Critical Assessment of the Role of Cannabinoid Receptor 2 in Inflammation

A thesis submitted for the degree of Doctor of Philosophy
at the University of Oxford



Michaelmas Term 2016

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Ιθάκη

Σα βγεις στον πηγαιμό για την Ιθάκη,
να εύχεσαι νάναι μακρύς ο δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις.
Τους Λαιστρυγόνας και τους Κύκλωπας,
τον θυμωμένο Ποσειδώνα μην φοβάσαι,
τέτοια στον δρόμο σου ποτέ δεν θα βρεις,
αν μέν' η σκέψις σου υψηλή, αν εκλεκτή
συγκίνησις το πνεύμα και το σώμα σου αγγίζει.
Τους Λαιστρυγόνας και τους Κύκλωπας,
τον άγριο Ποσειδώνα δε θα συναντήσεις,
αν δεν τους κουβανείς μες στην ψυχή σου,
αν η ψυχή σου δεν τους στήνει εμπρός σου.

Να εύχεσαι νάναι μακρύς ο δρόμος.
Πολλά τα καλοκαιρινά πρωιά να είναι
που με τι ευχαρίστησι, με τι χαρά
θα μπαίνεις σε λιμένας πρωτοειδωμένους,
να σταματήσεις σε εμπορεία Φοινικικά,
και τες καλές πραγμάτειες ν' αποκτήσεις,
σεντέφια και κοράλλια, κεχριμπάρια κ' έβενους,
και ηδονικά μυρωδικά κάθε λογής,
όσο μπορείς πιο άφθονα ηδονικά μυρωδικά,
σε πόλεις Αιγυπτιακές πολλές να πας,
να μάθεις και να μάθεις απ' τους σπουδασμένους.

Πάντα στον νου σου να έχεις την Ιθάκη.
Το φθάσιμον εκεί είν' ο προορισμός σου.
Αλλά μη βιάζεις το ταξείδι διόλου.
Καλλίτερα χρόνια πολλά να διαρκέσει
και γέρος πια να αράξεις στο νησί,
πλούσιος με όσα κέρδισες στο δρόμο,
μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη.

Η Ιθάκη σ'έδωσε τ'ωραίο ταξείδι.
Χωρίς αυτήν δε θάβγαινες στον δρόμο.
Άλλα δεν έχει να σε δώσει πια.

Και αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε.
Έτσι σοφός που έγινες, με τόση πείρα,
ήδη θα το κατάλαβες οι Ιθάκες τι σημαίνουν.

Κωνσταντίνος Π. Καβάφης

Abstract

Cannabinoid receptor 2 (CB2R) is a G protein-coupled receptor (GPCR) expressed in the periphery by immune cells, such as B cells, natural killer (NK) cells, neutrophils, macrophages and T cells. It belongs to the endocannabinoid system which also comprises CB1R, endogenous lipophilic agonists called endocannabinoids and the enzymes responsible for their synthesis and degradation.

During inflammatory diseases the endocannabinoid system is dysregulated and increased expression levels of endocannabinoids and cannabinoid receptors are observed. Therefore there is a continuing interest to target the endocannabinoid system to restore homeostasis in patients who suffer from a number of inflammatory diseases, such as multiple sclerosis, atherosclerosis, neuropathic pain and others. However, to date there has been no good anti-inflammatory drug in the market or clinical trials that acts via CB2R. The main aim of this DPhil thesis was to critically assess the role of this receptor in health and inflammation with a particular focus on innate immune cell recruitment.

Here I describe my findings regarding the immunophenotype of a CB2R knockout (KO) mouse strain at steady state and under acute systemic inflammation. I showed that CB2R KO mice display a trend in neutrophil accumulation and in monocyte egress in the bone marrow, as well as higher myeloperoxidase activity in the liver. These findings support the view that this cannabinoid receptor plays a pivotal role in innate immune cell trafficking during homeostasis.

Furthermore, I discovered that CB2R KO animals display exacerbated immune cell recruitment to peripheral tissues in a model of low dose endotoxemia. In particular, I found more neutrophils in the lungs and spleens of CB2R KO mice at the model's peak time point. These observations are consistent with a model in which CB2R plays a non-redundant role in the inhibition of neutrophil migration to injured tissues after an inflammatory insult.

My *in vitro* experiments showed that CB2R-selective agonists had no anti-inflammatory effects during macrophage activation. Looking for a mechanistic explanation for this result, I found that the expression levels of the receptor are severely downregulated upon challenge with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) which suggests that it is possible that CB2R is differentially regulated in immune cells during inflammation.

Collectively, the data presented in this thesis support the view that CB2R plays an important role in health and disease in pre-clinical models, but further studies are required to make more soluble CB2R-selective agonists and to understand which immune cells we should aim to target therapeutically.

Acknowledgments

It goes without saying that this work would not have been completed without the support and help of a few people. Here I would like to acknowledge their contributions and thank them with all my heart for what they have done in the past four years.

First, I would like to thank my supervisor, Prof. David Greaves for giving me the opportunity to do a DPhil in his lab. I learnt a lot under your supervision and I feel I have become a better scientist. I am thankful for all your guidance and support, especially in the last few months of my time in Oxford when job hunting became overwhelming.

Dr. Asif Iqbal (aka My boss) for acting like an older brother to me. You were always there to discuss science, give me your input, make suggestions and challenge me when needed. Most of my achievements in these four years I owe to you and there are no words to express my gratitude. I will always remember our dinners at the Uni club when I was under the weather and you encouraged me to never give up or when I woke up at 5 am to do the endotoxemia experiments with you at ZT=0. And of course, riding a horse carriage in rainy Killarney with you and Lewis was one of the craziest things I have ever done during scientific conferences.

Mr. (or Dr. depending on who passes their viva first) Lewis Taylor, my soul mate. Where to begin really? I will only mention the first pictures that come to my mind; the morning workouts in the gym with our "big" friend, the questions of life and death we used to ask each other at tissue culture, the table tennis games, the endless hours spent discussing about CB2R, Dub(u)lin, Girra (Utrecht) and Killarney (Riverdance). It is my pleasure to have met you and shared some ludicrous experiences with you.

All the members of the Greaves lab for making the DPhil experience less stressful and more pleasant. Gemma, Danny, Sophia, Max, Jamie, Carlota and Cherelle, I am lucky to have worked with you and spent quality time at college dinners and parties.

I tried to keep my family away from the everyday stress of a DPhil. And I did that because I know how much they care and how worried they would feel if they saw me tired or frustrated. However, I knew all the way through that they would listen and do whatever in their powers to help whenever asked. For that I am grateful and, in a sense, they laid a stone on this success with their love and support.

Last but definitely not the least, I want to thank the person who put up the most with my stress and insecurities (at least) in my final year. Cecilia had always something nice to say to comfort me and boost my confidence when I used to come home grumpy from the lab. She made my final year as joyful as it could have been, organising short trips to

escape Oxford and cooking the best sushi I have ever had in my life. Having someone like her so close to me during my DPhil has been a blessing.

This work would not have been possible without the financial support from the Edward P. Abraham Trust and the Medical Research Council who funded my stipend, university and college fees.

Abbreviations

ACEA- Arachidonyl-2'-chloroethylamide
ACPA- Arachidonylcyclopropylamide
AEA- N-arachidonoylethanolamine
2-AG- 2-arachidonoyl glycerol
AIDS- Acquired Immunodeficiency Syndrome
ANOVA- Analysis of Variance
APC- Antigen Precenting Cell
APC- Allophycocyanin
ATP- Adenosine Triphosphate
BCA- Bicinchoninic Acid Assay
BMDC- Bone Marrow-derived DC
BMDM- Bone Marrow-derived Macrophage
BSA- Bovine Serum Albumin
CARS- Compensatory Anti-inflammatory Response Syndrome
Cas9- CRISPR-associated Endonuclease 9
CASP- Colon Ascendens Stent Peritonitis
CBD- Cannabidiol
CB2R- Cannabinoid Receptor 2
CCL2- C-C motif ligand 2
CD- Cluster of Differentiation
cAMP- cyclic Adenosine Monophosphate
cDNA- complementary DNA
cGAS- cyclic GMP-AMP Synthase
CHO- Chinese Hamster Ovary
CLP- Caecal Ligation and Puncture
CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats
CSF-2- Colony Stimulating Factor 2
Ct- Cycle Threshold
COX-1- Cyclooxygenase-1
CXCL1- C-X-C motif ligand 1
Δ^9-THC- Δ^9-Tetrahydrocannabinol
DAG- Diacylglycerol
DAGL- Diacylglycerol Lipase
DAPI- 4',6-diamidino-2-phenylindole
DC- Dendritic Cell
DHA- Docosahexanoic Acid
DMEM- Dulbecco's Modified Eagle Medium
DMSO- Dimethyl Sulfoxide
DNA- Deoxyribonucleic Acid
ECAM- Epithelial Cell Adhesion Molecule

EDTA- Ethylenediaminetetraacetic acid
ELISA- Enzyme Linked Immunosorbent Assay
EMP- Erythro-myeloid Progenitor
EPA- Eicosapentanoic Acid
ERK1/2- Extracellular-signal Regulated Kinase 1/2
FAAH- Fatty Acid Amine Hydrolase
FAK- Focal Adhesion Kinase
FBS- Fetal Bovine Serum
FITC- Fluorescein isothiocyanate
FLAP- 5-LOX-activating Protein
fMLP- formyl Met Leu Phe
FS- Forward Scatter
Fw- Forward
GABA- Gamma-Aminobutyric Acid
GFP- Green Fluorescent Protein
GPCR- G protein-coupled receptor
HBSS- Hanks' Balanced Salt Solution
HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1- High Mobility Group Box 1
HMWK- High-Molecular-Weight-Kininogen
HPETE- Hydroperoxyeicosatetraenoic acid
HSC- Hematopoietic Stem Cell
HRP- Horseradish Peroxidase
HTAB- Hexadecyltrimethylammonium bromide
ICAM-1- Intercellular Adhesion Molecule
IFN-γ- Interferon γ
iNOS- Inducible NO Synthase
IL- Interleukin
i.p.- intraperitoneally
JAK- Janus Kinase
JAM- Junctional Adhesion Molecule
JNK- c-Jun N-terminal Kinase
KO- Knockout
LFA-1- Lymphocyte Function-associated Antigen -1
LOX- Lipoxygenase
LPS- Lipopolysaccharide
LTB_4- Leukotriene B_4
MAC- Membrane Attack Complex
MADCAM-1- Mucosal vascular Addressin Cell-adhesion Molecule 1
MAGL- Monoacylglycerol Lipase
MAPK- Mitogen Activated Protein Kinase
MBL- Mannose Binding Lectin

MFI- Mean Fluorescence Intensity
MHC-I- Major Histocompatibility Complex I
MIP-1α- Macrophage Inflammatory Protein 1α
moDC- monocyte-derived DC
moMΦ- monocyte-derived macrophage
MPO- Myeloperoxidase
mRNA- messenger Ribonucleic Acid
NADA- N-arachidonoyl Dopamine
NADPH- Nicotinamide Adenine Dinucleotide Phosphate
NArPE- N-arachidonoyl-phosphatidylethanolamine
NET- Neutrophil Extracellular Trap
NK- Natural Killer
NLR- NOD-like Receptor
O.D.- Optical Density
oxLDL- Oxidised Low Density Lipoprotein
PAA- Palmitoyl-ethanolamine-preferring Acid Amidase
PAF- Platelet-activating Factor
PBS- Phosphate Buffer Saline
pDC- Plasmacytoid DC
PE- Phycoerythrin
PEC- Peritoneal Exudate Cell
PerCP- Peridinin Chlorophyll Protein
PGK- Phosphoglycerine Kinase
PI3K- phosphoinositide-3 kinase
PLD- Phospholipase D
P/S- Penicillin/Streptomycin
PFA- Paraformaldehyde
PKA- Protein Kinase A
PLC- Phospholipase C
PRR- Pattern Recognition Receptor
PUFA- Polyunsaturated Fatty Acid
qPCR- quantitative Polymerase Chain Reaction
RIG-I- Retinoid acid-Inducible Gene 1
ROS- Reactive Oxygen Species
RT- Reverse Transcription
Rv- Reverse
SEM- Standard Error of the Mean
SIRS- Systemic Inflammatory Response Syndrome
SR-A- Scavenger Receptor A
SS- Side Scatter
STAT- Signal Transducer and Activator of Transcription
TGF-β- Transforming Growth Factor-β

TipDC- TNF and iNOS-producing DC
TLR- Toll-like Receptor
TMB- 3, 3', 5, 5'- tetramethylbenzidine
TNF-α- Tumour Necrosis Factor α
Treg- T regulatory
TRPV1- Transient Receptor Potential cation channel subfamily V member 1
VAP-1- Vascular Adhesion Protein-1
VCAM-1- Vascular Cell Adhesion Molecule
VLA- Very Late Antigen
WT- Wild Type

Brief table of contents

Chapter 1: Introduction 1

Chapter 2: Materials and methods 43

Chapter 3: The effects of a CB2R-selective agonist on leukocyte recruitment and mediator production in the zymosan-induced peritonitis model 64

Chapter 4: The effects of CB2R-selective agonists on macrophage functions during inflammation driven by LPS and IFN-γ 88

Chapter 5: The role of CB2R in the homeostasis of the immune system 117

Chapter 6: The role of CB2R in systemic acute inflammation 137

Chapter 7: General discussion 166

References 172

Table of contents

Abstract ii
Acknowledgments iii
Abbreviations v
Brief table of contents ix
Table of contents x
List of figures and tables xv
Chapter 1: Introduction 1
1.1 Acute and chronic inflammation 1
1.2 The main events of the inflammatory response 3
1.2.1 Vasculature activation 3
1.2.2 Oedema 3
1.2.3 Hemorrhage 4
1.2.4 The complement system 4
1.2.5 Coagulation and thrombosis 5
1.2.6 Leukocyte recruitment and effector functions 6
1.2.7 Wound healing 12
1.3 Inflammatory mediators 12
1.3.1 Vasoactive mediators 12
1.3.2 Cytokines 14
1.3.3 Chemokines 15
1.3.4 Lipid mediators 15
1.3.4.1 Eicosanoids 16
1.3.4.2 PAF 18
1.3.4.3 Specialised Pro-resolution Mediators: Resolvins, Protectins and Maresins 18
1.3.5 ROS 19
1.4. Cannabinoids; a distinct family of lipophilic mediators 20
1.4.1 Cannabis lipophilic components in inflammation: a historical perspective 20
1.4.2 Discovery, tissue distribution and functions of cannabinoid receptors 21
1.4.3 CB1R and CB2R molecular biology 24
1.4.4 Cell signalling by cannabinoid receptors 26
1.4.5 Gene polymorphisms of the human endocannabinoid system 28
1.4.6 Natural and synthetic cannabinoid agonists and antagonists 29

1.4.6.1 Endogenous cannabinoid agonists and the endocannabinoid system 29
1.4.6.2 Classification of CB1R and CB2R agonists 32
1.4.6.2.1 Classical agonists 32
1.4.6.2.2 Non-classical agonists 33
1.4.6.2.3 Aminoalkylindoles 33
1.4.6.2.4 Eicosanoids 34
1.4.6.3 CB1R and CB2R-selective antagonists 36
1.4.7 Therapeutic potential of cannabinoid agonists 36
1.5 Original hypothesis 41
1.6 Aims of this thesis 41
Chapter 2: Materials and methods 43
2.1 Materials 43
2.1.1 Media and buffers 43
2.1.2 Reagents 44
2.1.3 Commercial kits 45
2.1.4 Other materials 46
2.1.5 Animals 46
2.2 Methods 47
2.2.1 *In vivo* work 47
2.2.1.1 Animals 47
2.2.1.2 Models of inflammation 47
2.2.1.3 Tissue harvest and processing 48
2.2.1.4 Cell number quantification 49
2.2.2 *In vitro* work 50
2.2.2.1 Bone marrow-derived macrophages (BMDMs) 50
2.2.2.2 Activation assays 51
2.2.3 Techniques 51
2.2.3.1 cAMP assay 51
2.2.3.2 β-arrestin recruitment assay 52
2.2.3.4 ELISA 53
2.2.3.5 Griess assay 54
2.2.3.6 Confocal microscopy 55
2.2.3.7 RNA extraction 56
2.2.3.8 Primer design 56
2.2.3.9 Reverse transcription (RT) 57

2.2.3.10 qPCR 58
2.2.3.11 Flow cytometry 60
2.2.3.12 Genotyping 61
2.2.3.13 BCA assay 62
2.2.3.14 MPO assay 62
2.2.3.13 Luminex assay 62
2.2.4 Statistical analysis 63
Chapter 3: The effects of a CB2R-selective agonist on leukocyte recruitment and mediator production in the zymosan-induced peritonitis model 64
3.1 Introduction 64
3.1.1 *In vitro* and *in vivo* evidence for cannabinoid regulation of leukocyte recruitment 64
3.1.2 The zymosan-induced peritonitis model 66
3.2 Aim of the chapter 69
3.3 Results 69
3.3.1 DIAS2 reduces the number of recruited monocytes to the peritoneal cavity 69
3.3.2 DIAS2 induces the levels of pro-inflammatory mediators in the peritoneal cavity 72
3.3.3 Low dose DIAS2 administration does not inhibit monocyte recruitment 74
3.3.4 Low dose of DIAS2 still induces the secretion of pro-inflammatory mediators 76
3.3.5 Inactive compound on CB2R inhibits neutrophil recruitment to the peritoneal cavity 76
3.3.6 Inactive compound does not induce inflammation in the peritoneal cavity 78
3.4 Discussion 80
3.5 Limitations of the study 87
Chapter 4: The effects of CB2R-selective agonists on macrophage functions during inflammation driven by LPS and IFN-γ 88
4.1 Introduction 88
4.1.1.1 Origins and tissue distribution of the macrophage 88
4.1.1.2 Macrophage responses during inflammation 89
4.1.2 Effects of cannabinoids on macrophage activation 91

4.1.2.1 Macrophage cytokine and chemokine secretion 91
4.1.2.2 Macrophage oxidative burst 92
4.1.2.3 Macrophage antigen presentation 93
4.1.2.4 Macrophage phagocytosis 94
4.2 Aim of the chapter 95
4.3 Results 95
4.3.1 CB2R-selective ligands activate the receptor at nanomolar concentrations 95
4.3.2 Cytokine and chemokine secretion 98
4.3.3 Nitrite secretion 98
4.3.4 Surface expression of antigen presentation molecules 100
4.3.5 *E.coli*-triggered phagocytosis 102
4.3.6. CB2R KO BMDMs have a comparable inflammatory phenotype to littermate controls 104
4.3.7. Expression of *Cnr2* in macrophages is significantly reduced upon LPS and IFN-γ stimulation 104
4.4 Discussion 106
4.5 Limitations of the study 116
Chapter 5: The role of CB2R in the homeostasis of the immune system . 117
5.1 Introduction 117
5.1.1.1 Neutrophils 117
5.1.1.2 Monocytes/Macrophages 118
5.1.1.3 DCs 119
5.1.1.4 NK cells 121
5.1.1.5 Eosinophils, basophils and mast cells 121
5.1.2 The Buckley CB2R KO mouse strain 122
5.2 Aim of the chapter 124
5.3 Results 124
5.3.1 Genetic characterisation of the Deltagen CB2R KO mouse strain 124
5.3.2 Immunophenotyping of the Deltagen CB2R KO mouse strain 125
5.3.2.1 CB2R KO mice have more neutrophils in the bone marrow 125
5.3.2.2 WT and CB2R KO mice have similar numbers of neutrophils, Ly-6C^{lo} and Ly-6C^{hi} monocytes in the bloodstream 127
5.3.2.3 Splenic immune cell populations are similar in WT and CB2R KO animals 127

5.3.2.4 CB2R KO mice neutrophil populations in the liver differ significantly from WT littermates 129
5.4 Discussion 129
5.5 Limitations of the study 136
Chapter 6: The role of CB2R in systemic acute inflammation 137
6.1 Introduction 137
6.1.1 Local and systemic inflammation 137
6.1.2 Sepsis 137
6.1.3 Animal models of sepsis 139
6.1.3.1 Toxaemia models 140
6.1.3.2 Bacterial infection models 141
6.1.3.3 Host barrier disruption models 141
6.1.4 Effects of the endocannabinoid system on sepsis 142
6.2 Aim of the chapter 144
6.3 Results 144
6.3.1 The endotoxemia murine model is a model of acute systemic inflammation 144
6.3.2 Dexamethasone validates the endotoxemia *in vivo* model for reduction of inflammatory responses 147
6.3.3 CB2R KO animals have more neutrophils in the lungs compared to WT controls at the peak of inflammation 149
6.3.4 Neutrophil and monocyte numbers in the bone marrow of WT and CB2R KO mice are similar during the endotoxemia model time course 151
6.3.5 CB2R KO animals display enhanced neutrophil recruitment to the spleen at the peak time point 153
6.4 Discussion 155
6.5 Limitations of the study 164
Chapter 7: General discussion 166
7.1 Original hypothesis 166
7.2 Key findings 166
7.3 Future directions 167
7.4 Outstanding issues in the cannabinoid field 169
7.5 Final conclusion 171
References 172

List of figures and tables

Figure 1.1. Timeline of the acute inflammatory response 2
Figure 1.2. Schematic representation of perivascular extravasation of neutrophils 9
Figure 1.3. Overview of the acute inflammatory response 13
Figure 1.4. Biosynthetic pathways of lipid mediators in inflammation 17
Figure 1.5. Cannabinoid receptor signalling 27
Figure 1.6. Molecular structure of cannabinoid agonists 35
Figure 2.1. An overview of the Cellometer Auto T4 cell viability counter 49
Figure 2.2. Representative Forward scatter (FS)-Side scatter (SS) Flow Cytometry plot for cell number quantification from a murine blood sample 50
Figure 2.3. Typical standard curve for the quantification of the levels of IL-6 54
Figure 2.4. Typical standard curve for the quantification of the levels of nitrites 55
Figure 2.5. Representative melt curves from a qPCR experiment with *Cnr2* as the gene of interest (red curves) and *Actg1* as the reference gene (cyan curves) 59
Figure 2.6. Representative amplification curves from a qPCR experiment with *Cnr2* as the gene of interest (red curves) and *Actg1* as the reference gene (cyan curves) 59
Figure 3.1. DIAS2 reduces monocyte recruitment to the peritoneal cavity in the zymosan-induced peritonitis model 71
Figure 3.2. DIAS2 induces the production of pro-inflammatory cytokines and chemokines in the peritoneal cavity in the zymosan-induced peritonitis model 73
Figure 3.3. DIAS2 effect on monocyte recruitment to the peritoneal cavity is lost at a lower dose 75
Figure 3.4. DIAS2-induced pro-inflammatory cytokine and chemokine production in the peritoneal cavity in the zymosan-induced peritonitis model is attenuated at a lower dose 77
Figure 3.5. Chemical structure of CB2R-selective agonist DIAS2 and inactive analogue Compound 13 79
Figure 3.6. Inactive DIAS2 analogue also reduces monocyte recruitment to the peritoneal cavity in the zymosan-induced peritonitis model 81
Figure 3.7. Inactive DIAS2 analogue does not cause cytokine and chemokine production in the peritoneal cavity in the zymosan-induced peritonitis model 83
Figure 4.1. BMDMs generated in L929-conditioned medium cultures express *Cnr2* 97
Figure 4.2. CB2R-selective agonists induce distinct signalling pathways upon receptor activation 99
Figure 4.3. Cytokine and chemokine secretion is decreased by Dexamethasone in LPS- and IFN-γ-stimulated BMDMs 101
Figure 4.4. CB2R-selective agonists do not reduce pro-inflammatory levels produced by BMDMs following LPS and IFN-γ stimulation 103
Figure 4.5. Nitrite production by BMDMs is not modulated by CB2R-selective agonists 105

Figure 4.6. CB2R-selective agonists do not modulate antigen presentation molecule expression on macrophage surfaces 107
Figure 4.7. CB2R-selective agonists do not enhance *E.coli*-triggered phagocytosis 109
Figure 4.8. Deficiency of CB2R does not alter macrophage immune responses to LPS and IFN-γ 111
Figure 4.9. Expression of *Cnr2* is severely decreased upon LPS and IFN-γ administration 113
Figure 5.1. CB2R KO mice express receptor transcripts in lymphoid and non-lymphoid tissues 126
Figure 5.2. Experimental approach to genetically characterise CB2R KO mice 128
Figure 5.3. *Cnr2* gene exon 2-inserted neomycin cassette sequencing 130
Figure 5.4. CB2R KO mice have impaired T cell maturation in the thymus 132
Figure 5.5. CB2R KO mice have a bigger monocyte population in their bone marrow 134
Figure 6.1. CB2R KO mice have fewer neutrophils, Ly-6C^{lo} monocytes and T cells in their circulation 146
Figure 6.2. CB2R KO mice have significantly more neutrophils in their spleens in comparison with littermate control animals 148
Figure 6.3. CB2R KO mice have comparable T and B lymphocyte numbers in their spleens with littermate control animals 150
Figure 6.4. CB2R KO mice have fewer marginated neutrophils in their lungs, but more neutrophils in their livers compared to littermate control animals 152
Figure 6.5. CB2R KO mice have fewer B cells in their lymph nodes 154
Figure 6.6. Time course analysis of pro-inflammatory cytokines and chemokines in the serum of LPS-treated mice 156
Figure 6.7. Time course analysis of innate immune cell recruitment in LPS-treated mice 158
Figure 6.8. Time course analysis of pro-inflammatory cytokines and chemokines in the peritoneal fluid lavage, lung and liver homogenates of LPS-treated mice 160
Figure 6.9. CB2R KO animals display higher neutrophil recruitment in the spleen at 2 hours compared to littermate control animals 162

Table 1.1. Cannabinoid agonist affinities at CB1R and CB2R 37
Table 1.2. Pro- and anti-inflammatory effects of CB2R in disease models 39
Table 2.1. ELISA commercial kits 53
Table 2.2. Confocal microscopy antibodies 55
Table 2.3. PCR primer pair sequences 57
Table 2.4. RT reaction mastermix 57
Table 2.5. qPCR mastermix 58
Table 2.6. Flow Cytometry antibodies 60
Table 2.7. Flow Cytometry isotype control antibodies 61
Table 2.8. Genotyping PCR mastermix 61

Chapter 1: Introduction

1.1 Acute and chronic inflammation

Inflammation is a physiological process initiated in the host following injury of vascularised tissues (e.g. infection, trauma, stress, exposure to heat or cold) and aims to deliver defensive materials and restore the tissues to their pre-injured state. Inflammation has been traditionally described by its four cardinal signs, i.e. redness, swelling, heat and pain (Kumar *et al.* 2003).

Inflammatory responses are well structured. Soon after tissue injury, endothelial and tissue-resident cells (i.e. macrophages and mast cells) secrete soluble and lipid chemoattractants to induce neutrophil migration (Nathan 2002), and leakage of albumin, water, electrolytes and acute phase proteins (C-reactive protein, serum amyloid A and serum amyloid component P) from the blood is observed (Gabay and Kushner 1999;Steel and Whitehead 1994). In addition, the activation of platelets, complement and the coagulation cascade, and the release of proteases and oxidants potentiate acute inflammation and recruit cells of adaptive immunity (Nathan 2002). Eventually, an endogenous program of resolution is triggered by lipid mediators called lipoxins, resolvins, protectins and maresins which act to inhibit further migration of leukocytes and clear the tissue from apoptosing cells (Serhan 2011;Serhan and Savill 2005).

However, if inflammation fails to resolve, it can last for months or years and it becomes chronic (Figure 1.1) (Medzhitov 2008;Nathan and Ding 2010). Neutrophils in tissues are gradually replaced by macrophages, which fuse together to form granulomas in a final attempt to protect the host (Serhan *et al.* 2010). Moreover, T cells initiate the

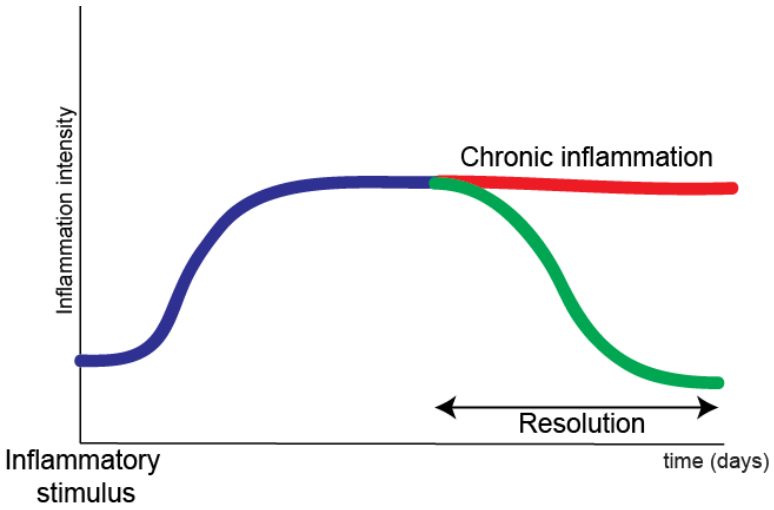


Figure 1.1. Timeline of the acute inflammatory response. Inflammation is initiated by exogenous and endogenous stimuli and is characterised by a period of amplification. In parallel, endogenous pro-resolution pathways are induced and are responsible for the termination of inflammation. If resolution fails, inflammation progresses and becomes chronic.

antigen-specific adaptive immune responses, the characteristics of which depend on their effector class (Murphy and Stockinger 2010).

1.2 The main events of the inflammatory response

1.2.1 Vasculature activation

The first step of the inflammatory response is the activation of the endothelium. Endothelial cells are sentinel cells and they respond rapidly upon sensing an inflammatory insult (Figure 1.2). They express a range of adhesion molecules, such as E-selectin, which they upregulate during inflammation, but also mobilise P-selectin from the Weibel-Palade bodies to their luminal surface (Bevilacqua *et al.* 1989;Geng *et al.* 1990;McEver *et al.* 1989).

Furthermore, pro-inflammatory cytokines, vasoactive peptides, neuropeptides, oxidised lipoproteins and reactive oxygen species (ROS) from activated tissue-resident macrophages, mast cells and fibroblasts promote pro-inflammatory gene transcription. In turn, endothelial cells release prostaglandins, cytokines, chemokines and lipid mediators (Pober and Sessa 2007). Recently, studies showed that altered laminar shear stress should also be considered as an inflammatory insult, as it induces a pro-adhesive phenotype in endothelial cells. Thus, endothelial cells are very sensitive to both biological and mechanical changes in vascular homeostasis (Givens and Tzima 2016).

1.2.2 Oedema

Oedema is a common feature of acute inflammation and is the result of an imbalance in the hydrostatic and osmotic forces acting on the endothelium (Sherwood and Toliver-Kinsky 2004). The endothelial lining of the inflamed tissue opens its tight junctions

reversibly and allows protein and fluid to leak into the extravascular space. This increases the hydrostatic forces that drive fluid into the extravascular space, whereas the osmosis that urges fluid back to the vessels declines (Wiig 2011).

Oedema functions to dilute microbial products, such as toxins and provides tissues with acute phase proteins and antibodies to the injured site. Moreover, it concentrates erythrocytes and increases blood viscosity (Sherwood and Toliver-Kinsky 2004).

1.2.3 Hemorrhage

Hemorrhage is defined as the passive transport of red blood cells from blood vessels to the extravascular space (Serhan *et al.* 2010). It can normally occur during oedema when red blood cells follow plasma proteins into the tissues. However, in extreme conditions, the destruction of the endothelial lining can result in excessive transport of red blood cells from the circulation into the tissues as a consequence of the vascular integrity loss.

1.2.4 The complement system

The complement system is responsible for pathogen lysis, phagocytosis and leukocyte recruitment. It is an integral component of innate immunity against bacteria, viruses and protozoa, and consists of three independent pathways with different origins, but identical downstream effector functions (Ricklin and Lambris 2013).

The classical complement pathway is initiated by immunoglobulin complexes on pathogen surfaces, the alternative complement pathway by autocleaved C3b peptide and the lectin complement pathway when mannose-binding lectin (MBL) binds to mannose-related carbohydrates on pathogen membranes (Wallis and Drickamer 1999).

Initiation of the complement cascade forms the C3 and C5 convertases, enzymatic complexes that cleave C3 and C5 into C3a and C3b and C5a and C5b, respectively. C5b molecules subsequently bind to C6, C7, C8 and several C9 complement peptides, and form the membrane attack complex (MAC) (Zipfel and Skerka 2009). The MAC integrates into microbial surfaces and promotes their lysis by rendering them permeable to extracellular fluid (Walport 2001).

Besides the lytic properties of complement, the cleaved fragments C3a, C4a and C5a, collectively known as anaphylatoxins, play a major role in inflammation (Zipfel and Skerka 2009). C3a induces mast cell degranulation and histamine release, whereas C5a is a potent neutrophil chemoattractant, induces ROS formation and enzyme release by phagocytes. Finally, C3b is an important opsonin, meaning that it can enhance phagocytosis and intracellular killing of microbes (Zipfel and Skerka 2009).

1.2.5 Coagulation and thrombosis

The coagulation cascade is triggered to restore hemostasis, i.e. prevent excessive blood loss (Figure 1.3). The coagulation cascade is divided into two pathways; the extrinsic and the intrinsic (Schenone *et al.* 2004;Smith *et al.* 2015). The extrinsic pathway is initiated when injured endothelial cells or leukocytes express tissue factor on their surface. Tissue factor comes into contact with Factor VII in the circulation which it activates, setting off the coagulation cascade via Factor X activation and thrombin conversion.

The intrinsic pathway is induced by collagen exposure. High-molecular-weight-kininogen (HMWK), prekallikrein and Hageman factor (Factor XII) form the primary complex which initiates the activation cascade. Factor XII converts to XIIa and

catalyses the activation of Factor XI and IX and the conversion or prekallikrein to kallikrein. Factor IXa then activates Factor X to convert prothrombin to thrombin and deposit fibrin on the endothelial wall, whilst kallikrein binds to HMWK and hydrolyses into bradykinin, a vasoactive peptide (Smith *et al.* 2015).

Platelets are also activated and convert prothrombin to thrombin. Their major function is to aggregate and cause thrombosis. However, they can also contribute to inflammation by releasing pro-inflammatory cytokines and chemokines (Levi *et al.* 2004).

1.2.6 Leukocyte recruitment and effector functions

Recruitment of leukocytes to the site of injury is a tightly regulated process. Endothelial cells are activated directly or indirectly by resident sentinel cells and synthesise the transmembrane glycoprotein E-selectin. In addition, endothelial P-selectin is translocated to the luminal surface and it initiates leukocyte arrest (Wang *et al.* 2014). Leukocytes also express L-selectin on their surface, which has been shown to regulate lymphocyte trafficking to the lymph nodes and secondary tethering to adhered leukocytes (Petri *et al.* 2008).

Endothelial cells also secrete chemokines or transport them from their abluminal to the luminal surface. A chemokine gradient is released by the injured endothelial cells and these chemoattractant proteins bind to GPCRs on rolling leukocytes. Downstream outside-in and inside-out signalling induces conformational changes from a bent to an extended position of transmembrane receptors expressed by leukocytes, called integrins (Nourshargh and Alon 2014).

Although the presence of selectins on the injured vasculature promotes leukocyte tethering and decreased rolling (Herter and Zarbock 2013;Kolaczkowska and Kubes 2013;Ley *et al.* 2007), it is the interactions of integrins with their ligands on the endothelium that facilitate cell arrest (Nourshargh and Alon 2014). Neutrophils express the leukocyte function-associated antigen 1 (LFA-1) and the β_2-integrin CD11b/CD18 and monocytes express the very late antigen 4 (VLA-4), which bind to intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) on the endothelium, respectively. Similarly, $\alpha_4\beta_7$-integrin associates with mucosal vascular addressin cell-adhesion molecule 1 (MADCAM-1) on the endothelium (Ley *et al.* 2007). Other molecules, such as CD44 and vascular adhesion protein-1 (VAP-1) can also facilitate leukocyte recruitment and adhesion (Herter and Zarbock 2013;Petri *et al.* 2008).

Upon activation and tight binding of the integrins on leukocytes to their ligands on the vasculature, subsequent signalling triggers ROS, cytokine secretion and differentiation of monocytes into macrophages (Herter and Zarbock 2013;Kolaczkowska and Kubes 2013).

Adhered leukocytes crawl toward the chemokine gradient that caused their attachment to the endothelium until they find an appropriate site for transmigration (Cook-Mills and Deem 2005). This can be the tricellular corners on the endothelium or tight junctions. Integrins, such as LFA-1 and CD11b/CD18 synergise in neutrophil and monocyte crawling, with CD11b/CD18 being more important for the final steps (Petri *et al.* 2008).

Once the leukocytes identify their preferred site of transmigration, they either cross the endothelium between two adjacent endothelial cells (paracellular route) or migrate

through the endothelial cells (transcellular route). The type of migration depends on the cell type, tissue and inflammatory stimulus, and cells may switch according to microenvironmental changes. However, in both transmigration routes, cells scan the preferred site for integrin-rich regions. LFA-1 and VLA-4, as well as cytoplasmic proteins and cytoskeletal components are well clustered and guide the leukocytes to the organs, whereas the endothelium forms a dome around the migrating leukocyte (Ley *et al.* 2007;Petri *et al.* 2008).

VLA-3 is responsible for allowing movement at the front of the transmigrating leukocytes and the final detachment from the abluminal surface of the endothelium (Herter and Zarbock 2013). In the paracellular route, endothelial cells redistribute tight junction molecules such as VE-cadherin, while tight junction molecules, such as CD31, CD99, junctional adhesion molecule (JAM) and epithelial cell adhesion molecule (ECAM) facilitate the transmigration of leukocytes between adjacent endothelial cells. Cells that transmigrate via the transcellular route make use of vesicular-vacuolar organelles, which form a continuum of membrane-associated passageways. ICAM-1 guides leukocytes to enter these membrane networks, while endothelial cell junction molecules may also participate in this process (Ley *et al.* 2007).

Leukocytes next need to make their way through pericytes lining the basement membrane. They prefer sites of the basement membrane where the expression of matrix proteins, such as laminins and collagens is lower than average or where the endothelium is very thin. In some cases, proteases, such as elastase have been reported to be released to facilitate leukocyte crossing of the basement membrane.

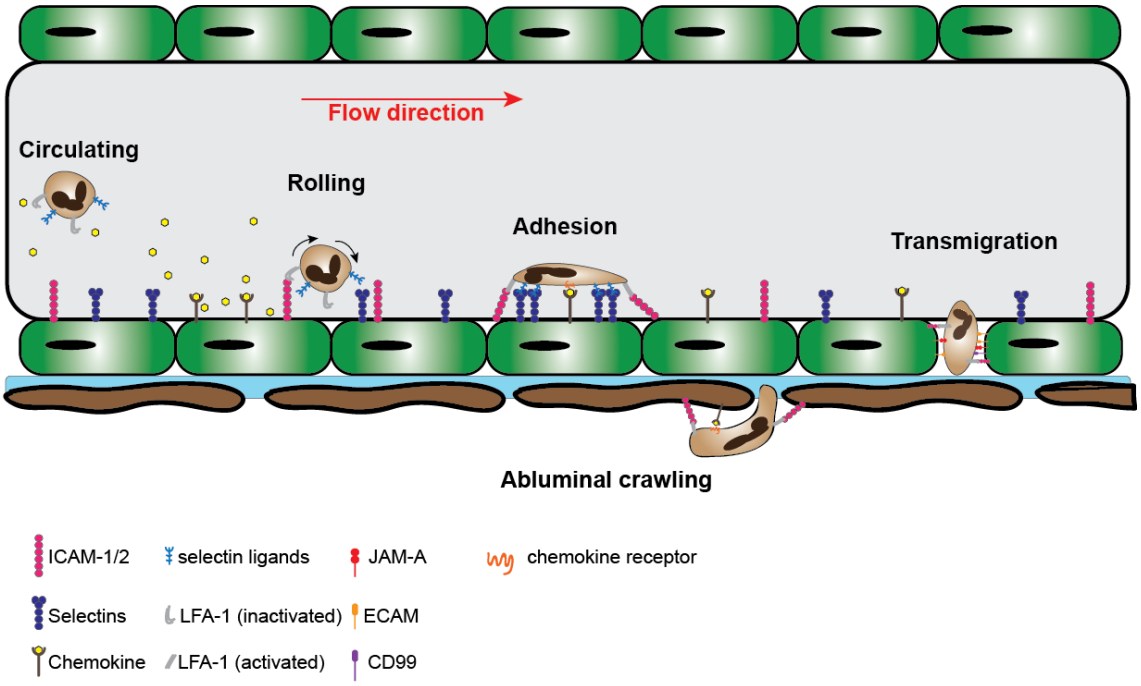


Figure 1.2. Schematic representation of perivascular extravasation of neutrophils. Upon the induction of inflammation by exogenous or endogenous stimuli, the endothelium is primed to overexpress adhesion molecules, such as L-selectin and translocate P-selectin to its luminal surface. In addition, endothelial cells secrete chemoattractant factors, such as chemokines which they immobilise on their surface creating a gradient to recruit leukocytes. Circulating neutrophils follow the chemokine gradient and roll on the endothelium making loose interactions via selectin ligands they express on their surface. Outside-in and in-outside chemokine signalling results in the activation of LFA-1 on neutrophils which engages ICAM-1 and ICAM-2 molecules on the endothelium to promote the adhesion of the neutrophils. The cells then crawl on the endothelial lining to find an appropriate site to transmigrate. They do so either through (paracellularly) or between (transcellularly) adjacent endothelial cells. In the transcellular transmigration pathway, the neutophils make contacts with tight junction molecules of the endothelial cells, such as JAM, ECAM and CD99. LFA-1 interactions with ICAM-1 facilitate the transmigration of the neutrophils to the basement membrane where they cross the layer of pericytes to gain entry to the injured tissue.

The first immune cell type to be recruited to an injured site is the neutrophil (Figure 1.2). Neutrophils respond to inflammatory products, such as the C5a complement fragment, bacterial formylated peptides, leukotriene B_4 (LTB_4), platelet-activating factor (PAF) and CXC family chemokines (Smith 1994). Once recruited, they recognise and kill bacteria by various mechanisms; phagocytosis, release of cytokines, chemokines, arachidonic acid metabolites, ROS, antibacterial proteins and proteolytic enzymes (Smith 1994;Witko-Sarsat *et al.* 2000). Recently, neutrophils were also shown to die by a process termed "NETosis", which is characterised by the release of extracellular traps (NETs) to their micro-environment consisting of nuclear histones, deoxyribonucleic acid (DNA) and granule proteins (Ermert *et al.* 2009).

Blood monocytes also respond to inflammatory signals secreted by the injured endothelium, tissue-resident cells and recruited neutrophils. Secreted cytokines (e.g. tumour necrosis factor and interleukin-6), chemokines (e.g. C-C motif ligand 2 (CCL2), CCL12), the presence of lipoproteins and pathogen oligosaccharides, complement peptides, immunoglobulins and lipid mediators are some of the molecules that activate monocytes (Shi and Pamer 2011) and guide them to tissues to mediate their effector functions (Dale *et al.* 2008).

In tissues, monocytes differentiate into macrophages (Figure 1.3) (Headland and Norling 2015). Apart from their secretory properties (Cailhier *et al.* 2005), macrophages are professional phagocytes and engulf intracellular microbes and apoptosing cells (a process called efferocytosis) to prevent secondary inflammation and autoimmunity (Headland and Norling 2015;Savill 1997;Serhan and Savill 2005).

In addition, macrophages are also antigen presenting cells (APCs) and like dendritic cells (DCs) and B cells, they bridge innate and adaptive immunity by processing and

presenting antigen to T cells. APCs recognise distinctive molecular patterns on microorganism surfaces or injury signals via both extracellular and intracellular receptors, such as the Toll-like receptors (TLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors, as well as scavenger molecules, e.g. CD36 and scavenger receptor A (SR-A) (Akira *et al.* 2001;Inohara and Nunez 2003;Taylor *et al.* 2005;Yoneyama *et al.* 2004) and act promptly to transfer the antigen to the lymph nodes.

Naïve T cells circulate in the blood and the lymphatic system in search of foreign antigen. During inflammation, activated DCs in lymph nodes present antigen to naive T cells via major histocompatibility complex I (MHC-I) or MHC-II and activate them (Delves and Roitt 2000). $CD4^+$ T helper cells then undergo activation and differentiate into T_H1 (intracellular bacteria), T_H2 (parasites), T_H17 (extracellular bacteria, fungi) or regulatory T (suppressors) subsets (Burkett *et al.* 2015;Mosmann *et al.* 1986;Smith-Garvin *et al.* 2009), whereas $CD8^+$ T cells which recognise viral and tumour epitopes differentiate into cytotoxic T cells (Weninger *et al.* 2002). Activated T cells then migrate to the inflamed tissues where they exert their effector functions, such as cytokine secretion, killing of virally-infected and tumour cells, B cell activation and innate immune cell priming to clear phagocytosed microbes (Murphy and Stockinger 2010).

Finally, B cell clones undergo proliferation after interactions with T cells in the lymph nodes (Rodriguez-Pinto 2005). This results in the generation and secretion of antigen-specific antibodies of an isotype that determines their functions (Ward and Ghetie 1995). For example, IgE antibodies activate mast cells in allergic responses, IgG antibodies are important for phagocytosis and neutralisation of microbes and IgM

antibodies activate complement. Antibodies neutralise and opsonise pathogens, but can also activate other immune cells, such as NK cells to initiate antibody-dependent cytotoxic responses (Raghavan and Bjorkman 1996).

1.2.7 Wound healing

Inflammation causes tissue damage and, if not controlled, it can become chronic and cause irreversible injury. If tissue damage is moderate, the host can replace dead parenchymal cells. However, in severe cases of extensive tissue damage, regeneration is not adequate and the process of wound healing is triggered (Serhan *et al.* 2010). Macrophages play a pivotal role in wound healing via the acquisition of a pro-repair phenotype and the secretion of transforming growth factor β (TGF-β) and other growth factors (Wynn and Vannella 2016).

During chronic inflammatory responses, fibroblasts secrete high levels of chemokines and cytokines to encourage leukocyte migration either directly or indirectly via endothelium recruitment ability modulation (McGettrick *et al.* 2012). However, macrophage-secreted TGF-β induces them to contribute to wound closure by collagen deposition (Shaw and Martin 2016). Recently, it has been shown that pluripotent mesenchymal stem cells also facilitate wound healing (Stappenbeck and Miyoshi 2009).

1.3 Inflammatory mediators

1.3.1 Vasoactive mediators

This family of mediators is responsible for vessel contraction and dilation during inflammation. They are stored pre-formed in granules in mast cells and platelets or

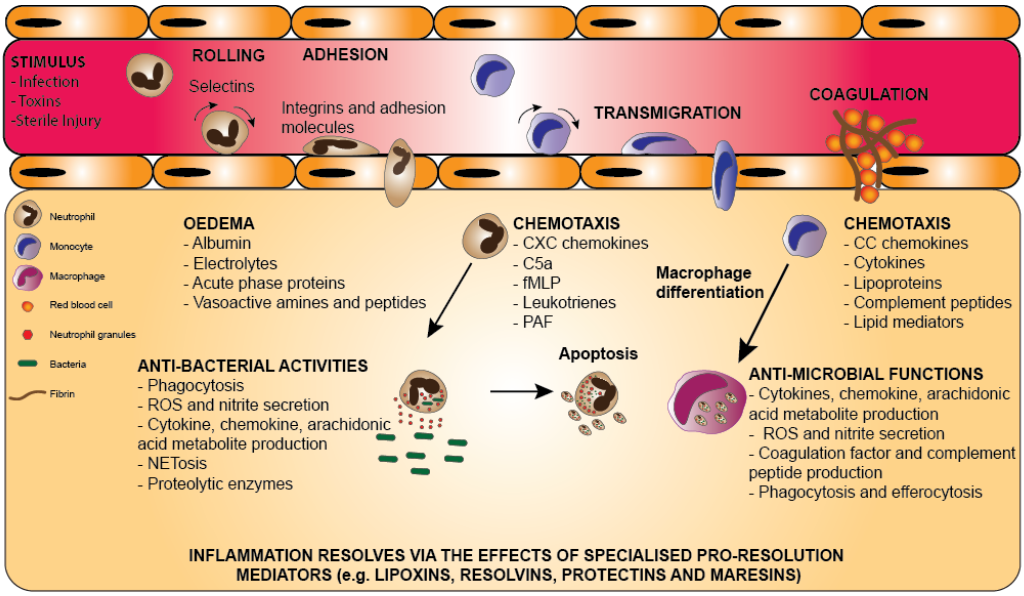


Figure 1.3. Overview of the acute inflammatory response. Inflammation is triggered by exogenous (infection, toxins) and endogenous (sterile injury) stimuli. Inflammatory stimuli prime endothelial cells to induce the expression of adhesion molecules and secrete chemokines to promote neutrophil migration to the tissue. Moreover, vasoactive mediators induce endothelial cell contraction which leads to oedema. The complement cascade is triggered by deposition of complement peptides on microbe surfaces and it results in pathogen lysis and complement peptide generation. Similarly, the coagulation cascade is initiated to restore hemostasis which is compromised by endothelial lining injury and hemorrhage. Neutrophils are attracted to chemokines and lipid mediators and adhere to the endothelium via tight interactions mediated by integrins and adhesion molecules on their surface. They then transmigrate either through (transcellularly) or between (paracellularly) cells and carry out their effector functions which include the phagocytosis of microbes, secretion of ROS, nitrites, cytokines, chemokines, arachidonic acid metabolites, NETosis and degranulation of proteolytic enzymes. Monocytes also follow a chemoattractant gradient to the injured tissue and transmigrate to differentiate into macrophages. They also possess anti-microbial properties and secrete cytokines, chemokines, arachidonic acid metabolites, complement and coagulation peptides, ROS and nitrites. In addition, they are professional phagocytes and ingest both foreign material and neutrophils that undergo apoptosis. This process, called efferocytosis, marks the initiation of the resolution phase of inflammation where further recruitment of leukocytes is inhibited and specialised pro-resolution mediators,such as lipoxins, resolvins, protectins and maresins take over to terminate inflammation.

circulate in an active form. Therefore they are activated before any other pathway (Serhan *et al.* 2010).

Histamine and serotonin are amines which have a wide distribution and are released by mast cells to regulate contraction and dilation of small and large arteries. Their long-term effects are either hypertension or hypotension (Larsen and Henson 1983;Ryan and Majno 1977). Similarly, vasoactive peptides of the kinin system, such as bradykinin are cleaved from their precursor forms by kallikreins which are activated during the coagulation cascade (Larsen and Henson 1983;Ryan and Majno 1977). Bradykinin mediates vascular permeability, vasoconstriction and vasodilation and gives the sensation of burning pain (Wallengren 1997).

1.3.2 Cytokines

Cytokines are polypeptides secreted by immune cells that regulate innate and adaptive immunity, hematopoiesis and tissue repair and regeneration. They act either in a paracrine or an endocrine manner (Dinarello 2007) and are potent at low concentrations, exhibit pleiotropy, redundancy or may be synergistic or antagonistic to other cytokines. Cytokines are divided into the tumour necrosis factor (TNF) superfamily and the families of interleukins (ILs), hematopoietins and interferons (Dinarello 2007;Liles and Van Voorhis 1995;Miyajima *et al.* 1992). They bind to multisubunit receptors that signal through Janus kinase (JAK)-signal transducer and activator protein (STAT) dimerisation (Miyajima *et al.* 1992).

During inflammation, the most relevant cytokines are members of the IL-1 (e.g. IL-1α, IL-1β, IL-18, IL-33), IL-2, IL-10 and IL-17 (e.g. IL-17A, IL-25, IL-17F) families, interferons (IFN-α, IFN-β, IFN-γ), the TNF superfamily (TNF-α and TNF-β) and TGF-

β (Dinarello 2007;Holdsworth and Gan 2015). However, despite their pivotal role in the initiation of innate immune responses and induction of adaptive immunity, they can be harmful if their levels exceed a certain threshold or their expression is prolonged.

1.3.3 Chemokines

Chemokines are short peptides with two disulfide bridges that regulate immune cell movement toward an inflamed site. They are produced in large amounts, are redundant and promiscuous, and apart from chemotaxis they have also been shown to regulate hematopoiesis, angiogenesis, lymphoid tissue development and organisation and tissue repair and regeneration (Griffith *et al.* 2014). Chemokines belong to one of four families; CC, CXC, C and CX_3C based on the position of the first two cysteine residues in their amino acid sequence. Hence, there are CCL2, CCL3 and CCL4 monocyte-recruiting, C-X-C motif ligand 1 (CXCL1) and CXCL2 neutrophil-recruiting chemokines, XCL1 (Lymphotactin) and CX_3CL1 (Fractalkine) which are the only members of their families (Griffith *et al.* 2014).

Chemokine receptor nomenclature follows the same logic. For example, CCR1 binds CCL3 and CCL5, CXCR2 binds CXCL1 and CXCL2, XCR1 ligates with XCL1 and CX_3CR1 with CX_3CL1 (Proudfoot 2002;White *et al.* 2013). Chemokine receptors are G protein-coupled and signal via Ca^{2+} influx, phosphoinositide and GTPase activation to mediate the chemokine-induced effects.

1.3.4 Lipid mediators

There are several classes of lipid mediators that play a role promoting or modulating inflammation (Figure 1.4). These are further divided into eicosanoids, PAF, resolvins,

protectins and maresins and are generated from plasma membrane phospholipids under the enzymatic activity of Phospholipase A_2 or from dietary essential fats. Phospholipase A_2 activity is increased during inflammation leading to higher levels of arachidonic acid, thus feeding forward to the generation of lipid mediators (Gilroy *et al.* 2004;Vadas *et al.* 1993).

1.3.4.1 Eicosanoids

Eisosanoids are arachidonic acid metabolites which are converted to prostaglandins, thromboxane, prostacyclin, leukotrienes and lipoxins based on the enzyme that catalyses the chemical reaction (Serhan *et al.* 2008). Prostaglandins are divided into three subseries; PG_1, PG_2 and PG_3. PG_2 prostaglandins are direct derivatives of arachidonic acid and can be cytoprotective, but also have pro-inflammatory and vasodilating properties (Serhan 2007;Williams and Peck 1977). The homeostatic prostaglandins are biosynthesised by cycloxygenase-1 (COX-1), whereas those involved in inflammation are generated by the inducible COX-2 (Dennis and Norris 2015).

Thromboxane A2 and B2 and prostacyclin (PGI_2) are eicosanoids with opposing functions; thromboxane is primarily synthesised by platelets and is a vasoconstrictor, whereas prostacyclin is the primary eicosanoid of the vascular endothelium and promotes vasodilation and inhibits platelet aggregation (Halushka *et al.* 1983). Hence, both eicosanoids are key regulators of systemic blood pressure and thrombogenesis.

Leukotrienes are biosynthesised by lipoxygenases (LOXs) from hydroperoxyeicosatetraenoic acids (HPETEs) (Dennis and Norris 2015). For instance, LTA_4 is synthesised by 5-LOX from 5-HPETE, whereas LTC_4, LTD_4 and LTE_4, also called cysteinyl leukotrienes, are synthesised in mast cells, eosinophils, basophils and

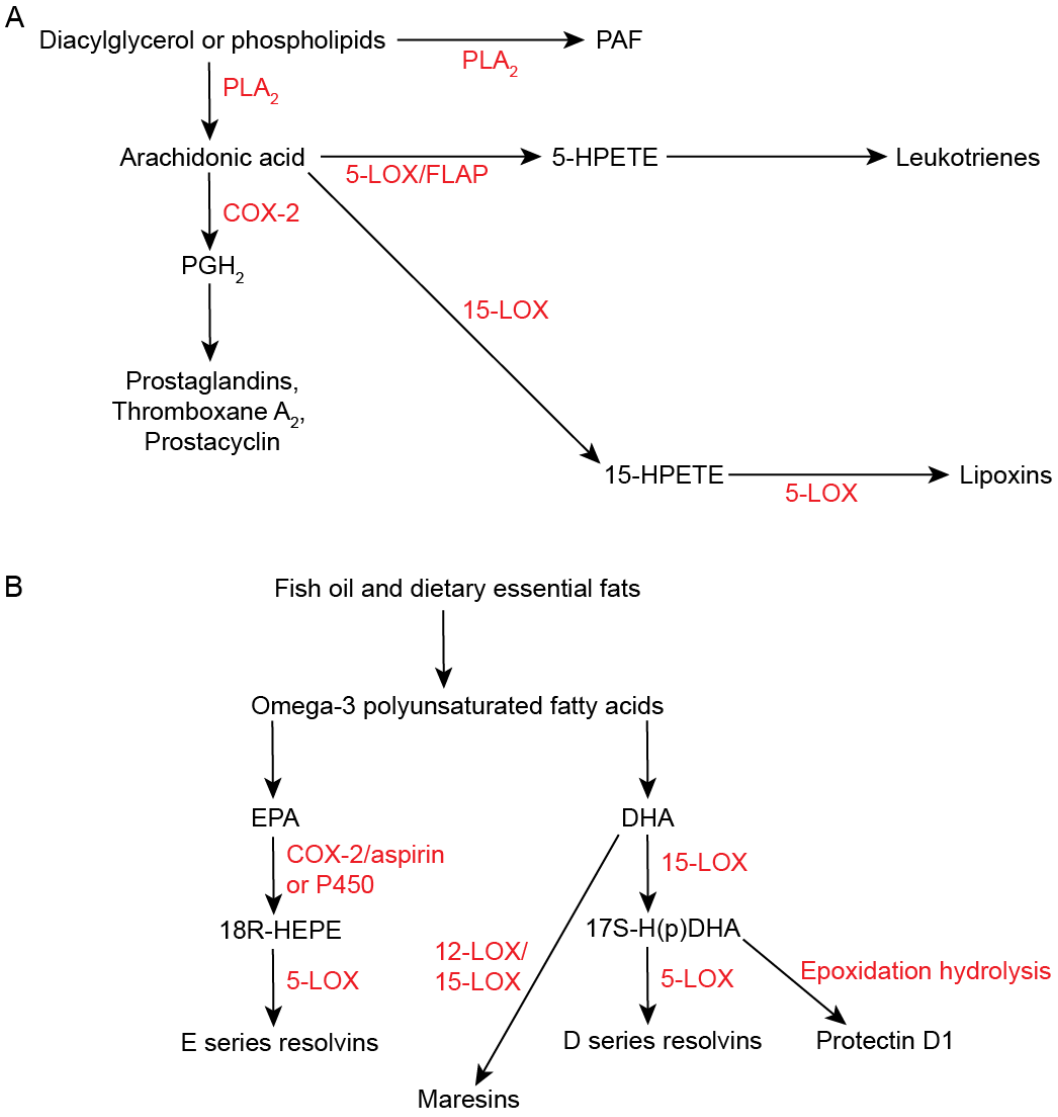


Figure 1.4. Biosynthetic pathways of lipid mediators in inflammation. During inflammation, diacylglycerol and plasma membrane phospholipids are converted to arachidonic acid or PAF by PLA_2. Arachidonic acid can further be converted to PGH_2 by COX-2 and a range of pro-inflammatory mediators, such as prostaglandins, Thromboxane A_2 and Prostacyclin which regulate vasodilation and vasoconstriction. 5-LOX and 5-LOX activating protein (FLAP) biosynthesise leukotrienes from 5-HPETE. Finally, 15-LOX and 5-LOX synthesise lipoxins in two steps with an intermediate product (15-HPETE) to modulate the actions of leukotrienes and cytokines (A). Fish oil and dietary essential fats are metabolised to EPA and DHA which are the source of the lipid mediator families of resolvins, protectins and maresins (B). In particular, EPA is converted to 18R-HEPE via either COX-2/aspirin or cytochrome P450 pathways and 5-LOX synthesises the E series resolvins. DHA is metabolised to 17S-H(p)DHA and 5-LOX synthesises the resolvins of the D series, while epoxidation hydrolysis leads to the biosynthesis of Protectin D1. The synergistic actions of 12-LOX and 15-LOX finally synthesise the maresins. These lipids are also termed specialised pro-resolution mediators for their ability to promote the termination of inflammation.

macrophages by 5-LOX and 5-LOX-activating protein (Kanaoka and Boyce 2004;Mandal *et al.* 2008).

Finally, lipoxins are synthesised by 5-LOX and 15-LOX and modulate the actions of leukotrienes and cytokines (Maderna and Godson 2009;Serhan *et al.* 1984). There are two main lipoxins, LXA_4 and LXB_4, secreted by eosinophils, monocytes/macrophages, DCs and the epithelium and they play a crucial role in the resolution of the inflammatory responses; they are synthesised following prostaglandin secretion to inhibit neutrophil recruitment and promote macrophage efferocytosis (Serhan 2007).

1.3.4.2 PAF

PAF is derived from lysophosphatidic acid by Phospholipase A_2. It is synthesised by macrophages, mast cells, platelets and non-hematopoietic cells, such as endothelial and epithelial cells (Venable *et al.* 1993) and it is responsible for platelet activation (hence its name), neutrophil migration and leukocyte adhesion onto the endothelial wall (Chignard *et al.* 1979;Prescott *et al.* 2000). Moreover, it has been shown that PAF regulates vasocontraction and vasopermeability (Ramirez *et al.* 1995).

1.3.4.3 Specialised Pro-resolution Mediators: Resolvins, Protectins and Maresins

These mediators were first identified in animal models of inflammation and derive from omega-3 polyunsaturated fatty acids (PUFAs) (Dennis and Norris 2015;Headland and Norling 2015). There are two resolvins series; E-series which derive from eicosapentanoic acid (EPA) and D-series which derive from docosahexaenoic acid (DHA). EPA is converted to 18R-HEPE and this is transformed to RvE1 and RvE2 by 5-LOX to reduce neutrophil migration (Serhan 2007).

On the other hand, DHA conversion by LOX enzymes generates RvD1-4, compounds that also suppress neutrophil infiltration of tissues (Serhan 2007). DHA can be also catabolised to 10, 17S-docosatriene, also called Protectin D1, and Maresins. These mediators negatively affect neutrophil and T cell migration, TNF and IFN-γ production and promote phagocytosis of apoptotic cells (Serhan 2007;Serhan *et al.* 2015;Serhan *et al.* 2009).

1.3.5 ROS

ROS are generated by leukocytes in response to inflammatory stimuli. They are important for cell migration, phagocytosis, inflammasome activation, control of microbiota and enteric pathogens, lymphocyte functions and tumour killing (Nathan and Cunningham-Bussel 2013). ROS can be generated by many sources; the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the respiratory chain, flavoenzyme in the endoplasmic reticulum, xanthine oxidase, cycloxygenases and lipoxygenases, cytochrome P450 and nitric oxide synthases. The generation of O_2^-, H_2O_2 and HO^- creates an arsenal of unstable and toxic compounds which form carbonyl groups in proteins, lipids and nucleic acids, and can act directly or engage in chemical reactions to convert to more potent compounds. However, if their levels are kept in control, ROS contribute to transcription factor induction, signalling and protein activity regulation (Nathan and Cunningham-Bussel 2013). Therefore ROS are emerging as useful mediators for both health and disease.

1.4. Cannabinoids; a distinct family of lipophilic mediators

1.4.1 Cannabis lipophilic components in inflammation: a historical perspective

The medical use of cannabis has a long history. The oldest reference we have is 5,000 years old and described its use as a remedy for malaria, constipation, rheumatic pains, child birth and as an analgesic (Robson 2005). However, it was not until the 19th century that cannabis was introduced to Europe as a therapeutic. The Irish scientist and doctor W.B. O'Shaughnessy tested it on humans and saw remarkable muscle-relaxant, anti-convulsant and analgesic effects (Robson 2001).

Soon after this, cannabis appeared in pharmacies across Britain and the US market as a home remedy. The notes of J.R. Reynolds, Queen Victoria's personal physician, reflect the revolutionary breakthrough cannabis-based prescriptions brought about. He described it as "incomparable" for senile insomnia, temper disease, neuralgia and migraine among others, but "not helpful" for melancholia and alcoholic delirium (Robson 2001).

Despite its original success, inconsistencies in cannabis potency from several herbal preparations, concerns about its recreational use, the lack of alternative synthetic agonists and difficulties with oral administration resulted in a considerable decline in its use in the early 20th century (Robson 2001). Eventually it was forbidden by law in several countries in Europe and around the world (Robson 2005).

However, anecdotal reports of beneficial effects of cannabis use in inflammatory and neurological diseases led to a number of studies which determined that cannabis use has the potential to be an anti-inflammatory remedy (Ware *et al.* 2005). The findings showed that many recreational smokers were relieved from chemotherapy-induced nausea, multiple sclerosis-associated spasticity and muscle pain (Consroe *et al.* 1997),

acquired immunodeficiency syndrome (AIDS)-related nausea and opportunistic infections, glaucoma symptoms, epilepsy and asthma (Grinspoon and Bakalar 1993;Robson 2001).

During this period, purification of cannabis components, such as Cannabinol, Cannabidiol (CBD) and the main psychoactive component Δ^9-tetrahydrocannabinol (Δ^9-THC) were successful (Robson 2001) and the quest for understanding the mechanisms of cannabis-related healing effects and drug development began again.

Thus, in the last 30 years the cannabinoid field has gained attention and the endocannabinoid system, as defined by its receptors, the endogenous ligands and the enzymes responsible for their biosynthesis, transport and degradation, has attracted a lot of interest as an endogenous pathway with anti-inflammatory properties.

1.4.2 Discovery, tissue distribution and functions of cannabinoid receptors

Until the late 20th century, it was believed that cannabinoids fused with target cell membranes to mediate their effects and the existence of a specific receptor was dismissed. However, in 1990, an endogenous cannabinoid receptor was discovered in a rat cerebral cortex complementary DNA (cDNA) library using oligonucleotide probes. This receptor was expressed, apart from the cerebral cortex, in the dentate gyrus and hippocampus of the rat brain (Matsuda *et al.* 1990), and cannabinoid agonists, such as CP55,940 inhibited adenylate cyclase activity in a dose-dependent and stereoselective manner (Matsuda *et al.* 1990).

Three years later, more excitement was brought to the field by the successful cloning of a second cannabinoid receptor (Munro *et al.* 1993). Using the previously described cannabinoid receptor as the probe and the human promyelocytic leukaemic line HL-60,

the new cannabinoid receptor was shown to engage the synthetic agonists WIN55,212-2 and CP55,940 and the natural agonist Δ^9-THC. Moreover, unlike the first cannabinoid receptor, the second one was expressed in the spleen, and more specifically by macrophages (Munro *et al.* 1993).

Eventually, the brain-expressed cannabinoid receptor was termed CB1R and the spleen-identified cannabinoid receptor was termed CB2R. Both receptors are expressed early in embryogenesis in tissues of the central nervous system and the periphery (Buckley *et al.* 1998). Interestingly, *Cnr1* expression changes during development; although the expression patterns are similar, fetal and neonatal human brain has markedly higher receptor density than the adult brain, especially in the basal ganglia, substantia nigra and the cerebellar cortex (Glass *et al.* 1997).

Cnr1 inter-species tissue distribution was extensively studied by autoradiography and *Cnr1* was found to be expressed in the globus pallidus, substantia nigra pars reticulata, cerebellum and dentate gyrus (Herkenham *et al.* 1991;Herkenham *et al.* 1990;Jansen *et al.* 1992;Mailleux *et al.* 1992;Rinaldi-Carmona *et al.* 1996). Dense autoradiography staining was also observed in the cerebral cortex and other formations of the hippocampus and striatum. Finally, the lowest *Cnr1* levels were measured in the remaining forebrain, brainstem and the spinal cord (Herkenham *et al.* 1991;Herkenham *et al.* 1990;Jansen *et al.* 1992). Hence, *Cnr1* localisation in the central nervous system suggests that this receptor might be involved in physiological and behavioural outcomes, such as cognition and movement.

Immunohistochemistry confirmed the autoradiography experiments; CB1R forebrain distribution was found to be wider, while its hindbrain and spinal cord was more restricted. In addition, CB1R-expressing cells were identified to be mainly neurons

(Tsou *et al.* 1998); microglia also express the receptor (Sinha *et al.* 1998), while expression in astroglia was not excluded (Rodriguez *et al.* 2001). Based on these findings, an early model of CB1R function in the brain proposed that signalling at pre-synaptic neurons, including gamma-aminobutyric acid (GABA)ergic interneurons is inhibited by endogenous ligands available at post-synaptic neurons (Hajos *et al.* 2001;Katona *et al.* 2000;Katona *et al.* 1999). This regulatory network also includes the expression of fatty acid amide hydrolase (FAAH) at post-synaptic areas to control the biosynthesis of the endogenous ligands (Egertova *et al.* 1998;Tsou *et al.* 1998).

However, not all neuron subpopulations express the same levels of *Cnr1*. For example, cholecystokinin-containing GABAergic (Tsou *et al.* 1999) and calbindin D28k-positive mid-proximal dendritic inhibitory interneurons express the highest levels, in contrast to projecting principal neurons in the amygdala, hippocampus and entorhinal cortex area, which express low *Cnr1* levels (Marsicano and Lutz 1999). This implies that a complex regulatory CB1R-dependent network exists in the brain and regulates physiological processes, such as motor behaviour, learning and memory, cognition and pain perception.

Finally, CB1R induces bradycardia and hypotension (Lake *et al.* 1997) and is also expressed in testis where it regulates luteinising hormone and testosterone levels (Gerard *et al.* 1991;Wenger *et al.* 2001). Similarly, a cannabinoid receptor with high presence in the cerebellum was also reported in the murine spleen (Kaminski *et al.* 1992), tonsils, heart, reproductive organs, lungs (Galiegue *et al.* 1995), thymus and peripheral blood mononuclear cells (Bouaboula *et al.* 1993). Among leukocyte populations, B cells express the highest degree CB1R levels (Bouaboula *et al.* 1993).

The second cannabinoid receptor, CB2R, has been reported to have a more restricted tissue distribution and was found in peripheral immune organs, such as the spleen, tonsils, thymus (Schatz *et al.* 1997), bone marrow, the pancreas and the heart (Galiegue *et al.* 1995;Valk *et al.* 1997). Autoradiography experiments showed that in the rat spleen, the marginal zone exhibits the highest binding levels. Similar results in germinal centers and Peyer's patches in the intestine indicated that B cells express the highest levels of peripheral cannabinoid receptors (Lynn and Herkenham 1994).

In addition, lymphocytes, monocytes and macrophages, polymorphonuclear neutrophils, mast cell lines, and NK cells express *Cnr2* messenger ribonucleic acid (mRNA), with B cells and NK cells exhibiting the highest levels and neutrophils, monocytes and T cells following (Carlisle *et al.* 2002;Condie *et al.* 1996;Facci *et al.* 1995;Galiegue *et al.* 1995;Schatz *et al.* 1997). Finally, a number of murine myeloid, T and B cell, mast cell lines also express the receptor (Valk *et al.* 1997).

1.4.3 CB1R and CB2R molecular biology

CB1R amino acid sequence is 98% identical between rat and human and 96% between mouse and rat or mouse and human. It is a 473 amino acid long GPCR (Chakrabarti *et al.* 1995;Gerard *et al.* 1990;Gerard *et al.* 1991) and the gene consists of two exons which code for two alternative splicing variants. These differ in 167 bp at the 5' end of the coding region which are spliced out creating a shorter protein isoform, present at low levels in the human brain (Shire *et al.* 1995). This variant, mentioned in the literature as $CB1_{(a)}$, presents with all CB1R properties, but to a smaller extent than the long variant of the receptor (Rinaldi-Carmona *et al.* 1996).

Murine CB2R shares 44% homology with CB1R (Munro *et al.* 1993) and is 437 amino acids long. Its transcript consists of two exons as well, with a short 5' untranslated region, a 1,041 bp open reading frame and 2.5 kb 3' untranslated region (Shire *et al.* 1996). It is 82% identical to the human protein, but has a shorter carboxyl terminus and a 1.7 kb longer 3' untranslated region (Shire *et al.* 1996). Rat CB2R is 93% identical and human is 81% identical to the murine homologue. The three homologues exhibit the highest amino acid sequence conservation in the transmembrane domains and greatest divergence in the carboxyl terminus (Griffin *et al.* 2000).

Like CB1R, human and murine CB2R have two and rat CB2R has four splice variants (Liu *et al.* 2009). Interestingly, the transcript variants in humans display differential expression with hCB2RA being expressed in the testis and hCB2RB being expressed in the spleen (Liu *et al.* 2009).

As GPCRs, both cannabinoid receptors consist of seven transmembrane domains. Mapping of the agonist binding sites at the receptor domains has been carried out with site-directed mutagenesis and shows that different classes of ligands engage the receptors at different transmembrane domains.

For example, substitution of a conserved lysine residue in the extracellular region of the third helix of CB1R showed that HU-210, CP55,940 and N-arachidonoylethanolamine (AEA), but not WIN55,212-2 use this site to engage with the receptor (Chin *et al.* 1998;Song and Bonner 1996). Using the same approach, Tao *et al*. demonstrated that mutation of the same lysine residue in the third transmembrane region of CB2R did not affect CP55,940 ligation (Tao *et al.* 1999). Furthermore, molecular modelling revealed that CP55,940 is retained in its binding pocket via interactions it makes with a unique to CB2R serine residue. Similarly, certain residues in the third transmembrane domain of

CB2R were responsible for the higher affinity of aminoalkylindoles to that receptor as opposed to CB1R, suggesting that CB1R/CB2R agonists exhibit selectivity to one over the other receptor based on the presence of specific anchor residues in their backbone (Chin *et al.* 1999).

The binding of antagonists to cannabinoid receptors is dependent on multiple transmembrane domains. For instance, SR141716A inhibits CB1R agonism via binding to the fourth and fifth transmembrane helices, whereas SR144528 uses two serine residues in transmembrane region 3 and made a number of interactions with aromatic residues in transmembrane regions 4 and 5 (Gouldson *et al.* 2000).

1.4.4 Cell signalling by cannabinoid receptors

Both CB1R and CB2R are $G_{i/o}$-coupled and as such their functions are the outcome of the pathways the G proteins are coupled with (Figure 1.5). Free α proteins can inhibit adenylate cyclase, whereas free βγ proteins are responsible for ion channel regulation and mitogen-activated protein (MAP) kinase and phosphoinositide 3-kinase (PI3K) activation (Howlett *et al.* 2002).

Work with CB1R-transfected and CB1R-expressing tumour cell lines has shown that one of the main downstream activated signalling pathways is the regulation of ion channel currents (Howlett *et al.* 2002;Pertwee 1997;Pertwee 2006). CB1R agonism modulates neurotransmitter release and signal transduction across neurons by reducing calcium and inward sodium channels and currents induced by 5-hydroxytryptamine receptor activation, while it augments inward and outward potassium channels (Pertwee 1997). There is also evidence that CB1R can mobilise intracellular calcium stores in

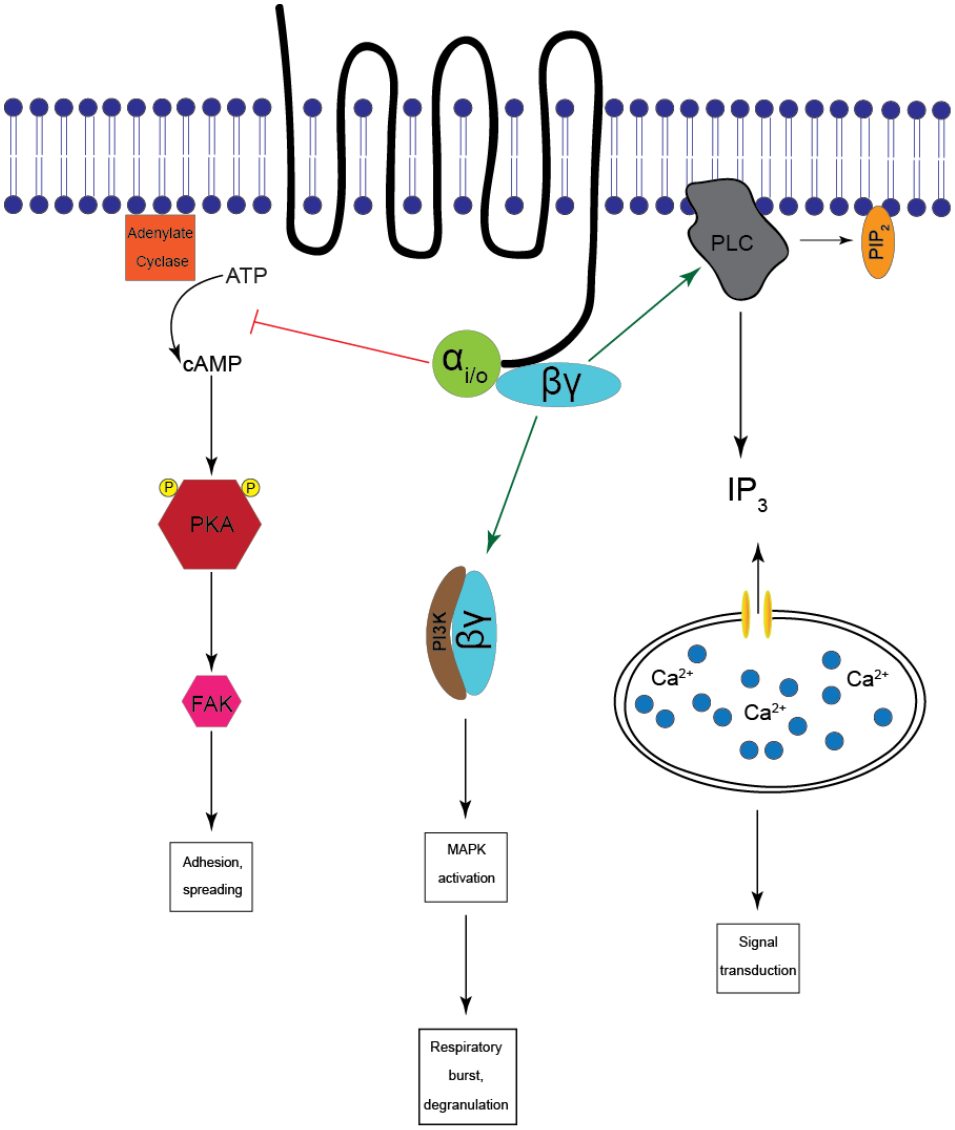


Figure 1.5. Cannabinoid receptor signalling. Cannabinoid receptors are GPCRs and are associated with $\alpha_{i/o}$ and βγ proteins. When a ligand binds to the receptor, it induces a change in the receptor's conformation and the βγ proteins dissociate from the receptor tail. The $\alpha_{i/o}$ protein inhibits the conversion of ATP to cAMP by the enzyme adenyl cyclase and subsequent phosphorylation of PKA and FAK. This outcome affects physiological processes, such as adhesion and spreading. In parallel, the βγ proteins activate phospholipase C (PLC) to cleave PIP_2 into IP_3 and diacylglycerol. IP_3 in turn induces the mobilisation of intracellular calcium stores and downstream signal transduction. Alternatively, βγ proteins can associate with PI3K and modulate MAPK activation and downstream oxidative burst and degranulation.

neurons and smooth muscle cells and hydrolyse sphingomyelin into ceramide via a $G_{i/o}$-independent mechanism (Howlett *et al.* 2002;Pertwee and Ross 2002).

Both CB1R and CB2R are negatively coupled with adenylate cyclase and their activation attenuates cyclic adenosine monophosphate (cAMP) intracellular levels (Howlett *et al.* 2002;Pertwee 1997;Pertwee 2006). This effect is pertussin toxin-sensitive which indicates the dependence on $G_{i/o}$ proteins. The deregulation of cAMP levels suppresses phosphorylation of protein kinase A (PKA), an important molecule for ion channel and focal adhesion kinase (FAK) function (Howlett *et al.* 2002). Another signalling pathway targeted by both receptors is that of MAP kinases (Howlett *et al.* 2002;Pertwee 1997;Pertwee 2006). Extracellular-signal regulated (ERK1/2), p38 and JNK phosphorylation are induced by the cannabinoid receptors, occasionally through the activation of PI3K (Howlett *et al.* 2002).

1.4.5 Gene polymorphisms of the human endocannabinoid system

A number of recently discovered polymorphisms in the two cannabinoid receptor genes are associated with human disease and imply that the endocannabinoid system has an active role in health and disease. Neuropsychiatric disorders (Gadzicki *et al.* 1999;Hoehe *et al.* 2000), schizophrenia subtypes (Leroy *et al.* 2001;Ujike *et al.* 2002), responses to social reward (Chakrabarti *et al.* 2006), alcoholism (Schmidt *et al.* 2002), cocaine addiction (Ballon *et al.* 2006), polysubstance abuse vulnerability (Comings *et al.* 1997;Zhang *et al.* 2004), depression (Barrero *et al.* 2005) and anorexia nervosa (Siegfried *et al.* 2004) have been shown to correlate with *Cnr1* polymorphisms which include single nucleotide polymorphisms and the copy number of a frequent AAT triple repeat.

In contrast to CB1R, CB2R polymorphisms are not linked to psychiatric predisposition. Instead, single polymorphisms have been identified as susceptibility factors for osteoporosis in women (Karsak *et al.* 2005), bone mineral density (Yamada *et al.* 2007), multiple sclerosis, rheumatoid arthritis, lupus erythematosus and myasthenia gravis (Sipe *et al.* 2005).

Finally, other components of the endocannabinoid system present with disease-associated polymorphisms. For example, a P129T substitution in FAAH in human peripheral blood T lymphocytes results in reduced activity of this enzyme due to posttranslational alterations in its stability (Chiang *et al.* 2004). This mutation is also associated with alcohol and recreational drug use (Sipe *et al.* 2002) and its expression significantly correlates with overweight and obese individuals (Sipe *et al.* 2005). Collectively, these data show that dysregulation of endocannabinoid levels due to mutations in their biosynthetic enzymes can have a serious impact on an individual's health.

1.4.6 Natural and synthetic cannabinoid agonists and antagonists

1.4.6.1 Endogenous cannabinoid agonists and the endocannabinoid system

Very soon after the cannabinoid receptors were cloned and their expression was demonstrated in various human and animal tissues, the hunt for endogenous cannabinoid agonists began. Two compounds were identified as the major endogenous ligands (endocannabinoids); AEA and 2-arachidonoyl glycerol (2-AG). Others have also been proposed and include 2-arachidonoyl-glycerol ether, O-arachidonoyl-ethanolamine (virodhamine) and N-arachidonoyl-dopamine (NADA) (De Petrocellis *et al.* 2004).

Both AEA and 2-AG are found in the brain at picomolar concentrations, but other tissues, such as the heart, spleen, kidney and liver also express these lipid compounds (Sugiura *et al.* 2002). As described earlier, endocannabinoids are believed to localise post-synaptically in the brain and their levels are elevated upon Ca^{2+} increases caused by neuron depolarisation. Consequently, pre-synaptic CB1R is triggered by AEA or 2-AG to suppress GABA release by hippocampal neurons (Pertwee and Ross 2002). Alterations in the endocannabinoid levels have been shown to be involved in many inflammation models, such as chronic relapsing experimental autoimmune encephalomyelitis, obesity and a rat model of Huntington's disease (Pertwee and Ross 2002).

AEA was discovered as an ethanolamide of a tetraenic C_{20} fatty acid able to inhibit murine vas deferens twitch response (Devane *et al.* 1992), whereas 2-AG was originally derived from canine gut (Mechoulam *et al.* 1995). Both agonists bind to CB1R and CB2R with low affinity. However, AEA has also been shown to exhibit off-target effects at other GPCRs (De Petrocellis *et al.* 2004). Some of these receptors still remain unidentified, however transient receptor potential cation channel subfamily V member 1 (TRPV1) and other non-CB1R, non-CB2R, non-TRPV1 receptors of the central nervous system, small intestine, mesenteric and coronary arteries have been shown to become activated by AEA under pathological conditions or when CB1R is blocked (De Petrocellis *et al.* 2004;Pertwee 2005;Pertwee and Ross 2002).

Endocannabinoids are not stored, but hey are synthesised on demand following cell stimulation and Ca^{2+}-dependent phospholipid precursor remodelling at the plasma membrane (Dimarzo *et al.* 1994). They are then quickly released into the micro-environment and mediate their signals through their re-uptake by target cells.

The biosynthetic pathways of AEA and 2-AG have gained a lot of attention in the last 30 years. AEA is synthesised as the product of N-arachidonoyl-phosphatidylethanolamine (NArPE) hydrolysis by phospholipase D (PLD) which is highly selective for these phospholipids (Di Marzo *et al.* 1996;Di Marzo *et al.* 1998;Sugiura *et al.* 2002). 2-AG can be synthesised from various sources. However, in most cases, 2-arachidonate-containing diacylglycerols (DAGs) are converted into 2-AG by an sn-1-selective DAG lipase (DAGL) (Di Marzo *et al.* 1996;Di Marzo *et al.* 1998;Sugiura *et al.* 2002). There are two described sn-1-specific DAGL enzymes at plasma membranes that regulate 2-AG biosynthesis, DAGLα and DAGLβ, and they are highly expressed in the brain (Bisogno *et al.* 2003).

Degradation of both AEA and 2-AG is catalysed by FAAH. This enzyme hydrolyses these endocannabinoids to arachidonic acid and ethanolamine or glycerol, respectively (Cravatt *et al.* 1996;Deutsch *et al.* 2002). FAAH inhibitors (Deutsch *et al.* 2002) and FAAH KO animals (Cravatt *et al.* 2001) have been generated to study the potential of FAAH inhibition in analgesic drug development. However, FAAH deficiency did not enhance 2-AG binding to CB1R and did not inhibit 2-AG-mediated hypothermia and locomotor activity loss (Lichtman *et al.* 2002). This finding suggests the presence of other hydrolases that degrade endocannabinoids.

Indeed, other intracellular enzymes were found to degrade endocannabinoids; N-palmitoyl-ethanolamine-preferring acid amidase (PAA), cyclooxygenase-2, lipoxygenases and cytochrome P450 also metabolise AEA (Pertwee 2008;Pertwee and Ross 2002), while monoacylglycerol lipases (MAGLs) are localised at the plasma membrane and cytosol of cells and degrade 2-AG and other monoacylglycerols (De Petrocellis *et al.* 2004;Dinh *et al.* 2002).

1.4.6.2 Classification of CB1R and CB2R agonists

CB1R and CB2R agonists have traditionally been divided into four groups; classical, non-classical agonists, aminoalkylindoles and eicosanoids. Each group of agonists has a distinct chemical structure and leading compounds that demonstrate the principal pharmacological characteristics of the group (Figure 1.6).

1.4.6.2.1 Classical agonists

This group consists of ABC-tricyclic dibenzopyran derivatives that are either found in *C.sativa* (phytocannabinoids) or are analogues of those (Howlett *et al.* 2002). Its best known members are Δ^9-THC, Δ^8-THC and HU-210, a more active synthetic analogue of Δ^8-THC (Howlett *et al.* 2002). Although Δ^9-THC partially agonises and possesses high CB1R and CB2R affinity at submicromolar concentrations, HU-210 is a more potent agonist of both cannabinoid receptors, presumably due to the presence of a dimethylheptyl side chain in its molecule (Howlett *et al.* 2002;Pertwee *et al.* 2010). Δ^9-THC agonises the cannabinoid receptors more potently than Δ^8-THC, CBD, Cannabigerol and Cannabinol, and can also act as an antagonist when receptor levels are very low (Pertwee 2008).

CBD, Cannabigerol and Cannabinol are phytocannabinoids with moderate affinity for CB1R. CBD lacks any psychoactive CB1R-mediated effects and also possesses CB2R inverse agonist/antagonist properties (Pertwee 2008;Pertwee *et al.* 2010). Cannabinol acts as a partial CB1R agonist, while Cannabigerol is a CB1R antagonist/inverse agonist (Pertwee *et al.* 2010). Finally, the n-propyl analogue of Δ^9-THC, Δ^9-THCV, is 4 to 5 times less potent than Δ^9-THC, but exhibits similar catalepsy effects and has both agonist and antagonist properties at CB1R (Pertwee 2008).

Classical cannabinoids are not selective to one or the other cannabinoid receptor. For this reason, small modifications to the THC molecule were made by JW Huffman and colleagues to synthesise the CB2R selective agonist JWH-133, and by Merck Frosst to synthesise L-759633 and L-759656 (Howlett *et al.* 2002). These agonists do not bind to CB1R or do so weakly (Table 1.1), and their synthesis has facilitated research and our understanding of CB2R pharmacology.

1.4.6.2.2 Non-classical agonists

The agonists of this group were developed by Pfizer and include AC-bicyclic and ACD-tricyclic compounds that lack the dihydropyran ring of Δ^9-THC (Howlett *et al.* 2002). The prototypical compound of this family is CP47,497, however it is the bicyclic derivative CP55,940 that is the best known member of this group. Less lipophilic than Δ^9-THC, it binds to both CB1R and CB2R and its name carries a long history as it was [^{3}H]CP55,940 studies that discovered CB1R in the rat brain (Pertwee *et al.* 2010). HU-308 is another non-classical CB2R-selective agonist which was synthesised by Huffman and colleagues and has been extensively used in studies to determine CB2R involvement in biological processes.

1.4.6.2.3 Aminoalkylindoles

Aminoalkylindoles comprise a group of cannabinoid agonists which differ substantially from classical and non-classical agonists. They were developed by Sterling Winthrop researchers as cannabimimetic compounds and have an increased ability to bind to CB1R (Howlett *et al.* 2002). The best known member, WIN55,212, exhibits agonism at both CB1R and CB2R, but has slightly higher affinity for CB2R (Howlett *et al.*

2002;Pertwee *et al.* 2010). Moreover, an S-(-)-enantiomer, WIN55,212-3, was synthesised and has been widely used in studies to determine stereospecificity, as it lacks *in vitro* and *in vivo* activity at cannabinoid receptors. Similar to the classical cannabinoid family, the substitution of the aminoalkyl substituent with n alkyl chains or the replacement of the indole by a pyrrole or an indene ring led to the synthesis of a few novel CB2R-selective agonists, such as JWH-015, AM1241 and L-768242 (Howlett *et al.* 2002).

1.4.6.2.4 Eicosanoids

The eicosanoid group of cannabinoid agonists is well represented by the endogenous cannabinoids AEA and 2-AG. They differ structurally from the classical, non-classical cannabinoids and aminoalkylindoles, and behave as CB1R and CB2R partial agonists (Table 1.1). AEA has slightly higher affinity for CB1R than CB2R (Howlett *et al.* 2002). In addition, structural modification of AEA led to the generation of CB1R-selective agonists, such as arachidonyl-2'-chloroethylamide (ACEA), arachidonylcyclopropylamide (ACPA) and the methylated derivative methylanandamide which is less degradable by FAAH.

2-AG is a more potent agonist of both receptors than AEA and has higher affinity for CB1R (Howlett *et al.* 2002;Pertwee *et al.* 2010). Derivatives of 2-AG may present similar (e.g. 1(3)-arachidonoylglycerol) or lack (e.g. 2-palmitoylglycerol, 2-linoleoylglycerol, 1(3)-palmitoylglycerol, 1(3)-stearoylglycerol) affinity for cannabinoid receptors (Howlett *et al.* 2002).

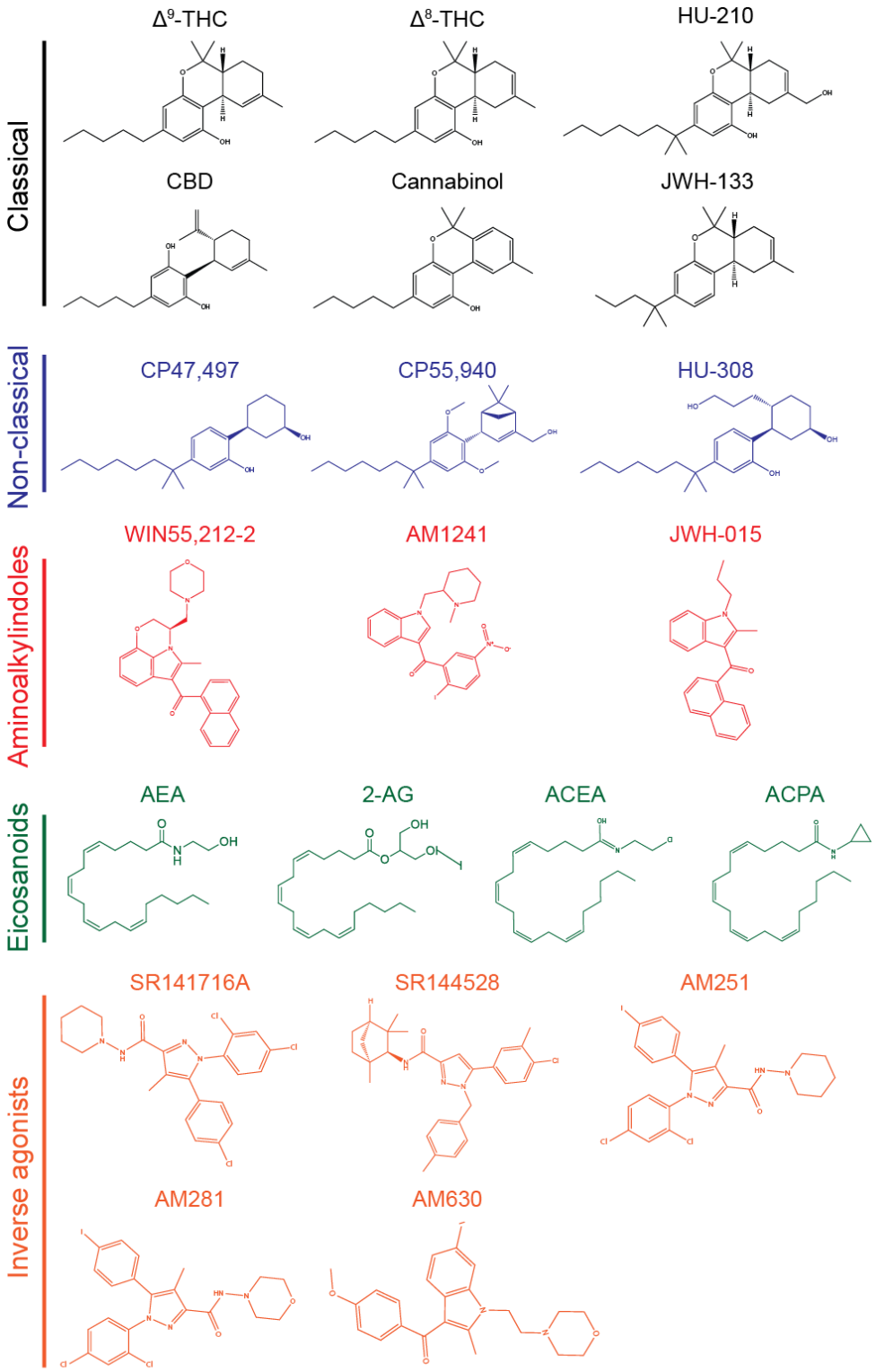


Figure 1.6. Molecular structure of cannabinoid agonists. The molecular structures of representative cannabinoid agonists from the four classification groups and the family of inverse agonists are shown; classical agonists (black), non-classical agonists (blue), aminoalkylindoles (red), eicosanoids (green) and inverse agonists (orange).

1.4.6.3 CB1R and CB2R-selective antagonists

There are several available CB1R and CB2R-selective antagonists, but the leading compounds are the Sanofi diarylpyrazoles SR141716A (Rimonabant) and SR144528, respectively (Howlett *et al.* 2002). Antagonists inhibit cannabinoid-mediated effects and are selective to one or the other cannabinoid receptor (Table 1.1). However, in some tissues CB1R-selective antagonists have been shown to carry out inverse agonist responses when they are administered alone and suppress the constitutive actions of cannabinoid receptors (Howlett *et al.* 2002;Pertwee 2005). For example, SR141716A is believed to bind with low affinity to an allosteric site on CB1R, which is responsible for its inverse agonist effects (Pertwee 2005). Conversely, CB2R antagonists are thought to act more as inverse agonists rather than neutral antagonists (Pertwee *et al.* 2010).

Structural analogues of SR141716A have been developed, such as AM251 and AM281 and have been used in studies to block CB1R agonist responses (Howlett *et al.* 2002). Similar to SR141716A, AM281 was also shown to possess inverse agonist potential when administered alone (Pertwee 2005). In contrast, CB2R-selective antagonists other than SR144528 have been synthesised and possess lower inverse agonist properties. One such compound is AM630 which antagonises CB2R, but also has low affinity for CB1R, making it an agonist with mixed pharmacological properties (Howlett *et al.* 2002).

1.4.7 Therapeutic potential of cannabinoid agonists

The synthesis of cannabinoid receptor-specific agonists tested in *in vitro* studies and experimental animal models prompted the development of drugs that target the endocannabinoid system to ameliorate inflammatory diseases. Due to its high

Table 1.1. Cannabinoid agonist affinities at CB1R and CB2R. Values were determined in dissociation studies with radiolabelled ligands (Howlett *et al.* 2002;Huffman *et al.* 2008;Pertwee 2005;Pertwee 2008)

Cannabinoid agonist	**CB1R K_i value (nM)**	**CB2R K_i value (nM)**
Δ^9-THC	53.3	75.3
Δ^8-THC	47.6	39.3
Δ^9-THCV	75.4	62.8
HU-210	0.0608	0.524
CBD	4,350	2,860
Cannabigerol	440	337
Cannabinol	211.2	126.4
JWH-133	677	3.4
L-759633	1,043	6.4
L-759656	4,888	11.8
CP47,497	2.2	ND
CP55,940	5	1.8
HU-308	>10,000	22.7
WIN55,212	9.94	16.2
JWH-015	383	13,8
AM1241	280	3.4
L-768242	1,917	12
AEA	472	1,400
2-AG	21.2	>3,000
ACEA	1.4	>2,000
ACPA	2.2	715
SR141716A	11.8	13,200
AM251	7.49	2,290
AM281	12	4,200
SR144528	437	0.6
AM630	52	31.2

prevalence in the brain and its involvement in the regulation of signal transmission (Pertwee and Ross 2002), CB1R was considered as a potential target for neurodegenerative diseases, such as Parkinson's (Brotchie 2003), Alzheimer's (Pazos *et al.* 2004) and schizophrenia (Ujike and Morita 2004).

Furthermore, CB1R agonists were attractive as neuroprotective and anti-cancer agents (Guzman 2003), modulators of glaucoma (Tomida *et al.* 2004), pain (Pertwee 2001) and as potential treatments for motor dysfunctions associated with multiple sclerosis (Pertwee 2002), Tourettes's syndrome and spinal cord injuries (Pertwee 2006;Pertwee and Ross 2002).

The first cannabinoid-related therapeutics licensed for clinical use as appetite stimulants and anti-emetics were Nabilone and Dronabinol (Pertwee 2006;Robson 2001). Nabilone is a synthetic Δ^9-THC analogue initially licensed for chemotherapy-induced nausea and vomiting treatment in 1981. In 1985 it became a medicine under the name Dronabinol and in 2005 became known as Sativex (Robson 2001). Dronabinol was prescribed as an anti-emetic and an apettite-stimulant for patients with AIDS. Sativex consists of equal amounts of Δ^9-THC and CBD, and since 2007 it has been prescribed for the symptomatic relief of neuropathic pain in patients with multiple sclerosis and advanced cancer. Other classical agonists, such as Δ^8-THC, Cannabinol and CBD have been used as treatments with mixed results so far (Abrahamov *et al.* 1995;Consroe *et al.* 1991;Grinspoon and Bakalar 1993).

A challenge of drug development to target the endocannabinoid system is that administration of high doses of cannabinoids can lead to off-target effects at irrelevant receptors or unwanted side effects, such as psychotropism. In many of the clinical trials

Table 1.2. Pro- and anti-inflammatory effects of CB2R in disease models (adapted from (Turcotte *et al.* 2016))

Disease	Effect	Reference
Ischemia-reperfusion	Suppresses leukocyte recruitment, inflammatory mediators, myeloperoxidase activity, tissue damage	(Batkai *et al.* 2012;Di Filippo *et al.* 2004;Duerr *et al.* 2015;Hegde *et al.* 2008;Zarruk *et al.* 2012)
Colitis	Suppresses inflammatory cytokines, oedema, leukocyte infiltration, disease score	(Alhouayek *et al.* 2011;Engel *et al.* 2010)
Sepsis	Promotes bacterial invasion and mortality, reduces leukocyte adhesion	(Csoka *et al.* 2009;Sardinha *et al.* 2014)
Brain and spinal cord injury	Reduces blood-brain permeability, inflammatory mediators, leukocyte infiltration	(Adhikary *et al.* 2011;Amenta *et al.* 2012;Amenta *et al.* 2014)
Ear inflammation	Promotes neutrophil recruitment and ear swelling	(Oka *et al.* 2005)
Dermatitis	Induces eosinophil recruitment, swelling and inflammatory mediators	(Oka *et al.* 2006)
Primary immunisation	Promotes delayed-type hypersensitivity, DC migration to lymph nodes	(Maestroni 2004)
Hypersensitivity	Suppresses neutrophil recruitment and ear swelling	(Karsak *et al.* 2007)
Atherosclerosis	Reduces disease score, macrophage infiltration and leukocyte adhesion, inflammatory mediators	(Steffens *et al.* 2005;Zhao *et al.* 2010)
Multiple sclerosis	Suppresses monocyte recruitment, T cell proliferation and inflammatory mediators	(Maresz *et al.* 2007;Wen *et al.* 2015)
Asthma	Suppresses mast cell degranulation, inflammatory mediators and myeloperoxidase	(Giannini *et al.* 2008)
Neuroinflammation	Reduces microglia accumulation, inflammatory mediators	(Tang *et al.* 2016;Zoppi *et al.* 2014)
Osteoarthritis	Suppresses leukocyte rolling, microvascular perfusion	(Krustev *et al.* 2014)
Inflammatory pain	Suppresses oedema, allodynia, inflammatory cytokines	(Booker *et al.* 2012)
Hepatitis	Reduces inflammatory cytokines	(Hegde *et al.* 2008)
Bacterial and viral nfection	Reduces inflammatory cytokines, leukocyte recruitment	(Buchweitz *et al.* 2008;Newton *et al.* 2009)
Acute inflammation	Suppresses leukocyte infiltration, inflammatory mediators, oedema, nociception	(Comelli *et al.* 2007;Costola-de-Souza *et al.* 2013)
Interstitial cystitis	Suppresses leukocyte recruitment, inflammatory cytokines and myeloperoxidase	(Tambaro *et al.* 2014)

to date, patients had to be excluded from the studies due to observed sedation effects, dizziness, panic, confused thinking, feeling of disturbance, disassociation, slurred speech, ataxia and blurred vision (Pertwee 2005). It is therefore desirable to use side effect-free agonists which bear similar pharmacological properties to the licensed phytocannabinoids. In that respect, Bayer developed a partial CB1R/CB2R agonist called BAY59-3074, which has been used in research for its antihyperalgesic and antiallodynic properties and the lack of typical cannabinoid-induced behavioural side effects (De Vry *et al.* 2004).

Alternatively, CB2R-selective agonists or cannabinoids that engage peripheral CB1Rs is another direction the field might need to take. CB2R activation has been shown pre-clinically to modulate neuropathic pain and does not exhibit the psychotropic effects of central CB1R. For instance, GlaxoSmithKlein characterised a CB2R-selective agonist called GW405833 for its ability to reverse neuropathic and chronic inflammatory pain clear of analgesic, sedative and cataleptic side effects (Clayton *et al.* 2002;Valenzano *et al.* 2005). There is also great potential for treatment of inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, atherosclerosis, sepsis, neuroinflammation, central nervous system injuries, brain ischemia and inflammatory bowel disease where CB2R agonists have been shown to attenuate the clinical outcome in animal models (Turcotte *et al.* 2016) and (Table 1.2).

Furthermore, an attractive concept is the use of CB1R and CB2R antagonists. Rimonabant passed clinical trials and was licensed as an anti-obesity drug in 2006 (Despres *et al.* 2005;Van Gaal *et al.* 2005). Similarly, CB2R antagonism is a promising idea given the ability of CB2R-selective antagonists to mitigate leukocyte trafficking *in vitro* and *in vivo* (Turcotte *et al.* 2016). Finally, a strategy which has gained more

attention is the indirect targeting of the endocannabinoid system via the biosynthetic enzymes of the endogenous ligands. For instance, FAAH and endocannabinoid uptake inhibitors with some selectivity have been developed to ameliorate pain, multiple sclerosis, Parkinson's disease and tumour growth in animal models (Pertwee 2005).

Classical cannabinoid agonists possess low affinity for the receptors. However, their binding becomes more potent under inflammatory conditions where their expression in tissues or specific cell types is upregulated. In fact, the endocannabinoid system has been reported to be dysregulated in some diseases, including multiple sclerosis, osteoporosis, obesity, endotoxemic shock, intestinal inflammation, hyperalgesia and stroke (Pertwee 2005). Collectively, this means that the endocannabinoid system is an important endogenous pathway that regulates homeostasis, and thus keeping up the battle to develop drugs that restore its balance in disease could relieve human patients.

1.5 Original hypothesis

CB2R possesses anti-inflammatory properties and plays a key role in acute inflammatory responses by modulating immune cell functions.

1.6 Aims of this thesis

- To investigate the anti-inflammatory effects of a CB2R-selective agonist in an *in vivo* animal model of leukocyte recruitment (Chapter 3)
- To study the potential anti-inflammatory effects of CB2R-selective ligands on macrophage biology in *in vitro* activation assays (Chapter 4)
- To look into the effects of CB2R depletion on immune cell trafficking at homeostasis in mice (Chapter 5)

- To assess the consequences of CB2R genetic deficiency in an animal model of acute systemic inflammation (Chapter 6)

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Media and buffers

DMEM (Dulbecco's Modified Eagle Medium) (Sigma-Aldrich, Dorset, UK)

Ear clip lysis buffer (Viagen Biotech, CA, USA)

EDTA (Ethylenediaminetetraacetic acid) (Sigma-Aldrich)

FACS block: 1:100 rat anti-mouse CD16/32 (Abd Serotec, Oxford, UK), 1:150 mouse Gamma Globulin (Jackson Immunoresearch Laboratories Inc., PA, USA) in FACS buffer

FACS buffer: 2 mM EDTA, 2% fetal bovine serum, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in PBS

HBSS (Hanks' Balanced Salt Solution) (Life technologies, MA, USA)

Live Cell Imaging Solution (Life technologies)

L929-conditioned medium: L929 fibroblasts were cultured in DMEM/10% Fetal Bovine Serum/1% Penicillin/Streptomycin for 10 days. Supernatants were then collected and filtered

L929 medium: 15% L929-conditioned medium, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin in DMEM

OptiMEM medium: 0.2% Bovine Serum Albumin (BSA), 1% Penicillin/Streptomycin in OptiMEM (Life technologies)

PBS (Phosphate Buffer Saline) (Lonza, Tewkesbury, UK)

PE buffer: 2 mM EDTA in PBS

Phosphate buffer (50 mM) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) (pH=5.4) for myeloperoxidase (MPO) assay: 0.6671% NaH_2PO_4, 0.0444% Na_2HPO_4

Phosphate buffer (80 mM) (pH=5.4) for MPO assay: 1.0674% NaH_2PO_4, 0.071% Na_2HPO_4

Red blood cell lysis buffers (BD Biosciences)

Saline 0.9% (Thermo Scientific, MA, USA)

2.1.2 Reagents

Agarose, Molecular Grade (Bioline, London, UK)

Bio-gel beads (Bio-Rad, Hertfordshire, UK)

Biomix red (2x) (Bioline)

BSA (Sigma-Aldrich)

Collagenase D (Roche, Welwyn Garden City, UK)

CountBright Absolute Counting Beads (Life Technologies)

Cremophor (Sigma-Aldrich)

Cytochalasin D (Sigma-Aldrich)

DAPI (4',6-diamidino-2-phenylindole) (Roche)

Dexamethasone (Sigma-Aldrich)

DMSO (Dimethyl Sulfoxide) (Sigma-Aldrich)

DNA molecular weight ladders (Life technologies)

Ethanol (VWR chemicals, Lutterworth, UK)

FBS (Fetal Bovine Serum) (Sigma-Aldrich)

FURA2-AM (Life technologies)

Griess reagent (Sigma-Aldrich)

H_2SO_4 (Sigma-Aldrich)

IFN-γ (Peprotech, NJ, USA)

LPS (Sigma-Aldrich)

MPO (Roche)

Penicillin/Streptomycin (P/S): 1/100 (Life Technologies)

PFA (Paraformaldehyde) solution: 1% in H_2O (Sigma-Aldrich)

pHrodo green *E.coli* BioParticles Conjugate for Phagocytosis (Life technologies)

Primers: *Tnf* and *Il6* (Qiagen, Hilden, Germany). The rest were synthesised by Eurofins Genomics (Ebersberg, Germany)

Proteinase K (Sigma-Aldrich)

Sybr Green Jumpstart Taq ReadyMix (Sigma-Aldrich)

Safeview Nucleic Acid stain (NBS Biologicals, Huntingdon, UK)

Trypan Blue 0.4% (Sigma-Aldrich)

Tween-20 (Sigma-Aldrich)

TMB (3, 3', 5, 5'- tetramethylbenzidine) (Thermo Scientific)

Zymosan (Sigma-Aldrich)

1400W (Sigma-Aldrich)

2.1.3 Commercial kits

Bicinchoninic acid (BCA) assay (Thermo Scientific)

Discoverx cAMP Hunter eXpress kit (Discoverx, Birmingham, UK)

Discoverx PathHunter eXpress β-Arrestin GPCR Assay kit (Discoverx)

DuoSet ELISA kit (R&D systems, MN, USA)

Magnetic Luminex Screening Assay kit (R&D systems)

QuantiTect Reverse (Qiagen)

RNeasy Mini kit (Qiagen)

2.1.4 Other materials

EASYstrainers 70 um^2 (Greiner Bio-one, Stonehouse, UK)

Needles (23G, 25G) (Terumo, Somerset, UK)

Nunc-Immuno MicroWell 96-well plates (Sigma-Aldrich)

100 mm petri dishes (Sterilin, Abergoed, UK)

Plastic tubes with caps (Qiagen)

Precellys 24 ceramic beads (Bertin technologies, France)

Scalpel blades size 24 (Swann-Morton, Sheffield, UK)

Syringes (1 ml, 5 ml and 10 ml) (Terumo)

Tissue culture plates (Corning, NY, USA)

Tubes (15 ml and 50 ml) (Greiner Bio-one)

Eppendorf tubes (0.5 ml and 1.5 ml) (Sigma-Aldrich)

2.1.5 Animals

Male 6-10 week old C57BL/6J mice were purchased from Harlan Laboratories (Bicester, UK). CB2R KO animals were purchased from the Jackson Laboratory (CA, USA).

2.2 Methods

2.2.1 *In vivo* work

2.2.1.1 Animals

All animal studies were conducted under PPL license 30/3053 Resolution of inflammation with ethical approval from the Dunn School of Pathology Local Ethical Review Committee and in accordance with the UK Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986). Experiments were designed with prior consideration of the 3R principles, i.e. reduction, refinement, replacement. Animals were housed in a 12 hour light/12 hour dark cycle unit with free access to food and water. CB2R KO animals were backcrossed four times to the C57BL/6J background and age- and sex-matched littermate wildtype (WT) controls were used in the studies.

2.2.1.2 Models of inflammation

In the zymosan-induced peritonitis experiments mice were injected intraperitoneally (i.p.) with 100 ug zymosan or PBS and 4 hours later, mice were divided into two groups which received vehicle or cannabinoid agonists, respectively. Animals were sacrificed via asphyxiation with a rising concentration of CO_2. The peritoneal cavities of animals were lavaged with 5 ml ice cold PE buffer and blood was retrieved from the hepatic vein.

Male C57BL/6J mice were injected i.p. with 1 ml of 2% sterile bio-gel polyacrylamide beads (P100 fine, 45-90 μM) in PBS and were sacrificed via asphyxiation with a rising

concentration of CO_2 4 days later. Elicited cells were collected in 10 ml ice cold PE buffer.

In the endotoxemia experiments mice were injected i.p. with 1 mg/kg LPS (serotype O127:B8) for a period of 2 to 8 hours. Animals were sacrificed via asphyxiation with a rising concentration of CO_2. Peritoneal cavities of animals were lavaged with 5 ml ice cold PE buffer and blood was retrieved from the hepatic vein. Serum was collected after blood was left to clot for 5 hours at 4^oC. Lungs, livers and bone marrows were harvested and inoculated in ice until further processing.

2.2.1.3 Tissue harvest and processing

Thymi, lymph nodes (mesenteric, inguinal and cervical), lungs, spleens, bone marrow and blood from male C57BL/6J mice were harvested and placed on ice. Thymi and lymph nodes were only used for *Cnr2* expression assessment. The remaining tissues were also processed for Flow Cytometry.

Lungs and spleens were cut into small pieces and incubated for 1 hour at $37^oC/5\%CO_2$ in 1 mg/ml Collagenase D diluted in HBSS medium. Lung lysates and livers were then passed through cell strainers and prepared for Flow Cytometry, whereas spleen lysates were re-suspended in 1 ml PBS, 200 ul of which were mixed with 2 ml BD Pharm Lyse (BD Biosciences) for 15 min at room temperature to lyse red blood cells. Cells were then washed twice with 1% FBS in PBS and re-suspended in FACS block buffer. The red blood lysis protocol was also carried out on bone marrow cells which had been flushed from harvested tibiae and femurs in 10 ml PBS.

A total of 50 ul of blood was stained according to the Flow Cytometry protocol followed by red blood cell lysis in 3 ml BD FACS lysis solution (BD Biosciences) for 5

min at room temperature. Two washes with 1 ml FACS buffer followed and cells were fixed in 1% PFA.

2.2.1.4 Cell number quantification

A total of 10 ul of peritoneal exudates cells (PECs) were diluted 1:10 (in 40 ul PBS and 50 ul Trypan Blue) and 20 ul were used for automated counting in a Cellometer Auto T4 cell viability counter (Nexcelom Bioscience, MA, USA). Briefly, the cells were pipetted in the chamber of a compatible plastic slide which was inserted into the instrument (Figure 2.1). Initiation of the software generated an image of the cell-containing chamber and the focus was adjusted to ensure sufficient contrast for Trypan blue exclusion-based cell quantification. The instrument then quantified the absolute number of live and dead cells and the concentration of live and total cells in the provided volume.

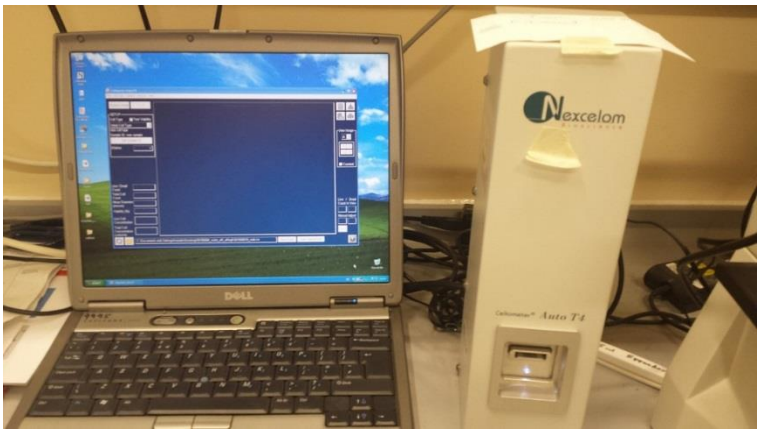


Figure 2.1. An overview of the Cellometer Auto T4 cell viability counter. Cell suspensions diluted 10x in Trypan Blue: PBS were pipetted in the chamber of a plastic slide which was inserted into the instrument. An image was taken by the software and the focus was adjusted where needed to increase the contrast and allow for precise discrimination of live from dead cells. The software then quantified live and dead cells and returned the concentration of live and total cells in the provided volume.

Blood leukocytes were counted with counting beads by mixing 50 ul of the beads with 300 ul of red blood cell-lysed samples before running the samples on a Flow Cytometer. To calculate the number of leukocytes per ul of blood, the number of leukocytes was divided by the number of beads in the Cytometry plots (Figure 2.2), multiplied by the concentration of the beads in the vial and divided by the volume of the samples (300 ul).

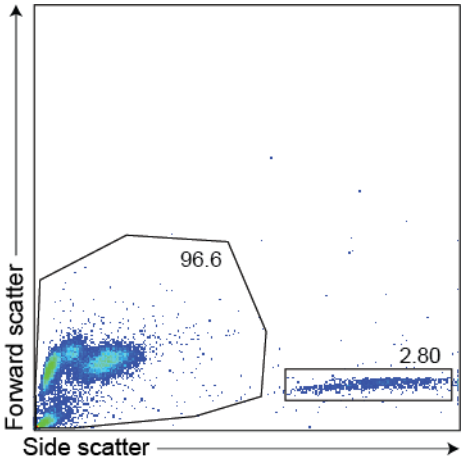


Figure 2.2. Representative Forward scatter (FS)-Side scatter (SS) Flow Cytometry plot for cell number quantification from a murine blood sample. Total leukocyte numbers were calculated by dividing the number of the gated cell and bead events in the FS-SS plot and multiplying with the concentration of beads in the vial divided by sample volume. Total leukocyte counts were further used to calculate individual immune cell population numbers using the appropriate Flow Cytometry gating strategies.

2.2.2 *In vitro* work

2.2.2.1 Bone marrow-derived macrophages (BMDMs)

Flushed bone marrow cells were passed through a 70 um cell strainer and spun at 5,000xg for 10 min. Pellets were resuspended in 10 ml L929 medium and cells were equally aliquoted into six 100 mm petri dishes for culture in 10 ml final volume L929 medium each. Five ml of fresh L929 medium were added on day 3 and cells were harvested on day 7 with PE buffer.

2.2.2.2 Activation assays

BMDMs were plated on 24-well tissue-cultured plates and were stimulated with 100 ng/ml LPS and 20 ng/ml IFN-γ in OptiMEM medium for 16 hours. Supernatants were collected to assess pro-inflammatory mediator levels with Enzyme Linked Immunosorbent Assay (ELISA) and nitrite levels with the Griess assay. Adherent cells were washed with PBS and cells were lysed for RNA extraction and quantitative polymerase chain reaction (qPCR) experiments.

In the phagocytosis experiments, $1x10^5$ BMDMs/well were incubated for one hour with pHrodo *E.coli*-conjugated particles and cells were washed three times with Live Cell Imaging solution. The PHERAstar FS plate reader (BMG Labtech, Aylesbury, UK) was used to read fluorescence at 509/533 nm. In another set of experiments, $6x10^5$ BMDMs were primed with LPS and IFN-γ in 15 ml tubes and 16 hours later they were assessed for surface marker expression with Flow Cytometry.

2.2.3 Techniques

2.2.3.1 cAMP assay

Intracellular cAMP levels were measured using the Discoverx cAMP Hunter eXpress kit. Chinese Hamster Ovary (CHO)-K1 cells ($3x10^4$) expressing human CB2R were plated on a half area 96 well plate and incubated at 37°C/5% CO_2 overnight. The next day, media were removed and replaced with 22.5 µl of assay buffer 2:1 cAMP capture antibody. Cells were then stimulated with 7.5 ul vehicle or serially diluted cannabinoid agonists in 20 uM Forskolin for 30 min at 37°C/5% CO_2. Following stimulation, 30 ul cell lysis buffer (3.8 ml Lysis buffer: 1 ml Substrate reagent 1: 0.2 ml Substrate Reagent 2: 5 ml cAMP Solution D) were added for 1 hour incubation in the dark. Finally, 30 ul

of cAMP Solution A were added for 3 hours in the dark before luminescence measurements were taken at a PHERAstar plate reader (BMG Labtech).

2.2.3.2 β-arrestin recruitment assay

Recruitment of β-arrestin was measured using the Discoverx PathHunter eXpress β-Arrestin GPCR Assay. CKO K1 cells co-expressing human CB2R tagged with the small 42 amino acid fragment of β-gal called Pro-Link and a fusion protein of β-arrestin with the larger N-terminal deletion mutant of β-gal ($3x10^4$) were plated on a half area 96 well plate and incubated at 37°C/5% CO_2 for 48 hours. Media were then removed and replaced with vehicle or serially diluted cannabinoid agonists in cell plating reagent for 90 min at 37°C/5% CO_2. A total of 55 ul of detection reagent (19:5:1, assay buffer:Substrate Reagent 1:Substrate Reagent 2) was subsequently added for 1 hour in the dark and luminescence measurements were taken at a PHERAstar plate reader (BMG Labtech).

2.2.3.3 Ca^{2+} influx assay

For the Ca^{2+} influx assay, $1x10^5$ BMDMs were treated with 4 uM FURA2-AM in Fluorobrite DMEM (Thermo Scientific) for 45 min at room temperature. Cells were washed with PBS and media were replaced with Fluorobrite DMEM. Vehicle or cannabinoids in DMEM media were added and changes in FURA-2 fluorescence were measured for 5 min at a PHERAstar FS plate reader (BMG Labtech) at 485/520 nm.

2.2.3.4 ELISA

All ELISAs were performed with BMDM supernatants and murine biological fluid supernatants according to the manufacturer's instructions. Briefly, 100 ul capture antibody diluted in sterile PBS were added per 96 well plate well and were incubated overnight at room temperature. The next day plates were washed with Tween 0.05% in PBS and 300 ul 1% BSA in PBS were added to each well for blocking. One hour later plates were washed and 100 ul diluted samples/standards diluted in 1% BSA in PBS were added (Table 2.1). Samples were incubated for 2 hours at room temperature after which they were washed with PBS/Tween and a biotinylated detection antibody (diluted in 1% BSA in PBS) was added to all samples. Plates were washed and 100 ul conjugated streptavidin-horseradish peroxidase (HRP) in 1% BSA in PBS were used to amplify the positive signal from the detection antibody. After an incubation of 20 min at room temperature and further wash step, 55 ul TMB-H_2O_2 substrate were added to each well. The reaction was stopped with 55 ul 2N H_2SO_4 per well when standards formed a colour gradient.

Table 2.1. ELISA commercial kits

Antigen	Cat No	Sample	Lower limit of detection (pg/ml)
IL-6	DY406	2x-4x	15.6
CCL2	DY479	10x	3.91
CCL3	DY450	2x	7.81
CXCL1	DY453	2x	15.6
CXCL2	DY452	2x	15.6
CXCL5	DY443	2x	15.6

Optical density (O.D.) values were read at 450 nm with correction at 630 nm at a PHERAstar FS plate reader (BMG Labtech) and concentration values were interpolated

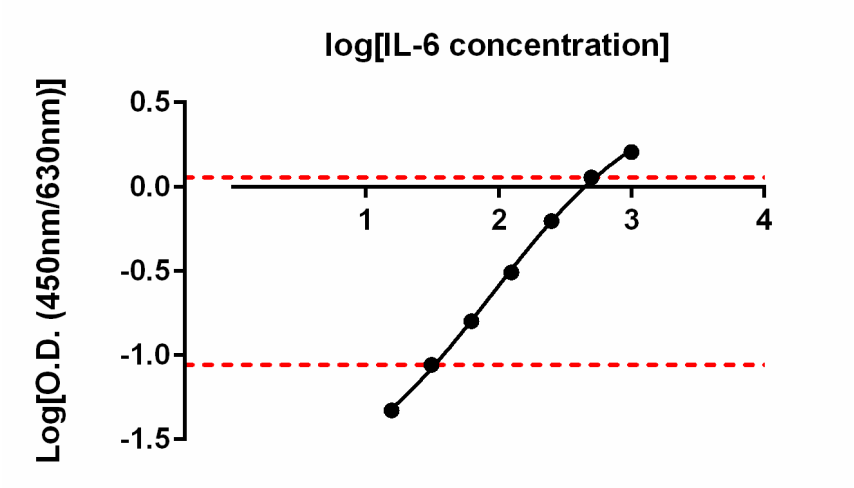


Figure 2.3. Typical standard curve for the quantification of the levels of IL-6. O.D. readings in the linear part of the curve (marked with dotted red lines) were interpolated to the log [concentration] values of the mediators using the standard curve. Dilution factor of the samples was taken into consideration for final IL-6 level calculation.

from a standard curve of known standards using a 4 parameter logistic nonlinear regression (Figure 2.3).

2.2.3.5 Griess assay

Supernatants from BMDMs ($1x10^5$ per well) stimulated with LPS and IFN-γ were mixed 1:1 with Griess reagent for a colorimetric measurement at 540 nm with correction at 630 nm after 10 min incubation at room temperature. A PHERAstar FS plate reader (BMG Labtech) was used to read O.D. values and concentration data were interpolated from a standard curve of known $NaNO_2$ standards using a 4 parameter logistic nonlinear regression (Figure 2.4).

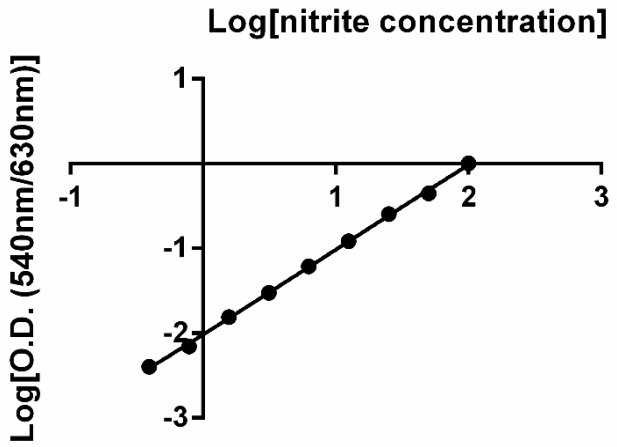


Figure 2.4. Typical standard curve for the quantification of the levels of nitrites. O.D. readings in the linear part of the curve were interpolated to the log [concentration] values of nitrite using the standard curve. Dilution factor of the samples was taken into consideration for final nitrite level calculation.

2.2.3.6 Confocal microscopy

BMDMs ($5x10^4$) incubated with pHrodo green *E.coli* bioparticles for 1 hour were washed with PBS to remove free particles and were fixed in 1% PFA for 10 min at room temperature. Cells were then blocked with 10% FBS in PBS for 45 min at room temperature and stained with 5 ug/ml goat anti-mouse Galectin-3 in 1% FBS in PBS for one hour (Table 2.2). Secondary donkey anti-goat antibody (5% v/v) in 1% FBS in PBS was added for 30 min at 4^oC and cells were washed a few times to remove excess antibody. Wells were incubated with 40 ug/ml DAPI for 3 min at room temperature to stain for nuclei and were then washed with PBS. Images were taken at an Olympus Fluoview FV100 confocal microscope (Olympus America Inc, PA, USA).

Table 2.2. Confocal microscopy antibodies

Antigen	Supplier	Cat No	Clone	Isotype	Fluorochrome
anti-mouse Galectin-3	R&D systems	AF1197	polyclonal	IgG	N/A
donkey anti-goat	R&D systems	F0108	polyclonal	IgG	Allophycocyanin (APC)

2.2.3.7 RNA extraction

Total RNA was extracted using the RNeasy Mini kit as instructed by the manufacturer. Briefly, $2x10^5$ cells were lysed with 350 ul buffer RLT and equal volume of 70% ethanol was added. Samples were then transferred to an RNeasy spin column and the lysates were spun at 8000xg for 15s. One wash with 700 ul of RW1 buffer and two washes with 500 ul of RPE followed. Finally, the samples were centrifuged for 1 min at maximum speed and the columns were transferred to new collection tubes for the elution step. A total of 20 ul of RNase free H_2O was added before centrifuging for 1 min at 8000xg. The RNA samples were analysed using ND-1000 spectrophotometer (Nanodrop, Thermo Scientific) to determine concentration and purity at 260/280 nm and 260/230 nm before being stored at -20^{o}C.

2.2.3.8 Primer design

Primers were designed for qPCR using Primer 3 (v.0.4.0) online software. Parameters selected were the product size (90-150 bases), primer size (18-22 bases), primer melting temperature (52^o-58^{o}C), primer GC% (40-60%), Max 3' stability (13 kcal/mol), Max Poly-X (3), Max Self and Max 3' Self Complementarity (as low as possible). Analysis for hairpins, dimer and cross-dimer formation, palindromes and repeats was carried out with the NetPrimer online software (Premier Biosoft, CA, USA). All primer pairs were then BLASTed with the *Mus musculus* genome database to determine whether they were likely to amplify other murine genes. All forward (Fw) and reverse (Rv) primer sequences used in this thesis can be found in Table 2.3.

Table 2.3. PCR primer pair sequences

	Sequence (5'→3')	Length	Gene Accession	Primer
Cnr1 Fw	GCCTTCCTACCACTTCATTG	20	NM_007726.3	exon 2
Cnr1 Rv	CACATTGGGACTATCTTTGC	20		
Cnr2 Fw	GGTCCTCTCAGCATTGATTTC	21	NM_009924.3	exon 2
Cnr2 Rv	GCCCAGTAGGTAGTCGTTAG	20		
Actg1 Fw	CCAACAGCAGACTTCCAGGATT	22	NM_001313923.1 NM_009609.3	exon 7
Actg1 Rv	CTGGCAAGAAGGAGTGGTAACTG	23		
Genotyping	GGAGTTCAACCCCATGAAGGAGTAC	25	NM_009924.3	exon 2
Genotyping	GGGGATCGATCCGTCCTGTAAGTCT	25	Neomycin cassette	exon 2
Genotyping	GACTAGAGCTTTGTAGGTAGGCGGG	25	NM_009924.3	exon 2
Ccl2 Fw	CAGCACCTTTGAATGTGAAGTTG	23	NM_011333.3	exon 3
Ccl2 Rv	TGCTTGAGGTGGTTGTGGAA	20	NM_011333.3	exon 3
Cxcl1 Fw	AAGTCATAGCCACACTCAAG	20	NM_008176.3	exons 2-3
Cxcl1 Rv	CAGACAGGTGCCATCAGA	18	NM_008176.3	exon 4

2.2.3.9 Reverse transcription (RT)

RNA (400-700 ng) was reverse transcribed to DNA with the QuantiTect Reverse Transcription kit following the manufacturer's instructions. Briefly, genomic DNA was eliminated from RNA with 2 ul of the kit's gDNA wipeout buffer in a 14 ul final volume reaction. Samples were incubated for 5 min at 42^{o}C and then put immediately on ice. After preparing the mastermix for RT (Table 2.4), the samples were incubated for 15 min at 42^{o}C. Inactivation of the Quantiscript Reverse Transcriptase was carried out by a 3 min incubation at 95^{o}C.

Table 2.4. RT reaction mastermix

	Volume (ul)
Quantiscript Reverse Transcriptase	1
Quantiscript RT buffer	4
RT primer mix	1
Genomic DNA elimination reaction	14
Total	20

2.2.3.10 qPCR

The detection chemistry used for the experiments was Sybr Green. Mastermixes were prepared for both the gene of interest and the reference gene (Table 2.5). The qPCR thermal profile included the initial incubation at 95^{o}C for 5 min followed by 40 cycles of 95^{o}C for 20s, 60^{o}C for 20s and 72^{o}C for 20s and the final elongation step at 72^{o}C for 5 min. The melting curve analysis was also performed (Figure 2.5).

Table 2.5. qPCR mastermix

	Volume (µl)	**Initial**	**Final**
Sybr Green mastermix	12.5	2x	x
Fw primer	1.25	10 uM	0.5 uM
Rv primer	1.25	10 uM	0.5 uM
H_2O	8	-	-
cDNA	2	20-35 ng/ul	1.6-2.8 ng/ul
Total	25		

Non-template control samples were run as negative controls of the reactions. Normalised Fluorescence threshold was arbitrarily set to 1 and fluorescence data were acquired at the end of each of the 40 cycles (Figure 2.6). The experiments were carried out in a Rotor-Gene RG-3000 (Qiagen) thermocycler. Samples were loaded in tubes with plastic caps and analysed with the Rotor Gene 6.1 software (Qiagen).

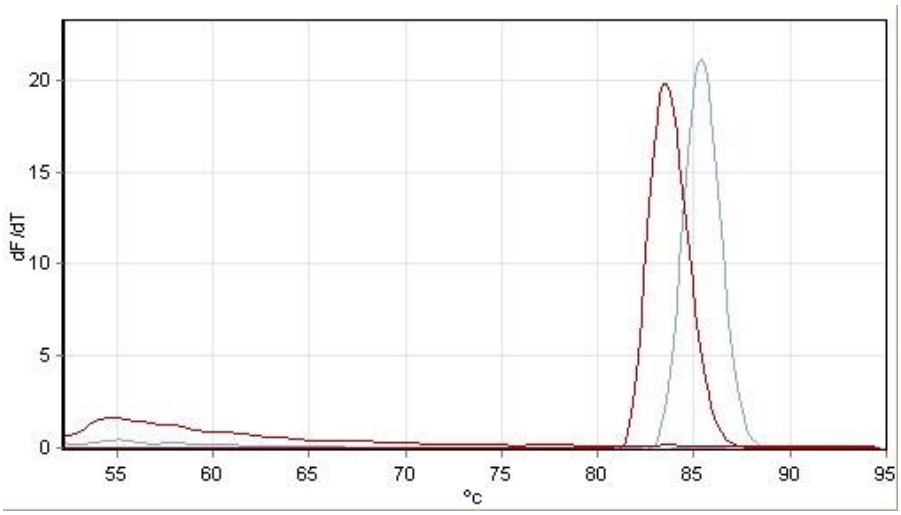


Figure 2.5. Representative melt curves from a qPCR experiment with *Cnr2* as the gene of interest (red curves) and *Actg1* as the reference gene (cyan curves). *Cnr2* curve has one peak at 85.35°C, whereas *Actg1* has a peak at 83.5°C, which suggest the qPCR primers amplified one, specific DNA product. Absence of fluorescence peaks at the non-template curves (seen at the bottom of the Y axis) suggests no DNA contamination was present in reaction mastermixes.

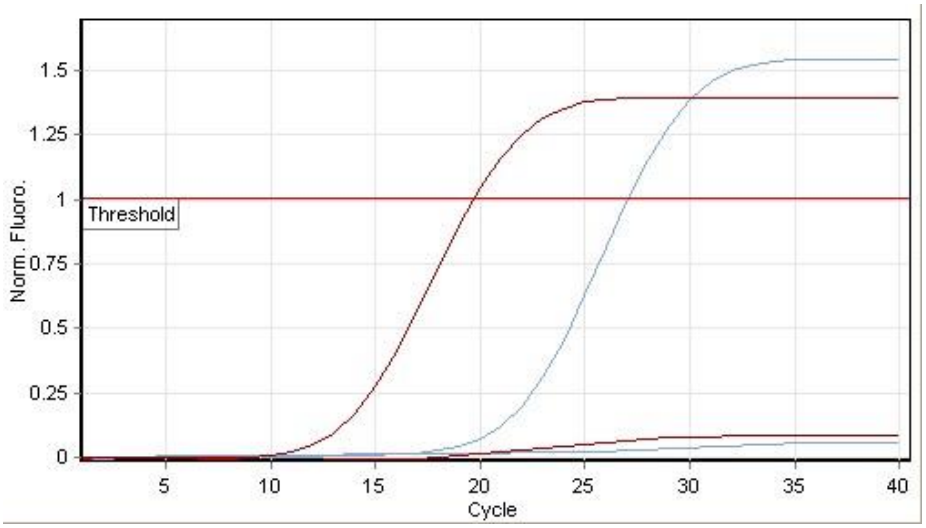


Figure 2.6. Representative amplification curves from a qPCR experiment with *Cnr2* as the gene of interest (red curves) and *Actg1* as the reference gene (cyan curves). Threshold was arbitrarily set to 1 and Ct values were calculated from the graph (19.69 for *Actg1* and 27.05 for *Cnr2*). Non-template controls (seen at the bottom of the Y axis) were also run to confirm the lack of DNA contamination in the reaction mastermixes.

Cycle threshold (Ct) values were determined and relative mRNA contents were inferred from normalization of the expression of the gene of interest to that of the housekeeping gene (ΔCt). Relative expression results were plotted as mRNA expression over actin ($2^{\wedge(-\Delta Ct)}$) (Pfaffl 2001).

2.2.3.11 Flow cytometry

All samples were spun at 2000xg for 10 min and the pellets were resuspended in 50 ul FACS block for a 10 min incubation on ice. A mastermix solution of the appropriate

Table 2.6. Flow Cytometry antibodies

Antigen	Supplier	Cat No	Clone	Isotype	Fluorochrome
CD11b	Biolegend	101230	M1/70	IgG2b	Peridinin Chlorophyll Protein (PerCP)
CD45	BD Pharmigen	557659	30-F11	IgG2b	APC-Cy7
CD80	Biolegend	104705	16-10A1	IgG	Fluorescein isothiocyanate (FITC)
CD86	Biolegend	105007	GL-1	IgG2a	Phycoerythrin (PE)
CD115	Biolegend	135505	AFS98	IgG2a	PE
F4/80	Abd Serotec	MCA497A647	CI:A3-1	IgG2b	Alexa Fluor 647
I-A/I-E	Biolegend	107607	M5/114.15.2	IgG2b	PE
Ly-6B.2	Abd Serotec	MCA771G	7/4	IgG2a	FITC
Ly-6C	Biolegend	128006	HK1.4	IgG2c	FITC
Ly-6G	Biolegend	127612	1A8	IgG2a	Pacific Blue
Ly-6G	BD Pharmigen	551461	1A8	IgG2a	PE

antibodies at 1:50 dilution in FACS buffer was prepared and 50 ul were added to each sample for 30 min incubation on ice in the dark. Samples were then centrifuged at 5,000xg for 10 min and the cell pellets were resuspended in 300 ul PFA solution. A Dako Cyan ADP flow cytometer (Beckman Coulter Ltd., High Wycombe, UK) was used to run the samples and analysis was carried out with FlowJo software 10.0.8 (Tree

Star Inc, Ashland, USA). A list of antibodies (and the respective isotype controls) is shown in Table 2.6 and Table 2.7.

Table 2.7. Flow Cytometry isotype control antibodies

Supplier	Cat No	Specificity	Clone	Isotype	Fluorochrome
Abd Serotec	MCA1125A647	Rat	-	IgG2b	Alexa Fluor 647
Abd Serotec	MCA1212F	Rat	-	IgG2a	FITC
BD Pharmigen	553930	Rat	R35-95	IgG2a	PE
Biolegend	400535	Rat	RTK2758	IgG2a	Pacific Blue
Biolegend	400623	Rat	RTK4530	IgG2b	APC-Cy7
Biolegend	400705	Rat	RTK4174	IgG2c	FITC
Biolegend	400929	Armenian Hamster	HTK888	IgG2b	PerCP
eBioscience	11-4888-81	Armenian Hamster	eBio299Arm	IgG	FITC

2.2.3.12 Genotyping

Ear clips from male WT and CB2R KO mice were incubated overnight with 1 mg/ml Proteinase K in tissue lysis buffer at 55^{o}C. Lysed samples were boiled at 85^{o}C to inactivate the enzyme and 2 ul were used in the following mastermix for a standard PCR reaction (Table 2.8). The PCR thermal profile included one step at 94^{o}C for 3 min followed by 35 cycles at 94^{o}C for 30 s, 62^{o}C for 1 min and 72^{o}C for 1 min and the final elongation step at 72^{o}C for 2 min. PCR products were collected and 20 ul were mixed with 4 ul Orange G (Sigma-Aldrich) and were run on a 2% agarose gel at 120V for 40 min to determine band size and mouse genotype.

Table 2.8. Genotyping PCR mastermix

	Volume (ul)	Initial	Final
Biomix red (2x)	12.5	2x	x
WT Fw primer	1.25	10 uM	0.5 uM
Mutant Fw primer	1.25	10 uM	0.5 uM
common Rv primer	1.25	10 uM	0.5 uM
H_2O	6.75	-	-
cDNA	2	N/A	N/A
Total	25		

2.2.3.13 BCA assay

Protein content was determined with a BCA assay using BSA standards mixing 10 ul sample/standard volume with 190 ul Reagent A 50:1 Reagent B. Samples were then incubated at 37^{o}C/5%CO_2 and measurements were taken at a PHERAstar FS plate reader at 560 nm (BMG Labtech).

2.2.3.14 MPO assay

Harvested murine livers were homogenised in 400 ul 0.5% HTAB-containing phosphate buffer (50 mM) at 6.5 m/s for 40 s using ceramic beads using a FastPrep-24 homogenizer (Oxford Biosystems, Milton Park, UK). The homogenates were centrifuged at 2,000xg for 10 min and the supernatants were diluted 10x in 80 mM phosphate buffer to determine protein contents using a BCA assay. Aliquots of 20 ul of 10x diluted samples were mixed with 160 ul TMB and 20 ul H_2O_2 (0.0005% in dH_2O) in 96 well plates and the assay was run for 5 min. Absorbance was measured at 620 nm every 50 s at a PHERAstar FS plate reader (BMG Labtech). MPO unit values were interpolated from a standard curve of known rMPO standards using a 4 parameter logistic nonlinear regression and were normalised to protein content (MPO units/mg protein).

2.2.3.13 Luminex assay

The Magnetic Luminex Screening Assay (R&D systems) was used to measure the *in vivo* levels of TNF-α, IL-1β, IL-6, IL-12p70, IFN-γ, CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL5 and CXCL10 in murine biological fluids. Briefly, the microparticle cocktail was diluted in Assay Diluent RD1W and 50 ul were added to each well of a 96

well plate. Another 50 ul of standards/samples were added for a 2 hour incubation at room temperature on a horizontal orbital shaker at 800 rpm. All samples were diluted 2x and standards were diluted 10x in calibrator diluent RD6-52. A magnetic device was then applied to the plate to wash the wells 3x with wash buffer. The antibody cocktail (50 ul) was then added for 1 hour at room temperature after which a wash step followed. Finally, 50 ul of streptavidin-PE were added to each well for 30 min at room temperature and the microparticles were washed and prepared for measurement at a Bio-Plex 200 system (Bio-rad, Hemel Hempstead, UK).

2.2.4 Statistical analysis

All experiments were statistically analysed with GraphPad Prism 6.0 (GraphPad, CA, USA). Data were first evaluated for statistical outliers with the Grubbs' test (GraphPad). They were then analysed with parametrical tests, if individual data points followed the normal distribution and their variance was not significantly different. Otherwise, non-parametrical tests were employed. Statistical significance was assumed when $p<0.05$ and mean+standard error of the mean (SEM) was plotted in all graphs. For two group comparisons, the parametrical student t test was applied with or without Welch's correction depending on whether variation within groups was non-significantly different from each other. For more than two groups, one-way analysis of variance (ANOVA) with Dunnett's post hoc comparison or the non-parametrical Kruskal-Wallis with Dunn's post hoc comparison was used. Finally, to assess the influence of two independent categorical variables in experiments with one continuous dependent variable, a two-way ANOVA with Sidak's post hoc multiple comparisons test was used.

Chapter 3: The effects of a CB2R-selective agonist on leukocyte recruitment and mediator production in the zymosan-induced peritonitis model

3.1 Introduction

3.1.1 *In vitro* and *in vivo* evidence for cannabinoid regulation of neutrophil and monocyte recruitment

A number of studies has been carried out to find out whether activation of CB2R affects leukocyte migration. Both natural and synthetic agonists have been used extensively to understand the pharmacology, kinetics and function of this GPCR. In addition, the use of receptor antagonists and KO mice have strengthened the view that CB2R is implicated in some of the reported anti-migratory effects *in vitro* and *in vivo*.

Cannabis pre-exposed human patients present with lower monocyte chemotaxis both when cells were used untreated or in the presence of phytocannabinoids (Sexton *et al.* 2013). This is in line with the anecdotal observations that cannabis pre-exposed human patients demonstrate ameliorated symptoms in a range of inflammatory diseases, such as inflammatory bowel disease (El Bakali *et al.* 2015).

Δ^9-THC, CBD and CP55,940 have been used *in vitro* and *in vivo* to reduce chemotaxis of macrophages in a CB2R-dependent manner (Raborn and Cabral 2010;Raborn *et al.* 2014;Raborn *et al.* 2008;Sacerdote *et al.* 2005;Sacerdote *et al.* 2000). Moreover, CBD decreases the recruitment of leukocytes and bone resorption in an experimental periodontitis model via TNF-α and IL-1β level reduction (Napimoga *et al.* 2009), whereas WIN55,212-2 and HU-308 reduce migration of leukocytes and ear swelling in mice (Oka *et al.* 2006).

In contrast, the endocannabinoid 2-AG has been widely reported as an inducer of monocyte chemotaxis. *In vitro*, 2-AG induces the migration of bone marrow cells (Patinkin *et al.* 2008) and HL-60 cells, U937, THP-1 cells and human monocytes in a CB2R-dependent manner (Kishimoto *et al.* 2003). A role for endocannabinoids has also been proposed in atherosclerosis. 2-AG is one of the endocannabinoid ligands synthesised during atherogenesis in visceral adipose tissue and it contributes to disease progression. Although these findings were not replicated *in vivo*, they observed enhanced monocyte migration and matrix metalloproteinase-9 production in response to this agonist (Montecucco *et al.* 2009).

Interestingly, AEA has been shown to inhibit cell migration (McHugh *et al.* 2008;Patinkin *et al.* 2008). In neutrophils, this effect was shown to be CB2R-independent and involve two GPCRs; CB1R at low AEA concentrations and an unidentified receptor at micromolar concentrations (McHugh *et al.* 2008).

CB2R may also exert its anti-migratory effects indirectly through endothelial cells. For example, JWH-133 and O-1966 reduce leukocyte migration to the brain via downregulation of VCAM-1 on the endothelium and restoration of blood brain barrier integrity (Ramirez *et al.* 2012). PCR arrays confirmed that a list of pro-inflammatory genes including cytokines/chemokines, adhesion molecules and transcription factors were targeted by these CB2R-selective agonists in activated primary human brain endothelial cell lines (Persidsky *et al.* 2015). Similarly, WIN55,212-2 decreases monocyte recruitment to atherosclerotic lesions via VCAM-1, ICAM-1 and P-selectin downregulation in plaques (Zhao *et al.* 2010).

The molecular mechanisms that cannabinoid agonists utilise in order to dampen macrophage and neutrophil migration have also been investigated. In an elegant *in vitro*

study with neutrophil-like HL-60 cells, Kurihara *et al.* showed that 2-AG and JWH-015 induce cytoskeletal rearrangements and the formation of pseudopodia to reduce the levels of RhoA kinase, contributing to reduced human neutrophil migration to formyl Met Leu Phe (fMLP) (Kurihara *et al.* 2006).

Neutrophil migration is not regulated only by CB2R. A $G\alpha_{13}$-coupled receptor, called GPR55, was shown to synergise with CB2R intracellularly to augment Cdc42 signalling and reduce translocation of Rac2 to the plasma membrane (Balenga *et al.* 2011). This suggests that the cannabinoid effects on innate immune cell chemotaxis might be the additive results at CB2R and other expressed GPCRs.

3.1.2 The zymosan-induced peritonitis model

To assess the role of CB2R-selective ligands on the recruitment of leukocytes, I employed the zymosan-induced peritonitis model of leukocyte recruitment. The zymosan-induced peritonitis model is triggered by killed *Saccharomyces cerevisiae* yeast particles when the latter are administered i.p. to animals. Zymosan is a TLR2 ligand, but it is also recognised by the pattern recognition receptor (PRR) Dectin-1 (Drummond and Brown 2011) and it induces a wave of acute local inflammation in the peritoneal cavity.

In its original report, the zymosan-induced peritonitis model was characterised by a rapid influx of plasma proteins and the presence of prostaglandins, such as PGE_2 and leukotrienes, such as LTC_4, LTD_4 and LTE_4, followed by accumulation of leukocytes (Doherty *et al.* 1985). Writhing response as a readout of pain was also evaluated, as was the extravasation of plasma proteins. Given their role in initiation and resolution of inflammation (Dennis and Norris 2015), the presence of eicosanoids in the peritoneum

marks the onset of inflammation brought about by resident cells aiming to clear the zymosan particles.

In a low dose (100 ug) zymosan-induced peritonitis, there is a rapid influx of neutrophils to the peritoneal cavity after the initiation of the disease, whereas the $F4/80^{+}$ macrophage population peaks at later time points and remained constant. Analysis of peritoneal cell-free exudates showed a range of pro-inflammatory mediators, such as cytokines and chemokines, including TNF-α, IL-1β, IL-12p40, macrophage inflammatory protein (MIP-1α), MIP-1β and CXCL1 (Bystrom *et al.* 2008). Interestingly, IL-10 levels elevate progressively after the peak of inflammation suggesting that anti-inflammatory endogenous networks are activated at later time points. Therefore the 100 ug zymosan-induced peritonitis is considered to be a self-resolving model of acute inflammation.

Conversely, the same group characterised a high dose (10 mg) zymosan-induced peritonitis, which fails to resolve and presents with persistent inflammatory responses (Bystrom *et al.* 2008;Newson *et al.* 2014). In particular, mice demonstrate a delayed recruitment of neutrophils to the peritoneal cavity. Comparison of the pro-inflammatory mediator levels in the peritoneal cell-free exudates showed that all cytokine and chemokine levels, apart from IL-10 were higher in this model.

As previously mentioned, the peritoneal resident immune populations play a pivotal role in the initiation and termination of inflammation. DCs, mast cells, lymphocytes and macrophages have been reported to reside in the peritoneal cavity and are commissioned to sense pathogen invasion (Ajuebor *et al.* 1999;Newson *et al.* 2014;Rajakariar *et al.* 2008). During inflammation, mast cells become activated and secrete chemokines, such as CCL2 and CXCL1, whereas resident macrophages express the anti-inflammatory

cytokine IL-10 (Ajuebor *et al.* 1999). After the resolution of inflammation, resident macrophages phagocytose apoptotic neutrophils in the cavity, but also migrate to the mesenteric lymph nodes where they induce the differentiation of effector T cells to regulatory T (Treg) cells in an inducible nitric oxide synthase (iNOS)-dependent manner (Newson *et al.* 2014).

Transcriptomic analysis confirmed the resolving nature of the resident macrophages (Stables *et al.* 2011). Key pathways defining their phenotype were shown to include expression of certain chemokines/cytokines, T and B cell chemoattraction and lymphocyte expansion and differentiation. In addition, Bystrom *et al.* found that these cells express high levels of iNOS, IL-10, mannose receptor, COX-2 and PGD_2, and that they exhibit reduced bactericidal properties.

On the contrary, resident DCs leave the peritoneal cavity and are replaced by monocyte-derived DCs (moDCs) and monocyte-derived macrophages (moMΦs) (Bystrom *et al.* 2008;Newson *et al.* 2014). These two blood-borne populations have a migratory phenotype and either trigger the inflammatory responses in the mesenteric lymph nodes or assist resolving macrophages in Treg cell induction.

Another feature of the resolving zymosan model is its biphasic nature (Rajakariar *et al.* 2008). Following the resolution of inflammation which is marked by the reduction in the number of total cells in the peritoneal cavity, there is a secondary mobilisation of lymphocytes and in particular $CD3^+$, $CD4^+$, $CD8^+$, γδ T cells, B cells and NK cells. These lymphocyte populations are recruited by moMΦs (Newson *et al.* 2014) and control the recruitment of neutrophils to the peritoneal cavity partially by regulating the TNF-α/IL-10 ratio.

Finally, leukocyte recruitment in the zymosan-induced peritonitis model is also triggered by pro-inflammatory lipid mediators. Both arachidonic acid and unsaturated fatty acid concentrations are increased early after zymosan administration and before the leukocyte recruitment peak (Lundy *et al.* 1990). This suggests that a portion of the unsaturated lipid mediators originate from plasma and/or resident cells in the peritoneal cavity.

3.2 Aim of the chapter

The aim of the experiments reported in this chapter was to study the potential anti-inflammatory effects of a novel in-house CB2R-selective agonist called DIAS2 in the resolving zymosan-induced peritonitis model. I hypothesised that DIAS2 is a CB2R-selective cannabinoid compound with anti-inflammatory properties. As such, it would suppress the secretion of pro-inflammatory cytokines and chemokines in the peritoneal cavity and thus fewer immune cells are recruited.

To address this question, I administered DIAS2 to mice undergoing zymosan-induced peritonitis and studied the deviations from the expected recruitment pattern. Particular emphasis was given to the recruitment of innate immune cells, such as neutrophils and monocytes as well as the production of pro-inflammatory mediators in the peritoneum.

3.3 Results

3.3.1 DIAS2 reduces the number of recruited monocytes to the peritoneal cavity

Previous work in the lab identified 4 hours as the ideal time point to study neutrophil recruitment in the zymosan-induced peritonitis model. Neutrophil migration to the peritoneal cavity reached its peak at 4 hours, but there is also significant migration of

blood monocytes (Regan-Komito 2015). Biochemical stability studies revealed that the CB2R-selective agonist DIAS2 has a very short half-life of approximately 45 min (Gianella-Borradori *et al.* 2015).

For the reasons mentioned above and with the goal to investigate DIAS2 effect on leukocyte recruitment in acute inflammation when administered as an intervention, I decided to inject this CB2R-selective agonist at the 4 hour time point and sacrifice mice 2 hours later (Figure 3.1A). Mice were sacrificed via CO_2 asphyxiation and the peritoneal fluid was harvested. PECs were counted and further analysed with Flow Cytometry. The cell-free peritoneal lavage fluid was used to determine the secreted pro-inflammatory mediator levels with ELISAs (Figure 3.1A).

I first asked whether the administration of DIAS2 into the peritoneal cavity could reduce the number of recruited neutrophils and monocytes. To answer this question, I injected male C57BL/6J animals with 100 ug zymosan i.p. and 4 hours later I administered vehicle or 20 mg/kg DIAS2 for 2 more hours. One group of animals received PBS and vehicle and served as a negative control (Figure 3.1A). DIAS2 was dissolved in Cremophor solution (0.9% saline: ethanol: Cremophor 18: 1: 1) which the lab has previously used due to the low solubility of cannabinoids and the fact that it does not induce inflammation (Christou 2012).

Analysis of the PEC populations revealed the migration of Ly-6G^{+}Ly-6B.2^{+} neutrophils and Ly-6G^{-}Ly-6B.2^{+} monocytes to the cavity in the vehicle-treated animals which also received zymosan (Figure 3.1B). DIAS2 administration decreased the percentage of recruited monocytes, while the percentage of neutrophils remained comparable between the two treatments. As expected, the PBS-treated animals showed no recruitment of

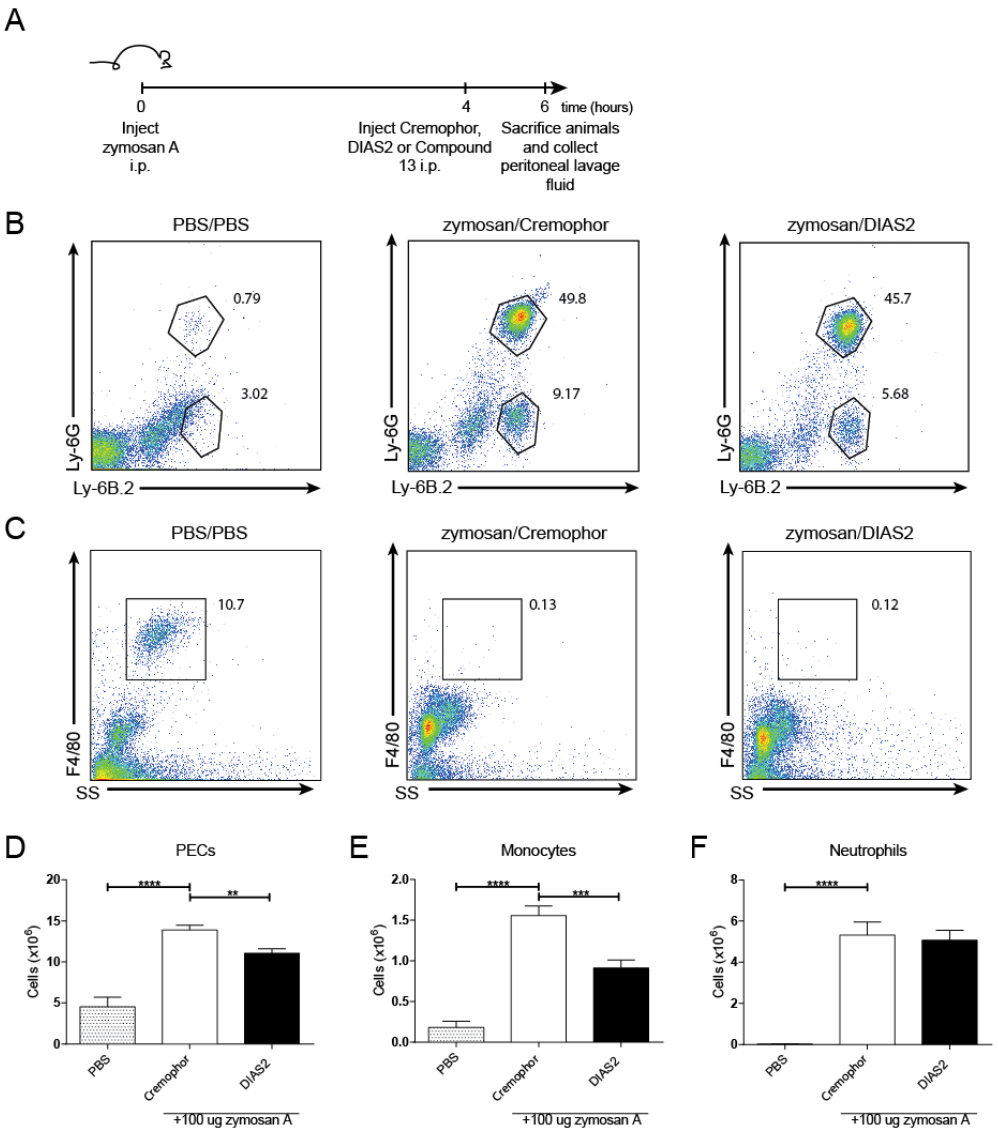


Figure 3.1. DIAS2 reduces monocyte recruitment to the peritoneal cavity in the zymosan-induced peritonitis model. PBS or 100 ug zymosan A were injected i.p. to male C57BL/6J mice (8-10 weeks old) and 4 hours later Cremophor solution or 20 mg/kg DIAS2 were administered i.p. for another 2 hours. Animals were sacrificed and peritoneal lavage fluid was collected for cytokine and chemokine level assessment and peritoneal cavity cell composition evaluation (A). Representative FACS plots from an animal from each group showing neutrophil (Ly-6B.2$^+$Ly-6G$^+$) and monocyte (Ly-6B.2$^-$Ly6G$^+$) populations (B). Representative FACS plots from an animal from each group showing the macrophage (F4/80$^+$) population (C). Total PEC (D), monocyte (E) and neutrophil (F) numbers from 2 independent experiments with 5-12 animals per group. Data are mean+SEM analysed with one-way ANOVA and Dunnett's post hoc test, ** p<0.01, *** p<0.001, ****p<0.0001

leukocytes at the time point studied (Figure 3.1B). PEC counts showed a significant reduction in the total cell number between the vehicle- and the DIAS2-treated mice (Figure 3.1D) which translated into a significant reduction in the number of monocytes in the DIAS2-treated animals (Figure 3.1E). On the contrary, neutrophil numbers remained comparable between the two treatments (Figure 3.1F).

To validate technical success in the experiment, I also stained the samples for F4/80 and Ly-6B.2 to identify the resident macrophage population of the peritoneal cavity (Figure 3.1C). Resident macrophages leave the peritoneal cavity upon an inflammatory insult (Regan-Komito 2015) and therefore experimental technical success would mean that all animals injected with zymosan would not stain for $F4/80^{+}Ly\text{-}6B.2^{-}$. In contrast, PBS-injected animals should retain intact resident macrophage population. Indeed, I could not detect resident macrophages in both the vehicle- and DIAS2-treated animals which also received zymosan. As anticipated, PBS-injected mice had a distinct $F4/80^{+}$ macrophage population as demonstrated by Flow Cytometry (Figure 3.1C).

3.3.2 DIAS2 induces the levels of pro-inflammatory mediators in the peritoneal cavity

I next examined the levels of pro-inflammatory mediators in the peritoneal fluid upon the administration of zymosan. We have previously identified a number of cytokines (e.g. IL-6) and chemokines (e.g. CCL2, CXCL1, CCL3) present in the cell-free exudate at this particular time point (Regan-Komito 2015).

Unexpectedly, the administration of 20 mg/kg DIAS2 induced the secretion of almost all the pro-inflammatory mediators I measured. In particular, the levels of IL-6, CCL2,

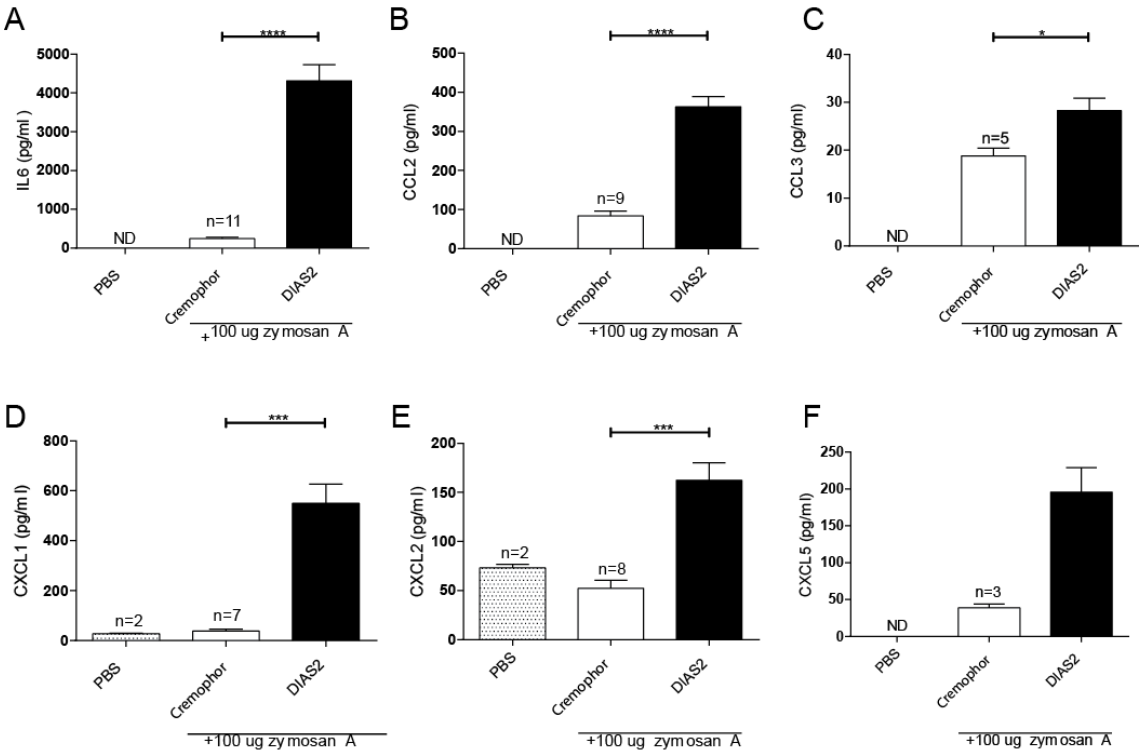


Figure 3.2. DIAS2 induces the production of pro-inflammatory cytokines and chemokines in the peritoneal cavity in the zymosan-induced peritonitis model. IL-6 (A), CCL2 (B), CCL3 (C), CXCL1 (D), CXCL2 (E) and CXCL5 (F) levels from 2 independent experiments with 5-12 animals per group unless otherwise stated on top of the bars. Data are mean +SEM analysed with one-way ANOVA and Dunnett's post hoc test, ND not detectable, * $p<0.05$, *** $p<0.001$, **** $p<0.0001$

CCL3, CXCL1, CXCL2 in the DIAS2-treated mice were significantly higher than vehicle-treated (Figure 3.2A-E). CXCL5 levels were comparable between the two treatments (Figure 3.2F).

3.3.3 Low dose DIAS2 administration does not inhibit monocyte recruitment

To test whether the elevated cytokine and chemokine secretion was an off-target effect of DIAS2, I decided to administer a lower dose of DIAS2 (2.5 mg/kg) (Figure 3.3A). Briefly, male C57BL/6J mice were injected with 100 ug zymosan i.p. and 4 hours later vehicle or 2.5 mg/kg DIAS2 were administered via the same route for 2 more hours. One group of animals received 2.5 mg/kg DIAS2 and vehicle to test whether DIAS2 *alone* could initiate a second wave of inflammation in the peritoneal cavity.

Administration of 2.5 mg/kg DIAS2 4 hours after zymosan did not affect the percentage of recruited neutrophils or monocytes, as shown in Figure 3.3B in comparison to the vehicle-treated group of animals. Interestingly, injection of DIAS2 followed by vehicle resulted in the induction of neutrophil recruitment and negligible migration of monocytes to the peritoneal cavity (Figure 3.3B). F4/80 staining of PECs confirmed that the zymosan-treated animals received the stimulus and that the administration was technically successful (Figure 3.3C). However, it is noteworthy that the mice which received DIAS2 *alone* also showed a significant reduction in the percentage of $F4/80^{+}$ staining as compared to Figure 3.1C.

DIAS2-treated animals which also received zymosan did not exhibit any reduction in PEC (Figure 3.3D) or monocyte (Figure 3.3E) numbers. At the same time, the total neutrophil numbers were comparable between the two treatments (Figure 3.3F). Interestingly, injection of DIAS2 and vehicle resulted in the recruitment of

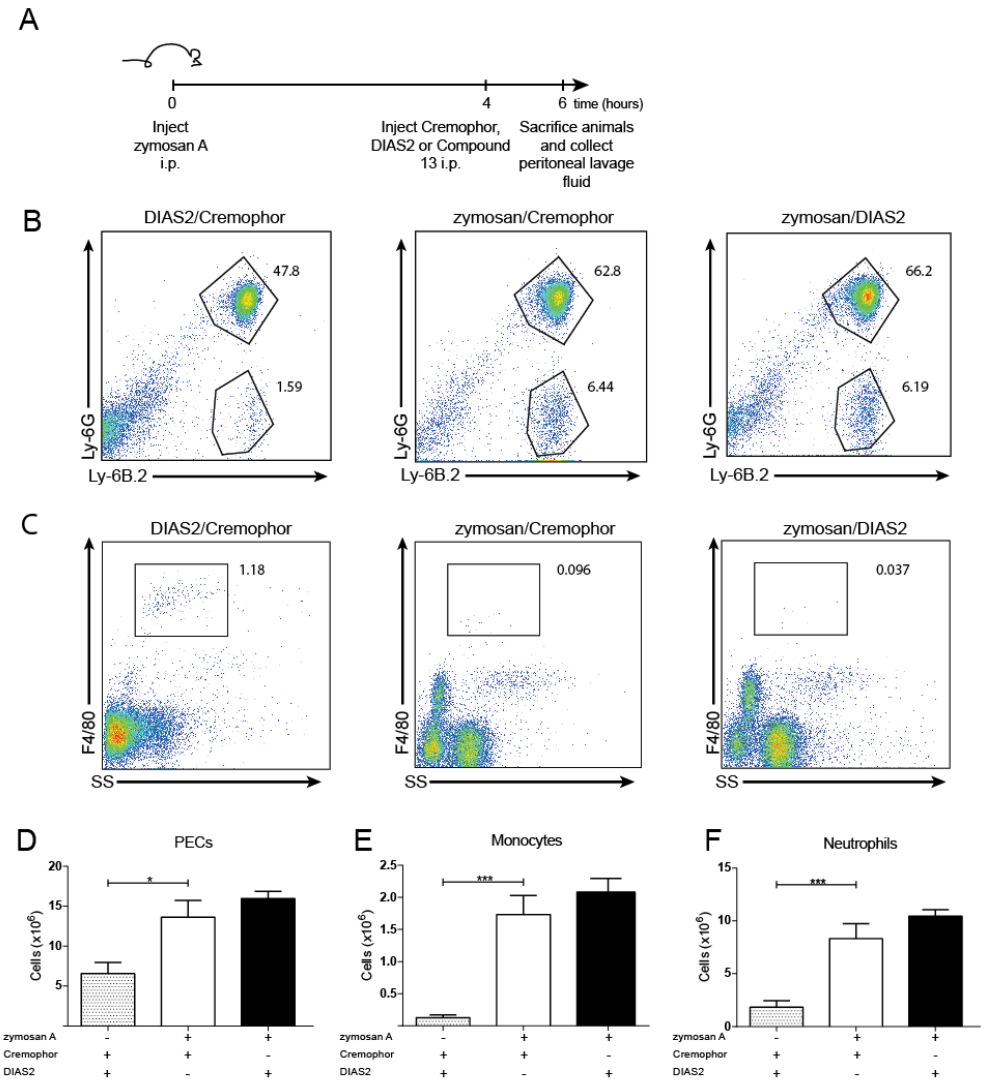


Figure 3.3. DIAS2 effect on monocyte recruitment to the peritoneal cavity in the zymosan-induced peritonitis model is lost at a lower dose. DIAS2 (2.5 mg/kg) or 100 ug zymosan A were injected i.p. to male C57BL/6J mice (8-10 weeks old) and 4 hours later Cremophor or DIAS2 (2.5 mg/kg) were administered i.p. for another 2 hours. Animals were sacrificed and peritoneal lavage fluid was collected for cytokine and chemokine level assessment and peritoneal cavity cell composition evaluation (A). Representative FACS plots from an animal from each group showing neutrophil (Ly-6B.2$^+$Ly-6G$^+$) and monocyte (Ly-6B.2$^-$Ly6G$^+$) populations (B). Representative FACS plots from an animal from each group showing the macrophage (F4/80$^+$) population (C). Total PEC (D), monocyte (E) and neutrophil (F) numbers from one experiment with 5 animals per group. Data are mean+SEM analysed with one-way ANOVA and Dunnett's post hoc test, * $p<0.05$, *** $p<0.001$

approximately 3 million neutrophils. Taking into account the absence of neutrophils in the peritoneal cavity in PBS-treated animals (Figure 3.1F), it seems that DIAS2 *alone* might be responsible for leukocyte recruitment to the peritoneal cavity.

3.3.4 Low dose of DIAS2 still induces the secretion of pro-inflammatory mediators

I next analysed the cell-free peritoneal fluid samples to test whether the low dose DIAS2 zymosan-injected mice were devoid of pro-inflammatory mediator secretion. As shown in Figure 3.4, DIAS2-treated animals which also received zymosan still had significantly higher levels of IL-6 (Figure 3.4A) and CCL2 (Figure 3.4B) compared to the vehicle-treated animals, whereas CCL3 (Figure 3.4C), CXCL1 (Figure 3.4D), CXCL2 (Figure 3.4E) and CXCL5 (Figure 3.4F) levels were comparable between the two treatments. IL-6 and CXCL5 levels of the 2.5 mg/kg DIAS2-treated animals which also received zymosan were lower compared to those which received 20 mg/kg (Figure 3.2A, F). Notably, the DIAS2 *alone* control mice secreted significantly higher levels of IL-6, CCL2, CXCL1 and CXCL2 than the mice which received zymosan and vehicle.

3.3.5 Inactive compound on CB2R inhibits neutrophil recruitment to the peritoneal cavity

To test whether this novel CB2R agonist affects monocyte recruitment selectively via CB2R, I used an inactive DIAS2 analogue side-by-side with DIAS2 (Gianella-Borradori *et al.* 2015). Substitution of the cyano group on DIAS2 to the third carbon atom results in a dramatic loss of its activity at the receptor as assessed by cAMP reduction in human CB2R-transfected CHO cells (Figure 3.5). I therefore administered

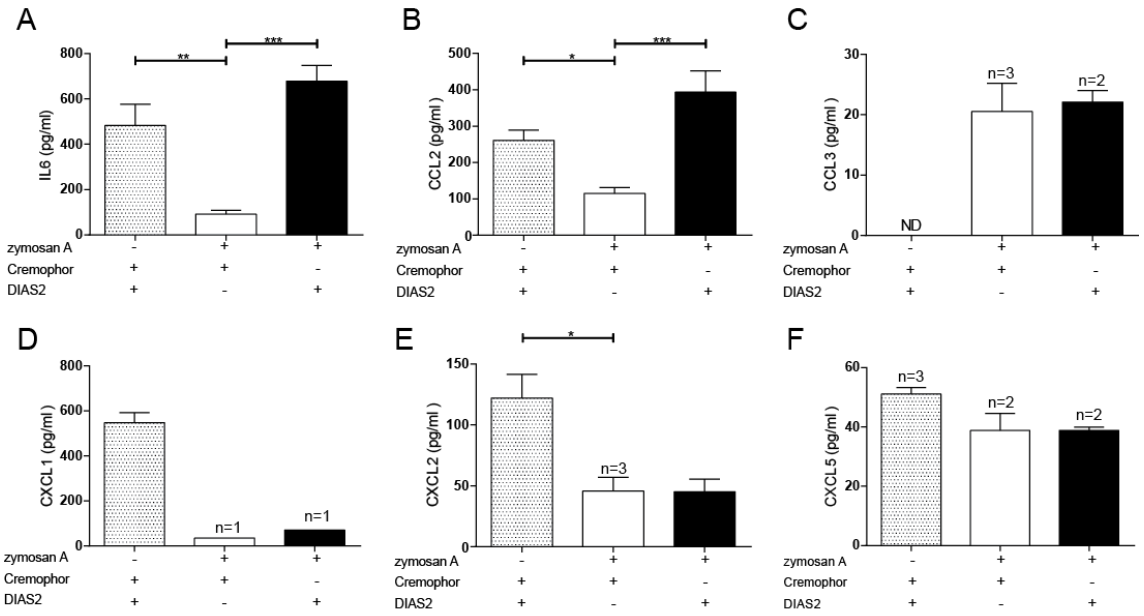


Figure 3.4. DIAS2-induced pro-inflammatory cytokine and chemokine production in the peritoneal cavity in the zymosan-induced peritonitis model is attenuated at a lower dose. IL-6 (A), CCL2 (B), CCL3 (C), CXCL1 (D), CXCL2 (E) and CXCL5 (F) levels from one experiment with 5 animals per group unless otherwise stated on top of the bars. Data are mean +SEM analysed with one-way ANOVA and Dunnett's post hoc test, ND not detectable, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

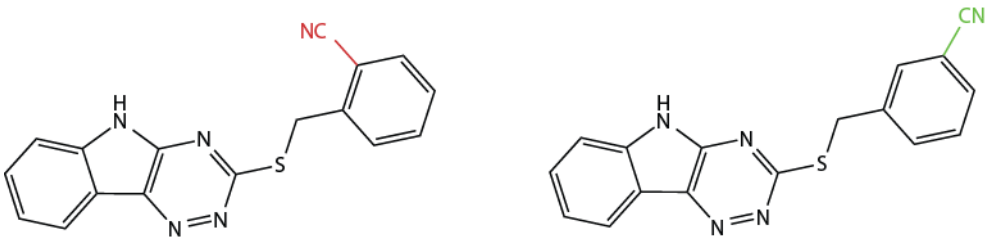
vehicle, 20 mg/kg DIAS2 or equimolar dose of Compound 13 for 2 hours after injection of 100 ug zymosan i.p. for 4 hours (Figure 3.6A). I then sacrificed the animals and proceeded with Flow Cytometry analysis and ELISA quantification of soluble mediators.

Both DIAS2- and Compound 13-treated mice had reduced percentages of monocytes compared to vehicle-treated animals, whereas the percentage of neutrophils remained unaffected between treatments (Figure 3.6B). Absence of F4/80 staining in mice from all three treatment groups confirmed the technical success of the experiments (Figure 3.6C). In addition, Compound 13, but not DIAS2 significantly reduced the numbers of PECs recruited to the peritoneal cavity in comparison with the vehicle-treated animals (Figure 3.6D). The reduction was significantly different from that induced by DIAS2.

However, the inactive compound behaved differently than DIAS2. Although I saw a reduction of the number of PECs in the peritoneum translating into a decrease in the number of monocytes recruited in DIAS2-treated animals, Compound 13 induced a subtle reduction in monocyte numbers (Figure 3.6E). Surprisingly, in this set of experiments, both DIAS2 and Compound 13 inhibited the number of neutrophils migrating to the peritoneal cavity compared to vehicle-treated mice (Figure 3.6F). This effect was statistically significant in both treatments.

3.3.6 Inactive compound does not induce inflammation in the peritoneal cavity

I next assessed the levels of pro-inflammatory mediators in the cell-free exudates. DIAS2-treated animals presented with the overt inflammatory response seen before with



DIAS2 Compound 13

Figure 3.5. Chemical structure of CB2R-selective agonist DIAS2 and inactive analogue Compound 13. 3-cyano substituted derivative exhibits significantly less activity at CB2R (EC_{50}=6450 nM) in comparison to the 2-cyano substituted counterpart DIAS2 (EC_{50}=112 nM) as assessed in cAMP reduction in vitro assays with hCB2R-transfected CHO cells.

increased secretion of IL-6, CCL2, CXCL1, CXCL2 and CXCL5 compared to vehicle-treated mice (Figure 3.7A-F). On the contrary, Compound 13 administration to mice after zymosan treatment did not enhance the levels of any of the cytokines or chemokines studied and all mediators had comparable levels with the vehicle-treated animals.

3.4 Discussion

The starting point for the *in vivo* experiments of this chapter was the potential anti-inflammatory effects of the novel CB2R-selective agonist DIAS2 on leukocyte recruitment to sites of acute inflammation. The model I used to answer this question has been widely used to assess leukocyte recruitment and pro-inflammatory mediator production. In addition, peptides and small compounds have been tested in anti-inflammatory or pro-resolution studies (McArthur *et al.* 2015;Perretti *et al.* 1993).

Cannabinoids are highly lipophilic compounds (Dewey 1986;Paton 1973) and therefore the design of drug formulation and delivery of DIAS2 to the animals was pivotal for the success of the study. In my hands, DIAS2 was soluble up to 80 uM in DMSO, slightly less soluble than the CB2R-selective agonists JWH-133 and HU-308. I tested DIAS2 solubility in a number of solvents including β-cyclodextrin, Tocrisolve, Cremophor solution and mineral oil. Cremophor solution is a well-travelled drug delivery vehicle and it did not induce inflammation when administered on its own *in vivo*. As a result, all my *in vivo* experiments with DIAS2 and Compound 13 in the zymosan-induced peritonitis model were carried out using Cremophor solution.

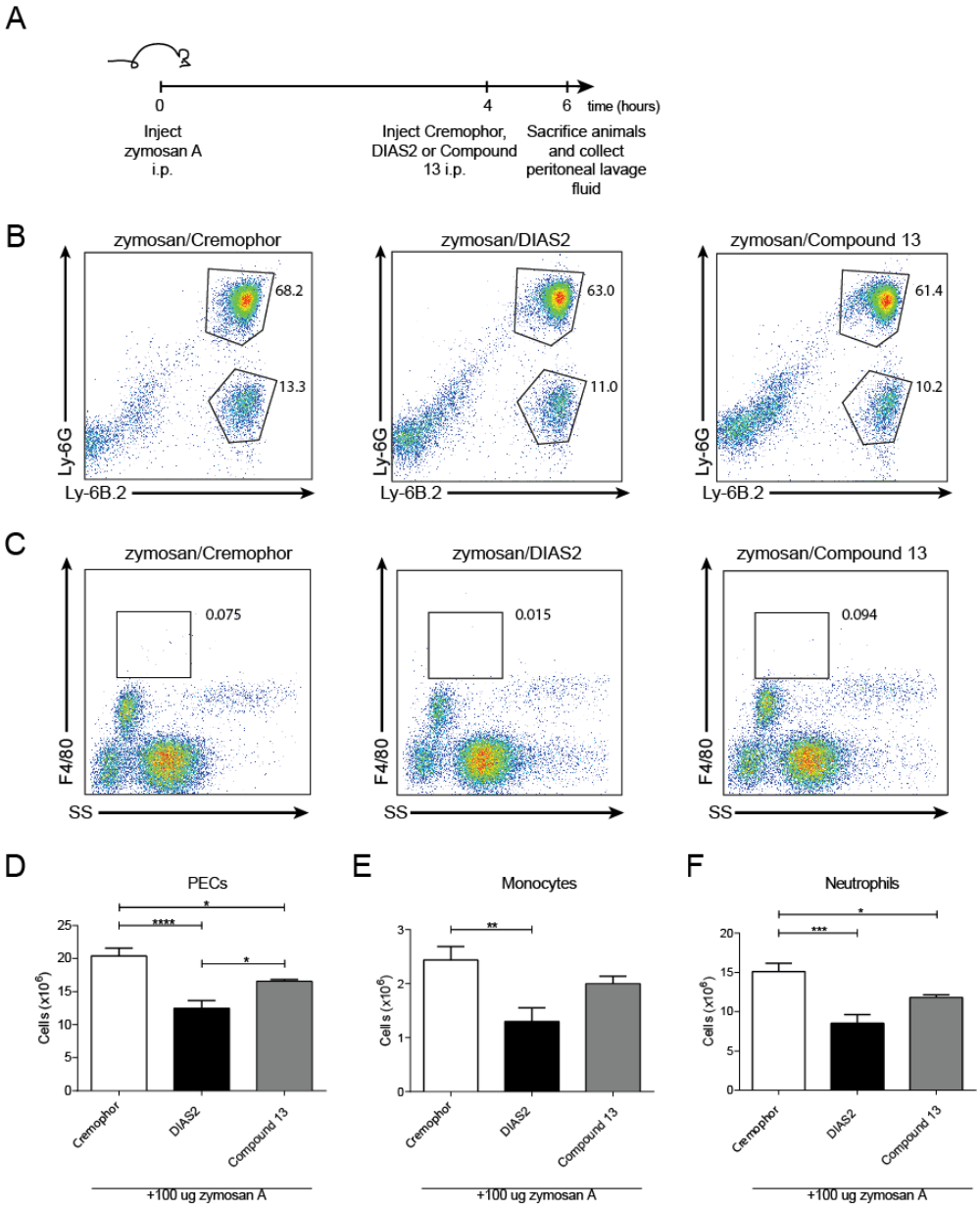


Figure 3.6. Inactive DIAS2 analogue also reduces monocyte recruitment to the peritoneal cavity in the zymosan-induced peritonitis model. 100 ug zymosan A were injected i.p. to male C57BL/6J mice (8-10 weeks old) and 4 hours later vehicle, DIAS2 (20 mg/kg) or Compound 13 (20 mg/kg) were administered i.p. for another 2 hours. Animals were sacrificed and peritoneal lavage fluid was collected for cytokine and chemokine level assessment and peritoneal cavity cell composition evaluation (A). Representative FACS plots from an animal from each group showing neutrophil (Ly-6B.2^{+}Ly-6G^{+}) and monocyte (Ly-6B.2^{-}Ly6G^{+}) populations (B). Representative FACS plots from an animal from each group showing the macrophage (F4/80^{+}) population (C). Total PEC (D), monocyte (E) and neutrophil (F) numbers from 2 independent experiments with 7-8 animals per group. Data are mean+SEM analysed with one-way ANOVA and Dunnett's post hoc test, * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001

Liver microsome experiments with DIAS2 revealed that the agonist's half-life is approximately 45 min (Gianella-Borradori *et al.* 2015). Using this knowledge, I chose to deliver DIAS2 for 2 hours at the 4 hour post zymosan time point where the number of neutrophils reach their peak. By the time the mice were sacrificed, monocytes would continue to accumulate creating an intervention window for DIAS2 to exert a monocyte-specific anti-migratory effect (Regan-Komito 2015).

Initial experiments by Dr. Ivy Christou determined that 20 mg/kg was a suitable dose to observe a reduction in total cell numbers as well as monocyte numbers (Christou 2012). This dose is also supported by the literature as animals have been shown to need higher doses of Δ^9-THC in order to show the similar effects as in humans because of their higher metabolic rate (Paton 1973).

DIAS2 showed a monocyte-specific effect as neutrophil numbers remained unaffected in the DIAS2-treated mice compared to the vehicle-treated controls. The effect size was remarkable and given the short half-life of the compound this result was promising. The ability of DIAS2 to impair monocyte recruitment to the peritoneal cavity might be explained in two possible ways; either DIAS2 inhibits monocyte-recruiting chemokines secreted by a cell type in the peritoneal cavity or it acts in a cell-intrinsic manner in monocytes.

The possibility that DIAS2 acts on other cell types present at the particular time point cannot be excluded. The peritoneal cavity is home to tissue-resident macrophages and mast cells which upon activation secrete pro-inflammatory factors to alert the host that it is under attack. Nevertheless, as I showed in this study resident macrophages leave

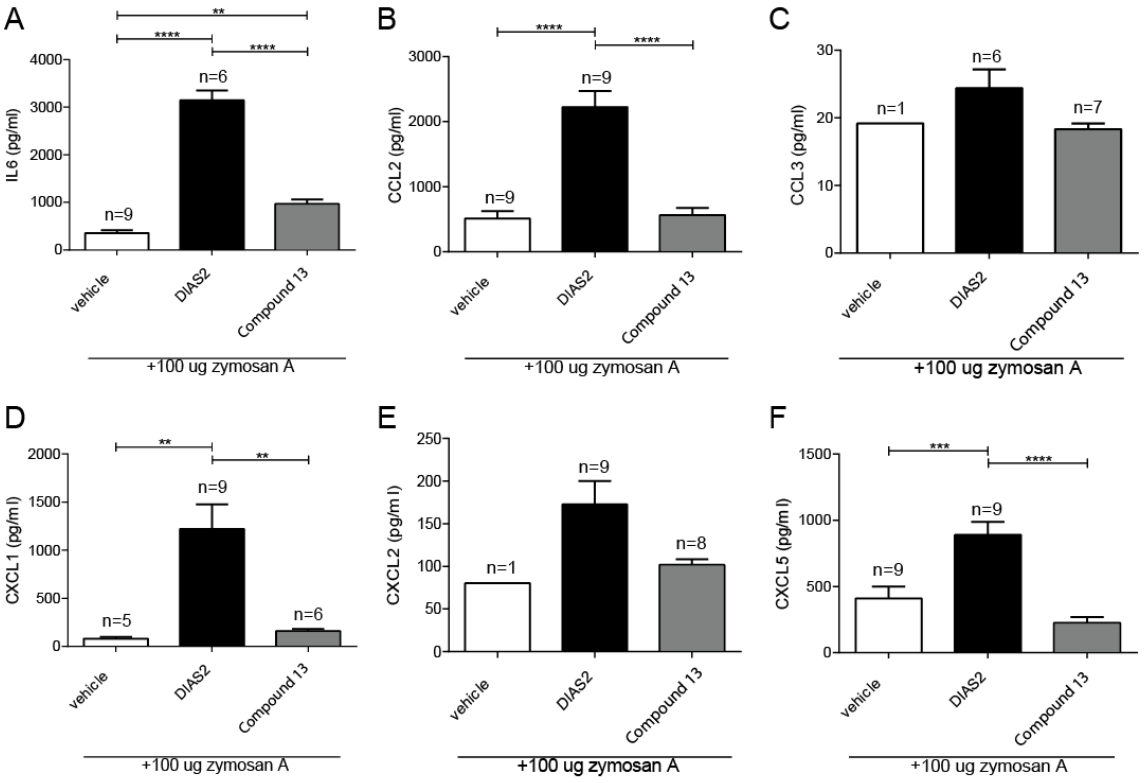


Figure 3.7. Inactive DIAS2 analogue does not cause cytokine and chemokine production in the peritoneal cavity in the zymosan-induced peritonitis model. IL-6 (A), CCL2 (B), CCL3 (C), CXCL1 (D), CXCL2 (E) and CXCL5 (F) levels from 2 independent experiments with 10 animals per group unless otherwise stated on top of the bars. Data are mean+SEM analysed with one-way ANOVA and Dunnett's post hoc test, ND not detectable, ** p<0.01, *** p<0.001, ****p<0.0001

the peritoneal cavity under inflammatory conditions so it is highly unlikely that DIAS2 might reduce the number of recruited monocytes via indirectly acting on resident macrophages. The possibility that DIAS2 might act on neutrophils to decrease the production of monocyte-recruiting chemokines should however be further investigated.

To gain insight on the mechanism via which DIAS2 mediates its anti-inflammatory effect, I assessed the levels of pro-inflammatory mediators in the peritoneal cavity. To my surprise, DIAS2-treated animals that also received zymosan had elevated levels of IL-6, CCL2, CCL3, CXCL1 and CXCL2 in comparison with the mice that received vehicle at 4 hours. This suggests that the mice that were treated with DIAS2 underwent a second wave of inflammation which was defined by the upregulation of the aforementioned soluble mediators.

To explain this unexpected result of reduced monocyte numbers, but increased monocyte- and neutrophil-recruiting mediators in DIAS2-treated animals, I titrated the dose of DIAS2 administered to the animals to 2.5 mg/kg. I therefore tested the hypothesis that the induction of the pro-inflammatory mediators in DIAS2-treated mice was a non-specific effect caused by the potentially high dose of the compound injected in a more hydrophilic environment than what it could tolerate.

The same experimental design was employed and analysis revealed that low dose of DIAS2 showed a partial upregulation of cytokines and chemokines in the peritoneal cavity compared to vehicle-treated animals, but the monocyte-specific effect was completely lost. Although the levels of the mediators measured here were lower than in the 20 mg/kg DIAS2-treated animals, the inflammation in the peritoneal cavity was still present.

In addition, in mice treated with 2.5 mg/kg DIAS2 and vehicle, a population of resident macrophages left the cavity which argues that DIAS2 was recognized as a threat by these cells. In fact, at the end of the experiments neutrophils were recruited to the cavity and the levels of neutrophil-recruiting chemokines, such as CXCL1 and CXCL2 were significantly higher compared to mice receiving zymosan and vehicle.

To finally conclude whether DIAS2-mediated reduction in monocyte numbers was specific, I used another compound with the same chemical structure backbone to DIAS2 which was inactive in CB2R cAMP assays. Compound 13 was the ideal control as it is a stereochemical DIAS2 analogue and its activity at CB2R is extremely low (Gianella-Borradori *et al.* 2015).

Side-by-side comparison between DIAS2 and Compound 13 demonstrated that DIAS2 biological effect was not due to CB2R activation, but was caused by a yet unidentified reason. What made me come to this conclusion was the fact that both agonists reduced the number of total cells and monocytes recruited to the peritoneal cavity. If DIAS2 was acting via CB2R, Compound 13 should show minimal, if any, reduction in both total cell and monocyte numbers.

Moreover, both DIAS2 and Compound 13 decreased the number of neutrophils migrating to the cavity. This result was even more alarming as I had not seen this effect in the previous experiments I carried out. If DIAS2 acted only via CB2R, I would have seen a reduction in the number of monocytes, but not neutrophils in this experiment. The lack of reproducibility in this experiment raised concerns about the selectivity of the compound *in vivo*.

Interestingly, Compound 13 did not induce any of the pro-inflammatory mediators assessed in the peritoneal cavity as compared to 20 mg/kg DIAS2. This finding is

difficult to interpret as lack of regulation of cytokines and chemokines by Compound 13 implies that the effect seen with DIAS2 is CB2R-associated. Therefore it is possible that CB2R promotes the secretion of cytokines and chemokines in the zymosan-induced peritonitis model. Relevant studies which utilised the peritonitis model have shown that HU-210 and WIN55,212-2 delay secretion of chemokines, such as CCL2, MIP-2 and CXCL1 and recruitment of neutrophils (Smith *et al.* 2001).

A possible explanation of DIAS2 behaviour *in vivo* is that it drops out of solution when exposed in the aqueous environment of the peritoneal cavity. Such a phenomenon would create insoluble aggregates in the peritoneum which may activate the inflammasomes in already recruited neutrophils, setting off a second wave of inflammation and thus masking any real anti-inflammatory effects DIAS2 might possess (Vanaja *et al.* 2015). This could be a significant technical limitation in my efforts to critically assess the potential anti-inflammatory effect of CB2R-selective ligands. From all my experiments using DIAS2 and Compound 13 in interventional studies, I conclude that the biggest technical hurdle to test my hypothesis that the CB2R is anti-inflammatory is their poor solubility.

In summary, in this chapter I have shown that DIAS2 reduces the recruitment of monocytes to the peritoneal cavity in a zymosan-induced peritonitis model. The enhancement of pro-inflammatory mediator levels observed in the peritoneal cavity and the chemical similarity in responses between DIAS2 and its inactive analogue, Compound 13, suggest that DIAS2 has a non-specific effect on monocyte migration, the side effects of which include the activation of peritoneal cells. In order to conclusively determine whether DIAS2 has anti-inflammatory effects on primary murine

macrophages, I proceeded with *in vitro* functional inflammation assays on BMDMs which are discussed in Chapter 4.

3.5 Limitations of the study

The experiments described in this chapter aimed to decipher the anti-inflammatory potential of our novel CB2R – selective agonist DIAS2. Unfortunately, as a cannabinoid agonist DIAS2 suffers from the same caveats all cannabinoids do. Its physicochemical characteristics are a major limitation. DIAS2 exhibits high lipophilicity and low solubility in hydrophilic solvents. Even though I used Cremophor as the optimal solvent, DIAS2 short half-life was a limiting factor in the *in vivo* experiments carried out. My experiments argue against DIAS2 being used in pre-clinical studies as a therapeutic agent.

Chapter 4: The effects of CB2R-selective agonists on macrophage functions during inflammation driven by LPS and IFN-γ

4.1 Introduction

4.1.1.1 Origins and tissue distribution of the macrophage

The macrophage is an immune cell of the host's innate immune system, originally studied by Elie Metchnikoff for its ability to ingest and kill microorganisms in a process called phagocytosis (Metchnikoff 1884). Research into the ontogeny of these immune cells has been the objective of elegant experimental designs, including fate-mapping studies, reporter animals, bone marrow transplantation and parabiosis techniques (Epelman *et al.* 2014). There are three waves of hematopoietic progenitors during embryonic development that give rise to tissue-resident macrophages (Perdiguero and Geissmann 2015).

First, a primitive population emerges at the E7 yolk sac in mice and has only a myeloid potential. At E8.25 the hemogenic endothelium of the yolk sac gives rise to the erythro-myeloid progenitors (EMPs) which migrate to the fetal liver from E9 (Perdiguero and Geissmann 2015) and differentiate into erythrocytes, macrophages, granulocytes, mast cells and megakaryocytes. Finally, hematopoietic stem cells (HSCs) from the hemogenic endothelium of the aorto-gonado-mesonephros region migrate to the fetal liver from E12.5 until definitive hematopoiesis shifts to the bone marrow (Perdiguero and Geissmann 2015). These cells co-exist with and replace EMP-derived erythrocytes, monocytes, granulocytes and mast cells postnatally with the exception of macrophages.

In mammals, macrophages reside in most, if not every, tissues; microglia in the brain, Kupffer cells in the liver, alveolar macrophages in the lungs are only some of the

populations of the diverse macrophage network (Murray and Wynn 2011). Their job is to maintain homeostasis and respond to environmental danger signals. In particular, under steady state conditions tissue-resident macrophages perform various roles; osteoclasts in the bones specialise in bone resorption, splenic macrophages process heme and iron from senescent red blood cells (Okabe and Medzhitov 2015) and intestinal macrophages constitutively secrete IL-10 to maintain a regulatory homeostatic environment in the gut.

The tissue-specific roles resident macrophages play are imprinted on them by their micro-environment via the expression of tissue-specific transcription factors. For instance, TGF-β and IL-34 in the brain induce *Mef2c* expression in microglia, retinoic acid in the peritoneum induces *Gata6* expression in peritoneal macrophages and colony stimulating factor 2 (CSF-2) in the lungs initiates expression of *Pparg* in alveolar macrophages (Amit *et al.* 2015). In this way, although all the different macrophage populations belong to the same lineage, they have their own identity which determines their ability to fully adjust to the needs of their niche.

4.1.1.2 Macrophage responses during inflammation

Although the homeostatic needs tissue-resident macrophages satisfy are crucial for development and maintenance of host tissues, these cells are key players in monitoring their environment for danger signals and rapidly deploy a panel of immune tools to combat any threats to the host.

Danger signals for macrophages can either be microbial components or endogenous molecules, presence of which implies loss of cell integrity (Glass and Natoli 2015). The macrophages can sense the existence of these threats via a plethora of surface and

intracellular PRRs which can detect microbial components, e.g. zymosan (TLR2), LPS (TLR4), double-stranded RNA (RIG-I), DNA (cyclic GMAP-AMP synthase (cGAS)); also products of sterile injury, such as high mobility group box 1 protein (HMGB1) and adenosine triphosphate (ATP) (Bianchi 2007;Brubaker *et al.* 2015;Takeuchi and Akira 2010).

Upon encounter of a danger signal, macrophages are activated and aim to restore homeostasis in the affected tissue. Traditionally, it was believed that macrophages could acquire two distinct phenotypes depending on the activating stimuli. The first was called M1 or classical pathway and is associated with priming of macrophages with LPS and IFN-γ, while the second was called M2 or alternative pathway and is linked to priming of macrophages with IL-4 and IL-13 (Ginhoux *et al.* 2015).

M1 macrophages have anti-microbial properties and secrete pro-inflammatory mediators, such as TNF-α, IL-6, CCL2, CXCL9, CXCL10. They produce ROS and NO which are toxic for microorganisms and engulf foreign material to present to MHC-II molecules on their surface and activate T cells (Unanue 1984). Antigen presentation together with secretion of IL-12 and IL-23 induces T cell polarisation to the T_H1 and T_H17 phenotype, respectively which promote inflammation and pathogen clearance (Biswas and Mantovani 2010;Ginhoux *et al.* 2015;Murray and Wynn 2011).

In contrast, M2 macrophages secrete CCL17, CCL22, CCL24, high levels of IL-10 and TGF-β, express IL-1ra on their surface and skew the adaptive immune responses to the T_H2 lineage. They are associated with parasite immunity, wound healing, fibrosis and resolution of inflammation (Biswas and Mantovani 2010;Ginhoux *et al.* 2015;Murray and Wynn 2011).

Recently, studies have revealed that the dichotomy of the M1/M2 paradigm is misleading; it is now becoming widely accepted that macrophages display a spectrum of phenotypes upon acute activation with LPS, IFN-γ, IFN-β, TNF-α and PGE_2 (M1 paradigm) or stimulation with IL-4, fatty acids and macrophage growth factors (M2 paradigm) (Xue *et al.* 2014). It has therefore been suggested that macrophage activation nomenclature should change and include the stimuli used to activate the macrophages *in vitro* or, if applicable, *in vivo* (Martinez and Gordon 2014).

4.1.2 Effects of cannabinoids on macrophage activation

The role of CB2R in macrophage activation has been extensively investigated with the use of a range of cannabinoid ligands. Broad anti-inflammatory effects of cannabinoids on macrophages have been reported and are summarised below, including the reduction of pro-inflammatory mediator secretion, decrease of cell oxidative burst, the impairment of antigen loading to antigen presentation molecules and the enhancement of pathogen phagocytosis.

4.1.2.1 Macrophage cytokine and chemokine secretion

The endocannabinoids 2-AG and AEA have been documented to reduce the uptake of oxidised low density lipoproteins (oxLDLs) by human foam cells, and like the selective CB2R agonist JWH-015, AEA ameliorates the secretion of TNF-α, IL-12, IL-23 and IL-10 (Chiurchiu *et al.* 2014;Correa *et al.* 2009). In addition, Tomar *et al*. found that JWH-133 polarises peritoneal macrophages to the M2 activation state via microRNA inhibition and subsequent IL-10 secretion increase (Correa *et al.* 2010;Tomar *et al.*

2015). In contrast, WIN55,212-2 was reported to reduce TNF-α secretion by oxLDL-stimulated macrophages in a CB2R-dependent way (Hao *et al.* 2010).

In accordance with these studies, CB2R KO BMDMs have higher CCR1, CCR5, and CCL2 expression in comparison with WT cells which leads to enhanced adherence of macrophages to extracellular matrix proteins and migration *in vitro* (Molica *et al.* 2012). Furthermore, CB2R KO animals have a more severe phenotype than WT controls in a sepsis model. Macrophage secretion of TNF-α, IL-6 and HMGB1 was reduced in macrophages treated with the CB2R-selective agonist GW405833 and this effect was lost in CB2R KO mice (Gui *et al.* 2013).

4.1.2.2 Macrophage oxidative burst

The effects of cannabinoids on macrophage oxidative burst during inflammation have been investigated in regards to both ROS and NO production. CB1R and CB2R have opposing roles in macrophage oxidative burst in mice. In particular, CB1R triggers p38 phosphorylation and functions to enhance ROS production, while CB2R activation inhibits CB1R-mediated ROS production via the Ras family protein Rap1 (Han *et al.* 2009).

Along these lines, WIN55,212-2 reduces ROS production by macrophages via CB2R (Hao *et al.* 2010). Furthermore, the non-psychotropic phytocannabinoid Cannabigerol reverses the clinical picture in a murine colitis model and can effectively reduce nitrite production by macrophages (Borrelli *et al.* 2013). Conversely, the cannabis component Δ^8-THC cytotoxic effects in the J774 macrophage cell line are mediated via CB2R, possibly via a ROS intermediate step (Yamaori, 2013, Toxicology).

NO production by activated macrophages is attenuated by CB2R-selective agonists. For example, JWH-133 decreases nitrite levels in LPS-stimulated thioglycollate-elicited macrophages (Romano *et al.* 2013) and JWH-015 reduces NO release in primary microglia cells (Merighi *et al.* 2012). WIN55,212-2 ameliorates the secretion of NO both in LPS-primed RAW264.7 cells, while like AEA, it can rescue neurons from neurotoxicity *in vivo* (Eljaschewitsch *et al.* 2006;Ross *et al.* 2000).

4.1.2.3 Macrophage antigen presentation

The effects of CB2R activation on macrophage antigen presentation has been intensely investigated. In particular, Δ^9-THC impairs T cell activation and memory cell generation by abrogating DC-released IL-12 and DC phagocytic capacity (Roth *et al.* 2015). Moreover, the same agonist ameliorates the antigen presentation machinery expression in bone marrow-derived DCs (BMDCs) (Karmaus *et al.* 2013). This defect may lead to impaired recruitment of antigen presenting cells to the lungs during influenza infection in mice and compromise the secretion of pro-inflammatory cytokines by T cells and NK cells. Finally, JWH-015 selectively inhibits IFN-γ-mediated CD40 expression by microglia cells in a CB2R-dependent manner via c-Jun N-terminal kinase (JNK)-STAT1 signalling modulation (Ehrhart *et al.* 2005).

McCoy *et al*. looked into the mechanistic aspects of this inhibition and they showed that Δ^9-THC effects are stereoselective and CB2R-dependent (McCoy *et al.* 1999), However, the agonists were found to target the processing of antigen in macrophages rather than antigen presentation itself (Mccoy *et al.* 1995). This means that cannabinoids affect macrophage and not T cell function.

4.1.2.4 Macrophage phagocytosis

The role of endocannabinoids in regulation of macrophage phagocytosis has been studied by several research groups. Endocannabinoids have been shown to augment macrophage phagocytosis *in vitro*. In particular, 2-AG enhances phagocytosis of zymosan particles by thioglycollate-elicited peritoneal macrophages in a PI3K- and CB2R-dependent manner (Shiratsuchi *et al.* 2008). In addition, it augments phagocytosis of opsonised zymosan in a CB2R- and β2 integrin-dependent manner (Gokoh *et al.* 2007). AEA treatment of primary macrophages and cell lines showed that this endocannabinoid also induces phagocytosis of zymosan bioparticles, but in a CB1R-dependent manner (Mai *et al.* 2015).

Synthetic CB2R agonists have been reported to behave like endocannabinoids to enhance macrophage phagocytosis. For instance, THP-1 cells remove Aβ amyloid peptides from frozen human Alzheimer's disease sections when treated with the CB2R-selective agonist JWH-015, an effect which is lost upon co-incubation of the cells with the CB2R-specific antagonist SR144528 (Tolon *et al.* 2009). Other mixed agonists, such as CP55,940 and WIN55,212-2 have also been reported to increase phagocytosis of zymosan (Gokoh *et al.* 2007).

Data from studies with JWH-133 are contradictory. Treatment of cirrhotic rats with JWH-133 reverses the disease manifestations and normalises the compromised phagocytosis of translocated bacteria (Yang *et al.* 2014). However, *in vitro* administration of JWH-133 to macrophage cell lines has no effect on zymosan phagocytosis (Mai *et al.* 2015).

4.2 Aim of the chapter

The aim of the experiments reported in this chapter was to study the effect of CB2R-selective agonists on macrophage behaviour during inflammatory conditions and they derived from the ambiguous *in vivo* results of DIAS2 in the zymosan-induced peritonitis model. Having reviewed the extensive literature on CB2R, I decided to use *in vitro* assays where cannabinoids were previously reported to bear anti-inflammatory properties and critically assess their functions. My original hypothesis was that selective agonists would activate CB2R and promote an anti-inflammatory phenotype and pro-phagocytic effects in macrophages.

I decided to carry out my experiments with primary cells and I therefore chose BMDMs. I used the TLR4 agonist LPS and measured secreted cytokine, chemokine and nitrite levels, antigen presentation and *E.coli* particle phagocytosis. Simultaneous treatment of macrophages with JWH-133, HU-308 and DIAS2 as representative CB2R-selective agonists was carried out to delineate whether CB2R-selective ligands modulate macrophage functions *in vitro*.

4.3 Results

The experiments shown in panels Figure 4.2A, B and D were performed in collaboration with Mr Lewis Taylor.

4.3.1 CB2R-selective ligands activate the receptor at nanomolar concentrations

To address the effects of CB2R-selective ligands on primary macrophages, I first compared the expression of *Cnr2* mRNA in three macrophage populations; Bio-gel-elicited murine macrophages, BMDMs and the murine microglia BV-2 cell line. I found

that Bio-gel-elicited macrophages and BMDMs express double the level of *Cnr2* mRNA in comparison to BV-2 cells (Figure 4.1A). I decided to use BMDMs in this study due to the higher cell yield and greater purity after differentiation.

For this purpose, I incubated bone marrow cells in 15% L929-conditioned medium and yielded 30 million BMDMs per mouse leg after 7 days (Figure 4.1B). Cells acquired the typical macrophage morphology (Figure 4.1C) and expressed the surface markers CD45, F4/80 and CD11b, but not the monocyte marker Ly-6B.2 (Figure 4.1D-E).

To find out the concentration where CB2R-selective agonists achieve maximal activation of the receptor, I treated human CB2R-transfected CHO cells with a range of concentrations of the CB2R-selective ligands JWH-133, HU-308 and DIAS2, and assessed signature intracellular assays for CB2R activation, such as β-arrestin recruitment (Figure 4.2A) and reduction of intracellular cAMP levels (Figure 4.2B). The cannabinoid ligands recruited β-arrestin [EC_{50} (JWH-133)= 17 nM, EC_{50} (HU-308)= 9.5 nM, EC_{50} (DIAS2)= 24 nM] and reduced intracellular levels of cAMP [IC_{50} (JWH-133)= 33 nM, IC_{50} (HU-308)= 100 nM, IC_{50} (DIAS2)= 72 nM] with different efficiencies.

Finally, I asked which downstream signalling pathways are activated upon CB2R triggering. CB2R-selective ligands failed to induce Ca^{2+} influx from intracellular stores (Figure 4.2C). Taken together, these data show that CB2R-selective ligands are active at the receptor at nanomolar concentrations and mediate both β-arrestin recruitment and reduction of intracellular cAMP levels.

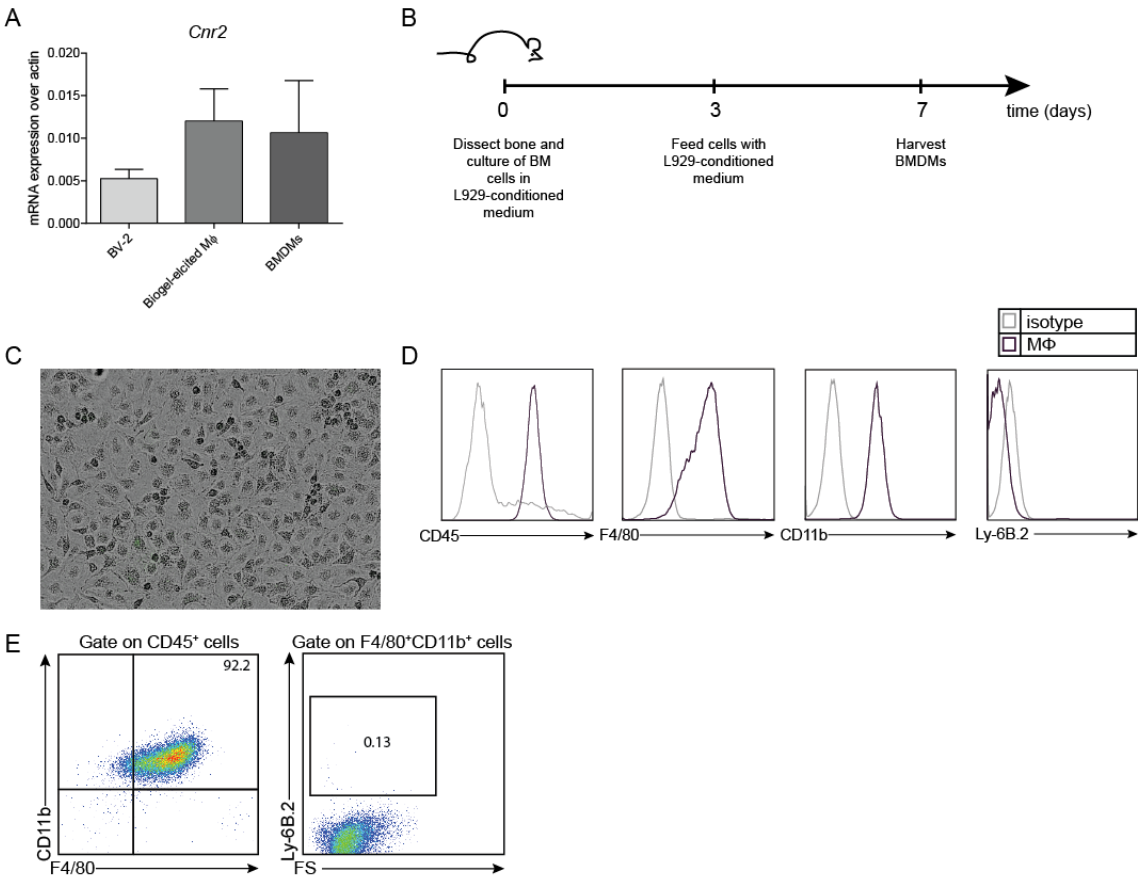


Figure 4.1. BMDMs generated in L929-conditioned medium cultures express *Cnr2*. *Cnr2* expression was assessed in murine BMDMs, Biogel-elicited macrophages and BV-2 microglia cells (A). Male C57BL/6J mice (6-8 weeks old) were sacrificed and bone marrow cells were differentiated to macrophages in L929-conditioned medium for 7 days (B). BMDMs were harvested on day 7 and used in experiments accordingly. Representative image of day 7 harvested BMDMs (20x magnification) (C). Day 7 harvested BMDMs were stained for CD45, F4/80, CD11b and Ly-6B.2 surface expression as a quality control (D and E). FACS plots from one representative experiment are demonstrated with grey curve showing isotype antibody staining and black line respective antigen-specific staining (D). FACS plot from one representative BMDM prep, gated on live, single cells (left panel) and $F4/80^{+}CD11b^{+}$ cells (right panel) (E).

4.3.2 Cytokine and chemokine secretion

Macrophages secrete a number of pro-inflammatory mediators, such as cytokines and chemokines when polarised towards the M1 phenotype. I sought to set up a functional assay to study the effects of CB2R-selective agonists on cytokine and chemokine secretion by BMDMs. I first performed a time course stimulation with LPS and IFN-γ (Figure 4.3A-E). This experiment revealed an optimal window for intervention for most of the chosen readouts at 16 hours. Moreover, stimulation with LPS/IFN-γ and 500 nM of the glucocorticoid Dexamethasone reduced TNF-α, IL-6 and CCL2 secreted levels and significantly decreased the secretion of CCL3 and CCL5, thus validating my assay (Figure 4.3F-J).

I next treated BMDMs with LPS and IFN-γ and 1 uM of the cannabinoids or 500 nM Dexamethasone for 16 hours, and collected the culture supernatants to look for anti-inflammatory effects of cannabinoids on the secretion of cytokines and chemokines. Administration of JWH-133, HU-308 or DIAS2 did not affect the levels of TNF-α, IL-6, CCL2, CCL3, CCL4 or CCL5 (Figure 4.4A-F). In contrast, Dexamethasone significantly reduced the levels of TNF-α, IL-6 and CCL2, and decreased the secreted levels of CCL4 and CCL5. Collectively, my results suggest that macrophage treatment with CB2R-selective ligands does not reduce the secretion of pro-inflammatory mediators by LPS-activated primary macrophages.

4.3.3 Nitrite secretion

One of the downstream signalling targets during inflammation is *Nos2* expression to generate secretion of bactericidal NO, causing a build up of nitrites in the macrophage

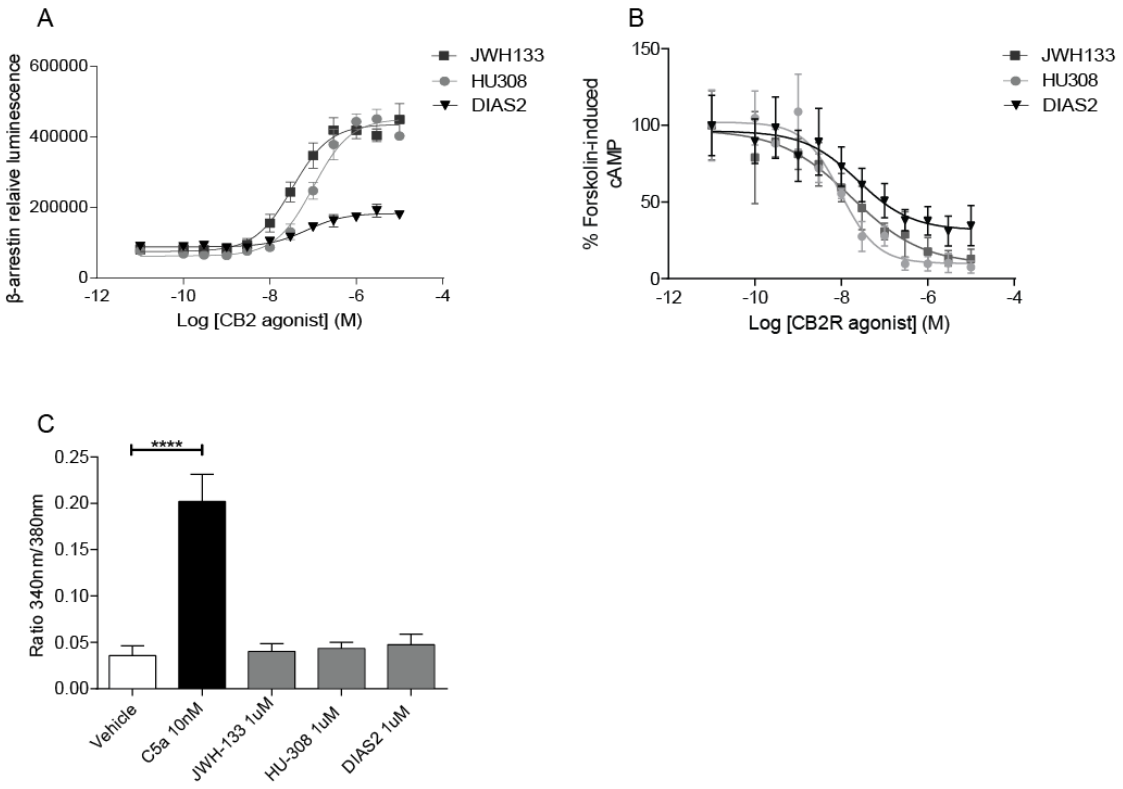


Figure 4.2. CB2R-selective agonists induce distinct signalling pathways upon receptor activation. Human CB2R-transfected CHO cells were treated with a range of CB2R-selective agonists concentrations, and β-arrestin recruitment (A) and intracellular cAMP levels (B) were measured. Calcium influx in murine BMDMs from male C57BL/6J mice (6-8 weeks old) treated with C5a or the CB2R-selective agonists JWH-133, HU-308 and DIAS2 was measured with a fluorescent probe (C). Data are from 3-4 independent experiments. Mean+SEM are represented and one-way ANOVA with Dunnett's post hoc multiple comparisons test was used for data analysis, **** $p<0.0001$

micro-environment (Stuehr and Marletta 1987). The next question I therefore asked was whether CB2R-selective ligands modulate nitrite production by BMDMs.

Time course of BMDMs with LPS and IFN-γ increased levels of nitrite found in the cell culture supernatants under baseline conditions (Figure 4.5A). Cells secreted micromolar levels of nitrites at 16 hours and the iNOS inhibitor 1400W reduced the secretion of nitrite by BMDMs in a dose-dependent manner (Figure 4.5B).

I next administered 1 uM JWH-133, HU-308, DIAS2 or 1400W to LPS and IFN-γ-treated BMDMs for 16 hours and measured the secretion of nitrites. None of the cannabinoids used decreased the secreted nitrite in the BMDM supernatants (Figure 4.5C), suggesting that the nitrite production pathway is not a downstream target of CB2R-selective ligands.

4.3.4 Surface expression of antigen presentation molecules

When macrophages are primed with an infectious stimulus, they upregulate MHC-II molecules and co-stimulatory molecules, such as CD80 and CD86 on their surface in order to present the captured antigen to cells of the adaptive immunity (Unanue 1984). I investigated whether CB2R-selective ligands dampen the ability of macrophages to express antigen presentation molecules on their surface.

BMDMs were treated with LPS and IFN-γ and vehicle or 1 uM of the cannabinoid ligands for 16 hours as before, and cells were stained with antibodies against MHC-II, CD80 and CD86 to quantify protein expression of these markers. As expected, treatment with LPS and IFN-γ significantly upregulated the surface expression of all three markers (Figure 4.6A, C, E and pooled Figure 4.6B, D, F). Nevertheless,

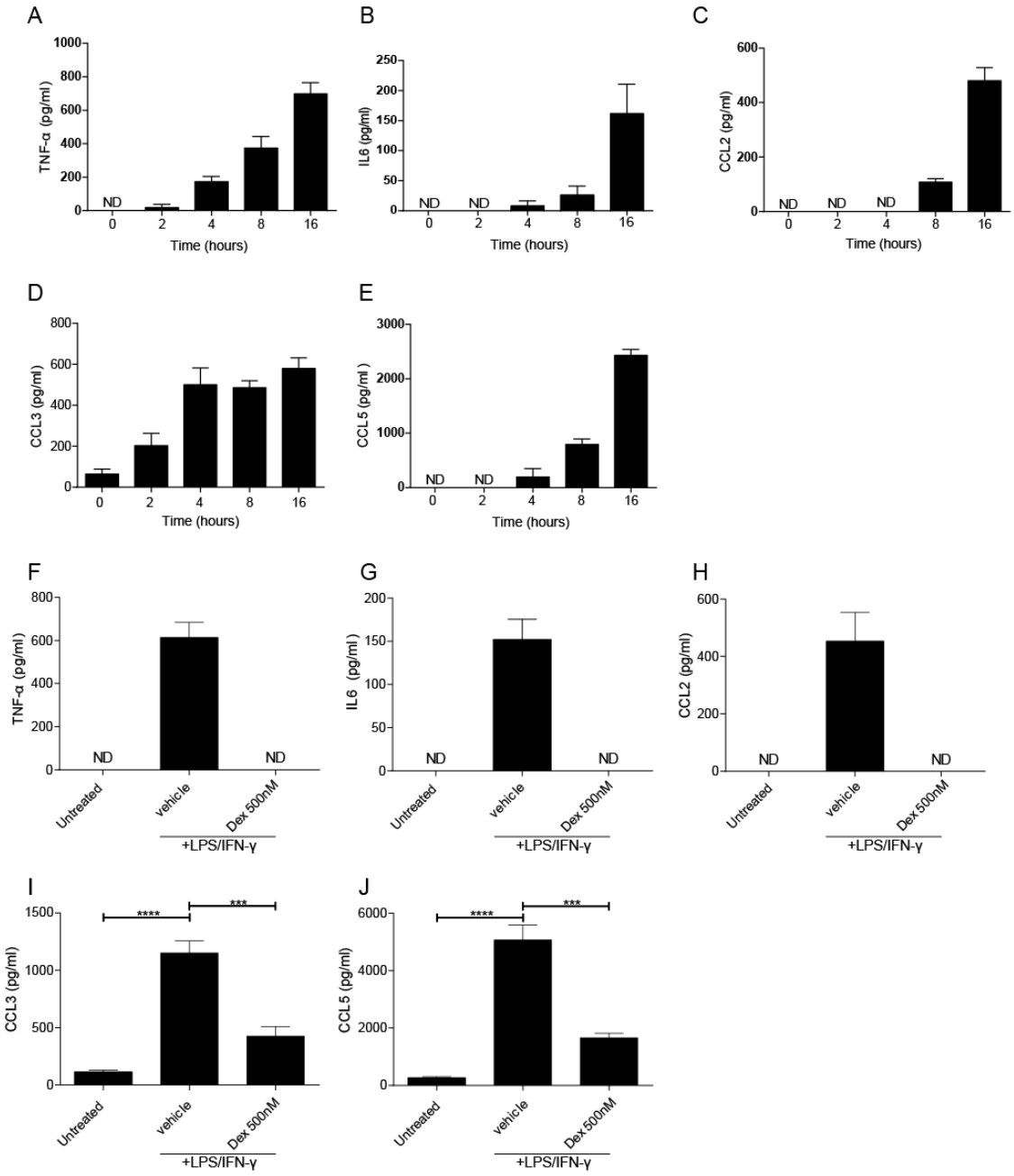


Figure 4.3. Cytokine and chemokine secretion is decreased by Dexamethasone in LPS and IFN-γ-stimulated BMDMs. Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were stimulated with LPS and IFN-γ for 2, 4, 8 and 16 hours, and supernatants were interrogated for TNF-α (A), IL-6 (B), CCL2 (C), CCL3 (D) and CCL5 (E). Dexamethasone decreases pro-inflammatory mediator levels in LPS and IFN-γ- stimulated BMDMs. Dexamethasone (500 nM) was administered alongside LPS and IFN-γ for 16 hours and TNF-α (F), IL-6 (G), CCL2 (H), CCL3 (I) and CCL5 (J) levels were determined in culture supernatants after 16 hours. Data are from 3-4 independent experiments analysed with one-way ANOVA with Dunnett's post hoc multiple comparisons test. Mean+SEM are represented, *** $p<0.001$, **** $p<0.0001$, ND not detected

administration of cannabinoids did not cause a reduction in the mean fluorescence intensity (MFI) levels of either MHC-II, CD80 or CD86 in comparison to the vehicle group (Figure 4.6A, C, E and pooled Figure 4.6B, D, F). In conclusion, CB2R-selective ligands did not modulate antigen presentation molecule and co-stimulatory molecule surface expression in BMDMs.

4.3.5 *E.coli*-triggered phagocytosis

A fundamental macrophage function is to engulf and eliminate pathogens or irritative compounds. To study the effects of CB2R-selective agonists on BMDM phagocytosis, I set up an assay of *E.coli* bioparticle phagocytosis. *E.coli* bioparticles were conjugated to beads carrying a pH-sensitive dye which only fluoresces when localised in an acidic environment, such as that of the phagolysosome.

BMDMs were fed the *E.coli*-conjugated beads during a time course for up to one hour and fluorescence was followed using a plate reader. The results showed that there is a significant induction of bead phagocytosis after 40 minutes (Figure 4.7A). As a negative control, I pre-treated the BMDMs for one hour with the actin inhibitor Cytochalasin D at a range of doses and then administered the bioparticles for a further hour. Cytochalasin D inhibited the fluorescent signal with an IC_{50} equal to 1.65 uM (Figure 4.7B), suggesting that cytoskeletal rearrangements are critical for macrophage phagocytosis.

To find out whether the CB2R-selective ligands enhance macrophage phagocytosis, I pre-treated BMDMs with 1 uM JWH-133, HU-308, DIAS2 or 10 uM Cytochalasin D for one hour and then fed the cells with the *E.coli*-conjugated beads. As shown in Figure 4.7C, the cannabinoids did not augment phagocytosis of this pathogen. Representative

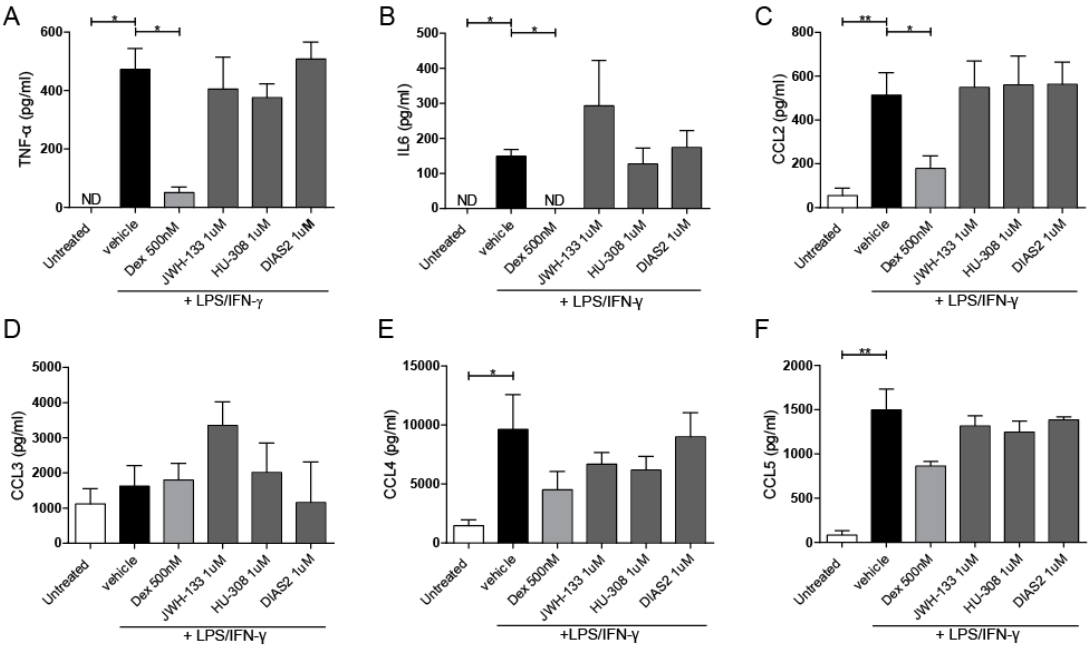


Figure 4.4. CB2R-selective agonists do not reduce pro-inflammatory cytokine and chemokine levels produced following LPS and IFN-γ activation. Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were stimulated with LPS and IFN-γ for 16 hours in the presence of vehicle, Dexamethasone or the CB2R-selective agonists JWH-133, HU-308 and DIAS2. Supernatants were assessed for TNF-α (A), IL-6 (B), CCL2 (C), CCL3 (D), CCL4 (E) and CCL5 (F) levels. Data are from 4-6 independent experiments. Mean+SEM are represented and one-way ANOVA (C, D, E) with Dunnett's post hoc multiple comparisons test or Kruskal-Wallis (A, B, F) with Dunn's post hoc multiple comparisons test were used for data analysis, ND not detected, *p<0.05, ** p<0.01

confocal microscopy pictures from one experiment confirm these findings (Figure 4.7D). Taken together, I conclude that the cannabinoids do not augment phagocytosis of *E.coli* bioparticles.

4.3.6. CB2R KO BMDMs have a comparable inflammatory phenotype to littermate controls

In Chapter 3 I showed that cannabinoids suffer from solubility issues. To rule out any false-negative results attributed to the agonists and to delineate the role of CB2R in macrophage activation, I asked whether deficiency of CB2R in murine BMDMs leads to altered inflammatory responses.

To answer this question, I generated CB2R KO BMDMs and compared them with macrophages from littermate control mice upon LPS and IFN-γ treatment for 16 hours. CB2R KO BMDMs showed a similar cytokine and chemokine profile to WT cells (Figure 4.8A-E). Furthermore, the secreted nitrites between the two genotypes were comparable (Figure 4.8F). Likewise, phagocytosis of *E.coli* bioparticles was similar between WT and CB2R KO BMDMs (Figure 4.8G).

To sum up, macrophage immune responses triggered by LPS and IFN-γ or *E.coli* bioparticles are not altered in cells deficient for CB2R, hinting that this GPCR does not regulate the inflammatory responses of macrophages upon LPS and IFN-γ treatment.

4.3.7. Expression of *Cnr2* in macrophages is significantly reduced upon LPS and IFN-γ stimulation

A plausible explanation for the lack of anti-inflammatory effects of CB2R-selective ligands on primary macrophages is decreased expression of the receptor mRNA which

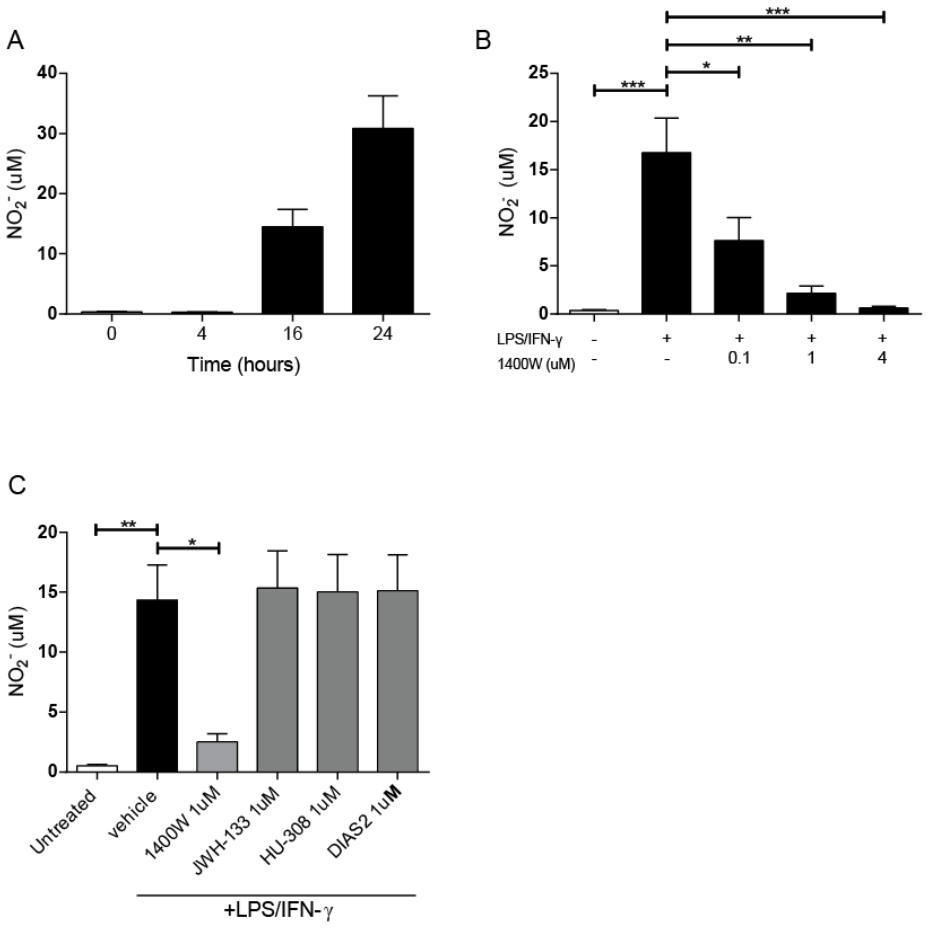


Figure 4.5. Nitrite production by BMDMs is not modulated by CB2R-selective agonists. Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were treated with LPS and IFN-γ for 4, 16 and 24 hours and nitrite levels in the culture supernatant were measured (A). Selective iNOS inhibitor 1400W dampens LPS and IFN-γ-mediated nitrite secretion. Murine BMDMs from male C57BL/6J (6-8 weeks old) were stimulated with LPS and IFN-γ and a range of 1400W concentrations for 16 hours, and nitrite levels were determined (B). Murine BMDMs from male C57BL/6J (6-8 weeks old) were treated with the iNOS inhibitor 1400W or the CB2R-selective agonists JWH-133, HU-308 and DIAS2 for 16 hours and nitrite levels were measured (C). Data are from 3-4 experiments. Mean+SEM are represented and one-way ANOVA with Dunnett's post hoc multiple comparisons test was used for data analysis, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

presumably leads to decreased CB2R cell surface expression. Unfortunately, there are no reliable commercial antibodies to detect CB2R so I relied on gene expression levels to address this hypothesis.

As shown in Figure 4.9A, *Cnr2* mRNA expression is rapidly downregulated in BMDMs upon LPS and IFN-γ administration and remains decreased until 16 hours. Assessment of the other member of the cannabinoid receptor family, *Cnr1*, showed no detectable expression in BMDMs under baseline conditions or following stimulation with LPS and IFN-γ. In contrast, pro-inflammatory mediators, such as TNF-α and IL-6 were significantly upregulated (Figure 4.9B-C).

4.4 Discussion

In this chapter I followed up the *in vivo* CB2R-selective agonist effect on murine monocyte recruitment in Chapter 3 with *in vitro* macrophage activation assays. I designed experiments to test the effects of cannabinoids on all aspects of macrophage biology during inflammation in an unbiased way. The inflammogens I chose to use are the M1 activation state prototypes and are commonly used in macrophage activation assays.

My data suggest that CB2R-selective agonists used at a dose to fully occupy CB2R do not display any anti-inflammatory properties, including cytokine and chemokine secretion, nitrite production, antigen presentation molecule expression and phagocytosis. In addition, the expression levels of *Cnr2* fall rapidly upon LPS and IFN-γ administration, providing a possible explanation why CB2R agonists proved unsuccessful in the experiments.

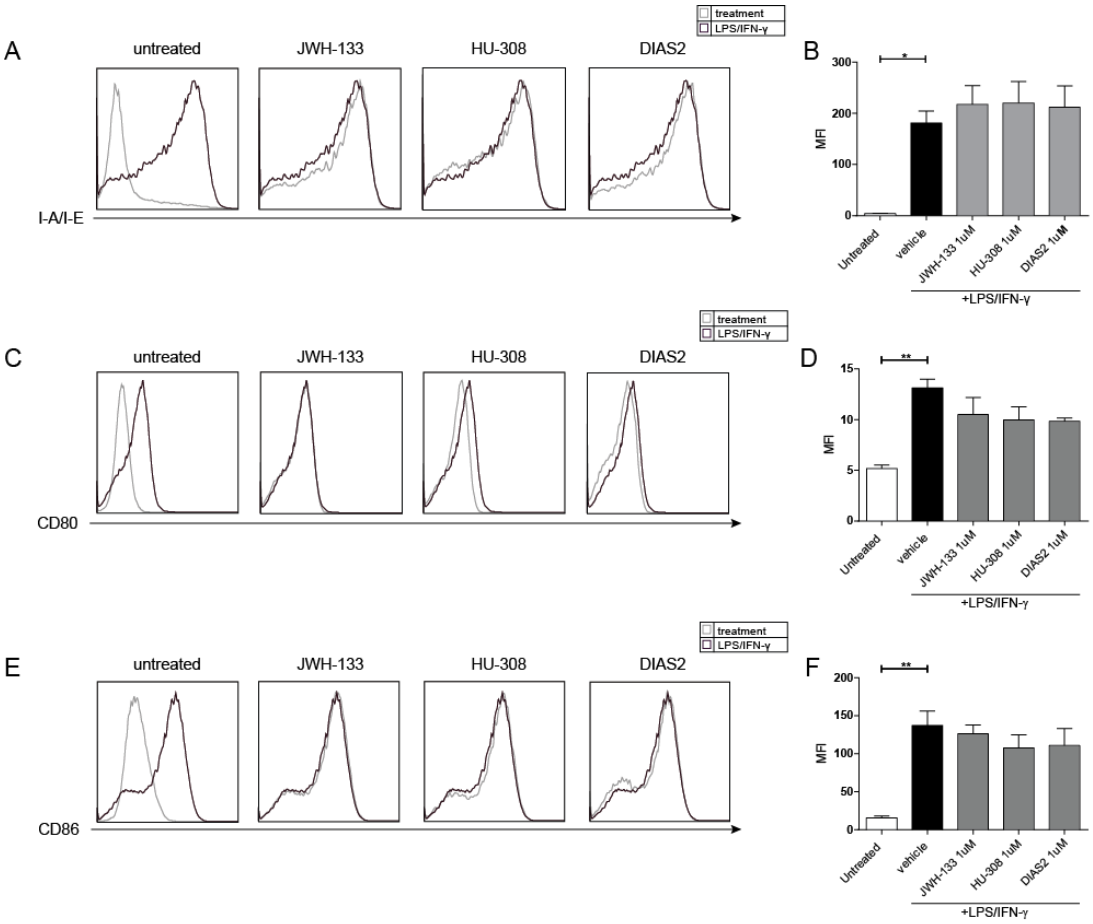


Figure 4.6. CB2R-selective agonists do not modulate antigen presentation molecule expression on macrophage cell surfaces. Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were treated with LPS and IFN-γ in the presence of vehicle or the CB2R-selective agonists JWH-133, HU-308 and DIAS2 for 16 hours and I-A/I-E (top), CD80 (middle) and CD86 (bottom) surface expression was assessed. A histogram plot from a single experiment is shown (A, C, E). Pooled MFI values from 3 independent experiments are shown (B, D, F). Mean+SEM are represented and one-way ANOVA with Dunnett's post hoc multiple comparisons test was used for data analysis, * $p<0.05$, ** $p<0.01$

The choice of the optimal dose of CB2R-selective ligands for my *in vitro* activation assays was crucial. In a report by Taylor *et al*., CB2R-selective agonists, such as JWH-133 and HU-308 used at 10 uM exhibited off-target effects at an unidentified $G\alpha_i$-coupled receptor and induced chemotaxis of Bio-gel-elicited macrophages (Taylor *et al.* 2015). To avoid reporting non-specific activation of other GPCR, I conducted the concentration-response assays of cAMP reduction and β-arrestin recruitment.

One possible criticism of the experiments in Chapter 4 is the ambiguous relevance of the expression levels of human CB2R in CHO-transfected cells as opposed to that in primary murine BMDMs. It is true that, although the level of *Cnr2* expression was normalised by the manufacturer to yield consistent results of high expression (personal communication), the *Cnr2* copy numbers in the cell line used may be significantly different from that in BMDMs, leading to an underestimation of the true optimal agonist doses to fully occupy macrophage CB2R.

The ability of the CB2R agonists I used to reduce cAMP levels and recruit β-arrestin with different efficiency reflects the idea of biased agonism which many GPCR ligands demonstrate at their cognate receptor (Rajagopal *et al.* 2010;Reiter *et al.* 2012). Atwood *et al*. carried out a side-by-side comparison of CB2R-selective and CB1R/CB2R agonist-induced activation of downstream signalling pathways and showed that JWH-133 and CP55-940 are more efficient in β-arrestin recruitment to the cell membrane in comparison with the other compounds used in that study (Atwood *et al.* 2012).

In addition, JWH-133, 2-AG and several cannabinoids activate ERK1/2 (Atwood *et al.* 2012). Moreover, JWH-015 activates JNK phosphorylation in a microglial cell line, a finding which suggests that members of the MAP kinase family could be activated in my experiments (Merighi *et al.* 2012).

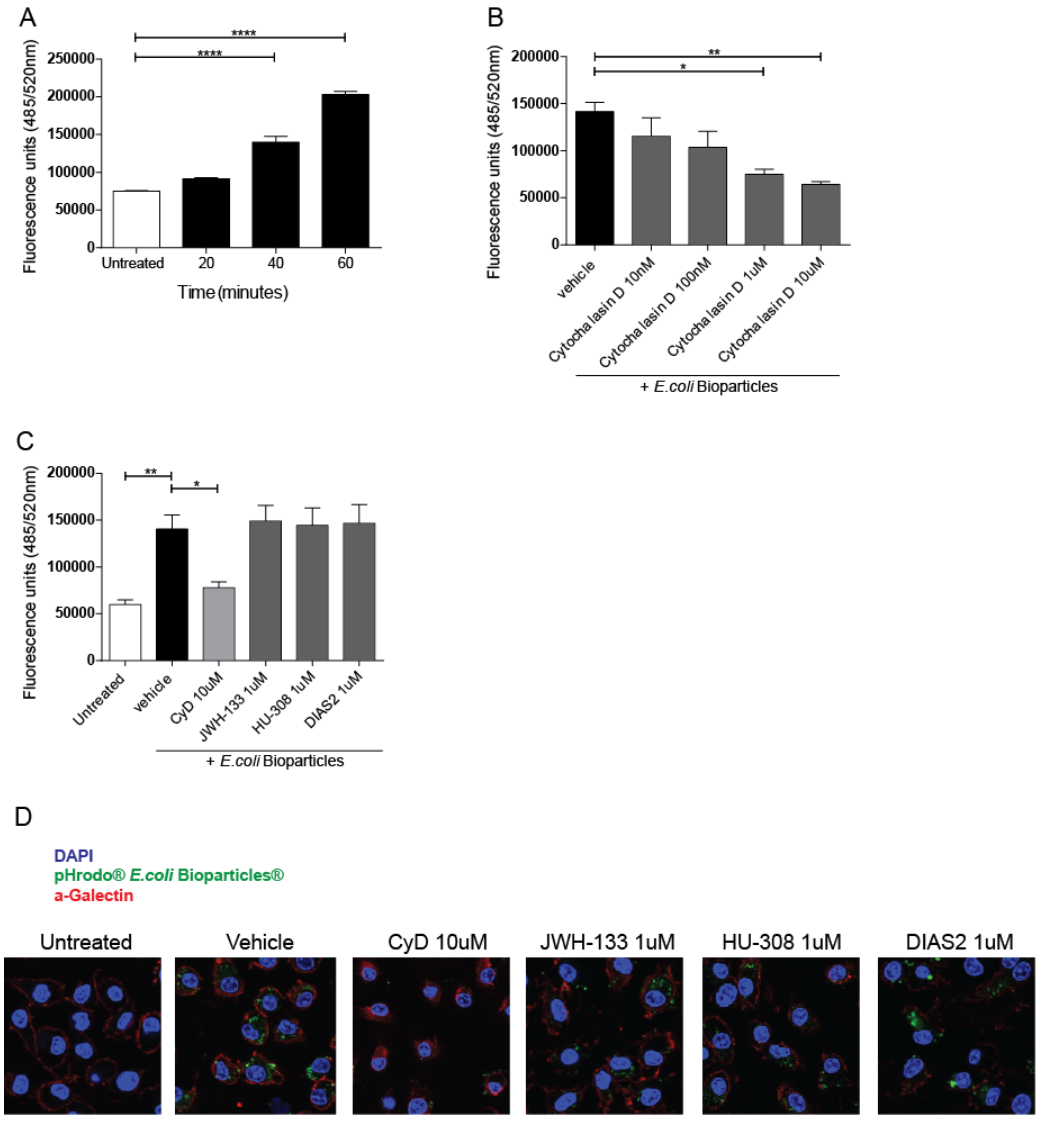


Figure 4.7. CB2R-selective agonists do not enhance *E.coli*-triggered phagocytosis. Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were treated with pHrodo *E.coli* Bioparticles for 20, 40 and 60 minutes and fluorescence was determined (A). Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were pre-treated with a 10-fold dose titration of Cytochalasin D for 1 hour before addition of pHrodo *E.coli* Bioparticles for a simultaneous treatment of one additional hour (B). Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were pre-treated with vehicle, Cytochalasin D or the CB2R-selective agonists JWH-133, HU-308 and DIAS2 for 1 hour before simultaneous treatment with pHrodo *E.coli* Bioparticles for one additional hour (C). Confocal microscope pictures from one representative experiment (D). Cell membranes were stained with an a-Galectin antibody and nuclei were stained with DAPI. Data are from 3-5 independent experiments. Mean+SEM are represented and one-way ANOVA with Dunnett's post hoc multiple comparisons test was used for data analysis, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$

The literature on cannabinoid effects in inflammation is contradictory. There is a notion that CB2R-selective agonists exhibit anti-inflammatory effects on macrophage cell lines and primary murine macrophages *in vivo*. For example, it has been reported that JWH-015 ameliorates the secretion of TNF-α, IL-12 and IL-10 in human macrophages and WIN55,212-2 reduces TNF-α secretion (Hao *et al.* 2010).

My data are in agreement with another body of the literature which implies that cannabinoid ligands can also mediate their effects independently of cannabinoid receptors when used at saturating doses. In LPS-stimulated microglia, CB1R/CB2R agonists and the phytocannabinoids CBD and Δ^9-THC were shown to decrease TNF-α, IL-1β, IL-6 and IFN-β levels via an unidentified cannabinoid receptor (Facchinetti *et al.* 2003;Kozela *et al.* 2010). Similarly, AEA dampens IL-12 secretion by BV-2 and RAW264.7 cells independently of CB1R and CB2R (Correa *et al.* 2008). Finally, a report by Puffenbarger *et al*. showed that Δ^9-THC reduces the expression of pro-inflammatory cytokines in rat microglia, possibly via direct effects on the cell membrane of cells (Puffenbarger *et al.* 2000).

Macrophages generate ROS and NO_2^- as an anti-microbial defense and a response to inflammatory stimuli. In my experiments, CB2R-selective agonists did not reduce inflammation-induced oxidative stress as assessed by secretion of NO_2^- in the micro-enivonment. There are conflicting results regarding the role of CB1R activation in ROS

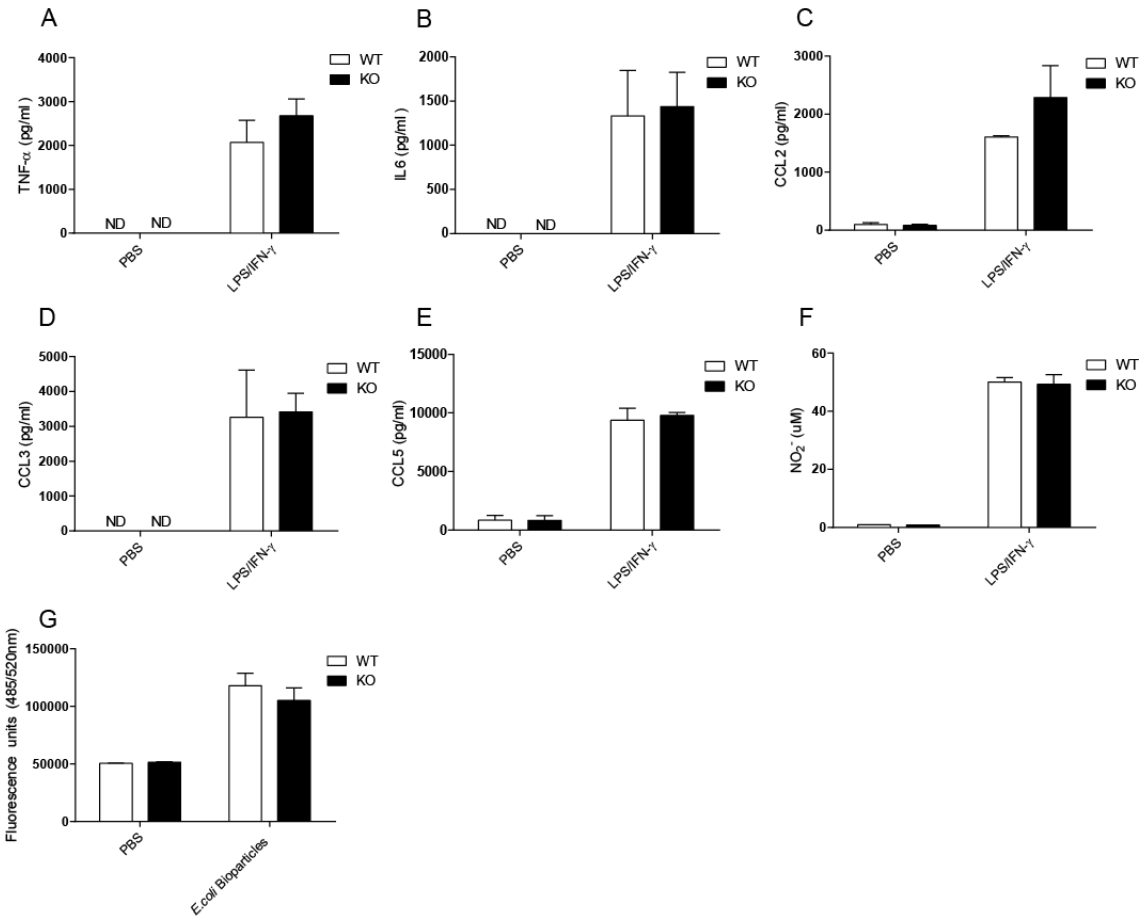


Figure 4.8. Deficiency of CB2R does not alter macrophage immune responses to LPS and IFN-γ. Murine BMDMs from WT and CB2R KO male mice (6-8 weeks old) were stimulated with LPS and IFN-γ for 16 hours, and supernatants were assessed for TNF-α (A), IL-6 (B), CCL2 (C), CCL3 (D), CCL5 (E) and nitrite (F) levels. Murine BMDMs from WT and CB2R KO male mice (6-8 weeks old) were treated with pHrodo *E.coli* Bioparticles and fluorescence emission was measured one hour later (G). Data are from 3 independent experiments. Mean+SEM are represented and two-way ANOVA with Sidak's post hoc multiple comparisons test were used for data analysis, ND not detected

production *in vivo* and *in vivo*, while CB2R reduces the secretion of oxygen and nitrogen radicals (Chung *et al.* 2011;Han *et al.* 2009),. In fact, CB2R-selective and mixed agonists are reported to contribute to ROS and NO level decrease in macrophages (Ribeiro *et al.* 2012). The most commonly used agonist, WIN55,212-2, reduces ROS and NO production by macrophages (Eljaschewitsch *et al.* 2006;Hao *et al.* 2010) and JWH-133 decreases nitrite levels in stimulated thioglycollate-elicited macrophages (Romano *et al.* 2013).

As far as surface expression of antigen presentation molecules is concerned, my data are in line with the work of McCoy *et al*. who showed that Δ^9-THC affects the processing of antigen in macrophages instead of antigen presentation (Mccoy *et al.* 1995). The expression of MHC-II, CD80 and CD86 on macrophage membranes is the net result of the process of antigen digestion in the phagolysosomes, loading on MHC molecules and translocation to the cell surface for presentation. It is therefore possible that JWH-133, HU-308 and DIAS2 might suppress antigen loading to MHC-II molecules during LPS and IFN-γ treatment, which was not assessed in my experiments and could be the focus of further investigation.

Finally, phagocytosis is a key feature of macrophage cell biology and the effects of CB1R and CB2R ligands on this process have been the subject of many studies to date. AEA treatment of macrophage cell lines showed that this endocannabinoid induces phagocytosis of zymosan bioparticles in a CB1R-dependent manner (Mai *et al.* 2015). Synthetic CB2R agonists have been reported to behave like endocannabinoids to enhance macrophage phagocytosis. For instance, THP-1 cells remove Aβ amyloid peptides from frozen human Alzheimer's disease sections when treated with JWH-015,

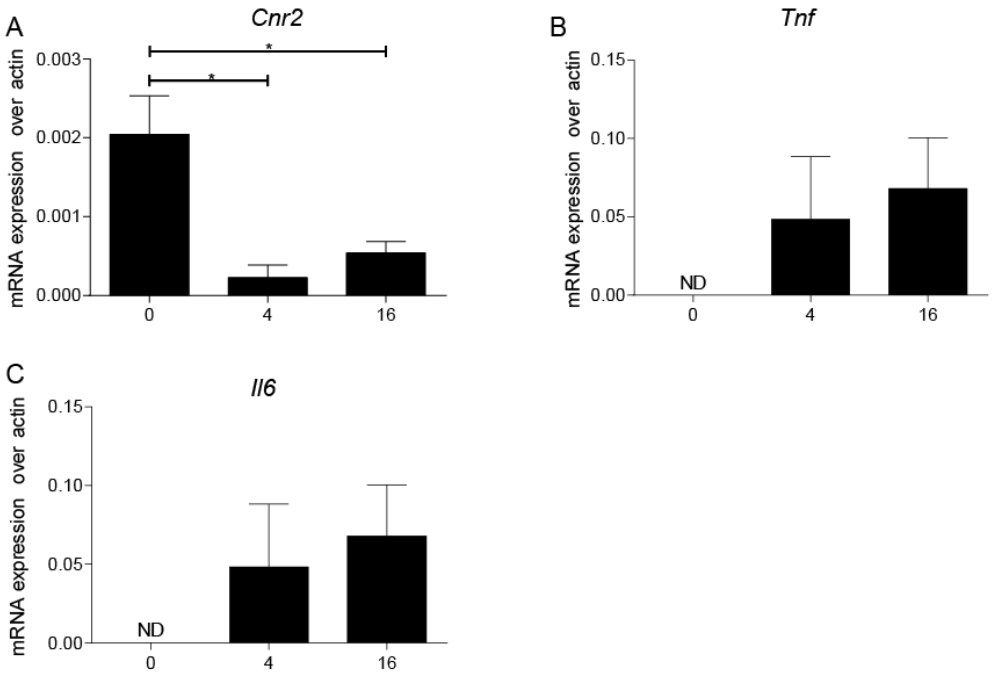


Figure 4.9. Expression of *Cnr2* is severely decreased upon LPS and IFN-γ administration.
Murine BMDMs from male C57BL/6J mice (6-8 weeks old) were stimulated with LPS and IFN-γ for 4 and 16 hours, supernatants were washed out and adherent cells were assessed for *Cnr2* (A), *Tnf* (B) and *Il6* (C) mRNA expression. Data are from 3 independent experiments. Mean+SEM are represented and one-way ANOVA with Dunnett's post hoc multiple comparisons test were used for data analysis, * $p<0.05$, ND not detected

an effect which is lost upon co-incubation of the cells with SR144528 (Tolon *et al.* 2009). Moreover, other CB1R/CB2R agonists, such as CP55,940 and WIN55,212-2 increase phagocytosis of zymosan (Gokoh *et al.* 2007). In contrast, *in vitro* administration of JWH-133 to macrophage cell lines has no effect on phagocytosis of zymosan (Mai *et al.* 2015). My results are in agreement with the latter observation as I did not observe any enhancement of *E.coli* phagocytosis upon treatment of BMDMs with JWH-133, HU-308 or DIAS2.

The involvement of CB2R in inflammation regulation can additionally be tested in two different ways; pharmacologically and biologically. In the experiments presented in Figures 4.1-4.5, I did not observe any effects on macrophage pro-inflammatory functions. Therefore, I next decided to compare the magnitude of inflammation between WT and CB2R KO mice and ask whether the presence of the receptor activates any endogenous anti-inflammatory pathways via the secretion of endocannabinoids in the cell micro-environment.

This approach has considerable advantages over pharmacological approaches; it tackles the hurdles of non-specific effects of insoluble compounds or inappropriate dosing, route of administration and compound half-life limitations. However, a common pitfall of KO animals in general is that they might allow misinterpretation of generated data because genetic loss of function might sometimes be compensated by other genes.

CB2R KO BMDMs have been previously reported to demonstrate higher CCR1, CCR5, and CCL2 expression compared to WT cells (Molica *et al.* 2012). In contrast, CB2R KO microglia have a reduced pro-inflammatory profile based on CD40 and ICAM expression and cytokine and chemokine secretion (Schmole *et al.* 2015).

The most likely explanation for the lack of anti-inflammatory effects of CB2R-selective agonists on macrophages derives from the fact that *Cnr2* mRNA levels are significantly reduced upon LPS/IFN-γ stimulation of macrophages. If *Cnr2* is downregulated, then its ligands will not be able to bind efficiently above the activation threshold and mediate their effects. CB2R expression levels have been reported to change during macrophage activation with different stimuli. For example, CD40 antibody ligation induces *Cnr2* overexpression in splenic macrophages (Lee *et al.* 2001), while LPS has the opposite effect (Carlisle *et al.* 2002;Lee *et al.* 2001). Therefore the fact that I did not observe the alleged anti-inflammatory effects documented in the literature for macrophages might be the outcome of a reduced CB2R cell surface expression.

Another possible reason for this discrepancy might be the particular animal strain used in the study. There are currently two available CB2R KO mouse strains; the first was made in Prof. Zimmer's lab (Buckley *et al.* 2000) and the second by Deltagen available by the Jackson Laboratories. It is possible that differences in the genetic engineering of these mice may affect the phenotype of immune cells in homeostasis and inflammation. Therefore, an investigation into the phenotype of the Deltagen CB2R KO mice in steady state and in inflammation was carried out and is the aim of Chapter 5 and Chapter 6.

In summary, this chapter showed that using optimal concentrations of CB2R-selective cannabinoids, BMDMs do not exhibit any alteration in cytokine and chemokine secretion, nitrite production, antigen presentation and co-stimulatory molecule expression and phagocytosis of *E.coli* bioparticles. More importantly, none of these macrophage functions was affected by genetic ablation of CB2R. A possible explanation for this lack of effects is the downregulation of *Cnr2* mRNA expession

upon the initiation of inflammation, implying that this gene and the receptor it encodes are less likely to be a good anti-inflammatory drug target in macrophages.

4.5 Limitations of the study

The experiments described in this chapter aimed to answer the scientific question of whether CB2R-selective ligands can have anti-inflammatory effects on macrophages. I hereby disproved my own original hypothesis, but the cannabinoid doses I used are open to criticism due to the use of artificial gene expression systems in the β–arrestin and cAMP assays.

The model proposed in this chapter suggests that macrophage *Cnr2* mRNA levels are decreased upon inflammation and therefore the cannabinoids cannot bind to and activate the receptor. This could explain the lack of a detectable effect in all of the *in vitro* assays I designed. However, with the lack of proper tools, e.g. specific monoclonal antibodies, whether protein levels of the receptor are also decreased or whether CB2R distribution on the plasma membrane of macrophages during inflammation is altered is a question which is difficult to answer.

Chapter 5: The role of CB2R in the homeostasis of the immune system

5.1 Introduction

Cells of the immune system are divided into cells of the innate and adaptive immunity based on the specificity they display for antigen infecting the host (Kumar *et al.* 2003). Innate immunity is triggered early upon infection and targets conserved patterns on pathogens, while adaptive immunity is initiated later during the course of infection and is specific for the antigen that caused its activation (Kumar *et al.* 2003). Below I describe the origin and tissue distribution of innate immune cellswhich this chapter will focus on and provide the phenotypical identification based on cell surface antigens.

5.1.1.1 Neutrophils

Neutrophils are the most abundant leukocytes in the circulation and they play a pivotal role in inflammation as they rapidly infiltrate inflamed tissues (Summers *et al.* 2010). They have been phenotyped as $CD11b^{+}Ly\text{-}6C^{+}Ly\text{-}6G^{+}$ cells (Rose *et al.* 2012) and they clear microbes by phagocytosis following the release of anti-microbial factors stored in pre-formed granules (azurophil, specific and gelatinase vesicles) (Faurschou and Borregaard 2003).

Neutrophils develop in the bone marrow which constantly replenishes the circulation pool during homeostasis. However, it has been shown that the bone marrow is not the only source of neutrophils. In particular, infusion of autologous radiolabelled neutrophils to human recipients showed that neutrophils are undetectable in the circulation after five to seven hours, implying that there are other neutrophil niches (Mauer *et al.* 1960).

Indeed, neutrophils have also been identified in murine spleen, liver and lungs (Summers *et al.* 2010). These neutrophil populations are in a dynamic equilibrium with the bloodstream and are exchanged in a two-way fashion (Lien *et al.* 1987;Peters *et al.* 1985;Ussov *et al.* 1995). During inflammation, neutrophils are mobilised from the bone marrow sinusoids and enter the circulation following chemokine and other chemoattractant cues.

5.1.1.2 Monocytes/Macrophages

Monocytes derive originally from bone marrow progenitors; $CD115^{+}CD117^{+}CD135^{+}$ high-dividing monocyte-macrophage/DC progenitors give rise to the common monocyte progenitor ($CD115^{+}CD117^{-}CD135^{+}CD11b^{-}Ly\text{-}6C^{hi}$ cells) which further differentiates into $CD115^{+}CD117^{-}CD135^{-}CD11b^{+}Ly\text{-}6C^{hi}$ or $Ly\text{-}6C^{lo}$ monocytes (Hettinger *et al.* 2013).

Mice have two monocyte populations in their blood; the $CD115^{+}CD11b^{+}CCR2^{+}CX_3CR1^{lo}Ly\text{-}6C^{hi}$ inflammatory and the $CD115^{+}CD11b^{+}CX_3CR1^{hi}Ly\text{-}6C^{lo}$ patrolling monocytes (Geissmann *et al.* 2003). $Ly\text{-}6C^{hi}$ monocytes are precursors for $Ly\text{-}6C^{lo}$ cells (Yona *et al.* 2013) and express high levels of chemokine receptors, complement peptides and annexins, while the $Ly\text{-}6C^{lo}$ monocytes express high levels of MHC-II molecules, growth factors, integrins and scavenger receptors (Hettinger *et al.* 2013;Ingersoll *et al.* 2010).

Patrolling monocytes migrate to tissues in a LFA-1- and CX_3CR1-dependent manner to monitor for tissue damage (Auffray *et al.* 2007;Geissmann *et al.* 2003). Upon encounter of inflammatory stimuli, they differentiate into macrophages and are responsible for endothelial cell necrosis and recruitment of neutrophils at the first stages of

inflammation (Carlin *et al.* 2013). On the contrary, Ly-6C^{hi} monocytes egress from the bone marrow in a CCR2-dependent manner and migrate to inflamed tissues where they differentiate into TNF and iNOS-producing (Tip) DCs (Serbina and Pamer 2006;Tsou *et al.* 2007).

In a classic paper by van Furth and Sluiter, monocytes were shown to be distributed in circulating and marginated populations in the blood (van Furth and Sluiter 1986). In addition, the skin, lungs, spleen and lymph nodes are home to monocytes and monocytes bear gene expression pattern similarities (Jakubzick *et al.* 2013;Robbins *et al.* 2012). Finally, marginated monocytes in the spleen are found in the red pulp and are mobilised under inflammatory conditions, e.g. atherosclerosis or myocardial infarction (Robbins *et al.* 2012;Swirski *et al.* 2009).

5.1.1.3 DCs

DCs are the immune cells that bridge T and B cell activation with innate immunity. Their ability to migrate to lymphoid organs, present antigens and crosstalk with T cells is of utmost significance and is reflected by their almost ubiquitous presence in lymphoid and non-lymphoid organs. DCs are divided into CD8α^{+}, CD103^{+} and CD11b^{+} cells. The CD8α^{+} DC subset resides in lymphoid organs (Vremec *et al.* 1992), whereas CD103^{+} DCs populate non-lymphoid tissues (del Rio *et al.* 2007). Finally, the CD11b^{+} DCs are a heterogeneous subset of which the role we do not fully understand yet (Lewis *et al.* 2011). CD8α^{+} are equipped to present antigen to CD8^{+} T cells and iNKT cells and can cross-present antigens (Arora *et al.* 2014;den Haan *et al.* 2000). CD11b^{+} DCs on the other hand can be further segregated into CD4^{+}Esam^{hi} and CD4^{-}Esam^{lo} DCs, and have been shown to be more efficient in priming CD4^{+} T cells and secreting pro-

inflammatory cytokines and chemokines (Dudziak *et al.* 2007;Persson *et al.* 2013;Proietto *et al.* 2004;Schlitzer *et al.* 2013).

Non-classical DCs have also been described in mice and humans, such as tissue moDCs and plasmacytoid DCs (pDCs) in the blood and peripheral organs. The former originate from infiltrating monocytes in inflamed tissues and bear surface markers of both monocytes and DCs (Serbina *et al.* 2003), whereas pDCs are a small subpopulation which is induced to rapidly secrete high amounts of type I interferons upon viral infections (Siegal *et al.* 1999).

DCs are distributed in several lymphoid organs. Bone marrow is a DC niche where they are considered to play an important role in providing survival signals for B cells (Sapoznikov *et al.* 2008). In the spleen, $CD8\alpha^+$, $Esam^{hi}CD11b^+$ and $Esam^{lo}CD11b^+$ subsets are all localised in the marginal zone, a strategic location to sample blood and lymph antigens and apoptotic or necrotic cells (Dudziak *et al.* 2007;Idoyaga *et al.* 2009;Iyoda *et al.* 2002). Lymph nodes also contain $CD8\alpha^+$ and $CD8\alpha^-$ DC populations, but the unique feature in this tissue is the population of migratory moDCs that come from the periphery to present antigen to $CD4^+$ T cells (Henri *et al.* 2001;Ruedl *et al.* 2000). Finally, thymic DCs consist primarily of $CD8\alpha^+$ DCs, although a small population of $CD8\alpha^-CD172^+$ cells also exist (Wu and Shortman 2005). Originally thought to play a role in negative T cell selection (Birnberg *et al.* 2008), $CD8\alpha^+$ DCs reside in the thymic medulla and can cross-present antigen to T cells, whereas the $CD8\alpha^-$ subset is thought to partially derive from the blood and carry antigen in a tolerogenic form (Bonasio *et al.* 2006).

5.1.1.4 NK cells

NK cells originate from the bone marrow where they rise from $NK1.1^{-}CD122^{+}DX5^{-}$ progenitors (Rosmaraki *et al.* 2001). They rely on IL-15 survival signals to function (Kennedy *et al.* 2000;Lodolce *et al.* 1998) and their maintenance and suppressor activities depend on the IL-2/IL-15 receptor common β subunit (CD122) (Suzuki *et al.* 1997). Immature NK cells acquire Ly49 and CD94/NKG2 receptors before they expand in numbers and these antigens remain on their surface until their full maturation (Kim *et al.* 2002). In addition, cell-surface integrin expression marks the maturation process of these cells. DX5 appears on NK cell surface early during their development, whereas CD11b is expressed after the NK expansion stage. CD11b levels are regulated during development as $NK1.1^{+}DX5^{+}CD122^{+}CD11b^{lo}$ NK precursors give rise to $NK1.1^{+}DX5^{+}CD122^{+}CD11b^{hi}$ cells (Kim *et al.* 2002). Like all leukocytes, mature NK cells leave the bone marrow and can be found in the mouse liver, spleen, lungs and the circulation under naive conditions.

5.1.1.5 Eosinophils, basophils and mast cells

Eosinophils differentiate in the bone marrow and fetal liver and circulate in the periphery as $CD4^{-}$Siglec $F^{+}CCR3^{+}CD62L^{+}FIRE^{+}$Pir-$A/B^{+}SSC^{hi}$ cells (Voehringer *et al.* 2007). During parasite infection and allergy, eosinophils are activated and recruited to peripheral tissues where they release anti-microbial and anti-helminth products from their granules, such as major basic protein, endothelin, eosinophilic cationic protein and erythropoietin (Uhm *et al.* 2012;Voehringer *et al.* 2007).

Basophils and mast cells are thought to originate from a common precursor cell in the bone marrow (Arinobu *et al.* 2005;Chen *et al.* 2005). However, basophils complete their

maturation in the bone marrow, whereas mast cells leave and mature in peripheral tissues (Kitamura *et al.* 1979). Because of their rapid turnover, mast cells are negligible in the circulation, but can be found in a number of tissues, such as the spleen, the cecum and the mesentery (Kitamura *et al.* 1979).

Recently basophil/mast cell progenitors were identified in peripheral tissues; c-Kit^{hi}FcγRII/III^{hi}β7^{hi} progenitors give rise exclusively to basophils and mast cells in the spleen, whereas c-Kit^{hi}FcεRαI^{hi} mast cells are generated in the intestine (Arinobu *et al.* 2005). On the other hand, c-Kit^{lo}CD49b^{+}FcεRI^{+} basophils are recruited from the bone marrow in the presence of activating signals, such as thymic stromal lymphopoietin and promote T_H2 immunity (Siracusa *et al.* 2011).

5.1.2 The Buckley CB2R KO mouse strain

The study of CB2R and its functions in the immune system has been facilitated by the use of KO animals. Buckley *et al.* reported the generation of a CB2R KO mouse strain where *Cnr2* is disrupted by homologous recombination (Buckley *et al.* 2000). A phosphoglycerine kinase (PGK) promoter-neomycin cassette is inserted to the 3' sequence of the CB2R coding exon at the embryonic stem cell level leading to the deletion of 341 bp. The mice are born healthy with similar weight and size to WT mice, are fertile and care for their offspring.

Validation of the CB2R KO mice came from autoradiography experiments where CP55,940 failed to bind to the splenic marginal zone of CB2R KO animals (Buckley *et al.* 2000). In addition, Δ^9-THC-pre-incubated CB2R KO peritoneal cells did not reduce IL-2 secretion by T cells in comparison with WT cells, but Δ^9-THC-induced hypothermia and catalepsy were persistent in the CB2R KO mice, suggesting that

another receptor (most likely CB1R) was involved in these cannabinoid activities (Buckley *et al.* 2000).

Finally, the authors assessed the immunophenotype of these mice and compared thymus, spleen and lymph node samples for the presence of T cells, B cells and macrophages (Buckley *et al.* 2000). The mice showed a comparable composition of these immune cells between the two genotypes and the authors concluded that CB2R does not affect homing of immune cells to primary and secondary lymphoid organs under baseline conditions. In a recent review, Buckley also summarised the studies this particular CB2R KO mouse has been used in (Buckley 2008). The Buckley CB2R KO mouse strain has been widely used in a range of different *in vivo* disease models, including atherosclerosis, multiple sclerosis, allergy and others.

Ofek *et al.* showed that CB2R is expressed in bone and that it promotes the differentiation of bone marrow cells to osteoblasts. In particular, CB2R KO animals under naive conditions showed increased trabecular bone loss and cortical expansion due to enhanced osteoclast differentiation (Ofek *et al.* 2006).

Under inflammatory conditions, CB2R KO mice generally exhibit an exacerbated phenotype, indicative of a protective role of this GPCR. For example, CB2R KO mice have an exacerbated ischemia-reperfusion phenotype with elevated pro-inflammatory mediator levels and tissue destruction (Batkai *et al.* 2007), while they display increased liver fibrosis after intoxication with carbon tetrachloride (Julien *et al.* 2005). Moreover, CB2R deficient mice have an exacerbated phenotype in a murine multiple sclerosis model, an effect attributed to CB2R deficiency in encephalitogenic T cells (Maresz *et al.* 2007).

In contrast, the same CB2R KO mouse strain was used in a IgE-mediated cutaneous reaction model and it was found that CB2R KO mice have ameliorated ear swelling compared to WT mice. Interestingly, treatment of CB2R KO mice with the CB2R inverse agonist SR144528 reversed the disease score, while endocannabinoid administration exacerbated inflammation (Ueda *et al.* 2007). In disagreement with this, another publication reported that CB1R, CB2R and CB1R/CB2R double KO animals show an exacerbated phenotype in allergic contact dermatitis (Karsak *et al.* 2007).

5.2 Aim of the chapter

The aim of the experiments reported in this chapter was to genotype the Deltagen CB2R KO mouse strain used in Chapter 4 and investigate whether genetic ablation of CB2R causes a defect in neutrophil and monocyte trafficking *in vivo* under naive conditions. Harvest of murine lymphoid and non-lymphoid organs and isolation of neutrophil and monocyte populations allowed me to assess the immune cell composition of these important tissues for health and disease when CB2R is absent.

5.3 Results

5.3.1 Genetic characterisation of the Deltagen CB2R KO mouse strain

According to the Deltagen CB2R KO mouse supplier's website (https://www.jax.org/strain/005786), the animals express *Cnr2* transcripts in a range of tissues, such as the thymus, spleen, lymph nodes and the bone marrow. I therefore extracted RNA from several tissues from WT, CB2R KO and heterozygous mice and assessed *Cnr2* expression levels. Indeed, CB2R KO and heterozygous mice also produce *Cnr2* transcripts, although at lower levels in comparison with WT mice (Figure

5.1A-F). The exception was the spleen and the liver where heterozygous animals expressed higher levels than both WT and CB2R KO mice (Figure 5.1C, F).

This result raised concerns that the CB2R KO mice might have not been successfully engineered. To verify that they were true CB2R KOs, I extracted DNA from murine earclips and used the commercially available primer pairs for genotyping. As shown in Figure 5.1G, WT mice yielded a PCR product of 385 bp and CB2R KO mice had a 550 bp PCR product when run on a 2% electrophoresis agarose gel. The heterozygous animals yielded both PCR products.

5.3.2 Immunophenotyping of the Deltagen CB2R KO mouse strain

5.3.2.1 CB2R KO mice have similar numbers of neutrophils and monocytes in the bone marrow with WT controls

I next looked at leukocyte populations in the bone marrow of WT and CB2R KO animals. I stained for neutrophils and monocytes with antibodies against CD45, CD11b, CD115, Ly-6C and Ly-6G. Flow Cytometry gating on $CD45^{+}CD11b^{+}$ cells distinguished neutrophils ($Ly\text{-}6C^{+}Ly\text{-}6G^{+}$) from monocytes ($Ly\text{-}6C^{+}Ly\text{-}6G^{-}$) (Figure 5.2A). CD115 staining of monocytes was not fruitful and was therefore excluded from the analysis.

My data showed that neutrophil and monocyte numbers are comparable between the two genotypes (Figure 5.2 B-C). However, neutrophils displayed a 45% increase trend in the bone marrow of CB2R KO mice, while monocytes displayed a 32% reduction trend (Figure 5.2C). None of these trends reached statistical significance. My results therefore suggest that CB2R does not affect the composition of the bone marrow in neutrophils and monocytes under naïve conditions.

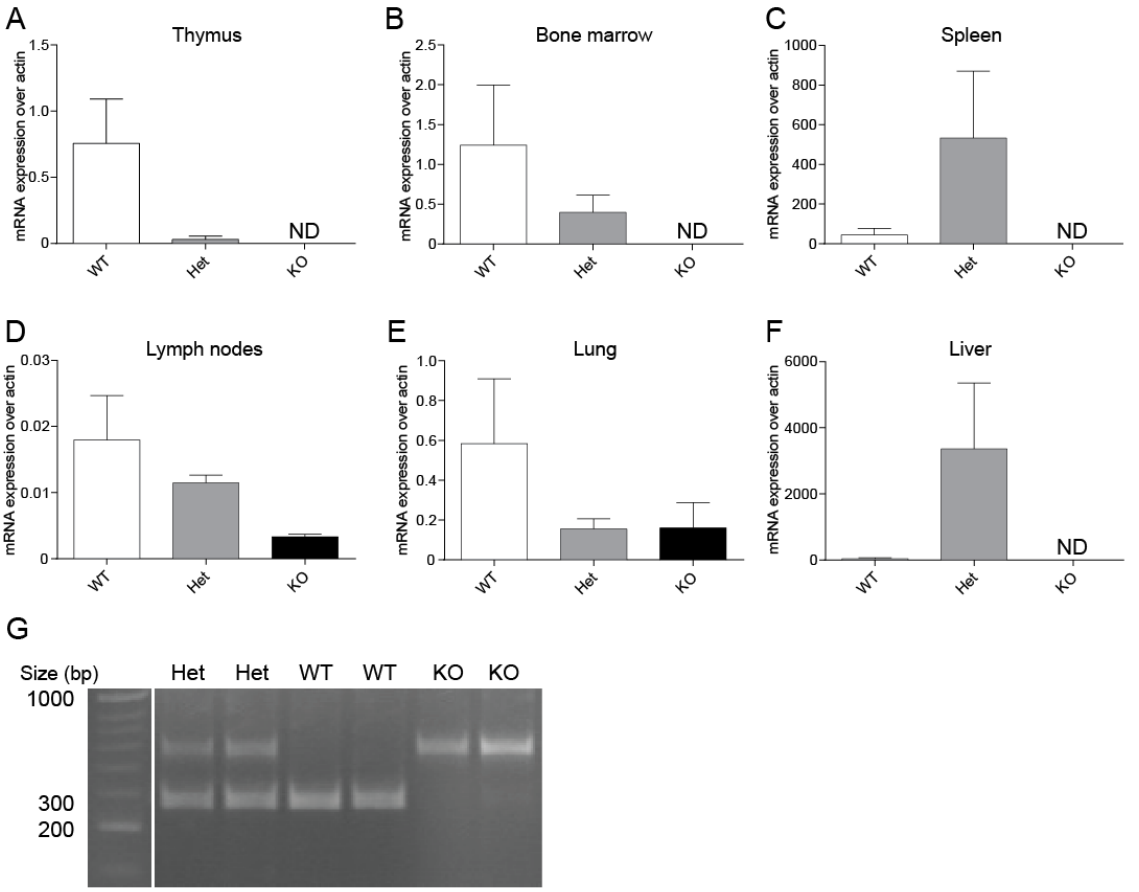


Figure 5.1. CB2R KO mice express receptor transcripts in lymphoid and non-lymphoid tissues. WT and CB2R KO mice (8-10 weeks old) were sacrificed and thymus, bone marrow, spleen, lymph nodes, lung and liver were harvested and disaggregated. Tissue homogenates were used to extract RNA to assess *Cnr2* expression levels using qPCR. Pooled data from thymus (A), bone marrow (B), spleen (C), lymph nodes (D), lung (E) and liver (F) tissues from 3-5 animals. Genotyping of CB2R KO mice (8-10 weeks old) and gel electrophoresis of the PCR products yields a band at 550 bp, whereas WT animals (8-10 weeks old) yield a 385 bp band. Heterozygotes have both PCR bands (G).

5.3.2.2 WT and CB2R KO mice have similar numbers of neutrophils, Ly-6C^{hi} and Ly-6C^{lo} monocytes in the bloodstream

I next collected blood from WT and CB2R KO mice and looked at the numbers of neutrophils and monocytes using a similar Flow Cytometry strategy. I stained for neutrophils and monocytes with antibodies against CD45, CD11b, CD115, Ly-6C and Ly-6G as in the bone marrow. Flow Cytometry analysis of CD45^{+}CD11b^{+} cells distinguished neutrophils (Ly-6C^{+}Ly-6G^{+}) from monocytes (Ly-6C^{+}Ly-6G^{-}) (Figure 5.3A). Monocyte subpopulation identification (Ly-6C^{hi} and Ly-6C^{lo}) was aided by the use of CD115 marker which is expressed in bone marrow-derived monocytes and successfully distinguished these two populations in the blood (Figure 5.3B).

Neutrophil numbers in CB2R KO animals displayed a non-statistically significant 55% increase trend (Figure 5.3C). Ly-6C^{hi} monocyte (Figure 5.3D) and Ly-6C^{lo} monocyte (Figure 5.3E) numbers were also comparable with those of littermate controls. Taken together, my data show that the populations of neutrophils and monocytes in the blood are not affected by CB2R deficiency.

5.3.2.3 Splenic immune cell populations are similar in WT and CB2R KO animals

The spleen is a marginating neutrophil and monocyte niche and thus I assessed the numbers of these two immune cell populations. I digested spleens, lysed the red blood cells and proceeded to Flow Cytometry analysis to identify neutrophils and monocytes (Ly-6C^{hi} and Ly-6C^{lo} populations).

I stained for neutrophils and monocytes with antibodies against CD45, CD11b, Ly-6G and Ly-6C. Gating on CD45^{+}CD11b^{+} cells revealed the neutrophil population (Ly-6C^{+}

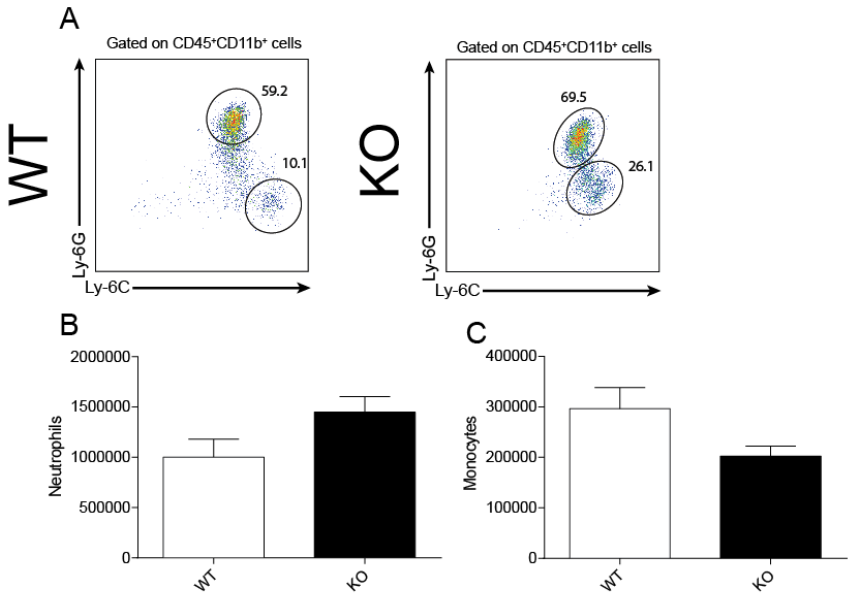


Figure 5.2. CB2R KO mice have a similar neutrophil and monocyte population in the bone marrow. Male WT and CB2R KO mice (8-10 weeks old) were sacrificed and bone marrow was harvested from tibiae and femurs. The cells were passed through cell strainers and red blood cells were lysed. The rest cells were stained for Flow Cytometry. The gating strategy followed is shown in representative histograms from one WT (left panel) and one CB2R KO (right panel) (A). Pooled graphs from 7 mice per group stained for neutrophil and monocyte populations are shown in (B)-(C). Mean+SEM are represented and student t test was used for data analysis

Ly-6G^{+}) (Figure 5.4A) and two monocyte populations (Ly-6C^{hi} and Ly-6C^{lo}) in combination with low SS values (Figure 5.4B).

Direct comparison of WT mice with CB2R KO animals showed that neutrophil displayes a 42% increase trend which, however, did not reach significance (Figure 5.4C). Ly-6C^{hi} and Ly-6C^{lo} monocyte subsets in the spleen were reduced by 67% in CB2R KO animals, but this effect also did not reach significance (Figure 5.4D-E). These results suggest that CB2R deficiency does not lead to neutrophil or monocyte retention in this secondary lymphoid organ.

5.3.2.4 CB2R KO mice neutrophil populations in the liver differ significantly from WT littermates

Finally, neutrophils have also been described to reside in peripheral non-lymphoid tissues, such as the liver. I thus homogenised livers from WT and CB2R KO animals and compared the myeloperoxidase levels between WT and CB2R KO mice in this organ. A liver MPO assay revealed that there are significantly higher myeloperoxidase levels in the livers of CB2R KO animals (Figure 5.5), suggesting that there are either more MPO^{+} cells or that the MPO^{+} cells express higher levels of this enzyme in comparison with littermate controls.

5.4 Discussion

In this chapter, I verified that *Cnr2* is interrupted in the Deltagen CB2R KO mouse strain. In addition, I studied the effect of CB2R deletion on innate immune cell populations under naive conditions *in vivo*. In particular, I employed Flow Cytometry to

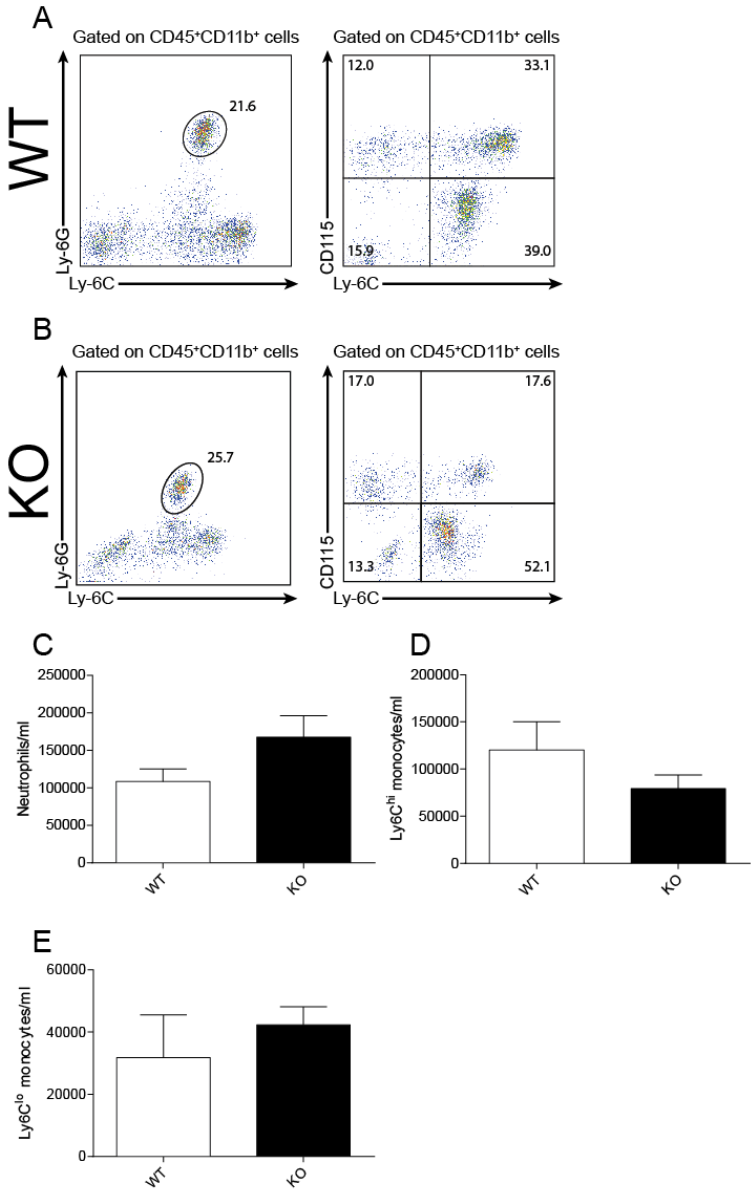


Figure 5.3. CB2R KO mice have similar in size neutrophil, Ly-6C^{hi} and Ly-6C^{lo} monocytes in the circulation. Male WT and CB2R KO mice (8-10 weeks old) were sacrificed and blood was collected in EDTA-treated tubes. Red blood cells were lysed and the rest cells were stained for Flow Cytometry. The gating strategy followed for neutrophils and monocytes is shown in representative histograms from one WT (A) and CB2R KO mouse (B). Pooled graphs from 7-8 WT and CB2R KO mice stained for neutrophils, Ly-6C^{hi} and Ly-6C^{lo} monocyte populations are shown in (C)-(E). Mean+SEM are represented and student t test was used for data analysis

study the numbers of neutrophils and monocytes in the bone marrow, blood, spleen and liver of WT and CB2R KO mice.

Genetic ablation of CB2R does not seem to have an effect on neutrophil or monocyte numbers in the bone marrow, blood, spleen or the liver. The data described in this chapter have not been previously reported for this mouse strain and reveal an unsuspected role of CB2R in immune cell homeostasis.

The verified insertion of the PGK neomycin cassette and the disruption of *Cnr2* exon 2 does not guarantee that the gene is non-functional. When an exogenous DNA sequence is inserted into an organism's genome, new open reading frames may be created, allowing for RNA polymerases to transcribe small but unstable mRNA transcripts. This could be a possible reason why *Cnr2* mRNA transcripts have been reported for this mouse strain before (https://www.jax.org/strain/005786).

If the generated mRNA transcripts are in frame with the remaining WT CB2R DNA sequence downstream of the neomycin cassette, they will code for WT CB2R amino acids. Given the fact that CB2R is a long GPCR molecule, folded in three dimensions to be embedded into the plasma membrane, it may occur that these mRNAs will code for domains important for ligand binding and/or signal transduction, and therefore will produce a functional CB2R protein. For these reasons, further assessment of the generated mRNAs in this CB2R KO mouse strain is needed. Identification of these mRNAs, cloning to expression vectors and transfection of cell lines will allow for assessment of their functionality using signature assays for CB2R activation and binding of labelled CB2R ligands.

CB2R is currently believed to be expressed in peripheral lymphoid organs where immune cells are generated and reside under naive conditions. Shortly after its initial

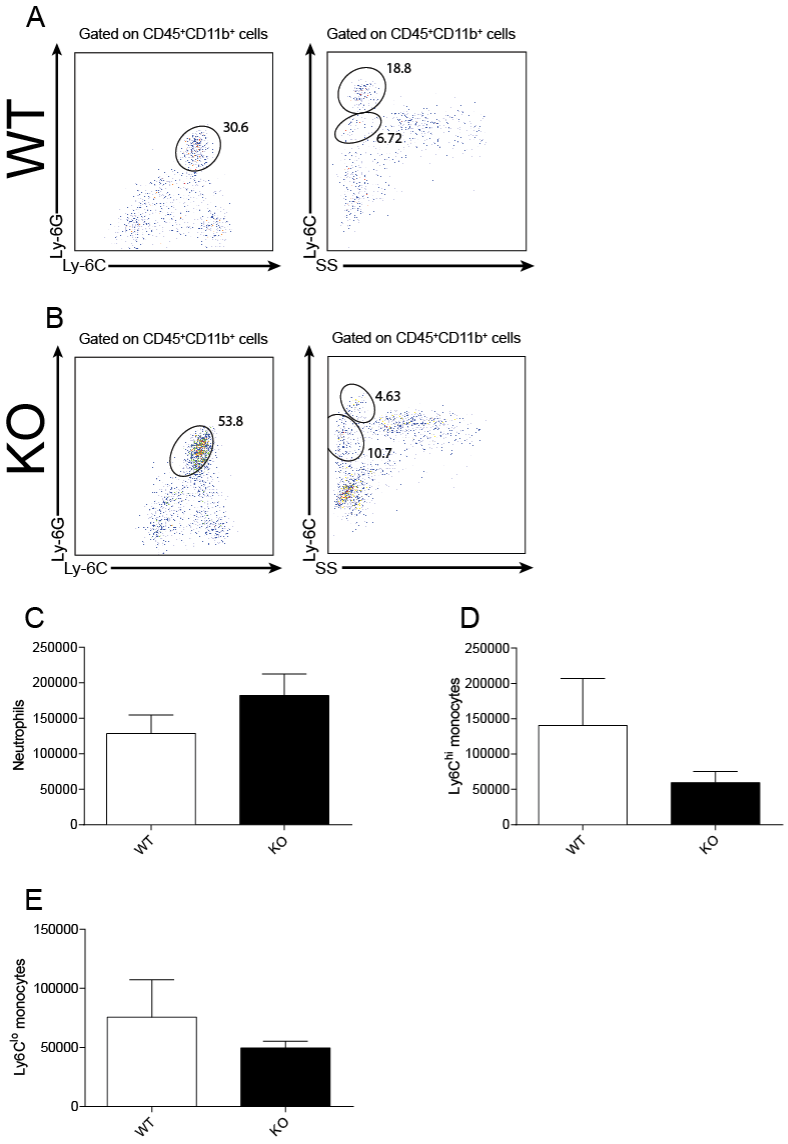


Figure 5.4. CB2R KO mice have comparable numbers of neutrophils and monocytes in the spleen compared to littermate control animals. Male WT and CB2R KO mice (8-10 weeks old) were sacrificed and spleens were harvested. The cells were passed through cell strainers and red blood cells were lysed. The rest cells were stained for Flow Cytometry. The gating strategy followed for neutrophils and monocytes is shown in representative histograms from one WT (A) and CB2R KO mouse (B). Pooled graphs from 7-8 mice per group stained for neutrophil, Ly-6C^{hi} and Ly-6C^{lo} monocyte populations are shown in (C)-(E). Mean+SEM are represented and student t test with (D, E) or without (C) Welch's correction was used for data analysis.

discovery, it was shown that CB2R is highly expressed in the rat and murine spleen and thymus (Schatz *et al.* 1997;Valk *et al.* 1997). Moreover, expression by a number of mast cell, myeloid and macrophage cell lines raised the possibility that the endocannabinoid system may have a potential role in regulating the homing of immune cells *in vivo* (Valk *et al.* 1997).

In fact, CB2R is expressed by splenic macrophages, B cells and T cells (Lee *et al.* 2001), whereas in humans CB2R is expressed in the spleen, thymus, pancreas, tonsils, bone marrow and peripheral blood leukocytes (Galiegue *et al.* 1995;Schatz *et al.* 1997). However, not all primary murine and human cells express the same levels of *Cnr2* mRNA. Galiegue *et al.* found that NK cells and B cells are among the highest CB2R-expressing immune cells, with neutrophils, monocytes and T cells following in descending order (Galiegue *et al.* 1995). It thus becomes apparent that the extent to which CB2R influences immune cell numbers will differ accordingly.

In this chapter, I looked at immune cell populations that express CB2R and I assessed their numbers in lymphoid and non-lymphoid organs by comparing their numbers in WT and CB2R KO mice. I did not look into DCs, mast cell, eosinophil and basophil subsets as these immune cells have not previously been reported to express CB2R.

Neutrophil and monocyte numbers in the bone marrow were not affected by the genetic deletion of CB2R in mice. However, I did observe an increase trend in neutrophil and reduction trend in monocyte numbers which, although not significant, are consistent with the literature. In particular, endocannabinoids have the capacity to enhance the IL-3-induced proliferation of bone marrow progenitors (Valk *et al.* 1997) and CB2R has been shown to block neutrophil differentiation (Jorda *et al.* 2003). It is thus plausible to hypothesise that CB2R KO animals, in which circulating levels of endocannabinoids do

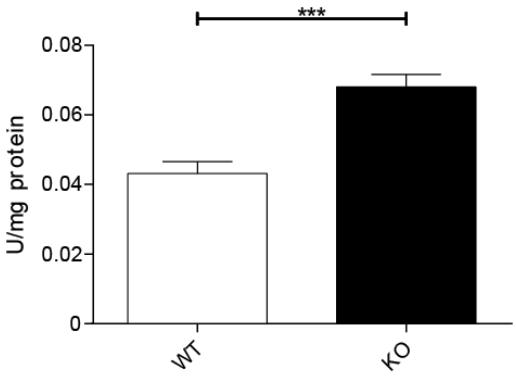


Figure 5.5. CB2R KO mice have fmore MPO^{+} cells in the liver compared to littermate control animals. Male WT and CB2R KO mice (8-10 weeks old) were sacrificed and livers were harvested. The tissues were homogenised and homogenates were used in a colorimetric MPO assay to assess the myeloperoxidase levels in this organ. Pooled graph from 8 mice per group is shown in (C). Mean+SEM are represented and student t test was used was used for data analysis, *** $p<0.001$

not bind to cognate cannabinoid receptors, have compromised proliferation of bone marrow cells, resulting in lower numbers of fully differentiated neutrophils.

On the other hand, given CB2R involvement in modulation of chemotaxis, a defect in neutrophil egress and enhanced release of monocytes from the bone marrow in the absence of CB2R warrants further investigation. To date, there are no published data demonstrating the involvement of this cannabinoid receptor in the regulation of neutrophil and monocyte trafficking from the bone marrow under naïve conditions. However, it has been shown that the endocannabinoids 2-AG and AEA can attract human neutrophils *in vitro* (McHugh *et al.* 2008). It would therefore be intriguing to look into this in future studies.

Neutrophils are also found in the lungs, spleen and liver under naive conditions (Summers *et al.* 2010), and are in a constant equilibrium with the bloodstream (Lien *et al.* 1987;Peters *et al.* 1985;Ussov *et al.* 1995). For this reason, I believe that the trend in the numbers of neutrophils observed in the blood and the spleen of CB2R KO mice is of particular interest. This finding suggests that the increase in the number of neutrophils in the bone marrow of CB2R KO mice is more likely to be attributed to enhanced retention in the tissues.

Finally, with regards to the augmented neutrophil trafficking in the liver of CB2R KO mice, it needs to be clarified that myeloperoxidase is mainly, but not exclusively, expressed by neutrophils (Tay *et al.* 1998;van der Veen *et al.* 2009). Monocytes also express low levels of myeloperoxidase which they downregulate when they differentiate to macrophages (van der Veen *et al.* 2009). However, the fact that myeloperoxidase in liver is only expressed by neutrophils in the liver (Amanzada *et al.* 2011)implies that the

MPO assay is sufficiently accurate to measure the trafficking of neutrophils in this organ.

In conclusion, here I report the phenotypical differences in the steady state immune system of CB2R KO male mice which can be summarised as an increase presence of neutrophils in the bone marrow, blood and spleen and a reduction trend in monocyte presence in the bone marrow and spleen. A plausible question that arises from this chapter's results is whether CB2R also affects immune cell composition of lymphoid and peripheral tissues during inflammation. To address this hypothesis, I compared WT and CB2R KO mice phenotype in an animal model of acute systemic inflammation (Chapter 6).

5.5 Limitations of the study

The limitations of the experiments of this chapter can be summarised in the choice of tissues to assess neutrophil and monocyte numbers. Neutrophils have also been described in the kidney, while monocyte populations have been found in lymph nodes and the skin. Due to time constraints, it was impossible to study neutrophil and trafficking in these tissues, meaning that this report is not complete.

Another limitation, which was discussed above, is the use of the MPO assay to assess neutrophil numbers in the liver. Use of a Flow Cytometry approach might have been more appropriate, although the current technique was used because of the exclusive expression of myeloperoxidase by neutrophils in the liver.

Chapter 6: The role of CB2R in systemic acute inflammation

6.1 Introduction

6.1.1 Local and systemic inflammation

Local inflammation occurs in one isolated part of the host, while systemic inflammation spreads to more than one organ or tissue, usually via the circulation. A local inflammatory response becomes systemic if it is excessive and uncontrolled, posing a greater threat to the individual's life (Serhan *et al.* 2010). One of the emerging systemic conditions that this chapter examines is sepsis.

6.1.2 Sepsis

Sepsis is a systemic inflammatory syndrome with worldwide prevalence. In the United States, it is responsible for one million cases per year of which 200,000 are fatal (Angus and van der Poll 2013). Genetics play a role as a risk factor with polymorphisms in cytokines, heat shock proteins (Angus and Wax 2001), lectins, TLRs, immunoglobulin receptors and proteins of the coagulation and fibrinolysis cascade being associated with increased risk (Chung and Waterer 2011).

The pathogens that cause human sepsis are mostly Gram-negative bacteria, although Gram-positive bacteria and fungal infections have also been reported (Vincent *et al.* 2009). Sepsis is initiated when the causative organisms break the first line of the host defence via the respiratory, intra abdominal or urinary tract (Vincent *et al.* 2009) and activate innate immune cells via PRRs (Deutschman and Tracey 2014). The inflammatory cascade is triggered leading to Systemic Inflammatory Response Syndrome (SIRS) which features tachycardia, fever, hyperventilation and leukocytosis

(Vincent *et al.* 2013). Leukocyte activation is followed by the secretion of pro-inflammatory cytokines, proteases and ROS, whereas the role of complement activation and the dysregulation of the coagulation cascade have been documented to result in the perpetuation of inflammation and the induction of collateral tissue damage (Angus and van der Poll 2013).

However, it is not only the overt inflammation that puts sepsis patients at risk. Inflammatory signals in organs are conducted along the afferent vagus nerve and descend from the brainstem via neuronal impulses to feed back to immune organs. For example, induction of norepinephrine in the spleen stimulates T cells to produce acetylcholine which binds to α_7 nicotinic acetylcholine receptors on macrophages and suppresses cytokine secretion (Wang *et al.* 2004;Wang *et al.* 2003). Similarly, norepinephrine release in tissues inhibits secretion of cytokines via beta-adrenergic receptor activation (Bosmann and Ward 2013). Finally, hormones, such as glucocorticoids, leptin, thyroid hormones, adrenocorticotropin and melanocyte-stimulating hormone have the ability to modulate lymphocyte and macrophage functions (Deutschman and Tracey 2014).

All these mechanisms cause immune cell tolerance to LPS and other infectious stimuli and initiate the Compensatory Anti-inflammatory Response Syndrome (CARS). CARS marks a prolonged period of immunosuppression where immune cell functions, such as cytokine secretion, antigen presentation, cytotoxicity and antibody production are repressed and anti-inflammatory cytokine secretion and the number of Tregs increase (Hotchkiss *et al.* 2013). Furthermore, enhanced immune cell apoptosis correlates with patient immunosuppression and organ failure (Hotchkiss *et al.* 2013).

Excessive production of pro-inflammatory mediators, such as HMGB1 leads to tissue barrier breakdown which, combined with the profound cell death of epithelial and endothelial cells, causes the loss of tissue integrity (Wolfson *et al.* 2011;Yang *et al.* 2006), microvascular leakage (London *et al.* 2010) and eventually multiple organ injury (Ye *et al.* 2008). Concomitantly, the oxidative stress induced in septic tissues causes mitochondria damage and compromises energy production (Galley 2011), a catastrophic event for multiple organs, including the lungs, the heart, the brain and the kidneys.

Currently, sepsis is handled in intensive care units by providing patients with cardiorespiratory resuscitation intravenous fluids, vasopressors and antibiotics, and in some cases mechanical ventilation (Dellinger *et al.* 2013). Attempts to design therapeutic drugs for sepsis have all failed in clinical trials to date. For instance, human activated protein C was licensed to control the dysregulated coagulation cascade, but was withdrawn due to the lack of benefits for patients (Ranieri *et al.* 2012). Another example was a TNF receptor fusion protein which, contrary to pre-clinical data, increased patient mortality in clinical trials and was abandoned (Fisher *et al.* 1996). These failures have challenged researchers to focus on targeted drug development based on more relevant animal models and better clinical trial design (Angus and van der Poll 2013).

6.1.3 Animal models of sepsis

Animal models of sepsis are divided into three categories; toxaemia, bacterial infection and host-barrier disruption. Toxaemia models are initiatied with chemical mediators, bacterial infection models with inoculation of animals with pure or mixed bacterial flora

and host-barrier disruption models by mechanical breakdown of protective barriers that separate the host from pathogens (Buras *et al.* 2005).

Although animal models have hugely contributed to our understanding of sepsis pathology and host defence mechanisms during infection, they suffer from a number of limitations. The use of young, healthy animals in *in vivo* studies, inter-species differences, comparative dosage, the lack of clinical management of the animals and environmental issues, such as tolerance due to coprophagy in rodents and baboons are some of the reasons why drugs successful in animal models fail to lead to approved therapies in humans (Esmon 2004;Riedemann *et al.* 2003). Therefore, animal model data should be interpreted with a degree of caution.

6.1.3.1 Toxaemia models

Toxaemia models are attractive because they are easy to carry out and studies have shown that low dose administration of LPS to humans induces similar pathophysiological changes to sepsis (Fink and Heard 1990). TLR ligands, with LPS being the most common, are the chemicals that have mostly been explored to date, often with the concomitant administration of D-galactosamine which lowers the threshold of hepatocyte susceptibility to immune cell cytotoxic products and induces liver failure (Xu *et al.* 1994). Despite their practicality, toxaemia models do not fully reflect the human pathology. In particular, low doses of stimuli fail to recapitulate the hyperdynamic phase observed in humans and for this reason high doses are frequently used (Buras *et al.* 2005). Another caveat has to do with the transient secretion of high levels of pro-inflammatory mediators in animal serum which is dissimilar to the prolonged but lower levels observed in human sepsis patients (Buras *et al.* 2005).

Finally, mice are more resistant to endotoxin and therefore experimental data cannot be directly extrapolated between species (Copeland *et al.* 2005).

6.1.3.2 Bacterial infection models

Bacterial infection models are physiologically more relevant than toxaemia models because they make use of pathogens that cause human sepsis. However, there is a lot of variability associated with the experimental design; pure versus mixed bacteria, different bacteria strains, bacterial load, route of administration and mouse strain specificity of host responses may complicate the interpretation of the results and affect the relevance of these models to human pathology (Buras *et al.* 2005).

6.1.3.3 Host barrier disruption models

Host barrier disruption models are initiated upon an artificial breach of the animal's protective barriers and the influx of pathogens into the peritoneal cavity. There are two models that fall into that category; Caecal Ligation and Puncture (CLP) and Colon Ascendens Stunt Peritonitis (CASP). In the first, the animal undergoes midline laparotomy and the caecum distal is ligated to the ileocaecal valve. Subsequently, it is punctured so that its contents are released into the peritoneal cavity. In the CASP model, a stent is inserted into the caecum ascendens so that intestinal bacteria are persistently leaked into the peritoneal cavity (Buras *et al.* 2005).

The CLP model is considered to be the gold standard model for sepsis studies, but has some key differences from the CASP model. In particular, bacterial leakage in the CASP model is continuous, whereas in the CLP model adhesive small-bowel loops are soon formed to limit bacterial release. That results in exacerbated inflammation in the

CASP model as determined by higher bacterial load, intestinal alterations and secreted pro-inflammatory mediators (Maier *et al.* 2004). Moreover, the active inflammatory pathways in the two models differ, with TNF-α being protective only in CLP (Echtenacher *et al.* 1990;Echtenacher *et al.* 1996), whereas IFN-γ is important for effective clearance of bacteria in the CASP model (Zantl *et al.* 1998).

Caveats of the host barrier disruption models are the high variability in animals to contain the disease (Buras *et al.* 2005), the surgery skills required to operate the animals, the diameter of the stent used and the percentage of caecum ligated in the CLP model (Singleton and Wischmeyer 2003). In addition, the lack of monitoring and resuscitation of the animals (Baker *et al.* 1983), and the fact that not all animals develop sepsis makes staging of individual animals challenging with respect to the severity of the disease.

6.1.4 Effects of the endocannabinoid system on sepsis

The literature on the contribution of CB2R in animal sepsis models is conflicting. Recently, Csoka *et al*. showed the important role CB2R plays in the progression of the CLP model in mice and suggested that it might be a GPCR to target to control pro-inflammatory mediator secretion, immune cell apoptosis and bacterial translocation (Csoka *et al.* 2009). In contrast, Lehmann *et al*. concluded that CB2R agonism with the selective agonists JWH-133 and HU-308 significantly decreased leukocyte adhesion within the intestinal microvasculature in both an endotoxemia and the CASP model (Lehmann *et al.* 2012).

In support of a protective role of CB2R in the CLP model, Tschop *et al*. found that CB2R KO animals presented with pronounced neutrophilia and elevated pro-

inflammatory chemokine levels in their lungs. Reflecting the compromised function of neutrophils, bacterial load and lung injury were higher in the CB2R deficient animals. However, when mice were injected with the CB2R-selective agonist Gp1a, the pathology was reversed (Tschop *et al.* 2009). In addition, CB2R activation with the selective ligand GW405833 leads to a reduction in pro-inflammatory cytokine levels in serum by splenocytes and peritoneal macrophages (Gui *et al.* 2013).

Interestingly, an earlier report which used a bacterial infection model showed that central CB1R also has the potential to modulate cytokine secretion, when HU-210 is administered intracerebroventricularly (Smith *et al.* 2001). CB1R antagonism reversed the effects which were also absent in CB1R KO animals. Moreover, SR144528 enhanced the anti-inflammatory effects of HU-210 and WIN55,212-2 when delivered concomitantly, implying that inhibition of CB2R can synergise with CB1R activation towards amelioration of sepsis. Consistent with the view that cannabinoid receptor antagonists may also act as inverse agonists at their targets, SR141716A modulates cytokine responses similarly to WIN55,212-2 and HU-210 when administered alone to animals (Smith *et al.* 2000).

It is apparent that CB1R involvement in sepsis is related more to the cardiovascular consequences of the disease. For instance, inhibition of CB1R with the antagonist AM251 prevents the LPS-induced tachycardia and vasodilation (Gardiner *et al.* 2005). The authors argued that this effect was not direct, but rather was mediated via the inhibition of β-adrenoreceptor. Nevertheless, it is possible that the AM251-induced effects observed on the hemodynamic measurements are independent of cannabinoid receptors (Batkai *et al.* 2004).

6.2 Aim of the chapter

In Chapter 5 I showed that CB2R deficiency in mice leads to dysregulated neutrophil and monocyte numbers in the bone marrow, suggesting that this GPCR is an important receptor that modulates immune cell trafficking during homeostasis. A plausible research question that emerged from Chapter 5 is whether the genetic deletion of CB2R also affects immune cell recruitment to sites of inflammation and kinetics of innate immune cell clearance post inflammation.

For these reasons, I decided to investigate the role of CB2R in the endotoxemia murine model of systemic inflammation. My original hypothesis was that CB2R KO animals would exhibit an exacerbated disease phenotype due to the absense of "brakes" in migration to injured tissues. Moreover, if CB2R is an anti-inflammatory receptor, its deletion would lead to increased secretion of pro-inflammatory mediators by innate immune cells, including cytokines and chemokines.

This chapter describes the setup and study of the kinetics of a low dose endotoxemia model, its validation with a well-characterised anti-inflammatory drug and the assessment of the immunophenotype of CB2R KO animals in comparison with littermate WT controls at several time points during the course of the endotoxemia model.

6.3 Results

6.3.1 The endotoxemia murine model is a model of acute systemic inflammation

To set up the endotoxemia murine model of systemic inflammation, I injected 1 mg/kg LPS i.p. into male C57BL/6J mice and carried out a time course analysis from 2 to 8 hours, including a cohort of naïve mice where baseline measurements were taken as

reference for the 0 hour time point. I chose to administer this low dose of LPS to prevent substantial adverse effects on animal welfare during the course of the experiments.

Administration of 1 mg/kg LPS led to the secretion of pro-inflammatory cytokines and chemokines in the serum (Figure 6.1) as demonstrated by TNF-α (A), IL-6 (B), CCL2 (C), CCL3 (D) and the neutrophil attracting chemokines CXCL1 (E), CXCL2 (F) and CXCL5 (G). All mediator production peaked by 2 hours upon LPS administration and, apart from CCL2 and CXCL5, levels fell to non-detectable levels by 8 hours. They readouts exhibited different kinetics. For example, TNF-α and CCL3 had a transient peak, whereas IL-6 and CCL2 levels decreased 4 hours upon LPS delivery. Interestingly, CXCL5 levels followed a different pattern and remained constant until the 4 hour time point.

I next looked at immune cell recruitment to peripheral tissues. The peritoneum, the lungs and the liver are the most studied tissues for cell recruitment in endotoxemia animal models. Due to the short time course of this model, I only assessed neutrophil and monocyte recruitment to these organs at the same time points. The data in Figure 6.2 show that neutrophils are found in all the tissues studied as soon as 2 hours post LPS administration. In the peritoneum, the neutrophil percentage was the highest at 2 hours after which it decreased (Figure 6.2A). In contrast, neutrophils in the lungs showed a delayed recruitment pattern and their percentage peaked at 4 hours. Monocytes were also recruited to the lungs and their percentage peaked at 2 hours post LPS delivery (Figure 6.2B). Neutrophil recruitment in the lungs did not decrease as fast as in the peritoneum with cells being present at 8 hours. Finally, neutrophils migrated to the liver within 2 hours and their kinetics seemed to be very similar with the peritoneum with

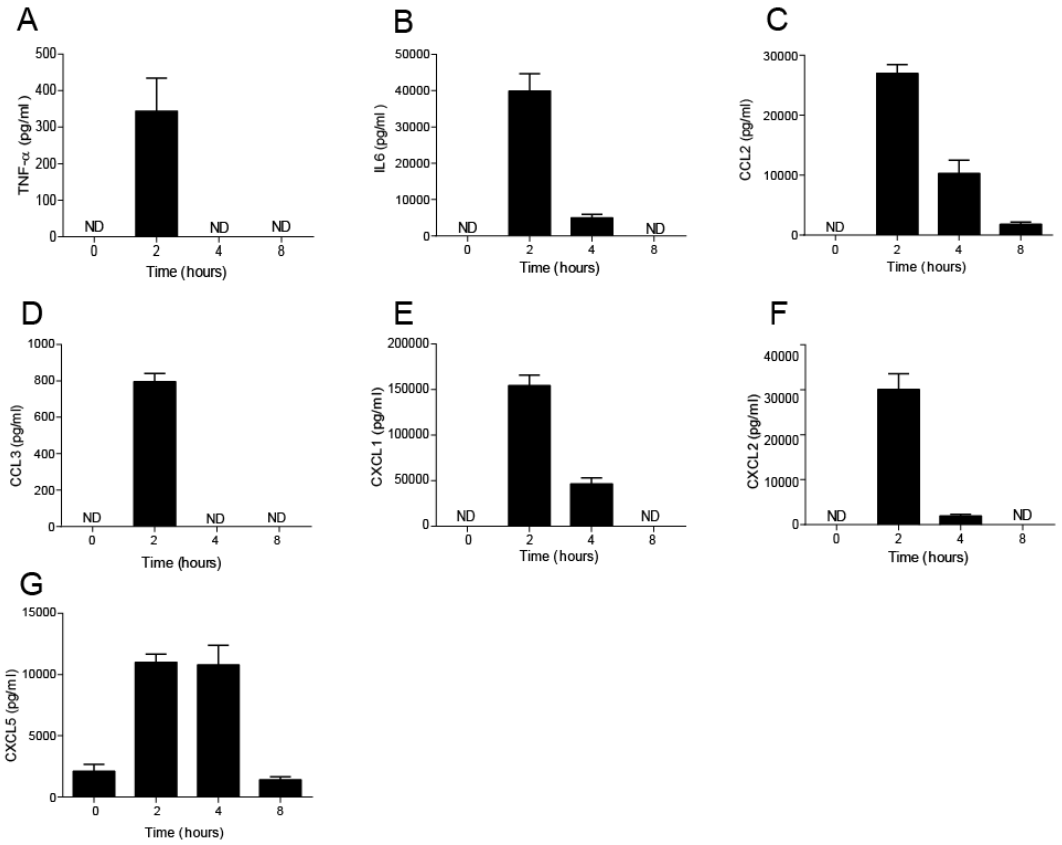


Figure 6.1. Time course analysis of pro-inflammatory cytokines and chemokines in the serum of LPS-treated mice. Male C57BL/6J (8-10 weeks old) mice were given 1 mg/kg LPS i.p. and the levels of TNF-α (A), IL-6 (B), CCL2 (C), CCL3 (D), CXCL1 (E), CXCL2 (F) and CXCL5 (G) were determined at 2, 4 and 8 hours in the serum. Naive animals were used for the 0 hour time point. Data are from one experiment with 4-5 animals per time point. Mean+SEM are shown, ND not detected

the exception that they were retained in the tissue for 4 hours (Figure 6.2C). Unlike the lungs, no monocytes were detected in the livers of endotoxemic mice.

To assess the disease score in these peripheral tissues, I lavaged the peritoneum and harvested lungs and livers from C57BL/6J mice injected i.p. with 1 mg/kg LPS. I measured the levels of pro-inflammatory mediators, such as IL-6, CCL2 and CXCL1 between 2 and 8 hours post LPS administration. Naïve mice were used for the 0 hour time point. The time kinetics of pro-inflammatory mediator expression in all tissues resembled that of soluble mediators in serum, and neutrophil and monocyte recruitment to peritoneum, lungs and liver. In fact, IL-6 and CXCL1 levels in the peritoneal lavage increased until 2 hours post LPS administration and fell under the detection limit at 8 hours (Figure 6.3A). Conversely, I was able to detect some residual CCL2 levels in mouse peritoneal lavage at 8 hours. Similarly, *Il6*, *Ccl2* and *Cxcl1* mRNA levels reached a peak of expression at 2 hours in the lungs and liver after which their levels significantly decreased until 8 hours (Figure 6.3B-C).

Taken together, my data show that the LPS dose and the route of administration I chose, induce endotoxemia in male C57BL/6J mice with secretion of a range of pro-inflammatory mediators in serum, peritoneum and peripheral tissues, such as the lungs and liver. In addition, mice exhibit innate immune cell recruitment to the peritoneum, lungs and liver.

6.3.2 Dexamethasone validates the endotoxemia *in vivo* model for reduction of inflammatory responses

To validate the *in vivo* endotoxemia murine model, I used the well-travelled anti-inflammatory glucocorticoid Dexamethasone which I administered at a dose of

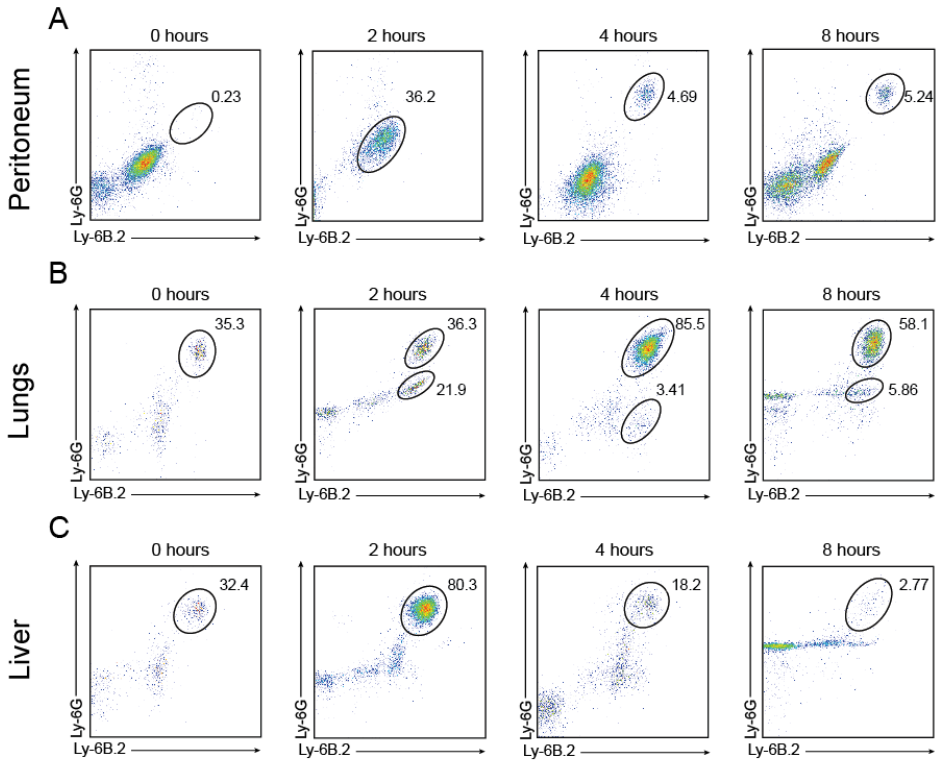


Figure 6.2. Time course analysis of innate immune cell recruitment in LPS-treated mice. Male C57BL/6J (8-10 weeks old) mice were given 1 mg/kg LPS i.p. and neutrophil (Ly-6B.2^{+}Ly-6G^{+}) and monocyte (Ly-6B.2^{+}Ly6G^{-}) population recruitment was assessed in the peritoneum, lungs and liver. PECs were isolated from lavaged peritoneal fluid, lungs were digested with collagenase D for one hour and, like livers, were disaggregated on a cell strainer to proceed to Flow Cytometry staining. Animals were sacrificed at 2, 4 and 8 hours, whereas naive animals were used for the 0 hour time point. Representative FACS plots with pooled 4-5 animals per time point from one experiment.

5 mg/kg i.p. to mice in DMSO 30 min before LPS. Mice were sacrificed at the 2 hour time point and serum and lungs were collected for further evaluation. The aim of these experiments was to test whether this *in vivo* model was appropriate to study anti-inflammatory effects of compounds or treatments.

Dexamethasone-pre-treated endotoxemic mice had lower levels of all pro-inflammatory mediators I measured in the serum compared to vehicle-treated animals (Figure 6.4). In particular, TNF-α levels were abrogated (Figure 6.4A), whereas IL-6, CCL2, CCL3, CXCL1 and CXCL2 were reduced by 50-67% (Figure 6.4B-F). CXCL5 levels showed a reduction trend in Dexamethasone-pre-treated endotoxemic mice, but did not reach statistical significance (Figure 6.4G).

Dexamethasone pre-treatment of endotoxemic mice led to a statistically significant reduction in the percentage of neutrophils (Figure 6.5B) and a significant increase of monocyte percentage in the lungs (Figure 6.5C) with a representative plot from one vehicle- and one Dexamethasone-treated mouse in Figure 6.5A. Coupled with the dramatic decrease in *Il6*, *Ccl2* and *Cxcl1* mRNA expression levels (Figure 6.5D-F), these data suggest that i.p. injection of Dexamethasone as a pre-treatment effectively reduced inflammation both locally and systemically.

6.3.3 CB2R KO animals have more neutrophils in the lungs compared to WT controls at the peak of inflammation

To assess whether CB2R genetic ablation affects the phenotype of mice during the endotoxemia model of systemic inflammation, I first assessed the clinical picture at the 2 hour peak time point.

I began by comparing the levels of pro-inflammatory mediators in the serum of WT and

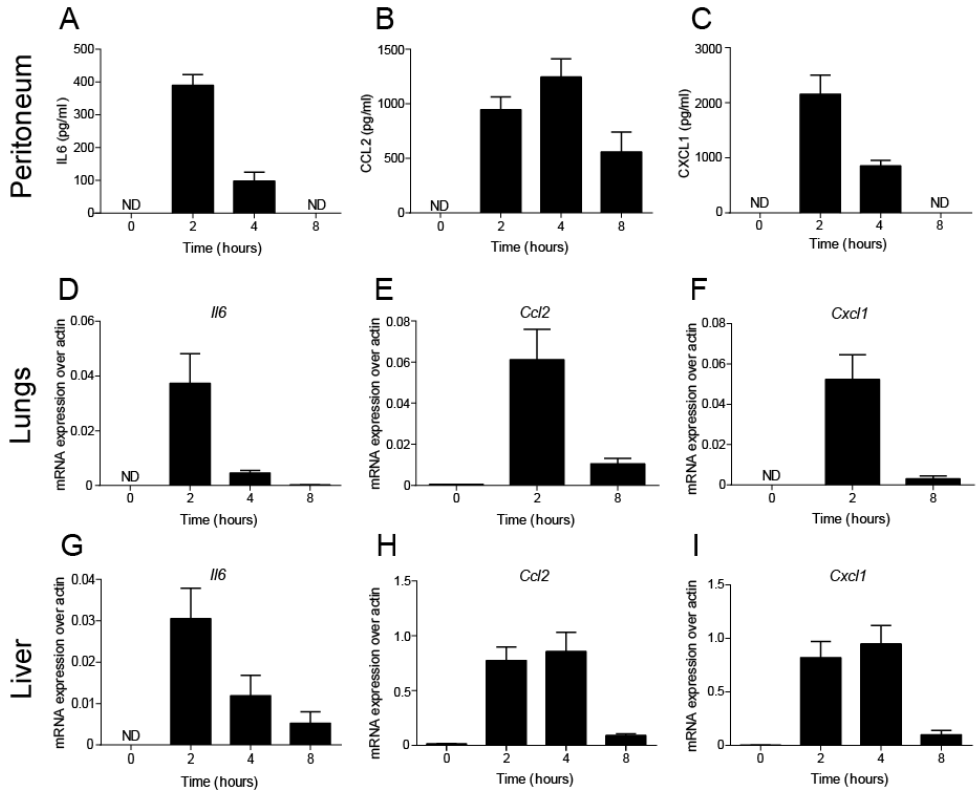


Figure 6.3. Time course analysis of pro-inflammatory cytokines and chemokines in the peritoneal fluid lavage, lung and liver homogenates of LPS-treated mice. Male C57BL/6J (8-10 weeks old) mice were given 1 mg/kg LPS i.p. and the levels of IL-6 (A, D, G), CCL2 (B, E, H) and CXCL1 (C, F, I) were assessed in lavaged peritoneal fluid and homogenised murine lungs and livers. Animals were sacrificed at 2, 4 and 8 hours, whereas naive animals were used for the 0 hour time point. Data are from one experiment with 4-5 animals per time point, ND not detected

CB2R KO mice. To unravel potential mechanisms that CB2R might be involved in, I measured pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12p70 and IFN-γ, monocyte-recruiting chemokines, such as CCL2, CCL3, CCL4 and CXCL10 and the IL-8 murine analogues of neutrophil-recruiting chemokines, CXCL1, CXCL2 and CXCL5. IL-1β, IL-12p70 and IFN-γ levels fell under the detection limit of the assay. The remaining cytokines and chemokines measured had comparable levels between the two genotypes (Figure 6.6A-I).

Next, I assessed leukocyte recruitment to the lungs in WT and CB2R KO animals at the 2 and 8 hour time point. I stained whole lung digests with antibodies against CD45, Ly-6G and Ly-6B.2, and identified neutrophils as $CD45^{+}Ly\text{-}6B.2^{+}Ly\text{-}6G^{+}$ and monocytes as $CD45^{+}Ly\text{-}6B.2^{+}Ly\text{-}6G^{-}$ cells. At 2 hours neutrophil numbers were significantly more in the lungs of CB2R KO mice, while monocytes had similar numbers in both genotypes (Figure 6.7C-D). However, at the 8 hour time point the numbers of both immune cell populations were comparable (Figure 6.7C-D). Representative plots from one WT and CB2R KO mouse are shown in Figure 6.7A and 6.7B, respectively.

The data show that, between neutrophils and monocytes, only the former are affected in the lungs in the absence of CB2R at the peak time point of the endotoxemia model.are significantly more than WT controls at the peak time point.

6.3.4 Neutrophil and monocyte numbers in the bone marrow of WT and CB2R KO mice are similar during the endotoxemia model time course

To test whether the bone marrow contributes to the recruitment of cells to the periphery as an emergency response, I stained harvested bone marrow cells with antibodies

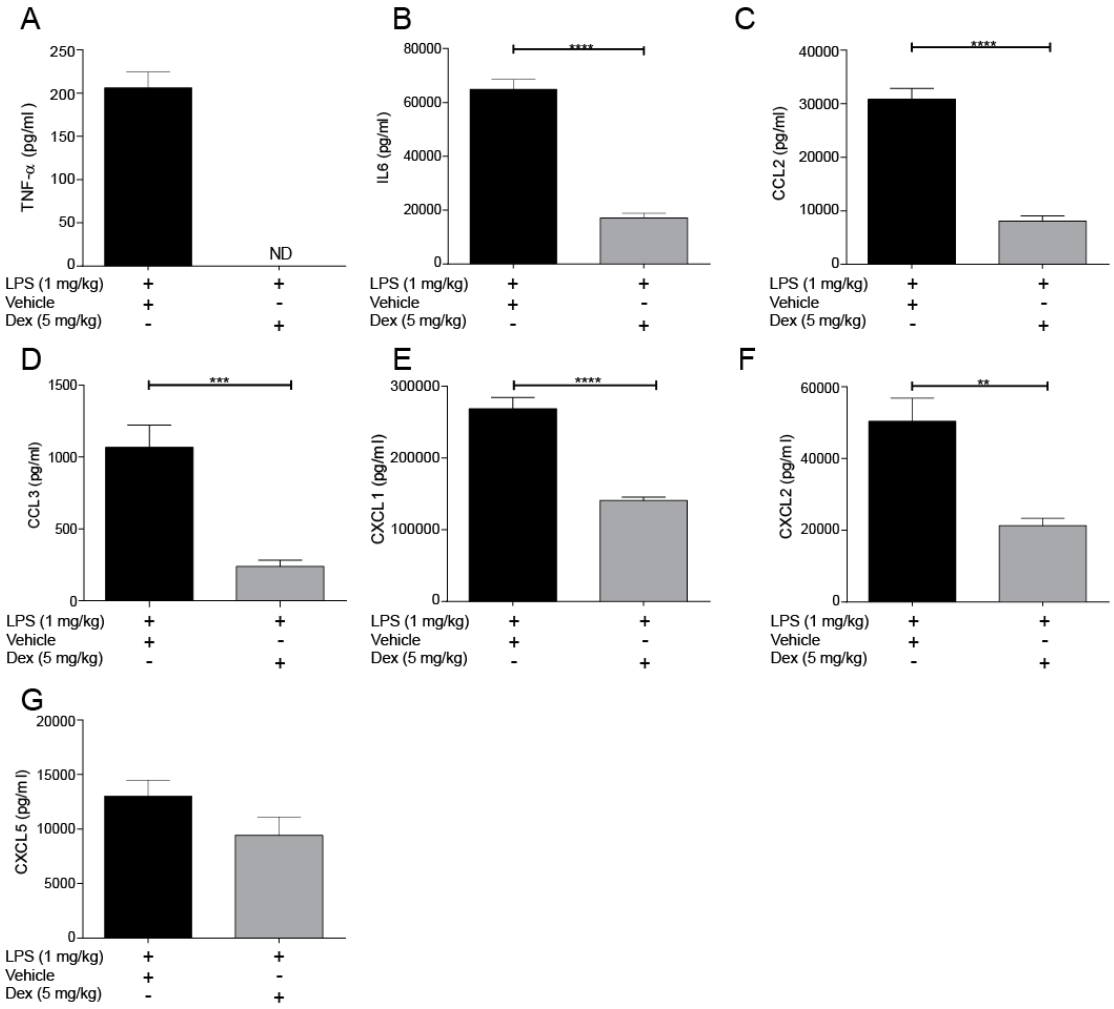


Figure 6.4. Dexamethasone mitigates levels of pro-inflammatory cytokines and chemokines in serum from LPS-treated mice. Male C57BL/6J (8-10 weeks old) mice were given 5 mg/kg Dexamethasone or vehicle 30 minutes before administration of 1 mg/kg LPS i.p. Two hours later the levels of TNF-α (A), IL-6 (B), CCL2 (C), CCL3 (D), CXCL1 (E), CXCL2 (F) and CXCL5 (G) were determined in the serum. Data are from two independent experiments with 6-8 animals per group. Mean+SEM are shown and data were analysed with student t test with (D-F), or without (A-C) Welch's correction ** p<0.01, *** p<0.001, **** p<0.0001, ND not detected

against CD45, CD11b, Ly-6C and Ly-6G. Flow Cytometry gating on $CD45^+CD11b^+$ cells distinguished neutrophils ($Ly\text{-}6C^+Ly\text{-}6G^+$) from monocytes ($Ly\text{-}6C^+Ly\text{-}6G^-$). Representative plots from one WT and CB2R KO mouse at 2 and 8 hours gated on $CD45^+CD11b^+$ cells are shown in Figure 6.8A-B.

Neutrophil and monocyte numbers in the bone marrow were decreased during the time course of the model in comparison with the baseline levels (Figure 5.2), suggesting that the tissue plays a major role in providing leukocytes to the periphery (Figure 6.8C-D). However, both immune cell populations in CB2R KO animals were similar to WT littermate control mice at the time points studied (Figure 6.8C-D), indicating that CB2R deficiency does not alter the contribution potential of the bone marrow to the periphery during endotoxemia.

6.3.5 CB2R KO animals display enhanced neutrophil recruitment to the spleen at the peak time point

Since the numbers of neutrophils in the lungs were significantly higher in CB2R KO mice, I assessed their numbers in the spleen as another potential organ where they might be recruited. I stained the tissue digests with antibodies against CD45, CD11b, Ly-6C and Ly-6G and gating at the $CD45^+CD11b^+$ cells identified the neutrophil populations as $Ly\text{-}6G^+Ly6C^+$. I found that neutrophil numbers were increased at 2 hours and were significantly more in the spleens of CB2R KO mice compared to their littermate controls (Figure 6.9C). On the contrary, at 8 hours they were reduced and were comparable between the two genotypes (Figure 6.9C). Taken together, my data suggest that neutrophil recruitment is enhanced in CB2R KO mice in another peripheral tissue apart from the lungs.

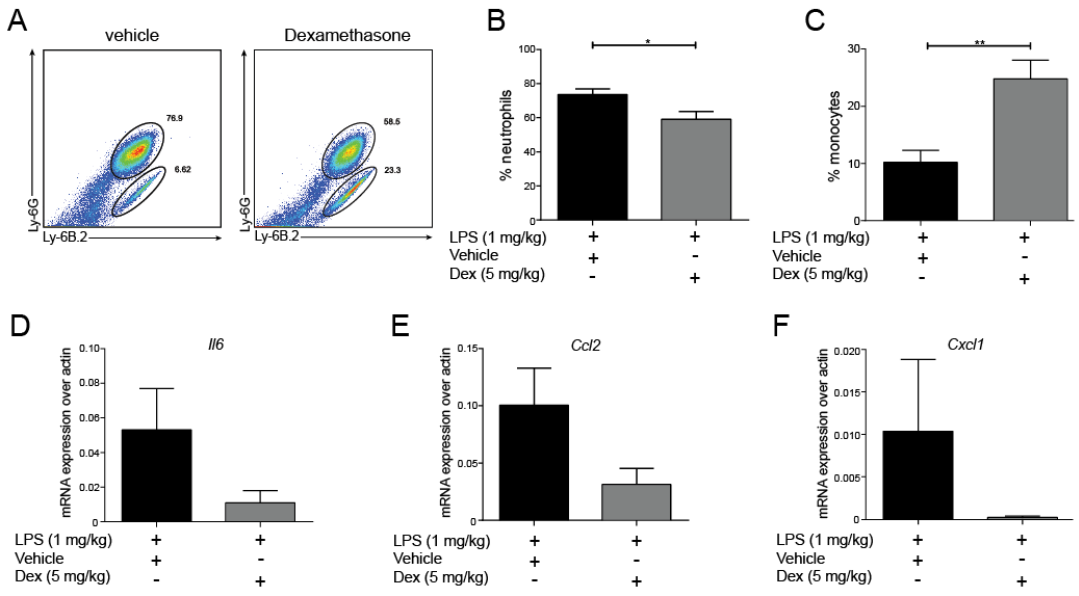


Figure 6.5. Dexamethasone promotes resolution of inflammation in LPS-treated mice lungs. Male C57BL/6J (8-10 weeks old) mice were given 5 mg/kg Dexamethasone or vehicle 30 minutes before administration of 1 mg/kg LPS i.p. Two hours later innate immune cell recruitment and pro-inflammatory mediator expression was determined in lungs. Representative Flow Cytometry plot from one animal from each group shows neutrophil (Ly-6B.2^{+}Ly-6G^{+}) and monocyte (Ly-6B.2^{+}Ly6G^{-}) population percentages (A). Pooled data from 8 animals per group are shown in (B) and (C). Levels of *Il6* (D), *Ccl2* (E) and *Cxcl1* (F) expression were determined by qPCR in lung homogenates. Data are from two independent experiments. Mean+SEM are shown and data were analysed with student t test, * $p<0.05$, ** $p<0.01$

6.4 Discussion

In this chapter, I wanted to test the hypothesis that CB2R genetic ablation disrupts endogenous immunosuppressive pathways resulting in an exacerbated inflammatory cascade. I chose a low dose endotoxemia inflammation model because this would allow me to study the effect of CB2R deficiency *in vivo* in context of systemic inflammation across an 8 hour time course.

The kinetic data support the view that this model is self-resolving and reaches its peak at 2 hours post i.p. LPS administration (as demonstrated by the measurement of several pro-inflammatory mediators in the serum, lungs, liver and peritoneal cavity). This finding is in line with the published literature in toxaemia models. For example, I also observed a rapid but transient upregulation of pro-inflammatory biomarkers in the serum which quickly decreased, but no obvious signs of septic shock, something that can be explained by the fact that I did not inject a high dose of LPS or co-inject D-galactosamine to induce liver injury (Buras *et al.* 2005). Given my emphasis on inflammatory mediators and innate immune cell mobilisation, it was not in my scope to assess haemodynamic changes in this model, but such an investigation could reveal common features of other established endotoxemia models, such as the lack of a hyperdynamic phase (Buras *et al.* 2005).

A cardinal feature of sepsis is multi-organ tissue destruction and failure (Abraham and Singer 2007). I therefore looked at peripheral organs most commonly affected in sepsis, such as the lungs and the liver. As the site of pathogen entry, the peritoneal cavity was inflamed, however the levels of the inflammatory cytokines and chemokines were lower than in the serum. This is probably due to the constant infusion of the injected LPS from the peritoneal cavity to the circulation which results in a suboptimal activation of

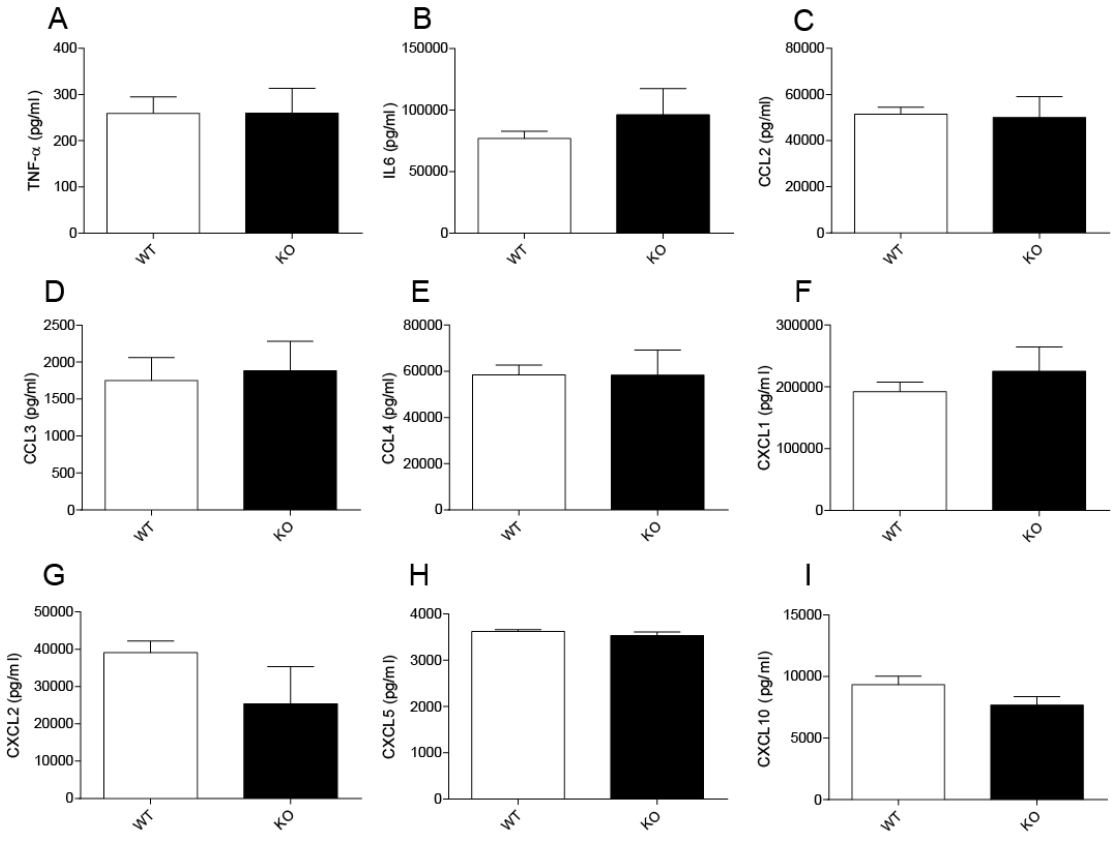


Figure 6.6. Pro-inflammatory mediator secretion in the serum of WT and CB2R KO animals administered i.p. with endotoxin for 2 hours. Male WT and CB2R KO (8-10 weeks old) mice were given 1 mg/kg LPS i.p. and the levels of TNF-α (A), IL-6 (B), CCL2 (C), CCL3 (D), CCL4 (E), CXCL1 (F), CXCL2 (G), CXCL5 (H) and CXCL10 (I) were determined. Data are from two independent experiments with 7-9 animals per group and were analysed with student t test. Mean+SEM are shown.

resident immune cells. Presumably, the bulk of TNF-α and IL-6 are synthesised in the liver.

Another reason could be that in the bloodstream there are more leukocyte subsets which respond to LPS challenge than in the peritoneum. In particular, the peritoneal cavity of naive mice is populated by resident macrophages and mast cells (Ajuebor *et al.* 1999) which can be activated by endotoxin. However, it has been observed that upon challenge with TLR ligands, resident macrophages rapidly leave the cavity and migrate to the mesenteric lymph nodes to carry out antigen presentation to lymphoid cells and that may account for less inflammation in the peritoneum.

In contrast, blood leukocytes are comprised of several innate immune cell populations which respond to endotoxin. The neutrophil pool can be activated directly or indirectly by the endothelium or platelets (Clark *et al.* 2007;Gill *et al.* 1998) to induce anti-microbial activities, including oxidative burst, degranulation and NETs (Chen *et al.* 2015;Liles *et al.* 2001;Nauseef and Borregaard 2014;Pugin *et al.* 1999). Although these immune cells have been shown to migrate to peripheral tissues, emergency myelopoiesis is initiated in the bone marrow and their numbers are constantly replenished, thus maintaining the higher mediator levels in the serum (Boettcher *et al.* 2014).

Analysis of the peritoneum, lungs and livers of endotoxemic mice found that the major recruited immune cell is the neutrophil. Their arrival coincides with the peak of the pro-inflammatory mediator expression in these tissues, and in particular that of *Cxcl1* which is a well characterised neutrophil chemoattractant protein that plays a crucial role in neutrophil mobilisation (Jin *et al.* 2014). Moreover, in the lungs I found a monocyte population which was not present in the liver. From my results, it is not clear whether

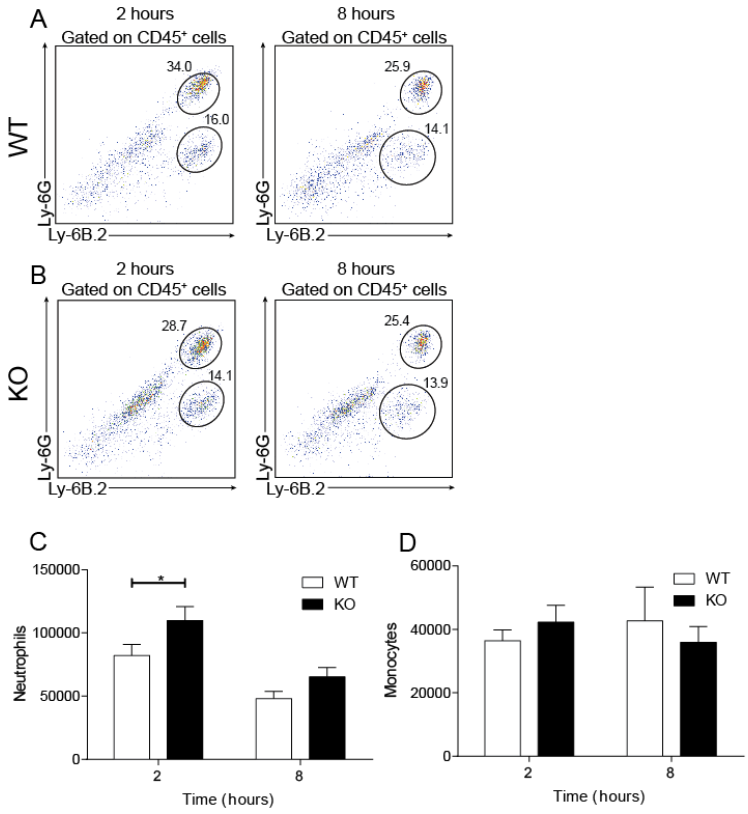


Figure 6.7. CB2R KO mice recruit more neutrophils to the lungs at the 2 hour time point. Male WT and CB2R KO mice (8-10 weeks old) were administered 1 mg/kg LPS i.p and neutrophil and monocyte recruitment to the lungs at 2 and 8 hours was assessed with Flow Cytometry. Representative Flow Cytometry plots from one WT and CB2R KO animal at the two time points are shown in (A) and (B). Pooled neutrophil (Ly-6B.2^{+}Ly-6G^{+}) and monocyte (Ly-6B.2^{+}Ly6G^{-}) population numbers from 9 animals per group are shown in (C) and (D). Mean+SEM are shown and data were analysed with two-way ANOVA with Sidak's post hoc multiple comparisons test, * $p<0.05$

these monocytes are resident lung monocytes or whether they are recruited to the lungs from the circulation.

Validation of the endotoxemia model was carried out to test whether this system is appropriate to study the anti-inflammatory potential of drugs. For this purpose, I chose to use Dexamethasone as glucocorticoids have been demonstrated to modulate cytokine release, leukocyte trafficking and coagulation in animal models (de Kruif *et al.* 2007), and it has already been used therapeutically in sepsis patients with some success (Marik 2011). In animal models, Dexamethasone has been shown to inhibit neutrophil migration to a dorsal air pouch of zymosan-treated mice (Perretti *et al.* 2002) and to the intestines of splanchnic artery occluded and reperfused rats (Cuzzocrea *et al.* 1997).

Because the endotoxemia model I set up is acute, I decided to pre-treat animals with the glucocorticoid for 30 min to allow the compound to enter the circulation before the administration of the inflammatory stimulus. The data clearly demonstrate that Dexamethasone serves as an effective anti-inflammatory agent, ablating TNF-α levels in serum and significantly reducing the other inflammatory markers I measured. Interestingly however, CXCL5 levels in the serum were not significantly affected. This is agreement with the literature where CXCL5 has been shown to exhibit different kinetics than other murine IL-8 isoforms and its levels remain unaffected in the lungs of mice treated with a similar dose of Dexamethasone (Rovai *et al.* 1998).

Having validated the low dose endotoxemia model, I proceeded to test my original hypothesis. My results show that CB2R deficiency at 2 hour peak time point does not affect the production of a range of pro-inflammatory cytokines and chemokines. This is in contrast to previous reports regarding the role of the endocannabinoid system in

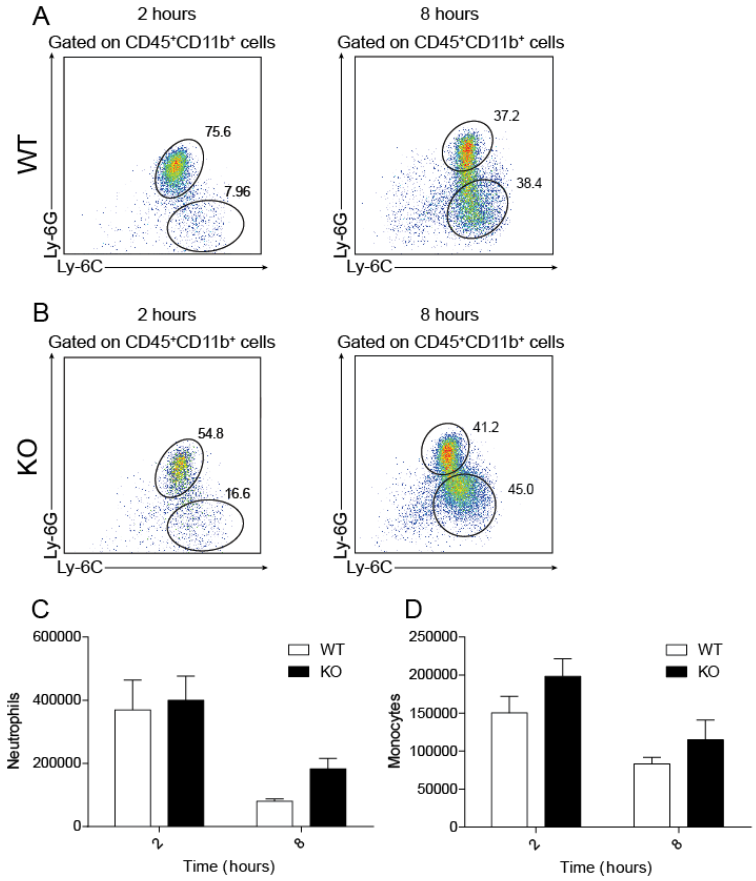


Figure 6.8. CB2R KO mice have a similar neutrophil and monocyte population in the bone marrow at 2 and 8 hours with littermate control animals. Male WT and CB2R KO mice (8-10 weeks old) were administered 1 mg/kg LPS i.p and neutrophil and monocyte numbers in the bone marrow were assessed at 2 and 8 hours with Flow Cytometry. Bone marrow was harvested from tibiae and femurs, the cells were passed through cell strainers and red blood cells were lysed. Representative Flow Cytometry plots from one WT and CB2R KO animal at the two time points are shown in (A) and (B). Pooled neutrophil (Ly-6B.2$^+$Ly-6G$^+$) and monocyte (Ly-6B.2$^+$Ly6G$^-$) population numbers from 7-10 animals per group are shown in (C) and (D). Mean+SEM are shown and data were analysed with two-way ANOVA with Sidak's post hoc multiple comparisons test

sepsis animal models. In particular, I did not see a protective role of CB2R as reported previously in studies where CB2R agonism with several natural and synthetic ligands was used (Berdyshev *et al.* 1998;Gui *et al.* 2013;Lehmann *et al.* 2012;Tschop *et al.* 2009) or a contributory role as Csoka *et al.* reported in CB2R KO mice (Csoka *et al.* 2009).

In contrast, I found differences in neutrophil recruitment to the lungs between WT and CB2R KO mice similar to other reports. Neutrophils play a key role in lung inflammation during sepsis both via their effector functions and crosstalk with other immune cells. In fact, Tsujimoto *et al.* showed that neutrophil elastase induces the expression of TLR4 in macrophages and secretion of CXCL2 in the lungs of CLP mice. CXCL2 then feeds back and is potentially responsible for the augmented neutrophil recruitment and tissue damage (Tsujimoto *et al.* 2005). My results are in agreement with Tschop *et al.* who also reported augmented recruitment of neutrophils to the lungs of CLP mice, while they identified that the cells were more activated than in WT animals (Tschop *et al.* 2009).

Monocytes have also been demonstrated to play a crucial role in promoting inflammation in sepsis as depletion of the classical monocyte-recruiting chemokine CCL2 leads to a reduction in LTB_4 levels and leukocyte recruitment to the peritoneum (Matsukawa *et al.* 1999). In my experiments I did not observe a change in the recruiting pattern of monocytes to the lungs in the absence of CB2R. Similarly, neither CB2R deletion, nor agonism with the CB2R-selective agonist Gp1a affected macrophage numbers in the lungs in a recent report (Tschop *et al.* 2009). Why CB2R deficiency affects neutrophil and not monocyte infiltration into the lungs is a question which warrants further investigation.

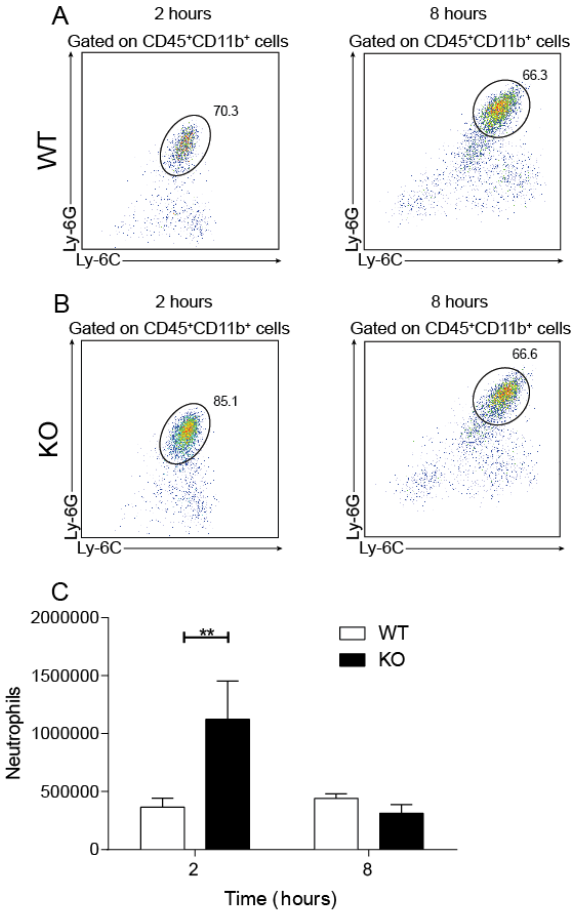


Figure 6.9. CB2R KO mice display higher neutrophil recruitment in the spleen at 2 hours compared to littermate control animals. Male WT and CB2R KO mice (8-10 weeks old) were administered 1 mg/kg LPS i.p and neutrophil numbers in the spleen were assessed at 2 and 8 hours with Flow Cytometry. Spleens were harvested, the cells were passed through cell strainers and red blood cells were lysed. The rest cells were stained for Flow Cytometry. Representative Flow Cytometry plots from one WT and CB2R KO animal at the two time points are shown in (A) and (B). Pooled neutrophil (Ly-6B.2^{+}Ly-6G^{+}) and monocyte (Ly-6B.2^{+}Ly6G^{-}) population numbers from 7-10 animals per group are shown in (C) and (D). Mean+SEM are shown and data were analysed with two-way ANOVA with Sidak's post hoc multiple comparisons test

Emergency myelopoiesis is initiatied in the bone marrow and the spleen via colony stimulating growth factor signals (Boettcher *et al.* 2014;Lundblad *et al.* 1995). Progenitor cells generate neutrophils and monocytes in a Jagged-1-dependent manner (Pasquevich *et al.* 2015;Skirecki *et al.* 2015) and this physiological process is essential for efficient immune responses and pathogen clearance (Lundblad *et al.* 1995).

The substantial drop in the numbers of bone marrow neutrophils and monocytes in both genotypes compared to baseline confirms the significance of this tissue during endotoxemia for both genotypes. However, the fact that the numbers of both immune populations studied are comparable between the two genotypes during endotoxemia, given the differences observed under naïve conditions, hints that there are more neutrophils and fewer monocytes egressing from the bone marrow in CB2R KO mice. Consistent with the literature current state presented in Chapter 3 these data support the idea that CB2R might regulate migration of neutrophils and monocytes differently.

A plausible destination for neutrophils and monocytes during endotoxemia is the spleen as this organ hosts both populations (Robbins *et al.* 2012;Summers *et al.* 2010;Swirski *et al.* 2009). Indeed, I found more neutrophils in the spleens of CB2R KO animals at the peak time point. This finding has not been reported in the cannabinoid literature before and suggests that neutrophil infiltration into other peripheral tissues might also be regulated by CB2R.

It is, for instance, known that neutrophils migrate to the liver in a CXCL2- and Ron receptor tyrosine kinase-dependent manner in sepsis models (Caldwell *et al.* 2008;Dorman *et al.* 2005) where they interact with Kupffer cells to clear bacteria (Gregory *et al.* 2002). Similarly, neutrophils have been found in the peritoneal cavity and the kidney in the CLP model (Castoldi *et al.* 2012). Studying the recruitment of

neutrophils to these tissues in WT and CB2R KO animals is therefore a plausible future direction.

The main weakness of this chapter is the lack of mechanistic data to explain the substantial increase in neutrophil migration to the lungs and spleen of CB2R KO mice. The comparable levels of pro-inflammatory mediators in the serum of WT and CB2R KO mice rule out a systemic differential secretion of neutrophil-recruiting chemokines and it is very likely that the soluble mediators that cause the selective upregulation of neutrophils in CB2R KO mice are secreted locally. A and comparison between WT and CB2R KO animal cytokine and chemokine levels in the lungs and spleen is therefore needed to reveal which molecules are important for the enhanced infiltration of neutrophils in these tissues of CB2R KO mice.

In summary, in this chapter I have shown that CB2R KO mice present with enhanced recruitment of neutrophils to the lungs and spleen in the endotoxemia model at the peak of the inflammatory response. In contrast, mobilisation of monocytes to the lungs is similar between the two genotypes. Thus, my data support a role of CB2R in regulating leukocyte recruitment during inflammatory responses.

6.5 Limitations of the study

The limitations of the experiments described in this chapter are primarily related to the model itself. This low dose endotoxemia model does not fully map on to human sepsis in that it is a resolving model of acute inflammation. Second, the dose used in this study was largely dictated by limitations of the animal project licence which is the reason why no severe pathology was observed at any of the time points assessed. Collectively, my

data suggest that this model is suitable to study the kinetics of systemic inflammation, but not sepsis.

In addition, more information regarding cell recruitment could have been extracted from Flow Cytometry studies of blood samples, however this was technically challenging because blood collected from the hepatic vein of endotoxemic mice clotted rapidly.

Chapter 7: General discussion

7.1 Original hypothesis

The original hypothesis of my DPhil was that CB2R is involved in endogenous anti-inflammatory pathways. Therefore, activation of this receptor would ameliorate the outcome of immune responses. Congruent with this, CB2R activation would lead to the acceleration of endogenous pro-resolution pathways to terminate inflammation cascade and initiate wound healing and tissue repair. Based on my hypothesis, I would expect that pharmacological inhibition or genetic ablation of CB2R would lead to exacerbated immune responses *in vitro* and *in vivo* and possibly delay resolution of inflammation.

7.2 Key findings

- My *in vivo* experiments with the CB2R-selective agonist synthesised in the Greaves lab (Chapter 3) highlighted the limitations in the pharmacological properties of these compounds. I found that CB2R-selective agonists can be very lipophilic and cause secondary inflammation due to formulation issues. Therefore development of new solvents or more soluble agonists should be a priority in the field of cannabinoid research in pre-clinical studies.
- I ruled out the involvement of CB2R in the activation of endogenous anti-inflammatory pathways in primary macrophages during inflammation (Chapter 4). The use of both CB2R-selective ligands and CB2R KO cells confirmed that CB2R cannot be an anti-inflammatory target in these cells. Moreover, I speculated that the lack of anti-inflammatory effects may be the result of the dramatic downregulation of *Cnr2* mRNA levels under inflammatory conditions.

- However, CB2R deficiency in mice causes a dysregulation of immune cell trafficking under homeostatic conditions (Chapter 5). I showed that CB2R KO mice disaply a trend for more neutrophils and fewer monocytes in the bone marrow and significantly more neutrophils in the liver.
- In my final results chapter, I showed that CB2R is implicated in the inhibition of neutrophil infiltration to the lungs and the spleen of male mice at the peak of the inflammatory model. My data therefore suggest that CB2R might be an appropriate molecule to target therapeutically.

7.3 Future directions

My findings open new research avenues to be explored in the cannabinoid field. Had I more time, I would like to carry out additional experiments to understand more about the functions of CB2R in inflammation.

First, I would test the functions of endocannabinoids and CB1R/CB2R agonists in macrophage activation assays to discern whether CB1R is part of anti-inflammatory endogenous pathways. There are reports that *Cnr1* levels are upregulated during inflammation (Miller and Devi 2011), therefore the use of these ligands together with the evaluation of its mRNA levels would provide an insight into the role of the other cannabinoid receptor in the periphery. For example, CB1R KO keratinocytes secrete more pro-inflammatory chemokines and exacerbate disease in a murine contact allergic inflammation model (Gaffal *et al.* 2013).

Moreover, I would like to extend my studies on BMDMs reported in Chapter 4. It is intriguing that *Cnr2* levels decline dramatically following LPS and IFN-γ treatment. It would be worthwhile to test several different inflammatory stimuli to test how the

inflammatory insult alters the expresion of cannabinoid receptors and endocannabinoid system components. I could use different TLR ligands, such as the TLR2 agonist zymosan used in Chapter 3 or the TLR3 and TLR5 ligands Poly I:C and flagellin.

Importantly, in Chapter 4 I discussed my findings on CB2R agonism under LPS and IFN-γ, the prototype M1 stimulus. However, it is not yet understood whether CB2R is involved in macrophage alternative activation by cytokines, such as IL-4 and IL-10 or immune complexes. Given the reported macrophage plasticity, it is possible that CB2R-selective agonists may mediate their effects under a different activation pattern.

I would like to characterise the immune cell populations in the CB2R KO mouse strain in more detail. In Chapter 5, I focused mainly on tissues where neutrophils and monocytes reside under naïve conditions. However, there are other organs which are of interest due to the micro-environments they create. For example, mucosal tissues are at the interface of host with the outer environment, while the brain, the liver, white adipose tissue and the peritoneum are home to tissue-resident macrophages which, apart from protection from pathogens, mediate key homeostatic functions (Hill *et al.* 2014;Varol *et al.* 2015). Alterations in the numbers of these immune cells in CB2R KO mice might impair these physiological processes. Therefore the evaluation of their numbers could potentially reveal novel roles for CB2R at homeostasis.

Finally, further work is needed to understand the mechanism by which CB2R deficiency accelerates neutrophil recruitment to the periphery in the endotoxemia model. One hypothesis is to look at locally produced neutrophil-recruiting chemokines in the tissues of interest to explain the augmented neutrophil migration in CB2R KO mice. Based on the findings, the use of blocking antibodies or recombinant peptides could take the story forward to confirm the involvement of certain mediators.

Furthermore, whether CB2R activation by endocannabinoids inhibits proliferation and differentiation of myeloid progenitors in the bone marrow is an important question that arose from Chapter 6 and warrants further investigation. In more detail, I would like to investigate a possible antagonistic effect of CB2R on growth factor-mediated proliferation of myeloid progenitors.

7.4 Outstanding issues in the cannabinoid field

After critically assessing the current published literature on CB2R and the endocannabinoid system in general, I feel that we do not understand its implications in health and disease in enough depth. Contributing to this, is the lack of tools to convincingly determine which cell types express the receptor and how its levels are regulated in inflammatory diseases. For example, the commercially available antibodies have been reported to exhibit non-specific staining and lot-to-lot variability (Cecyre *et al.* 2014) making unequivocal identification of CB2R protein in native tissues tough (Marchalant *et al.* 2014). As a result mRNA expression assessment is the only currently reliable way to measure changes in CB1R/CB2R expression.

To this end, the use of the novel genome editing techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR) coupled with CRISPR-associated endonuclease (Cas9) could prove revolutionary (Jinek *et al.* 2012). A reporter mouse strain with the genetic sequence of a fluorescent protein knocked in-frame at the *Cnr2* locus would allow for CB2R haplosufficient (one allele) or CB2R KO (two alleles) fluorescent cell sorting using a no-touch approach. A similar approach was recently utilised to create a CB2R-green fluorescent protein (GFP) transgenic mouse strain (Schmole *et al.* 2015).

Furthermore, although different classes of CB2R ligands exist and a new generation of agonists has been synthesised, many of them come with formulation issues which makes the prospect of using them therapeutically in clinical trials troublesome. More work to understand the changes in receptor conformation upon each ligand binding will not only be useful from an academic perspective, but will also link the agonist molecular structure with the amino acid residues important for binding to CB2R. If we could determine which signalling pathways are critical for the mediation of CB2R-selective ligand anti-inflammatory effects in pre-clinical animal models, we could perform virtual screens using them as templates to synthesise novel compounds with high affinity at CB2R and more desirable pharmacological characteristics, e.g. higher solubility and longer half-life (Renault *et al.* 2013).

Lastly, I believe that despite numerous reports of anti-inflammatory effects of CB2R-selective ligands during the past decades, basic research and target validation of this GPCR in animals and humans are still in their infancy. There are many crucial unanswered questions regarding CB2R biology, the answers to which could facilitate the design of appropriate therapeutics. Hereby I only mention a few; is CB2R a pro-inflammatory, anti-inflammatory and/or a pro-resolution receptor? What does its transcriptional regulation during inflammation tell us about its role in this physiological process? Would CB2R activation/inhibition be desirable for all or groups of inflammatory illnesses? Is there a cause and effect association of CB2R and endocannabinoid system polymorphisms/mutations in inflammatory diseases? How is the endocannabinoid system related to the lipid mediator networks active in inflammation? Is there a sexual dimorphism in the responses of male and female

individuals to CB2R agonists? Do the effects of CB2R activation decline with age? Are CB2R-mediated responses controlled by circadian rhythms?

Understanding CB2R biology is important to clarify whether this receptor is one we want to target in our attempts to alleviate inflammatory diseases, and if so, what the most appropriate intervention designs should be (e.g. diseases to be targeted, age groups, disease stage, route of administration, dosage, formulation). I believe that only when we acquire this basic knowledge, will we be ready to deliver efficient drugs based on CB2R which will manipulate the endocannabinoid system to treat inflammatory diseases.

7.5 Final conclusion

Despite numerous studies on the endocannabinoid system roles in inflammation, the field has not lived up to the high expectations created after the discovery of the cannabinoid receptors. My DPhil work highlights some of the limitations of CB2R-selective ligands in animal models of disease, but also brings new data to our attention regarding the potential homeostatic and inflammatory role of CB2R which will hopefully lead to a re-evaluation of the role of this receptor in immunity and contribute to novel drug discovery based on the endocannabinoid system.

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