Figure 3.9B, bottom row).

Thus together this data indicates that HD6 expression under standard conditions is dependent on high HLA-B27 expression levels, independent of HLA-B27 subtype and inhibited by the addition of a C-terminal HA tag.

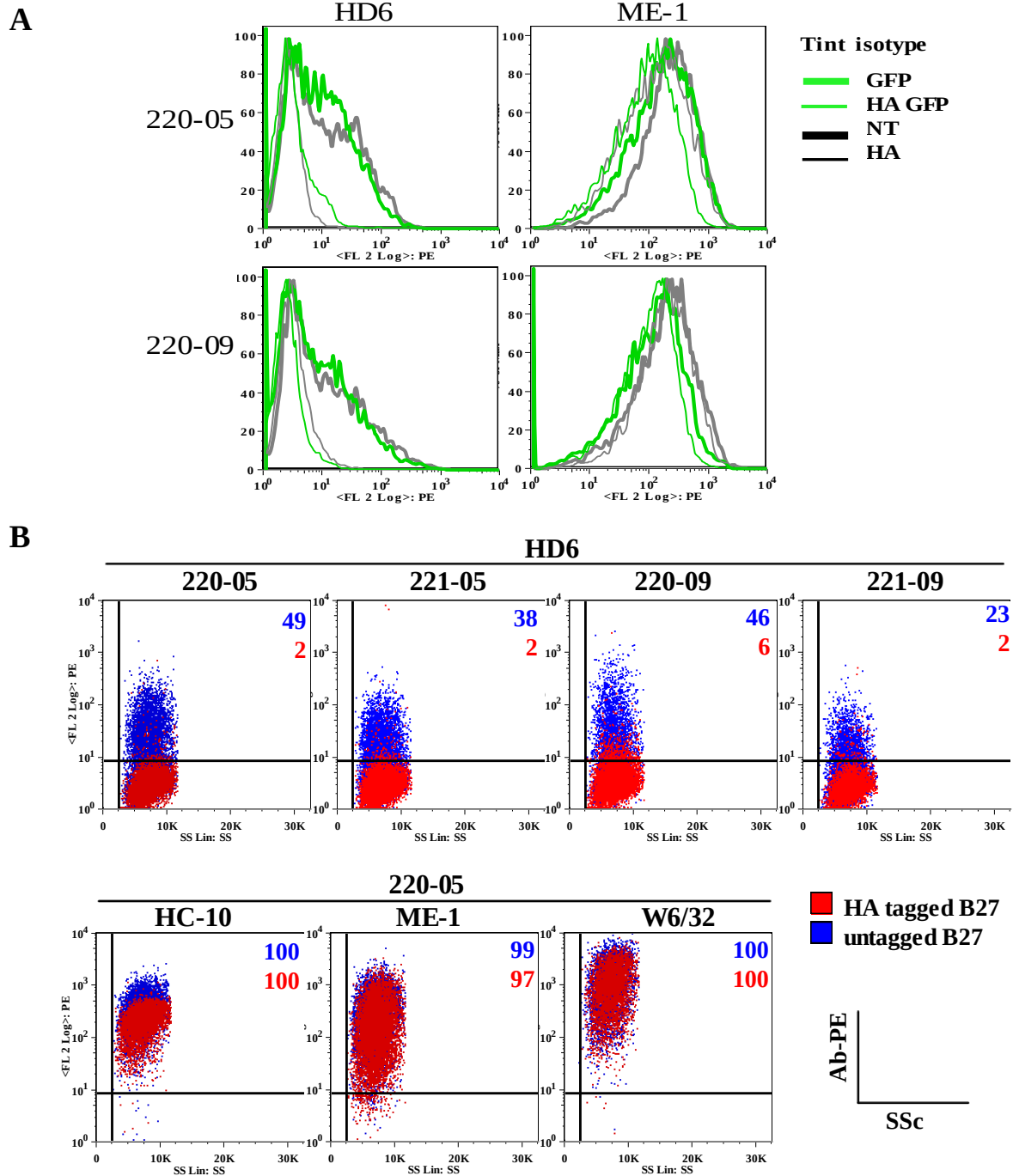


Figure 3.9 The presence of a C-terminal HA tag abrogates HD6 epitope expression.

(A) HD6 staining in .220 cells transduced with HLA-B*27:05 (top row) or HLA-B*27:09 (bottom row) LV that are untagged (NT, thick grey histogram), HA-tagged (thin grey histogram), untagged and express GFP (GFP, thick green histogram) or tagged and express GFP (HA GFP, thin green histogram). (B) Antibody expression as indicated vs SSc for .220 or .221 cells transduced with HA tagged (red) or untagged (blue) HLA-B*27:05 (top row, 220-05 and 221-05) or HLA-B*27:09 (220-09 and 221-09) LV. Numbers indicate the MFI of the Ab-PE staining. Data is representative of n=2 independent experiments.

3.3 HD6 staining of non-B cell lines transduced or transfected to express HLA-B27

3.3.1 HD6 does not stain HLA-B27-transfected U937, CEM and KG-1 cells or lentivirally-transduced C58 cells

HD6 epitope expression was next analysed in cell lines of both human and rat origin. The rat basophil line RBL transduced with the HLA-B*27:05 NT lentivirus (05 LV), the U937 human monocyte/macrophage-like cell line transfected with HLA-B27 or a control allele HLA-A2, the human T-LCL CEM and the CEM-derived calnexin-deficient NK-resistant NKR cell line both transfected with HLA-B27 were all stained with monoclonal antibodies and analysed by flow cytometry. No significant HD6 staining was observed for any of these cell lines (Figure 3.10). For the U937 cell line, ME-1 expression was increased after PMA treatment, however this did not result in any HD6 expression (data not shown).

The KG-1 cell line is a dendritic cell-like human cell line that, when transfected with HLA-B27, has previously been shown to express HC-10 reactive dimers both intracellularly and at the surface upon DC maturation with PMA and ionomycin stimulation (Santos et al., 2008). Therefore, HD6 expression was analysed in these cells both before and after similar PMA/ionomycin-induced stimulation (Figure 3.11). As reported by Santos and colleagues, this stimulation resulted in upregulation of cell surface CD11c (top row) and increased ME-1 expression (bottom row), representing HLA-B27 and endogenous HLA-B35 expression, which is weakly recognised by ME-1. However, no noticeable HD6 expression was observed under either condition (second row).

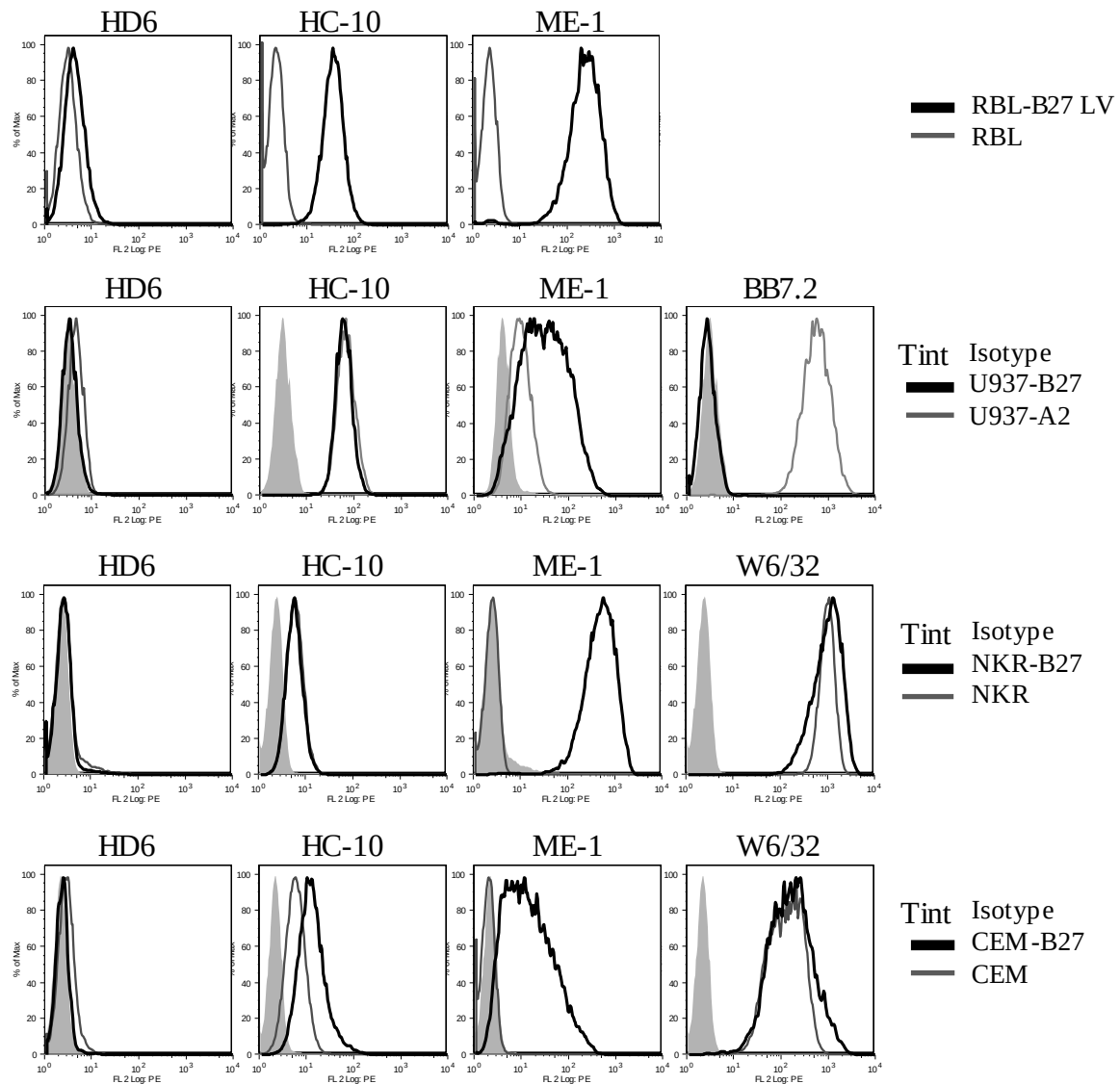


Figure 3.10 The HD6 epitope is not detectable in several non-B cell lines. RBL, U937, NKR and CEM cell lines with (black histogram) and without (grey histogram) HLA-B27 expression were stained with the monoclonal antibodies HD6, HC-10, ME-1, W6/32 or the HLA-A2 specific antibody BB7.2 as shown. Tinted histograms represent staining of the B27-expressing cells with IgG1 isotype control. Data shown are representative of at least n=3 independent experiments.

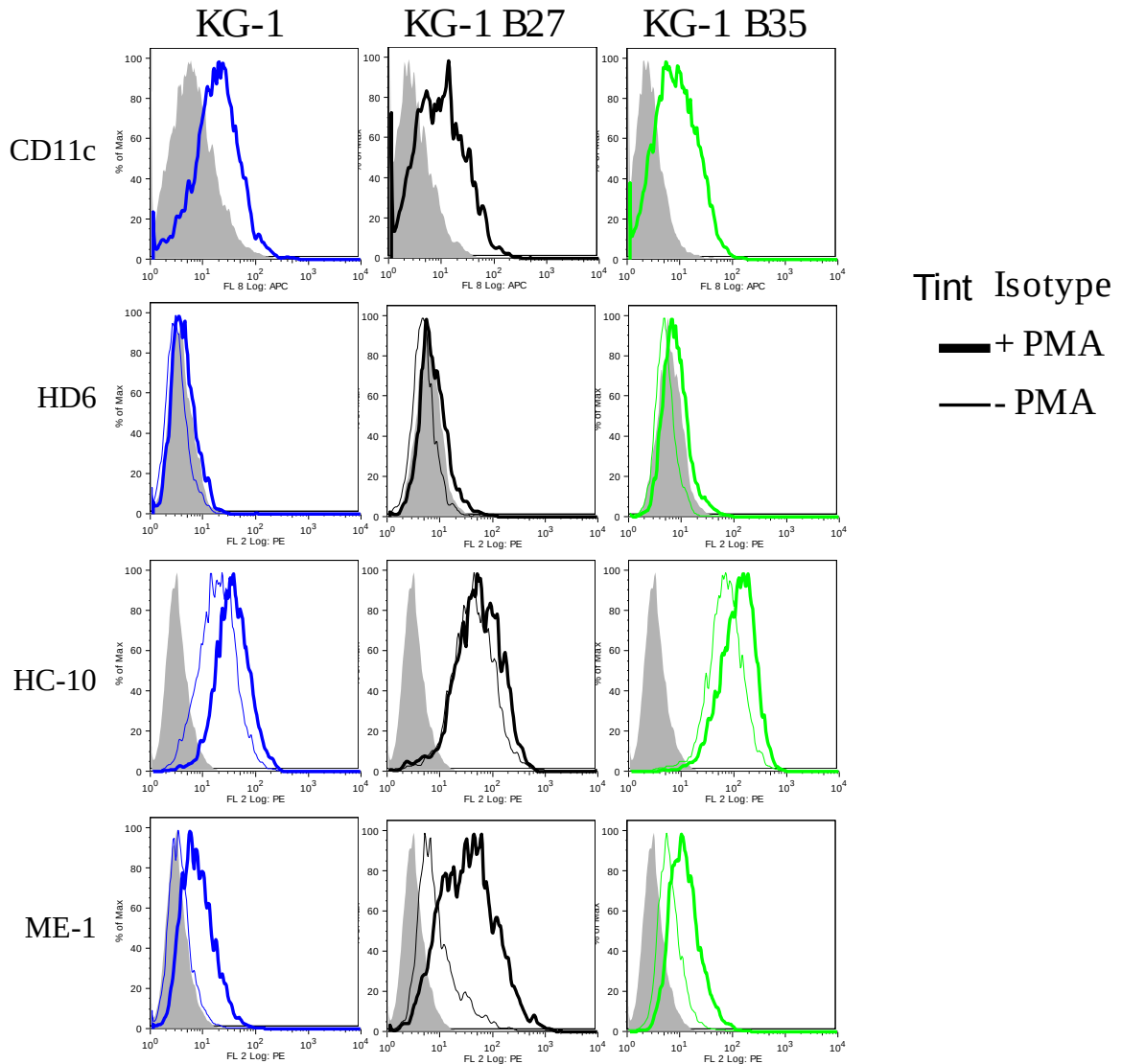


Figure 3.11 The HD6 epitope is not expressed by KG-1 cell lines transfected with HLA-B27 before or even after PMA-induced maturation. Untransfected KG-1 cells (left column) and those transfected to express HLA-B27 (middle column) or HLA-B35 (right column) were either untreated (thin histograms) or treated with PMA for 72h to induce cell maturation. Cells were stained with CD11c-APC or the monoclonal antibodies HD6, HC-10 and ME-1 as shown. Data is representative of n=2 independent experiments.

The C58 rat thymoma cell line has also been used in several studies to investigate the folding behaviour of HLA-B27 (Antoniou et al., 2004; Fussell et al., 2008; Santos et al., 2006). In particular, Antoniou *et al.* have shown HC-10 reactive dimers detectable both within the ER and at the cell surface in C58-B27 cells. In addition, Antoniou *et al.* have generated a number of mutant B27 molecules expressed in the C58 cell line. These have enabled the investigation of i) B27 subtypes and ii) the contribution of cysteine residues in HLA-B27 to dimer formation (unpublished data). I therefore, tested these transfected C58 cells for HD6 reactivity. No HD6 expression was observed in C58 cells or those transfected with –A2, or –B27 (left column, black histograms, Figure 3.12). C58 cells transfected with –B7, –B35 and an Sv5-tagged B27 were also HD6 negative (data not shown). Furthermore, in an attempt to increase the HLA-B27 expression levels to determine whether this may induce HD6 expression, I transduced these cell lines with the untagged HLA-B27 (NT) lentivirus (blue histograms). No changes in HD6 reactivity were observed, despite an increase in HC-10 expression (Figure 3.12, 2nd panel from left). Similarly, the C58 cells expressing HLA-B27 mutants received from Dr. Antoniou (UCL, London) were also HD6 negative; this included cells expressing i) HLA-B27 molecules with the single amino acid substitution D116H (representing HLA-B*27:09), D116Y, or the double substitution H114D D116Y (representing the peptide pocket of HLA-B*27:06) and ii) HLA-B27 molecules with either the single cysteine mutations C67S, C308S, or C325S C67S or with all three of these cysteine mutations (triple mutant, TM) (data not shown). These lines are investigated further in Chapter Four.

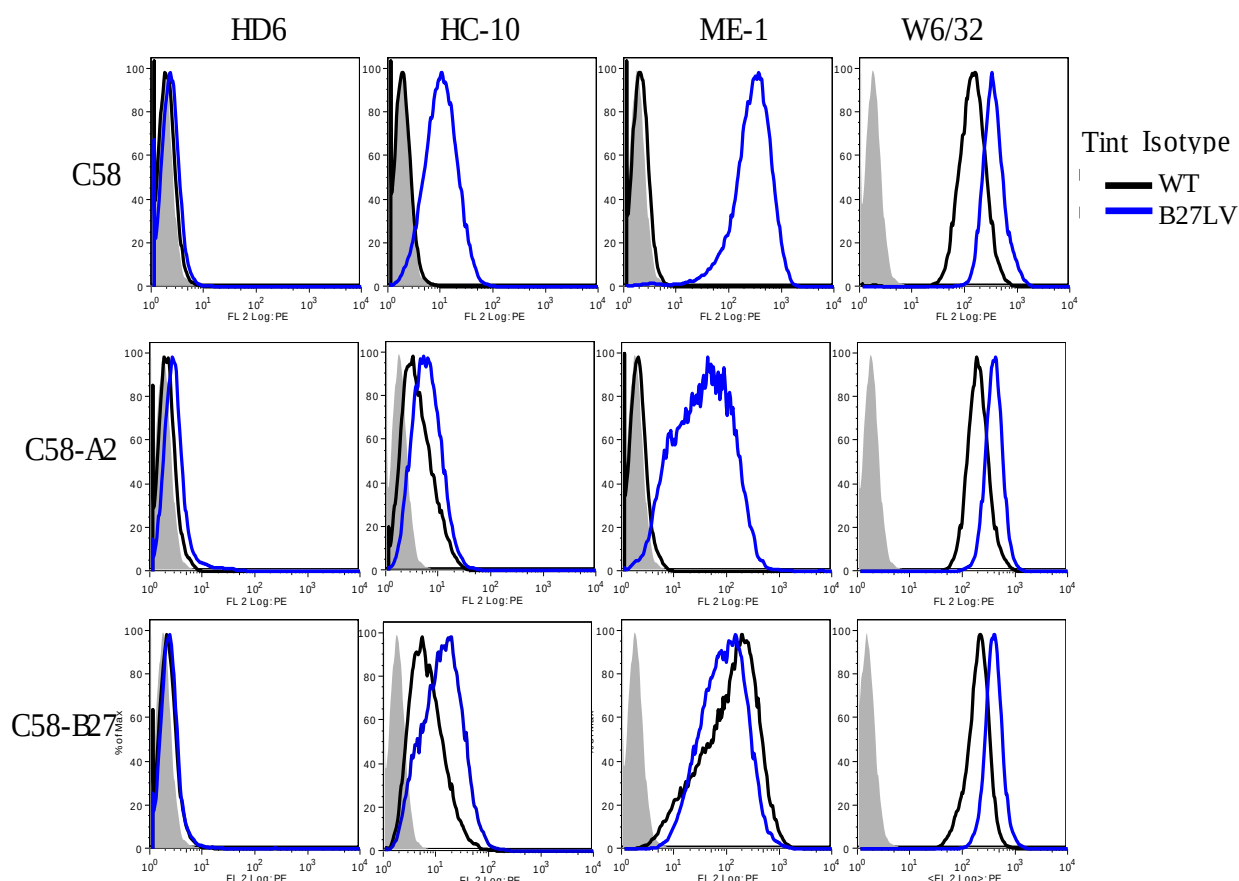


Figure 3.12 HD6 does not stain the C58 rat thymoma cell line transfected with HLA-B27. Untransfected C58 cells (top row), or C58 cells transfected with HLA-A2 (middle row) or HLA-B27 (bottom row) cDNA were left untreated (WT, black histogram) or transduced with the untagged HLA-B*27:05 lentivirus (B27 LV, blue histograms) and then stained with the monoclonal antibodies HD6, HC-10, ME-1 and W6/32. IgG1 isotype staining is shown as grey tinted histogram. Data are representative of n=2 independent experiments. Cells supplied by Dr. Antoniou (UCL, London).

To investigate HD6 epitope expression in the human epithelial HeLa cell line, I transduced the cells with the HLA-B27 NT lentivirus and compared staining with untransduced cells (Figure 3.13). HD6 reactivity was observed for the HLA-B27-expressing cells (black histogram, left panel) but not untransduced cells (grey histogram). As expected, the transduced cells also expressed an increased number of unfolded (HC-10, second panel) and folded (W6/32, fourth panel) MHC class I molecules at the surface, the latter of which can be attributed to correctly folded HLA-B27 (ME-1, third panel).

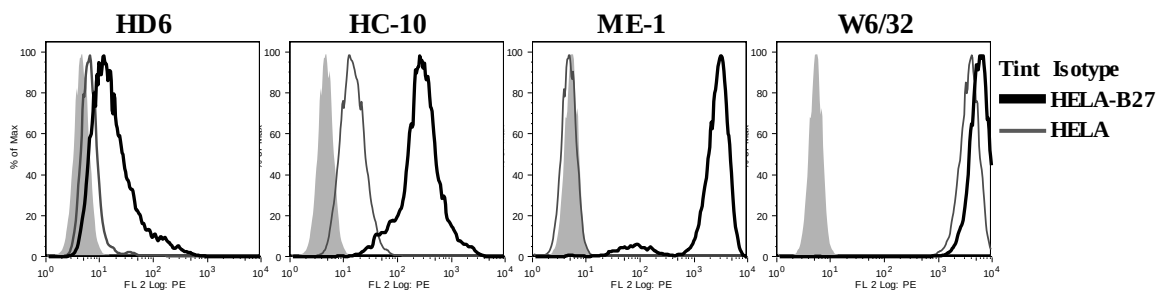


Figure 3.13 HD6 staining of LV-transduced HeLa cells. WT HeLa cells (HELA, grey histogram) or those transduced with the untagged HLA-B*27:05 lentivirus (HELA-B27, black histogram) were stained with the monoclonal antibodies HD6, HC-10, ME-1 and W6/32 as shown. IgG1 isotype staining is shown as tinted histograms. Data is representative of at least n=4 experiments

Given that the transduced HeLa cell line was the only non-B cell line found to consistently express the HD6 epitope, it was of interest to further investigate HD6 expression in HeLa cells that express HLA-B27 as a single-chain trimer (SCT) construct. Here, an SCT consists of an Sv5-tagged MHC class I heavy chain (either -B27 or -B35 HC), a (GGGGGS)₃ linker, human β_2m , a second GGGGGG(SGG)₃ linker, and a 9mer peptide, which for HLA-B27 is the influenza virus nucleoprotein peptide (NP) epitope SRYWAIRTR. This was carried out in collaboration with Keith Gould, Antony Antoniou,

and Izabela Lenart, who generated HLA-B27 WT and cysteine mutant SCTs, a control HLA-B35 SCT and expressed them in HeLa cells. A cartoon schematic of SCTs and heavy chain (HC) only constructs is shown in Figure 3.14A. Single chain molecules for several human and mouse MHC class I have been previously described (Hansen et al., 2009; Primeau et al., 2005; Yu et al., 2002). In particular, studies have demonstrated that class I molecules in the SCT form are highly expressed at the surface and as intact molecules, are recognised by conformation-dependent monoclonal antibodies, and are functionally recognised by their cognate CTLs (Truscott et al., 2008). In addition, when examining the level of steady-state folding, this study found that almost all B27 SCT molecules were present in a folded and mature state, with few peptide-empty forms present, indicating their rapid folding and egress from the ER. This was consistent with a study of the murine K^b SCT that found the SCT form to have a lower propensity to misfold than K^b HC (Yu et al., 2002). Furthermore, when examining a B27 SCT expressed in the C58 cell line, B27 SCT appeared less susceptible to reduction than non-SCT B27 molecules, further indicating the efficient folding of this molecule (Fussell et al., 2008). Finally, these studies have shown that surface expressed SCTs have a significantly prolonged half-life compared with native class I molecules bound by endogenous peptides, but are prone to peptide-exchange at the cell surface, a feature dependent on the affinity of the linked peptide for the class I molecule.

Therefore, on the basis of these previous studies with SCT molecules, I wished to test the hypothesis that expression of HLA-B27 molecules in an SCT form may affect the surface expression of the HD6 epitope.

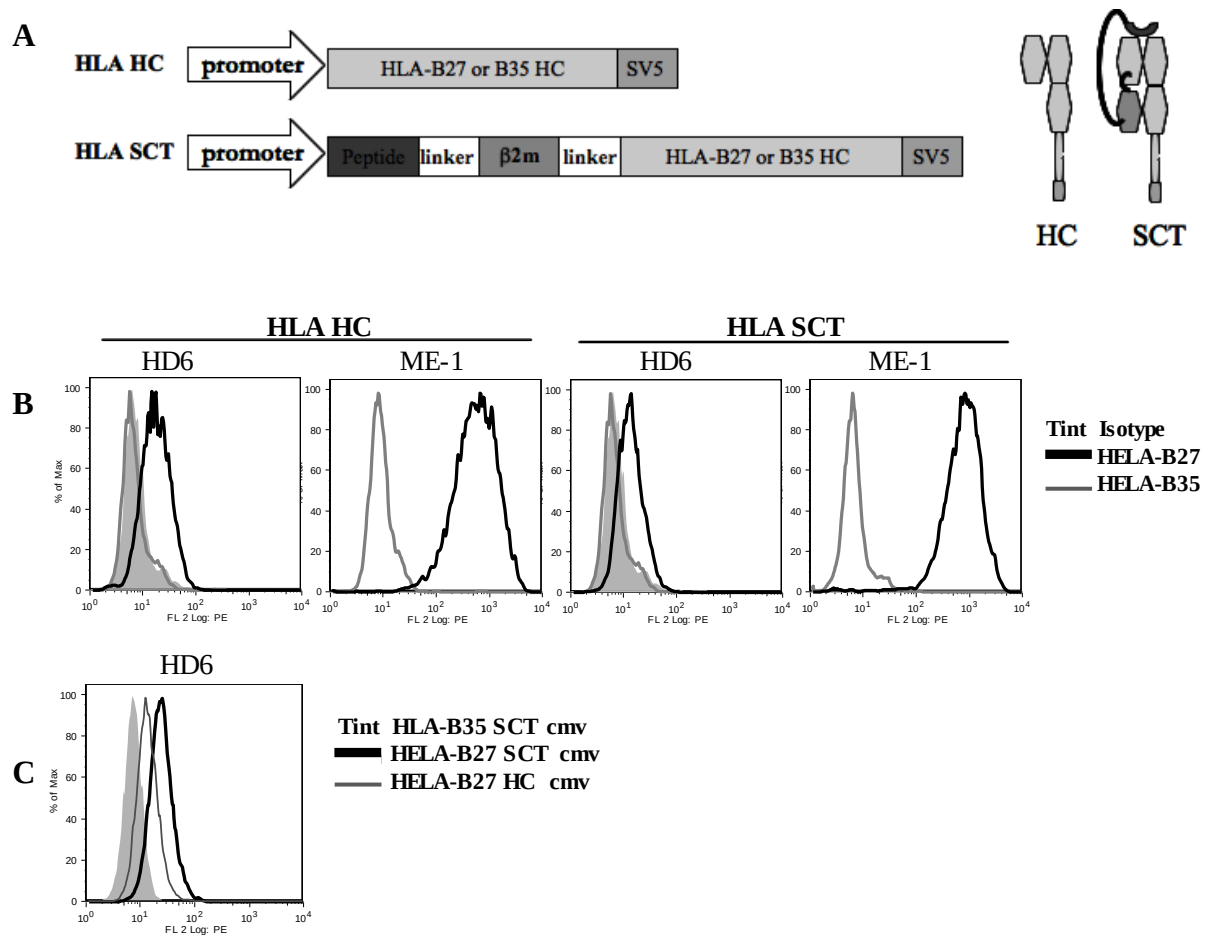


Figure 3.14 HD6 staining of HeLa cells expressing HLA-B27 SCT molecules. (A) Cartoon schematic of class I HC and SCT lentiviral constructs, depicting the order in which the domains are linked and how they assemble into class I molecules (right). (B) HeLa cell lines expressing HLA-B27 (black histograms) or HLA-B35 (grey histograms) under the eIF1 α promoter were stained with HD6 or ME-1 monoclonal antibodies as shown. First two panels are HLA HC-expressing cells, and second two panels are the HLA SCT-expressing cells. IgG1 isotype staining is shown as tinted histogram (C) HD6 expression in the HeLa cells expressing either HLA-B35 SCT (tinted histogram), HLA-B27 SCT (black histogram) or HLA-B27 HC (grey histogram) all under the CMV promoter. Data is representative of at least n=2 independent experiments.

Consistent with the previous experiments (Figure 3.14), HD6 recognised HeLa cells when transduced with a lentivirus construct expressing the HLA-B27 heavy chain (black histogram) but not the HLA-B35 HC (grey histogram) (Figure 3.14B). ME-1 staining confirmed the presence of correctly folded surface HLA-B27 molecules in these cells. In addition, HD6 also bound to cells expressing the B27 SCT (black histogram) but not the B35 SCT. Furthermore, HD6 also bound to cells expressing the B27 HC and SCT molecules when under the control of an alternative CMV promoter, which had been incorporated to replace the eIF1 α promoter (Figure 3.14C).

To confirm that HLA-B27 SCT molecules under the two alternative promoters were immunologically functional, their ability to upregulate CD107a expression on a Flu-specific B27⁺ restricted CTL line was tested (see Appendix I for generation of CTLs). For the first set of HeLa cells, under an eIF1 α promoter, incubation with a Flu-specific CTL line resulted in increased CD107a expression for the B27 SCT but not B35 SCT target cells, without exogenous Flu NP peptide addition (Figure 3.15A and B, top panels). As a control, the B27 HC but not B35 HC were shown to exhibit similar CD107a upregulation, but only after pre-pulsing with exogenous peptide (bottom panels). For the second set of HeLa cells, under a CMV promoter, target HeLa cells were incubated with a second Flu-specific CTL and similar results were observed, with CD107a upregulation triggered in the presence of B27 SCT without peptide, and B27 HC only when pre-pulsed with exogenous FLU peptide (Figure 3.16). In addition, no difference in CD107a upregulation was observed between the single cysteine mutant SCTs, C308S and C325S, and the B27 SCT WT, indicating that recognition of HLA-B27 in the SCT form was not altered when these

cysteine residues were mutated. These B27 mutant SCTs and their reactivity with HD6 are further addressed in Chapter Four.

3.4 Preliminary studies suggest HD6 may additionally recognise an intracellular epitope not confined to HLA-B27

HD6 surface expression was not observed in all HLA-B27 expressing cell lines, despite previous studies detecting HC-10 reactive dimers in several of these lines, including some at the surface. In order to determine whether HD6 negative cell lines expressed the HD6 epitope intracellularly, U937 cells were permeabilised and washed with BD Cytofix/CytopermTM reagents with 10% rabbit serum as a blocking reagent, and stained for HD6 expression (thick black histogram) (Figure 3.17). HC-10 (thin black histogram) and an isotype-matched IgG1 monoclonal antibody (tinted histogram) were used as a positive and negative control respectively. .220, .221, C1R and T2 cells with and without HLA-B27 expression were also analysed. Interestingly, HD6 not only recognised an epitope within the B27-expressing cells but also in the control cell lines. This was the case for all cell lines tested.

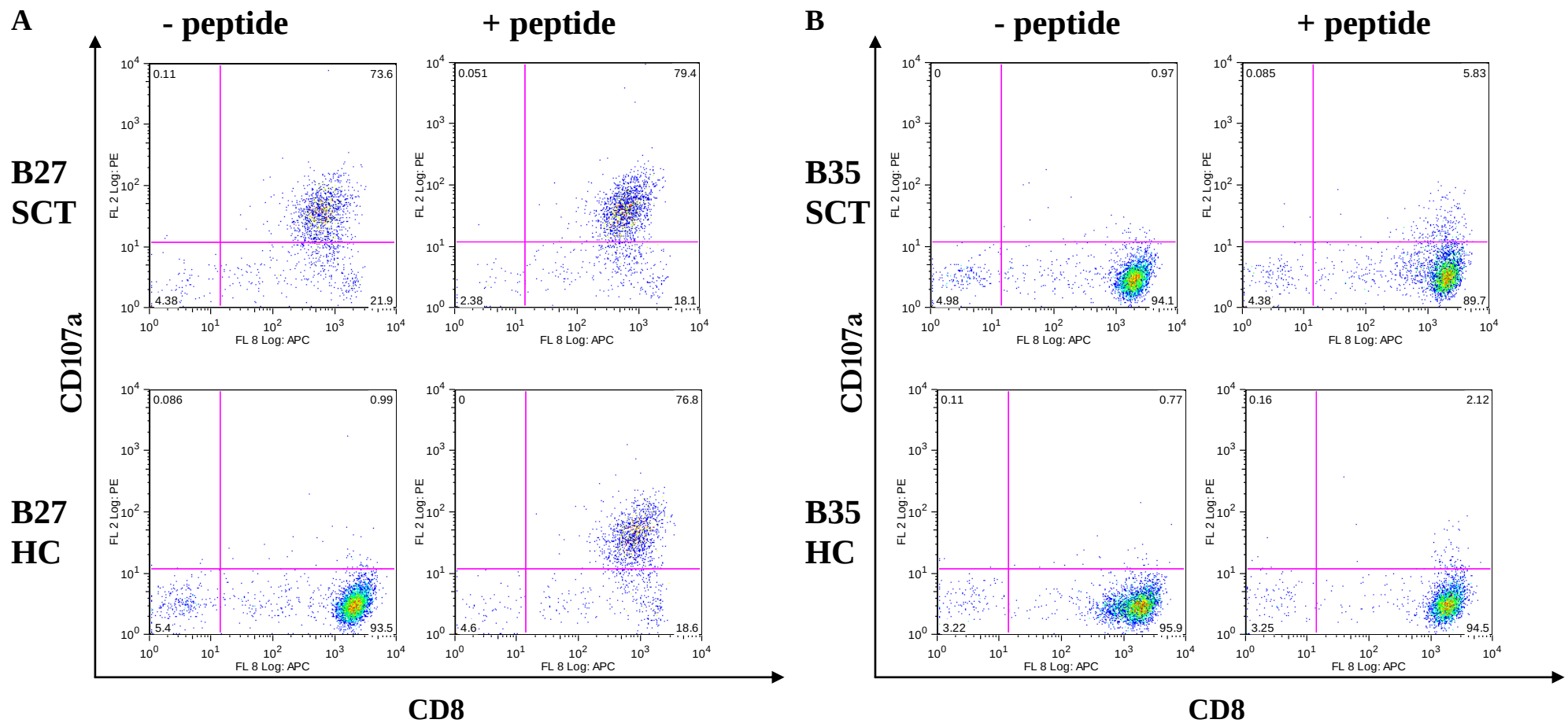


Figure 3.15 HeLa cells expressing B27 molecules as SCTs under an eIF1 α promoter can activate a specific CTL response. HeLa cells expressing (A) HLA-B27 single chain construct (B27 SCT) or heavy chain (B27 HC) or (B) HLA-B35 single chain construct (B35 SCT) or heavy chain (B35 HC) were un-pulsed or pulsed with FLU peptide and incubated with a FLU-specific HLA-B27 restricted CD8⁺ CTL line and an anti-CD107a-APC antibody for 4h before further staining with CD8-APC and analysing by flow cytometry. Data is representative of 3 independent experiments: 2 carried out with an E:T ratio of 0.5:1 and 1 at an E:T ratio of 1:1.

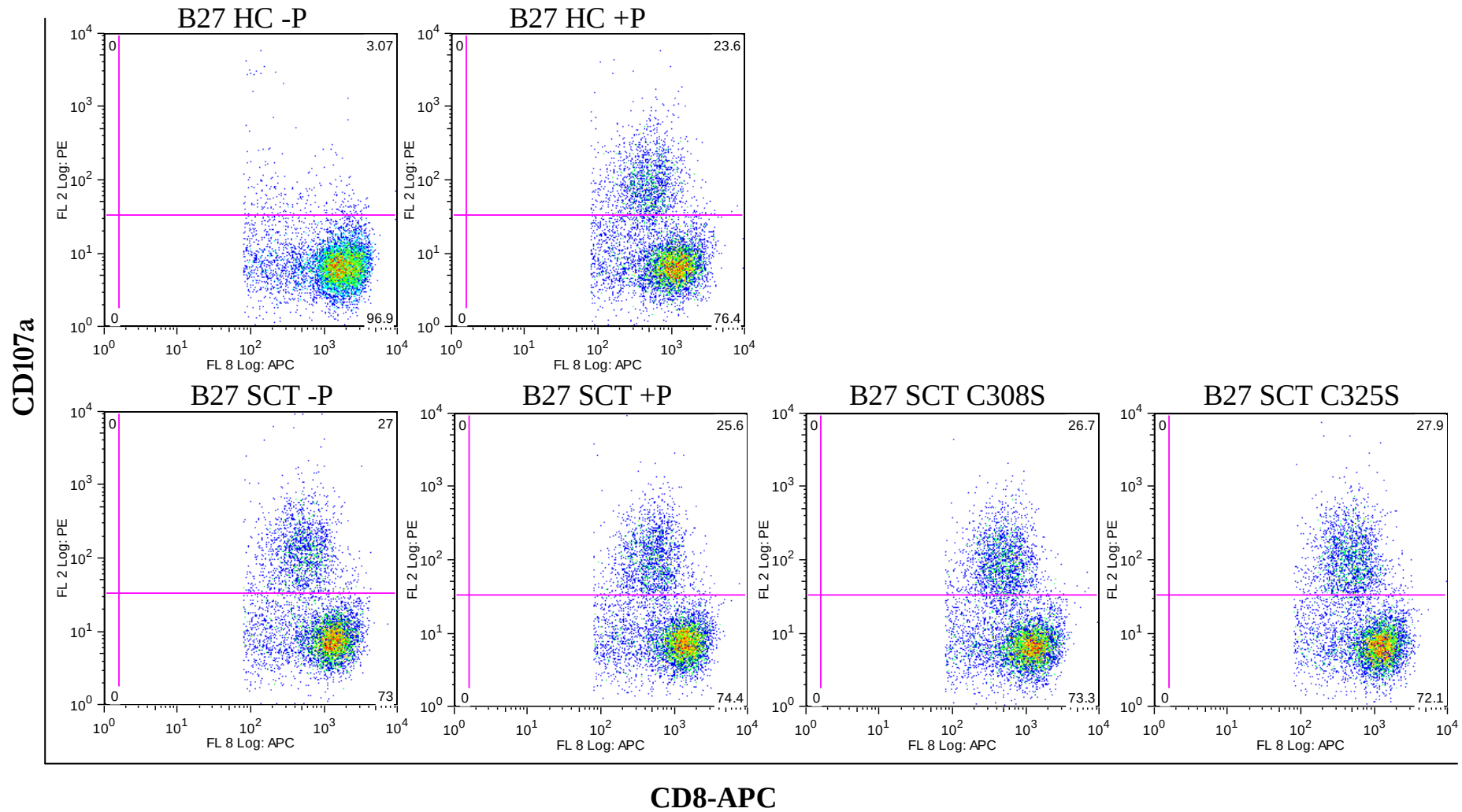


Figure 3.16 HeLa cells expressing B27 molecules as SCTs under a CMV promoter can activate a specific CTL response. HeLa cells expressing HLA-B27 as heavy chains (B27 HC) (top row) or as single chain constructs (B27 SCT) were un-pulsed or pulsed with FLU peptide and incubated with a FLU-specific HLA-B27 restricted CD8⁺ CTL line and an anti-CD107a-APC antibody for 4h before further staining with CD8-APC and analysing by flow cytometry. The single cysteine mutants B27 SCT C308S and B27 SCT C325S were un-pulsed and treated as the WT SCT.

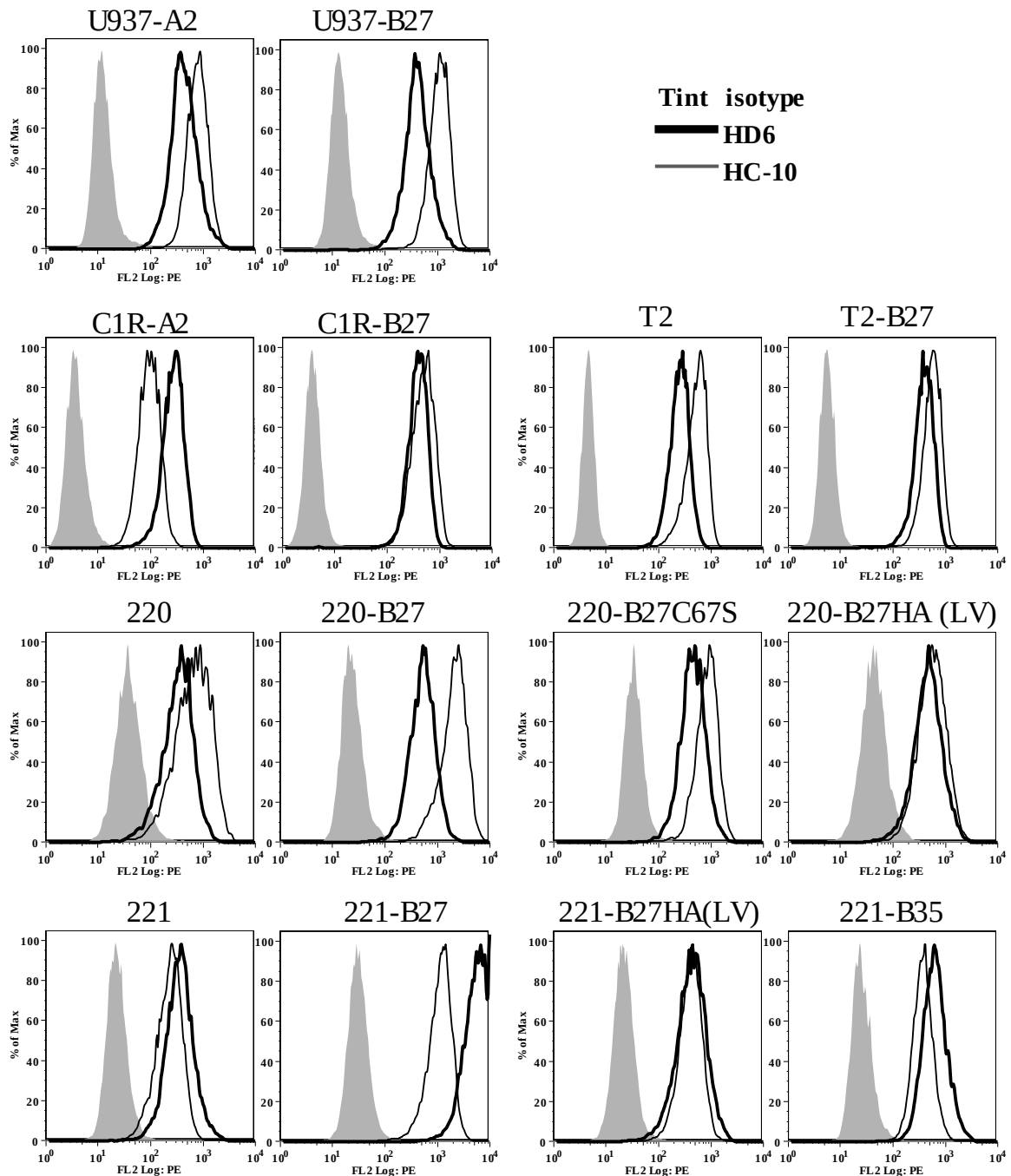


Figure 3.17 Intracellular staining of cell lines with HD6. Intracellular expression of the HD6 epitope for the cell lines U937, C1R, .220, .221, and T2 cells with and without expression of HLA-B27. Cells were permeabilised with BD Cytofix/Cytoperm™ reagents prior to staining with HD6 (thick black histogram), HC-10 (thin black histogram) or isotype control (tinted histogram). Data is representative of at least n=2 independent experiments.

3.5 Discussion

In collaboration with Christoph Renner and colleagues, our group have generated a novel monoclonal antibody, HD6, that binds in ELISA to *in vitro* refolded HLA-B27 homodimeric but not peptide- and β_2m -associated HLA-B27 heterotrimeric complexes (Bowness *et al.* unpublished data). Thus, the principal aim of this chapter was to characterise the binding specificity of this candidate B27₂ antibody by flow cytometry. This was addressed by examining the staining of HD6 to a number of cell lines expressing either HLA-B27 or control HLA alleles. Together, the data presented here demonstrates that i) HD6 binds to a novel B27-specific cell surface epitope, distinct from that bound by HC-10, ii) the HD6 epitope is present on some but not all HLA-B27-expressing cell lines, iii) HD6 binding to B27⁺ cells is strongly correlated with levels of HLA-B27 expression, and iv) HD6 binding to B27 is independent of subtype polymorphism at position 116. Furthermore, HD6 binding was abrogated by the presence of C-terminal HA tag on HLA-B27. HD6 may additionally bind a non-B27 epitope expressed intracellularly in several human cell lines.

i) HD6 binds a novel B27-specific cell surface epitope, distinct from that bound by HC10

I have shown HD6 binds to .220, and .221 B lymphoblastoid cell lines when transfected with HLA-B27 but not other HLA-B or HLA-A alleles, indicating its nature as a B27-specific reagent. Moreover, the observed HC-10 staining of these lines transfected with control alleles reveals the HD6 binding profile to be distinct from that of HC-10. In addition, in tapasin-deficient .220 cells, co-expression of human tapasin (TPN) reduced HD6 staining. Differential staining by HC-10 and other monoclonal antibodies of HLA-B27-transfected .220 cells with (.220TPNB27) and without (.220B27) co-transfection of

TPN has been reported in several studies (Peh et al., 1998; Purcell et al., 2000). Firstly, Peh *et al.* demonstrated increased staining of .220B27 compared with .220TPNB27 by HC-10 and decreased staining by B27M1, a mAb relatively HLA-B27-specific and known to react with some but not all B27-peptide complexes (Wang et al., 1994). In contrast, staining with the conformation-specific antibodies ME-1, ABC-m3 and 116.5.28 (anti-Bw4) was comparable, indicating similar expression of folded HLA-B27 molecules in these cells. Extending these findings, Purcell *et al* also found quantitative differences in staining with Ye-2 and MARB4, B27-specific mAbs also shown to bind only a subset of peptide-associated B27 molecules. As with B27M1, both Ye-2 and MARB4 exhibited higher staining to .220TPNB27 cells. This increased presence of free heavy chains and differential staining by the peptide-dependent mAbs in the absence of tapasin was attributed to differences in the peptide repertoires in these cells and the presence of a cohort of less stable B27 complexes in the tapasin-defective cell line.

The fact that HD6 staining was increased in the absence of tapasin, in a similar manner to HC-10 staining, supports the hypothesis that HD6-reactive molecules derive from the expression of less stable B27 complexes at the cell surface. These molecules could represent the previously identified HC-10-reactive dimers, which have also been detected at increased levels in the absence of tapasin (Bird et al., 2003; Dangoria et al., 2002). Consistent with this idea, I have also shown HD6 binding to C1R cells expressing HLA-B27 (C1RB27), another cell line known to form HC-10-reactive dimers. Alternatively, however, it is also conceivable that HD6-reactive molecules represent a novel subset of HLA-B27 molecules that share a conformation epitope with *in vitro* refolded B27₂ complexes, but are not necessarily B27₂ molecules themselves. To distinguish between

these possibilities, further studies using alternative methods such as combining HD6 surface staining or surface labelling with immunoprecipitation experiments are required that will help establish whether HD6-reactive surface molecules comprise monomeric or multimeric forms of B27 HC.

It is worth noting here that initial attempts to address this issue were carried out but met with little success for several reasons. Firstly, as will be discussed later, the HD6 epitope appears to be conformation-dependent, and failed to work as a detection reagent in immunoblots of cell lysates (data not shown). Secondly, HD6 is a chimeric antibody comprising a human Fab portion combined with a mouse Fc region. This proved problematic for detection of HD6-immunoprecipitated molecules in immunoblots by the HC-10 and W6/32 antibodies (both of mouse origin) as the secondary anti-mouse HRP antibody also picked up the HD6 antibody (data not shown). One method to overcome this issue in the future will be to directly conjugate HC-10 with HRP prior to use. Thirdly, another approach initially tested was using the cells expressing HA-tagged HLA-B27 molecules. Following immunoprecipitation with HD6, an anti-HA HRP-conjugated antibody was used for detection of bound B27 molecules. When looking at U937-B27HA cells, bands were observed that indicated the presence of both dimeric and monomeric species under non-reducing conditions, with the former disappearing upon addition of DTT. However, additional bands of unknown identity were also present, and it was not possible to reproduce this data in the .220 or .221 cell lines. Furthermore, a major caveat of this, other than limiting the investigation to cells expressing HA-tagged B27 molecules, is that the HA tag was found to affect the formation of HD6-reactive molecules. These issues are by no means insurmountable, and overcoming these obstacles

should provide valuable information about the nature of the surface HD6-reactive molecules.

At least two forms of HLA-B27 homodimers have been observed in human cell lines that are distinguishable by several factors, one of which being their differential recognition by the conformation-specific antibodies HC-10 and W6/32. As mentioned above, HC-10-reactive dimers have been shown to accumulate in .220B27 cells, and are detected in C1RB27 cells and also at low levels in T2B27 and in the 5.2.4 cell line, a TAP-deficient derivative of the T5-1 B lymphoblastoid cell line that expresses HLA-B27 as well as –A1, –A2 and –B8 alleles (Bird et al., 2003; Dangoria et al., 2002). Therefore, expression of HC-10-reactive dimers appears to be favoured under conditions of sub-optimal peptide loading. In contrast, Dangoria *et al* have reported W6/32-reactive dimers in .220TPNB27 but not .220B27 cells, and in T2 cells when expressing both functional TAP and HLA-B27 (T2TAPB27) but not HLA-B27 alone (T2B27), indicating a requirement for a fully functional peptide-loading complex. Interestingly, unlike the HC-10-reactive dimers, all W6/32-reactive dimers in that study were found to be endo-H resistant, suggesting they form outside of the ER. Using surface biotinylation methods, both forms of dimer were shown to appear at the surface of C1RB27 cells, although the extent to which this held true for the other cell lines was not investigated.

Despite the evidence indicating that HC-10-reactive and W6/32-reactive dimers represent two distinct pools, it is conceivable that both sets of dimers may be bound by HD6. Although my data cannot rule out the possibility that HD6 recognises surface W6/32-reactive dimers, the lack of detection of these forms in .220B27 cells, which stain strongly

with HD6, suggests that this is less likely. This conclusion is also supported by the *in vitro* binding of HD6 to refolded HLA-B27 homodimer complexes, which are known to be both β_2m - and peptide-free and are bound by HC-10 but not W6/32. Since W6/32 binds to folded class I complexes containing peptide and β_2m , it follows that binding to dimers would also require at least the presence of peptide and/or β_2m for recognition.

To further investigate the role of the peptide-loading machinery in the formation of HD6-reactive molecules, T2 cells transfected with HLA-B27 or HLA-A3 were investigated. Under standard conditions, I observed no HD6 staining above background for T2B27 cells, which would support the hypothesis that TAP-dependent peptide loading is a prerequisite for HD6 reactivity. This appears to contrast with the situation for HC-10-reactive dimers, which, as mentioned in the above, were detected in two TAP-deficient cell lines, albeit at low concentrations. Notably, however, the authors did not distinguish between cell surface and ER-localised dimers (Dangoria et al., 2002). Thus, it is possible that these cell surface and ER-localised dimers represent distinct HC-10-reactive dimer subsets are differentially influenced by TAP, the former failing to form in the absence of TAP. Furthermore, although HC-10-reactive dimers were detected in T2B27 cells, the authors note that 4 times as many cells were needed compared with the number of .220B27 cells, and suggested this indicated at least a partial-dependence on peptide-loading for the formation of HC-10-reactive dimers.

Previous studies have incubated cell lines deficient in antigen processing overnight at 26°C to stabilise ‘empty’ peptide-free MHC class I molecules, thereby enabling their egress to the cell surface in the absence of peptide (Ljunggren et al., 1990). This results in

an increase in the number of free heavy chains at the surface that form as a result of the dissociation of the less stable 'empty' surface MHCI complexes. Consistent with this, I have shown that overnight incubation of both T2 and T2B27 cells at 26°C resulted in a marked increase in HC-10 staining. In addition, and in agreement with the findings of Vazquez *et al.* (Vazquez and Lopez de Castro, 2005), I also observed increased ME-1 staining in the T2B27, which is likely due to the stabilisation of 'empty' HLA-B27 molecules by TAP-independent peptide ligands. Most notable, however, was the effect of temperature treatment on HD6 staining of two batches of T2B27 cells that differ in their levels of ME-1 staining, possibly as a consequence of reduced HLA-B27 expression over time in the first batch. Upon lower temperature treatment, HD6 staining remained negative in the cells with lower ME-1 staining upon temperature treatment. Yet, in the cells with higher ME-1 staining, treatment induced HD6 staining. Thus, culturing cells at a lower temperature appears to overcome the postulated TAP-dependence of HD6 epitope expression, but only when a sufficient number of HLA-B27 molecules are expressed.

One interpretation of this result is that the lack of HD6 staining in TAP-deficient cells is a result of insufficient levels of, but not an inability to form, the precursor molecules from which the HD6 epitope is formed. In the absence of TAP, the inability to load peptides onto newly formed B27:β₂m complexes via the peptide-loading machinery results in a pool of B27 molecules with reduced stability that are retained in the ER. This pool may contain the HD6-reactive precursor molecules, however they fail to egress to the cell surface and therefore remain undetected. Lowering the temperature stabilises these molecules enough to allow their transport to the surface, however an additional threshold level must be met before HD6 staining is observed. This threshold may represent a

minimum number of unstable B27 molecules required to leave the ER for HD6-reactive molecules/precursor molecules to be present, or the minimum number of B27 molecules required to reach the cell surface for HD6-reactive molecules to form.

Further insight into the role of TAP in HD6 epitope expression may be gained in future through the use of TAP inhibitors, such as ICP47, as well as the study of the HLA-B27+ T5-1 B cell line and its derivative cell lines 8.1.6 (lacking a single copy of TAP1 and TAP2 genes) and 5.2.4 (lacking both copies of TAP1 and TAP2 genes), and may provide further support for this proposal.

ii) the HD6 is present on some but not all HLA-B27 expressing cell lines

Given that the cell lines described above are either defective in antigen presentation and/or are mutants lacking the full complement of endogenous HLA alleles, it was important to establish whether HD6 could also bind to cell lines without these deficits. Somewhat surprisingly, no staining was detected in a range of HLA-B27 transfected cell lines, including U937, KG-1, RBL, C58, CEM, and CEM.NKR cells as well as in EBV-transformed HLA-B27 positive B cell lines, including the B27 homozygous Jethom BCL. This raised the question whether HD6 staining demands certain deficiencies in cell lines for its surface expression. Yet, this was addressed when HeLa cells lentivirally-transduced for HLA-B27 expression, were shown to stain with HD6. Notably, HeLa cells have previously been reported to form HLA-B27 multimeric species, at least intracellularly, when transiently transfected with HLA-B27 (Antoniou et al., 2004). Intriguingly, I also found that HeLa cells expressing single chain trimer constructs (SCTs) of HLA-B27, but not a B35 SCT, were also bound by HD6. Importantly, I also confirmed

that surface B27 in the form of an SCT were functional through B27-restricted Flu-specific CTL recognition.

While SCTs have been shown to have a prolonged half-life when compared with their naturally folded MHCI heavy chain counterpart, they also appear more susceptible to binding by exogenous peptide, particularly when linked to lower affinity binding peptides (Truscott et al., 2008). This has been attributed to a disruption in the H-bonding network of the peptide-binding groove at the peptide C-terminus caused by the protruding peptide-linker region. Significantly, the linker-attached peptide is not cleaved by proteases, and all three components remain linker attached when detected in western blots. On the basis of these findings, there are at least two possibilities that could explain the B27 SCT reactivity with HD6. Firstly, B27 SCTs may form disulfide-bonded heavy chain dimers, whereby the flexible linkers in the SCT enable the peptide and β_2m to remain attached but so that do not disrupt dimer formation. Alternatively, peptide dissociation from the peptide binding groove may in some cases lead to a partial unfolding of the B27 heavy chain that results in a conformation of the HC that is recognised by HD6. Further investigation is warranted in order to distinguish between these possibilities.

iii) HD6 binding to B27+ cells is strongly correlated with levels of HLA-B27 expression,

One explanation for the lack of HD6 staining seen in several HLA-B27-expressing cell lines is that a threshold number of HLA-B27 molecules are required for the expression of the HD6 epitope, which is not met in these cell lines. This is in agreement with my previous findings whereby HD6 staining of T2B27 cells at 26°C required high HLA-B27 surface expression. Consistent with this theory, I found HD6 staining in HLA-B27

lentivirus (LV)-transduced .220 and .221 cells to depend on transduction levels. Firstly, in cells co-expressing B27 and GFP, HD6 staining was only detected in those cells with high levels of GFP. Secondly, when these cells were transduced with an intermediate titre or a high-titre LV, the latter of which result in substantially higher surface ME-1 staining, HD6 staining was only observed for the cells transduced with the high-titre LV. Together, these data indicate that HD6 staining requires a high level of HLA-B27.

Given that the levels of B27 expression required for HD6 staining in these cell lines may be a consequence of over-expression of HLA-B27, this could bring into question the biological relevance of an epitope derived under such conditions. However, in the transgenic rodent models of SpA, disease symptoms only manifest in rats with a high copy number of the B27 transgene. Therefore, at least in the animal model, it seems over-expression of B27 is a prerequisite for disease pathogenesis, and, for this reason, study of the behaviour of B27 molecules under these conditions is highly justified.

iv) HD6 binding to B27 is independent of subtype polymorphisms at position 116

As outlined in Chapter One, HLA-B27 is a highly polymorphic MHC class I molecule with its subtypes differentially associated with AS. The less disease-associated subtype HLA-B*27:09 (09) differs from the disease-associated subtype HLA*B27:05 (05) by a single amino acid residue at position 116, implicating this residue as a key determinant in the pathogenicity of HLA-B27. Interestingly, Galocha *et al.* have shown that polymorphism at this position in B27 correlates with both the rate of assembly and the thermostability of the major B27 subtypes; those containing a D116 (02, 04 and 05) exhibited prolonged assembly kinetics and increased stability compared to those with

either Y116 (06 and 07), or H116 (09) (Galocha and de Castro, 2008; Galocha and Lopez de Castro, 2010). It follows that these differences in stability and assembly kinetics of B27 molecules could impact on the presence and/or formation of novel conformers of B27 at the surface. It was therefore of interest to establish whether polymorphism at position 116 affected HD6 binding.

To address this issue, I compared the staining of lentivirally transduced .220 and .221 cells expressing comparable levels of the 05 and 09 subtypes. This demonstrated that HD6 could bind to a subset of HLA-B*27:09 molecules at the cell surface. Moreover, HD6 staining of .220-09 and .221-09 cells was comparable to the staining of .220-05 and .221-05, respectively. As with 05, HD6 staining of 09 was found to correlate with its expression levels. These findings indicate that HD6 can bind to both 05 and 09 B27 molecules at the cell surface, and that the ability to form HD6-reactive surface molecules is not abrogated by the D116H amino acid substitution. The similar surface recognition by HD6 of the two subtypes is analogous to a previously reported finding with HC-10 and B27 subtypes (Vazquez and Lopez de Castro, 2005). Furthermore, consistent with the idea that 05 and 09 can both form the novel B27 conformer bound by HD6, our group have shown that both 05 and 09 are capable of forming *in vitro* refolded dimers, which are recognised by HD6 by ELISA and SPR (Jacqueline Shaw and Joanna Giles, unpublished data). However, in quantitative analyses, 05 was found to form more *in vitro* refolded dimers than 09, a difference not emulated in the HD6 surface staining I carried out. One possible explanation to account for this discrepancy is that the propensity of 05 to form more dimers than 09 *in vitro* is reflective of the situation in the ER but not at the

cell surface. Alternatively, HD6 staining may represent a subset of HLA-B27 surface dimers and/or alternative novel conformers of HLA-B27.

Nevertheless, these data imply that, if HD6-reactive molecules are involved in disease pathogenesis, the reason for the lack of association of the 09 subtype, if it is truly not disease-associated, is not simply due to an intrinsic inability to form the HD6-reactive molecules. Rather, it is possible that the differential disease associations of 05 and 09 are due to differences in the kinetics of formation of the epitope, which cannot be measured under steady state conditions by flow cytometry.

Lastly, it was intriguing to find that when HD6 was used in intracellular staining experiments with BD Cytofix/CytopermTM reagents, it stained all cells tested, irrespective of their HLA-B27 status. As a negative control both IgG1 and IgG2a isotype controls, as well as secondary-only control were used. However, while these controls all exhibited minimal background staining to all cells tested, a more suitable control would have been a chimeric control antibody, which was not available. Nevertheless, a tentative conclusion from these data is that it is possible that the HD6 epitope is expressed within all cell lines expressing class I molecules, but only reaches the surface in the HLA-B27-expressing cells. One explanation for this may be that HD6 recognises an epitope common to multiple HLA alleles but that is restricted to expression within cells, perhaps specifically limited to localisation within the ER or folding compartments. This could be an intermediate in the HLA class I folding pathway that shares a conformational epitope with HLA-B27 homodimers, perhaps a peptide-receptive conformer or a chaperone-associated intermediate. However, this is highly speculative and thus to clarify this issue, further

studies using different approaches such as confocal microscopy and ImageStream analysis are merited.

Chapter Four: Characterisation of the HLA-B27 epitope recognised by HD6

4.1 Introduction

HLA-B27 homodimers assemble both intracellularly during maturation and are expressed at the cell surface following endosomal recycling of B27 heterotrimers. HD6 binds to refolded B27₂ complexes but not refolded B27 HT complexes in ELISA experiments, yet the exact nature of the B27 conformer recognised by HD6 in a cellular setting remains unclear. Moreover, whether HD6 specifically recognises B27₂ or whether B27₂ is one of several alternative B27 conformers recognised needs to be clarified. If HD6 is dimer-specific, is recognition strictly dependent on the presence of the unpaired C67 residue, and is the recognised epitope generated as a result of the C67 disulfide bonding of two B27 heavy chains during endosomal recycling, as the homodimer theory would posit? To address these questions, this chapter endeavours to further characterise the nature of the epitope recognised by HD6, assessing whether particular factors contribute to its formation. In particular, I examine the importance of B27-binding peptides and several components of the peptide-loading complex, as well as the requirement of unpaired cysteines in HD6 epitope formation. In addition, I have included the results of the preliminary epitope mapping studies carried out with the aim of better defining the exact molecular nature of the HD6 epitope.

4.2 The effect of exogenous peptide addition on HLA-B27 expression

4.2.1 Addition of exogenous HLA-B27-binding peptides reduces cell HD6 staining of HLA-B27-expressing lymphoblastoid B cell lines

Previous studies have shown that the addition of exogenous peptide can stabilise MHC class I molecules at the cell surface (Baas et al., 1992; Townsend et al., 1989). In addition, Carreno *et al.* reported that B27 peptide ligands increase the half life of W6/32 reactive molecules (Carreno and Hansen, 1994). Moreover, our group have previously shown that incubation of 220-B27 cells with high affinity B27-binding peptide epitopes decreases the level of surface HC-10-reactive homodimers (Bird et al., 2003). I therefore sought to determine whether the addition of exogenous B27-binding peptides to stabilise cell surface heterodimeric complexes similarly affected the level of HD6-reactive molecules. To address this, .220, .221 and C1R B cell lines expressing HLA-B27 or control HLA alleles were incubated overnight (12-16h) in the presence or absence of B27-binding peptides (SRYWAIRTR and RRYQKSTEL) or control peptides (the B7-binding peptide RPMTYKAAV and the A2-binding peptide SLYNTVATL) and subsequently stained with antibodies. For all B27-expressing cells, addition of the B27-binding peptides (grey dashed and dotted histograms) but not control peptides (grey line histogram) reduced HD6 staining by at least 50% (Figure 4.1). For example, for C1R-B27 cells, B27-binding peptide addition reduced the MFI of HD6 binding from a value of 17 to 6. HC-10 staining was also reduced for all B27-expressing cells, with an observed 40% reduction for the C1R-B27 cells, but no notable change in ME-1 was detected (data not shown). These experiments were also carried out with two particular B27-binding peptides, GRWRGWYTY or GQWRGWYTY, reported by Malik *et al.* to generate a novel peptide-dependent B27 conformer recognised by the MARB4 antibody (Malik et

al., 2002). Incubation of cells with either of these peptides also reduced HD6 epitope expression (Figure 4.2).

4.2.2 Peptide addition abrogates HD6 reactivity for up to 24h

To further investigate the effect of exogenous peptide addition on the surface expression of HLA-B27, the B27-binding peptide SRYWAIRTR was added to .220B27, .220B27TPN and C1RB27 cells and samples were taken for staining over a period of 48h (black histograms, Figures 4.3-4.5). At each time point, staining was compared with untreated cells (grey tinted histograms). A decrease in HD6 staining was observed 1h after peptide addition (1st column), with the maximal decrease at 9h (4th column), after which a recovery in staining levels was seen for all cell lines tested. Similarly, HC-10 staining levels decreased 1h after peptide addition, but recovered over the 48h time course. No detectable changes in ME-1 or W6/32 staining were observed (3rd and 4th rows).

To determine whether the recovery of HD6 epitope expression after peptide addition was delayed by the continued presence of peptide in the media, a further time course experiment was carried out with the B27-binding peptide RRYQKSTEL. .220B27 and .220B27TPN cells were incubated for 1h with peptide, washed twice with PBS to remove peptide, and then incubated for a further 25h in fresh R10 media with or without 100uM peptide (black and green histograms respectively, Figure 4.6). Washing of peptide-pulsed cells allowed a faster recovery of HD6 surface expression. Furthermore, addition of extra peptide at t=8h maintained the loss of HD6 reactivity for a further 12h (data not shown).

4.2.3 Exogenous peptide addition reduces HD6 staining in HeLa cells lentivirally-transduced to express HLA-B27

Adherent HeLa cells transduced with lentiviruses for B27 heavy chain (B27 HC), B27 single chain trimer (B27 SCT), or B35 single chain trimer (B35 SCT) were trypsinised for 10 min, washed and incubated at 37C for 4h in media alone or with 100uM B27-binding peptide (SRYWAIRTR) or an irrelevant peptide. Cells were stained with the monoclonal antibodies HC-10, HD6, ME-1, and W6/32 and compared with cells stained immediately after trypsin treatment. Unexpectedly, the combination of trypsin treatment followed by the 4h incubation period resulted in an increase in HC-10 staining for all cells analysed, including untransduced HeLa cells (black histograms in left column, Figure 4.7). Similarly, but confined to B27-expressing cells only, HD6 staining was markedly increased following treatment and a 4h incubation period compared with cells stained immediately after trypsin treatment (black histograms, 2nd column). In contrast, no detectable difference was observed for ME-1 and W6/32 staining (3rd and 4th columns, respectively). Notably, however, the addition of exogenous B27-binding peptide (dashed grey histograms), but not irrelevant peptide, (dotted grey histograms) inhibited this effect. This was also seen for HeLa cells expressing SCTs with the single cysteine mutations C308S (4th row) and C325S (data not shown), which and have been implicated in dimer formation within exosomes and, most recently, in B27-expressing cell lines exposed to an altered redox environment (Lynch et al., 2009; Makhadiyeva et al., 2011).

In summary, these data show that addition of B27-binding peptides can inhibit HD6 staining, even when B27 is expressed as a SCT, while mutation of the cytoplasmic cysteines in B27 SCT does not affect HD6 epitope expression.

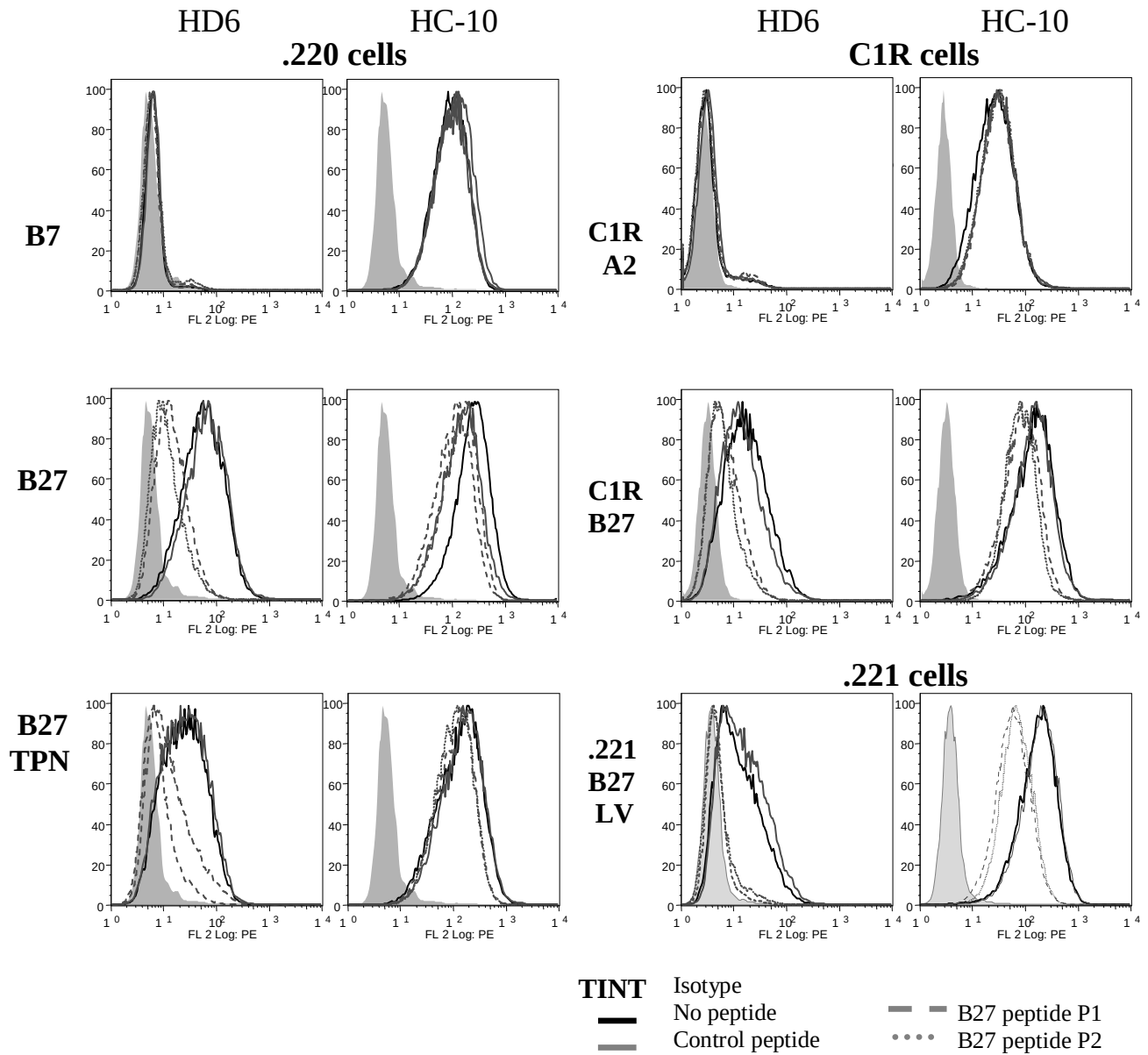


Figure 4.1 Overnight addition of B27-binding peptides reduces HD6 binding to HLA-B27-expressing B cell lines. Transfected .220 cells expressing HLA-B7, HLA-B27, or co-expressing HLA-B27 and human tapasin (B27.TPN), transfected C1R cells expressing HLA-A2 or HLA-B27, and transduced .221 cells expressing HLA-B27 were incubated overnight with 100uM control peptide (grey histograms) or B27-binding peptides P1 (SRYWAIRTR, grey dashed histograms) and P2 (RRYQKSTEL, grey dotted histograms) or without peptide (black histograms). Cells were stained with HD6 and HC-10 as indicated and IgG1 isotype control (grey tinted histogram). Data is representative of 3 B27-binding peptides, 2 control peptides from 3 independent experiments.

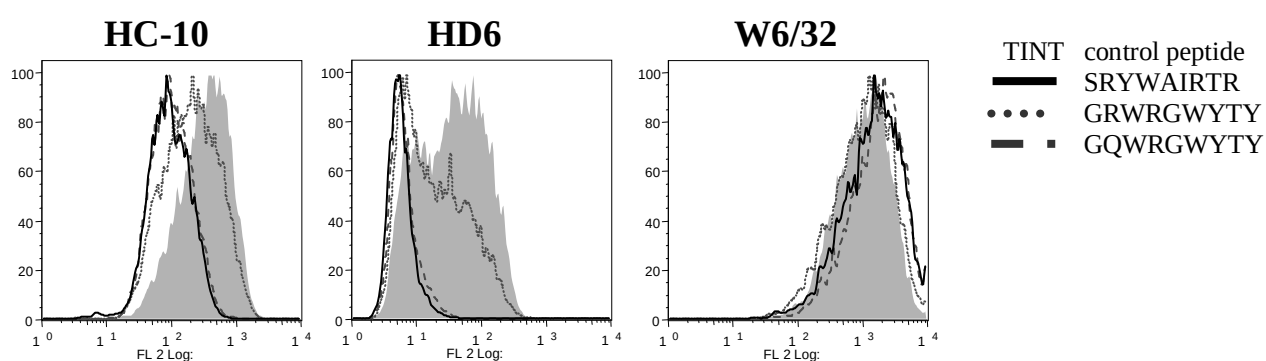


Figure 4.2 Overnight incubation of .220B27 cells with the B27-peptides recognised by the peptide-dependent B27-specific MARB4 antibody reduces HD6 staining. 220B27 cells were incubated overnight with 100uM of the B27-binding peptides SRYWAIRTR, GRWRGWYTY and GQYRGWYTY or the control peptide SLYNTVATL and then stained with the monoclonal antibodies HC-10, HD6 and W6/32 as indicated. Data is representative of two independent experiments.

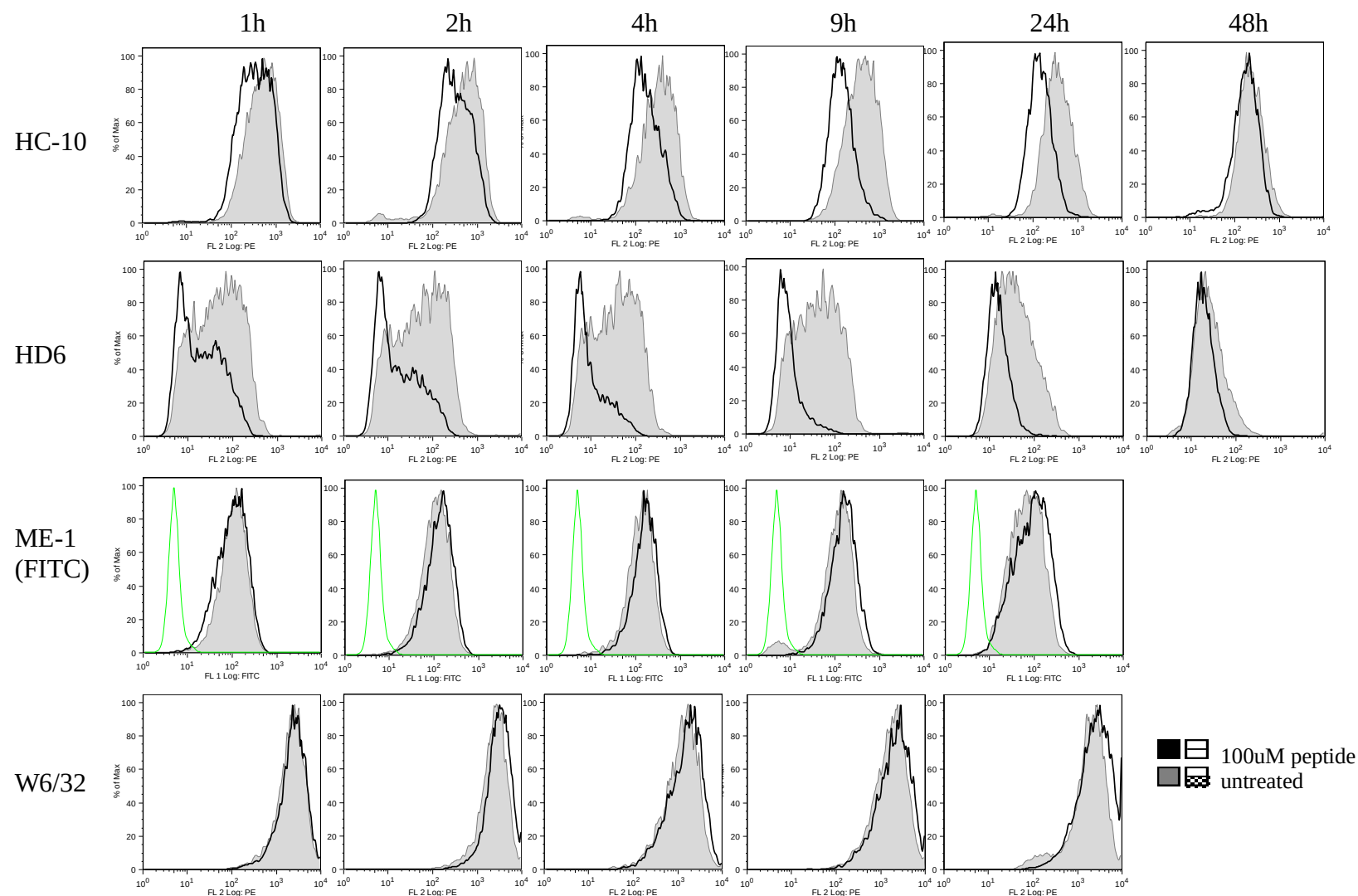


Figure 4.3 Peptide addition to .220B27 cells abrogates HD6 binding for up to 24h. .220B27 cells were incubated with (black histograms) or without (grey tinted histograms) 100uM of the B27-binding peptide SRYWAIRTR and samples were taken over a period of 48h and stained with the monoclonal antibodies HC-10, HD6, and W6/32 as indicated. Cells were also stained with FITC-conjugated ME-1 or isotype control (green histograms).

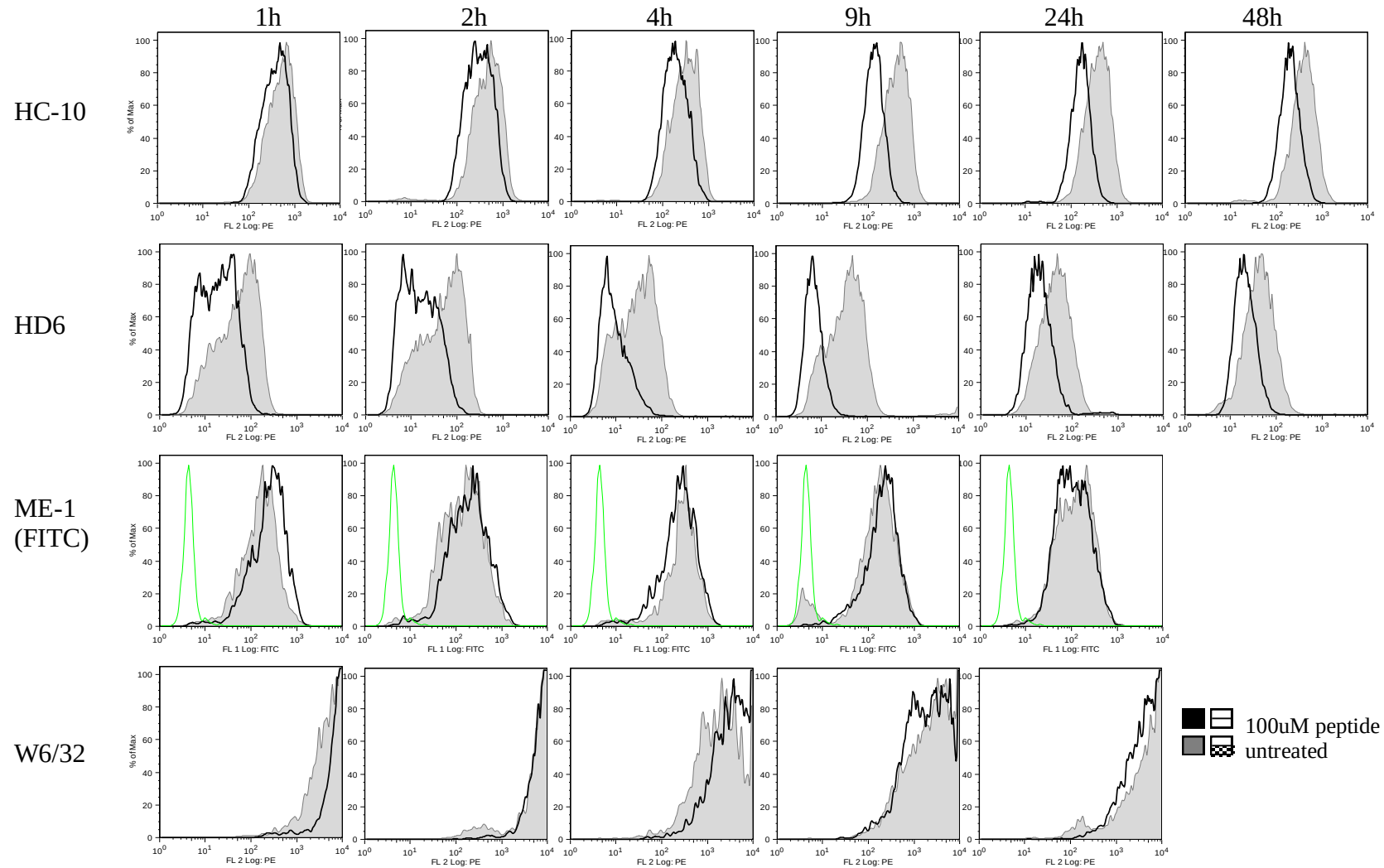


Figure 4.4 Peptide addition to .220B27TPN cells abrogates HD6 binding for up to 24h. .220B27 cells were incubated with (black histograms) or without (grey tinted histograms) 100uM of the B27-binding peptide SRYWAIRTR and samples were taken over a period of 48h and stained with the monoclonal antibodies HC-10, HD6, and W6/32 as indicated. Cells were also stained with FITC-conjugated ME-1 or isotype control (green histograms).

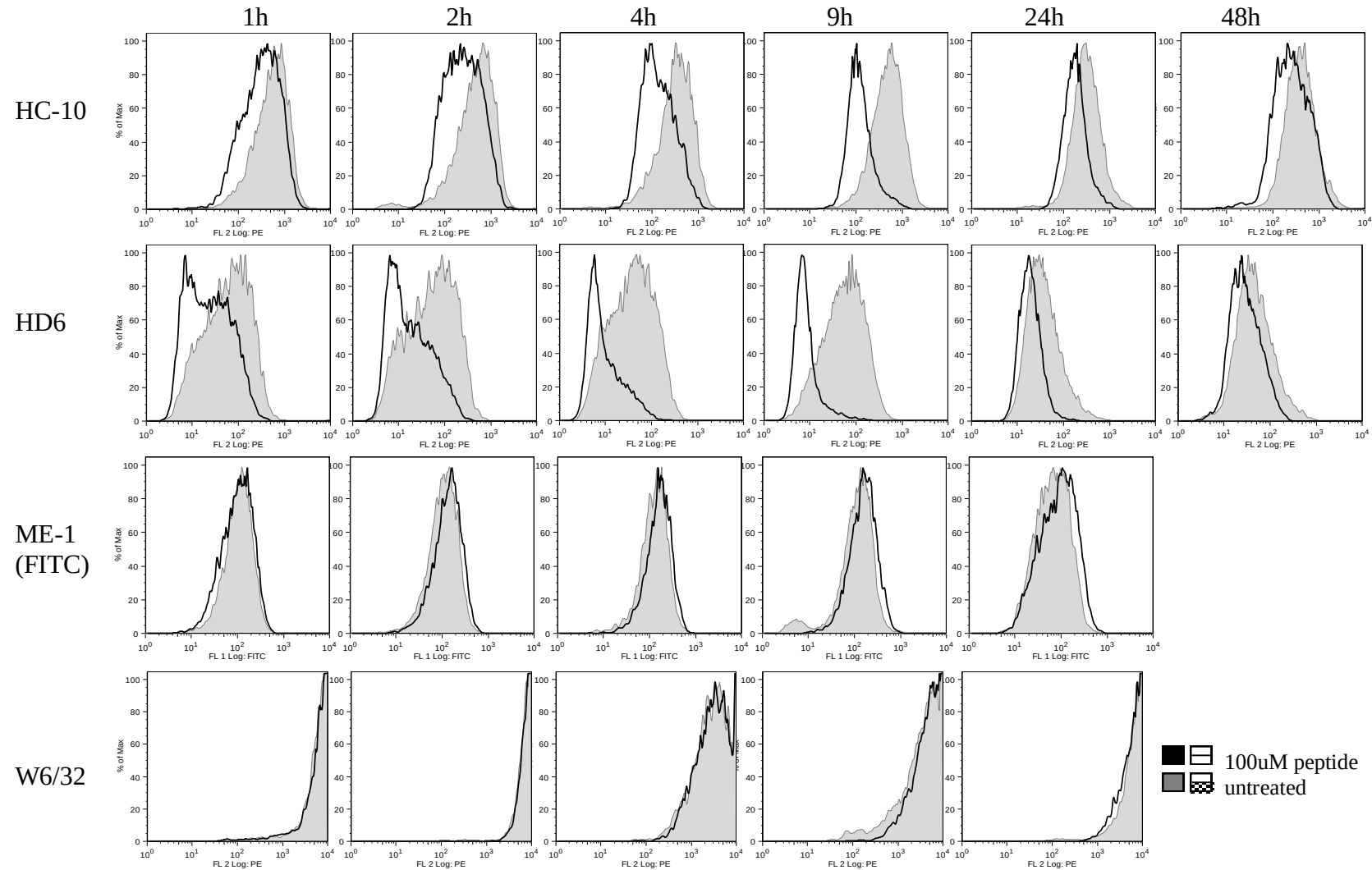


Figure 4.5 Peptide addition to C1RB27 cells abrogates HD6 binding for up to 24h. .220B27 cells were incubated with (black histograms) or without (grey tinted histograms) 100uM of the B27-binding peptide SRYWAIRTR and samples were taken over a period of 48h and stained with the monoclonal antibodies HC-10, HD6, and W6/32 as indicated. Cells were also stained with FITC-conjugated ME-1 or isotype control (green histograms).

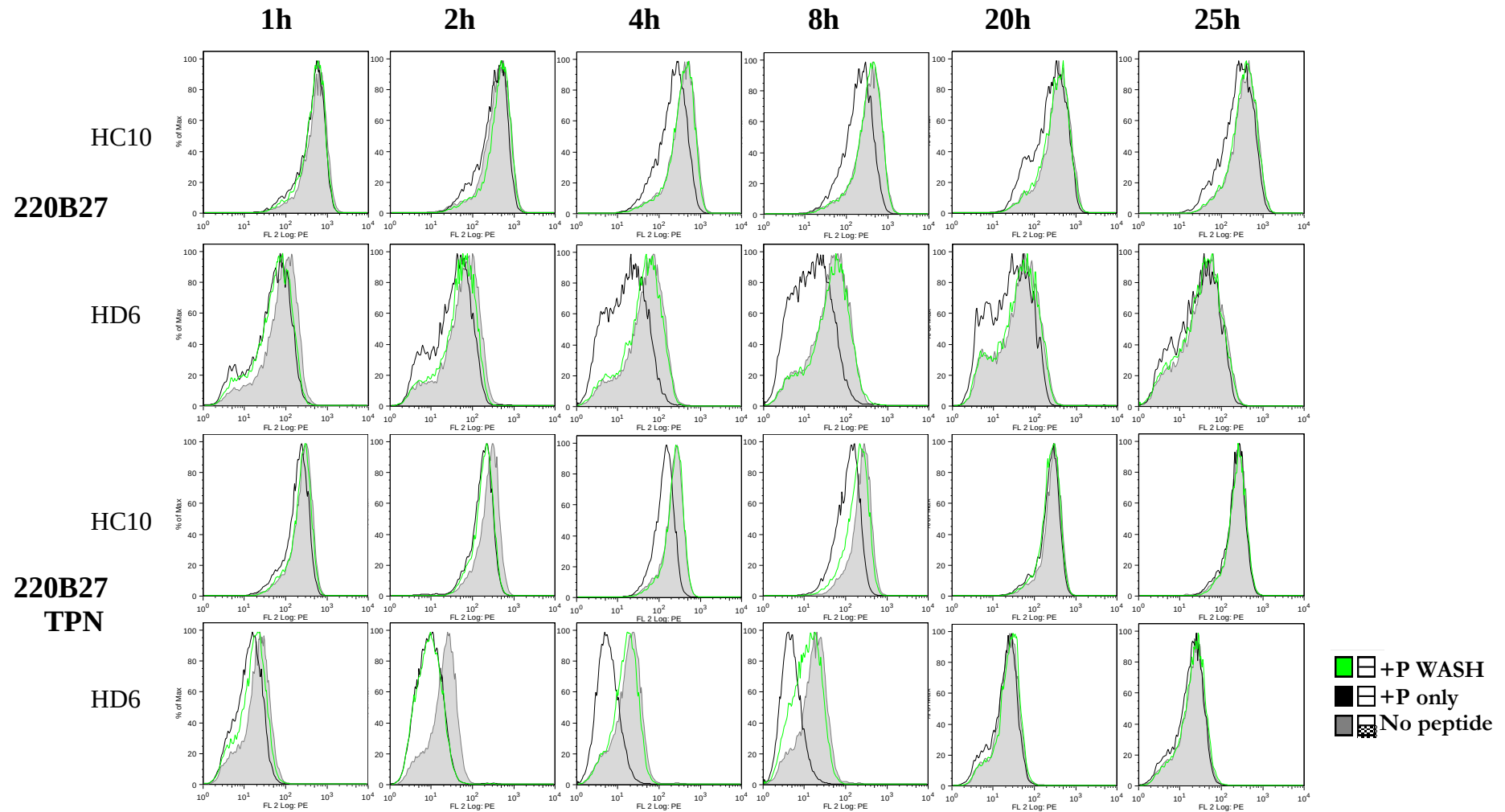


Figure 4.6 Recovery of HD6 staining is delayed by the presence of peptide in the media. .220B27 and .220B27TPN cells were incubated for 1h with 100uM of the B27-binding peptide RRYQKSTEL, washed twice with PBS, then incubated for a further 25h in the presence (black histogram) or absence (green histogram) of 100uM peptide. Samples were taken over a period of 25h and stained with the monoclonal antibodies HC-10 and HD6 as indicated. Staining is compared to cells left untreated (grey tinted histograms).

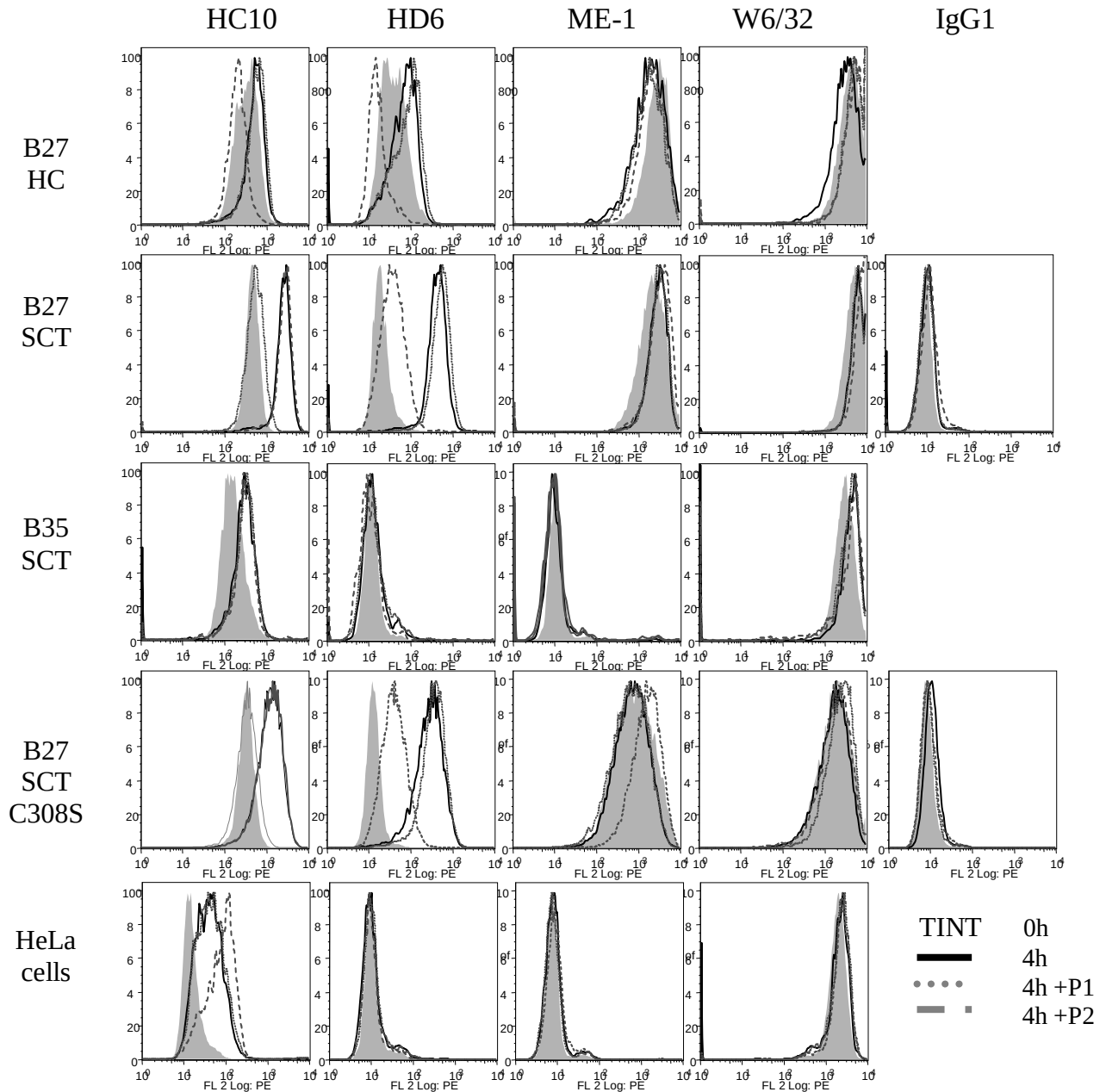


Figure 4.7 Incubation of HLA-B27-expressing HeLa cells with peptide abrogates HD6 reactivity. HeLa cells and lentiviral-transduced HeLa cells expressing B27 heavy chain (HC), or single chain trimers of HLA-B27 (B27 SCT), HLA-B35 (B35 SCT) and the B27 single cysteine mutant C308S were trypsinised for 10min, washed and either directly placed on ice (0h, grey tinted histograms) or resuspended in fresh R10 media alone (black histogram), with 100uM of the B7-binding peptide RPMTYKAAV (P1, grey dashed histograms) or with 100uM of the B27-binding peptide SRYWAIRTR (P2, grey dotted histograms). Data is representative of 2 independent experiments.

4.3 The effect of iso-osmotic low pH treatment of cells on HD6 reactivity

To further assess the contribution of bound peptide to HD6 recognition of HLA-B27 molecules, iso-osmotic low pH treatment (acid treatment) of cells was carried out as described previously (Zeh et al., 1994). This method has been shown to denature cell surface HLA class I complexes, removing peptide and β_2m and thereby reducing W6/32 and BBM.1 reactivity while increasing the number of HC-10 reactive molecules. Furthermore, Malik *et al.* have used this method to test whether monoclonal antibodies recognise conformation-dependent epitopes on class I heavy chains (Malik et al., 2002). Low pH treatment of cells can therefore allow the differentiation between peptide-free and peptide-containing heavy chain conformations. Thus, I sought to investigate the effect of low pH treatment on the expression of the HD6 epitope in B27-expressing cell lines. Unless otherwise stated, acid treatment was performed by resuspending cells in a low pH glycine buffer (300mM glycine, 2% BSA, pH 2.4) for 2 minutes, before neutralisation and washing steps with RPMI-1640, prior to subsequent staining with monoclonal antibodies for FACS analysis.

4.3.1 Low pH treatment of transfected .221 and C1R BCLs increases HD6 reactivity

I first examined the effect of low pH treatment on HD6 staining of the .221 and C1R B cell lines, which I have shown in Chapter Three and here to stain with HD6 when transfected with HLA-B27 but not control alleles (grey filled histograms, 2nd panel, Figure 4.8). Low pH treatment increased HD6 staining for both .221 and C1R cells expressing HLA-B27 compared with untreated cells left (2nd column, black histograms). Furthermore, treatment reduced staining with the conformation-dependent mAbs ME-1 and W6/32 (3rd and 4th columns) and increased staining with HC-10, consistent with the

findings of previous reports. Low pH treatment had no effect on HD6 staining of untransfected cells or those expressing control alleles, which remained HD6-negative.

4.3.2 HD6 staining of BCLs expressing HLA-B27 is induced by low pH treatment

EBV-transformed BCLs expressing HLA-B27 or other HLA-B alleles, which did not stain with HD6 under normal conditions, were next subjected to low pH treatment: 6 HLA-B27+ BCLs derived from AS patient PBMCs, of which 1 had the HLA-B*27:02 subtype, 1 B27+ reference cell line (Jesthom cells) and 4 B27- BCLs derived from healthy donor PBMCs were tested. HD6 staining was consistently induced in the HLA-B27+ BCLs but not in the HLA-B27- BCLs (black histograms in 2nd column, Figure 4.9. Data is shown for 4/7 B27+ and 1/4 B27- BCLs, respectively). As before, treatment also increased HC-10 staining, while reducing ME-1 and W6/32 reactivity on all BCLs.

4.3.3 Acid-induced HD6 staining is independent of HLA-B27 subtype polymorphisms in the F pocket

To investigate whether the observed acid-induced increase in HD6 binding was specific to disease-associated B27 subtypes, the transduced .221 cells expressing HLA-B*27:05 or HLA-B*27:09 were acid treated before mAb staining and analysis as before. Low pH treatment increased HD6 and HC-10 staining for both subtypes, with concomitant decreases in ME-1 and W6/32 reactivity (Figure 4.10). To further verify this, I generated the untagged B*27:05 and B*27:09 lentiviruses described earlier, and used them to transduce C58 cells, which were subsequently treated with acid. In addition, C58 cells expressing Sv5-tagged B27 and B27 variants with the single amino acid substitutions D116H, or D116Y or with the double substitution H114D D116Y were also treated.

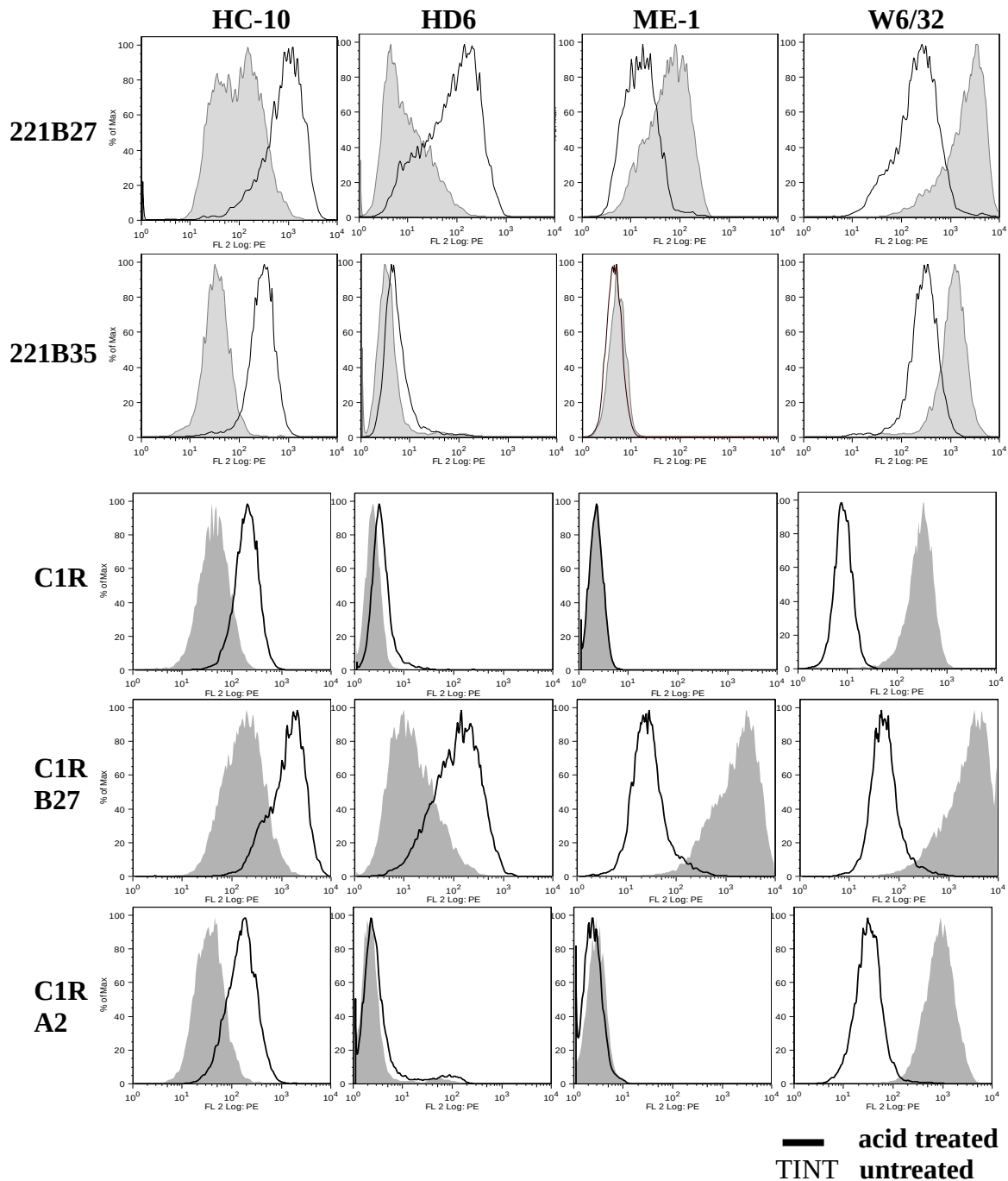


Figure 4.8 HD6 staining is increased in .221 and C1R BCLs expressing HLA-B27 but not control HLA alleles after acid treatment. (A) Transfected .221 cells expressing HLA-B27 or HLA-B35 and (B) untransfected C1R cells or transfected C1R cells expressing HLA-B27 or HLA-A2 were treated with low pH glycine buffer for 2 minutes, washed and stained with the monoclonal antibodies HC-10, HD6, ME-1 and W6/32 as indicated (black histograms). Staining was compared with untreated cells (grey tint histograms) and is representative of at least 3 independent experiments.

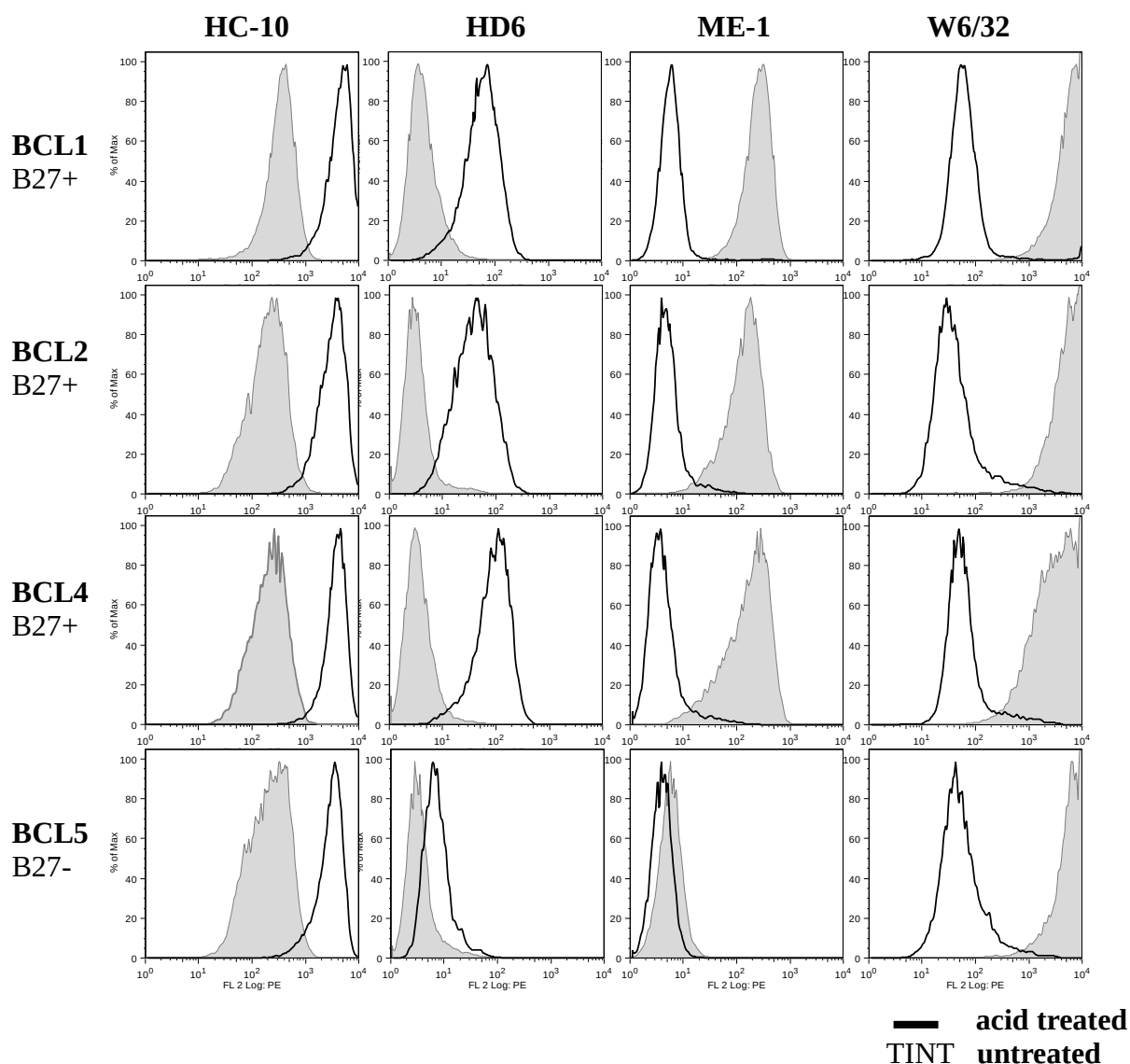


Figure 4.9 Low pH treatment of EBV-transformed BCLs induces HD6 epitope expression in cells expressing HLA-B27 but not control alleles. EBV-transformed B cell lines known to express HLA-B27 (BCL1-BCL4) and a B27 negative BCL (BCL5) were treated with low pH glycine buffer for 2 minutes, washed and stained with the monoclonal antibodies HC-10, HD6, ME-1 and W6/32 as indicated (black histograms). Staining was compared with untreated cells (grey tint histograms). Data is representative of 2 independent experiments and a further 3 B27+ BCLs and 3 B27- BCLs (kindly donated by Tim Powell (HLA-B haplotyping B*40:01/B58, B*40:02/B38, B5/B55)). Where known, HLA haplotyping for BCL1-BCL5 is shown in Figure 3.5B.

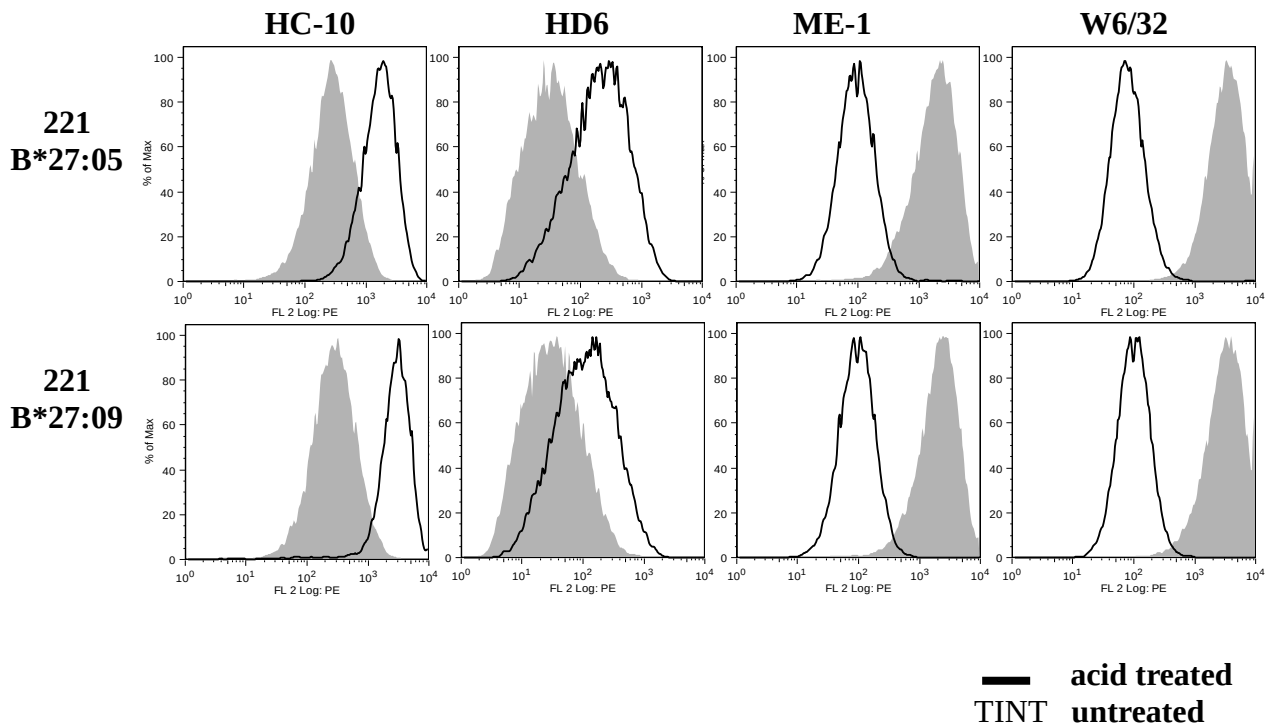


Figure 4.10 Acid-induced HD6 reactivity is independent of HLA-B27 subtype polymorphism at position 116 in the F pocket. .HLA-B*27:05 and HLA-B*27:09 lentivirus was generated in 293T cells and used to transduce .221 cells. Transduced cells were then treated with low pH acid buffer for 2 minutes, washed, and stained with monoclonal antibodies HC-10, HD6, ME-1 and W6/32 as indicated. Staining of acid treated cells (black histograms) is compared with untreated cells (grey tint histograms) and is representative of 3 independent experiments.

These B27 variants enable further examination of the contribution of the polymorphic residues in the F pocket to HD6 staining. In the previous chapter, C58 cells expressing HLA-B27 were shown to not stain with HD6. Here, after acid treatment, HD6 staining was detected in the transduced C58 cells expressing either the HLA-B*27:05 or HLA-B*27:09 subtype but not in cells expressing HLA-A2 (black histograms, 3rd, 4th and 1st rows on left respectively, Figure 4.11A). Surprisingly, no HD6 staining was induced in the transfected C58 cells expressing Sv5-tagged B27 wild type (WT), despite an increase in HC-10 reactivity (2nd row on left). This does not appear to be due to the presence of the C-terminal Sv5 tag, as HD6 staining was also induced in cells expressing the Sv5-tagged B27 variants (Figure 4.11B). Notably, staining with ME-1 indicated that the Sv5-tagged B27 WT had the lowest B27 surface expression (Figure 4.11C). These data suggest that the generation of acid-inducible HD6 may depend on the number of HLA-B27 molecules at the surface, prompting the further investigations outlined in 4.3.4.

4.3.4 Acid-induced HD6 staining is dependent on surface expression levels of HLA-B27

To further investigate whether acid-induced HD6 staining of C58 cells is dependent on surface expression levels of HLA-B27, cells were incubated overnight at 26°C before acid treatment. Low temperature treatment has been used by several groups to increase surface expression of class I molecules, and in the previous chapter low temperature treatment of the T2 cell line expressing HLA-B27 induced low levels of HD6 staining (Figure 3.5). Here, low temperature treatment also induced low levels of HD6 staining on one of the C58 cell lines expressing HLA-B27 (D116H variant) (Figure 4.12A, 3rd row, black histograms). Notably, these cells had the highest levels of ME-1 staining both when

grown at 37°C (grey tint histogram) and overnight at 26°C (black histogram). Low temperature treatment increased HC-10 and ME-1 staining on all C58 cells expressing HLA-B27 molecules but not untransfected C58 cells (Figure 4.12B). Acid treatment after incubation at 26°C induced HD6 staining on C58 cells expressing the SV5-tagged B27 WT after incubation at 26°C (black dotted histogram) but not when cells were grown at 37°C (grey dashed histogram). Furthermore, acid treatment resulted in higher levels of HD6 staining of the C58 cells expressing the B27 variants 116H and 114D 116H following low temperature treatment compared with cells grown at 37°C. This was a more marked difference than that seen for HC-10 staining.

4.3.5 The unpaired cysteine residues at positions 67, 308, and 325 of HLA-B27 are not necessary for acid-induced HD6 reactivity

Our group have previously shown the unpaired C67 residue to be critical for the formation of both soluble recombinant B27 disulfide-bonded homodimers *in vitro* and cell surface homodimers in stably transfected .220 cells (Allen et al., 1999; Bird et al., 2003). In Chapter Three, .220 cells expressing HLA-B27 with the single amino acid substitution C67S were shown to have undetectable HD6 staining. Yet, given the dependency of HD6 staining on surface HLA-B27 levels and the lower levels of ME-1 in the .220B27 C67S cells, the contribution of this unpaired cysteine to formation of the HD6 epitope remained unclear. To address this and to assess whether two other unpaired cysteines in the C-terminal tail of B27 affect HD6 staining, C58 cells expressing the B27 single cysteine mutants C67S, C308S, C325S or B27 with all three of these mutations (triple mutant, TM) were incubated at 37°C or 26°C overnight before acid treatment and subsequent analysis (Figure 4.12). At 37°C, none of the mutants stained with HD6

(middle column, grey tint histograms), however acid treatment induced detectable binding on the C308S and C325S but not the C67S mutant cells (grey dashed histograms), which had lower ME-1 staining before acid treatment (3rd column). After incubation at 26C, HD6 staining was detected on all mutants (black histograms), which was further increased upon acid treatment (black dotted histograms), suggesting that HD6 binding does not depend on cysteine residues at these positions.

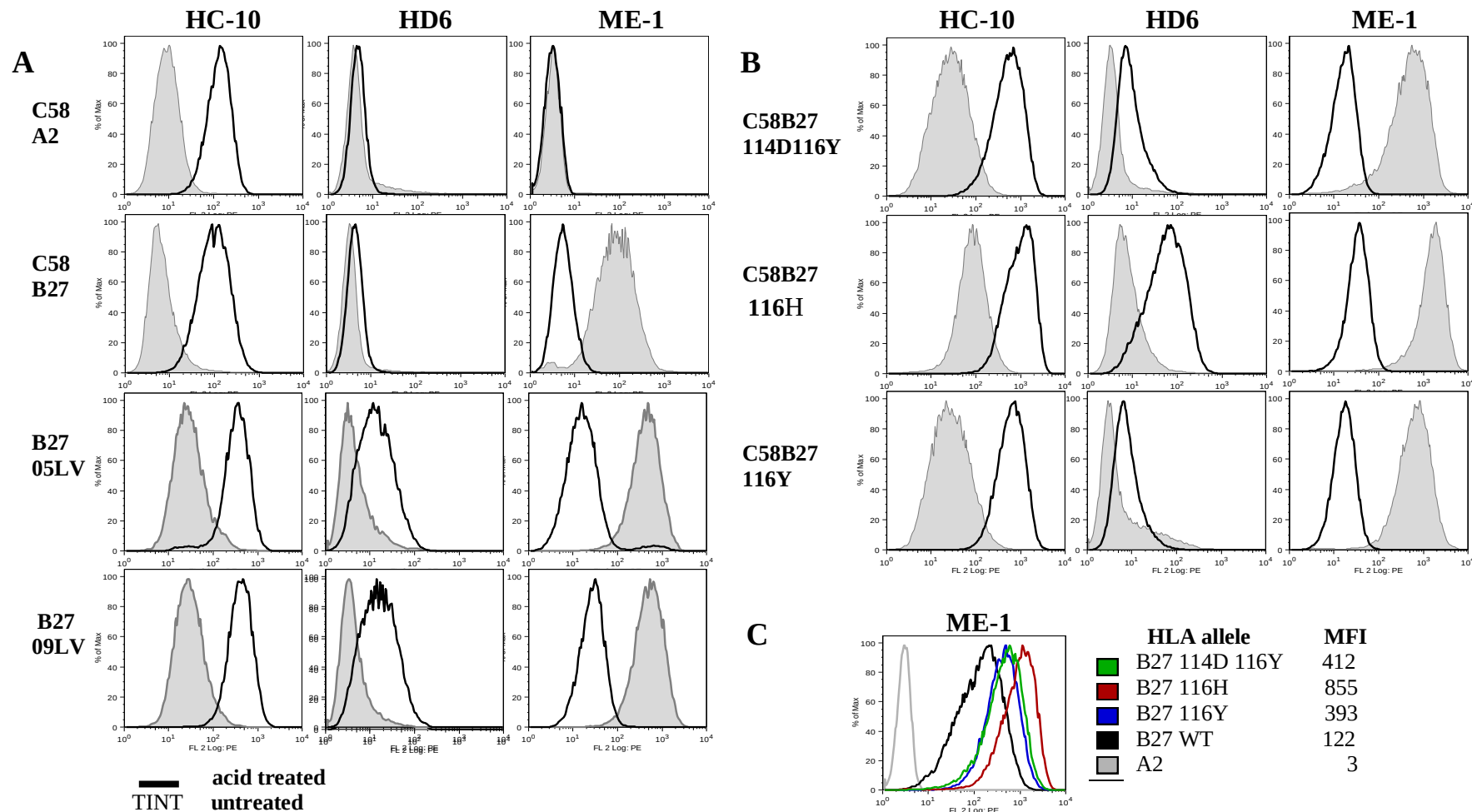


Figure 4.11 Acid-induced HD6 correlates with surface expression levels of HLA-B27 in the rat C58 cell line. (A) Transfected C58 cells expressing HLA-A2 or Sv5-tagged HLA-B27 or transduced C58 cells expressing B*27:05 (05LV) or B*27:09 (09LV) and (B) transfected C58 cells expressing the Sv5-tagged mutants 114D116Y, 116H and 116Y were treated with low pH glycine buffer for 2 minutes before staining with HC-10, HD6 and ME-1 antibodies as indicated (black histograms). Staining was compared with untreated cells (grey tinted histograms) and data is representative of two independent experiments. (C) A comparison of the ME-1 surface expression levels with corresponding MFI levels.

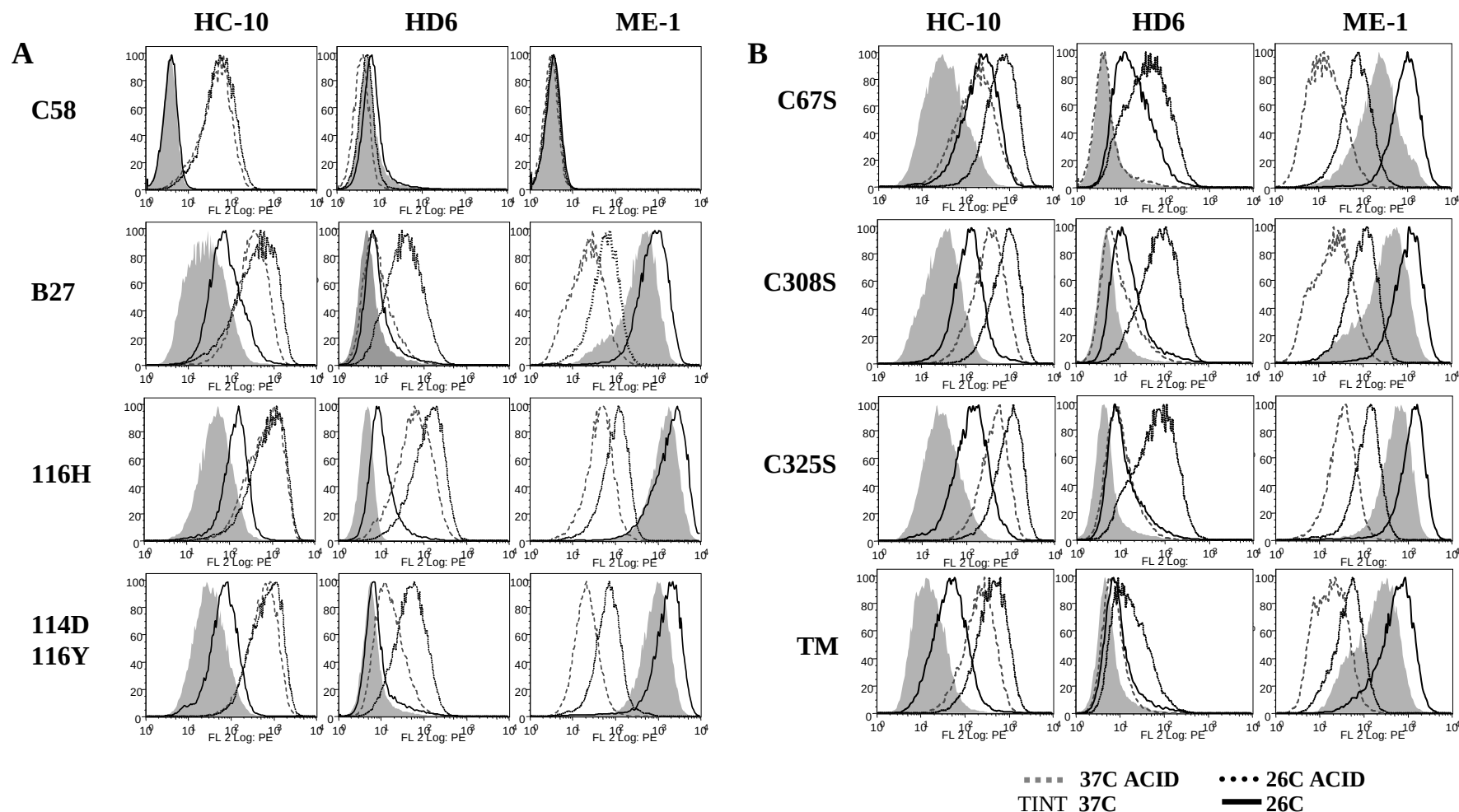


Figure 4.12 HD6 staining can be induced in C58 cells expressing HLA-B27 by low temperature treatment, is augmented upon low pH treatment, and does not require cysteine residues at positions 67, 308 and 325. C58 cells expressing (A) Sv5-tagged WT B27 or the mutants 116H and 114D116Y or (B) untagged single cysteine mutants C67S, C308S, C325S or with all three cysteine mutations (TM) were incubated overnight at 37C (grey tint histogram) or 26C (black histogram) before treating with low pH glycine buffer (grey dashed histograms and black dotted histograms respectively) and staining with antibodies as indicated. Data is representative of 2 independent experiments.

4.3.6 Acid-induced HD6 staining requires key components of the peptide loading complex, tapasin and TAP, but does not require calnexin

Stably transfected .220 cells expressing HLA-B27 or HLA-B7 were also treated with low pH glycine buffer to investigate the effect on the surface expression of HD6-reactive molecules. Surprisingly, and in contrast to the increase in HD6 staining of .221B27 after acid treatment, no change in HD6 staining after acid treatment was observed for .220B27 cells (Figure 4.13, top row, 2nd column, black histogram vs. grey filled histogram). This was despite an increase in HC-10 staining, and concomitant decreases in ME-1 and W6/32 staining. This was also seen for .220 cells transduced with lentiviruses to express WT HLA-B*27:05 and B*27:09 and several single cysteine mutants (data not shown). To determine whether the difference in response to acid treatment between the .220 and .221 cells could be attributed to the lack of functional tapasin, .220 cells transfected with both HLA-B27 and human tapasin constructs were acid treated. .220B27TPN cells responded to acid treatment in the same manner as .221B27 cells, with a considerable increase in HD6 staining observed (Figure 4.13B, 2nd row). This data suggests a key role for tapasin in the formation of a pool of precursor HLA-B27 molecules that are able to react with HD6 after acid treatment.

Next, 220 and 221 cells expressing HLA-B27 carrying a C-terminal HA-tag, which I have shown to inhibit surface HD6 reactivity in these cells (Figure 3.9), were tested for HD6 reactivity after acid treatment. Acid treatment induced surface HD6 staining in the .221B27HA but not in the .220B27HA cells (Figure 4.13B), reiterating the importance of tapasin. Moreover, this indicates that the capacity of the HA tag to inhibit surface HD6 formation in these cell lines is overcome upon acid treatment.

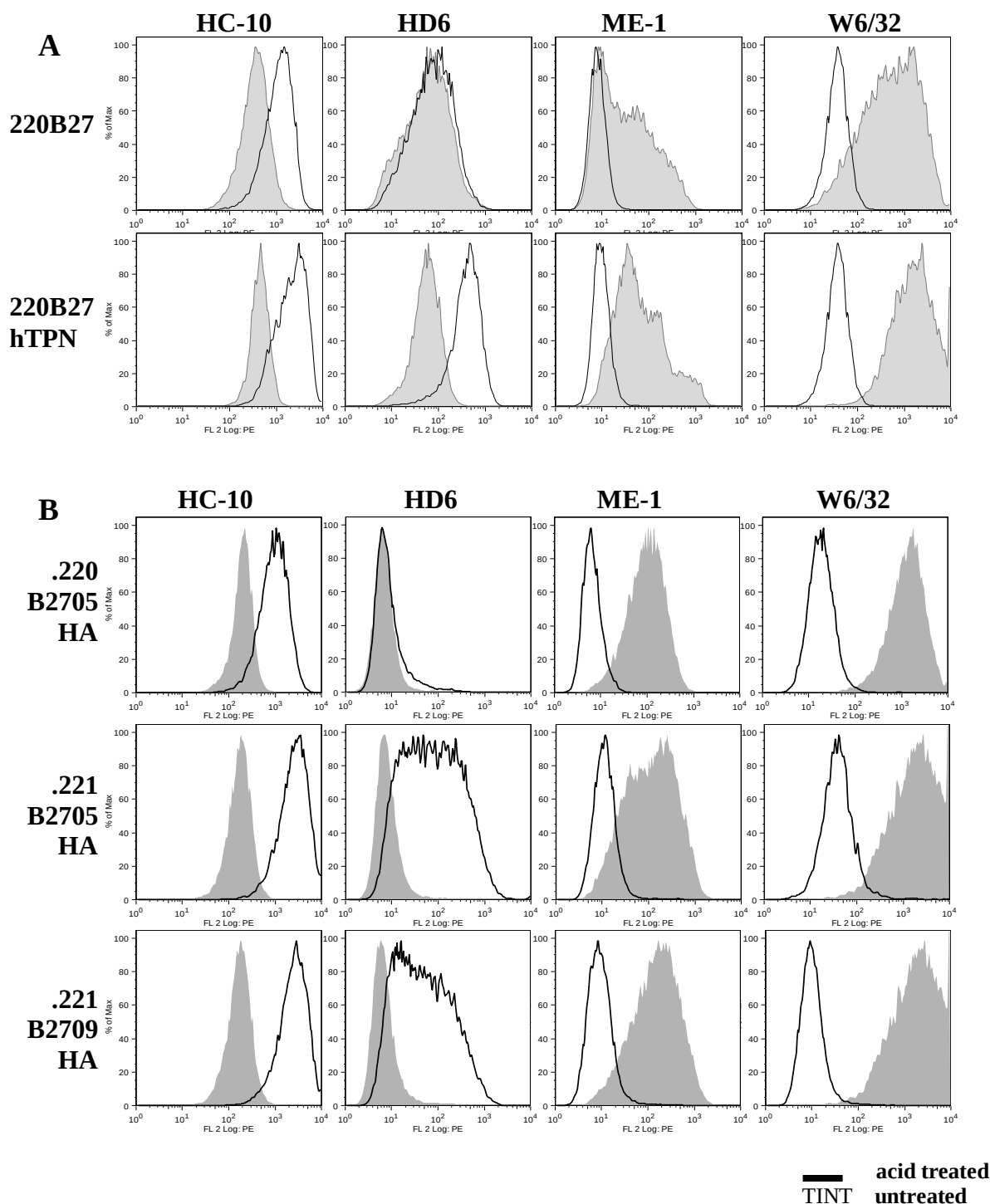


Figure 4.13 Acid-induced HD6 staining is dependent on tapasin and not inhibited by a C-terminal HA tag. (A) Transfected 220 cells expressing B27 with and without tapasin and (B) lentiviral-transduced .220 and .221 cells expressing either HA-tagged B*27:05 or 09 were treated with low pH glycine buffer for 2 minutes, washed and stained with the monoclonal antibodies HC-10, HD6, ME-1 and W6/32 as indicated (black histograms). Staining was compared with untreated cells (grey tinted histograms) and is representative of 3-5 independent experiments, depending on the cell line under investigation.

The TAP-deficient T2 cell line and the calnexin-deficient NKR cell line enable the investigation of two further components of the peptide loading machinery that have both been shown to interact with tapasin. These cell lines were used here to investigate whether TAP and calnexin were similarly required for the formation of acid-induced HD6-reactive molecules. Given that low levels of HD6 staining were observed for one T2 cell line expressing HLA-B27 after low temperature incubation (Figure 3.4), these cells were next subjected to acid treatment after an overnight incubation at 26°C. No HD6 staining was observed when cultured at 37°C, as before (grey tinted histogram, Figure 4.14A), yet no change was seen upon subsequent acid treatment (grey dotted histogram). In addition, although low temperature treatment induced low levels of HD6 staining (black histogram), this was not increased with acid treatment (black dashed histogram). Notably, little change in HC-10 staining was observed with and without acid treatment at both 37°C and 26°C, despite considerable reductions in ME-1 levels. Moreover, pulsing cells with B27-binding peptides overnight at both 37°C and 26°C, a method commonly used with T2 cells to increase surface expression of selected class I molecules, also failed to result in any increase in HD6 staining upon acid treatment, despite increasing the number of surface HLA-B27 ME-1 reactive molecules before treatment (data not shown). Peptide incubation at 26°C did however inhibit the low steady state levels of HD6 staining in these cells, consistent with previous findings. In contrast, acid treatment of NKR cells and the parent CEM cells induced HD6 staining only when the cells expressed HLA-B27 (Figure 4.14B). These findings implicate an important role for tapasin and TAP, but not calnexin, in the formation of a pool of HLA-B27 molecules that, upon acid treatment, bind to HD6.

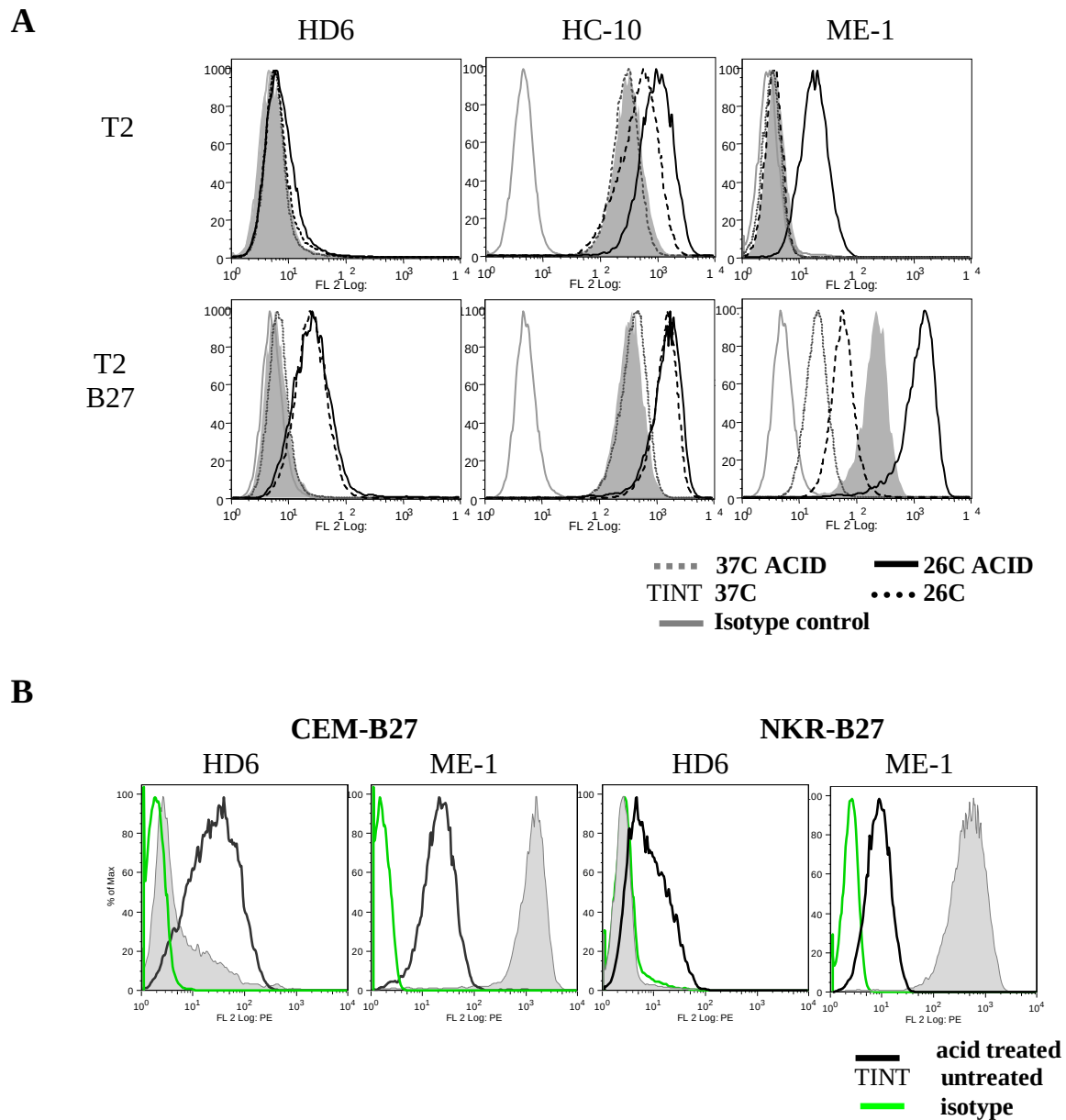


Figure 4.14 Acid treatment of HLA-expressing TAP-deficient but not calnexin-deficient cells induces HD6 epitope expression. (A) T2 and T2B27 cells cultured at 37°C (grey tinted histograms) and acid treated (grey dashed histograms) or incubated overnight at 26°C (black histograms) and acid treated (black dotted histograms) were stained with the antibodies HD6, HC-10 and ME-1 as indicated. (B) CEM and NKR cells expressing HLA-B27 were treated with low pH glycine buffer for 2 minutes, washed and stained with the monoclonal antibodies HD6 or ME-1 as indicated (black histograms) and compared with untreated cells (grey filled histograms and IgG1 isotype control (green histograms)). Data is representative of two independent experiments.

4.4 The use of papain to investigate the formation of surface HD6-reactive B27 molecules

Several investigators have proposed that endocytosis of MHC class I molecules is necessary for the appearance of misfolded class I heavy chains at the surface, although the mechanism by which this is achieved remains unclear (Abdel Motal et al., 1993; Pickl et al., 1996). Investigation of Nef-induced down-modulation of MHCI surface expression has identified tyrosine Y320, present in the cytoplasmic tail of all classical class I molecules, as a key residue in an unconventional tyrosine-based motif important for this process and thereby an attractive candidate motif for MHCI endosomal recycling (Greenberg et al., 1998; Le Gall et al., 1998). In agreement with this, Santos and colleagues have demonstrated that the Y320 residue in HLA-B27 is involved in the regulation of its spontaneous endocytosis, at least in HLA-B27-transfected rat C58 cells (Santos et al., 2006). Importantly, however, this residue was not required for the formation of surface HC-10-reactive species. This conflicting data could be explained by the existence of multiple recycling pathways for β_2 m-associated surface molecules, one of which involves the Y320 residue but does not contribute to the conversion of β_2 m-associated molecules into β_2 m-free molecules. Alternatively, whether recycling is required for formation of HC-10 reactive species may be less stringent under certain conditions or in particular cell lines.

To follow the fate of cell surface HLA-B27 molecules, our group and others have used the protease papain to strip class I molecules from the surface, thereby enabling their surface recovery to be monitored (Bird et al., 2003). This resulted in the identification of a pool of cell surface HC-10 reactive B27 homodimers, distinct from those found in the

ER, that form through the endosomal recycling of surface β_2m -associated complexes. In this section I investigate whether HD6-reactive HLA-B27 molecules exhibit similar behaviour to HC-10-reactive molecules following papain treatment.

4.4.1 HD6-reactive B27 surface molecules are susceptible to digestion by papain in the absence of cysteine

Papain is a cysteine protease isolated from the plant *Carica papaya* in an inactive form, in which the sulfhydryl group of the catalytic cysteine residue is unavailable for enzyme catalysis. Activation can be achieved through the addition of mild reducing agents, including free cysteine itself, which results in intramolecular disulfide exchange that liberates the blocked cysteine sulfhydryl in the active site. Papain digestion has been widely used to solubilise MHC class I molecules, owing to the presence of at least one papain cleavage site in the membrane-proximal region of the $\alpha 3$ domain in the region of position 281. Papain cleavage of MHCI molecules results in the loss of its transmembrane region and cytoplasmic tail, but does not disturb its interaction with β_2m (Springer and Strominger, 1976). Interestingly, treatment of cells with papain alone is sufficient to strip β_2m -free class I heavy chains while leaving β_2m -associated complexes intact. In contrast, pre-incubation of papain with 10mM cysteine is required for the removal of both forms of MHC class I molecules (Pickl et al., 1996).

Thus, to determine whether HD6-reactive molecules were similarly susceptible to cleavage by papain alone, HLA-B27-transfected B cells were incubated for 45min at 37C with either 3mg/ml papain alone (P), or with 3mg/ml papain pre-activated with 10mM cysteine for 15min (P*). Samples were taken for antibody staining immediately after

treatment (0h) and after a re-culture period, which involved resuspending cells in fresh R10 and incubating at 37°C for a further 3.5h. Staining was compared with untreated cells (Figure 4.15, grey tinted histograms). Treatment with papain alone was sufficient to abrogate HD6 staining (black histograms, top row), and was observed in all cell lines tested, including .220B27TPN and C1RB27 as shown and also 220B27 and 221B27 cells (data not shown). Papain alone also cleaved the majority of HC-10 reactive B27 molecules (black histograms, second row), whereas little change was observed for ME-1 and W6/32 staining, in agreement with previous studies (black histograms, third and fourth rows). In addition, recovery of HD6 and HC-10 staining was observed following a re-culture period of 3.5h (dashed histograms). No differences were observed in HD6 staining following treatment with papain alone (P) or activated papain (P*), which recovered to levels similar to those of untreated cells after the re-culture period. As expected, treatment with activated papain (P*) reduced ME-1 and W6/32 staining, and further decreased HC-10 levels, all of which recovered following a re-culture period.

4.4.2 Cell surface HD6-reactivity partially recovers in the presence of BFA after treatment with papain alone but not cysteine-activated papain

Our group have also shown that the re-acquisition of surface HC-10 staining after treatment with cysteine-activated papain can be blocked by inhibiting the egress of newly synthesised molecules from the ER with Brefeldin A (BFA) (Bird et al., 2003). This indicates that the HC-10 reactive B27 molecules that emerge following papain treatment require the presence of cell surface heterodimers. Thus, to determine whether this is also a prerequisite for HD6-reactive molecules, experiments were carried out with .220B27, .221B27 and C1RB27 cells in the presence and absence of 10ug/ml BFA (Figure 4.16).

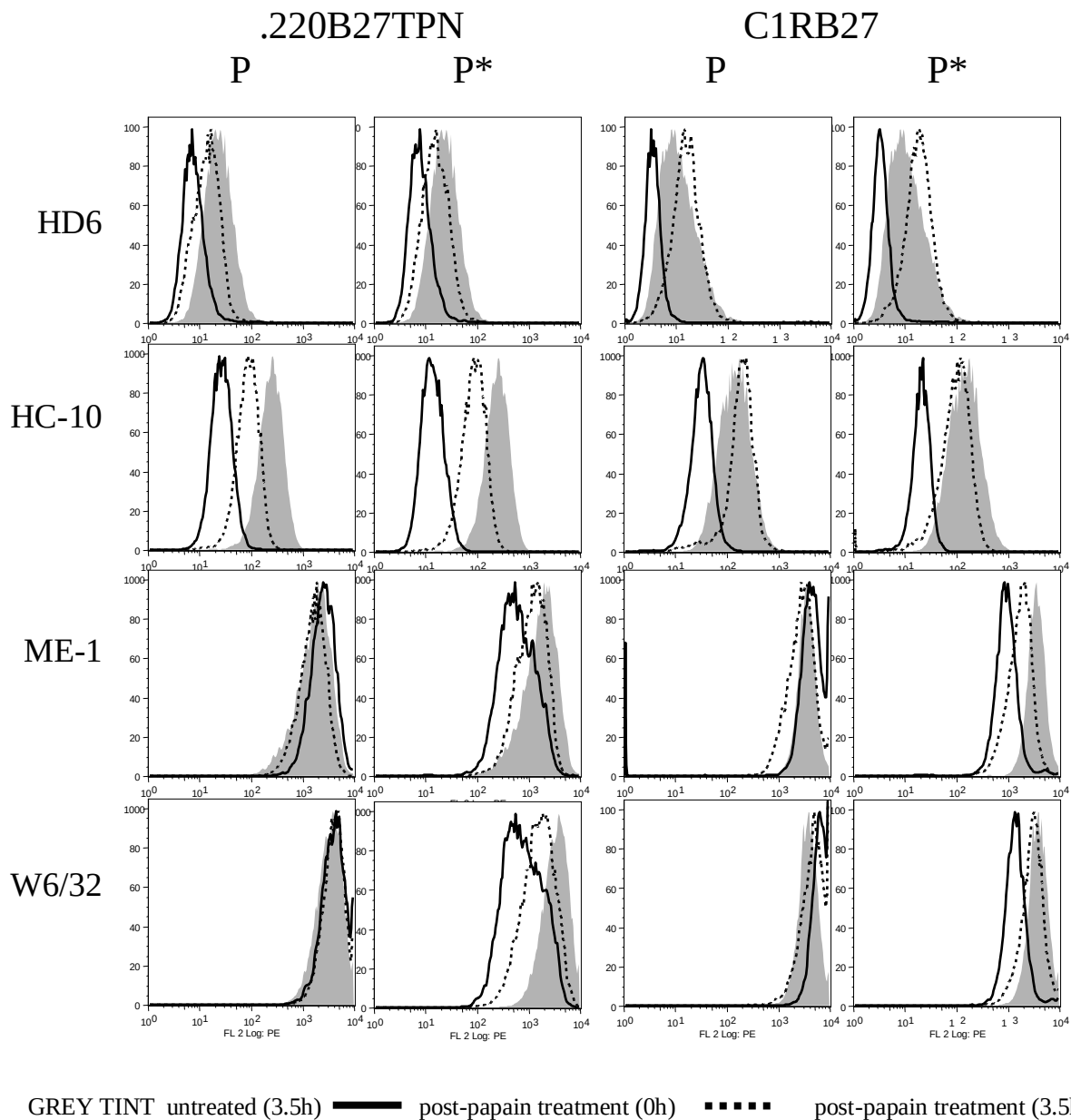


Figure 4.15 HD6-reactive molecules are susceptible to papain stripping in the absence of cysteine. .220B27TPN and C1RB27 cells were treated with papain alone (P) or cysteine-activated papain (P*) for 45min, washed then resuspended in R10 and incubated at 37C for a further 3.5h. Samples were taken at immediately after papain treatment (t=0h, black histograms) and 3.5h after treatment (dashed histograms) for staining with the monoclonal antibodies HD6, HC-10, ME-1 and W6/32 as indicated. Data is representative of 3 independent experiments.

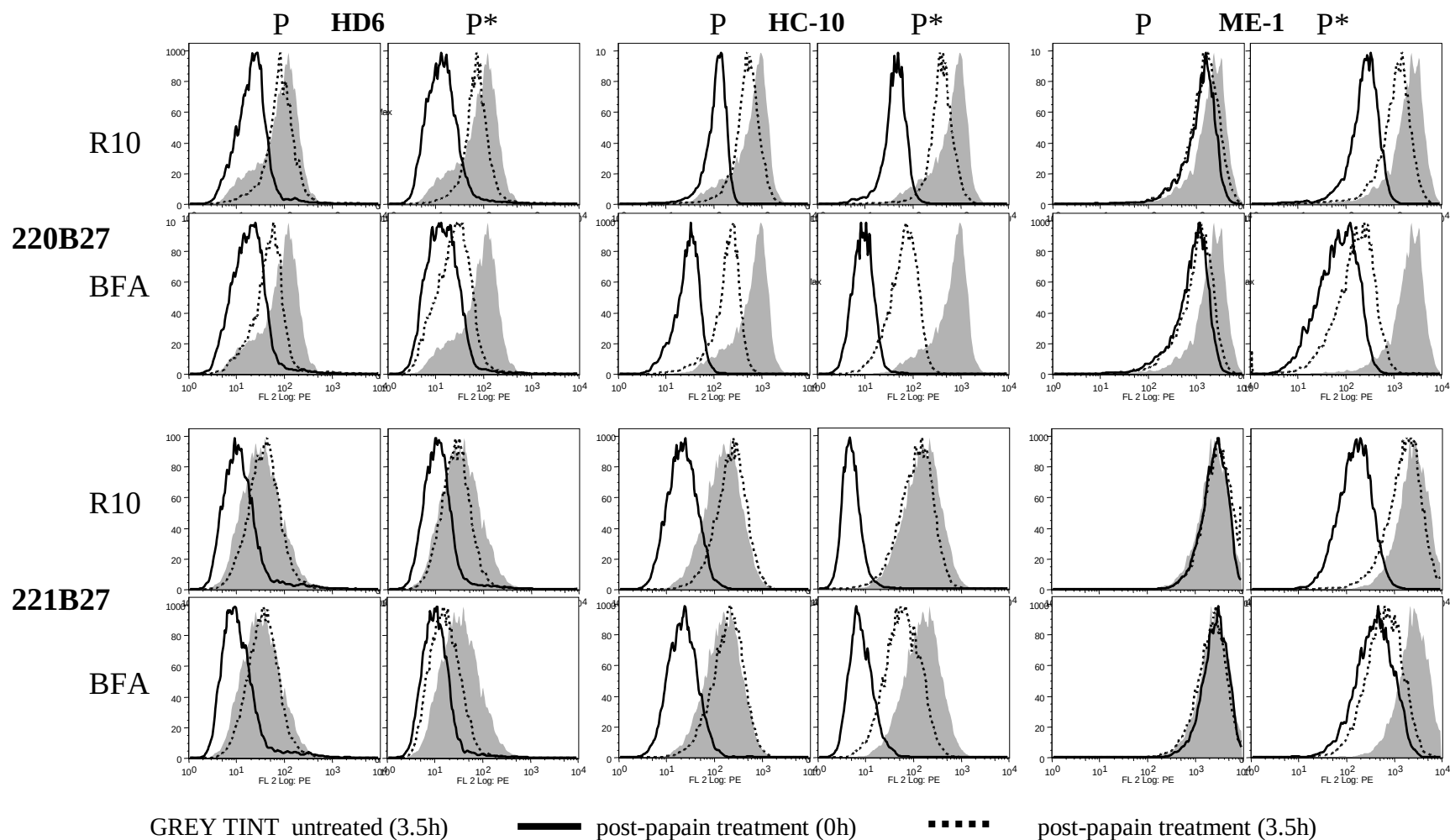


Figure 4.16 Recovery of HD6 and HC-10 surface staining is inhibited by BFA only after treatment of cells with cysteine-activated papain. 220B27 and 221B27 cells were treated with papain alone or cysteine-activated papain as before in the presence and absence of BFA (10ug/ml). Samples were taken immediately post-papain treatment (0h, black histograms) and 3.5h after treatment (dashed histograms), stained with the monoclonal antibodies HD6, HC-10 and ME-1 and compared to untreated cells (grey tint histograms). Data is representative of three independent experiments

As before, HD6 and HC-10 staining recovered following treatment with both papain alone (P) or activated papain (P*) and reached levels similar of those in untreated cells (first and third rows). This recovery was also observed when papain alone was combined with BFA treatment, albeit to a slightly lesser extent (second and fourth rows), indicating HD6-reactive molecules are not newly synthesised molecules direct from the ER. A more marked inhibition of recovery was observed when BFA was combined with activated papain treatment for both HD6 and HC-10 (second and fourth columns, dashed histograms). Notably, complete inhibition was not observed in these experiments, although this is likely a result of remaining surface HLA-B27 molecules not cleaved by P*, which could be detected by ME-1 following treatment (last row, black histograms). Re-acquisition of ME-1 staining following activated papain treatment was almost completely inhibited in the presence of BFA, confirming that the egress of newly synthesised class I molecules was inhibited.

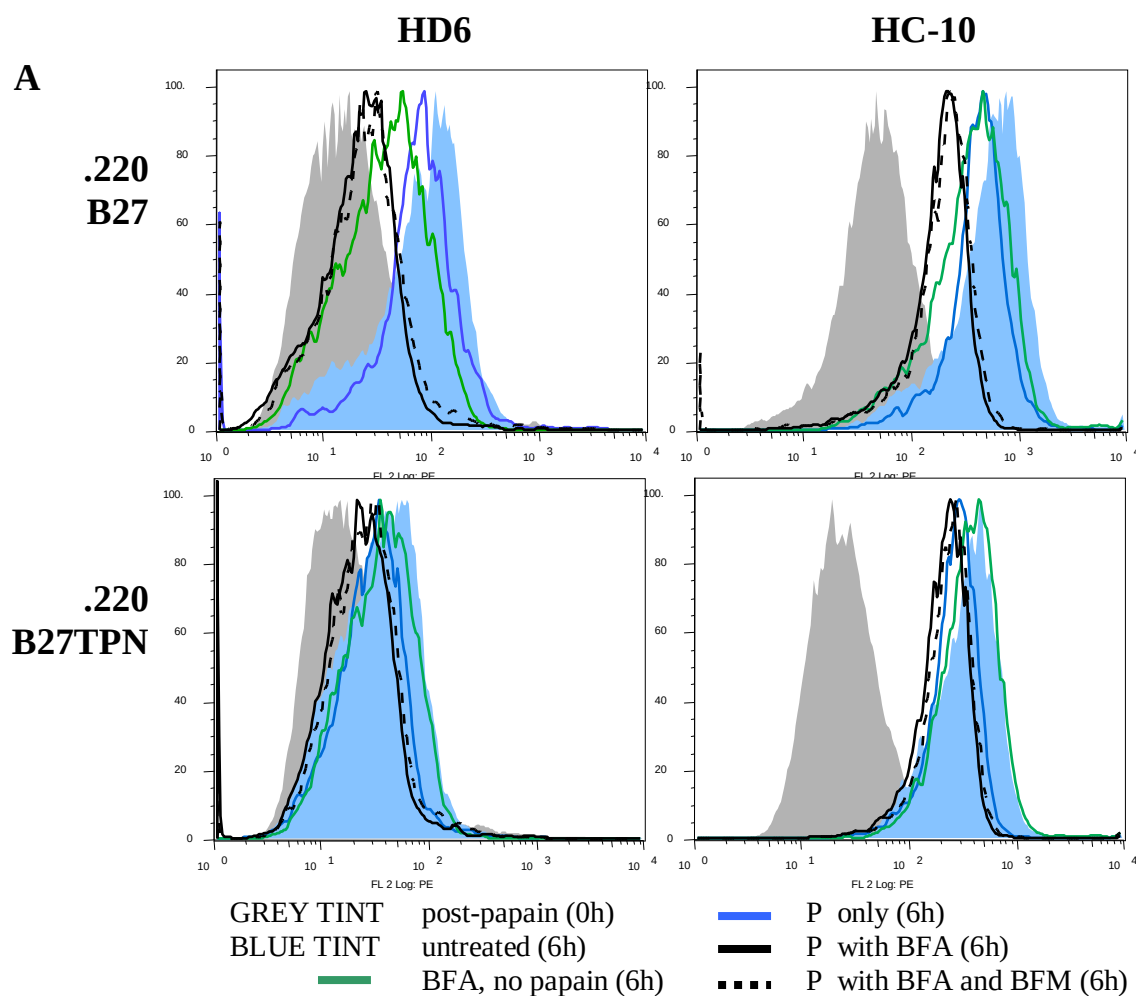
4.4.3 The absence of functional tapasin in .220B27 cells influences the recovery of surface HLA-B27 molecules following papain treatment and protein transport inhibition

Several studies have shown that .220 cells expressing HLA-B27 have increased free heavy chains on the surface compared with those co-expressing HLA-B27 and functional human tapasin (Peh et al., 1998; Purcell et al., 2000). In chapter three, I demonstrated that HD6-reactive molecules are also increased on the surface of .220B27 cells compared with .220B27TPN cells. It has been suggested that the increase in HC-10 reactive molecules could be attributed to the denaturation of less stable HLA-B27 complexes during egress to the cell surface (Purcell et al., 2000).

Thus, on the basis of these findings, I next sought to establish whether HD6-reactive molecules, as well as HC-10-reactive molecules, could form during the egress of HLA-B27 molecules to the surface in the absence of tapasin. .220B27 and .220B27TPN cells were treated with papain alone (P) with and without BFA and surface recovery of HD6 and HC-10 staining was monitored after 6h. For both cell lines, HD6 staining recovered to levels similar to those of untreated cells 6h after papain treatment in the absence of BFA (Figure 4.17A, first column, blue line histograms). This was also the case for HC-10 staining (second column, blue line histograms). In the 220B27TPN cells, recovery of HD6 staining was slightly reduced by addition of BFA, with a 75% recovery seen in the presence of BFA compared with a 95% recovery without BFA (Figure 4.17B). Similarly, for HC-10, recovery of staining was reduced from 72% to 63% when BFA was added. In contrast, staining of 220B27 cells exhibited a more marked difference with BFA treatment. For HD6 staining, BFA treatment reduced the recovery from 95% to 29% and from 79% to 33% for HD6 staining and HC-10 staining, respectively. Notably, both the HC-10 and HD6 staining levels of .220B27 cells following recovery in the presence of BFA resembled those seen in the .220B27TPN cells. Furthermore, BFA treatment alone had more of an impact on .220B27 cells, resulting in a 31% reduction of HC-10 staining compared with a 10% reduction in .220B27TPN cells. Additionally, and in contrast with the previous observations of our group, treatment of cells with either of the endosomal recycling inhibitors chloroquine and bafilomycin at the same concentrations previously described in (Bird et al., 2003) did not alter the recovery kinetics of HC-10 reactive molecules (Figure 4.17A, black dashed histograms, and data not shown). Similarly no detectable change was observed for HD6 staining.

Experiments were repeated to allow the re-acquisition of surface molecule after papain treatment to be monitored at several further time points (Figure 4.18). These data confirm the previous findings, as recovery of both HD6 (left graphs) and HC-10 staining (right graph) was inhibited by BFA following treatment with cysteine-activated papain (grey filled triangles with long dashed lines) but not papain alone (black filled diamonds with long dashed lines) over the time course, and this was not affected by the addition of endosomal inhibitors. These data have also reproduced the finding that the absence of tapasin in .220B27 cells appears to result in a cohort of HC-10 reactive and HD6-reactive molecules (not present in .220B27TPN cells) that do not recover following treatment with activated-papain and BFA.

In summary, these data indicate that i) HD6 epitope is susceptible to papain digestion in a similar manner to HC-10 ii) HD6-reactive molecules can arise from cell surface heterodimers in the presence of BFA iii) a subset of HD6-reactive molecules are present in .220B27 but not 220B27TPN, which may form during the egress of HLA-B27 molecules to the cell surface. Although a role for endosomal recycling in HD6 epitope formation, or indeed HC-10 epitope formation, was not demonstrated, further clarification of this is required.



B

Cell Treatment	% recovery (compared with untreated cells)			
	220B27		220B27TPN	
	HD6	HC-10	HD6	HC-10
Untreated	100	100	100	100
P only (0h)	17	10	50	7
P only (6h)	95	79	95	72
P + BFA (6h)	29	33	75	63
P + BFA + BFM (6h)	31	34	75	57
BFA only (6h)	50	69	88	90

Figure 4.17 Recovery after treatment with activated-papain and BFA reveals of a cohort of HD6- and HC-10-reactive molecules in .220B27 cells not present in .220B27TPN cells.

(A).220 cells expressing HLA-B27 alone (B27) or with tapasin (B27TPN) were treated as before with papain alone (P), papain with BFA, or papain with BFA and bafilomycin (BFM). Cells were washed, and resuspended in fresh R10 supplemented with the appropriate inhibitors where required. Samples were taken at 0h and 6h after treatment and staining as indicated. (B) % recovery was calculated by converting the MFI value of treated cells into a percentage of the MFI value of untreated cells.

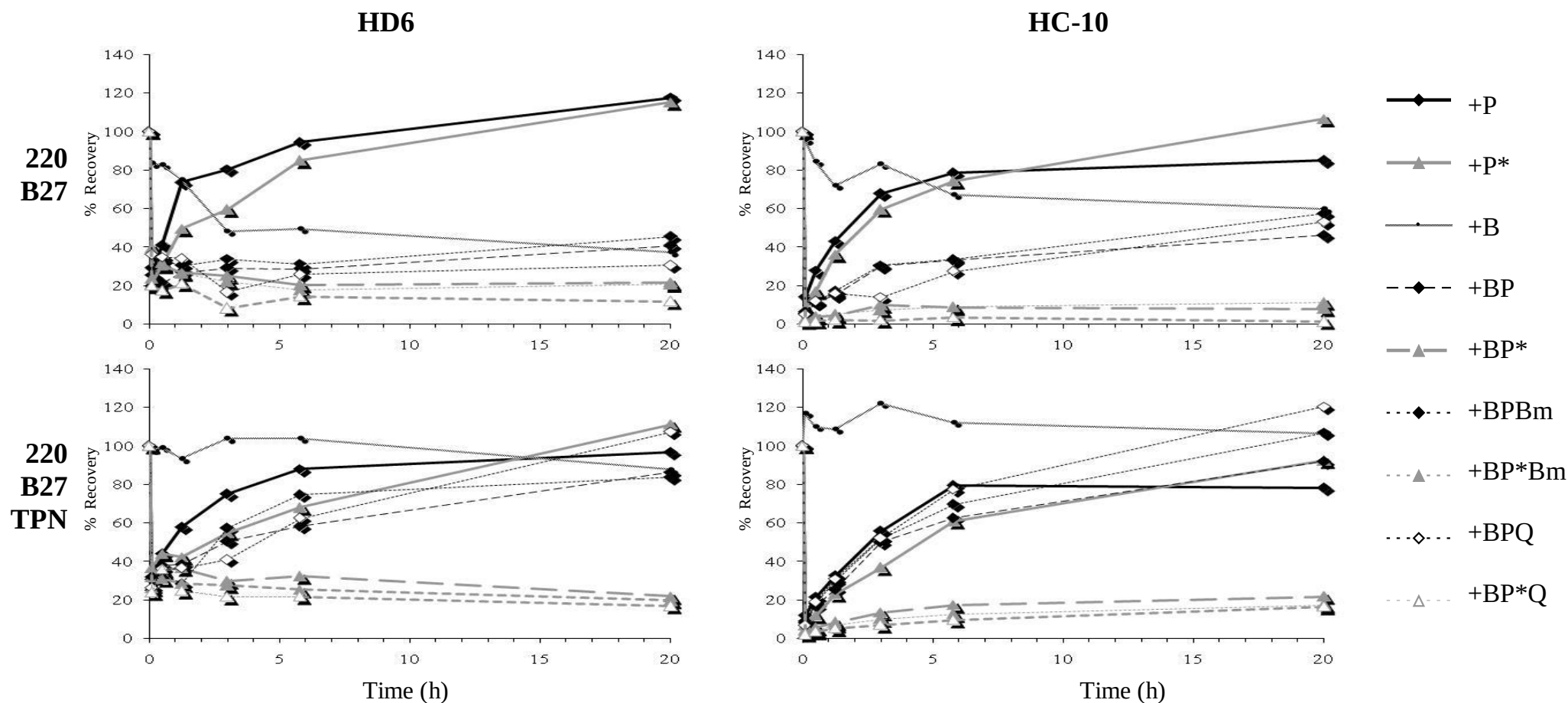


Figure 4.18 Re-expression of cell surface HD6 reactivity following stripping with activated papain requires egress of newly synthesised proteins from the ER. 220B27 and 220B27TPN cells were treated with papain alone (+P), cysteine-activated papain (+P*), BFA alone (+B), or a combination of papain with BFA (BP and BP*), with BFA and bafilomycin (BPBm and BP*Bm) or with BFA and chloroquine (BPQ and BP*Q). After washing, cells were re-cultured in R10 supplemented with inhibitors where required and samples were taken for staining with monoclonal antibodies 0, 30, 75, 180, 350, 1800 min after treatment. Data is representative of two independent time course experiments.

4.5 Preliminary Epitope Mapping of the HD6 epitope

To determine whether the epitope recognised by HD6 is a linear sequence in the HLA-B27 heavy chain, overlapping 20mer peptides were synthesised covering residues 1-275 of B27 HC and used in peptide blocking assays and a peptide ELISA. These methods have previously been used in antibody epitope mapping, with the former being employed to help elucidate the HC-10 epitope (Perosa et al., 2003). In the following experiments, the HC-10 epitope, PXXWDR, was used as a positive control. The sequences of the overlapping peptides are shown in Table 4.1.

PEPTIDE		AMINO ACID SEQUENCE
P1	1-20	G S H S M R Y F H T S V S R P G R G E P
P2	16-35	G R G E P R F I T V G Y V D D T L F V R
P3	31-50	T L F V R F D S D A A S P R E E P R A P
P4	46-65	E P R A P W I E Q E G P E Y W D R E T Q
P5	61-80	D R E T Q I C K A K A Q T D R E D L R T
P6	76-95	E D L R T L L R Y Y N Q S E A G S H T L
P7	91-110	G S H T L Q N M Y G C D V G P D G R L L
P8	106-125	D G R L L R G Y H Q D A Y D G K D Y I A
P9	121-140	K D Y I A L N E D L S S W T A A D T A A
P10	136-155	A D T A A Q I T Q R K W E A A R V A E Q
P11	151-170	R V A E Q L R A Y L E G E C V E W L R R
P12	166-185	E W L R R Y L E N G K E T L Q R A D P P
P13	181-200	R A D P P K T H V T H H P I S D H E A T
P14	196-215	D H E A T L R C W A L G F Y P A E I T L
P15	211-230	A E I T L T W Q R D G E D Q T Q D T E L
P16	226-245	Q D T E L V E T R P A G D R T F Q K W A
P17	241-260	F Q K W A A V V V P S G E E Q R Y T C H
P18	256-275	R Y T C H V Q H E G L P K P L T L R W E

Table 4.1 Overlapping peptides used in epitope mapping experiments.

Overlapping 20mer peptides were generated based on the linear sequence of HLA-B27.

For the peptide blocking assay, the HD6 antibody was incubated with molar excess of each peptide (P1-P18) on ice for 1h. As a positive control, HC-10 was either incubated with peptide P4, containing the PXXWDR motif (PEYWDR), or with control peptides P3 or P5. The blocked antibodies were then used to stain .220B27, .221B27 and C1RB27 cells as normal. Pre-incubation of HC-10 with peptide P4, but not control peptides P3 and P5, abrogated HC-10 binding to all B27-expressing cell lines tested (green histograms, Figure 4.19A). In contrast, none of the peptides were able to reduce the ability of HD6 to bind to cells (Figure 4.19B). To verify this, HD6 binding to the B27-derived linear peptides was tested by ELISA. An ELISA plate was coated overnight with the linear peptides (P1-P18), blocked in assay diluent, and then probed with the HD6 or HC-10 antibodies followed by anti-mouse HRP for detection. Wells were set up in duplicate at two different concentrations for each peptide tested, as shown in Figure 4.19C. A signal was only detected for the wells coated with peptide P4 and probed with HC-10. Absorbance readings are shown for peptides P3-P5 probed with HC-10 (blue) and HD6 (purple).

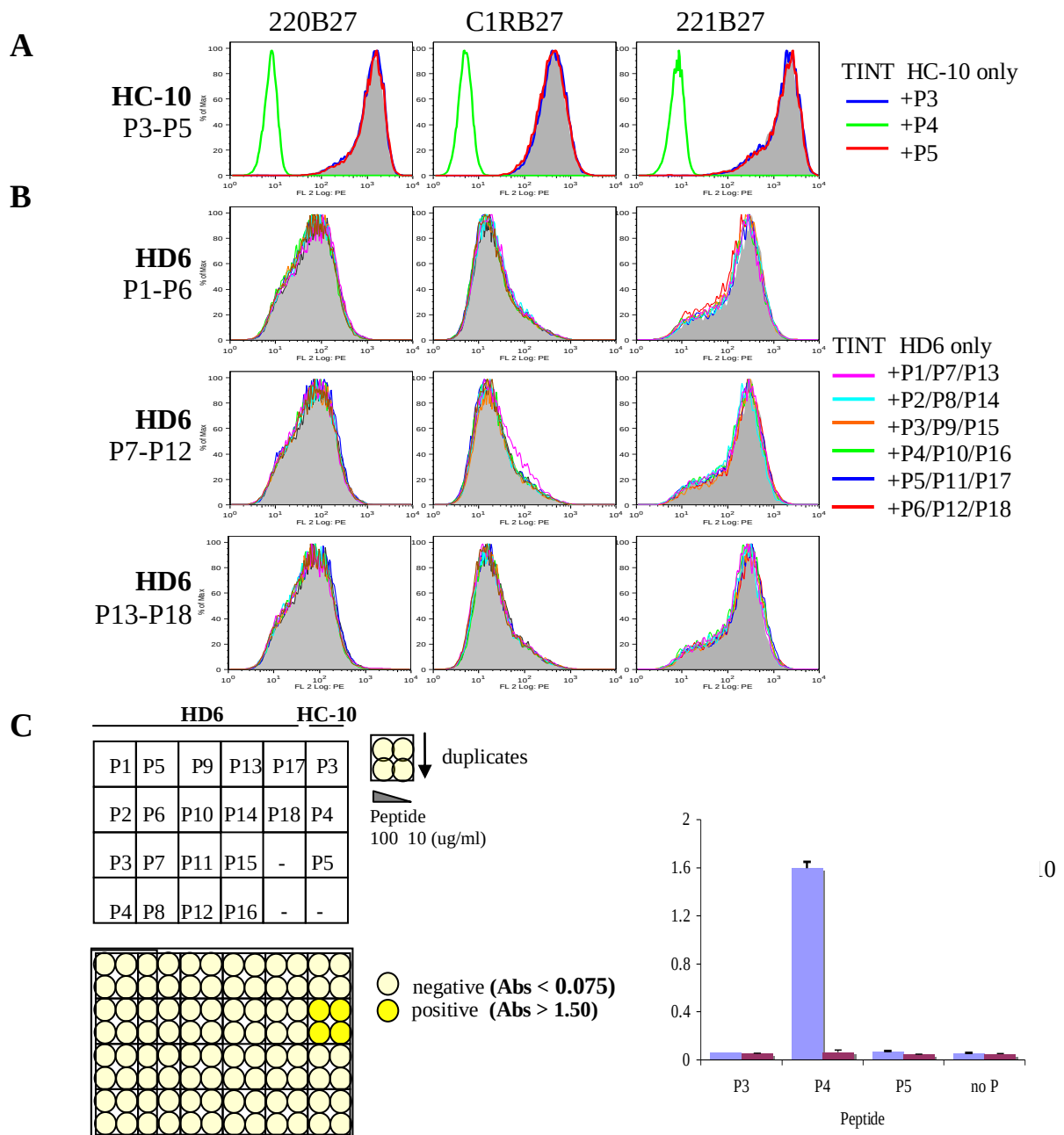


Figure 4.19 Mapping of the HD6 epitope. (A) Molar excess of B27-derived linear peptides were incubated with HD6 or HC-10 for 1h on ice, before the mAbs were used to stain 220B27, C1RB27 and 221B27 as indicated. Antibody alone was used as a control (grey tinted histograms). (B) B27-derived peptides were tested by ELISA for their ability to bind to HD6 or HC-10. Peptides in coating buffer were added to ELISA plate wells at 100ug/ml or 10ug/ml in duplicate (left schematic). The plate was blocked and then probed with HD6 or HC-10 and anti-mouse HRP for detection at 405nm. Positive wells are indicated in dark yellow. Absorbance readings are shown on right for peptides P3-P5.

4.6 Discussion

What form or forms of HLA-B27 are bound by HD6? HD6 binding to *in vitro* refolded B27₂ complexes has been established in ELISA experiments, and, as discussed in the previous chapter, binding to several transfected B cell lines in a B27-restricted manner has been demonstrated. Yet, whether cell-surface HD6-reactive molecules represent B27 heavy chain dimer complexes and/or alternative novel forms of B27 was not determined. While it is anticipated that suitable biochemical methods will offer a means to distinguish between these possibilities, further insight can also be gained from investigation of factors that influence the expression of the HD6 epitope. In particular, the origin and fate of these molecules at the cell surface can be explored. In this chapter, the role of peptide and components of the peptide-loading complex as well as the role of unpaired cysteines in B27 have been examined. Together, the findings in this chapter indicate i) HD6 binding can be abrogated by the presence of B27-binding peptides, ii) HD6 likely binds to a peptide-free conformer of HLA-B27, iii) tapasin and TAP, but not calnexin are required for the formation of a pool of 'acid-inducible HD6' molecules that bind to HD6 following acid treatment, iv) HD6-reactive molecules are sensitive to digestion by the proteases papain and trypsin and can arise from cell surface heterotrimers in the presence of BFA, resembling the behaviour of HC-10-reactive molecules, and v) epitope formation is not dependent on the unpaired C67 residue in the B pocket of the peptide-binding groove, or on the C308 and C325 residues in cytoplasmic tail. In addition, preliminary epitope mapping studies demonstrate that HD6 does not bind to HLA-B27-derived linear peptide sequences, suggesting that the HD6 epitope is a

conformation-dependent discontinuous peptide sequence. This may explain the failure of HD6 to react in western blots, unlike HC-10. On the basis of these findings, I propose a possible model as an explanation for the observed HD6 binding behaviour, which will be discussed.

i) HD6 binding can be abrogated by the presence of B27-binding peptides

Previous studies have reported that the stability of MHC class I molecules at the surface can be enhanced upon culturing cells in the presence of appropriate peptide ligands (Lie et al., 1991; Lie et al., 1990; Ljunggren et al., 1990; Schumacher et al., 1990). Moreover, several groups have demonstrated this specifically for HLA-B27 surface expression (Bird et al., 2003; Carreno and Hansen, 1994; Vazquez and Lopez de Castro, 2005). For example, Carreno and colleagues have shown that overnight culture of HLA-B27 transfected murine L cells with HLA-B27-binding peptide ligands i) reduces the ratio of surface-free (HC-10-reactive) HLA-B27 molecules to surface-folded (W6/32-reactive) HLA-B27 molecules, and ii) increases the half-life of W6/32-reactive molecules, while decreasing the half-life of HC-10 reactive molecules. Similarly, our group have shown that incubation of human .220B27 cells with a high-affinity B27 peptide ligand increased the steady-state level of W6/32-reactive molecules, while substantially decreasing the level of surface HC-10-reactive molecules. Importantly, the latter subset of molecules included HC-10-reactive homodimers. In this chapter I present an extension of these studies, whereby I have shown that, as well as reducing HC-10 staining, addition of B27-binding peptides abrogates HD6 binding to .220B27, .221B27 and C1RB27 cell lines. How exogenous peptide achieves these effects could be explained by several possible mechanisms: i)

peptide binds to unstable B27 complexes at the surface, preventing their dissociation and the generation of free heavy chains and/or the HD6-reactive molecules, ii) peptide stabilises B27 complexes in the ER, preventing the egress of unstable B27 molecules to the cell surface that subsequently dissociate to form free heavy chains and/or the HD6-reactive molecules iii) peptide induces the refolding of HC-10- and/or HD6-reactive conformers in the presence of β_2m , or iv) peptide induces partial refolding of HC-10-reactive and/or HD6-reactive molecules, independent of β_2m , thereby blocking their respective epitopes.

Although one study has reported TAP-independent delivery of exogenous fluorescently-labelled peptides to the ER, subsequent egress of these peptides to the cell surface could not be confirmed, and thus it has been proposed that exogenous peptides are unlikely to exert their influence within the ER (Day et al., 1997). In agreement with this, I observed a reduction in both HC-10 and HD6 staining within 1h of peptide addition, which would appear an insufficient amount of time for peptides to reach the ER and stabilise the exiting MHC class I molecules. Similarly, in experiments where unbound exogenous peptides were washed away after a 1h incubation period, both HC-10 and HD6 staining recovered within a further 1h. If peptide were to act on ER-localised molecules, I would have expected a longer lag period (>1h) before recovery of staining was observed. Whether peptide acts by stabilising folded molecules, or by inducing full or partial refolding of free heavy chains is less clear. In agreement with Vazquez *et al.* (Vazquez and Lopez de Castro, 2005), I found addition of peptide to have little effect on ME-1 staining, indicating that complete refolding of B27 molecules does not occur to a significant extent. My

data cannot exclude the possibility that peptide-induced partial refolding of HC-10- and HD6-reactive molecules occurs, which remain undetected by ME-1. However, I suggest that it is more likely that peptide stabilises folded surface molecules given the previously reported increased half-life of W6/32-reactive molecules in the presence of peptide and the comparable kinetics of HC-10 and HD6 recovery shown here. This also suggests that a similar mechanism applies for both HC-10-reactive and HD6-reactive molecules. This would be consistent with a model whereby HD6-reactive molecules derive from unstable cell surface B27 heterotrimers in a similar manner to HC-10, wherein the stability of the HLA-B27 molecules is central in determining the amount of HD6 formed.

ii) HD6 likely binds to a peptide-free conformer of HLA-B27

The data in this chapter strongly suggest that HD6 recognises a peptide-free conformer of HLA-B27. This is in contrast to a previously characterised B27-specific antibody, MARB4, which also recognises a novel subset of β_2m -free HLA-B27 molecules distinct from HC-10-reactive molecules (Urban et al., 1994). Incubation of T2B27 and .221B27 cells with a subset of B27-binding peptides was shown by Malik *et al.* to induce MARB4 staining (Malik et al., 2002). Yet, I have shown here that under the same conditions and with the same peptides, no HD6 staining is induced, confirming the distinct reactivities of these B27-specific mAbs. Moreover, acid treatment of B27-expressing cells, which Malik *et al.* reported to abrogate MARB4 binding as a result of peptide dissociation, had the opposite effect on HD6 staining, further emphasising the peptide-free nature of the HD6 epitope. Low pH treatment of cells has often been used to denature MHC class I molecules at the surface, disrupting

the association of peptide and β_2m with the class I heavy chain. This can be detected through the loss of staining with W6/32, BBM.1 and, in the case of HLA-B27 molecules, ME-1, as well as through the substantial increase in staining with HC-10. These changes were seen for all cells I treated with acid. Additionally, with two notable exceptions, HD6 staining was induced and/or increased on all B27+ cell lines when acid-treated, including EBV-transformed BCLs and cells expressing HA-tagged B27 molecules. This suggests that a cohort of B27 molecules exists on the surface of these cell lines, which I have termed ‘acid-inducible HD6’ molecules, that undergo some sort of conformational change upon acid treatment to reveal the HD6 epitope. Moreover, the presence and/or conversion of this cohort into HD6-reactive molecules is not impaired by an HA-tag, and was similar for both the 05 and 09 subtypes. Also, given the time frame in which this effect occurs, it is likely that conversion of ‘acid-inducible HD6’ molecules into HD6-reactive molecules occurs directly at the surface and not through a recycling compartment, ruling out the possibility that acid treatment triggers increased endosomal recycling events. Importantly, however, ‘acid-inducible HD6’ molecules are not present on the surface of all B27+ cells, as discussed below.

iii) tapasin and TAP, but not calnexin, are required for the formation of a pool of ‘acid-inducible HD6’ molecules that bind to HD6 following acid treatment

I have demonstrated that acid treatment has no effect on HD6 staining of two sets of B27+ cells: 220B27 and T2B27 cells, which have inherent deficiencies in the peptide loading complex, and in C58B27 cells with low surface ME-1 staining. This indicates firstly that not all ME-1-reactive B27 molecules are capable of undergoing the acid-

induced conformational change that results in HD6-reactivity, consistent with the proposal that only a cohort of B27 molecules are ‘acid-inducible HD6’ molecules. Furthermore, the presence of this cohort requires functional peptide loading machinery, in particular functional tapasin and the TAP transporter. This was best exemplified in the .220B27 and .221B27 cells expressing HA-tagged B27 molecules, with comparable ME-1 staining. As a result of the HA tag, no HD6 staining was observed prior to acid treatment. However, following acid treatment, HD6 staining was only detected in the .221B27 cells. In contrast, calnexin is not required, as shown through acid treatment of the calnexin-deficient NKR-B27 transfected cells.

The dependency on tapasin for the formation of ‘acid-inducible HD6’ molecules is intriguing, given the relative independency of HLA-B27 on tapasin for normal surface expression. Although no crystal structure of tapasin in complex with an MHCI molecule has been obtained, information relating to their interaction within the PLC has been gained from both mutagenesis studies and molecular modelling studies, which have been aided in light of the recently solved structure of the tapasin-Erp57 heterodimer. The interactions between the components comprising the PLC have recently been reviewed in detail (Van Hateren et al., 2010). Together, these studies have shown two regions of the MHCI heavy chain are key for its interaction with tapasin: a site in the $\alpha 3$ domain (residues 227/229), which is also important for CD8 recognition, and a site in the $\alpha 2$ domain (residues 128-136), which has been proposed to be important for the ability of tapasin to monitor the conformation of the H chain when peptide binds (Garstka et al., 2011). In particular, molecular dynamic simulations with two HLA-B44 subtypes differing in their dependence on tapasin for

surface expression, B*44:02 (tapasin-dependent) and B*44:05 (tapasin-independent), have provided insight into how conformational changes within the $\alpha 2$ domain above the tapasin binding site as a consequence of peptide binding could translate to this region and how this is affected by polymorphism at position 116 (Garstka et al., 2011). Applying this to HLA-B27 would suggest that the relative tapasin independence of B27 could be explained by the presence of residues at positions 114 and 116 that preserve the integrity of the F-pocket in the absence of bound peptide in a similar manner to B*44:05, enabling it to maintain a peptide-receptive conformation in the absence of tapasin. Tapasin serves as a molecular bridge between MHC class I: β_2m heterodimers and formation of the full peptide loading complex, thus in its absence PLC assembly is severely impaired. For example, Peh *et al.* demonstrated that only low levels of TAP-associated MHC class I heavy chains could be detected by anti-TAP immunoprecipitations of .220B27 cells (Peh et al., 1998). While peptide loading can be achieved in the absence of tapasin, and thus presumably without the association of MHCI: β_2m heterodimers with TAP, this tapasin-independent assembly pathway is likely to differ markedly in its association of MHCI: β_2m with other components of the PLC compared with the tapasin-dependent pathway. Intriguingly, tapasin may also fulfill a chaperone role itself outside of the ER. On the basis of these properties relating to B27 and tapasin, I propose several possible mechanisms as explanations. Firstly, one possibility to address the lack of ‘acid-inducible HD6’ molecules in tapasin-deficient cells is that these molecules require transit through the PLC for their assembly. This could result in the formation of chaperone-associated B27 molecules that escape to the cell surface only in cells with a functional PLC. Upon acid treatment, the associated chaperone may dissociate

from the B27 heavy chain with β_2m and peptide to reveal the HD6 epitope. Alternatively, tapasin-dependent association of a chaperone with B27 heavy chains may occur within endosomal recycling compartments, that masks the HD6 epitope but can be revealed upon acid treatment. This chaperone may be tapasin itself or another known chaperone associated with the PLC, such as BiP, calreticulin, ERp57, or PDI, or an as yet unidentified chaperone. Lending support to these proposals are the findings that have demonstrated the presence of some of these chaperones in association with MHC class I molecules outside of the ER (Arosa et al., 1999; Ghanem et al., 2010; Spiro et al., 1996; Tran et al., 2004). For example, Howe *et al.* have recently proposed that calreticulin binds to suboptimally loaded class I molecules in the ER, then transits through the ERGIC and *cis*-Golgi with them before mediating their retrieval via its C-terminal KDEL sequence (Howe et al., 2009). Although calreticulin has been shown to bind MHCI in the absence of tapasin (Solheim et al., 1997), it is also possible that some of this retrieval occurs within the context of other PLC components, including tapasin. On this basis, Howe *et al.* suggest two possible retrieval pathways: a calreticulin-mediated pathway that operates independent of the PLC, and a PLC-retrieval pathway that may be especially important for MHCI molecules binding tightly to the PLC. I suggest that it is this latter pathway, or one similar, that could allow the escape of some chaperone-associated molecules to the surface that form the pool of ‘acid-inducible HD6’ molecules, which are absent in some PLC-deficient cells. It would be interesting to see whether acid treatment of the calreticulin-deficient cell line K42 expressing HLA-B27 yields similar results to tapasin-deficient cells. A third possible mechanism may simply involve a conformational change in a cohort of B27 molecules that transit

through the tapasin-dependent assembly pathway that renders these molecules capable of forming the HD6 epitope upon acid treatment. Further investigation into the interactions of surface HLA-B27 molecules with PLC members is required to provide more insight into the credibility of these models.

The lack of 'acid-inducible HD6' molecules in the TAP-deficient T2B27 cells may be a consequence of reduced surface expression of the class I molecules in the absence of optimum peptide loading, even after incubation at low temperature. This would be consistent with the findings in the C58 cells discussed below. Alternatively, the absence of TAP results in a failure to form a functional PLC that is a prerequisite for the formation of 'acid-inducible HD6' B27 molecules, as suggested in the above.

Finally, the presence of 'acid-inducible HD6' molecules at the surface appears dependent on the expression levels of B27 in C58 cells. In C58B*27:05 and C58B*27:09 cells that I generated with our untagged lentivirus constructs, HD6 staining was induced upon acid treatment, indicating the presence of the 'acid-inducible HD6' molecules at the surface of these cells. Yet, this cohort was not seen in other C58B27 cells with lower ME-1 staining. The importance of high B27 surface expression levels for 'acid-inducible HD6' molecules was confirmed when these latter cells were incubated overnight at 26°C, resulting in the appearance of acid-induced HD6 staining. Again, HLA-B27 subtype polymorphisms did not inhibit this phenomenon, as C58 cells expressing the variants 116Y, 116H (B*27:09) and 114D116Y (B*27:06) all stained with HD6 following acid treatment, which was increased upon overnight incubation at 26°C. Importantly, despite the requirement for

a seemingly high level of HLA-B27 in the rat C58 cells, the threshold level of HLA-B27 surface expression required for formation of the pool of ‘acid-inducible HD6’ molecules appears to be in the physiological range in human cell lines, as shown for the EBV-transformed B cell lines derived from B27+ individuals. Thus, it is plausible that this artificial acid treatment may mimic the effect of long-term exposure of surface B27 molecules to the decreased pH conditions that are associated with inflammation of affected tissues (Steen et al., 1995). This is in line with one study that has demonstrated moderate changes in pH values have the potential to destabilise B27 heterotrimeric complexes in a subtype dependent manner (Fabian et al., 2011).

In summary, these data are consistent with a model whereby surface HLA-B27 molecules can be divided into two subsets based on their ability to form the HD6-recognised epitope following acid treatment: those that can, which I have termed ‘acid-inducible HD6’ molecules, and those that can’t. The ‘acid-inducible HD6’ molecules are absent in tapasin-deficient cells and TAP-deficient cells, suggesting that their formation could either require transit through a tapasin-dependent assembly pathway, or is a result of a tapasin-dependent endosomal recycling pathway.

iv) HD6-reactive molecules are sensitive to digestion by the proteases papain and trypsin and can arise from cell surface heterotrimers in the presence of BFA, resembling the behaviour of HC-10-reactive molecules

How do HC-10-reactive and HD6-reactive molecules derive from unstable heterotrimeric precursor molecules? It is widely accepted that surface free heavy

chains can derive from sub-optimally loaded MHC class I molecules, yet whether they form directly as a result of peptide and β_2m dissociation at the surface, whether they require transit through an endosomal recycling pathway, or whether it is through a combination of both these mechanisms is unclear. Moreover, the contribution of recycling pathways toward formation of HD6-reactive and HC-10-reactive HLA-B27 species may differ. To address this, our group and others have used the protease papain, which can selectively remove β_2m -free but not β_2m -associated class I heavy chains from the surface, thereby enabling their recovery to be monitored in the presence of inhibitors (Bird et al., 2003; Pickl et al., 1996; Santos et al., 2006). Similar studies have also been carried out with the protease trypsin, which has also been shown to selectively remove surface free heavy chains (Demaria et al., 1992; Pickl et al., 1996). Together, these studies have confirmed that newly expressed β_2m -free heavy chains originate from surface β_2m -associated MHCI molecules. Moreover, through use of chloroquine, a lysosomotropic agent reported to neutralise the pH of endosomal/lysosomal compartments, they have indicated a central role for acidification of endosomes in this process, at least within the first few hours of recovery. In Bird *et al.*, chloroquine treatment resulted in almost complete inhibition of HC-10 recovery following papain treatment in both the Hom2 and HLA-B27 transfected C1R B cell lines. However, under similar conditions, Pickl *et al.* demonstrated that, for the HUT-102 T cell line, chloroquine only partially inhibited the recovery of LA45 staining, another β_2m -free heavy chain mAb, suggesting that acidification of endosomes may not be the only mechanism of β_2m -dissociation. These differences seen in the surface recovery of β_2m -free forms of class I HC and the extent to which endosomal

acidification is involved, may be due to different rates of internalisation and recycling in these cells. Additionally, it is worth noting that in order to distinguish between newly synthesised molecules and recycling molecules, both these studies use BFA to inhibit egress of the former. Yet, this itself may impact on the recycling of class I molecules, either by affecting the availability of components of the recycling machinery, or by reducing the number of cell surface molecules and therein relieving the pressure place on recycling pathways.

Taking the above into consideration, this thesis similarly investigated the susceptibility of HD6-reactive molecules to the proteases papain and trypsin, and how recovery of HD6 staining after treatment was affected by BFA and also the endosomal acidification inhibitors chloroquine and bafilomycin. As for HC-10, HD6 staining was abrogated by treatment with these proteases. This is likely due to both forms of molecule lacking an association with β_2m that would otherwise offer some protection against protease cleavage. In addition, and in line with previous findings, these data provide further evidence that newly expressed HD6-reactive molecules also originate from surface β_2m -associated HLA-B27 heterotrimers. Specifically, BFA treatment failed to prevent the reappearance of HD6-reactive molecules following their removal with papain. However, BFA treatment did diminish this reappearance if both free heavy chains and a substantial proportion of ME-1-reactive material were removed by activated papain. In the presence of the inhibitors bafilomycin and chloroquine, I did not observe any inhibition of the recovery of either HC-10-reactive or HD6-reactive molecules following papain treatment above that induced by BFA treatment. This apparent inconsistency with the previous data

reported by Bird *et al.* could be explained by differences in the cell lines investigated. The .220B27 and .220B27TPN cells investigated here may not heavily depend upon endosomal acidification in the recycling compartments for β_2 m-dissociation, as seen for the HUT-102 T cells described in the above. Alternatively, other mechanisms for β_2 m-dissociation such as the direct dissociation at the cell surface may compensate in the absence of endosomal acidification. This would be consistent with the findings in Santos *et al.*, whereby the mutant B27-Y320F molecule, which failed to undergo spontaneous endocytosis, retained comparable HC-10 staining to cells expressing WT and exhibited similar recovery of HC-10 staining following papain treatment in the presence of BFA. Finally, although these inhibitors were used as described previously and in multiple independent experiments, it is also possible that the functionality of these inhibitors may have been compromised, resulting in their reduced/inhibited activity. Whether endosomal acidification is needed for HD6 epitope formation therefore requires further investigation.

Interestingly, recent data has suggested that ubiquitination may promote MHCI HC internalisation following peptide or β_2 m dissociation at the surface (Boname *et al.*, 2010; Duncan *et al.*, 2010), while an earlier study has indicated the involvement of tyrosine phosphorylation in regulation of misfolded surface MHCI (Santos *et al.*, 2004). Furthermore, dissociation of β_2 m from MHCI HC has been shown to correlate with acquisition of epitopes in the HC cytoplasmic tail that could be used to mediate its internalisation (Little *et al.*, 1995). Although endocytosis and recycling of MHCI is poorly understood, recent studies have indicated it involves clathrin- and dynamin-independent Arf-mediated mechanisms (Naslavsky *et al.*, 2004a; Naslavsky *et al.*,

2004b; Radhakrishna and Donaldson, 1997). Thus, it will be interesting to see whether the same mechanisms apply for recycling of β_2m -free MHCI HC and whether quality control mechanisms exist at the surface, perhaps analogous to those involved in ERAD, or in the recently identified retrieval mechanisms, that distinguish between misfolded and folded MHCI and enable regulation of its surface expression.

Consistent with Pickl *et al.* (Pickl *et al.*, 1996), I have shown removal of adherent cells with trypsin resulted in reduced HC-10 staining compared to cells treated with a lidocaine/EDTA solution or scraped cells. In addition, HD6 staining of the trypsin-treated cells was negligible. Upon re-culturing of trypsinised cells in round bottom plates, HD6 and HC-10 staining were markedly increased. Interestingly, these exceeded the levels seen in the immediately stained lidocaine/EDTA-treated cells. In contrast, no differences were seen for ME-1 staining, indicating this effect is specific for β_2m -free conformers. This could be explained in several ways: i) lidocaine/EDTA treatment also removes some unfolded surface molecules, but to a lesser extent than trypsin ii) reculturing HeLa cells as non-adherent cells stimulates the recycling of β_2m -free molecules from the surface, resulting in a net gain in surface free heavy chains but no change in folded molecules, iii) trypsin itself stimulates the recycling of β_2m -free molecules, iv) fewer peptides are available in the fresh culture media that were previously stabilising heterotrimeric B27 molecules. Consistent with my previous findings, addition of B27-binding peptides to the fresh culture media abrogated this recovery in HD6 staining, which, although doesn't exclude any of the possibilities, would appear to favour (iv). Nevertheless, this re-emphasises the effect peptide has on the inhibition of HD6 staining of cells.

v) *HD6 epitope formation is not dependent on the unpaired C67 residue, nor on the C308 and C325 residues in the cytoplasmic tail*

Our group have previously reported that *in vitro* refolding of HLA-B27 homodimer complexes is strictly dependent on the presence of C67. In agreement, Dangoria *et al.* have shown this residue crucial for HC-10-reactive dimer formation in cells (Dangoria et al., 2002). However, in this chapter, I have shown that this residue is not required for the formation of ‘acid-inducible HD6’ molecules. Moreover, in rat C58 cells expressing the B27 C67S mutant, I demonstrated that overnight incubation at 26°C was able to induce HD6 staining without acid treatment. Consistent with this, our group have since shown HD6 staining in lentivirally-transduced .220 cells expressing the B27 C67S mutant (Simon Kollnberger, unpublished observations). I had not seen this for the transfected .220B27 C67S cells examined in Chapter Three, however the expression levels of HLA-B27 in these cells was markedly lower. Thus, together, these data indicate the HD6 epitope does not require the C67 residue, which suggests that, at least in C67S cells, HD6-reactive molecules are not B27 heavy chain homodimers stabilised by a C67 disulfide bond. It is possible that other cysteine residues may stabilise homodimers in the absence of C67 residues, however, this is unlikely to involve the C308 and C325 residues given that similar HD6 staining in C58 cells was seen for cells expressing the C308S, C325S and C67S/C308S/C325S triple mutant B27 molecules. It seems more feasible that the C67 residue helps stabilise *in vitro* refolded B27 dimer molecules in an HD6-reactive conformation in the absence of a cell membrane. Additionally, these data also indicate that it is unlikely that acid treatment promotes disulfide bond formation of two heavy chains at the cell surface, given the similar results obtained for the various cysteine mutants.

Thus, it appears that dimerisation via the unpaired C67 residue is not a critical factor in determining the pathogenicity of HLA-B27, if HD6-reactive molecules are indeed critical players in the pathogenesis of SpA. Consistent with this is the finding that rats transgenic for the C67S mutant still develop a disease phenotype, and that the lower prevalence of arthritis in these rats is simply a result of fewer copies of the B27 C67S transgene. Interestingly, two studies have shown the C67S mutation leads to B27 complexes with decreased thermodynamic and cell surface stability, as a consequence of weakened pocket B interactions (Alvarez et al., 2001; Reinelt et al., 2001). If formation of HD6-reactive molecules is favoured in the presence of less stable heterotrimeric B27 surface complexes, as would appear to be the case in the absence of tapasin, then it is conceivable that the C67S mutation could promote HD6 staining. This is currently being investigated by our group using the .221 cell line and lentiviral transduction with mutant constructs.

Preliminary epitope mapping

The epitope bound by HC-10 is an unfolded linear epitope within the heavy chain of all HLA-B alleles – PXXWDR (residues 57-62) (Perosa et al., 2003; Stam et al., 1986). Similarly, 4 other notable Mabs, generated to recognise peptide-free or denatured forms of various human MHCI molecules, also detect linear epitopes in regions near the HC-10 epitope: 64-3-7, which binds to a linear stretch of amino acids including 48Q and 50P only exposed in peptide-empty forms of several MHC class I molecules (Yu et al., 1999); LA45 binds to residues 62-63 (Madrigal et al., 1991); L31 binds to residues 66-68 (Grassi et al., 1991); and HCA2 binds to residues 77-84 (Stam et al., 1986). Thus, it was plausible that HD6 also bound to a linear epitope

near this region that is specific for unfolded B27 molecules. Yet, in this chapter I have shown that the HD6 epitope does not appear to be a linear epitope. Using overlapping 20mer peptides derived from the HLA-B27 sequence, I demonstrated in both ELISA and flow cytometry experiments that none of these linear peptides sequences were able to bind to HD6 or block HD6 binding to cells. In contrast, the peptide containing the PXXWDR motif, P4, was able to bind to HC-10 and block HC-10 binding to cells. This suggests that the HD6 epitope is more likely a discontinuous sequence, such as that seen for W6/32. Moreover, given its failure to work as a detection reagent in western blots, it is also likely that the epitope is at least partially conformation-dependent. This is somewhat surprising given that acid denaturation of surface B27 is associated with producing ligand-free MHCI HC that exhibit a molten globule state (MGS), with a much less compact conformation than folded molecules (Bouvier and Wiley, 1998). Yet I have shown acid induces the HD6 epitope, suggesting that at least some acid-denatured B27 molecules retain some partial folding. Whether this is a result of some stabilisation exerted by the association of two HC in a dimer conformation, with or without cysteine disulfide bonds, or by the association of a chaperone, remains to be determined.

A model for the formation of HD6-reactive B27 molecules

On the basis of these findings, I propose the following two part model to account for the formation of steady-state HD6-reactive molecules (Figure 4.20), and for the formation of acid-induced HD6-reactive molecules (Figure 4.21). For steady-state HD6 staining, over-expression of B27 is required, as is seen for .220B27, .221B27, and C1RB27 cells. This results in the saturation of the ERAD machinery that, under

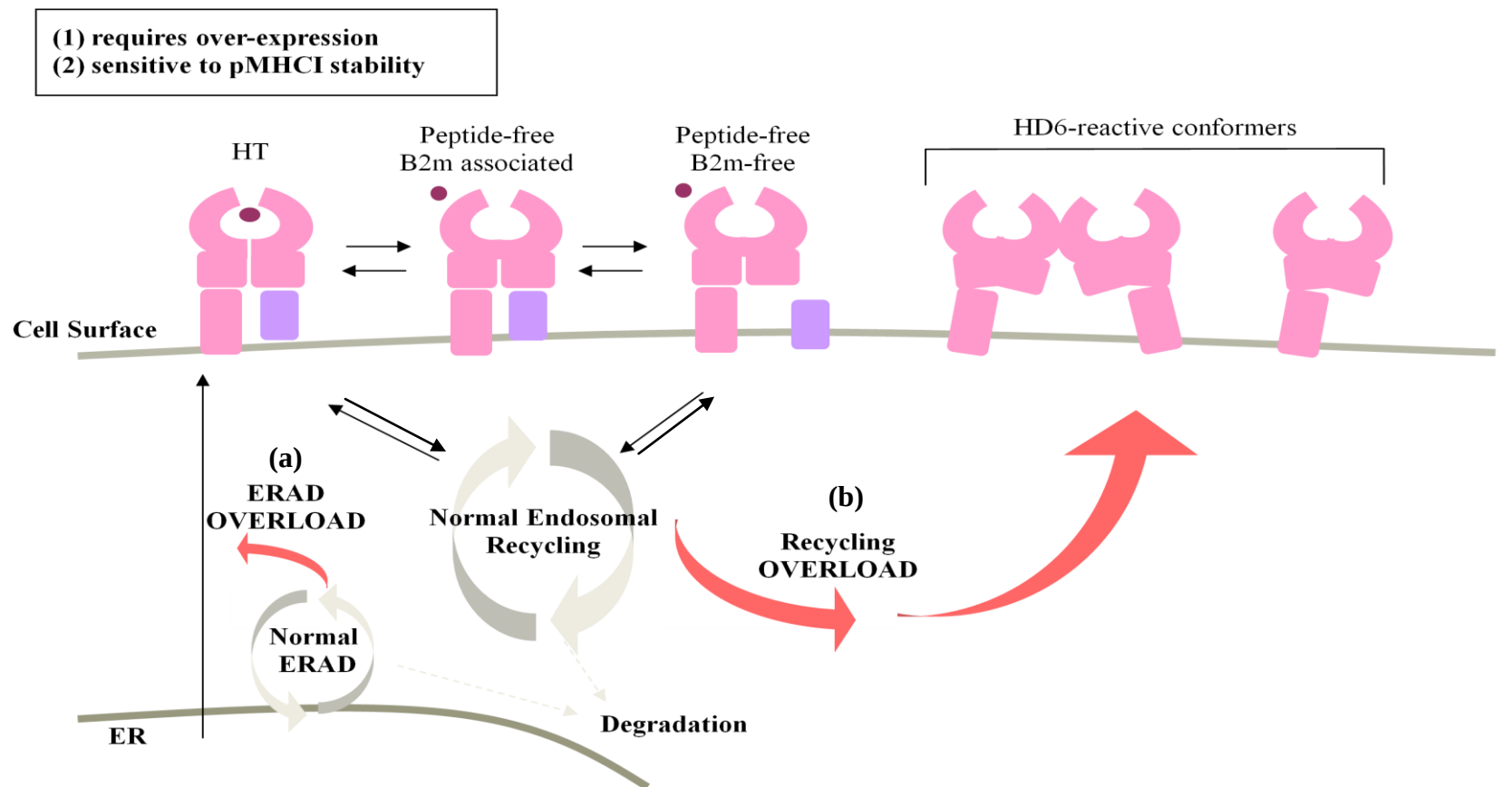
normal conditions, serves as the quality control checkpoint that ensures few suboptimally loaded or misfolded MHCI molecules escape the ER and egress to the surface (a). With an increased number of unstable pMHCI complexes reaching the surface, the normal endosomal recycling pathways also become overloaded as a result of an increased number of pMHCI surface molecules falling apart (b). Under normal conditions, these pathways may serve as similar quality control mechanisms to ERAD, whereby misfolded or empty MHCI molecules that fail to re-load with appropriate antigen are sent for degradation. Yet, saturation of these pathways can result in the escape of misfolded B27 conformers back to the cell surface. The exact nature of these recycling pathways is unknown, and it is also possible that some HD6-reactive conformers may form directly at the cell surface. In C58B27 and T2B27 cells, lowering the temperature induced HD6 staining. This can be explained by the stabilisation effect the lower temperature has on suboptimally loaded pMHCI molecules, enabling their ER escape and surface expression, contributing to the recycling pool of peptide-free B27 conformers. Addition of a C-terminal HA tag to B27 abrogates HD6 binding. This inhibitory effect may be acting at the level of ER egress and its quality control mechanisms, whereby the presence of the tag enables better optimisation of cargo or enables the ERAD machinery to detect the suboptimal-peptide loaded complexes, perhaps by slowing down the egress of folded molecules through the ERGIC and cis-Golgi compartments. Alternatively, the tag may interfere with the endosomal recycling of B27 molecules, preventing their re-appearance at the cell surface as HD6-reactive molecules. However, given that Sv5-tagged B27 molecules in C58B27 cells can form the HD6-reactive epitope under conditions of lower temperature, I suggest the former is more likely i.e. the ability of

the tag to allow more stable complexes to form prior to surface expression is overcome at lower temperature. Finally, although the presence of a C67-C67 disulfide bond may stabilise dimer molecules at the surface, it is not a requirement to have this unpaired C67 residue for HD6 epitope formation.

For the formation of acid-induced HD6-reactive molecules, cells require a functional PLC, which explains why these molecules are not detected in .220B27 and T2B27 cells (Figure 4.21). Under normal conditions, cell surface B27 molecules can be divided into two subsets: (1) a subset of regularly folded heterotrimers and that are bound by HC-10 but not HD6 following acid treatment (Figure 4.21 (a)), and (2) a subset of 'Acid-inducible HD6' molecules that are bound by HD6 following acid treatment (Figure 4.21 (b-d)). The formation of this latter subset is dependent on their transit through the tapasin-dependent assembly pathway and/or post-ER association with tapasin-associated chaperones. This subset is absent in tapasin-deficient and TAP-deficient cells. I propose two possibilities to account for the formation of these 'acid-inducible HD6- molecules: (i) the Conformational change model and (ii) the Chaperone model. In the Conformational change model, a cohort of B27 complexes that undergo tapasin-dependent assembly acquire a conformational change, perhaps as a result of prolonged association with the PLC or the acquisition of a particular pool of peptide epitopes. Acid-induced dissociation of peptide and β 2m then reveal the conformational change in the B27 free heavy chain, which is now able to bind HD6 (Figure 4.21 (b)). Alternatively, the Chaperone model proposes that a cohort of B27 complexes, are chaperone-associated. This unidentified chaperone may be bound to free B27 HC, folded B27 molecules, or B27 dimers (not limited to C67 disulfide

bonded). This interaction may have formed in the ER, following PLC assembly, during ERAD, or as part of an endosomal recycling mechanism (blue dashed arrows). Upon acid treatment, the chaperone may dissociate from a B27 HC along with β 2m and peptide to reveal the hidden HD6 epitope, or may remain attached holding the B27 HC in an HD6-reactive conformation (Figure 4.21 (c)). Alternatively, if the chaperone was interacting with a B27 dimer, acid treatment could result in its dissociation and concomitant exposure of the HD6 epitope (Figure 4.21 (d)).

Although several important questions concerning the nature of the HD6 epitope remain, not least whether it recognises a heavy chain monomer and/or a multimeric form, this tentative model for the formation of HD6-reactive molecules provides a framework within which future study can be carried out. In particular, although highly speculative, the involvement of folding chaperones such as tapasin and calreticulin in quality control mechanisms outside of the ER to ensure regulated expression of folded surface B27 molecules is an intriguing possibility that warrants further investigation that would be relatively straightforward. Most notable was the identification of two distinct pools of surface B27 molecules in tapasin-competent cells vs tapasin-deficient cells with distinct Mab binding reactivities following acid treatment. It is possible that in the former, the presence of the functional PLC masks the appearance of HD6-reactive molecules under physiological conditions. Yet, the capacity of these cells to form HD6-reactive molecules is revealed upon acid treatment, which may reflect similar situations triggered in a disease-setting.



- (a) *ERAD Overload* - overexpression of B27 results in more unstable pMHCI leaving the ER/ escaping ERAD degradation
 (b) *Recycling Overload* - normal endosomal recycling is saturated, allowing escape of misfolded HD6-reactive B27 complexes back to the surface

Lower temperature: increases number of unstable complexes getting to the cell surface, saturating the recycling compartment
C-terminal HA tag: reduces number of unstable complexes leaving ER and/or interferes with recycling to inhibit appearance of misfolded surface B27 conformers

Figure 4.20 Proposed model for the formation of steady-state HD6-reactive B27 molecules (.220B27, .221B27 and C1RB27 cells)

- (1) Requires a functional PLC
- (2) Independent of C-terminal Tag
- (3) Does not require C67

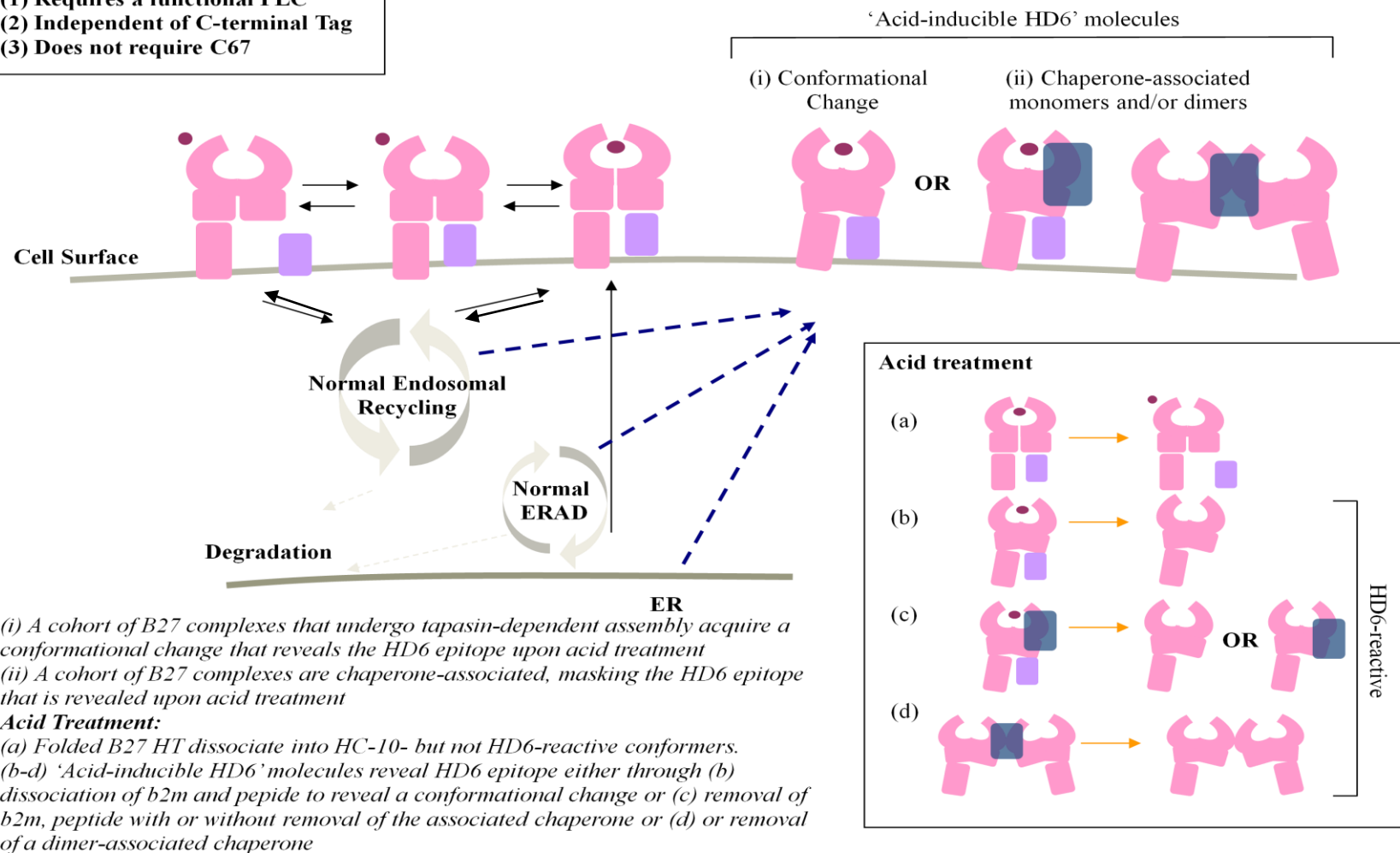


Figure 4.21 Proposed model for the formation of 'Acid-inducible HD6' molecules at the cell surface.

Chapter Five: HD6 recognition of HLA-B27 in an animal model of SpA and in SpA patients

5.1 Introduction

Prior to genome-wide association studies, the generation of HLA-B27 transgenic rodent models of SpA, outlined in Chapter One, provided the most compelling evidence for a direct role for HLA-B27 in disease pathogenesis. Moreover, these established animal models have enabled the investigation of the expression and behaviour of B27 in the context of a disease setting, which in turn has provided further support for two of the leading theories of pathogenesis that postulate a non-classical role for B27 molecules – the B27 misfolding theory and the B27 homodimer theory. In particular, one study with HLA-B27 transgenic rats found a proportion of B27 heavy chains recognised by HC-10 to be expressed as β_2m -free homodimers and multimers, both intracellularly and at the cell surface (Kollnberger et al., 2004). Consistent with this, Tran *et al.* also observed disulfide-linked intracellular heavy chain complexes in HLA-B27 but not HLA-B7 transgenic rats (Tran et al., 2004). Extending these findings to AS patients has proved more difficult, nevertheless a limited number of studies have provided additional support for the homodimer theory. Firstly, HC-10-reactive homodimers have been detected in freshly isolated PBMCs and SFMCs from AS patients (Kollnberger et al., 2002), and in DCs of HLA-B27+ individuals after activation with LPS (Santos et al., 2008). However, a more recent publication has also detected HC-10 reactive dimers in LPS-activated DCs derived from healthy controls (B27+ and B27-) (Campbell et al., 2011). Secondly, several groups have shown an increased level of HC-10 staining of PBMCs from HLA-B27+

AS patients compared with controls (Raine et al., 2006; Tsai et al., 2002). Yet, a major drawback in all these studies is their use of HC-10 as a detection reagent, which, given the heterogeneity of molecules it recognises, does not allow the specific recognition of HLA-B27 molecules. Moreover, the detection of HC-10 reactive dimers in DCs from healthy individuals as well as from AS patients appears to largely rule out its use as a biomarker for AS. Thus, the exact nature of the HC-10 reactive molecules detected both intracellularly and at the cell surface in these studies remains poorly defined. Notably, evidence that these non-classical B27 conformers directly play a role in disease pathogenesis is lacking. To satisfy the homodimer theory, firm evidence that B27₂ have functional effects on immune receptor recognition is required.

In the previous chapters I have shown that binding of HD6 to human HLA-B27-expressing cell lines is distinct from HC-10, and strongly correlates with HLA-B27 expression levels. Therefore, the main aim of this chapter was to extend these findings by determining whether HD6 can bind to B27 molecules in the context of a disease setting, such as in the HLA-B27 transgenic rat model and in AS patient PBMCs. Additionally, as in Chapter Four, the contribution of the cell surface stability of folded B27 molecules to HD6 epitope formation in transgenic rat and AS patient samples is investigated by using exogenous peptide addition and acid-induced surface MHC class I denaturation as previously described.

5.2 Expression of HD6-reactive HLA-B27 molecules in an HLA-B27 transgenic rat model of Spondyloarthritis

5.2.1 HD6 binds to HLA-B27 transgenic rat cell subsets

Staining of rat splenocytes was carried out in collaboration with Simon Milling's group. HLA-B27 transgenic (B27 TG) rats from the founder disease-susceptible 33-3 rat line, derived by Hammer *et al.* (see chapter 1), which bear a high copy number of the HLA-B27 transgene, were obtained from Taconic and housed under conventional pathogen-free conditions at Glasgow University. For comparison, initial controls were disease-free non-transgenic PVG inbred rats, denoted Wild Type (WT). Spleen mononuclear cells from age-matched B27 TG and WT rats were isolated by Lotta Utriainen and Dawn Firmin and sent overnight on ice in R10 for subsequent antibody staining and analysis by myself. Samples were first taken from transgenic rats with established inflammatory disease (aged 7-9 months). An increased percentage of cells in the granulocyte gate relative to that in the lymphocyte gate was consistently observed for the B27 TG samples compared to the age-matched WT controls, symptomatic of the ongoing inflammation in the diseased rats (Figure 5.1). HD6 staining of splenocytes was then compared to HC-10 and HLA-ABC-m2 staining, the latter recognising correctly folded B27 molecules in a similar manner to ME-1. Cells were gated according to typical forward-scatter/side-scatter (FSC/SSC) characteristics of lymphocytes, monocytes/macrophages/DCs (M/DC), large mononuclear cells (MNC) and granulocytes (Figure 5.2A). As expected, folded B27 molecules were detected in all gates for B27 transgenic rats samples (black histograms) but not for WT (grey histograms) (Figure 5.2B top row). For HD6, staining was consistently seen in the lymphocyte, M/DC and large MNC gates for B27 TG but not WT. Notably, however, background only staining was observed in the granulocyte gate

(Figure 5.2B, middle row), which was also the case for HC-10 staining (Figure 5.2B, bottom row).

HD6 surface staining of cells in both the lymphocyte and M/DC gates consistently showed a bimodal pattern, therefore I next carried out multi-colour staining of splenocytes with phenotypic markers to look for heterogeneity of HD6 staining among distinct leukocyte populations within these gates. In the lymphocyte gate, B cells and T cells were identified by the markers CD45R and CD4 or CD8 respectively. No CD3 antibody was available at the time of staining; therefore it was not possible to distinguish the T lymphocytes from other CD4⁺ or CD8⁺ subsets including some NK and NKT cell subsets, which have not been investigated here. To identify monocytes/macrophages and DCs, the OX-42 antibody was used, which recognises a common epitope shared by CD11b and CD11c and is expressed on these subsets. HD6 bound to all these subsets in the B27 TG samples (Figure 5.3). In the lymphocyte gate, comparable staining was seen for the CD4⁺ and CD8⁺ cells. This was increased in the CD45R⁺ population, although these cells also had higher WT background HD6 staining (Figure 5.3A and B). Notably, HD6 staining of CD11b/c⁺ cells in the lymphocyte gate resulted in a double peak (Figure 5.3C, 3rd panel). Using the MHC class II (CII) antibody, OX6, the higher peak could be attributed to double positive CD11b⁺ CII⁺ cells (Figure 5.3, blue histogram, bottom right). In contrast, a single peak was seen for CD11b⁺ cells in the M/DC gate. As before, no detectable staining was seen in the granulocyte gate (Figure 5.3, top row, far right).

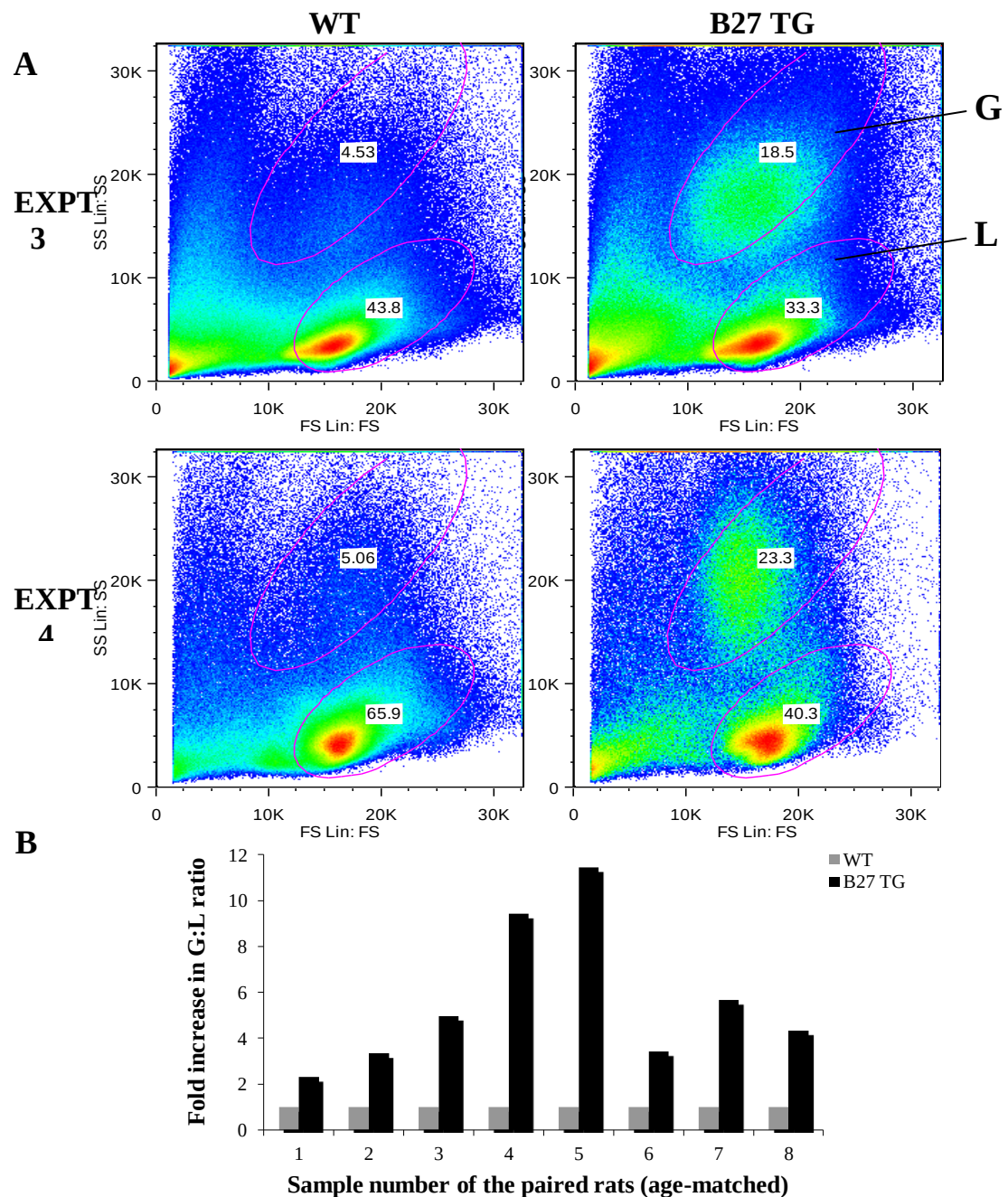


Figure 5.1 HLA-B27 transgenic rat splenocytes have an increased relative percentage of cells in the granulocyte gate. (A) Splenocytes isolated from age-matched WT rats and HLA-B27 transgenic rats aged 7-9 months are analysed by flow cytometry and plotted as SSc vs FSc. Representative data from 2 of 8 experiments are shown. (B) For 8 pairs of age-matched rat splenocyte samples, the ratio of percentage cells in the granulocyte gate compared with the lymphocyte gate (G:L) is calculated and the fold increase is calculated by comparing this value to the WT value (TG ratio / WT ratio).

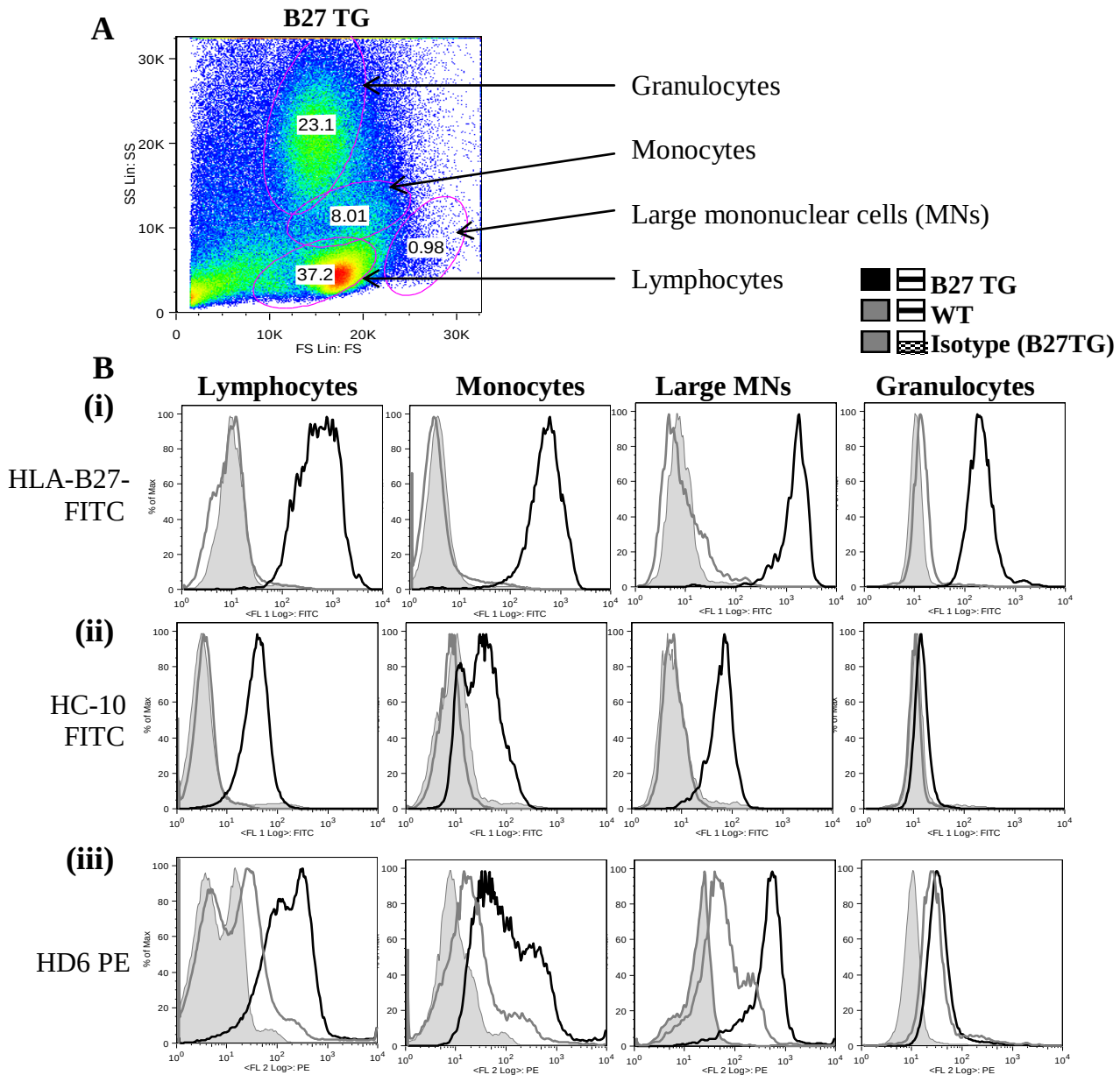


Figure 5.2 HD6 stains splenocytes from an adult disease-prone HLA-B27 transgenic rat but not from an age-matched WT rat. (A) SSc vs FSc plot of showing the gating strategy for the rat cell subsets analysed. (B) Splenocytes from an 8 month old HLA-B27 transgenic rat (black histograms) or an age-matched WT rat (grey histograms) were stained with (i) a FITC-conjugated HLA-B27 antibody (HLA-ABC-m3) or a FITC-conjugated matched isotype control (grey filled histograms), (ii) HC-10 or an IgG2a isotype control followed by a FITC-conjugated secondary antibody, and (iii) HD6 or an IgG1 isotype control followed by a PE-conjugated secondary antibody.

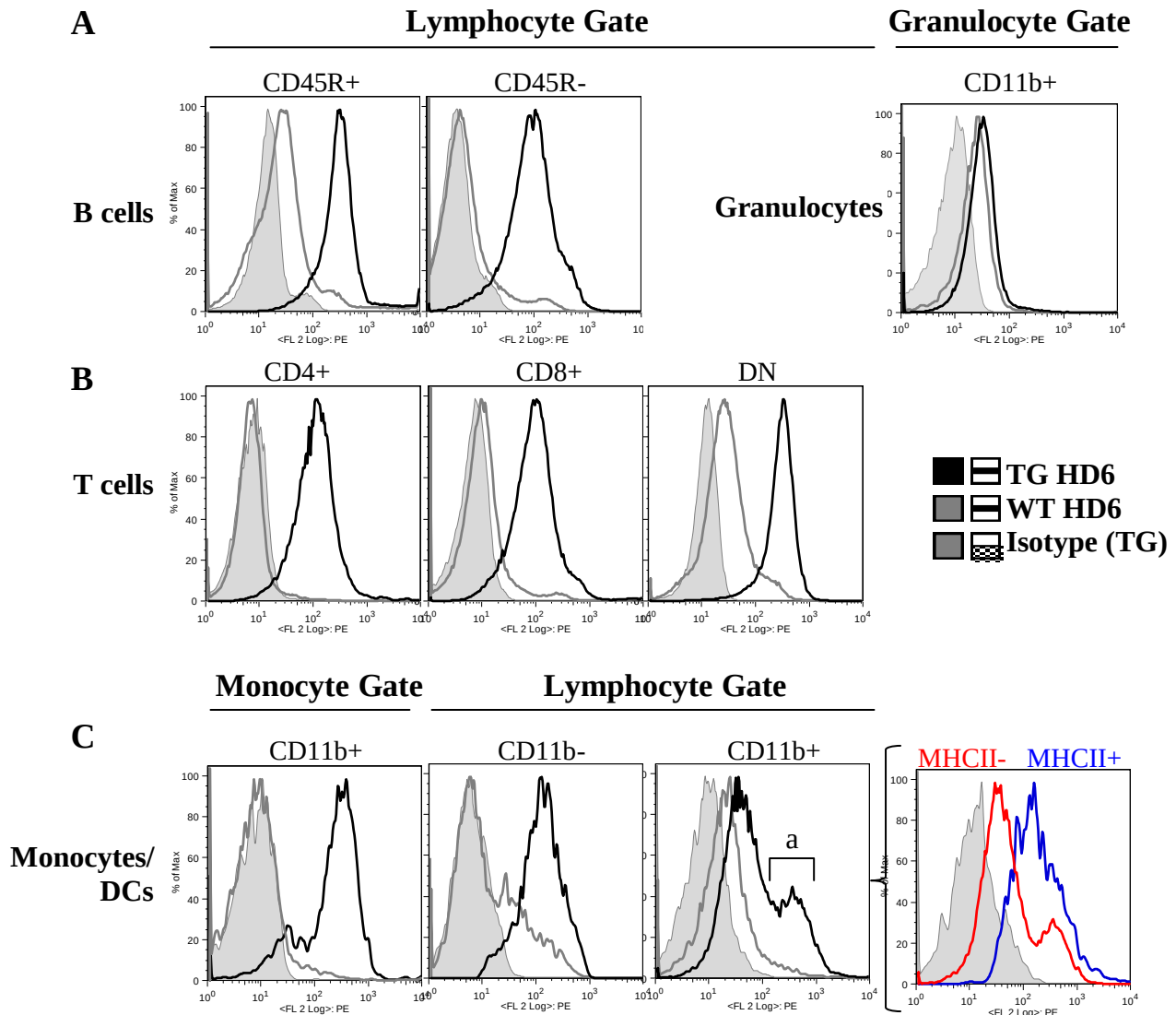


Figure 5.3 HD6 stains lymphocyte and monocyte but not granulocyte cell subsets in splenocytes from an adult disease-prone HLA-B27 transgenic rat. Splenocytes from an 8 month old HLA-B27 transgenic rat (black histograms) and an age matched WT rat (grey histograms) were stained with HD6 or a matched isotype control antibody (grey filled histograms) followed by a PE-conjugated secondary antibody. Cells were co-stained for the subset markers (A) CD45R, (B) CD4, CD8, (C) CD11b and MHC class II as indicated. In (C), the 4th panel shows HD6 staining of MHCII- and MHCII+ cells from gate a (in 3rd panel). Staining is representative of data from splenocytes of 7 age-matched adult WT and B27 TG (aged 7-9 months).

In summary, this data has shown that HD6 binds splenocytes from adult HLA-B27 transgenic rats but not non-transgenic controls. Specifically, HD6 stained a number of distinct cell subsets including CD4+, CD8+ and CD45R+ cells in the lymphocyte gate, and CD11b+ cells in the monocyte/DC gate. However, HD6 did not bind to cells in the granulocyte gate. In addition, the adult HLA-B27 transgenic rats had an increased percentage of cells in the granulocyte gate compared to the lymphocyte gate, indicative of the ongoing inflammation.

5.2.2 HD6 binding to HLA-B27 transgenic rat cell subsets is dependent on HLA-B27 expression levels

On the basis of studies with the disease-prone and disease-resistant B27 TG rats, Taurog *et al.* have suggested that disease onset results from the expression of B27 above a critical threshold level. In addition, by comparing young clinically healthy rats from the disease-prone line with older rats with established inflammatory disease, they demonstrated that B27 surface expression on lymphoid cells, detected by ME-1 staining, increases in parallel with development of disease (Taurog *et al.*, 1993). Given that HD6 staining of human cell lines in the previous chapters was shown to strongly correlate with HLA-B27 levels, I next sought to determine whether this is also true for the disease-prone transgenic rats, predicting that the younger clinically healthy rats with lower B27 would also have lower HD6 epitope expression. This could also explain why the granulocyte population, which had the lowest B27 levels, did not stain with HD6. Therefore, samples were taken from young rats (aged 2-3 months) and compared with the older diseased rats.

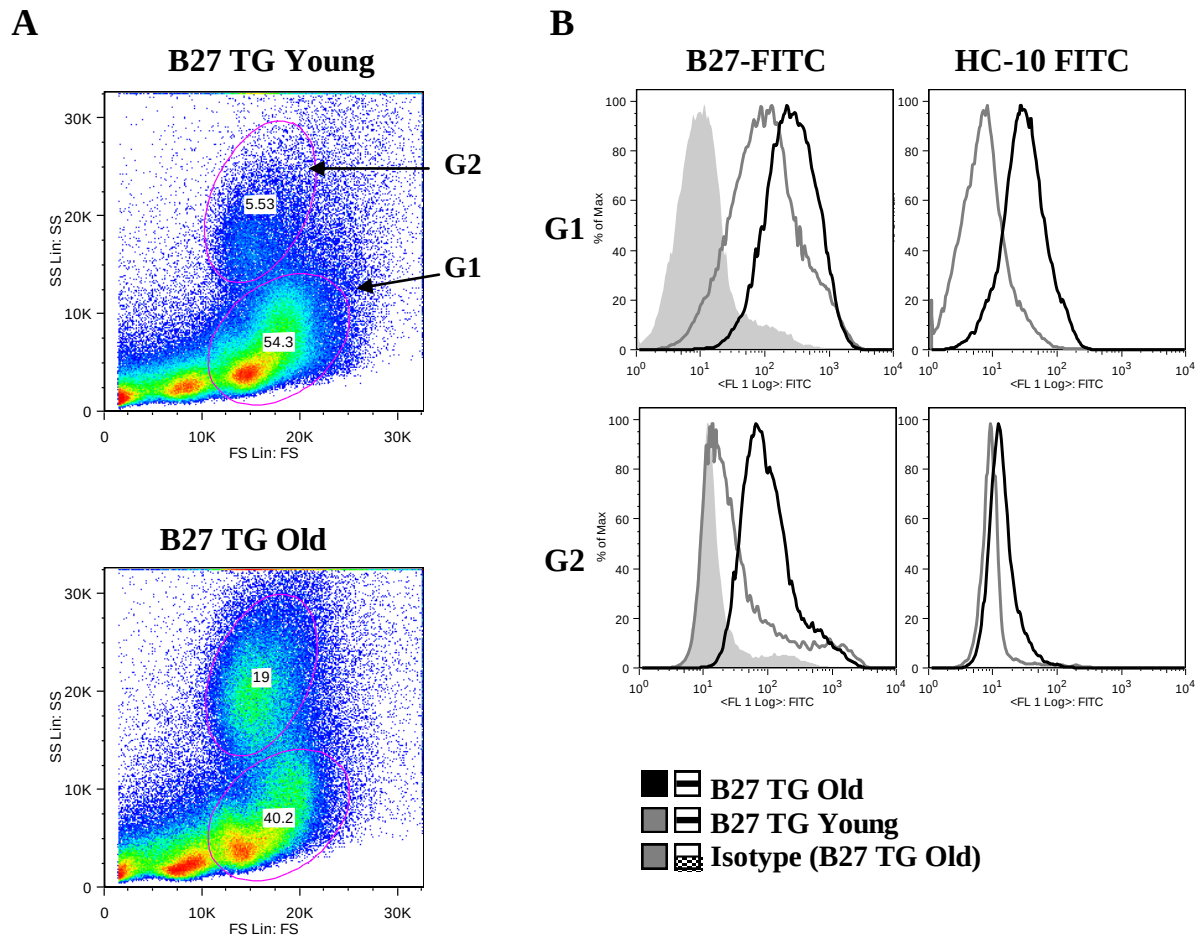


Figure 5.4 Cell surface expression of HLA-B27 on rat splenocytes is increased with age in disease-prone HLA-B27 transgenic rats. Splenocytes were simultaneously isolated from a 9 week old HLA-B27 transgenic rat (B27 TG Young, grey histograms) and from an 8 month old HLA-B27 transgenic rat (B27 TG Old, black histograms) for analysis. (A) SSc vs FSc plots with labelled lymphocyte/monocyte (G1) and granulocyte (G2) gates. (B) Splenocytes were stained with a FITC-conjugated B27 mAb (HLA-ABC-m3) or a FITC-conjugated isotype control (grey filled histograms), or HC-10 followed by a FITC-conjugated anti-mouse IgG2a secondary antibody. Similar results were obtained by direct comparisons with different sets of animals in 2 further separate experiments.

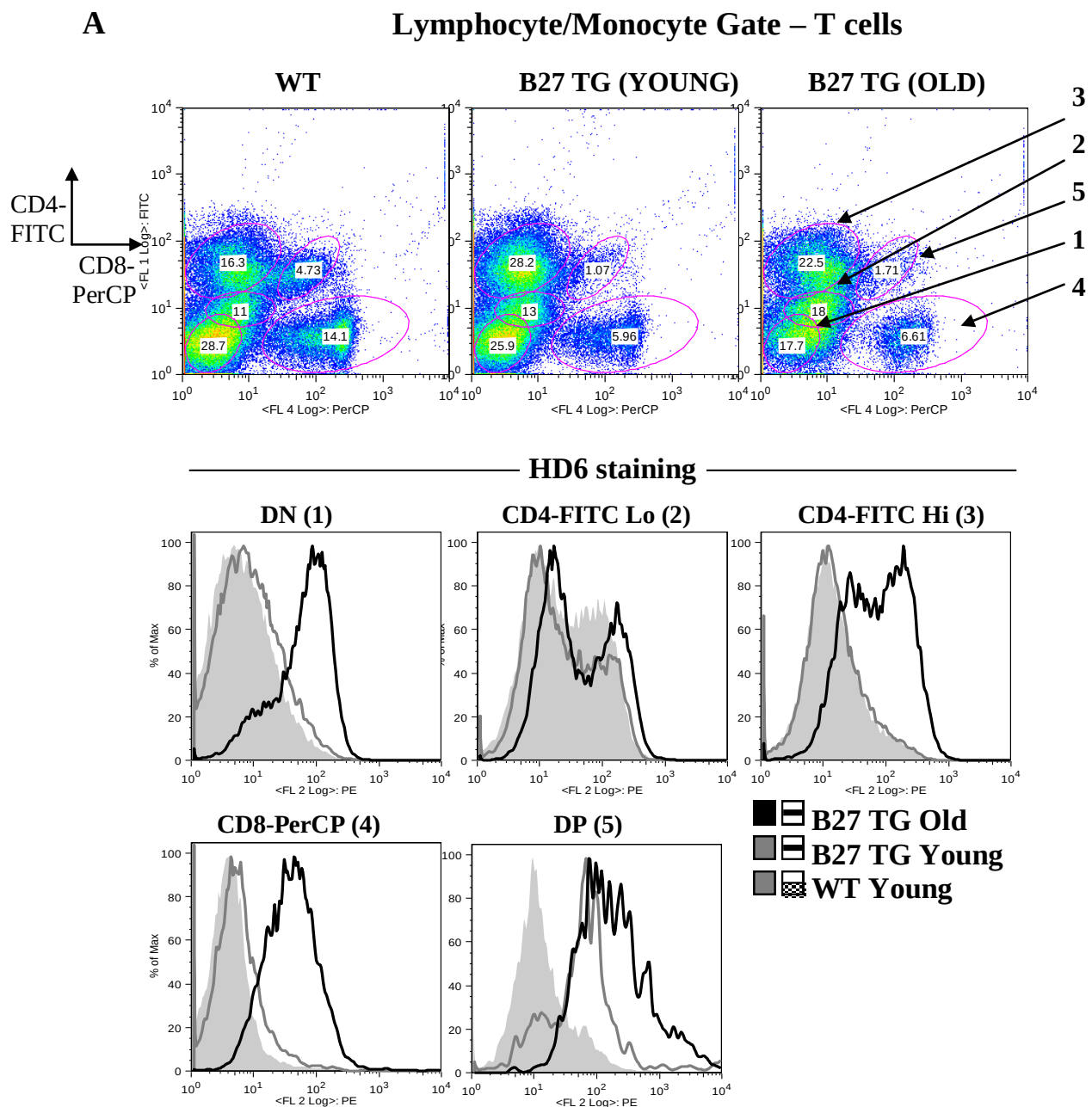


Figure 5.5 HD6 staining of splenocyte subsets from disease-prone HLA-B27 transgenic rats is increased with age. Splenocytes were isolated from a 9 week old HLA-B27 transgenic rat (B27 TG Young, grey histograms), a 9 month old HLA-B27 TG rat (B27 TG Old, black histograms), and an 8 month old WT rat. Cells were stained with subset markers and HD6 as follows: (A) For T cells, cells were stained with anti-CD4-FITC and anti-CD8-PerCP antibodies and gated on double negative (DN, 1), CD4-FITC low single positive (2), CD4 FITC high single positive (3), CD8-PerCP single positive (4) and double positive (DP, 5) populations as indicated. HD6 staining in each gate is shown.

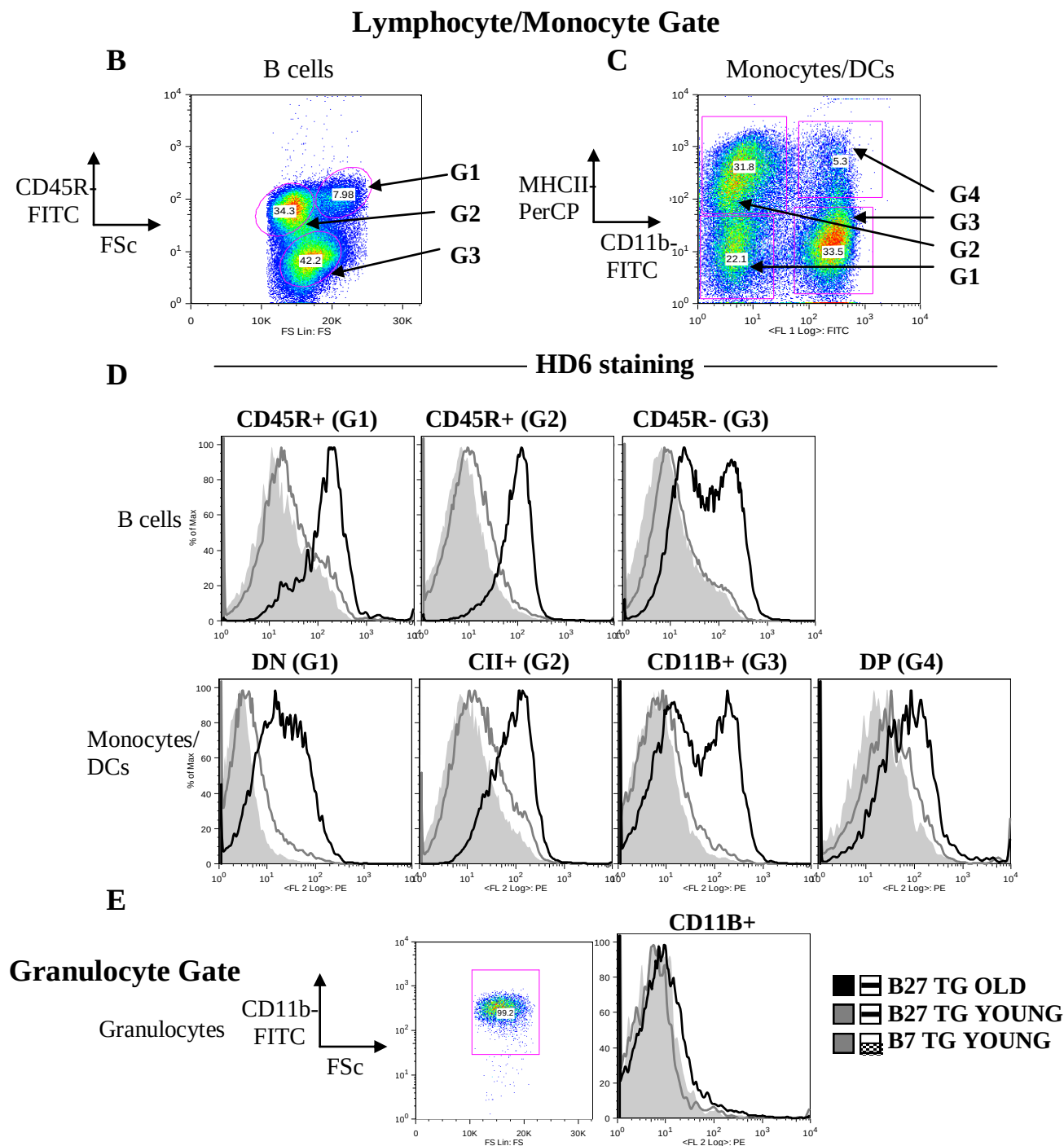


Figure 5.5 continued. (B) For B cells, cells were stained with anti-CD45R-FITC and gated on large CD45R+ (G1), small CD45R+ (G2) and CD45R- (G3) populations as indicated. (C) For monocytes/DCs, cells were stained with anti-CD11b and anti-MHCII and gated on double negative (DN, G1), MHC class II single positive (CII+, G2), CD11b single positive (G3), and double positive (DP) populations as indicated. (D) HD6 staining in each gate is shown for B cells and Monocytes/ DCs. (D) For granulocytes, cells in the granulocyte gate were gated on the CD11b+ population as indicated.

The percentage of cells in the granulocyte gate (G2) relative to the lymphocyte/monocyte gate (G1) was consistently lower in the samples from young rats compared to older rats, indicative of the lack of inflammation in the former (Figure 5.4A). Expression of folded B27 molecules, as detected by the HLA-ABC-m3 antibody, was increased in the older rats (black histograms) compared to the younger rats (grey histograms), consistent with previous reports (Figure 5.4B, left column). This was also the case for free heavy chain expression, as detected by HC-10 (Figure 5.4B, right column). For HD6 staining, cells were also stained with lineage markers. In the lymphocyte/monocyte gate (G1), cells were further gated according to CD4 and CD8 expression as shown (Figure 5.5A). HD6 staining was observed in all the gates for samples from older rats (B27 TG Old, black histograms). In contrast, HD6 staining of the younger rat cells (B27 TG Young, grey histograms) was absent for most cells, resembling that of the WT samples (grey-filled histograms). An exception to this was seen in the double positive (DP) cells, which showed considerable HD6 staining, albeit still at lower levels than in the older rats. For B cells and monocytes/DCs, which were gated as shown in Figure 5.5B and 5.5C respectively, HD6 staining was detected in the older but not younger rat samples (Figure 5.5D). For granulocytes, no HD6 staining was observed for either young or old rats, consistent with the previous results (Figure 5.5E). This lack of expression of HD6-reactive B27 molecules in younger transgenic rat splenocytes was reproduced in three further experiments, one of which is shown in Figure 5.6. Here, although low levels of HD6 staining were observed in some of the subsets (blue histograms), this was substantially lower than that of the older rat cells (black histograms).

5.2.3 HD6 stains cells from HLA-B27 but not HLA-B7 transgenic rats

To confirm that the observed HD6 staining of transgenic rats was HLA-B27 specific, splenocytes from HLA-B7 transgenic rats were next screened with the antibody. These rats derived from the 120-4 LEW strain described in Taurog *et al.*, which were crossed for 1-2 generations onto a PVG background to become B7 hemizygous (Taurog *et al.*, 1999) (Milling *et al.* unpublished data). The number of copies of the B7 and β_2m genes in these animals has yet to be confirmed. Figure 5.7 shows the staining of splenocytes from a young B7 TG rat (green histograms) compared with age matched B27 transgenic rat (black histograms) and non-transgenic rat (grey histograms) splenocytes (Figure 5.7). HLA-B7 TG rats expressed similar levels of free heavy chains and correctly folded human MHC class I molecules to the HLA-B27 TG rats, as determined by HC-10, ME-1 and W6/32 staining (Figure 5.7A). In contrast, no HD6 staining above that seen for WT cells was seen in the HLA-B7 cells for all subsets analysed (Figure 5.7B). Next, staining was compared with samples from both young and old B7 (light blue and dark blue histograms, respectively) and B27 (grey and black histograms, respectively) TG rats. Again, no considerable HD6 staining of the B7 TG cells was observed, despite similar HC-10 and ME-1 staining to the corresponding age-matched B27 TG rats (Figure 5.8).

In summary, this data has shown that HD6 can recognise a subset of HLA-B27 molecules in TG rats. Expression of these molecules is strongly correlated with the levels of surface B27, and correlates with age in a disease-prone rat strain. Its specificity for HLA-B27 is confirmed by the negative staining demonstrated in HLA-B7 TG rats.

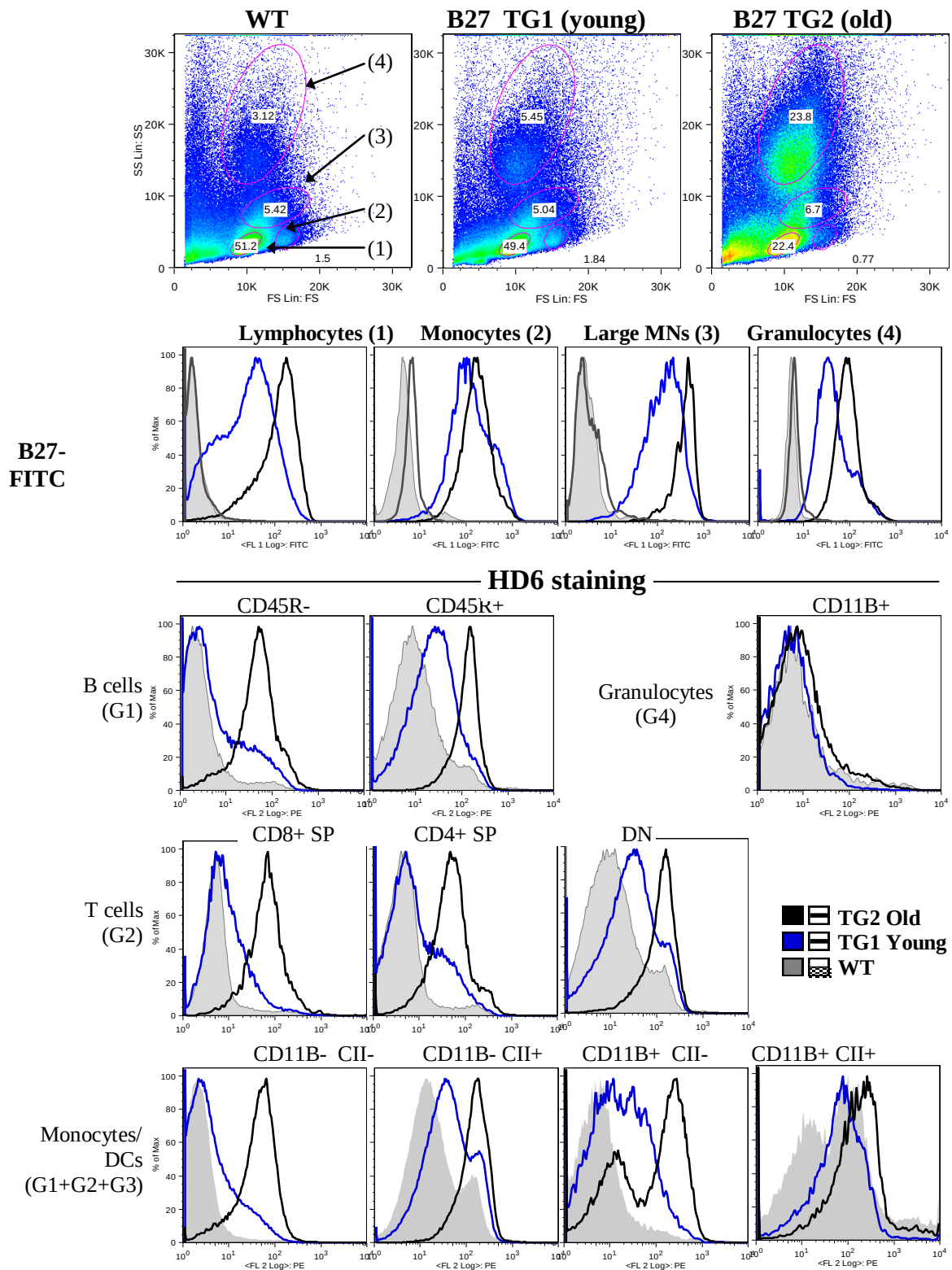


Figure 5.6 HD6 staining of cell subsets is increased with age in disease-prone HLA-B27 transgenic rats. Staining of splenocyte subsets as described in figure 5.6. Cells were taken from a 10 week old HLA-B27 TG rat (TG1 Young, blue histograms), an 8 month old HLA-B27 TG (TG2 Old, black histograms) and an 8 month old WT rat (grey filled histograms).

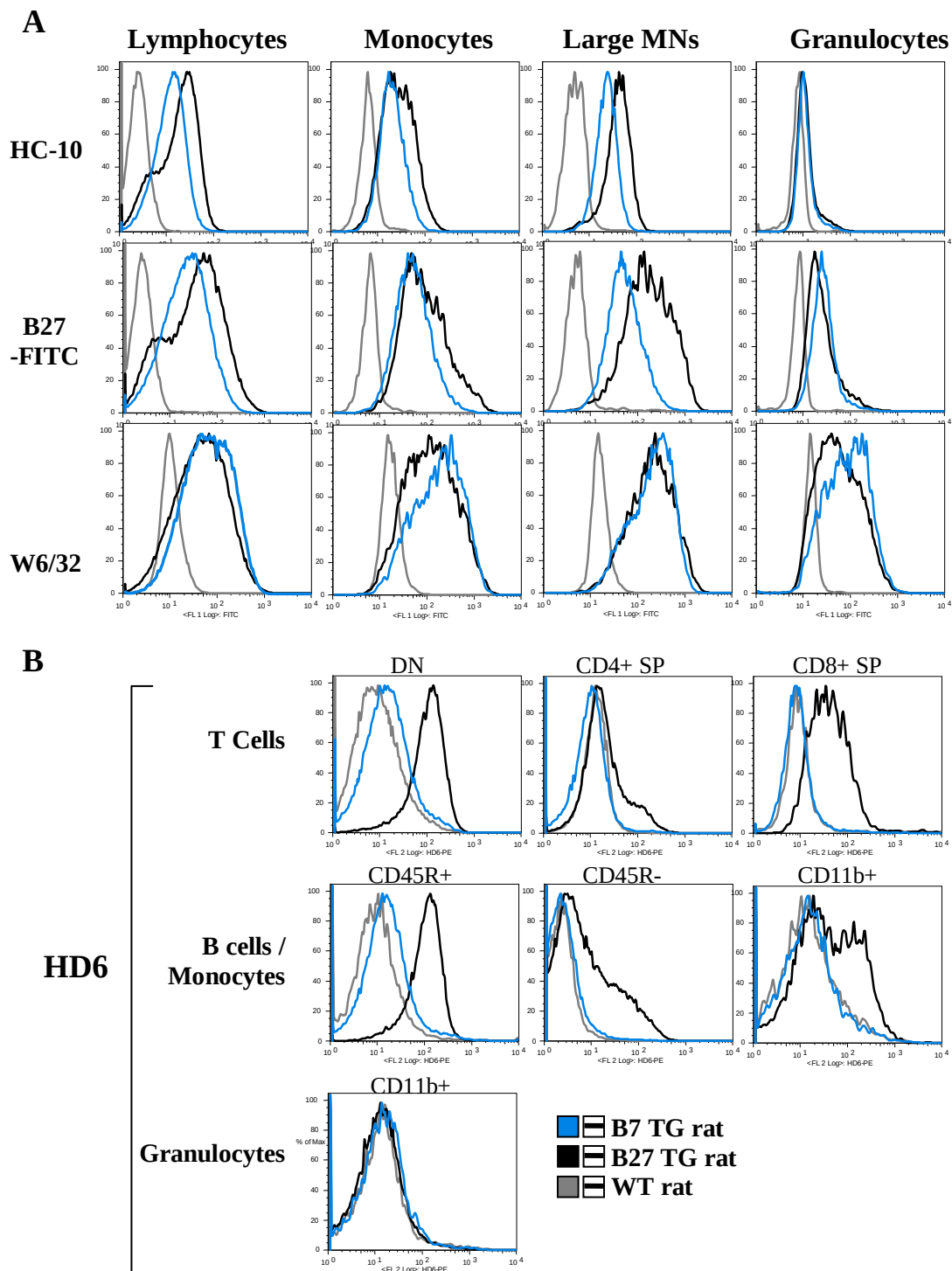


Figure 5.7 HD6 stains cell surface molecules on splenocytes from young HLA-B27 TG rats but not young HLA-B7 TG rats. Splenocytes were isolated from an HLA-B27 TG rat (10 weeks old) an HLA-B7 TG rat (11 weeks old) and a WT rat (10 weeks old) and stained with (A) subset markers and HC-10, HLA-ABC-m3 (B27-FITC), or W6/32 as indicated or (B) subset markers and HD6.

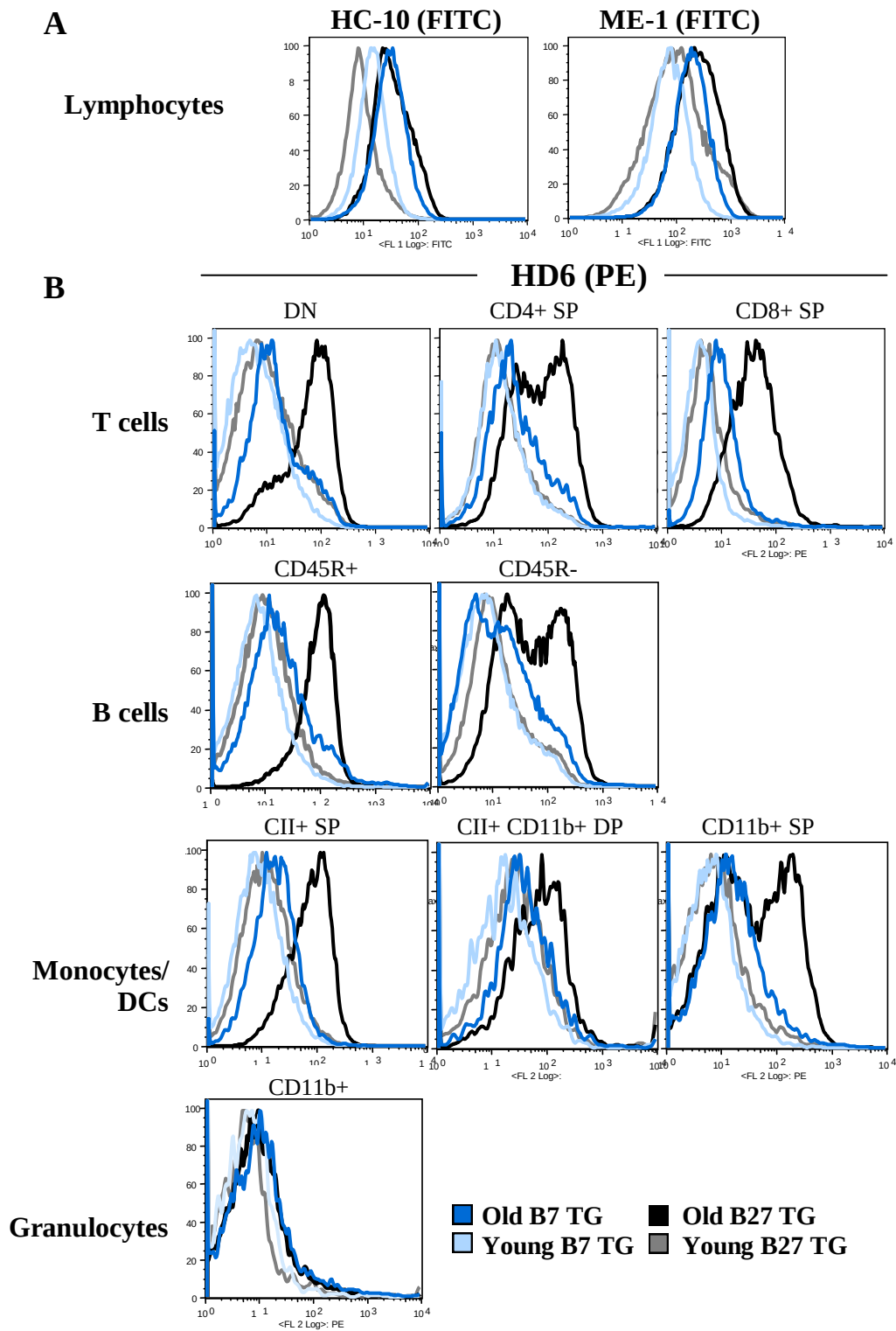


Figure 5.8 HD6 recognises cell surface molecules on splenocytes from HLA-B27 but not HLA-B7 TG rats. Splenocytes were isolated from two HLA-B27 TG rats (5 weeks and 5 months old), a young HLA-B7 TG rat (6 weeks old) and an old HLA-B7 TG rat (9 months old) and stained with (A) HC-10 or ME-1, or (B) subset markers and HD6 as indicated.

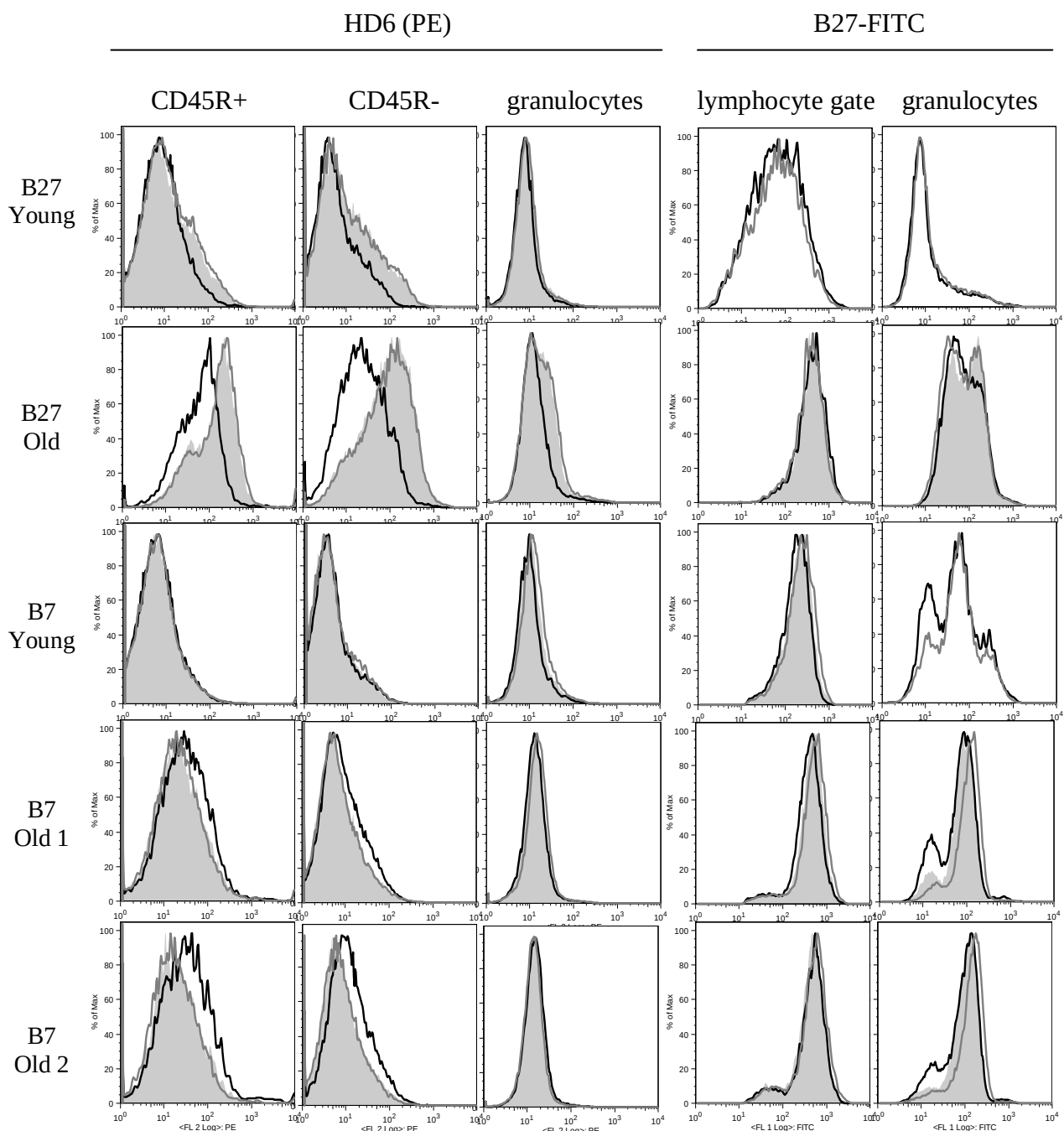


Figure 5.9 HD6 staining of adult HLA-B27 transgenic rat splenocytes is reduced upon addition of a B27-binding peptide but not a control peptide. Splenocytes isolated from 1 young (6 weeks) and 1 older (5 months) B27 TG rat, and from 1 young (5 weeks), and 2 older (11 months) B7 TG rats were incubated in R10 only (grey filled histograms) or R10 supplemented with 100uM of the B27-binding peptide SRYWAIRTR (black histogram) or the B7-binding peptide RPMTYKAAV (grey line histogram) for 4 hours before staining with HD6 and anti-CD45R, or HLA-ABC-m3 (B27-FITC) as indicated.

5.3 The effect of B27 stability on HD6 staining in transgenic rats

5.3.1 HD6 staining is reduced by addition of exogenous HLA-B27 binding peptides

Splenocytes isolated from both young and old B27 and B7 transgenic rats were incubated for 4h at 37C with 100uM of B27-binding peptide (black histograms), 100uM of a control A2-binding peptide (grey histograms) or in R10 media alone (grey-filled histograms), before staining with HLA-ABC-m3, or HD6 and an anti-CD45R antibody. Cells were then gated on lymphocytes and granulocytes as before (see Figure 5.1). In the lymphocyte gate, considerable HD6 staining of both CD45R+ and CD45R- cells was observed for the splenocytes from the older B27 TG rat (B27 Old), which were markedly reduced in splenocytes from the younger B27 TG rat (B27 Young), consistent with previous findings (Figure 5.9, top two rows). Significantly, this staining was reduced by incubation with the B27-binding peptide but not the control peptide. In contrast, neither peptide had an effect on the levels of correctly folded HLA-B27 surface molecules. For the HLA-B7 TG rat samples, no HD6 staining was observed, consistent with previous data, and this was not changed under conditions of exogenous peptide addition (Figure 5.9, bottom three rows).

5.3.2 HD6 staining of B27 TG rat splenocytes is increased by low pH treatment

As previously discussed, HD6 staining of most HLA-B27-expressing human cell lines is increased upon treatment with acid to denature the folded cell surface class I molecules. This approach was therefore used again here to investigate transgenic rat cells. Firstly, splenocytes from a young B27 TG rat were examined by comparing HD6 staining of untreated cells with those treated with low pH glycine buffer for 2 minutes, as described in Chapter Four (Figure 5.10). As before, only low levels of

HD6 staining were observed in untreated cell subsets (black histograms). However, this was markedly increased after acid treatment for each subset, including the granulocyte population (black dashed histograms). In contrast, acid treatment had no effect on staining with isotype control, as shown in the top row for the B cells (grey filled and dashed histograms). Furthermore, and as expected, treatment removed the majority of correctly folded HLA-B27 molecules, as detected by the HLA-ABC-m3 (B27-FITC) antibody, while increasing the presence of free heavy chains (HC-10 staining, bottom row). I next repeated this with splenocytes from an older B27 TG rat, which showed moderate HD6 staining without acid treatment in all but the granulocyte populations, consistent with previous findings (black histograms, Figure 5.11). Again, acid treatment markedly increased HD6 staining on all subsets (black dashed histograms), while reducing the presence of correctly folded surface molecules (bottom row). To confirm these acid-induced HD6-reactive molecules are derivatives of HLA-B27 molecules, acid treatment of splenocytes from a young B7 TG rat was also carried out (Figure 5.12). No changes in HD6 staining were detected after acid treatment (grey histograms), although an increase in HC-10 staining confirmed the efficacy of acid treatment of these cells (bottom row). A further experiment was carried out splenocytes from both young and old HLA-B27 and HLA-B7 TG rats as shown in Figure 5.13. This data was in agreement with the previous findings, demonstrating that acid treatment induced increased HD6 staining in the splenocytes from young and old B27 TG rats (bottom row, grey and black histograms, respectively), the latter of which showing the most substantial staining levels. No notable differences in HD6 staining were seen after acid treatment of splenocytes from young and old B7 TG.

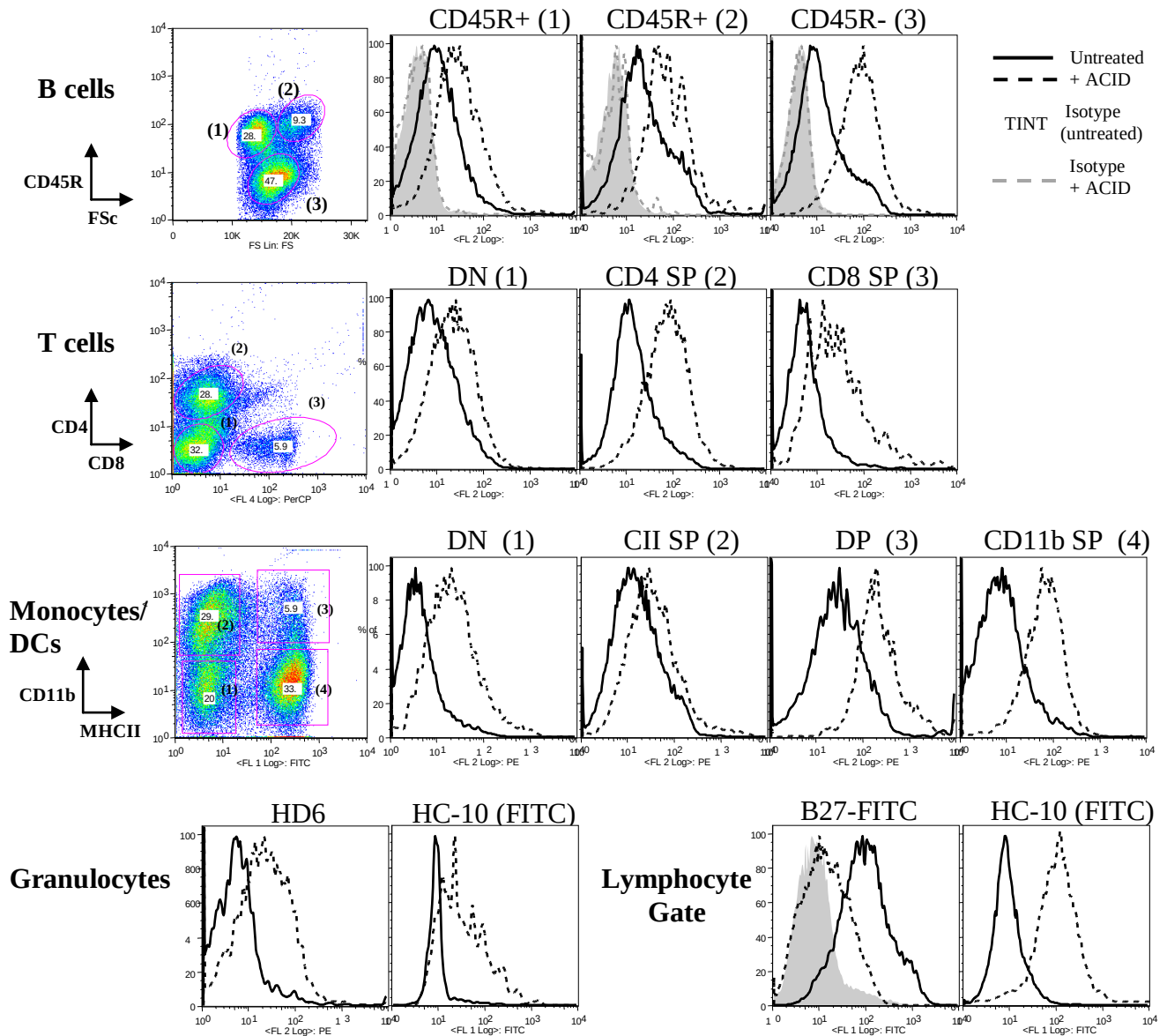


Figure 5.10 Low pH treatment of splenocytes from a young HLA-B27 transgenic rat induces HD6 staining. Splenocytes isolated from a 10 week old B27 transgenic rat were left untreated (black histograms) or treated with low pH glycine buffer as previously described (dashed histograms) Cells were subsequently stained with HD6 and phenotype markers (CD45R, CD4, CD8, MHC class II (CII), CD11b), HC-10, or FITC-conjugated HLA-ABC-m3 as indicated. Where carried out, staining with isotype control is shown as grey filled histograms.

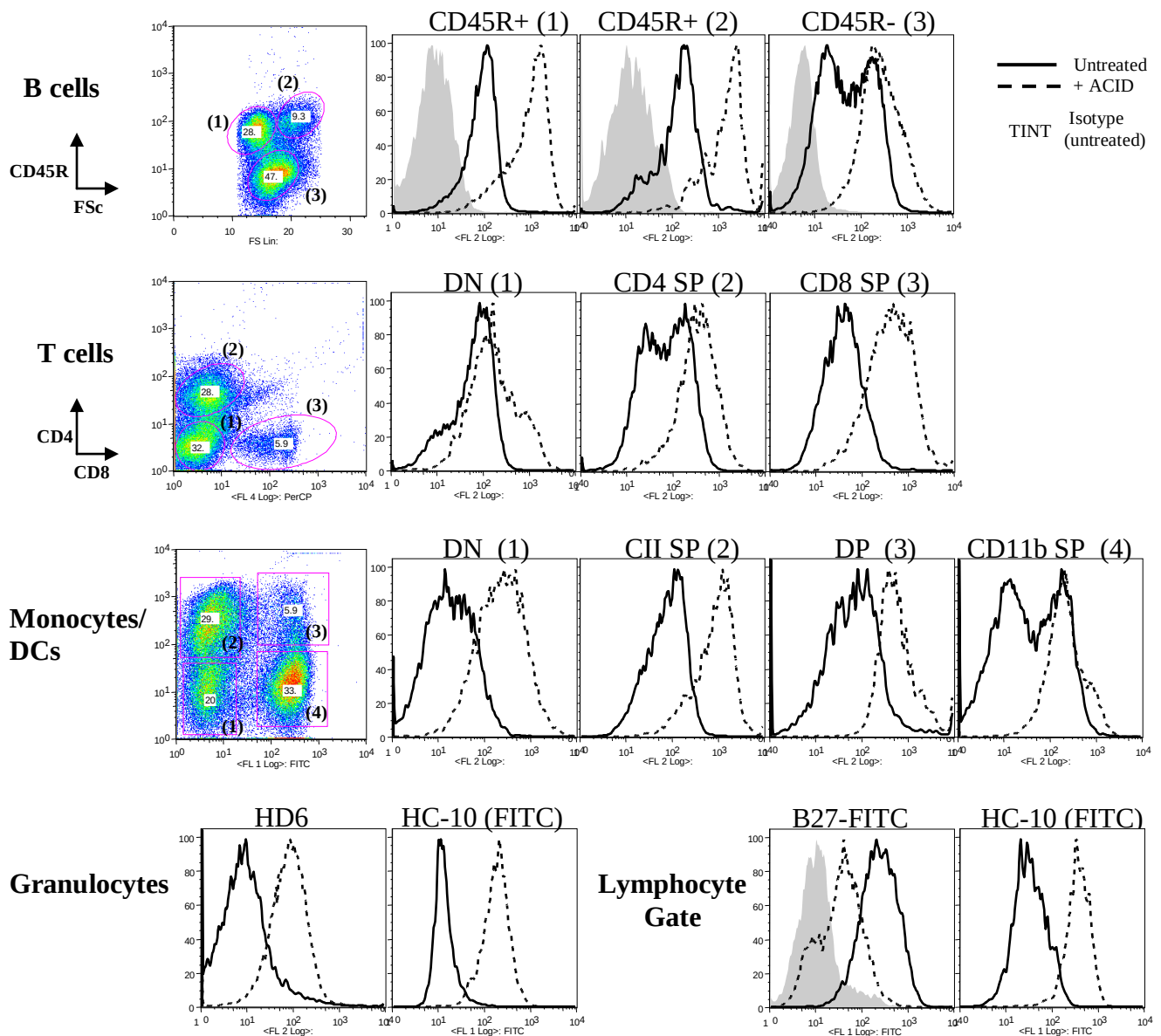


Figure 5.11 Low pH treatment of splenocytes from an older HLA-B27 transgenic rat increases and/or induces HD6 staining. Splenocytes isolated from an 8 month old B27 TG rat were left untreated (black histograms) or treated with low pH glycine buffer as previously described (dashed histograms) Cells were subsequently stained with HD6 and phenotype markers (CD45R, CD4, CD8, MHC class II (CII), CD11b), HC-10, or FITC-conjugated HLA-ABC-m3 as indicated. Where carried out, staining with isotype control is shown as grey filled histograms.

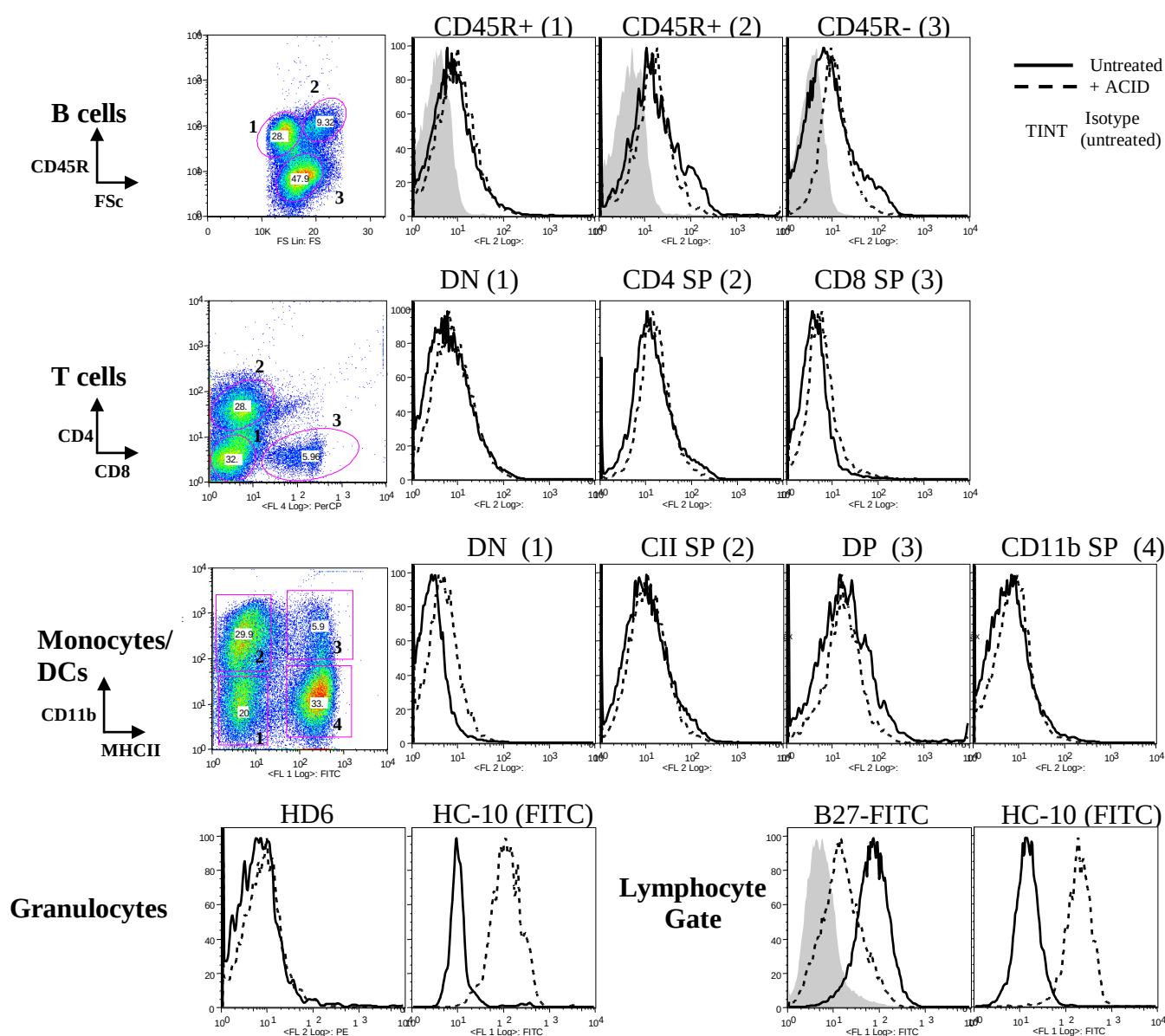


Figure 5.12 Low pH treatment of splenocytes from a young HLA-B7 transgenic rat increases surface free heavy chains but does not induce HD6 staining. Splenocytes isolated from a 10 week old B7 transgenic rat were left untreated (black histograms) or treated with low pH glycine buffer as previously described (dashed histograms) Cells were subsequently stained with HD6 and phenotype markers (CD45R, CD4, CD8, MHC class II (CII), CD11b), HC-10, or FITC-conjugated HLA-ABC-m3 as indicated. Where carried out, staining with isotype control is shown as grey filled histograms.

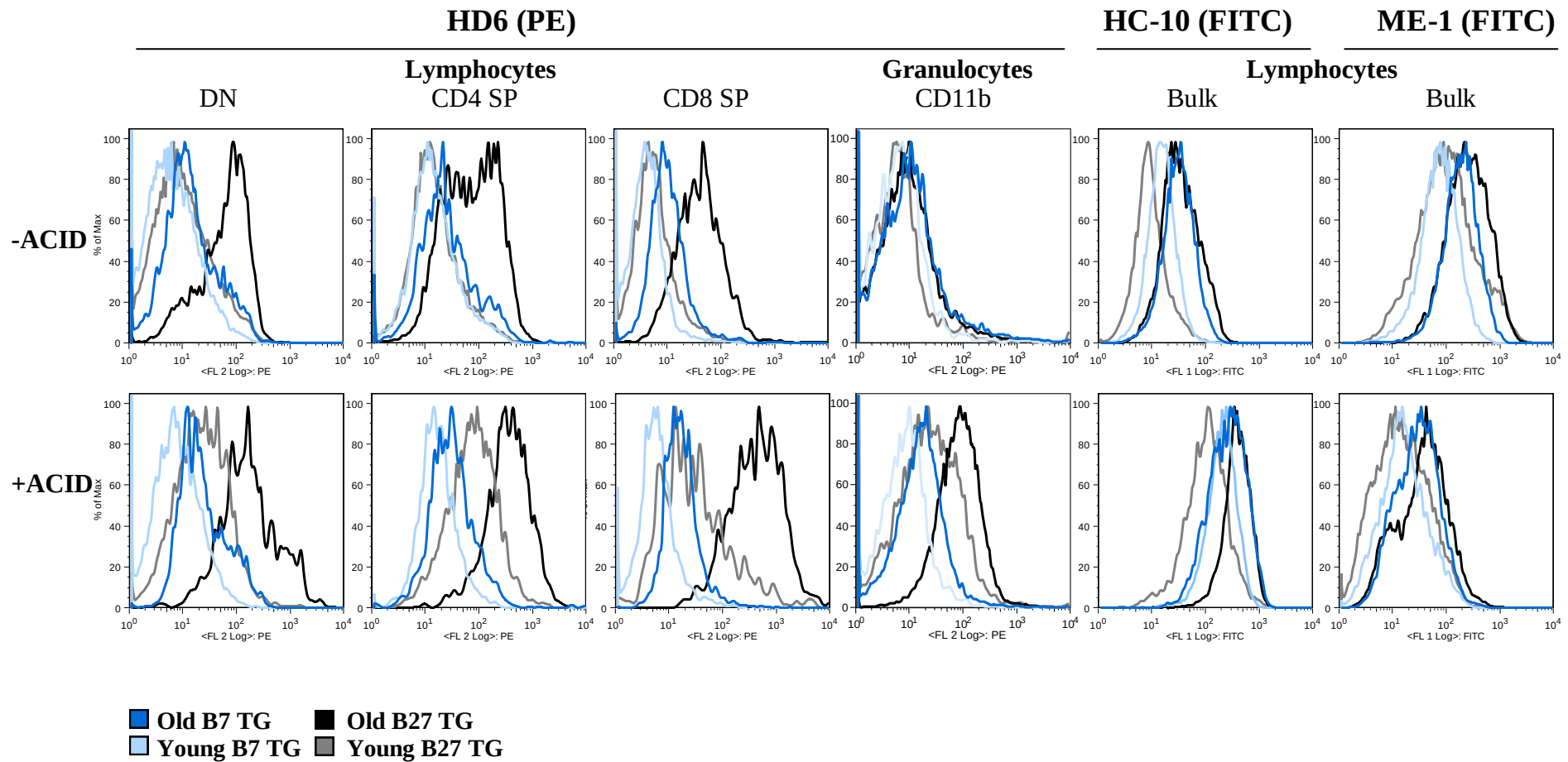


Figure 5.13 A comparison of the effect of acid treatment on HLA-B27 and HLA-B7 transgenic rat splenocytes. Splenocytes isolated from 1 young (6 weeks) and 1 older (5 months) B27 TG rat, and from 1 young (5 weeks), and 2 older (11 months) B7 TG rats were left untreated (top row) or treated with low pH glycine buffer for 2 minutes prior to staining with HD6 and lineage markers (CD4, CD8 or CD11b) as shown, or with HC-10 or ME-1. Cells were gated on lymphocytes and granulocytes according to typical forward scatter/side scatter characteristics.

rats, providing further supporting that HD6 is specific for a subset of surface HLA-B27 molecules.

In Summary, this data has shown that addition of exogenous HLA-B27 binding peptides but not control peptides reduces HD6 staining to several cell subsets in splenocytes from B27 TG rats. Furthermore, acid-induced denaturation of surface molecules increases the HD6 staining on all cell subsets in the lymphocyte/monocyte cell gate and induces expression on granulocytes in splenocyte samples taken from both young and old B27 TG rats but not B7 TG controls.

5.4 HD6 epitope expression on peripheral blood mononuclear cells from patients with Ankylosing Spondylitis

5.4.1 HD6 staining of AS patient PBMCs

To determine whether the HD6-reactive epitope is expressed in individuals with SpA, peripheral blood mononuclear cells (PBMCs) were obtained from an HLA-B27 positive AS patient (AS1) and an HLA-B27 negative healthy control individual (HC1). Cells were stained with the FITC-conjugated HLA-ABC-m3 antibody to confirm the AS patient was B27 positive (Figure 5.14, top row). For HD6 staining, cells were also stained with an anti-CD14 antibody in order to identify the monocytes, as elevated levels of HC-10 in monocytes of AS patients have been described previously (Raine et al., 2006). No HD6 staining was detected for the patient PBMCs in either the lymphocyte (G1) or monocyte/granulocyte (G2) gates above the background staining levels seen for the HLA-B27 negative control sample (Figure 5.14, bottom row). This lack of staining was then reproduced in three further

B27+ AS patient PBMCs, one B27+ healthy individual and 2 further B27- healthy individuals. Furthermore, preliminary data from cells infected with a strain of *S. typhimurium* found this to have no effect on HD6 epitope expression (data not shown).

5.4.2 HD6 staining is induced in AS patient PBMCs upon acid treatment

To determine whether AS patient PBMCs can be artificially induced to express HD6-reactive molecules at the cell surface PBMCs from a B27+ AS patient, and a non-B27 healthy control were treated with acid as previously described before staining with monoclonal antibodies as before. Treatment with acid induced HD6 staining in the B27+ PBMCs but not the B27- control (Figure 5.15A). HC-10 staining was increased for both samples, while W6/32 and BBM.1 staining were both reduced, consistent with the findings of previous experiments. Next, this acid-induced HD6 staining was further examined with lineage markers, to investigate whether there was any heterogeneity in staining between cell subsets (Figure 5.16B). Similar levels of HD6 binding were seen for the CD4+ and CD8+ populations, with the smallest increase seen for the CD11b+ population.

In summary, this data has shown HD6 does not bind to PBMCs from patients with AS under standard conditions. However, staining can be induced by denaturing surface molecules with acid treatment, and was seen in all cell subsets analysed.

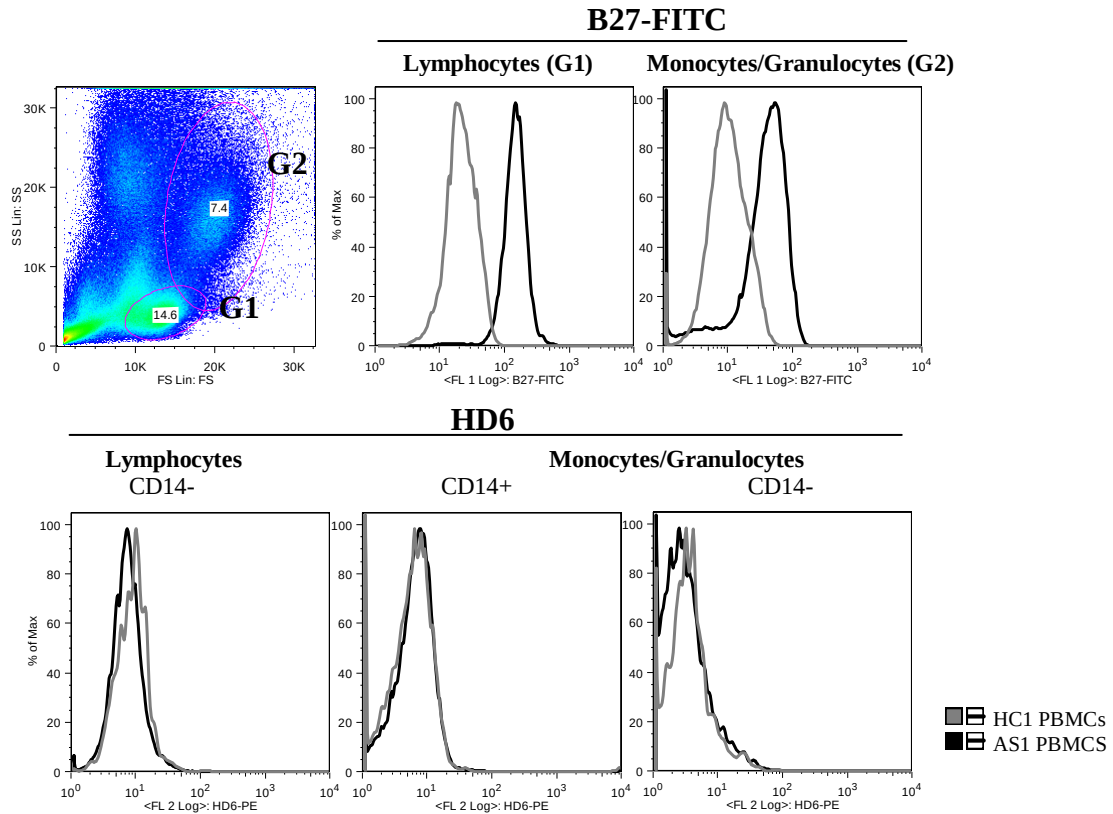


Figure 5.14 HD6 does not bind to peripheral blood mononuclear cells from B27+ AS patients under standard conditions. PBMCs were isolated from freshly isolated venous blood samples from a B27+ AS patient (AS1, black histograms) and a non-B27+ healthy control (HC1, grey histograms) and stained with B27-FITC or HD6 and a Pacific Blue-conjugated CD14 antibody. Data shown is representative of 3 independent experiments with staining of PBMCs from 4 B27+ AS patients and 3 B27- healthy controls.

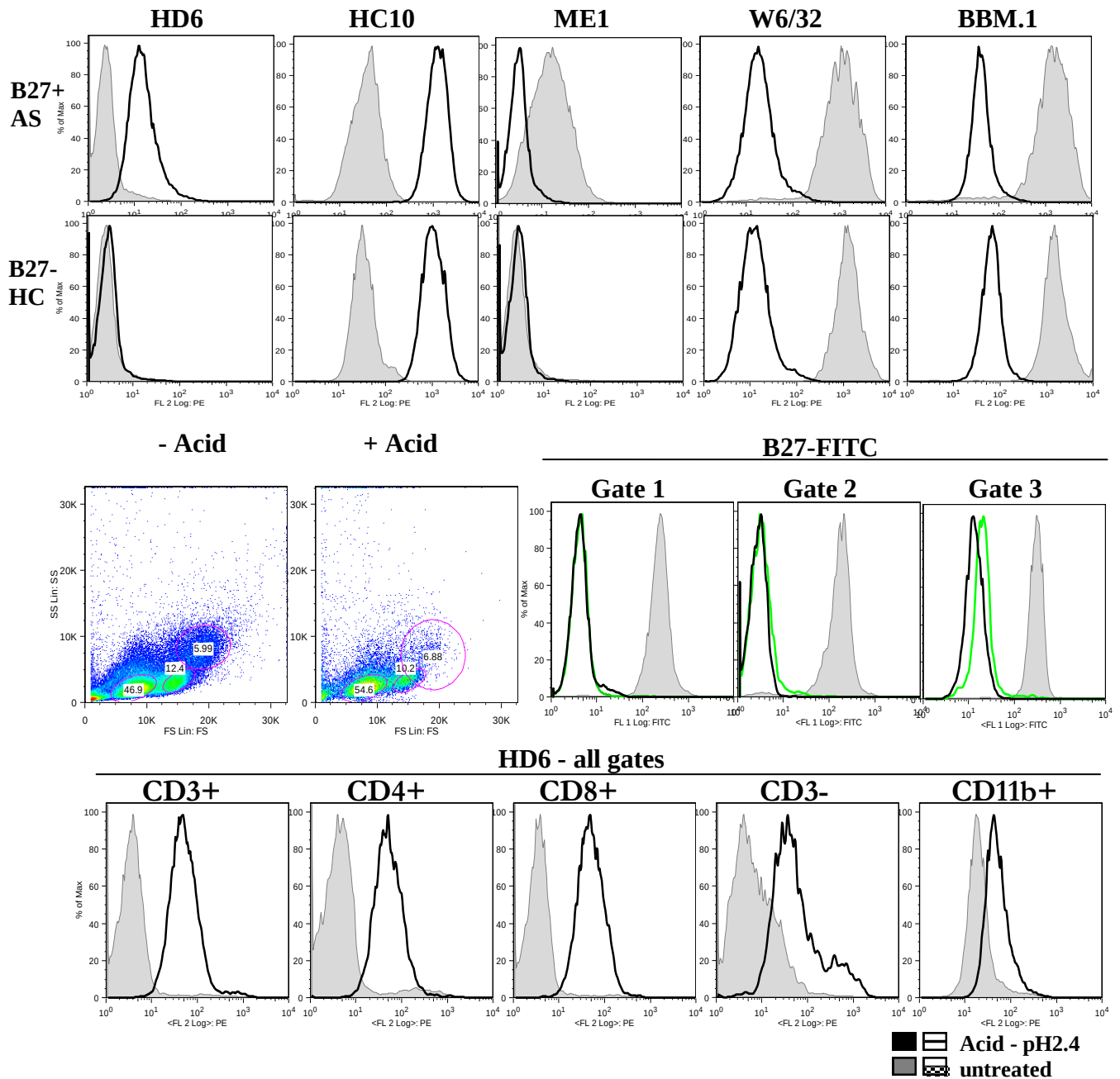


Figure 5.15 HD6 staining is induced by low pH treatment on PBMCs from B27+ AS patients but not controls. PBMCs from a B27+ AS patient (B27+ AS) and a B27- healthy control (B27-HC) were isolated and left untreated (grey histograms) or treated for 2 min with a low pH glycine buffer (pH 2.4) as previously described. Samples were washed twice before antibody staining. (A) Cells stained with the mAbs HD6, HC-10, ME-1, W6/32, and BBM.1 as indicated, gating on all live cells. (B) Cells stained with B27-FITC or isotype control (green histograms) and gated on the 3 populations shown (C) Cells stained with HD6 and the subset markers as indicated, gating on all live cells.

5.5 Discussion

The purpose of this chapter was to determine whether HD6 binds to B27 molecules in the context of a disease setting. To address this, splenocytes isolated from the HLA-B27 transgenic rat model of SpA and PBMCs isolated from AS patients were analysed by flow cytometry. The results presented here demonstrate that i) HD6 binds to splenocyte cell subsets from HLA-B27 transgenic rats but not age-matched WT or HLA-B7 transgenic rats, ii) both HD6 and HC-10 binding are strongly correlated with the levels of surface HLA-B27 and age of the B27 TG rat, iii) HD6 staining of HLA-B27 TG rats is reduced by addition of exogenous B27-binding peptides and increased by low pH treatment, iv) HD6 did not bind to PBMCs from AS patients under standard conditions, but was induced upon acid treatment in multiple subsets.

i) HD6 binds to splenocyte cell subsets from HLA-B27 transgenic rats but not age-matched WT or HLA-B7 transgenic rats

As outlined at the start of this chapter, two studies have reported the presence of HC-10-reactive B27 homodimers in B27 transgenic rat samples (Kollnberger et al., 2004; Tran et al., 2004). In the first, Tran *et al.* observed these dimers in lysates of spleen cells isolated from several strains of B27 TG rats, including the C67S mutant rat cell line 133-1, but not from the B7 TG rat line 120-4. Similarly, Kollnberger *et al.* found HC-10-reactive dimers in lysates from both lymph node and spleen cells from B27 TG rats, the levels of which correlated with the rat B27 transgene copy number. Furthermore, this study also detected multimeric species, including dimers, at the surface of B27 TG rat DCs. Notably, however, low levels of HC-10-reactive dimers were also detected in lysates and at the surface of B7 TG rat cells. These low levels

were comparable to those detected in the disease-resistant B27 TG rat line 21-4L, which have fewer copies of the B27 (6 copies) and human β_2m transgenes (6 copies) than the 33-3 B27 TG rats (55 and 66, respectively). Consistent with these findings, in this chapter I have shown that HD6 stains to splenocytes from disease-prone B27 TG rats but not age-matched WT or B7 TG rats. These results provide further evidence that HD6 recognition is B27-specific. In addition, the steady-state HD6 staining observed is in accordance with my proposal that expression of HD6-reactive molecules requires over-expression of HLA-B27. This is also supported by the differences in subset staining I observed for the adult B27 TG rats, which suggest a correlation between the levels of surface HLA-B27 and the presence of misfolded B27 conformers, as detected by HD6 and HC-10 mAbs. Both HC-10 and HD6 stained lymphocyte and monocyte cell populations, but not granulocytes, the latter of which had markedly lower HLA-B27 surface expression levels.

Interestingly, when compared to age-matched WT rats, the splenocytes from the B27 TG rats had an increased relative percentage of cells within the granulocyte gate, the majority of which were CD11b⁺. An enhanced granulocyte infiltration has previously been observed macroscopically in the GI tract of B27 TG rats (Aiko and Grisham, 1995). Furthermore, Qian *et al.* have reported TG rat spleens to contain a higher proportion of CD11b/c cells compared to non-TG littermates (Qian et al., 2008). In this study, the authors claim these cells are primarily DCs and macrophages, although given that no gating on FSC/SSC appears to have been carried out, it is perhaps surprising that granulocytes were overlooked. Nevertheless, despite their increased prevalence, it appears that the low levels of folded HLA-B27 and absence of

misfolded B27 molecules on the surface of granulocytes rules them out as key instigators in HLA-B27-mediated pathology. Instead, their increased infiltration is more likely symptomatic of the ongoing inflammation in diseased rats, or they may be recipients of a B27 interaction, through the expression of PIR.

ii) both HD6 and HC-10 binding are strongly correlated with the levels of surface HLA-B27 and age of the B27 TG rat

Taurog *et al.* have previously demonstrated that disease susceptibility in B27 TG rats correlates with the B27 transgene copy number, and that B27 surface expression is increased with age and/or disease onset in disease-prone lines (Taurog *et al.*, 1993). Therefore, to further investigate the correlation between HD6 staining and B27 surface levels described above, staining of splenocytes from young and old disease-prone B27 TG rats were compared. In these experiments, I demonstrated HLA-B27 surface expression was consistently higher in the older B27 TG rat cell subsets, as reported by Taurog *et al.*. Similarly, I observed increased HC-10 staining levels in the older B27 TG rat cells compared to the younger B27 TG rat cells. For HD6, a marked difference was also seen for the samples. As before, with the exception of granulocytes, all cell subsets from the older B27 TG rat samples stained with HD6. In contrast, no HD6 staining was observed above background levels for any of the cell subsets analysed in the young B27 TG rat samples in two sets of experiments. In a third experiment, HD6 staining was observed on some of the cell subsets, but still at markedly lower levels than in the older B27 TG cells. In particular, in the lymphocyte gate, CD45R+ B cells exhibited the highest staining, with some CD4+ SP T cells also

staining. Whereas in the monocyte/DC gate, HD6 staining was most notable in the CD11b⁺ MHCII⁻ subset.

Together, these data are consistent with the theory that the level of B27 expression determines whether HD6-reactive molecules are expressed. Moreover, they are compatible with the idea that HD6-reactive molecules contribute to disease pathogenesis, given their appearance at low levels in the young rats precedes the onset of disease symptoms. If HD6-reactive molecules represent a pathogenic form of HLA-B27, these data raise the interesting question of whether the cells that exhibit HD6 staining earliest are effector cells that mediate disease pathogenesis. To address this, further investigation into the time course and pattern of HD6 epitope expression in these B27 TG rats is required. It will also be interesting to further investigate the HD6 staining of DC subsets, such as OX62⁺ cells, in more detail. Several studies have reported defective DC function in B27 TG rats as being a consequence of high HLA-B27 expression, but which may in fact be a result of the increased presence of HD6-reactive molecules in these cells (Dhaenens et al., 2009; Fert et al., 2008; Hacquard-Bouder et al., 2007; Hacquard-Bouder et al., 2004).

iii) HD6 staining of HLA-B27 TG rats is reduced by addition of exogenous B27 binding peptides and increased by low pH treatment

In Chapter Four, I established that exogenous peptide abrogates HD6 staining and reduces HC-10 staining in human cell lines. In this chapter, these findings have been extended to the HLA-B27 transgenic rat, wherein I have shown addition of a B27-binding peptide also considerably reduced HD6 staining. As in the human cell lines, no change in folded B27 molecules was observed, consistent with the proposal that

peptide does not refold molecules at the cell surface, but may either stabilise surface molecules prior to and/or during endosomal recycling events or simply bind to HD6-reactive conformers and block the epitope. Intriguingly, Taurog's group have reported that the introduction of a minigene, NP1, encoding a B27-binding peptide (SRYWAIRTR) to disease-prone B27 TG rat lines resulted in a 50% reduction in the prevalence of arthritis (Zhou, 2000 ; Zhou et al., 1998). Conversely, no change in disease phenotype was seen when a control minigene was introduced (SLYWAIRTR). To explain their findings, these authors propose that the high-affinity NP1 peptide may suppress the level of presentation of a putative arthritogenic peptide by competing with it for loading in the ER. However, on the basis of more recent findings in transgenic rats that strongly suggest CD8⁺ T cells are not required for disease pathogenesis, this appears unlikely. An alternative explanation, consistent with my findings, is that the presence of high-affinity B27-binding peptides at a high concentration ensures that the B27 molecules transported to the cell surface are always optimally loaded stable complexes, reducing the expression of unstable and unfolded surface B27 molecules that contribute to disease pathogenesis. Notably, the minigene constructs were designed to target the peptide to the ER, whereas the exogenously added peptides used in my studies likely exert their effect at the cell surface. Nevertheless, both could result in stabilisation of B27 heterotrimeric complexes and/or a reduction in the appearance of HC-10- and HD6-reactive surface molecules. Unfortunately, although these authors reported that the NP1 minigene did not affect expression of folded B27 molecules, its effect on the expression of free heavy chains was not investigated, which could have provided further support for this mechanism.

These results provide further evidence in support of the theory that the surface stability of peptide-loaded B27 complexes is a critical to its role in enhancing susceptibility to SpA. In addition, this highlights the potential for peptide therapy as a means to specifically target HLA-B27 molecules either for stabilisation or to block potentially pathogenic aberrantly folded forms.

Consistent with the data presented in Chapter Four, low pH treatment of B27 TG rat splenocytes markedly increased HD6 staining on all cell subsets. This was particularly apparent in the splenocytes of young B27 TG rats, and the granulocyte populations of old B27 TG rats, which had little or no HD6 staining prior to acid treatment. Importantly, despite increases in HC-10 staining, no HD6 staining was observed upon acid treatment of the B7 TG rat cells. Again, when simultaneously investigated, HD6 staining was consistently higher in the older B27 TG rats both with and without acid treatment, suggesting that the level of expression of folded B27 molecules is an important factor in determining the number of 'acid-inducible HD6' molecules present. These data are consistent with the model proposed in Chapter Four, and enable it to be extended to the HLA-B27 transgenic rat model of SpA.

Future investigation should also compare HLA-B27 expression in disease-prone rats derived under normal conventional housing conditions, which develop disease, and those derived under specific pathogen-free conditions, which remain disease free. This may provide further insight into the link between environmental triggers and potentially pathogenic HD6-reactive molecules. For example, if HD6-reactive

molecules are central to the onset of disease in the TG model, one prediction could be that fewer molecules are expressed in the SPF-derived disease-free TG rats compared with their conventionally-housed TG littermates. Introducing the former to conventional housing conditions may then result in detectable increases in HD6-reactive molecules, which may correlate with disease onset. Alternatively, the role of the environmental trigger may not be linked to the formation of HD6-reactive molecules, which would predict similar HD6 staining levels for these differently housed TG rat groups.

v) HD6 did not bind to PBMCs from AS patients under standard conditions, but was induced upon acid treatment in multiple subsets

Investigation of B27₂ expression in SpA individuals has been more difficult, but has met with limited success. Using the combination of ¹²⁵I surface labelling, HC-10 immunoprecipitation, and isoelectric focusing, one study first reported HC-10-reactive dimer expression at the surface of PBMCs from an HLA-B27+ AS patient (Kollnberger et al., 2002). HC-10-reactive dimers were also detected by western blot in lysates of PBMCs and SFMCs from an HLA-B27+ ReA patient. However, as only 2 SpA patients and no healthy controls were examined, it is impossible to conclude that this phenomenon is specific for HLA-B27 and restricted to HLA-B27+ SpA individuals. An alternative approach for B27₂ detection in humans has been to investigate whole cell lysates of monocyte-derived DCs derived from HLA-B27+ individuals (Campbell et al., 2011; Santos et al., 2008). These studies demonstrated that a transient population of HC-10-reactive dimers in MD-DCs could be induced upon LPS activation. In the first report, which compared cells derived from 2 HLA-

B27+ individuals with those from 2 HLA-B27- individuals, the presence of HC-10-reactive dimers appeared HLA-B27 specific (Santos et al., 2008). However, in the most recent study by the same group, these dimers were also present in non-B27 controls. Moreover, no significant difference was observed in the levels of these dimers or the kinetics of their formation when comparing the B27+ AS patient and the healthy control cohorts (Campbell et al., 2011).

Thus, on the basis of the research to date, it has been difficult to draw firm conclusions regarding the expression of HLA-B27 homodimers in the context of human disease and their role in SpA pathogenesis. This could be better understood through the development of reagents specific for B27₂. Rather unexpectedly, no HD6 staining was detected on any of the cell subsets analysed in any of the 4 AS patients PBMC samples. However, given that the cell activation status was important for detection of HC-10-reactive dimers in MD-DCs, it is perhaps not surprising that under steady state conditions no HD6-reactive molecules were detected in bulk PBMCs. Therefore, future work needs to look at whether HD6 staining is affected by altering the activation status of immune cell subsets, such as MD-DCs, by investigating the effect of TLR ligands such as LPS. Moreover, it will be important to ensure that an appropriate time course is chosen, given the transient nature of the HC-10-reactive dimers in MD-DCs described above. In this regard, preliminary experiments I undertook with *S.typhimurium* found infection of PBMCs did not appear to induce HD6 epitope expression after 24h (data not shown). However, further experiments are required to confirm this.

Another possible explanation for the lack of detectable HD6-reactive molecules on AS patient PBMCs is that abnormal expression of HLA-B27 molecules may be limited to the affected tissues of patients, such as hip and knee joints, sacroiliac joints, and intervertebral joints. In this regard, more insight may be provided through the analysis of synovial fluid samples and synovial tissue cells. These studies have been approved and will begin later this year. Furthermore, HD6-epitope expression may correlate with disease activity and disease severity, a theory supported by the findings in the transgenic rat model of SpA. It will therefore be important to ensure that disease activity scores are recorded (BASDAI, BASFI, and BASMI scores) together with the HLA-B27 status and levels of inflammatory markers (CRP and ESR) at the time when samples are obtained from patients. This may also explain the lack of HD6 staining observed in the 4 AS patient PBMC samples, for which there was limited information regarding their disease status. If present in PBMCs, surface HD6-reactive molecules may only be detectable at certain periods in disease, such as immediately prior to a flare-up of inflammation. Some support for this concept is also provided in the B27 TG rat model, whereby the ongoing inflammation could account for the continued presence of HD6-reactive molecules.

As seen for HLA-B27+ human cell lines and HLA-B27 TG rat splenocytes, HD6-reactive molecules could be artificially induced on the surface of AS patient PBMCs through acid treatment. This suggests that, although not present at steady state, these cells have the capacity to express HD6-reactive molecules, albeit under the extreme low-pH conditions used here. However, as discussed for the TG rats, this artificial

induction may be reflective of the possible outcome of long-term exposure of cells to an inflammatory setting, or the acidic and hypoxic conditions of the joints.

Concluding remarks and future perspectives

The central aim of this thesis was to investigate the cell surface expression of aberrant forms of HLA-B27, such as B27₂, by means of a novel and potentially B27₂-specific monoclonal antibody, HD6. In addressing this, I have shown that cell surface HD6-reactive molecules are expressed in several cell lines expressing HLA-B27, and in splenocytes isolated from HLA-B27 transgenic rats. In addition, comparable HD6 binding was detected in cell lines expressing the disease-associated B*27:05 subtype and the less disease-associated B*27:09 subtype, and HD6-reactive molecules were also detected in cells expressing the mutant B27 C67S form. Importantly, HD6 staining is strongly correlated with the degree of HLA-B27 expression in these cells. Furthermore, I have shown that cells expressing physiologic levels of HLA-B27, including EBV-transformed B cell lines and AS patient PBMCs, are capable of expressing the HD6 epitope upon treatment with low pH. Interestingly, these ‘acid-inducible HD6’ molecules are absent from cells lacking tapasin and TAP. Finally, I have demonstrated that HD6-reactive molecules can derive from pre-existing folded B27 molecules at the cell surface. This may be inhibited by incubation of HLA-B27 binding peptides, which I have shown to abrogate HD6 surface staining. Together, these findings raise several questions relating to the role of misfolded B27 molecules in SpA pathogenesis that warrant further investigation in future studies.

Firstly, if aberrantly folded B27 molecules such as the HD6-reactive B27 molecules are indeed pathogenic, a key question is whether it is the intracellular or extracellular misfolded molecules that underlie disease pathogenesis. This distinguishes the HLA-

B27 misfolding and UPR theory from the B27 homodimer theory, although support for either theory could be garnered through the development and use of reagents that specifically recognise B27₂, such as the HD6 antibody. Immunoprecipitation experiments combined with surface labelling techniques should provide straightforward approaches to determine the cellular localisation of HD6-reactive molecules, as well as allowing further investigation into the role of components of the PLC and their interactions with misfolded B27 conformers. Moreover, these methods should also allow the exact nature of HD6-reactive molecules to be determined. In the B27 misfolding and UPR theory, disease is proposed to be a result of the accumulation of aberrantly folded B27 molecules in the ER, resulting in the production of an inflammatory response. Therefore, immunoprecipitations with HD6 should enable quantification of the levels of a subset of misfolded B27 molecules within the ER. In doing so, this provides a means to verify whether a correlation between levels of misfolded B27 in the ER and occurrence of disease holds.

The homodimer theory proposes that B27₂ contribute to disease pathogenesis by altering T cell and/or APC responses through their interaction with innate immune receptors such as those of the KIR and LILR families. In particular, whereas B27HT and B27₂ both bind to KIR3DL1 and LILRB2, only B27₂ binds to KIR3DL2. Therefore, to investigate the physiological relevance of the surface HD6-reactive molecules detected in my studies and their functional effects on immune receptor recognition, our group are developing an IL-2 reporter system in transfected Jurkat T cells that express LILRB2-CD3 ϵ and KIR3DL2-CD3 ϵ fusion receptors. Through co-culturing of these T cells with APCs expressing different HLA alleles, the functional

consequences of the interaction of these receptors with HLA-B27 can be compared with other HLA alleles. Moreover, the functional consequences of interaction of HD6-reactive molecules with LILRB2 and KIR3DL2 can be specifically examined by co-culturing these T cells with .220B27 cells (express HD6-reactive molecules) and comparing the response to co-culture with .220B27 cells expressing HA-tagged B27, which I have shown in this thesis to lack surface HD6-reactive molecules despite high HLA-B27 surface expression. In addition, this system provides a direct means to assess the ability of HD6 antibody to specifically block innate immune receptor interactions. Our group have also investigated the proliferation, survival and cytokine production of KIR3DL2⁺ NK and CD4 T cells in co-culture experiments with HLA-B27 expressing APCs or controls (Chan et al., 2005). The KIR3DL2⁺ NK cells were protected from apoptosis in the presence of B27₂-expressing APCs, whereas the KIR3DL2⁺ CD4 T cells demonstrated enhanced survival and proliferation. These results provided indirect evidence for a role for B27₂:KIR3DL2 interactions in disease. Therefore, an obvious extension to this is to test the ability of HD6 to inhibit the protection from apoptosis of KIR3DL2⁺ NK cells and the enhanced survival and proliferation of KIR3DL2⁺ CD4 T cells mediated by the B27₂-expressing APCs. This would offer definitive evidence that the observed effects were a consequence of interaction of HD6-reactive molecules with KIR3DL2. Together, these approaches could provide further support for the hypothesis that increased cell surface expression of B27₂ and/or other novel B27 conformers results in interactions with immune receptors that culminate in the survival of pro-inflammatory leukocytes in disease.

Our group have also shown that KIR3DL2⁺ CD4 T cells from SpA patients are enriched for the Th17 phenotypic markers CCR6, CD161 and, most notably, IL23R compared with controls (Bowness et al., 2011). Moreover, these cells provide an important source of IL-17, and have been shown to be enriched in the peripheral blood and synovial fluid of SpA patients. In addition, KIR3DL2⁺ CD4 T cells from AS patients produced more IL-17 than the equivalent cells from healthy controls when stimulated with B27-expressing APCs. Whether the increased production of IL-17 is a consequence of the interaction with HD6-reactive molecules is of great interest, especially given the strong genetic association of IL23R with SpA. This relationship can also be tested through similar co-culture assays that incorporate HD6 blockade. Moreover, as described above, the IL-17 production upon co-culture of KIR3DL2⁺ CD4 T cells with .220B27 cells (HD6 positive) could be compared to co-culture of KIR3DL2⁺ CD4 T cells with .220B27HA cells (HD6 negative). Lower IL-17 production would be expected in the latter, if HD6-reactive molecules are important in the process. The findings of this thesis also present low-pH treatment of cells as an intriguing possible means to assess the functional recognition of HD6-reactive molecules by innate immune receptors in the context of the cell assays described above. For example, the functional consequences of KIR3DL2⁺ CD4 T cells co-cultured with either acid-treated or untreated APCs can be compared with each other. Although the effect of acid treatment on other cell surface molecules would need to be taken into consideration, this could provide compelling evidence for the potential functional relevance of these HD6-reactive molecules in SpA pathogenesis.

LILRB2 expression is restricted to cells of the myeloid lineage, which suggests that its interaction with HD6-reactive molecules could affect the functioning of APCs themselves as oppose to T and NK cells. Notably, several studies have shown that immunoreceptor interactions with MHC class I molecules can occur on the same cell surface ‘in cis’ (Doucey et al., 2004; Masuda et al., 2007; Mori et al., 2008; Scarpellino et al., 2007). For example, Masuda *et al.* have shown by FRET analyses the co-localisation of MHC class I molecules with LILRB2 in a human cell line, and co-localisation with the murine ortholog PIR-B in a murine cell line (Masuda et al., 2007). Moreover, the cis interaction between PIR-B and MHCI appeared to provide a constitutive negative signal to the cell, which they suggest to be a novel mechanism of inhibitory auto-regulation of cell activation. These authors have postulated that the cis-binding of these inhibitory receptors by MHCI delivers self-regulating signalling and maintains a threshold for cell activation. Thus, an attractive hypothesis is that cis interactions between LILRB2 and B27₂ may have an effect on the threshold for cell activation that differs from the B27HT interaction. This could interfere with the constitutive negative signalling by LILRB2, altering the activation threshold in favour of a proinflammatory response. In relation to this, Huang *et al* have shown that preferential recognition of a subset of HLA-B35 alleles by LILRB2 is associated with DC dysfunction (decreased maturation and cytokine production) in *in vitro* functional assays (Huang et al., 2010). This highlights the impact that LILRB2 interactions can have on APC function. In addition, DCs isolated from disease-prone HLA-B27 transgenic rats have been shown to form defective interactions with T cells (Dhaenens et al., 2009; Fert et al., 2008; Hacquard-Bouder et al., 2007; Hacquard-Bouder et al., 2004; Stagg et al., 1995). The possibility that this is a consequence of

LILRB2:B27₂ cis interactions, although highly speculative, merits further investigation.

The observation that over-expression of HLA-B27 appears to be a pre-requisite for steady-state HD6 staining raises the question of how this could translate to human disease. Notably, B27 expression, at both the mRNA and protein level, has been shown to be increased in cells from B27+ patients compared to healthy B27+ controls (Cauli et al., 2002; Liu et al., 2006). In the first study, Liu *et al.* also showed that quantitative measurement of B27 mRNA in patients with AS correlated with clinical activity, as determined by BASDAI data. This is supported by the findings in Cauli *et al.*, which reported statistically higher levels of B27 expression in lymphocytes of patients with AS compared with B27+ healthy controls. The cause of the increased B27 expression in AS patients is unclear. However, further insight could be gained through a better understanding of the B27 promoter region, its polymorphisms (if any) and the components of the upstream signalling pathways, including transcription factors, that culminate in transcriptional activity. MHC class I gene promoters contain three major regulatory elements, one of which is the IFN stimulated response element (ISRE), which, through binding of transcription factors in the IFN regulatory (IRF) family, mediates the induction of transcription by type I and type II IFN. Notably, the ISRE of most HLA-B alleles, including HLA-B27, is flanked by a putative E box that is a binding site for upstream stimulatory factors (USF), which have been suggested to be inhibitors of constitutive HLA-B gene transcription (Gobin et al., 1999). Enhancer A is a second regulatory element that contains the binding sites for NF- κ B/Rel transcription factors and mediates the TNF α -induced transactivation of MHC

class I. One group have investigated the promoter region of B27 by cloning the 5'UTR region of B27 into a luciferase reporter gene construct. In this study, the effect of a panel of cytokines on the promoter of B27 was examined, identifying TNF α and IFN β as major participants in upregulating promoter activity (Zhao et al., 2008). Therefore, it is conceivable that in the context of SpA, an initial trigger releases inflammatory cytokines that augment the expression of HLA-B27 to the threshold level required for formation of HD6-reactive molecules, which then contribute to a persisting inflammatory setting. This could also be relevant to the observed efficacy of anti-TNF α agents in SpA treatment. These observations justify the investigation of cell surface expression of both folded and misfolded forms of HLA-B27 in response to TNF α and type I and type II IFN stimulation, as well as to other reagents reported to impact on HLA-B27 transcription (Zhao et al., 2011).

I have shown HD6 staining to be independent of HLA-B27 subtype polymorphisms at position 116, indicating that the lack of association between B*27:09 and disease is not due to an inability to form HD6-reactive dimers. However, it is possible that B*27:09 is expressed at lower levels in cells, either in general or in specific disease-relevant cell subsets, compared to other disease-associated subtypes. Therefore, although the threshold level for formation of HD6-reactive molecules is reached in cell lines over-expressing HLA-B*27:09, this may not be the case in B*27:09 individuals. Future investigation of this would first require the identification of triggers of HD6 epitope expression in B*27:05 individuals, given that no steady-state HD6 staining of PBMCs from B*27:05 individuals was detected here. Alternatively, a titration of HLA-B27 expression levels of the two subtypes in human cell lines may

provide insight into the relationship between mRNA levels of B27, surface levels of B27 and the appearance of HD6-reactive molecules. The generation of HLA-B*27:09 transgenic rats has been proposed by several groups. Although it is possible that these rats will fail to develop disease, I consider it more likely that over-expression of B*27:09 in these rats would be enough to trigger disease development, particularly given the strong correlation between expression levels and disease in the B*27:05 transgenic rats.

The spondyloarthropathies are complex diseases in which genetic factors other than HLA-B27 as well as environmental factors play a significant role in the development and pathogenesis. The role of the environment is particularly apparent in the HLA-B27 transgenic rat model, whereby derivation of rats under SPF conditions prevents development of an inflammatory phenotype. This is despite over-expression of HLA-B27 in these rats. Further investigation should address the expression of aberrant forms of HLA-B27 in SPF-free rats as well as in the HLA-B27 TG rats expressing the NP1 minigene construct described in Chapter Five. What then is the role of environmental triggers in SpA pathogenesis? Environmental factors could be important in promoting HLA-B27 expression. For example, intracellular bacteria such as Salmonella and Chlamydia species, as well as many viruses, can induce production of type I IFN (Rothfuchs et al., 2001), providing a possible means of increasing HLA-B27 expression and, in doing so, promoting the formation of HD6-reactive molecules. Alternatively, bacteria may overload or interfere with components in the ERAD or endosomal recycling pathways, impairing the quality control mechanisms and thereby promoting the escape to the surface of aberrantly folded

and/or suboptimally loaded HLA-B27 molecules. Interference of pathogens with the proteasome and other cellular proteases involved in the processing of the peptide cargo for HLA-B27 may also contribute to impaired folding and subsequent overload of ERAD. These theories could be investigated through specific targeting of components of the ERAD and the endosomal recycling pathways. For example, it may be possible to enhance the activity of ERAD pathways by over-expression of components such as the E3 ligases TRC8 and Hrd1. This could conceivably increase the threshold of HLA-B27 required before HD6-reactive molecules are formed. Alternatively, siRNA-mediated down-regulation of ERAD components may be predicted to lower this threshold and result in increased HD6 staining. siRNA-mediated down-regulation of components of the PLC such as tapasin is also worth pursuing, as it could provide further evidence for the role of B27HT stability in HD6 epitope expression.

A possible therapeutic option for treatment of SpA that is supported by the findings in this thesis and that warrants further investigation is the targeting of HD6-reactive molecules with B27-binding peptide analogues. Given the short half-lives of exogenously administered peptides and supraphysiologic levels that would be required, administration of B27-binding peptides themselves is may to be ineffective *in vivo*. However, the possibility of targeting the peptide binding groove of HLA-B27 molecules to either prevent the formation of HD6-reactive molecules by stabilising the precursors, or to block the interaction of already formed HD6-reactive molecules with innate immune receptors is intriguing. Further justification for this line of

investigation and proof of concept could be provided by antibody staining of the HLA-B27 TG rats expressing the NP1 mini-transgene.

In conclusion, the work presented in this thesis has validated the use of a novel monoclonal antibody, HD6, as a B27-specific detection reagent in a cellular setting. Importantly, its recognition of aberrantly folded HLA-B27 conformers, which may include B27₂, is distinct from that of HC-10. Moreover, the detection of cell surface HD6-reactive molecules in both human cell lines and in a transgenic rat model of SpA provides evidence to support a role for these potentially pathogenic forms of B27 in disease pathogenesis. HD6 will serve as a powerful tool that can be used in future studies to investigate the formation, interactions and proposed pathogenic role of misfolded HD6-reactive B27 molecules in SpA. Moreover, its potential to be used as a therapeutic agent to treat disease will be addressed in the HLA-B27 transgenic rat model, which in turn will provide insight into the feasibility of targeting misfolded B27 molecules in this manner.

Appendix I

Generation of HLA-B27 peptide-specific tetramers

HLA-B27 heterotrimeric tetramers were generated with well-described HLA-B27 restricted viral epitopes as previously reported (Stewart-Jones et al., 2005). The extracellular domain of HLA-B27 was refolded with either the RRIYDLIEL peptide from EBV (EBNA3C residues 258-266, 'EBV' peptide) or the SRYWAIRTR peptide from influenza virus (nucleoprotein residues 383-391, 'FLU' peptide) and β_2 -m for 48h, and refolded complexes were then analysed by gel filtration (Figure 7.1 A and B). Black arrows indicate the peaks corresponding to the refolded heterotrimeric complexes (elution volume 160-175ml), which were concentrated to 1ml and analysed by reducing and non-reducing SDS-PAGE (Figure 7.1C). For both the EBV and FLU refolds, bands corresponding to heterotrimeric complexes (43kDa) and a β_2 -m dimer (26kDa) are seen in the non-reduced gel, the latter of which is resolved to a 13kDa monomeric β_2 -m band under reducing conditions. Binding of these peptide-HLA-B27 heterotrimeric tetramers was then tested by FACS staining to confirm that the refolded complexes were structurally intact molecules (Figure 7.1D). Baf cells transfected with LILRB2 (left panel) or KIR3DL1 (right panel) were stained with 5ug of tetramer. As previously shown in (Stewart-Jones et al., 2005), FLU tetramer bound to both LILRB2 and KIR3DL1 whereas EBV tetramer only bound to LILRB2.

Generation of antigen-specific CD8⁺ cytotoxic T cell lines from HLA-B27⁺ PBMCs

HLA-B27 heterotrimeric tetramer complexes were used to derive viral peptide-specific HLA-restricted CD8⁺ cytotoxic T cell lines from HLA-B27⁺ PBMCs. Cells that had been cultured for 14 days with Flu peptide were first sorted on the basis of Flu-tetramer and CD3 staining (Figure 7.2A, black circle highlights sorted cells). Sorted cells were cultured for a further 14 days in 'feeder' mix, and then stained with Flu-tetramer and either anti-CD3-APC or anti-CD8-APC (figure 7.2B top and bottom panels respectively), which showed the cultured CD3⁺ population (98.2%) to comprise 2 tetramer positive sub-populations (top panel). The tetramer high population corresponded to a CD8⁺ subpopulation (lower panel), which was subsequently selected for further sorting (black circle). Double-sorted FLU-specific CD8⁺ T cells (figure 7.2C, upper quadrant 94.4%) were then checked for specificity and shown to stain with the FLU-tetramer but not the EBV-tetramer (figure 7.2C right panel). An EBV-specific CD8⁺ T cell line was also generated in the same manner (data not shown). These cell lines were generated for use in subsequent CTL killing assays.

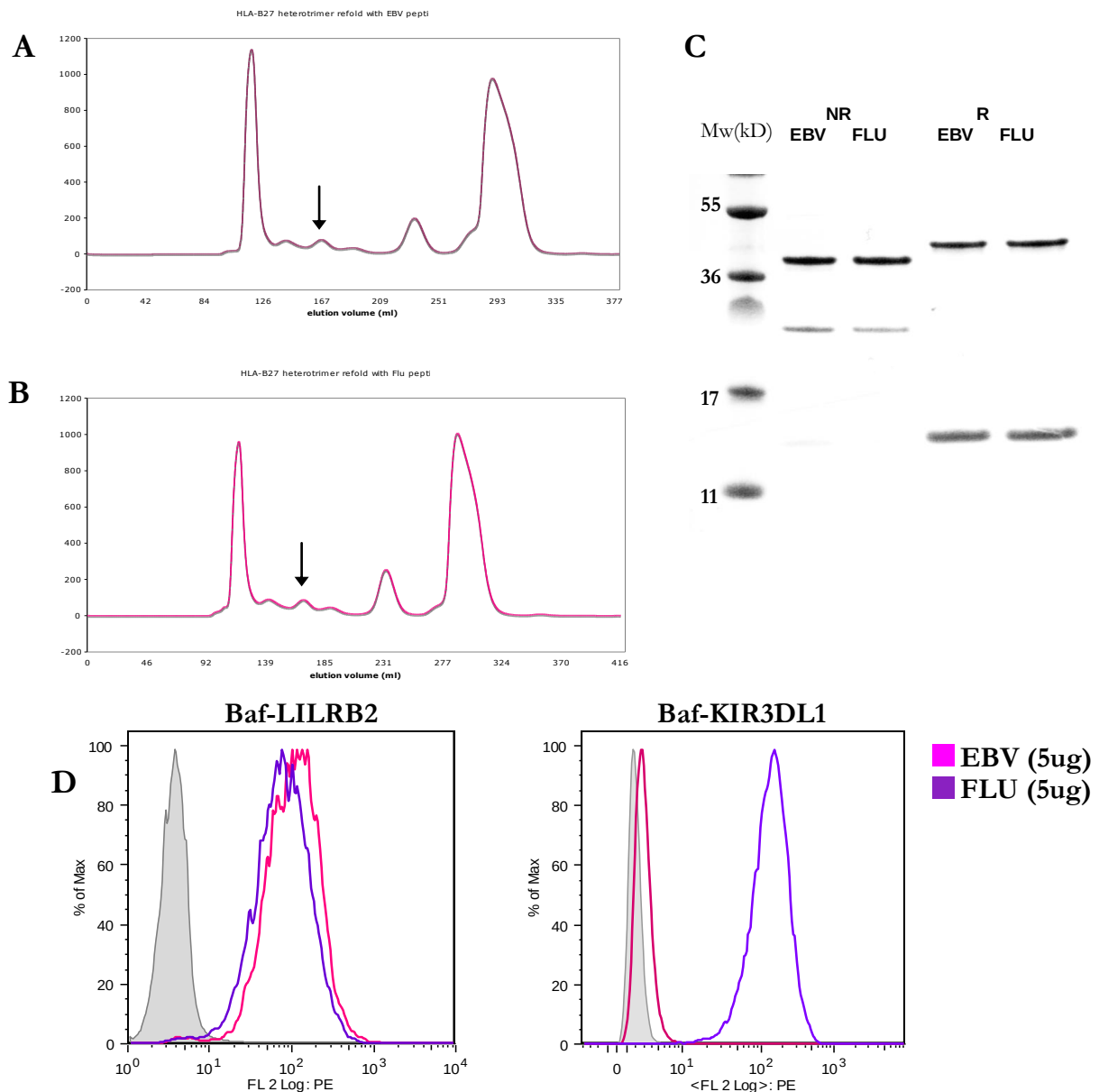


Figure 7.1 LILRB1 and KIR3DL1 binding of HLA-B27 heterotrimeric tetramers refolded with viral peptides. HLA-B27 heavy chains were refolded with β_2m and either (A) RRIYDLIEL peptide (EBV) or (B) SRYWAIRTR peptide (FLU) and analysed on a Superdex sd75 gel filtration column. (C) SDS-PAGE analysis of concentrated fractions (arrow, elution volume 160-175ml) under non-reducing and reducing (+DTT) conditions. (D) FACS analysis of tetramer staining of Baf cells transfected with either LILRB1 (ILT2) or KIR3DL1. Results are representative of 2 independent staining experiments.

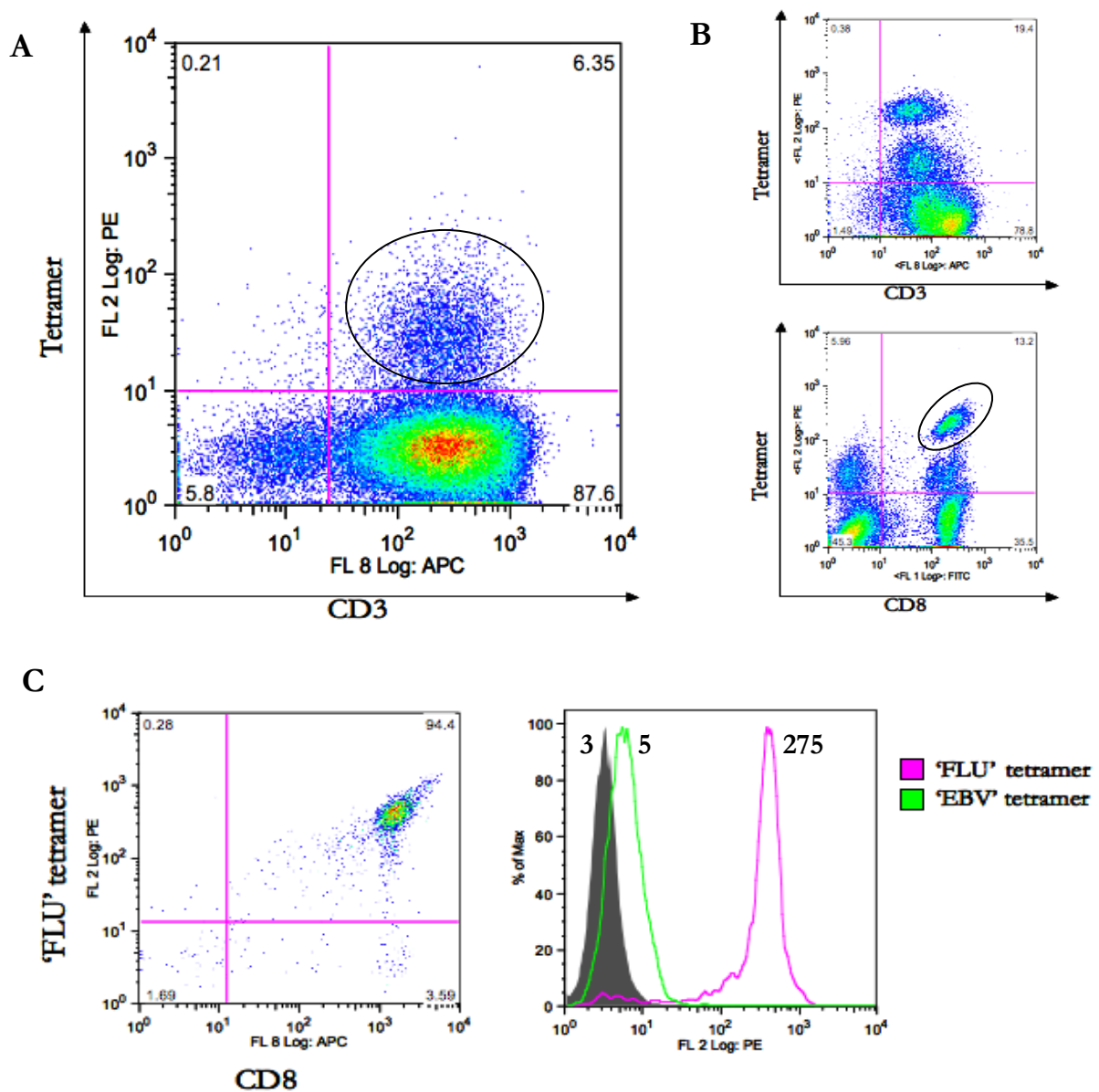


Figure 7.2 The generation of an HLA-B27-restricted 'Flu'-specific CD8+ T cell line. PBMCs were collected from HLA-B27+ individuals, cultured in 24 well plates with SRYWAIRTR ('Flu') or RRIYDLIEL ('EBV') peptide for 14d, and then stained with anti-CD3-APC and the appropriate heterotrimeric tetramer for sorting of double positive cells, as shown for Flu tetramer in (A). (B) Sorted cells were grown in 'feeder' mix for 14d, stained for CD3 (top) or CD8 (bottom) expression and sorted again for CD8+ tetramer+ double positive cells (black ring). (C) Double-sorted Flu-specific CD8 T cells (left) stained with Flu tetramer and EBV tetramer (right). The MFI are displayed on histogram; the grey filled histogram is the negative control (extravidin-PE). Results are representative for 3 HLA-B27+-restricted cell lines (1 EBV-specific and 2 Flu-specific).

Appendix II

Trypsin treatment of HLA-B27-expressing HeLa cells reduces their reactivity with mAbs

Untransduced HeLa cells and HeLa cells lentivirally transduced for HLA-B27 expression were treated with reagents used commonly for the removal of adherent cells, including trypsin, EDTA/Lidocaine, and AccutaseTM (data not shown), or were scraped from the surface of 6 well plates. 2ml of removal reagent was added to cells, which were then incubated at 37°C until floating cells were observed. Cells were washed twice with R10 and stained with mAbs as before. Data is shown for HeLa-B27 cells, but similar reduction in HC-10 expression of untransduced HeLa cells was observed following trypsin treatment. Cell scraping resulted in fewer viable cells and considerable cell clumping.

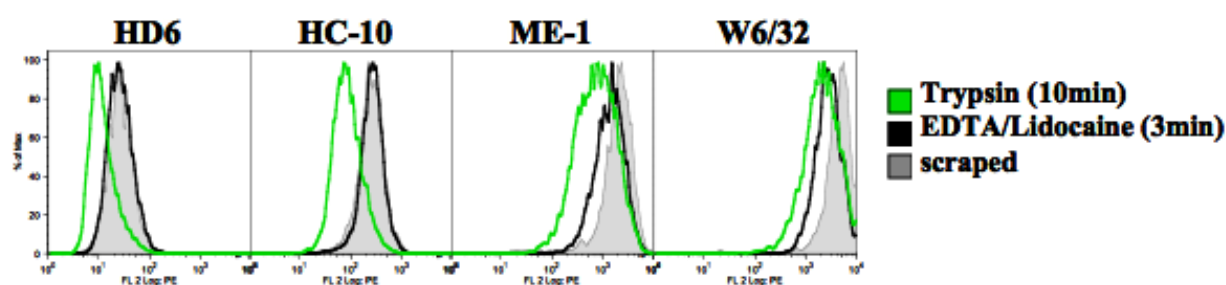


Figure 7.3 Removal of HeLa cells with trypsin treatment reduces staining with mAbs

Appendix III

Generation of B27₂-specific monoclonal antibodies using phage display technology

The generation of biotinylated B27 HT and B27₂ complexes was undertaken by members of the Bowness Group. These complexes were then used by Renner and colleagues to generate candidate dimer-specific antibodies as follows.

Selection of B27₂-specific human Fab antibodies using a phage library

Biotinylated B27₂ refolded complexes were incubated with streptavidin-coated dynalbeads (M-280, Dynal, Oslo, Norway) and phage particles from a fully human Fab antibody phage library (provided by Dyax, MA, USA) for 1h. After multiple washes with 0.3% Tween20-PBS, binding phages were eluted with 100mM triethanolamine (pH 10.5) for 5 min and neutralized with 0.5 ml of 1M Tris-HCl (pH 7.4) (**Step 1**, Figure 7.4). Eluted phage particles were rescued by infecting *E. Coli* strain TG1 (Zymo Research, Switzerland) grown in 2YT broth (BD/DIFCO, Switzerland), supplemented with 100µg/ml ampicillin and 2% glucose (2YT-AG) (**Step 2**, Figure 7.4). This was followed by a negative selection step, whereby rescued phages were incubated with biotinylated B27 HT refolded complexes incubated and streptavidin-coated dynal beads for 1h to remove any cross-reacting phage particles (**Step 3**, Figure 7.4). Remaining phage particles then underwent two further rounds of positive selection with biotinylated B27₂ complexes as before (**Step 4**, Figure 7.4).

Rescued clones from the third selection round were cultured on 2YT-AG agar plates overnight at 30°C. Individual TG1 colonies were infected with helper phage M13K07 (NEB, Switzerland) at 1:20 ratio and grown in 2YT broth supplemented with ampicillin and 25µg/ml of kanamycin over night at 30°C. Supernatants were tested for binders by ELISA with B27₂ complexes. Diversity of the selected binders was determined by colony PCR (Red-Taq readymix, Sigma, Switzerland), finger printing (BstNI digestion) and confirmed by DNA sequencing with heavy and light chain specific primers.

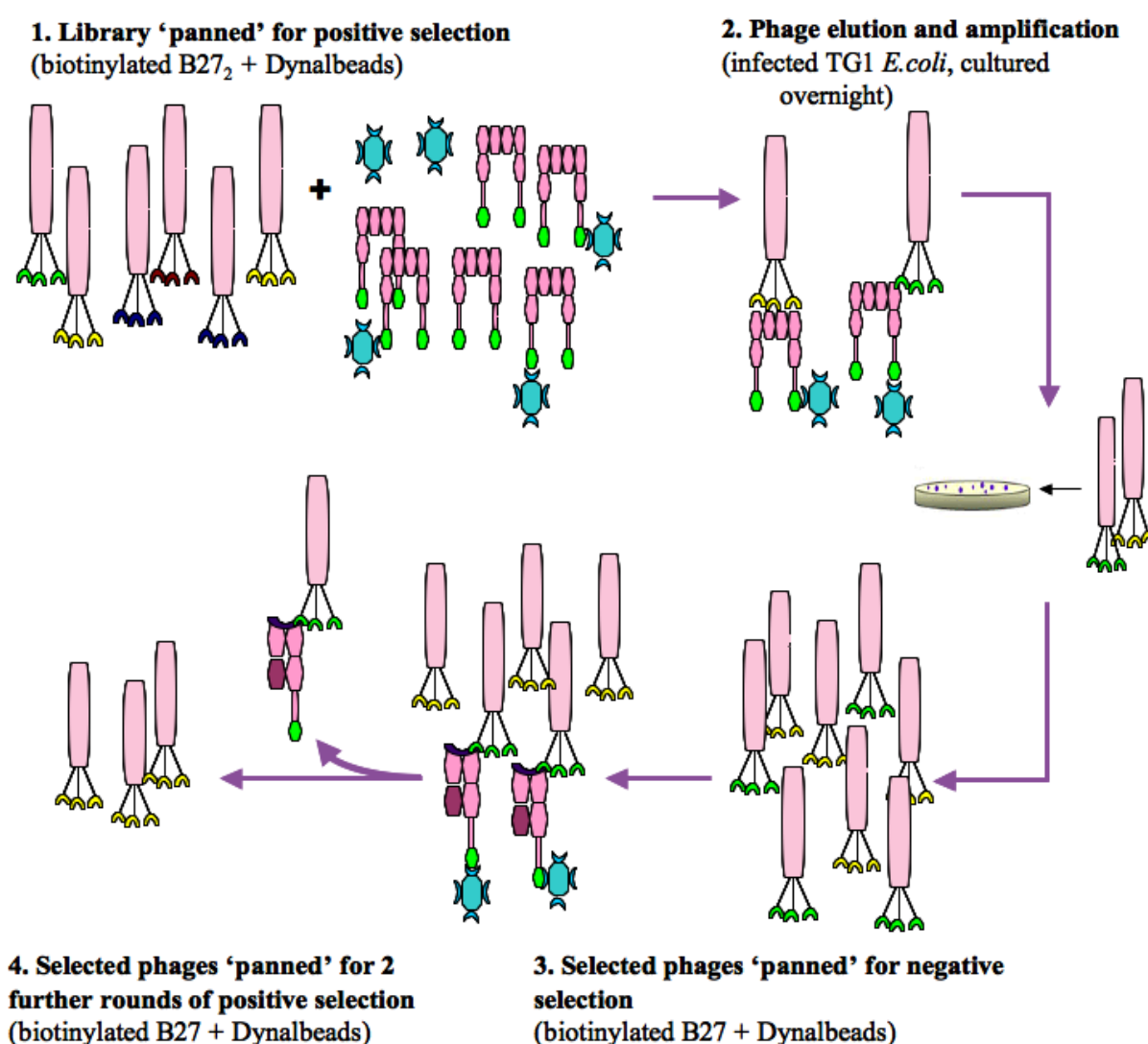


Figure 7.4. Cartoon schematic of the selection stages in the generation of candidate B27₂-specific monoclonal antibodies. See text for details.

Expression and purification of Fab fragments and the generation of a chimeric IgG

Fabs were expressed and purified as described previously (Hoogenboom et al., 1999). Briefly, TG1 cultures were grown at 37°C until the OD reached 0.8-1.0 at A600nm in 2YT supplemented with ampicillin and 0.1% glucose. Cultures were induced with 1mM IPTG (Roche, Switzerland) and further incubated at 30°C for 4h. Periplasmic portions were isolated using 2M sucrose and Fab molecules were purified with Talon resin (BD Clontech, Switzerland). Purity and molecular weight were confirmed by SDS-PAGE. Specific human Fab binders were converted into chimeric IgG molecules exhibiting mouse Fc using a GS (Glutamine Synthetase) gene expression system (LONZA, Switzerland). Briefly, the variable regions of heavy chain and light chain were amplified with DraIII or RsrII primers. Variable chain amplicons of approximately 400 bp were digested with respective enzymes and were cloned into the pEE-12.4 vector possessing mouse Fc and human kappa light chain. 40µg of plasmid DNA was linearized with Pvu-I and transfected into NS0 cells by electroporation at 250V and 400µF with 6-7.5 millisecond time constant (Genepulser II, BioRad, Switzerland). Clones were grown in glutamine free DMEM (GIBCO, Germany) supplemented with 10% FBS, penicillin, streptomycin and GS supplement (Invitrogen, Switzerland). After electroporation, cells were grown in DMEM containing 7.5µM of MSX (Methionine Sulfoximine - a glutamine synthetase inhibitor, sigma, Switzerland) supplemented with glutamine. Secreted IgGs were purified with Capture Select Fab kappa affinity matrix (BAC, Netherlands). Purity and molecular weights were determined by SDS-PAGE.

References

- Abdel Motal, U.M., Zhou, X., Joki, A., Siddiqi, A.R., Srinivasa, B.R., Stenvall, K., Dahmen, J., and Jondal, M. (1993).** Major histocompatibility complex class I-binding peptides are recycled to the cell surface after internalization. *European journal of immunology* 23, 3224-3229.
- Aiko, S., and Grisham, M.B. (1995).** Spontaneous intestinal inflammation and nitric oxide metabolism in HLA-B27 transgenic rats. *Gastroenterology* 109, 142-150.
- Allen, R.L., O'Callaghan, C.A., McMichael, A.J., and Bowness, P. (1999).** Cutting edge: HLA-B27 can form a novel beta 2-microglobulin-free heavy chain homodimer structure. *J Immunol* 162, 5045-5048.
- Alvarez, I., Marti, M., Vazquez, J., Camafeita, E., Ogueta, S., and Lopez de Castro, J.A. (2001).** The Cys-67 residue of HLA-B27 influences cell surface stability, peptide specificity, and T-cell antigen presentation. *The Journal of biological chemistry* 276, 48740-48747.
- Antoniou, A.N., Ford, S., Taurog, J.D., Butcher, G.W., and Powis, S.J. (2004).** Formation of HLA-B27 homodimers and their relationship to assembly kinetics. *The Journal of biological chemistry* 279, 8895-8902.
- Appel, H., Kuon, W., Kuhne, M., Wu, P., Kuhlmann, S., Kollnberger, S., Thiel, A., Bowness, P., and Sieper, J. (2004).** Use of HLA-B27 tetramers to identify low-frequency antigen-specific T cells in Chlamydia-triggered reactive arthritis. *Arthritis research & therapy* 6, R521-534.
- Arosa, F.A., de Jesus, O., Porto, G., Carmo, A.M., and de Sousa, M. (1999).** Calreticulin is expressed on the cell surface of activated human peripheral blood T lymphocytes in association with major histocompatibility complex class I molecules. *The Journal of biological chemistry* 274, 16917-16922.
- Baas, E.J., van Santen, H.M., Kleijmeer, M.J., Geuze, H.J., Peters, P.J., and Ploegh, H.L. (1992).** Peptide-induced stabilization and intracellular localization of empty HLA class I complexes. *The Journal of experimental medicine* 176, 147-156.
- Barnstable, C.J., Bodmer, W.F., Brown, G., Galfre, G., Milstein, C., Williams, A.F., and Ziegler, A. (1978).** Production of monoclonal antibodies to group A

erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell* 14, 9-20.

Baxter, A.G., and Smyth, M.J. (2002). The role of NK cells in autoimmune disease. *Autoimmunity* 35, 1-14.

Benjamin, R., and Parham, P. (1990). Guilt by association: HLA-B27 and ankylosing spondylitis. *Immunology today* 11, 137-142.

Benjamin, R.J., Madrigal, J.A., and Parham, P. (1991). Peptide binding to empty HLA-B27 molecules of viable human cells. *Nature* 351, 74-77.

Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P., and Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature cell biology* 2, 326-332.

Bird, L.A., Peh, C.A., Kollnberger, S., Elliott, T., McMichael, A.J., and Bowness, P. (2003). Lymphoblastoid cells express HLA-B27 homodimers both intracellularly and at the cell surface following endosomal recycling. *European journal of immunology* 33, 748-759.

Bjorkman, P.J., and Parham, P. (1990). Structure, function, and diversity of class I major histocompatibility complex molecules. *Annual review of biochemistry* 59, 253-288.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987a). The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 329, 512-518.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987b). Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329, 506-512.

Boname, J.M., Thomas, M., Stagg, H.R., Xu, P., Peng, J., and Lehner, P.J. (2010). Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains. *Traffic* 11, 210-220.

Bouvier, M., and Wiley, D.C. (1998). Structural characterization of a soluble and partially folded class I major histocompatibility heavy chain/beta 2m heterodimer. *Nat Struct Biol* 5, 377-384.

Bowness, P., Ridley, A., Shaw, J., Chan, A.T., Wong-Baeza, I., Fleming, M., Cummings, F., McMichael, A., and Kollnberger, S. (2011). Th17 cells expressing

KIR3DL2+ and responsive to HLA-B27 homodimers are increased in ankylosing spondylitis. *J Immunol* 186, 2672-2680.

Bowness, P., Zaccai, N., Bird, L., and Jones, E.Y. (1999). HLA-B27 and disease pathogenesis: new structural and functional insights. *Expert reviews in molecular medicine* 1999, 1-10.

Boyle, L.H., Goodall, J.C., Opat, S.S., and Gaston, J.S. (2001). The recognition of HLA-B27 by human CD4(+) T lymphocytes. *J Immunol* 167, 2619-2624.

Braun, J., Brandt, J., Listing, J., Zink, A., Alten, R., Golder, W., Gromnica-Ihle, E., Kellner, H., Krause, A., Schneider, M., et al. (2002). Treatment of active ankylosing spondylitis with infliximab: a randomised controlled multicentre trial. *Lancet* 359, 1187-1193.

Braun, J., and Sieper, J. (2007). Ankylosing spondylitis. *Lancet* 369, 1379-1390.

Breban, M., Fernandez-Sueiro, J.L., Richardson, J.A., Hadavand, R.R., Maika, S.D., Hammer, R.E., and Taurog, J.D. (1996). T cells, but not thymic exposure to HLA-B27, are required for the inflammatory disease of HLA-B27 transgenic rats. *J Immunol* 156, 794-803.

Breban, M., Hammer, R.E., Richardson, J.A., and Taurog, J.D. (1993). Transfer of the inflammatory disease of HLA-B27 transgenic rats by bone marrow engraftment. *The Journal of experimental medicine* 178, 1607-1616.

Brewerton, D.A., Caffrey, M., Nicholls, A., Walters, D., and James, D.C. (1973a). Acute anterior uveitis and HL-A 27. *Lancet* 302, 994-996.

Brewerton, D.A., Caffrey, M., Nicholls, A., Walters, D., and James, D.C. (1974a). HL-A 27 and arthropathies associated with ulcerative colitis and psoriasis. *Lancet* 1, 956-958.

Brewerton, D.A., Caffrey, M., Nicholls, A., Walters, D., and James, D.C. (1974b). Letter: Acute anterior uveitis and HL-A 27. *Lancet* 1, 464.

Brewerton, D.A., Hart, F.D., Nicholls, A., Caffrey, M., James, D.C., and Sturrock, R.D. (1973b). Ankylosing spondylitis and HL-A 27. *Lancet* 1, 904-907.

Brodsky, F.M., Bodmer, W.F., and Parham, P. (1979). Characterization of a monoclonal anti-beta 2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. *European journal of immunology* 9, 536-545.

- Brown, D., Trowsdale, J., and Allen, R. (2004).** The LILR family: modulators of innate and adaptive immune pathways in health and disease. *Tissue antigens* 64, 215-225.
- Brown, M., Jin, R., Wordsworth, B.P., Stone, M.A., Ward, M.M., and Weisman, M.H. (2009).** HLA Class I and II Associations of Ankylosing Spondylitis [abstract]. *Arthritis and rheumatism* 2009, Suppl 10:1171.
- Brown, M.A. (2008).** Breakthroughs in genetic studies of ankylosing spondylitis. *Rheumatology (Oxford, England)* 47, 132-137.
- Brown, M.A., Jepson, A., Young, A., Whittle, H.C., Greenwood, B.M., and Wordsworth, B.P. (1997a).** Ankylosing spondylitis in West Africans--evidence for a non-HLA-B27 protective effect. *Annals of the rheumatic diseases* 56, 68-70.
- Brown, M.A., Kennedy, L.G., Darke, C., Gibson, K., Pile, K.D., Shatford, J.L., Taylor, A., Calin, A., and Wordsworth, B.P. (1998).** The effect of HLA-DR genes on susceptibility to and severity of ankylosing spondylitis. *Arthritis and rheumatism* 41, 460-465.
- Brown, M.A., Kennedy, L.G., MacGregor, A.J., Darke, C., Duncan, E., Shatford, J.L., Taylor, A., Calin, A., and Wordsworth, P. (1997b).** Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment. *Arthritis and rheumatism* 40, 1823-1828.
- Brown, M.A., Laval, S.H., Brophy, S., and Calin, A. (2000).** Recurrence risk modelling of the genetic susceptibility to ankylosing spondylitis. *Annals of the rheumatic diseases* 59, 883-886.
- Brown, M.A., Pile, K.D., Kennedy, L.G., Calin, A., Darke, C., Bell, J., Wordsworth, B.P., and Cornelis, F. (1996).** HLA class I associations of ankylosing spondylitis in the white population in the United Kingdom. *Annals of the rheumatic diseases* 55, 268-270.
- Brown M.A. (2011).** Progress in the genetics of ankylosing spondylitis. *Brief Funct Genomics* 10, 249-57.
- Burr, M.L., Cano, F., Svobodova, S., Boyle, L.H., Boname, J.M., and Lehner, P.J. (2011).** HRD1 and UBE2J1 target misfolded MHC class I heavy chains for endoplasmic reticulum-associated degradation. *Proceedings of the National Academy of Sciences of the United States of America* 108, 2034-2039.

Burton, P.R., Clayton, D.G., Cardon, L.R., Craddock, N., Deloukas, P., Duncanson, A., Kwiatkowski, D.P., McCarthy, M.I., Ouwehand, W.H., Samani, N.J., et al. (2007). Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nature genetics* 39, 1329-1337.

Calin, A., Marder, A., Becks, E., and Burns, T. (1983). Genetic differences between B27 positive patients with ankylosing spondylitis and B27 positive healthy controls. *Arthritis and rheumatism* 26, 1460-1464.

Calin, A., Taurog, J. D. (1998). The Spondylarthritides (Oxford University Press, USA; the illustrated edition).

Campbell, E.C., Fettke, F., Bhat, S., Morley, K.D., and Powis, S.J. (2011). Expression of MHC class I dimers and ERAP1 in an ankylosing spondylitis patient cohort. *Immunology* 133, 379-385.

Capps, G.G., Van Kampen, M., Ward, C.L., and Zuniga, M.C. (1989). Endocytosis of the class I major histocompatibility antigen via a phorbol myristate acetate-inducible pathway is a cell-specific phenomenon and requires the cytoplasmic domain. *The Journal of cell biology* 108, 1317-1329.

Capps, G.G., and Zuniga, M.C. (1993). The cytoplasmic domain of the H-2Ld class I major histocompatibility complex molecule is differentially accessible to immunological and biochemical probes during transport to the cell surface. *The Journal of biological chemistry* 268, 21263-21270.

Carreno, B.M., and Hansen, T.H. (1994). Exogenous peptide ligand influences the expression and half-life of free HLA class I heavy chains ubiquitously detected at the cell surface. *European journal of immunology* 24, 1285-1292.

Cascio, P., Hilton, C., Kisselev, A.F., Rock, K.L., and Goldberg, A.L. (2001). 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *The EMBO journal* 20, 2357-2366.

Cauli, A., Dessole, G., Fiorillo, M.T., Vacca, A., Mameli, A., Bitti, P., Passiu, G., Sorrentino, R., and Mathieu, A. (2002). Increased level of HLA-B27 expression in ankylosing spondylitis patients compared with healthy HLA-B27-positive subjects: a possible further susceptibility factor for the development of disease. *Rheumatology (Oxford, England)* 41, 1375-1379.

- Cauli, A., Vacca, A., Mameli, A., Passiu, G., Fiorillo, M.T., Sorrentino, R., and Mathieu, A. (2007).** A Sardinian patient with ankylosing spondylitis and HLA-B*2709 co-occurring with HLA-B*1403. *Arthritis and rheumatism* 56, 2807-2809.
- Chan, A.T., Kollnberger, S.D., Wedderburn, L.R., and Bowness, P. (2005).** Expansion and enhanced survival of natural killer cells expressing the killer immunoglobulin-like receptor KIR3DL2 in spondylarthritis. *Arthritis and rheumatism* 52, 3586-3595.
- Chang, C.C., Ciubotariu, R., Manavalan, J.S., Yuan, J., Colovai, A.I., Piazza, F., Lederman, S., Colonna, M., Cortesini, R., Dalla-Favera, R., *et al.* (2002).** Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nature immunology* 3, 237-243.
- Chang, S.C., Momburg, F., Bhutani, N., and Goldberg, A.L. (2005).** The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a "molecular ruler" mechanism. *Proceedings of the National Academy of Sciences of the United States of America* 102, 17107-17112.
- Chen, J.H., Kono, D.H., Yong, Z., Park, M.S., Oldstone, M.M., and Yu, D.T. (1987).** A *Yersinia pseudotuberculosis* protein which cross-reacts with HLA-B27. *J Immunol* 139, 3003-3011.
- Ciccia, F., Bombardieri, M., Principato, A., Giardina, A., Tripodo, C., Porcasi, R., Peralta, S., Franco, V., Giardina, E., Craxi, A., *et al.* (2009).** Overexpression of interleukin-23, but not interleukin-17, as an immunologic signature of subclinical intestinal inflammation in ankylosing spondylitis. *Arthritis and rheumatism* 60, 955-965.
- Clavin, N.W., Fernandez, J., Schonmeyr, B.H., Soares, M.A., and Mehrara, B.J. (2008).** Fractionated doses of ionizing radiation confer protection to mesenchymal stem cell pluripotency. *Plastic and reconstructive surgery* 122, 739-748.
- Colbert, R.A. (2000).** HLA-B27 misfolding: a solution to the spondyloarthropathy conundrum? *Molecular medicine today* 6, 224-230.
- Colbert, R.A., Rowland-Jones, S.L., McMichael, A.J., and Frelinger, J.A. (1993).** Allele-specific B pocket transplant in class I major histocompatibility complex protein changes requirement for anchor residue at P2 of peptide. *Proceedings of the National Academy of Sciences of the United States of America* 90, 6879-6883.

Colonna, M., Nakajima, H., Navarro, F., and Lopez-Botet, M. (1999). A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. *Journal of leukocyte biology* 66, 375-381.

Colonna, M., Navarro, F., Bellon, T., Llano, M., Garcia, P., Samaridis, J., Angman, L., Cella, M., and Lopez-Botet, M. (1997). A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *The Journal of experimental medicine* 186, 1809-1818.

Colonna, M., Samaridis, J., Cella, M., Angman, L., Allen, R.L., O'Callaghan, C.A., Dunbar, R., Ogg, G.S., Cerundolo, V., and Rolink, A. (1998). Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J Immunol* 160, 3096-3100.

Corthay, A., Hansson, A.S., and Holmdahl, R. (2000). T lymphocytes are not required for the spontaneous development of enthesal ossification leading to marginal ankylosis in the DBA/1 mouse. *Arthritis and rheumatism* 43, 844-851.

Cui, X., Hawari, F., Alsaaty, S., Lawrence, M., Combs, C.A., Geng, W., Rouhani, F.N., Miskinis, D., and Levine, S.J. (2002). Identification of ARTS-1 as a novel TNFR1-binding protein that promotes TNFR1 ectodomain shedding. *The Journal of clinical investigation* 110, 515-526.

Cui, X., Rouhani, F.N., Hawari, F., and Levine, S.J. (2003a). An aminopeptidase, ARTS-1, is required for interleukin-6 receptor shedding. *The Journal of biological chemistry* 278, 28677-28685.

Cui, X., Rouhani, F.N., Hawari, F., and Levine, S.J. (2003b). Shedding of the type II IL-1 decoy receptor requires a multifunctional aminopeptidase, aminopeptidase regulator of TNF receptor type 1 shedding. *J Immunol* 171, 6814-6819.

D'Amato, M., Fiorillo, M.T., Carcassi, C., Mathieu, A., Zuccarelli, A., Bitti, P.P., Tosi, R., and Sorrentino, R. (1995). Relevance of residue 116 of HLA-B27 in determining susceptibility to ankylosing spondylitis. *European journal of immunology* 25, 3199-3201.

Dangoria, N.S., DeLay, M.L., Kingsbury, D.J., Mear, J.P., Uchanska-Ziegler, B., Ziegler, A., and Colbert, R.A. (2002). HLA-B27 misfolding is associated with aberrant intermolecular disulfide bond formation (dimerization) in the endoplasmic reticulum. *The Journal of biological chemistry* 277, 23459-23468.

Davis, J.C., Jr., Van Der Heijde, D., Braun, J., Dougados, M., Cush, J., Clegg, D.O., Kivitz, A., Fleischmann, R., Inman, R., and Tsuji, W. (2003). Recombinant human tumor necrosis factor receptor (etanercept) for treating ankylosing spondylitis: a randomized, controlled trial. *Arthritis and rheumatism* 48, 3230-3236.

Day, P.M., Yewdell, J.W., Porgador, A., Germain, R.N., and Bennink, J.R. (1997). Direct delivery of exogenous MHC class I molecule-binding oligopeptides to the endoplasmic reticulum of viable cells. *Proceedings of the National Academy of Sciences of the United States of America* 94, 8064-8069.

Dayer, J.M., de Rochemonteix, B., Burrus, B., Demczuk, S., and Dinarello, C.A. (1986). Human recombinant interleukin 1 stimulates collagenase and prostaglandin E2 production by human synovial cells. *The Journal of clinical investigation* 77, 645-648.

DeMars, R., Chang, C.C., Shaw, S., Reitnauer, P.J., and Sondel, P.M. (1984). Homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens. *Human immunology* 11, 77-97.

Dhaenens, M., Fert, I., Glatigny, S., Haerinck, S., Poulain, C., Donnadieu, E., Hacquard-Bouder, C., Andre, C., Elewaut, D., Deforce, D., *et al.* (2009). Dendritic cells from spondylarthritis-prone HLA-B27-transgenic rats display altered cytoskeletal dynamics, class II major histocompatibility complex expression, and viability. *Arthritis and rheumatism* 60, 2622-2632.

Doucey, M.A., Scarpellino, L., Zimmer, J., Guillaume, P., Luescher, I.F., Bron, C., and Held, W. (2004). Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nature immunology* 5, 328-336.

Duncan, L.M., Nathan, J.A., and Lehner, P.J. (2010). Stabilization of an E3 ligase-E2-ubiquitin complex increases cell surface MHC class I expression. *J Immunol* 184, 6978-6985.

Ellis, S.A., Taylor, C., and McMichael, A. (1982). Recognition of HLA-B27 and related antigen by a monoclonal antibody. *Human immunology* 5, 49-59.

Fabian, H., Loll, B., Huser, H., Naumann, D., Uchanska-Ziegler, B., and Ziegler, A. (2011). Influence of inflammation-related changes on conformational

characteristics of HLA-B27 subtypes as detected by IR spectroscopy. *FEBS J* 278, 1713-1727.

Feldtkeller, E., Khan, M.A., van der Heijde, D., van der Linden, S., and Braun, J. (2003). Age at disease onset and diagnosis delay in HLA-B27 negative vs. positive patients with ankylosing spondylitis. *Rheumatology international* 23, 61-66.

Fernandez-de-Las-Penas, C., Alonso-Blanco, C., Alguacil-Diego, I.M., and Miangolarra-Page, J.C. (2006). One-year follow-up of two exercise interventions for the management of patients with ankylosing spondylitis: a randomized controlled trial. *American journal of physical medicine & rehabilitation / Association of Academic Physiatrists* 85, 559-567.

Fert, I., Glatigny, S., Poulain, C., Satumtira, N., Dorris, M.L., Taurog, J.D., and Breban, M. (2008). Correlation between dendritic cell functional defect and spondylarthritis phenotypes in HLA-B27/HUMAN beta2-microglobulin-transgenic rat lines. *Arthritis and rheumatism* 58, 3425-3429.

Fiorillo, M.T., Maragno, M., Butler, R., Dupuis, M.L., and Sorrentino, R. (2000). CD8(+) T-cell autoreactivity to an HLA-B27-restricted self-epitope correlates with ankylosing spondylitis. *The Journal of clinical investigation* 106, 47-53.

Fiorillo, M.T., Meadows, L., D'Amato, M., Shabanowitz, J., Hunt, D.F., Appella, E., and Sorrentino, R. (1997). Susceptibility to ankylosing spondylitis correlates with the C-terminal residue of peptides presented by various HLA-B27 subtypes. *European journal of immunology* 27, 368-373.

Fiorillo, M.T., Ruckert, C., Hulsmeier, M., Sorrentino, R., Saenger, W., Ziegler, A., and Uchanska-Ziegler, B. (2005). Allele-dependent similarity between viral and self-peptide presentation by HLA-B27 subtypes. *The Journal of biological chemistry* 280, 2962-2971.

Fiorillo, M.T., and Sorrentino, R. (2009). T-cell responses against viral and self-epitopes and HLA-B27 subtypes differentially associated with ankylosing spondylitis. *Advances in experimental medicine and biology* 649, 255-262.

Flodstrom, M., Shi, F.D., Sarvetnick, N., and Ljunggren, H.G. (2002). The natural killer cell -- friend or foe in autoimmune disease? *Scandinavian journal of immunology* 55, 432-441.

- Fussell, H., Nesbeth, D., Lenart, I., Campbell, E.C., Lynch, S., Santos, S., Gould, K., Powis, S.J., and Antoniou, A.N. (2008).** Novel detection of in vivo HLA-B27 conformations correlates with ankylosing spondylitis association. *Arthritis and rheumatism* 58, 3419-3424.
- Galocha, B., and de Castro, J.A. (2008).** Folding of HLA-B27 subtypes is determined by the global effect of polymorphic residues and shows incomplete correspondence to ankylosing spondylitis. *Arthritis and rheumatism* 58, 401-412.
- Galocha, B., and Lopez de Castro, J.A. (2010).** Mutational analysis reveals a complex interplay of peptide binding and multiple biological features of HLA-B27. *The Journal of biological chemistry* 285, 39180-39190.
- Garstka, M., Borchert, B., Al-Balushi, M., Praveen, P.V., Kuhl, N., Majoul, I., Duden, R., and Springer, S. (2007).** Peptide-receptive major histocompatibility complex class I molecules cycle between endoplasmic reticulum and cis-Golgi in wild-type lymphocytes. *The Journal of biological chemistry* 282, 30680-30690.
- Garstka, M.A., Fritzsche, S., Lenart, I., Hein, Z., Jankevicius, G., Boyle, L.H., Elliott, T., Trowsdale, J., Antoniou, A.N., Zacharias, M., et al. (2011).** Tapasin dependence of major histocompatibility complex class I molecules correlates with their conformational flexibility. *FASEB J*.
- Ghanem, E., Fritzsche, S., Al-Balushi, M., Hashem, J., Ghuneim, L., Thomer, L., Kalbacher, H., van Endert, P., Wiertz, E., Tampe, R., et al. (2010).** The transporter associated with antigen processing (TAP) is active in a post-ER compartment. *J Cell Sci* 123, 4271-4279.
- Girschick, H.J., Guilherme, L., Inman, R.D., Latsch, K., Rihl, M., Sherer, Y., Shoenfeld, Y., Zeidler, H., Arienti, S., and Doria, A. (2008).** Bacterial triggers and autoimmune rheumatic diseases. *Clinical and experimental rheumatology* 26, S12-17.
- Gitter, B.D., Labus, J.M., Lees, S.L., and Scheetz, M.E. (1989).** Characteristics of human synovial fibroblast activation by IL-1 beta and TNF alpha. *Immunology* 66, 196-200.
- Gobin, S.J., van Zutphen, M., Woltman, A.M., and van den Elsen, P.J. (1999).** Transactivation of classical and nonclassical HLA class I genes through the IFN-stimulated response element. *J Immunol* 163, 1428-1434.

Goldstein, S.A., and Mescher, M.F. (1986). Cell-sized, supported artificial membranes (pseudocytes): response of precursor cytotoxic T lymphocytes to class I MHC proteins. *J Immunol* 137, 3383-3392.

Gorman, J.D., Sack, K.E., and Davis, J.C., Jr. (2002). Treatment of ankylosing spondylitis by inhibition of tumor necrosis factor alpha. *The New England journal of medicine* 346, 1349-1356.

Goss, G.D., Wittwer, M.A., Bezwoda, W.R., Herman, J., Rabson, A., Seymour, L., Derman, D.P., and Mendelow, B. (1985). Effect of natural killer cells on syngeneic bone marrow: in vitro and in vivo studies demonstrating graft failure due to NK cells in an identical twin treated by bone marrow transplantation. *Blood* 66, 1043-1046.

Grassi, F., Meneveri, R., Gullberg, M., Lopalco, L., Rossi, G.B., Lanza, P., De Santis, C., Brattsand, G., Butto, S., Ginelli, E., *et al.* (1991). Human immunodeficiency virus type 1 gp120 mimics a hidden monomorphic epitope borne by class I major histocompatibility complex heavy chains. *The Journal of experimental medicine* 174, 53-62.

Greenberg, M., DeTulleo, L., Rapoport, I., Skowronski, J., and Kirchhausen, T. (1998). A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. *Curr Biol* 8, 1239-1242.

Gu, J., Rihl, M., Marker-Hermann, E., Baeten, D., Kuipers, J.G., Song, Y.W., Maksymowych, W.P., Burgos-Vargas, R., Veys, E.M., De Keyser, F., *et al.* (2002). Clues to pathogenesis of spondyloarthritis derived from synovial fluid mononuclear cell gene expression profiles. *The Journal of rheumatology* 29, 2159-2164.

Hacquard-Bouder, C., Chimenti, M.S., Giquel, B., Donnadieu, E., Fert, I., Schmitt, A., Andre, C., and Breban, M. (2007). Alteration of antigen-independent immunologic synapse formation between dendritic cells from HLA-B27-transgenic rats and CD4⁺ T cells: selective impairment of costimulatory molecule engagement by mature HLA-B27. *Arthritis and rheumatism* 56, 1478-1489.

Hacquard-Bouder, C., Falgarone, G., Bosquet, A., Smaoui, F., Monnet, D., Ittah, M., and Breban, M. (2004). Defective costimulatory function is a striking feature of

antigen-presenting cells in an HLA-B27-transgenic rat model of spondylarthropathy. *Arthritis and rheumatism* 50, 1624-1635.

Hammer, G.E., Gonzalez, F., Champsaur, M., Cado, D., and Shastri, N. (2006). The aminopeptidase ERAAP shapes the peptide repertoire displayed by major histocompatibility complex class I molecules. *Nature immunology* 7, 103-112.

Hammer, G.E., Gonzalez, F., James, E., Nolla, H., and Shastri, N. (2007). In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides. *Nature immunology* 8, 101-108.

Hammer, R.E., Maika, S.D., Richardson, J.A., Tang, J.P., and Taurog, J.D. (1990). Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta 2m: an animal model of HLA-B27-associated human disorders. *Cell* 63, 1099-1112.

Hansen, T., Yu, Y.Y., and Fremont, D.H. (2009). Preparation of stable single-chain trimers engineered with peptide, beta2 microglobulin, and MHC heavy chain. *Current protocols in immunology* / edited by John E Coligan [et al *Chapter 17*, Unit17 15.

Hart, G.W. (1982). Biosynthesis of glycosaminoglycans by thymic lymphocytes. Effects of mitogenic activation. *Biochemistry* 21, 6088-6096.

Harvey, D., Pointon, J.J., Evans, D.M., Karaderi, T., Farrar, C., Appleton, L.H., Sturrock, R.D., Stone, M.A., Oppermann, U., Brown, M.A., et al. (2009). Investigating the genetic association between ERAP1 and ankylosing spondylitis. *Human molecular genetics* 18, 4204-4212.

Hayer, S., Niederreiter, B., Nagelreiter, I., Smolen, J., and Redlich, K. (2010). Interleukin 6 is not a crucial regulator in an animal model of tumour necrosis factor-mediated bilateral sacroiliitis. *Annals of the rheumatic diseases* 69, 1403-1406.

Herberts, C., Reits, E., and Neefjes, J. (2003). Proteases, proteases and proteases for presentation. *Nature immunology* 4, 306-308.

Hermann, E., Yu, D.T., Meyer zum Buschenfelde, K.H., and Fleischer, B. (1993). HLA-B27-restricted CD8 T cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis. *Lancet* 342, 646-650.

Hochman, J.H., Shimizu, Y., DeMars, R., and Edidin, M. (1988). Specific associations of fluorescent beta-2-microglobulin with cell surfaces. The affinity of

different H-2 and HLA antigens for beta-2-microglobulin. *J Immunol* 140, 2322-2329.

Hoogenboom, H.R., Lutgerink, J.T., Pelsers, M.M., Rousch, M.J., Coote, J., Van Neer, N., De Bruine, A., Van Nieuwenhoven, F.A., Glatz, J.F., and Arends, J.W. (1999). Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. *Eur J Biochem* 260, 774-784.

Howe, C., Garstka, M., Al-Balushi, M., Ghanem, E., Antoniou, A.N., Fritzsche, S., Jankevicius, G., Kontouli, N., Schneeweiss, C., Williams, A., et al. (2009). Calreticulin-dependent recycling in the early secretory pathway mediates optimal peptide loading of MHC class I molecules. *The EMBO journal* 28, 3730-3744.

Howell, D.N., Andreotti, P.E., Dawson, J.R., and Cresswell, P. (1985). Natural killing target antigens as inducers of interferon: studies with an immunoselected, natural killing-resistant human T lymphoblastoid cell line. *J Immunol* 134, 971-976.

Huang, J., Burke, P., Yang, Y., Seiss, K., Beamon, J., Cung, T., Toth, I., Pereyra, F., Lichterfeld, M., and Yu, X.G. (2010). Soluble HLA-G inhibits myeloid dendritic cell function in HIV-1 infection by interacting with leukocyte immunoglobulin-like receptor B2. *J Virol* 84, 10784-10791.

Huet, S., Nixon, D.F., Rothbard, J.B., Townsend, A., Ellis, S.A., and McMichael, A.J. (1990). Structural homologies between two HLA B27-restricted peptides suggest residues important for interaction with HLA B27. *International immunology* 2, 311-316.

Hulsmeyer, M., Fiorillo, M.T., Bettosini, F., Sorrentino, R., Saenger, W., Ziegler, A., and Uchanska-Ziegler, B. (2004). Dual, HLA-B27 subtype-dependent conformation of a self-peptide. *The Journal of experimental medicine* 199, 271-281.

Inman, R.D., Davis, J.C., Jr., Heijde, D., Diekman, L., Sieper, J., Kim, S.I., Mack, M., Han, J., Visvanathan, S., Xu, Z., et al. (2008). Efficacy and safety of golimumab in patients with ankylosing spondylitis: results of a randomized, double-blind, placebo-controlled, phase III trial. *Arthritis and rheumatism* 58, 3402-3412.

Ismail, N., Chamberlain, J., and Inman, R. (1994). Persistence and dissemination of *Yersinia enterocolitica* 0:8 after intragastric challenge in B27-transgenic mice [abstract]. *Arthritis and rheumatism* 37, S322.

- Jaakkola, E., Herzberg, I., Laiho, K., Barnardo, M.C., Pointon, J.J., Kauppi, M., Kaarela, K., Tuomilehto-Wolf, E., Tuomilehto, J., Wordsworth, B.P., et al. (2006).** Finnish HLA studies confirm the increased risk conferred by HLA-B27 homozygosity in ankylosing spondylitis. *Annals of the rheumatic diseases* 65, 775-780.
- Jandus, C., Bioley, G., Rivals, J.P., Dudler, J., Speiser, D., and Romero, P. (2008).** Increased numbers of circulating polyfunctional Th17 memory cells in patients with seronegative spondylarthritides. *Arthritis and rheumatism* 58, 2307-2317.
- Jardetzky, T.S., Lane, W.S., Robinson, R.A., Madden, D.R., and Wiley, D.C. (1991).** Identification of self peptides bound to purified HLA-B27. *Nature* 353, 326-329.
- Jarvinen, P. (1995).** Occurrence of ankylosing spondylitis in a nationwide series of twins. *Arthritis and rheumatism* 38, 381-383.
- Kanaseki, T., Blanchard, N., Hammer, G.E., Gonzalez, F., and Shastri, N. (2006).** ERAAP synergizes with MHC class I molecules to make the final cut in the antigenic peptide precursors in the endoplasmic reticulum. *Immunity* 25, 795-806.
- Karaderi, T., Harvey, D., Farrar, C., Appleton, L.H., Stone, M.A., Sturrock, R.D., Brown, M.A., Wordsworth, P., and Pointon, J.J. (2009).** Association between the interleukin 23 receptor and ankylosing spondylitis is confirmed by a new UK case-control study and meta-analysis of published series. *Rheumatology (Oxford, England)* 48, 386-389.
- Karlhofer, F.M., Ribaldo, R.K., and Yokoyama, W.M. (1992).** MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* 358, 66-70.
- Khare, S.D., Bull, M.J., Hanson, J., Luthra, H.S., and David, C.S. (1998).** Spontaneous inflammatory disease in HLA-B27 transgenic mice is independent of MHC class II molecules: a direct role for B27 heavy chains and not B27-derived peptides. *J Immunol* 160, 101-106.
- Khare, S.D., Hansen, J., Luthra, H.S., and David, C.S. (1996).** HLA-B27 heavy chains contribute to spontaneous inflammatory disease in B27/human beta2-

microglobulin (beta2m) double transgenic mice with disrupted mouse beta2m. The Journal of clinical investigation 98, 2746-2755.

Khare, S.D., Lee, S., Bull, M.J., Hanson, J., Luthra, H.S., Hammerling, G.J., and David, C.S. (1999). Peptide binding alpha1alpha2 domain of HLA-B27 contributes to the disease pathogenesis in transgenic mice. Human immunology 60, 116-126.

Khare, S.D., Luthra, H.S., and David, C.S. (1995). Spontaneous inflammatory arthritis in HLA-B27 transgenic mice lacking beta 2-microglobulin: a model of human spondyloarthropathies. The Journal of experimental medicine 182, 1153-1158.

Kievits, F., Ivanyi, P., Krimpenfort, P., Berns, A., and Ploegh, H.L. (1987). HLA-restricted recognition of viral antigens in HLA transgenic mice. Nature 329, 447-449.

Kim, S., Poursine-Laurent, J., Truscott, S.M., Lybarger, L., Song, Y.J., Yang, L., French, A.R., Sunwoo, J.B., Lemieux, S., Hansen, T.H., et al. (2005). Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature 436, 709-713.

Kim, T.J., Na, K.S., Lee, H.J., Lee, B., and Kim, T.H. (2009). HLA-B27 homozygosity has no influence on clinical manifestations and functional disability in ankylosing spondylitis. Clinical and experimental rheumatology 27, 574-579.

Kingsbury, D.J., Mear, J.P., Witte, D.P., Taurog, J.D., Roopenian, D.C., and Colbert, R.A. (2000). Development of spontaneous arthritis in beta2-microglobulin-deficient mice without expression of HLA-B27: association with deficiency of endogenous major histocompatibility complex class I expression. Arthritis and rheumatism 43, 2290-2296.

Kisselev, A.F., Akopian, T.N., Woo, K.M., and Goldberg, A.L. (1999). The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. The Journal of biological chemistry 274, 3363-3371.

Kochan, G., Krojer, T., Harvey, D., Fischer, R., Chen, L., Vollmar, M., von Delft, F., Kavanagh, K.L., Brown, M.A., Bowness, P., et al. (2011). Crystal structures of the endoplasmic reticulum aminopeptidase-1 (ERAP1) reveal the molecular basis for N-terminal peptide trimming. Proceedings of the National Academy of Sciences of the United States of America 108, 7745-7750.

Kollnberger, S., Bird, L., Sun, M.Y., Retiere, C., Braud, V.M., McMichael, A., and Bowness, P. (2002). Cell-surface expression and immune receptor recognition of HLA-B27 homodimers. *Arthritis and rheumatism* 46, 2972-2982.

Kollnberger, S., Bird, L.A., Roddis, M., Hacquard-Bouder, C., Kubagawa, H., Bodmer, H.C., Breban, M., McMichael, A.J., and Bowness, P. (2004). HLA-B27 heavy chain homodimers are expressed in HLA-B27 transgenic rodent models of spondyloarthritis and are ligands for paired Ig-like receptors. *J Immunol* 173, 1699-1710.

Krimpenfort, P., Rudenko, G., Hochstenbach, F., Guessow, D., Berns, A., and Ploegh, H. (1987). Crosses of two independently derived transgenic mice demonstrate functional complementation of the genes encoding heavy (HLA-B27) and light (beta 2-microglobulin) chains of HLA class I antigens. *The EMBO journal* 6, 1673-1676.

Kuang, E., Wan, Q., Li, X., Xu, H., Liu, Q., and Qi, Y. (2005). ER Ca²⁺ depletion triggers apoptotic signals for endoplasmic reticulum (ER) overload response induced by overexpressed reticulon 3 (RTN3/HAP). *Journal of cellular physiology* 204, 549-559.

Kuon, W., Holzhutter, H.G., Appel, H., Grolms, M., Kollnberger, S., Traeder, A., Henklein, P., Weiss, E., Thiel, A., Lauster, R., *et al.* (2001). Identification of HLA-B27-restricted peptides from the *Chlamydia trachomatis* proteome with possible relevance to HLA-B27-associated diseases. *J Immunol* 167, 4738-4746.

Lan, C.C., Tsai, W.C., Wu, C.S., Yu, C.L., and Yu, H.S. (2004). Psoriatic patients with arthropathy show significant expression of free HLA class I heavy chains on circulating monocytes: a potential role in the pathogenesis of psoriatic arthropathy. *The British journal of dermatology* 151, 24-31.

Lanier, L.L. (1998). Follow the leader: NK cell receptors for classical and nonclassical MHC class I. *Cell* 92, 705-707.

Layh-Schmitt, G., and Colbert, R.A. (2008). The interleukin-23/interleukin-17 axis in spondyloarthritis. *Current opinion in rheumatology* 20, 392-397.

Le Gall, S., Erdtmann, L., Benichou, S., Berlioz-Torrent, C., Liu, L., Benarous, R., Heard, J.M., and Schwartz, O. (1998). Nef interacts with the mu subunit of

clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity* 8, 483-495.

Li, P., and Schwarz, E.M. (2003). The TNF-alpha transgenic mouse model of inflammatory arthritis. *Springer seminars in immunopathology* 25, 19-33.

Lie, W.R., Myers, N.B., Connolly, J.M., Gorka, J., Lee, D.R., and Hansen, T.H. (1991). The specific binding of peptide ligand to Ld class I major histocompatibility complex molecules determines their antigenic structure. *The Journal of experimental medicine* 173, 449-459.

Lie, W.R., Myers, N.B., Gorka, J., Rubocki, R.J., Connolly, J.M., and Hansen, T.H. (1990). Peptide ligand-induced conformation and surface expression of the Ld class I MHC molecule. *Nature* 344, 439-441.

Lilley, B.N., and Ploegh, H.L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 429, 834-840.

Little, A.M., Nossner, E., and Parham, P. (1995). Dissociation of beta 2-microglobulin from HLA class I heavy chains correlates with acquisition of epitopes in the cytoplasmic tail. *J Immunol* 154, 5205-5215.

Liu, S.Q., Yu, H.C., Gong, Y.Z., and Lai, N.S. (2006). Quantitative measurement of HLA-B27 mRNA in patients with ankylosing spondylitis-- correlation with clinical activity. *The Journal of rheumatology* 33, 1128-1132.

Liu, Y., Jiang, L., Cai, Q., Danoy, P., Barnardo, M.C., Brown, M.A., and Xu, H. (2010). Predominant association of HLA-B*2704 with ankylosing spondylitis in Chinese Han patients. *Tissue antigens* 75, 61-64.

Ljunggren, H.G., and Karre, K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology today* 11, 237-244.

Ljunggren, H.G., Stam, N.J., Ohlen, C., Neefjes, J.J., Hoglund, P., Heemels, M.T., Bastin, J., Schumacher, T.N., Townsend, A., Karre, K., *et al.* (1990). Empty MHC class I molecules come out in the cold. *Nature* 346, 476-480.

Lopez-Larrea, C., Sujirachato, K., Mehra, N.K., Chiewsilp, P., Isarangkura, D., Kanga, U., Dominguez, O., Coto, E., Pena, M., Setien, F., *et al.* (1995). HLA-B27 subtypes in Asian patients with ankylosing spondylitis. Evidence for new associations. *Tissue antigens* 45, 169-176.

Lynch, S., Santos, SG., Campbel, EC., Nimmo, AM., Botting, C., Prescott, A., Antoniou, AN., Powis, SJ., (2009) Novel MHC class I structures on exosomes. *J Immunol* 183 1884-91

Madden, D.R., Gorga, J.C., Strominger, J.L., and Wiley, D.C. (1991). The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature* 353, 321-325.

Madrigal, J.A., Belich, M.P., Benjamin, R.J., Little, A.M., Hildebrand, W.H., Mann, D.L., and Parham, P. (1991). Molecular definition of a polymorphic antigen (LA45) of free HLA-A and -B heavy chains found on the surfaces of activated B and T cells. *The Journal of experimental medicine* 174, 1085-1095.

Makhadiyeva, D., Lam, L., Moatari, M., Vallance, J., Zheng, Y., Campbell, EC., Powis, SJ., (2011) MHC class I dimer formation by alteration of the cellular redox environment and induction of apoptosis. *Immunology In Press*.

Malik, P., Klimovitsky, P., Deng, L.W., Boyson, J.E., and Strominger, J.L. (2002). Uniquely conformed peptide-containing beta 2-microglobulin-free heavy chains of HLA-B2705 on the cell surface. *J Immunol* 169, 4379-4387.

Marti, M., Alvarez, I., Montserrat, V., and Lopez de Castro, J.A. (2001). Large sharing of T-cell epitopes and natural ligands between HLA-B27 subtypes (B*2702 and B*2705) associated with spondyloarthritis. *Tissue antigens* 58, 351-362.

Martin, T.M., Smith, J.R., and Rosenbaum, J.T. (2002). Anterior uveitis: current concepts of pathogenesis and interactions with the spondyloarthropathies. *Current opinion in rheumatology* 14, 337-341.

Masuda, A., Nakamura, A., Maeda, T., Sakamoto, Y., and Takai, T. (2007). Cis binding between inhibitory receptors and MHC class I can regulate mast cell activation. *The Journal of experimental medicine* 204, 907-920.

May, E., Dorris, M.L., Satumtira, N., Iqbal, I., Rehman, M.I., Lightfoot, E., and Taurog, J.D. (2003). CD8 alpha beta T cells are not essential to the pathogenesis of arthritis or colitis in HLA-B27 transgenic rats. *J Immunol* 170, 1099-1105.

McGonagle, D., Gibbon, W., and Emery, P. (1998). Classification of inflammatory arthritis by enthesitis. *Lancet* 352, 1137-1140.

McMichael, A., and Bowness, P. (2002). HLA-B27: natural function and pathogenic role in spondyloarthritis. *Arthritis research 4 Suppl 3*, S153-158.

Mear, J.P., Schreiber, K.L., Munz, C., Zhu, X., Stevanovic, S., Rammensee, H.G., Rowland-Jones, S.L., and Colbert, R.A. (1999). Misfolding of HLA-B27 as a result of its B pocket suggests a novel mechanism for its role in susceptibility to spondyloarthropathies. *J Immunol* 163, 6665-6670.

Merino, E., Galocha, B., Vazquez, M.N., and Lopez de Castro, J.A. (2008). Disparate folding and stability of the ankylosing spondylitis-associated HLA-B*1403 and B*2705 proteins. *Arthritis and rheumatism* 58, 3693-3704.

Miyazaki, J., Appella, E., Zhao, H., Forman, J., and Ozato, K. (1986). Expression and function of a nonglycosylated major histocompatibility class I antigen. *The Journal of experimental medicine* 163, 856-871.

Moffett-King, A. (2002). Natural killer cells and pregnancy. *Nature reviews* 2, 656-663.

Montserrat, V., Marti, M., and Lopez de Castro, J.A. (2003). Allospecific T cell epitope sharing reveals extensive conservation of the antigenic features of peptide ligands among HLA-B27 subtypes differentially associated with spondyloarthritis. *J Immunol* 170, 5778-5785.

Mori, Y., Tsuji, S., Inui, M., Sakamoto, Y., Endo, S., Ito, Y., Fujimura, S., Koga, T., Nakamura, A., Takayanagi, H., et al. (2008). Inhibitory immunoglobulin-like receptors LILRB and PIR-B negatively regulate osteoclast development. *J Immunol* 181, 4742-4751.

Mungall, A.J., Palmer, S.A., Sims, S.K., Edwards, C.A., Ashurst, J.L., Wilming, L., Jones, M.C., Horton, R., Hunt, S.E., Scott, C.E., et al. (2003). The DNA sequence and analysis of human chromosome 6. *Nature* 425, 805-811.

Naslavsky, N., Boehm, M., Backlund, P.S., Jr., and Caplan, S. (2004a). Rabenosyn-5 and EHD1 interact and sequentially regulate protein recycling to the plasma membrane. *Mol Biol Cell* 15, 2410-2422.

Naslavsky, N., Weigert, R., and Donaldson, J.G. (2004b). Characterization of a nonclathrin endocytic pathway: membrane cargo and lipid requirements. *Mol Biol Cell* 15, 3542-3552.

Nasution, A.R., Mardjuadi, A., Kunmartini, S., Suryadhana, N.G., Setyohadi, B., Sudarsono, D., Lardy, N.M., and Feltkamp, T.E. (1997). HLA-B27 subtypes

positively and negatively associated with spondyloarthropathy. *The Journal of rheumatology* 24, 1111-1114.

Nguyen, T.T., Chang, S.C., Evnouchidou, I., York, I.A., Zikos, C., Rock, K.L., Goldberg, A.L., Stratikos, E., and Stern, L.J. (2011). Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1. *Nature structural & molecular biology* 18, 604-613.

Nickerson, C.L., Luthra, H.S., Savarirayan, S., and David, C.S. (1990). Susceptibility of HLA-B27 transgenic mice to *Yersinia enterocolitica* infection. *Human immunology* 28, 382-396.

Nixon, D.F., Townsend, A.R., Elvin, J.G., Rizza, C.R., Gallwey, J., and McMichael, A.J. (1988). HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature* 336, 484-487.

Nurzia, E., Panimolle, F., Cauli, A., Mathieu, A., Magnacca, A., Paladini, F., Sorrentino, R., and Fiorillo, M.T. (2010). CD8⁺ T-cell mediated self-reactivity in HLA-B27 context as a consequence of dual peptide conformation. *Clinical immunology (Orlando, Fla)* 135, 476-482.

Ogata, M., Hino, S., Saito, A., Morikawa, K., Kondo, S., Kanemoto, S., Murakami, T., Taniguchi, M., Tanii, I., Yoshinaga, K., *et al.* (2006). Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* 26, 9220-9231.

Oldstone, M.B. (1987). Molecular mimicry and autoimmune disease. *Cell* 50, 819-820.

Olivieri, I., Padula, A., Cianco, G., Moro, L., Durante, E., Guadiano, C., Masciandaro, S., Pozzi, S., and Ferrara, G.B. (2000). The HLA-B*2709 subtype in a patient with undifferentiated spondarthritis. *Annals of the rheumatic diseases* 59, 654-655.

Orchard, T.R., Thiyagaraja, S., Welsh, K.I., Wordsworth, B.P., Hill Gaston, J.S., and Jewell, D.P. (2000). Clinical phenotype is related to HLA genotype in the peripheral arthropathies of inflammatory bowel disease. *Gastroenterology* 118, 274-278.

- Orr, M.T., Murphy, W.J., and Lanier, L.L. (2010).** 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nature immunology* 11, 321-327.
- Pahl, H.L. (1999).** Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiological reviews* 79, 683-701.
- Pahl, H.L., and Baeuerle, P.A. (1997).** The ER-overload response: activation of NF-kappa B. *Trends in biochemical sciences* 22, 63-67.
- Parham, P., and Brodsky, F.M. (1981).** Partial purification and some properties of BB7.2. A cytotoxic monoclonal antibody with specificity for HLA-A2 and a variant of HLA-A28. *Human immunology* 3, 277-299.
- Paulsson, K.M., Jevon, M., Wang, J.W., Li, S., and Wang, P. (2006).** The double lysine motif of tapasin is a retrieval signal for retention of unstable MHC class I molecules in the endoplasmic reticulum. *J Immunol* 176, 7482-7488.
- Paulsson, K.M., Kleijmeer, M.J., Griffith, J., Jevon, M., Chen, S., Anderson, P.O., Sjogren, H.O., Li, S., and Wang, P. (2002).** Association of tapasin and COPI provides a mechanism for the retrograde transport of major histocompatibility complex (MHC) class I molecules from the Golgi complex to the endoplasmic reticulum. *The Journal of biological chemistry* 277, 18266-18271.
- Pedersen, O.B., Svendsen, A.J., Ejstrup, L., Skytthe, A., and Junker, P. (2008).** On the heritability of psoriatic arthritis. Disease concordance among monozygotic and dizygotic twins. *Annals of the rheumatic diseases* 67, 1417-1421.
- Peh, C.A., Burrows, S.R., Barnden, M., Khanna, R., Cresswell, P., Moss, D.J., and McCluskey, J. (1998).** HLA-B27-restricted antigen presentation in the absence of tapasin reveals polymorphism in mechanisms of HLA class I peptide loading. *Immunity* 8, 531-542.
- Penttinen, M.A., Ekman, P., and Granfors, K. (2004).** Non-antigen presenting effects of HLA-B27. *Current molecular medicine* 4, 41-49.
- Penttinen, M.A., Holmberg, C.I., Sistonen, L., and Granfors, K. (2002).** HLA-B27 modulates nuclear factor kappaB activation in human monocytic cells exposed to lipopolysaccharide. *Arthritis and rheumatism* 46, 2172-2180.
- Perez-Villar, J.J., Melero, I., Navarro, F., Carretero, M., Bellon, T., Llano, M., Colonna, M., Geraghty, D.E., and Lopez-Botet, M. (1997).** The CD94/NKG2-A

inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1. *J Immunol* 158, 5736-5743.

Perosa, F., Luccarelli, G., Prete, M., Favoino, E., Ferrone, S., and Dammacco, F. (2003). Beta 2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. *J Immunol* 171, 1918-1926.

Pickl, W.F., Holter, W., Stockl, J., Majdic, O., and Knapp, W. (1996). Expression of beta 2-microglobulin-free HLA class I alpha-chains on activated T cells requires internalization of HLA class I heterodimers. *Immunology* 88, 104-109.

Pimlott, N.J., and Miller, R.G. (1986). The use of tunicamycin to study the role of cell surface oligosaccharides in lymphocyte recognition. *J Immunol* 137, 2455-2459.

Ploegh, H.L., Orr, H.T., and Stominger, J.L. (1981). Biosynthesis and cell surface localization of nonglycosylated human histocompatibility antigens. *J Immunol* 126, 270-275.

Powis, S.J., Santos, S.G., and Antoniou, A.N. (2009). Biochemical features of HLA-B27 and antigen processing. *Advances in experimental medicine and biology* 649, 210-216.

Primeau, T., Myers, N.B., Yu, Y.Y., Lybarger, L., Wang, X., Truscott, S.M., Hansen, T.H., and Connolly, J.M. (2005). Applications of major histocompatibility complex class I molecules expressed as single chains. *Immunol Res* 32, 109-121.

Purcell, A.W., Kelly, A.J., Peh, C.A., Dudek, N.L., and McCluskey, J. (2000). Endogenous and exogenous factors contributing to the surface expression of HLA B27 on mutant APC. *Human immunology* 61, 120-130.

Qian, B.F., Tonkonogy, S.L., and Sartor, R.B. (2008). Aberrant innate immune responses in TLR-ligand activated HLA-B27 transgenic rat cells. *Inflamm Bowel Dis* 14, 1358-1365.

Radhakrishna, H., and Donaldson, J.G. (1997). ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *The Journal of cell biology* 139, 49-61.

Rahman, P., Inman, R.D., Gladman, D.D., Reeve, J.P., Peddle, L., and Maksymowych, W.P. (2008). Association of interleukin-23 receptor variants with ankylosing spondylitis. *Arthritis and rheumatism* 58, 1020-1025.

- Raine, T., Brown, D., Bowness, P., Hill Gaston, J.S., Moffett, A., Trowsdale, J., and Allen, R.L. (2006).** Consistent patterns of expression of HLA class I free heavy chains in healthy individuals and raised expression in spondyloarthropathy patients point to physiological and pathological roles. *Rheumatology (Oxford, England)* 45, 1338-1344.
- Ramos, M., Paradela, A., Vazquez, M., Marina, A., Vazquez, J., and Lopez de Castro, J.A. (2002).** Differential association of HLA-B*2705 and B*2709 to ankylosing spondylitis correlates with limited peptide subsets but not with altered cell surface stability. *The Journal of biological chemistry* 277, 28749-28756.
- Rath, H.C., Herfarth, H.H., Ikeda, J.S., Grenther, W.B., Hamm, T.E., Jr., Balish, E., Taurog, J.D., Hammer, R.E., Wilson, K.H., and Sartor, R.B. (1996).** Normal luminal bacteria, especially *Bacteroides* species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. *The Journal of clinical investigation* 98, 945-953.
- Rath, H.C., Wilson, K.H., and Sartor, R.B. (1999).** Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with *Bacteroides vulgatus* or *Escherichia coli*. *Infection and immunity* 67, 2969-2974.
- Redlich, K., Gortz, B., Hayer, S., Zwerina, J., Kollias, G., Steiner, G., Smolen, J.S., and Schett, G. (2004).** Overexpression of tumor necrosis factor causes bilateral sacroiliitis. *Arthritis and rheumatism* 50, 1001-1005.
- Rehakova, Z., Capkova, J., Stepankova, R., Sinkora, J., Louzecka, A., Ivanyi, P., and Weinreich, S. (2000).** Germ-free mice do not develop ankylosing enthesopathy, a spontaneous joint disease. *Human immunology* 61, 555-558.
- Rein, R.S., Seemann, G.H., Neefjes, J.J., Hochstenbach, F.M., Stam, N.J., and Ploegh, H.L. (1987).** Association with beta 2-microglobulin controls the expression of transfected human class I genes. *J Immunol* 138, 1178-1183.
- Reinelt, S., Dedier, S., Asuni, G., Folkers, G., and Rognan, D. (2001).** Mutation of Cys-67 alters the thermodynamic stability of the human leukocyte antigen HLA0-B*2705. *The Journal of biological chemistry* 276, 18472-18477.
- Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., Janssen, H., Calafat, J., Drijfhout, J.W., and Neefjes, J. (2003).** Peptide diffusion,

protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* 18, 97-108.

Reits, E.A., Vos, J.C., Gromme, M., and Neefjes, J. (2000). The major substrates for TAP in vivo are derived from newly synthesized proteins. *Nature* 404, 774-778.

Reveille, J.D. (2011). The genetic basis of spondyloarthritis. *Annals of the rheumatic diseases* 70 Suppl 1, i44-50.

Reveille, J.D., and Maganti, R.M. (2009). Subtypes of HLA-B27: history and implications in the pathogenesis of ankylosing spondylitis. *Advances in experimental medicine and biology* 649, 159-176.

Reveille, J.D., Sims, A.M., Danoy, P., Evans, D.M., Leo, P., Pointon, J.J., Jin, R., Zhou, X., Bradbury, L.A., Appleton, L.H., et al. (2010). Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. *Nature genetics* 42, 123-127.

Ristich, V., Liang, S., Zhang, W., Wu, J., and Horuzsko, A. (2005). Tolerization of dendritic cells by HLA-G. *European journal of immunology* 35, 1133-1142.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78, 761-771.

Rothfuchs, A.G., Gigliotti, D., Palmblad, K., Andersson, U., Wigzell, H., and Rottenberg, M.E. (2001). IFN-alpha beta-dependent, IFN-gamma secretion by bone marrow-derived macrophages controls an intracellular bacterial infection. *J Immunol* 167, 6453-6461.

Rubin, L.A., Amos, C.I., Wade, J.A., Martin, J.R., Bale, S.J., Little, A.H., Gladman, D.D., Bonney, G.E., Rubenstein, J.D., and Siminovitch, K.A. (1994). Investigating the genetic basis for ankylosing spondylitis. Linkage studies with the major histocompatibility complex region. *Arthritis and rheumatism* 37, 1212-1220.

Ruckert, C., Fiorillo, M.T., Loll, B., Moretti, R., Biesiadka, J., Saenger, W., Ziegler, A., Sorrentino, R., and Uchanska-Ziegler, B. (2006). Conformational dimorphism of self-peptides and molecular mimicry in a disease-associated HLA-B27 subtype. *The Journal of biological chemistry* 281, 2306-2316.

Rudwaleit, M., Landewe, R., van der Heijde, D., Listing, J., Brandt, J., Braun, J., Burgos-Vargas, R., Collantes-Estevez, E., Davis, J., Dijkmans, B., et al. (2009a). The development of Assessment of SpondyloArthritis international Society classification criteria for axial spondyloarthritis (part I): classification of paper patients by expert opinion including uncertainty appraisal. *Annals of the rheumatic diseases* 68, 770-776.

Rudwaleit, M., Schwarzlose, S., Hilgert, E.S., Listing, J., Braun, J., and Sieper, J. (2008). MRI in predicting a major clinical response to anti-tumour necrosis factor treatment in ankylosing spondylitis. *Annals of the rheumatic diseases* 67, 1276-1281.

Rudwaleit, M., van der Heijde, D., Khan, M.A., Braun, J., and Sieper, J. (2004). How to diagnose axial spondyloarthritis early. *Annals of the rheumatic diseases* 63, 535-543.

Rudwaleit, M., van der Heijde, D., Landewe, R., Listing, J., Akkoc, N., Brandt, J., Braun, J., Chou, C.T., Collantes-Estevez, E., Dougados, M., et al. (2009b). The development of Assessment of SpondyloArthritis international Society classification criteria for axial spondyloarthritis (part II): validation and final selection. *Annals of the rheumatic diseases* 68, 777-783.

Rueda, B., Orozco, G., Raya, E., Fernandez-Sueiro, J.L., Mulero, J., Blanco, F.J., Vilches, C., Gonzalez-Gay, M.A., and Martin, J. (2008). The IL23R Arg381Gln non-synonymous polymorphism confers susceptibility to ankylosing spondylitis. *Annals of the rheumatic diseases* 67, 1451-1454.

Saklatvala, J. (1986). Tumour necrosis factor alpha stimulates resorption and inhibits synthesis of proteoglycan in cartilage. *Nature* 322, 547-549.

Salter, R.D., Howell, D.N., and Cresswell, P. (1985). Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* 21, 235-246.

Santos, S.G., Antoniou, A.N., Sampaio, P., Powis, S.J., and Arosa, F.A. (2006). Lack of tyrosine 320 impairs spontaneous endocytosis and enhances release of HLA-B27 molecules. *J Immunol* 176, 2942-2949.

Santos, S.G., Lynch, S., Campbell, E.C., Antoniou, A.N., and Powis, S.J. (2008). Induction of HLA-B27 heavy chain homodimer formation after activation in dendritic cells. *Arthritis research & therapy* 10, R100.

Santos, S.G., Powis, S.J., and Arosa, F.A. (2004). Misfolding of major histocompatibility complex class I molecules in activated T cells allows cis-interactions with receptors and signaling molecules and is associated with tyrosine phosphorylation. *The Journal of biological chemistry* 279, 53062-53070.

Saper, M.A., Bjorkman, P.J., and Wiley, D.C. (1991). Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 Å resolution. *Journal of molecular biology* 219, 277-319.

Saric, T., Chang, S.C., Hattori, A., York, I.A., Markant, S., Rock, K.L., Tsujimoto, M., and Goldberg, A.L. (2002). An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. *Nature immunology* 3, 1169-1176.

Scarpellino, L., Oeschger, F., Guillaume, P., Coudert, J.D., Levy, F., Leclercq, G., and Held, W. (2007). Interactions of Ly49 family receptors with MHC class I ligands in trans and cis. *J Immunol* 178, 1277-1284.

Schett, G. (2008). Review: Immune cells and mediators of inflammatory arthritis. *Autoimmunity* 41, 224-229.

Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., and Bennink, J.R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404, 770-774.

Schumacher, T.N., Heemels, M.T., Neefjes, J.J., Kast, W.M., Melief, C.J., and Ploegh, H.L. (1990). Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell* 62, 563-567.

Serwold, T., Gonzalez, F., Kim, J., Jacob, R., and Shastri, N. (2002). ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature* 419, 480-483.

Sesma, L., Montserrat, V., Lamas, J.R., Marina, A., Vazquez, J., and Lopez de Castro, J.A. (2002). The peptide repertoires of HLA-B27 subtypes differentially associated to spondyloarthropathy (B*2704 and B*2706) differ by specific changes at three anchor positions. *The Journal of biological chemistry* 277, 16744-16749.

Shen, H., Goodall, J.C., and Hill Gaston, J.S. (2009). Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. *Arthritis and rheumatism* 60, 1647-1656.

Shimizu, Y., and DeMars, R. (1989). Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. *J Immunol* 142, 3320-3328.

Sieper, J., van der Heijde, D., Landewe, R., Brandt, J., Burgos-Vagas, R., Collantes-Estevez, E., Dijkmans, B., Dougados, M., Khan, M.A., Leirisalo-Repo, M., *et al.* (2009). New criteria for inflammatory back pain in patients with chronic back pain: a real patient exercise by experts from the Assessment of SpondyloArthritis international Society (ASAS). *Annals of the rheumatic diseases* 68, 784-788.

Singh, R., Aggarwal, A., and Misra, R. (2007). Th1/Th17 cytokine profiles in patients with reactive arthritis/undifferentiated spondyloarthropathy. *The Journal of rheumatology* 34, 2285-2290.

Sinkorova, Z., Capkova, J., Niederlova, J., Stepankova, R., and Sinkora, J. (2008). Commensal intestinal bacterial strains trigger ankylosing enthesopathy of the ankle in inbred B10.BR (H-2(k)) male mice. *Human immunology* 69, 845-850.

Solheim, J.C., Harris, M.R., Kindle, C.S., and Hansen, T.H. (1997). Prominence of beta 2-microglobulin, class I heavy chain conformation, and tapasin in the interactions of class I heavy chain with calreticulin and the transporter associated with antigen processing. *J Immunol* 158, 2236-2241.

Spiro, R.G., Zhu, Q., Bhoyroo, V., and Soling, H.D. (1996). Definition of the lectin-like properties of the molecular chaperone, calreticulin, and demonstration of its copurification with endomannosidase from rat liver Golgi. *The Journal of biological chemistry* 271, 11588-11594.

Springer, T.A., and Strominger, J.L. (1976). Detergent-soluble HLA antigens contain a hydrophilic region at the COOH-terminus and a penultimate hydrophobic region. *Proceedings of the National Academy of Sciences of the United States of America* 73, 2481-2485.

Stagg, A.J., Breban, M., Hammer, R.E., Knight, S.C., and Taurog, J.D. (1995). Defective dendritic cell (DC) function in a HLA-B27 transgenic rat model of spondyloarthropathy (SpA). *Advances in experimental medicine and biology* 378, 557-559.

- Stam, N.J., Spits, H., and Ploegh, H.L. (1986).** Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J Immunol* 137, 2299-2306.
- Steen, K.H., Steen, A.E., and Reeh, P.W. (1995).** A dominant role of acid pH in inflammatory excitation and sensitization of nociceptors in rat skin, in vitro. *J Neurosci* 15, 3982-3989.
- Stewart-Jones, G.B., di Gleria, K., Kollnberger, S., McMichael, A.J., Jones, E.Y., and Bowness, P. (2005).** Crystal structures and KIR3DL1 recognition of three immunodominant viral peptides complexed to HLA-B*2705. *European journal of immunology* 35, 341-351.
- Stodulkova, E., Pohl, J., Man, P., Votruba, J., Capkova, J., Sedlackova, M., Ivaskova, E., Ivanyi, P., and Flieger, M. (2006).** Comparison of amino acid compositions of peptides eluted from HLA-B27 molecules of healthy individuals and patients with ankylosing spondylitis. *Immunology letters* 103, 135-141.
- Strange, A., Capon, F., Spencer, C.C., Knight, J., Weale, M.E., Allen, M.H., Barton, A., Band, G., Bellenguez, C., Bergboer, J.G., et al. (2010).** A genome-wide association study identifies new psoriasis susceptibility loci and an interaction between HLA-C and ERAP1. *Nature genetics* 42, 985-990.
- Suarez-Almazor, M.E., and Russell, A.S. (1990).** Sacroiliitis in psoriasis: relationship to peripheral arthritis and HLA-B27. *The Journal of rheumatology* 17, 804-808.
- Sung, I.H., Kim, T.H., Bang, S.Y., Kim, T.J., Lee, B., Peddle, L., Rahman, P., Greenwood, C.M., Hu, P., and Inman, R.D. (2009).** IL-23R polymorphisms in patients with ankylosing spondylitis in Korea. *The Journal of rheumatology* 36, 1003-1005.
- Taurog, J.D. (1998).** Arthritis in HLA-B27 transgenic animals. *The American journal of the medical sciences* 316, 250-256.
- Taurog, J.D., Dorris, M.L., Satumtira, N., Tran, T.M., Sharma, R., Dressel, R., van den Brandt, J., and Reichardt, H.M. (2009).** Spondylarthritis in HLA-B27/human beta2-microglobulin-transgenic rats is not prevented by lack of CD8. *Arthritis and rheumatism* 60, 1977-1984.

- Taurog, J.D., and Hammer, R.E. (1996).** Experimental spondyloarthropathy in HLA-B27 transgenic rats. *Clinical rheumatology* 15 Suppl 1, 22-27.
- Taurog, J.D., Lowen, L., Forman, J., and Hammer, R.E. (1988).** HLA-B27 in inbred and non-inbred transgenic mice. Cell surface expression and recognition as an alloantigen in the absence of human beta 2-microglobulin. *J Immunol* 141, 4020-4023.
- Taurog, J.D., Maika, S.D., Satumtira, N., Dorris, M.L., McLean, I.L., Yanagisawa, H., Sayad, A., Stagg, A.J., Fox, G.M., Le O'Brien, A., et al. (1999).** Inflammatory disease in HLA-B27 transgenic rats. *Immunological reviews* 169, 209-223.
- Taurog, J.D., Maika, S.D., Simmons, W.A., Breban, M., and Hammer, R.E. (1993).** Susceptibility to inflammatory disease in HLA-B27 transgenic rat lines correlates with the level of B27 expression. *J Immunol* 150, 4168-4178.
- Taurog, J.D., Richardson, J.A., Croft, J.T., Simmons, W.A., Zhou, M., Fernandez-Sueiro, J.L., Balish, E., and Hammer, R.E. (1994).** The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *The Journal of experimental medicine* 180, 2359-2364.
- Thomas, G.P., and Brown, M.A. (2010).** Genetics and genomics of ankylosing spondylitis. *Immunological reviews* 233, 162-180.
- Todd, D.J., Lee, A.H., and Glimcher, L.H. (2008).** The endoplasmic reticulum stress response in immunity and autoimmunity. *Nature reviews* 8, 663-674.
- Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H.G., Foster, L., and Karre, K. (1989).** Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* 340, 443-448.
- Tran, T.M., Dorris, M.L., Satumtira, N., Richardson, J.A., Hammer, R.E., Shang, J., and Taurog, J.D. (2006).** Additional human beta2-microglobulin curbs HLA-B27 misfolding and promotes arthritis and spondylitis without colitis in male HLA-B27-transgenic rats. *Arthritis and rheumatism* 54, 1317-1327.
- Tran, T.M., Satumtira, N., Dorris, M.L., May, E., Wang, A., Furuta, E., and Taurog, J.D. (2004).** HLA-B27 in transgenic rats forms disulfide-linked heavy chain oligomers and multimers that bind to the chaperone BiP. *J Immunol* 172, 5110-5119.

Truscott, S.M., Wang, X., Lybarger, L., Biddison, W.E., McBerry, C., Martinko, J.M., Connolly, J.M., Linette, G.P., Fremont, D.H., Hansen, T.H., et al. (2008). Human major histocompatibility complex (MHC) class I molecules with disulfide traps secure disease-related antigenic peptides and exclude competitor peptides. *The Journal of biological chemistry* 283, 7480-7490.

Tsai, W.C., Chen, C.J., Yen, J.H., Ou, T.T., Tsai, J.J., Liu, C.S., and Liu, H.W. (2002). Free HLA class I heavy chain-carrying monocytes--a potential role in the pathogenesis of spondyloarthropathies. *The Journal of rheumatology* 29, 966-972.

Turner, M.J., Delay, M.L., Bai, S., Klenk, E., and Colbert, R.A. (2007). HLA-B27 up-regulation causes accumulation of misfolded heavy chains and correlates with the magnitude of the unfolded protein response in transgenic rats: Implications for the pathogenesis of spondylarthritis-like disease. *Arthritis and rheumatism* 56, 215-223.

Turner, M.J., Sowders, D.P., DeLay, M.L., Mohapatra, R., Bai, S., Smith, J.A., Brandewie, J.R., Taurog, J.D., and Colbert, R.A. (2005). HLA-B27 misfolding in transgenic rats is associated with activation of the unfolded protein response. *J Immunol* 175, 2438-2448.

Uderhardt, S., Diarra, D., Katzenbeisser, J., David, J.P., Zwerina, J., Richards, W., Kronke, G., and Schett, G. (2010). Blockade of Dickkopf (DKK)-1 induces fusion of sacroiliac joints. *Annals of the rheumatic diseases* 69, 592-597.

Ugolini, S., Arpin, C., Anfossi, N., Walzer, T., Cambiaggi, A., Forster, R., Lipp, M., Toes, R.E., Melief, C.J., Marvel, J., et al. (2001). Involvement of inhibitory NKRs in the survival of a subset of memory-phenotype CD8⁺ T cells. *Nature immunology* 2, 430-435.

Ugrinovic, S., Mertz, A., Wu, P., Braun, J., and Sieper, J. (1997). A single nonamer from the Yersinia 60-kDa heat shock protein is the target of HLA-B27-restricted CTL response in Yersinia-induced reactive arthritis. *J Immunol* 159, 5715-5723.

Urban, R.G., Chicz, R.M., Lane, W.S., Strominger, J.L., Rehm, A., Kenter, M.J., UytdeHaag, F.G., Ploegh, H., Uchanska-Ziegler, B., and Ziegler, A. (1994). A subset of HLA-B27 molecules contains peptides much longer than nonamers. *Proceedings of the National Academy of Sciences of the United States of America* 91, 1534-1538.

van der Heijde, D., Da Silva, J.C., Dougados, M., Geher, P., van der Horst-Bruinsma, I., Juanola, X., Olivieri, I., Raeman, F., Settas, L., Sieper, J., et al. (2006). Etanercept 50 mg once weekly is as effective as 25 mg twice weekly in patients with ankylosing spondylitis. *Annals of the rheumatic diseases* 65, 1572-1577.

van der Linden, S., Valkenburg, H., and Cats, A. (1983). The risk of developing ankylosing spondylitis in HLA-B27 positive individuals: a family and population study. *British journal of rheumatology* 22, 18-19.

van der Linden, S., Valkenburg, H.A., and Cats, A. (1984). Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis and rheumatism* 27, 361-368.

Van Hateren, A., James, E., Bailey, A., Phillips, A., Dalchau, N., and Elliott, T. (2010). The cell biology of major histocompatibility complex class I assembly: towards a molecular understanding. *Tissue antigens* 76, 259-275.

Varnavidou-Nicolaidou, A., Karpasitou, K., Georgiou, D., Stylianou, G., Kokkofitou, A., Michalis, C., Constantina, C., Gregoriadou, C., and Kyriakides, G. (2004). HLA-B27 in the Greek Cypriot population: distribution of subtypes in patients with ankylosing spondylitis and other HLA-B27-related diseases. The possible protective role of B*2707. *Human immunology* 65, 1451-1454.

Vazquez, M.N., and Lopez de Castro, J.A. (2005). Similar cell surface expression of beta2-microglobulin-free heavy chains by HLA-B27 subtypes differentially associated with ankylosing spondylitis. *Arthritis and rheumatism* 52, 3290-3299.

Vembar, S.S., and Brodsky, J.L. (2008). One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol* 9, 944-957.

Wanders, A., Heijde, D., Landewe, R., Behier, J.M., Calin, A., Olivieri, I., Zeidler, H., and Dougados, M. (2005). Nonsteroidal antiinflammatory drugs reduce radiographic progression in patients with ankylosing spondylitis: a randomized clinical trial. *Arthritis and rheumatism* 52, 1756-1765.

Wang, J., Yu, D.T., Fukazawa, T., Kellner, H., Wen, J., Cheng, X.K., Roth, G., Williams, K.M., and Raybourne, R.B. (1994). A monoclonal antibody that recognizes HLA-B27 in the context of peptides. *J Immunol* 152, 1197-1205.

- Wang, X., Lin, Z., Wei, Q., Jiang, Y., and Gu, J. (2009).** Expression of IL-23 and IL-17 and effect of IL-23 on IL-17 production in ankylosing spondylitis. *Rheumatology international* 29, 1343-1347.
- Wei, J.C., Tsai, W.C., Lin, H.S., Tsai, C.Y., and Chou, C.T. (2004).** HLA-B60 and B61 are strongly associated with ankylosing spondylitis in HLA-B27-negative Taiwan Chinese patients. *Rheumatology (Oxford, England)* 43, 839-842.
- Weinreich, S., Eulderink, F., Capkova, J., Pla, M., Gaede, K., Heesemann, J., van Alphen, L., Zurcher, C., Hoebe-Hewryk, B., Kievits, F., *et al.* (1995).** HLA-B27 as a relative risk factor in ankylosing enthesopathy in transgenic mice. *Human immunology* 42, 103-115.
- Weinreich, S.S., Hoebe-Hewryk, B., van der Horst, A.R., Boog, C.J., and Ivanyi, P. (1997).** The role of MHC class I heterodimer expression in mouse ankylosing enthesopathy. *Immunogenetics* 46, 35-40.
- Weiss, E.H., Schliesser, G., Botteron, C., McMichael, A., Riethmuller, G., Kievits, F., Ivanyi, P., and Brem, G. (1990).** HLA class-I-transgenic mice as model system to study MHC-restricted antigen recognition in man. *Scandinavian journal of rheumatology* 87, 91-96.
- Wendling, D., Cedoz, J.P., Racadot, E., and Dumoulin, G. (2007).** Serum IL-17, BMP-7, and bone turnover markers in patients with ankylosing spondylitis. *Joint Bone Spine* 74, 304-305.
- Whelan, M.A., and Archer, J.R. (1993).** Chemical reactivity of an HLA-B27 thiol group. *European journal of immunology* 23, 3278-3285.
- Williams, A.P., Peh, C.A., Purcell, A.W., McCluskey, J., and Elliott, T. (2002).** Optimization of the MHC class I peptide cargo is dependent on tapasin. *Immunity* 16, 509-520.
- Wong, P.K., Quinn, J.M., Sims, N.A., van Nieuwenhuijze, A., Campbell, I.K., and Wicks, I.P. (2006).** Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis. *Arthritis and rheumatism* 54, 158-168.
- Wright, C., Edelmann, M., diGleria, K., Kollnberger, S., Kramer, H., McGowan, S., McHugh, K., Taylor, S., Kessler, B., and Bowness, P. (2009).** Ankylosing

spondylitis monocytes show upregulation of proteins involved in inflammation and the ubiquitin proteasome pathway. *Annals of the rheumatic diseases* 68, 1626-1632.

Yewdell, J.W., Anton, L.C., and Bennink, J.R. (1996). Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules? *J Immunol* 157, 1823-1826.

York, I.A., Chang, S.C., Saric, T., Keys, J.A., Favreau, J.M., Goldberg, A.L., and Rock, K.L. (2002). The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. *Nature immunology* 3, 1177-1184.

Yu, Y.Y., Myers, N.B., Hilbert, C.M., Harris, M.R., Balendiran, G.K., and Hansen, T.H. (1999). Definition and transfer of a serological epitope specific for peptide-empty forms of MHC class I. *International immunology* 11, 1897-1906.

Yu, Y.Y., Netuschil, N., Lybarger, L., Connolly, J.M., and Hansen, T.H. (2002). Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potently stimulate antigen-specific T cells and B cells. *J Immunol* 168, 3145-3149.

Zeh, H.J., 3rd, Leder, G.H., Lotze, M.T., Salter, R.D., Tector, M., Stuber, G., Modrow, S., and Storkus, W.J. (1994). Flow-cytometric determination of peptide-class I complex formation. Identification of p53 peptides that bind to HLA-A2. *Human immunology* 39, 79-86.

Zemmour, J., Little, A.M., Schendel, D.J., and Parham, P. (1992). The HLA-A,B "negative" mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. *J Immunol* 148, 1941-1948.

Zhao, L., Fong, Y., Granfors, K., Gu, J., and Yu, D. (2008). Identification of cytokines that might enhance the promoter activity of HLA-B27. *The Journal of rheumatology* 35, 862-868.

Zhao, L., Liu, C.H., and Yu, D. (2011). High-throughput screening of chemical libraries for modulators of gene promoter activity of HLA-B2705: environmental pathogenesis and therapeutics of ankylosing spondylitis. *The Journal of rheumatology* 38, 1061-1065.

Zhou, M. (2000). ERRATUM - correction to The specificity of peptides bound to human histocompatibility leukocyte antigen (HLA)-B27 influences the prevalence of

arthritis in HLA-B27 transgenic rats. The Journal of experimental medicine 21, following 746.

Zhou, M., Sayad, A., Simmons, W.A., Jones, R.C., Maika, S.D., Satumtira, N., Dorris, M.L., Gaskell, S.J., Bordoli, R.S., Sartor, R.B., *et al.* (1998). The specificity of peptides bound to human histocompatibility leukocyte antigen (HLA)-B27 influences the prevalence of arthritis in HLA-B27 transgenic rats. The Journal of experimental medicine 188, 877-886.

Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L., and Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nature biotechnology 15, 871-875.