The Regulation of Interleukin-10 and Interleukin-12 in Macrophages: Investigating the differential regulation of IL-10 and IL-12 in C57BL/6 and BALB/c mice

Ashleigh Frances Howes

June 2013

Division of Immunoregulation,
MRC National Institute for Medical Research,
The Ridgeway, Mill Hill,
London, NW7 1AA

A thesis submitted to University College London
for the Degree of Doctor of Philosophy

Declaration

I Ashleigh Frances Howes confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Pattern recognition receptors (PRR) detect microbial products and induce cytokines which shape the immunological response. Interleukin-12 (IL-12) is a proinflammatory cytokine important for the differentiation of T helper 1 (Th1) cells which produce IFN-γ to activate macrophages and eradicate intracellular pathogens. In contrast, interleukin-10 (IL-10) is an immunosuppressive cytokine that minimises immune-driven host pathology, but can also lead to defective pathogen clearance. The regulation of IL-10 and IL-12 is therefore of interest due to their central roles in the orchestration of an effective but regulated immune response. C57BL/6 and BALB/c mice differ significantly in their resistance to several pathogens. We observed that macrophages generated from these mice produce reciprocal levels of IL-10 and IL-12 in response to the bacterial ligands LPS and Pam3CSK4, which activate TLR4 and TLR2 respectively, and heat-killed *Burkholderia pseudomallei*, a Gram-negative bacterium which activates TLR2 and TLR4. We have investigated this differential cytokine production in order to further dissect the molecular mechanisms underlying the regulation of IL-10 and IL-12. Detailed analyses of protein production, signal transduction and transcriptional kinetics have identified a type I IFN dependent, but IL-27 independent mechanism for the differential production of IL-10 in LPS and heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages. Microarray analysis of LPS stimulated C57BL/6 and BALB/c macrophages further revealed potential regulatory networks that may differ between these mouse strains. These findings highlight key pathways responsible for the regulation of IL-10 and IL-12, and may provide valuable information on factors contributing to the ability of C57BL/6 and BALB/c mice to clear bacterial infections.

Acknowledgement

I would like to begin by thanking my principal supervisor, Dr Anne O'Garra, for taking me on as a PhD student in her lab, for her guidance throughout the course of this project and for her support towards my future career. I would additionally like to thank the members of my thesis committee Dr Gregory Bancroft, Dr Steve Ley and Dr Jean Langhorne, for their helpful suggestions and discussions at all stages of my PhD. I would like to thank Dr John Ewbank for the time he took to train me in the lab at the start of my PhD. I thank Dr Xuemei Wu and Dr Margarida Saraiva for their contributions to the initial findings which gave rise to this project, and their continued advice throughout this investigation. I am also very grateful to Dr Xuemei Wu and Dr Damian Carragher for their organisation of mouse breeding and for doing their utmost to ensure I had the mice required to carry out my experiments. I thank Dr Christine Graham and Dr Harsha Jani for their help with RNA preparation and microarray processing, and Evangelos Stavropoulos for his help with *in vivo* experiments and going out of his way to make sure I was always prepared for my experiments. I would like to thank Dr Simon Blankley and Dr Krzysztof Potempa for their helpful discussions on microarray analysis. My gratitude also goes to Dr Leona Gabrysova, Dr Finlay McNab and Dr Jonathan Pitt for always having time to give advice and discuss science. I would additionally like to thank all members of the O'Garra lab for making the day to day environment of the lab a friendly and lively place to work. I also thank Dr Anna Gibson for her tireless efforts to sort out all our lab needs, and the animal facility at NIMR for breeding and housing the animals used in this project.

On a personal note, I would like to thank my past and present housemates Tris, Hollie, Meghan and Hannah for being great company, providing encouragement when needed and always checking I'm OK when I'm running late in the lab. I also thank Jay for his continuing support, particularly in the writing of this thesis. Finally I would like to say a big thank you to my parents and my brother who have always had confidence in me and have given me their utmost support throughout all of my endeavours.

Table of Contents

Abstract…… **3**

Acknowledgement **4**

Table of Contents **6**

Table of figures **14**

List of tables **18**

Abbreviations **20**

Chapter 1. General Introduction **23**

1.1 An overview of the Immune Response 24

1.2 The role of IL-12 in the immune response 25

1.2.1 Cellular sources and targets of IL-12 26

1.2.2 IL-12 and the regulation of immune responses 27

1.3 The role of IL-10 in the immune response 30

1.3.1 Cellular sources and targets of IL-10 32

1.3.2 The regulation of immune responses by IL-10 34

1.3.2.1 *The role of IL-10 in protection against immunopathology* 35

1.3.2.2 *The inhibition of protective immune responses by IL-10* 37

1.3.2.3 *The complex roles of IL-10 in autoimmune diseases and anti-tumour immune responses* 38

1.4 Macrophages and the immune response 40

1.5 The recognition of infection and signal transduction by PRRs 43

1.5.1 TLR-induced signal transduction 43

1.5.1.1 *MyD88 dependent TLR signalling* 45

1.5.1.2 *TRIF dependent TLR signalling* 47

1.5.1.3 *PI(3)K/AKT/mTOR signalling induction by TLRs* 47

1.5.2 Non-TLR PRR signal transduction 48

1.5.2.1 *CLRs* 48

1.5.2.2 *NLRs* 48

1.5.2.3 *RLRs* 49

1.6 The molecular mechanisms of IL-10 regulation 50

1.6.1 The regulation of IL-10 production in macrophages and mDCs 50
1.6.1.1 *The induction of IL-10 by PRRs* 51
1.6.1.2 *The negative regulation of IL-10 in macrophages and DCs* 57
1.6.1.3 *The regulation of IL-10 in macrophages and DCs by autocrine and paracrine factors* 58
1.6.2 The regulation of IL-10 in other cell types 60
1.7 The molecular mechanisms of IL-12 regulation 61
1.7.1 The induction of IL-12 by PRRs 62
1.7.2 The negative regulation of IL-12 66
1.7.3 The modulation of IL-12 production by T cell signals, and autocrine and paracrine factors 68
1.8 The coordinate regulation of IL-10 and IL-12 69
1.9 The role of type I IFN in the immune response 70
1.9.1 Cellular sources and targets of type I IFN 70
1.9.2 The regulation of immune responses by type I IFN 72
1.9.3 The regulation of type I IFN production in macrophages and DCs 73
1.10 Using inbred strains of mice to study the immune response 75
1.10.1 The impact of genetic background: C57BL/6 and BALB/c mice 75
1.10.2 *Burkholderia pseudomallei* as a model of C57BL/6 and BALB/c resistance and susceptibility 77
1.11 Project perspective: Using C57BL/6 and BALB/c mice to study the regulation of IL-10 and IL-12 81

Chapter 2. Materials and Methods 87
2.1 Mice 88
2.2 Reagents 88
2.2.1 Cell culture medium 88
2.2.2 PRR ligands and heat-killed bacteria 89
2.2.3 Recombinant cytokines and monoclonal antibodies 89
2.2.4 Inhibitors 89
2.3 *In vitro* differentiation and stimulation of macrophages and DCs 90
2.3.1 Generation of bone marrow derived macrophages (BMDM) 90

2.3.2 Generation of bone marrow derived DCs (BMDC) 90
2.4 Quantification of cytokine production by ELISA 91
2.5 Western blotting analysis of cellular proteins 92
2.5.1 Protein extraction and quantification 92
2.5.2 Protein separation and visualisation 93
2.6 RNA isolation and purification 94
2.7 cDNA preparation and real-time quantitative PCR (qPCR) analysis 94
2.7.1 Primer Design for the quantification of premature *Il10* mRNA 96
2.8 Microarray analysis 97
2.8.1 Verification of RNA quality 97
2.8.2 Preparation of RNA for microarray analysis 97
2.8.3 Microarray processing 98
2.8.4 Microarray data analysis 98
2.8.4.1 *Pre-analysis data processing, normalisation, fold change and statistical analyses* 98
2.8.4.2 *Clustering analyses* 99
2.8.4.3 *Gene Ontology analysis* 99
2.8.4.4 *Pathway and upstream regulator analyses by IPA* 100
2.8.4.5 *Transcription factor binding site enrichment analysis by PSCAN* 100
2.8.4.6 *Quality control analysis of microarray samples* 101
2.8.4.7 *Comparison of basal gene expression in C57BL/6 and BALB/c macrophages* 102
2.9 *In vivo* experiments 102
2.9.1 LPS administration 103
2.9.2 Spleen processing and RNA extraction 103
2.9.3 Serum processing and cytokine quantification 103
2.10 Statistical analysis 104

Chapter 3. The regulation of IL-10 in C57BL/6 and BALB/c macrophages 107
3.1 Background 108
3.2 Investigating the regulation of IL-10 in C57BL/6 and BALB/c macrophages: Study Aims 111

3.3 Results 112
3.3.1 Investigation into TLR ligand and heat-killed bacteria induced IL-10 production in C57BL/6 and BALB/c macrophages 112
3.3.1.1 *C57BL/6 and BALB/c macrophages produce distinct levels of IL-10 when stimulated with TLR ligands or heat-killed bacteria* 112
3.3.1.2 *TLR2, TLR4, MyD88 and TRIF contribute to HkBps induced IL-10 production* 114
3.3.2 Investigation into the mechanisms of differential IL-10 production in LPS, Pam3CSK4 and HkBps stimulated C57BL/6 and BALB/c macrophages. 116
3.3.2.1 *C57BL/6 macrophages produce higher levels of IL-10 compared to BALB/c macrophages throughout the response to LPS, Pam3CSK4 and HkBps*.116
3.3.2.2 *C57BL/6 mDCs do not produce higher levels of IL-10 than BALB/c mDCs when stimulated with LPS, Pam3CSK4 or HkBps* 116
3.3.2.3 *The level of* Tlr *mRNA expression does not correlate with the level of IL-10 production in C57BL/6 and BALB/c macrophages.* 117
3.3.2.4 *ERK1/2 and p38 are central regulators of IL-10, but are similarly activated in TLR2 and TLR4 stimulated C57BL/6 and BALB/c macrophages*.... 118
3.3.2.5 *LPS, Pam3CSK4 and HkBps differentially induce* Il10 *transcription in C57BL/6 and BALB/c macrophages.* 120
3.3.2.6 *LPS and HkBps stimulated C57BL/6 macrophages express higher levels of* Ifnb1 *mRNA and IFN-β protein than BALB/c macrophages.* 121
3.3.2.7 *Type I IFN signalling mediates the second peak of* Il10 *mRNA and contributes to higher levels of IL-10 protein production in LPS and HkBps stimulated C57BL/6 macrophages* 122
3.3.2.8 *Treatment with exogenous IFN-β enhances IL-10 production in both C57BL/6 and BALB/c macrophages.* 124
3.3.3 Investigation into the mechanisms of type I IFN mediated regulation of IL-10 in C57BL/6 and BALB/c macrophages. 125
3.3.3.1 *The second peak of* Il10 *mRNA in C57BL/6 macrophages is likely to be mediated by a transcriptional mechanism* 125
3.3.3.2 *IL-27 is not required for type I IFN mediated enhancement of IL-10 production in macrophages.* 126

3.3.3.3 *STAT-1 has an important role in the type I IFN mediated induction of IL-10*…… 129
3.3.3.4 *STAT-1 is phosphorylated in LPS stimulated C57BL/6 and BALB/c macrophages but not C57BL/6* Ifnar1$^{-/-}$ *macrophages*. 131
3.3.4 Investigation into the genetic factors regulating differential IL-10 production in LPS, Pam3CSK4 and HkBps stimulated C57BL/6 and BALB/c macrophages. 132
3.3.4.1 *C57BL/6 x BALB/c F1 macrophages produce intermediate levels of IL-10*…… 132
3.3.4.2 *Preliminary analysis of LPS, Pam3CSK4 and HkBps stimulated C57BL/6 x BALB/c F2 macrophages shows segregation of the genetic factors controlling IL-10 production.* 133
3.3.5 Investigation into IL-10 production in C57BL/6 and BALB/c mice *in vivo*.134
3.3.5.1 *LPS treatment induces higher* Il10 *and* Ifnb1 *mRNA expression in the spleen of C57BL/6* Rag1$^{-/-}$ *mice compared to BALB/c* Rag2$^{-/-}$ *mice* 134
3.4 Discussion 136
3.4.1 Mechanisms of differential IL-10 production in C57BL/6 and BALB/c macrophages 136
3.4.2 The mechanisms of type I IFN mediated IL-10 regulation in LPS and heat-killed *B.pseudomallei* stimulated cells 143
3.4.3 Genetic factors underlying differential IL-10 production in C57BL/6 and BALB/c macrophages 146
3.4.4 Differential IL-10 and type I IFN production in C57BL/6 and BALB/c macrophages in the context of bacterial infection 147
3.4.5 IL-10 and type I IFN production in C57BL/6 and BALB/c mice *in vivo* 149

Chapter 4. The Regulation of IL-12 in C57BL/6 and BALB/c macrophages 171
4.1 Background 172
4.2 Investigating the regulation of IL-12 in C57BL/6 and BALB/c macrophages: Study Aims 175
4.3 Results 176

4.3.1 IL-12p40 production is not consistently different between C57BL/6 and BALB/c macrophages, and IL-12p70 production is difficult to detect. 176
4.3.2 LPS, Pam3CSK4 and HkBps induce higher levels of *Il12a* mRNA expression in BALB/c macrophages. 177
4.3.3 Higher levels of IL-12p70 in BALB/c macrophages are revealed at low cell density, and are independent of IL-10 in LPS and HkBps stimulated macrophages. 177
4.3.4 IL-12p70 production in LPS or HkBps stimulated C57BL/6 macrophages is not affected by endogenous type I IFN. 179
4.3.5 BALB/c macrophages produce higher levels of TNF-α and IL-1β in response to LPS, Pam3CSK4 and HkBps. 180
4.3.6 IL-10 accounts for differential proinflammatory cytokine production in response to Pam3CSK4 but not LPS or HkBps. 181
4.3.7 Type I IFN may contribute to differential IL-1β production in LPS and HkBps stimulated C57BL/6 and BALB/c macrophages. 182
4.4 Discussion 183
4.4.1 Differential IL-12 production in C57BL/6 and BALB/c macrophages and the effect of cell density. 184
4.4.2 Mechanisms of differential IL-12 production in C57BL/6 and BALB/c macrophages. 185
4.4.3 Mechanisms of differential TNF and IL-1β production in C57BL/6 and BALB/c macrophages 186
4.4.4 Differential proinflammatory cytokine production in C57BL/6 and BALB/c macrophages in the context of infection and inflammatory diseases 188

Chapter 5. Microarray Analysis of the LPS induced Transcriptional Response in C57BL/6 and BALB/c Macrophages 197
5.1 Background 198
5.2 Investigating the LPS induced transcriptional response of C57BL/6 and BALB/c macrophages: Study aims 200
5.3 Results 201
5.3.1 Experimental design 201

5.3.2 LPS induces a dynamic transcriptional response in C57BL/6 and BALB/c macrophages 201
5.3.3 Identification of significantly differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages 203
5.3.4 C57BL/6 macrophages may have a stronger LPS induced transcriptional response than BALB/c macrophages 205
5.3.5 *k*-means clustering of differentially expressed genes identifies the groups of genes that most clearly distinguish LPS induced C57BL/6 and BALB/c transcriptional profiles 206
5.3.5.1 *Clusters with higher expression in LPS stimulated C57BL/6 macrophages* 206
5.3.5.2 *Clusters with higher expression in LPS stimulated BALB/c macrophages* 210
5.3.6 Ingenuity pathway analysis of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages. 212
5.3.7 Identification of candidate transcription factors that may be responsible for differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages 214
5.3.7.1 *Identification of upstream transcriptional regulators associated with differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages by IPA* 215
5.3.7.2 *Transcription factor binding motif enrichment analysis of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages* 217
5.3.7.3 *Differential transcription factor mRNA expression in LPS stimulated C57BL/6 and BALB/c macrophages.* 219
5.3.7.4 *Transcription factor candidates mediating differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages identified by comparison of IPA, PSCAN and transcription factor mRNA expression* 221
5.4 Discussion 223
5.4.1 The overall transcriptional profiles of LPS stimulated C57BL/6 and BALB/c macrophages 223
5.4.2 Basal gene expression in C57BL/6 and BALB/c macrophages 224

5.4.3 Differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages 225
5.4.4 Putative transcriptional regulators involved in the differential gene expression of LPS stimulated C57BL/6 and BALB/c macrophages 228

Chapter 6. Summary and Future Perspectives 247
6.1 Summary 248
6.2 Future perspectives 250
6.2.1 Continuing to use C57BL/6 and BALB/c macrophages to study the molecular regulation of IL-10 and IL-12 250
6.2.1.1 *Further investigation into the mechanisms of type I IFN dependent regulation of IL-10* 250
6.2.1.2 *Independent validation of microarray analysis and understanding the C57BL/6 and BALB/c genetic differences that are dependent on type I IFN* 252
6.2.1.3 *Studies towards understanding the genetic variation underlying differential IL-10 and IL-12 production in C57BL/6 and BALB/c macrophages* .253
6.2.2 Extending differential cytokine production in C57BL/6 and BALB/c macrophages to models of *B.pseudomallei* infection 254
6.3 Concluding remarks 256

Chapter 7. Appendix 258

Reference List 281

Table of figures

Chapter 1

Figure 1.1 Summary of TLR signalling in macrophages and DCs. 83
Figure 1.2 The induction of IL-10 by TLRs in macrophages, monocytes and DCs. 84
Figure 1.3 The induction of IL-12 by TLRs (and IFN-γ) in macrophages and DCs. 85
Figure 1.4 The co-ordinate regulation of IL-10 and IL-12 in macrophages and DCs. ...86

Chapter 2

Figure 2.1 Quality control analysis of microarray samples. 105
Figure 2.2 Genes differentially expressed at baseline in C57BL/6 and BALB/c macrophages associate with MHC protein and immune response GO terms. 106

Chapter 3

Figure 3.1 C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages when stimulated with LPS, Pam3CSK4, Poly I:C, HkBps or HkLm. 151
Figure 3.2 TLR4, TLR2, TRIF and MyD88 contribute to IL-10 production in HkBps stimulated macrophages. 152
Figure 3.3 LPS, Pam3CSK4 and HkBps stimulated C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages at all time-points during the response. 153
Figure 3.4 C57BL/6 DCs produce higher levels of IL-10 than BALB/c DCs when stimulated with Curdlan, but not LPS or Pam3CSK4. 154
Figure 3.5 The level of *Tlr* mRNA expression in C57BL/6 and BALB/c macrophages does not correlate with IL-10 production. 155
Figure 3.6 ERK and p38 are important regulators of TLR2 and TLR4 induced IL-10 in C57BL/6 and BALB/c macrophages. 156
Figure 3.7 LPS and Pam3CSK4 induced phosphorylation of ERK1/2 and p38 is similar in C57BL/6 and BALB/c macrophages. 157
Figure 3.8 *Il10* mRNA expression differs in C57BL/6 and BALB/c macrophages stimulated with LPS, Pam3CSK4 and HkBps. 158

Figure 3.9 LPS and HkBps stimulated C57BL/6 macrophages produce higher levels of *Ifnb1* mRNA and IFN-β protein than BALB/c macrophages. 159
Figure 3.10 The second peak of *Il10* mRNA in LPS and HkBps stimulated C57BL/6 macrophages is dependent on type I IFN signalling, and in the absence of type I IFN signalling, C57BL/6 IL-10 production is similar to BALB/c. 160
Figure 3.11 Exogenous IFN-β enhances IL-10 production in C57BL/6 and BALB/c macrophages.....161
Figure 3.12 Premature *Il10* mRNA follows similar dynamics to mature *Il10* mRNA in LPS stimulated C57BL/6 and BALB/c macrophages. 162
Figure 3.13 IL-27 is induced by LPS and HkBps stimulated C57BL/6 and BALB/c macrophages and is positively regulated by type I IFN. 163
Figure 3.14 The addition of IL-27 does not modulate IL-10 production in C57BL/6, BALB/c or C57BL/6 *Ifnar1*$^{-/-}$ macrophages. 164
Figure 3.15 IL-10 production is not affected by the absence of IL-27 signalling. 165
Figure 3.16 The enhancement of IL-10 by IFN-β is predominantly STAT1 dependent. 166
Figure 3.17 STAT-1 is phosphorylated in C57BL/6 and BALB/c macrophages stimulated with LPS and is dependent on type I IFN signalling. 167
Figure 3.18 C57BL/6 x BALB/c F1 macrophages produce intermediate levels of IL-10. 168
Figure 3.19 C57BL/6 x BALB/c F2 macrophages show segregation in the level of IL-10 production. 169
Figure 3.20 LPS treatment induces higher levels of *Il10* and *Ifnb1* mRNA in C57BL/6 compared to BALB/c mice *in vivo*. 170

Chapter 4

Figure 4.1 Kinetics of IL-12p40 and IL-12p70 production in C57BL/6 and BALB/c macrophages.....190
Figure 4.2 *Il12a* mRNA is more highly expressed in BALB/c macrophages than C57BL/6 macrophages. 191

Figure 4.3 Higher IL-12p70 production in BALB/c macrophages is revealed at low cell density due to negative autocrine regulation by IL-10 and in the absence of IL-10, C57BL/6 IL-12p70 production is rescued in Pam3CSK4 stimulated cells.....192
Figure 4.4 The absence of type I IFN signalling or combined absence of type I IFN and IL-10 signalling, does not rescue IL-12p70 production in LPS or HkBps stimulated C57BL/6 macrophages.....193
Figure 4.5 The production of TNF-α and IL-1β is higher in LPS, Pam3CSK4 and HkBps stimulated BALB/c macrophages compared to C57BL/6 macrophages. 194
Figure 4.6 IL-10 accounts for the differential proinflammatory cytokine production in Pam3CSK4 stimulated C57BL/6 and BALB/c cells, but not LPS or HkBps stimulated cells. 195
Figure 4.7Abrogation of type I IFN signalling enhances IL-1β production in LPS and HkBps stimulated C57BL/6 macrophages. 196

Chapter 5

Figure 5.1 Experimental design for microarray analysis of differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages. 235
Figure 5.2 LPS induces dynamic transcriptional changes in C57BL/6 and BALB/c macrophages.....236
Figure 5.3 Identification of significantly differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages. 237
Figure 5.4 C57BL/6 macrophages may have a stronger transcriptional response to LPS than BALB/c macrophages. 238
Figure 5.5 *k*-means clustering of differentially expressed genes at each time-point identifies the groups of genes that most clearly distinguish C57BL/6 and BALB/c transcriptional profiles. 239
Figure 5.6 C57BL/6^{hi} *k*-means clusters of interest.....240
Figure 5.7 BALB/c^{hi} *k*-means clusters of interest. 241
Figure 5.8 IPA pathway analyses of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages. 242

Figure 5.9 IPA transcriptional regulators associated with the differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages.....243
Figure 5.10 PSCAN transcription factor binding site (TFBS) enrichment analysis of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages. 244
Figure 5.11 Differential expression of transcription factor genes in LPS stimulated C57BL/6 and BALB/c macrophages. 245

Chapter 7 - Appendix

Figure 7.1 The levels of IL-10 production are variable in CpG1668 and R848 stimulated C57BL/6 and BALB/c macrophages. 259
Figure 7.2 Differential IL-10 production is consistent in C57BL/6 and BALB/c macrophages derived from mice bred at NIMR or Jackson Laboratories. 280

List of tables

Chapter 1

Table 1.1 The TLR family in mouse and man. 82

Chapter 2

Table 2.1 Assay details for ELISA 92
Table 2.2 Reaction mixture for cDNA synthesis 95
Table 2.3 qPCR reaction mixture (per well) 95
Table 2.4 TaqMan primer-probes for qPCR 96
Table 2.5 Premature *Il10* mRNA primer probe sequences 97

Chapter 5

Table 5.1 Candidate transcriptional regulators mediating differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages. 246

Chapter 7 - Appendix

Table 7.1 0.5 h, *k*-means C3 (C57BL/6^{hi}). Refers to Figure 5.6. 260
Table 7.2 0.5 h, *k*-means C4 (C57BL/6^{hi}). Refers to Figure 5.6. 261
Table 7.3 1 h, *k*-means C1 (C57BL/6^{hi}). Refers to Figure 5.6. 262
Table 7.4 1 h, *k*-means C5 (C57BL/6^{hi}). Refers to Figure 5.6. 263
Table 7.5 3 h, *k*-means C1 (C57BL/6^{hi}). Refers to Figure 5.6. 264
Table 7.6 3 h, *k*-means C3 (C57BL/6^{hi}). Refers to Figure 5.6. 265
Table 7.7 5 h, *k*-means C0 (C57BL/6^{hi}). Refers to Figure 5.6. 266
Table 7.8 5 h, *k*-means C3 (C57BL/6^{hi}). Refers to Figure 5.6. 267
Table 7.9 8 h *k*-means C0 (C57BL/6^{hi}). Refers to figure 5.6. 269
Table 7.10 1 h *k*-means C4 (BALB/c^{hi}). Refers to Figure 5.7. 270
Table 7.11 3 h *k*-means C4 (BALB/c^{hi}). Refers to Figure 5.7. 271
Table 7.12 5 h *k*-means C2 (BALB/c^{hi}). Refers to Figure 5.7. 272
Table 7.13 8 h *k*-means C3 (BALB/c^{hi}). Refers to Figure 5.7. 273
Table 7.14 0.5 h differentially expressed genes associated with IPA pathways. Refers to figure 5.8. 274

Table 7.15 1 h differentially expressed genes associated with IPA pathways. Refers to figure 5.8. 275
Table 7.16 3 h differentially expressed genes associated with IPA pathways. Refers to figure 5.8. 276
Table 7.17 5 h differentially expressed genes associated with IPA pathways. Refers to figure 5.8. 277
Table 7.18 8 h differentially expressed genes associated with IPA pathways. Refers to figure 5.8. 278
Table 7.19 Transcription factors differentially expressed in LPS stimulated C57BL/6 and BALB/c macrophages. Refers to Figure 5.11. 279

Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	analysis of variance
APC	antigen presenting cell
B.pseudomallei	*Burkholderia pseudomallei*
BH-FDR	Benjamini-Hochberg False Detection Rate
BMDC	bone marrow derived dendritic cell
BMDM	bone marrow derived macrophage
CLR	C-type lectin receptor
cDNA	complementary deoxyribonucleic acid
DC	dendritic cell
DMSO	dimethyl sulfoxide
EBV	Epstein–Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
h	hour
HkBps	heat-killed *B.pseudomallei*
HRP	horseradish peroxidase
i.p.	intra-peritoneal
IFN	interferon
IL-	interleukin
IPA	Ingenuity Pathway Analysis
IRF	interferon regulatory factor
JAK	Janus kinase
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MDP	muramyl dipeptide

med	media
MHC	major histocompatibility complex
min	minute
ml	millilitre
MRC	Medical Research Council
MyD88	myeloid differentiation primary response gene (88)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NIMR	National Institute for Medical Research
NK	natural killer
NLR	NOD-like receptors
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCA	principle component analysis
pDC	plasmacytoid DC
pg	picogram
PRR	pattern recognition receptor
qPCR	quantitative PCR
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RT	room temperature
RU	relative units
SD	standard deviation
SDS	sodium dodecyl sulfate
STAT	signal transducer and activator of transcription
TFBS	transcription factor binding site
Th	T helper
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF-α	Tumour necrosis factor α
TRIF	TIR-domain-containing adapter-inducing interferon-β

TYK	tyrosine kinase
µl	microliter
vs.	versus
WT	wild type

Chapter 1. General Introduction

1.1 An overview of the Immune Response

A complete immune response consists of both innate and adaptive phases. The innate immune system is composed of many cell types including macrophages, dendritic cells (DC), natural killer (NK) cells, eosinophils, basophils and mast cells. Macrophages and DCs are phagocytic cells which have particularly important functions in the recognition of infection due to their expression of pattern recognition receptors (PRRs). These receptors are germ-line encoded, and recognise conserved microbial motifs present on the surface or within microorganisms (Medzhitov 2007). The recognition of infection by these cells initiates the inflammatory response by inducing the production of cytokines and chemokines and recruiting other immune cells to the site of infection (Medzhitov 2007). Macrophages, of which there are several specialised subsets, additionally play a first role in the containment of infection through their antimicrobial activities such as the production of reactive oxygen species (ROS) (Gordon *et al.* 2005). There are also several subsets of DC including conventional DCs, (cDC, also known as myeloid DC (mDC)), specialised in alerting components of the adaptive immune system by migrating to lymphoid organs and activating T cells, and plasmacytoid DCs (pDCs), specialised in the production of type I interferon (IFN) (Banchereau *et al.* 1998; Colonna *et al.* 2004; Hashimoto *et al.* 2011). Antigen presenting cells (APCs, which can include both DCs and macrophages), stimulate $CD4^+$ T cells through the presentation of antigen in the context of major histocompatibility complex (MHC) class II, and the ligation of co-stimulatory molecules (Steinman 2007). Under the additional influence of innate cytokine production, $CD4^+$ T cells expand and differentiate into distinct T helper (Th) subsets, each with specialised effector functions (Murphy *et al.* 2000). Th1 cells express the master transcription factor Tbet, produce the hallmark cytokine IFN-γ, and

are particularly important in immune defence against intracellular pathogens (Mosmann *et al.* 1986; Sher *et al.* 1992; Szabo *et al.* 2000; Flynn *et al.* 2001). Th2 cells express the master transcription factor GATA3, produce the hallmark cytokines IL-4, IL-5 and IL-13, and are important in immune responses against parasitic helminths (Mosmann *et al.* 1986; Zheng *et al.* 1997; Maizels *et al.* 2012). Th17 cells express the master transcription factor RORγT, produce the hallmark cytokines IL-17A and IL-17F, and are important in immune response against fungal infections and some extracellular bacteria (Ivanov *et al.* 2006; Veldhoen *et al.* 2006; O'Quinn *et al.* 2008). Forkhead box protein 3 (FOXP3) expressing regulatory T cells (Treg) are an additional subset of $CD4^+$ T cell which have an important role in regulating immune responses to maintain immunological tolerance and homeostasis (Bacchetta *et al.* 2007; Rudensky 2011). $CD8^+$ T cells are activated by antigen presentation in the context of MHC class I and through their cytotoxic functions can mediate the killing of infected cells, but also produce cytokines such as IFN-γ (Harty *et al.* 2000). B cells constitute an additional critical arm of the adaptive immune system through their production of antibodies, and their increasingly appreciated immunoregulatory functions (Mauri *et al.* 2012). Thus, the immune response is a complex network of cells, the activity of which must be carefully coordinated to ensure effective pathogen clearance with minimal damage to the host. Two cytokines, which contribute to the orchestration of many of these processes are interleukin-10 (IL-10) and interleukin-12 (IL-12).

1.2 The role of IL-12 in the immune response

IL-12 was first isolated and characterised in the late 1980's as a 70-kD product of an Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell line (Kobayashi *et al.*

1989). It was initially named 'NK cell stimulatory factor' due to its ability to induce cytotoxic activity, IFN-γ production, and enhance phytohaemahglutinin (PHA) induced proliferation of human peripheral blood lymphocytes (Kobayashi *et al.* 1989). In this study, IL-12 was also found to be a heterodimeric cytokine, the first described of its kind, composed of disulphide linked 40-kD and 35-kD subunits which are now known as p40 and p35 (Kobayashi *et al.* 1989). Since then, a substantial amount of research has been dedicated to understanding the regulation and the biological activities of IL-12. IL-12 is the founding member of the IL-12 family cytokines which also includes IL-23, IL-27 and IL-35 (Vignali *et al.* 2012). All cytokines in this family are heterodimeric, and subunits are shared between family members. For example, p40 is also a component of IL-23 when bound to p19 (Oppmann *et al.* 2000), and p35 is a component of IL-35 when bound to Ebi3 (Collison *et al.* 2007; Niedbala *et al.* 2007). Ebi3 additionally dimerises with p28 to form IL-27 (Pflanz *et al.* 2002).

1.2.1 Cellular sources and targets of IL-12

IL-12 is produced predominantly by phagocytic cells including monocytes, macrophages and DCs in response to microbial products (D'Andrea *et al.* 1992; Macatonia *et al.* 1995). Activated T and NK cells are the main cell types that express the IL-12 receptor and therefore respond to this cytokine (Desai *et al.* 1992). The IL-12 receptor is composed of two subunits, IL-12Rβ1 and IL-12Rβ2 (Presky *et al.* 1996). Signalling downstream of the IL-12 receptor activates the Janus kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway, in which activated receptor associated Janus family tyrosine kinases induce the phosphorylation and activation of STAT molecules. STATs then homo- or hetero-dimerise and translocate to the nucleus

where they can regulate gene expression (Leonard *et al.* 1998). In the case of the IL-12 receptor, JAK2 and tyrosine kinase 2 (TYK2) are associated with the IL-12 receptor (Bacon *et al.* 1995a) and upon the binding of IL-12, mediate the activation of STAT4 (Bacon *et al.* 1995b; Jacobson *et al.* 1995). Other STATs have been shown to be activated downstream of the IL-12 receptor (Jacobson *et al.* 1995), however most of the biological activity of IL-12 on T and NK cells is dependent on STAT4 (Kaplan *et al.* 1996; Thierfelder *et al.* 1996).

1.2.2 IL-12 and the regulation of immune responses

One of the most widely known roles of IL-12 is to promote the differentiation of naïve T cells into IFN-γ producing Th1 cells (Hsieh *et al.* 1993; Manetti *et al.* 1993; Macatonia *et al.* 1995). IL-12 therefore enhances immune system activation, and is consequently classed as a proinflammatory cytokine, bridging innate and adaptive immune systems. IL-12 can also drive IFN-γ production from NK cells (Kobayashi *et al.* 1989; Chan *et al.* 1991). Importantly, the synergy of IL-12 with other activation signals such IL-2 and in the case of T cells, T cell receptor/CD3 and CD28 signalling, ensures robust IFN-γ production from these cells (Trinchieri 2003). IFN-γ is a potent activator of macrophages (Varesio *et al.* 1984), which possess many antimicrobial effector functions (Gordon *et al.* 2005). Thus, this network forms a positive feedback loop where macrophage or DC derived IL-12, drives the differentiation of Th1 cells to produce IFN-γ which further activates effector mechanisms of the innate immune system.

The ability of IL-12 to induce the differentiation of Th1 cells and promote IFN-γ production from NK cells is critically important in protective immune responses against several intracellular parasitic and bacterial pathogens. For example, in the mouse model of infection with the intracellular protozoan parasite *Leishmania major* (*L.major*), the blockade of IL-12 in resistant C57BL/6 mice resulted in a reduction of IFN-γ production from lymph node cells, and exacerbated disease (Sypek *et al.* 1993). Conversely, the treatment of susceptible BALB/c mice with recombinant IL-12 lessened disease severity and this was dependent on IFN-γ (Heinzel *et al.* 1993; Sypek *et al.* 1993). In further support of a central role for IL-12 in protection against *L.major* infection, IL-12p40 deficient mice are unable to control *L.major* infection, and this can be rescued with the administration of IL-12 (Park *et al.* 2000). This study also demonstrated the need for IL-12 to be present throughout the infection to maintain a protective Th1 response (Park *et al.* 2000).

Similarly, in the mouse model of *Toxoplasma gondii* (*T.gondii*) infection, another intracellular protozoan parasite, Th1 derived IFN-γ is important for the generation of a protective immune response (Gazzinelli *et al.* 1992). Further, treatment of mice with anti-IL-12 or anti-IFN-γ antibodies led to 100% mortality during the acute phase of infection, whereas all control mice survived demonstrating an important role for both cytokines in protective immunity (Gazzinelli *et al.* 1994). The presence of IL-12 throughout the infection has also been shown to be necessary for the maintenance of the Th1 response in this model (Yap *et al.* 2000). IL-12 also induces IFN-γ production from NK cells during this infection (Gazzinelli *et al.* 1994). This has a protective role, illustrated by the finding that the survival of *T.gondii* infected severe combined

immunodeficient (SCID) mice was enhanced by IL-12 treatment in an IFN-γ and NK cell dependent manner (Gazzinelli *et al.* 1993). IL-12, in synergy with TNF-α, also induces IFN-γ production from NK cells in response to infection with the intracellular bacterium *Listeria monocytogenes* (*L.monocytogenes*) (Tripp *et al.* 1993). This is essential for the early containment of infection (Tripp *et al.* 1994), although $CD4^+$ Th1 and $CD8^+$ cytotoxic T cell responses are also important for *L.major* clearance and long term resistance to reinfection (Pamer 2004).

The acid-fast bacillus *Mycobacterium tuberculosis* (*M.tuberculosis*) provides a further example of the importance of IL-12 induced Th1 responses in protection against intracellular bacterial infection (Flynn *et al.* 2001; O'Garra *et al.* 2013). In the mouse model, IFN-γ is essential for protection against *M.tuberculosis* infection (Cooper *et al.* 1993; Flynn *et al.* 1993). IL-12p35 deficient mice, which specifically lack the bioactive form of IL-12 (IL-12p70), were less able to control *M.tuberculosis* bacterial replication than wild type (WT) mice, and had reduced recruitment of IFN-γ producing $CD4^+$ T cells to the lung (Cooper *et al.* 2002). Of note, in this study, IL-12p40 deficient mice were much more severely affected (Cooper *et al.* 2002), highlighting the additional roles of IL-12p40 outside of the formation of bioactive IL-12. In humans, mutations in *Ifngr1*, *Ifngr2*, *Il12rb1* and *Il12b* (which encodes IL-12p40) have been linked to susceptibility to mycobacterial disease (Alcais *et al.* 2005). However, as IL-12p40 is a component of IL-23 and IL-12Rβ1 is additionally a component of the IL-23 receptor (Oppmann *et al.* 2000), a role for IL-23 cannot be excluded.

In comparison to intracellular bacterial and parasitic infections, IL-12 may have less of an important role in generating protective immune responses against viral infections. In a murine model of flu, IL-12p40 blockade reduced IFN-γ production, but only at early time points (Monteiro *et al.* 1998). In a model of murine lymphocytic choriomeningitis virus (LCMV) infection, IL-12p35 deficient mice had no defect in the generation of anti-viral responses (Cousens *et al.* 1999). However, in the absence of type I IFN, an important mediator of anti-viral responses (Garcia-Sastre *et al.* 2006), IL-12 was necessary to induce IFN-γ production from $CD8^+$ T cells in an alternative immune pathway which offered some protection (Cousens *et al.* 1999). In addition, treatment with exogenous IL-12 has been shown to enhance IFN-γ production from NK cells and anti-viral immune responses against murine cytomegalovirus (Orange *et al.* 1995).

Thus, IL-12 has roles that are beneficial to the host in several infection settings. Moreover, owing to its ability to enhance cellular immune responses including $CD8^+$ T cell and NK cell cytotoxic function (Gately *et al.* 1994), IL-12 may have therapeutic potential in anti-cancer treatments (Colombo *et al.* 2002). However, proinflammatory immune responses, including those driven by IL-12, can be harmful to the host and give rise to infection induced immunopathology or autoimmunity if not appropriately regulated (Moore *et al.* 2001). It is therefore critical that these pathways are carefully controlled in order to provide protection to the host.

1.3 The role of IL-10 in the immune response

IL-10 was initially characterised as a 'cytokine synthesis inhibitory factor' (CSIF) secreted from a murine Th2 clone (Fiorentino *et al.* 1989). At this time, IL-10 was

shown to inhibit IFN-γ production from Th1 clones, proposed to be most likely by an indirect mechanism (Fiorentino *et al.* 1989). IL-10 was also found to inhibit proinflammatory cytokine production from macrophages, suggesting a wider role for IL-10 in the regulation of inflammation (Fiorentino *et al.* 1991a). It was soon found that the inhibitory effect of IL-10 was mediated through the dampening of APC activity (Fiorentino *et al.* 1991b; Macatonia *et al.* 1993), solidified by the finding that IL-10 inhibits IFN-γ production from Th1 cells by suppressing macrophage derived IL-12 (Hsieh *et al.* 1993; Murphy *et al.* 1994).

IL-10 is now considered a key immunoregulatory cytokine, produced by most cells of the immune system (Saraiva *et al.* 2010). IL-10, a homodimeric cytokine, is the founding member of the IL-10 family cytokines. IL-19, IL-20, IL-24, IL-22, IL-26, IL-28A, IL-28B and IL-29 are also within this cytokine family although of these, only IL-10 has clearly established anti-inflammatory activity (Ouyang *et al.* 2011). Of note, IL-28A, IL28B and IL-29 are also members of the type III IFN family (Trinchieri 2010). A viral homologue of the *Il10* gene, ebvIL-10, has been described in EBV (Moore *et al.* 1990). IL-10 homologues are also present in the genomes of other viruses, implying that 'capture' of the *Il10* gene may facilitate viral immune evasion by dampening the host immune response (Slobedman *et al.* 2009). In keeping with this, while retaining immunosuppressive activity (Hsu *et al.* 1990), ebvIL-10 does not retain all of the immunostimulatory activities of mammalian IL-10 (Vieira *et al.* 1991). In addition to well characterised anti-inflammatory function, IL-10 can enhance the activity of cytotoxic $CD8^+$ T cells (Chen *et al.* 1991) and the survival and antibody production of B cells, particularly in the human system (Rousset *et al.* 1992).

1.3.1 Cellular sources and targets of IL-10

IL-10 is produced by innate and adaptive immune cells including macrophages, mDCs, mast cells, neutrophils, eosinophils, NK cells, B cells all subsets of $CD4^+$ T cells and $CD8^+$ T cells (Saraiva *et al.* 2010). To date, pDCs have not been described to produce IL-10 (Boonstra *et al.* 2006; Kaiser *et al.* 2009). The IL-10 receptor is expressed on most haematopoietic cells however, the highest level of expression is on macrophages and DCs (Murray 2006), in keeping with these populations being the main target of IL-10 activity (Moore *et al.* 2001). The IL-10 receptor is composed of IL-10R1 (Liu *et al.* 1994) and IL-10R2 subunits (Kotenko *et al.* 1997). IL-10R1 is important for the binding of IL-10, whereas IL-10R2, also used in the signalling of other IL-10 cytokine family members including IL-22, IL-26, IL-28A, IL-28B and IL-29 (Ouyang *et al.* 2011), is critical for signal transduction (Kotenko *et al.* 1997). Downstream of the IL-10 receptor, JAK/STAT signalling is activated involving the activation of JAK1 and TYK2 receptor associated tyrosine kinases and subsequent activation of STAT3 (Finbloom *et al.* 1995). Other STATs can be activated downstream of the IL-10 receptor (Moore *et al.* 2001), however STAT3 is considered most important for immunosuppressive activity (Takeda *et al.* 1999; Lang *et al.* 2002; Williams *et al.* 2004).

Exactly how IL-10 mediates its suppressive effects is not yet fully understood, although the expression of STAT3 dependent genes is essential. Thus, as opposed to STAT3 directly inhibiting gene expression, IL-10 is thought to induce the expression of inhibitory factors through STAT3 activation, which then in turn inhibit the expression of proinflammatory cytokines and chemokines (Murray 2005). Importantly, IL-10 only

inhibits a subset of genes expressed in activated macrophages and DCs showing that there is some specificity in the inhibitory mechanisms of IL-10 signalling (Lang *et al.* 2002). The action of these inhibitory factors has been shown to be mainly at the level of transcription (Murray 2005; Smallie *et al.* 2010). For example, in human macrophages, IL-10 inhibits the elongation of the tumour necrosis factor (*Tnf*) transcript (Smallie *et al.* 2010). IL-10 has also been shown to inhibit the transcription of *Il12b* (IL-12p40) and *Il12a* (IL-12p35) (Aste-Amezaga *et al.* 1998). More recently, the transcription factor nuclear factor, interleukin 3 regulated (NFIL3) has been identified as a STAT3 dependent target of IL-10 that mediates the inhibition of *Il12b* (IL-12p40) transcription (Smith *et al.* 2011). The transcriptional repressors Ets variant 3 (ETV3) and Strawberry notch homologue 2 (SBNO2) have also been identified as downstream targets of IL-10 signalling, and may be involved in mediating the anti-inflammatory effects of IL-10 (El Kasmi *et al.* 2007). A more recent study in which IL-10 induced STAT3 binding sites were analysed on a genome-wide scale, proposed over 40 additional transcription factor targets of IL-10 signalling (Hutchins *et al.* 2012). Further studies will be required to confirm the relevance of these factors in the induction of IL-10 mediated anti-inflammatory responses (Hutchins *et al.* 2012). There is however also evidence for IL-10 post-transcriptionally regulating target genes as IL-10 mediated expression of microRNA-187 has been shown to directly negatively regulate TNF-α production in human monocytes (Rossato *et al.* 2012).

Although most studies have focused on the effects of IL-10 on macrophages and DCs, recent findings have suggested that IL-10 may also act directly on $CD4^+$ T cells. In a model of intestinal inflammation, Th17 cells were found to express the IL-10 receptor,

and inhibition of IL-10 receptor activity specifically on these cells lead to an increase in proinflammatory IL-17A expressing effector T cells (Huber *et al.* 2011). It has also been suggested that IL-10 may directly act on Tregs to maintain their expression of FOXP3 (Murai *et al.* 2009). Of note, a later study also reported that IL-10 receptor expression on Tregs was necessary for their suppressive function, although FOXP3 expression was not affected (Chaudhry *et al.* 2011). Thus, IL-10 may act directly on T cells, although the implications of this are currently unclear.

1.3.2 The regulation of immune responses by IL-10

A proinflammatory immune response is critical in the protection against infectious diseases. In the absence of adequate regulation however, an immune response can become over exuberant and cause damage to the host. Inappropriate immune responses can also cause host damage when they occur in response to self, or non-harmful stimuli such as commensal bacteria or innocuous antigens e.g. pollen. The production of IL-10 can prevent this scenario due to its immunosuppressive properties (Moore *et al.* 2001). However, not every immune response has the potential to cause damage to the host and in these situations, the inappropriate production of IL-10 can inhibit what would otherwise be a protective response. The consequence of this can be the onset of chronic infection (Moore *et al.* 2001). The production of IL-10 is therefore essential, but the balance of IL-10 is important to ensure protection from immunopathology, without an inappropriate inhibition of the immune response.

1.3.2.1 *The role of IL-10 in protection against immunopathology*

In the context of infection, pathogens which strongly stimulate the immune system can induce potentially damaging inflammatory responses. In these situations, the production of IL-10 is beneficial to the host and likewise a deficiency in IL-10 production is detrimental. An example of this is septic shock, where inflammatory mediators can drive multi-organ failure and even death during severe bacterial infections (De Kock *et al.* 2010). Mice with a disruption in the *Il10* gene are more susceptible to this syndrome, at least in part due to their enhanced production of TNF-α and IFN-γ (Berg *et al.* 1995). Conversely, the treatment of mice with IL-10 protects them from lipopolysaccharide (LPS)-induced shock and this correlates with a decrease in TNF-α production (Howard *et al.* 1993). In the murine model of endotoxic shock, it has been shown that non-T cells are an important source of protective IL-10 (Roers *et al.* 2004). IL-10 also prevents immunopathology during infections with the parasites *Plasmodium chabaudi chabaudi* (*P.chabaudi*) (Li *et al.* 1999), the causative agent of malaria, and *T.gondii* (Gazzinelli *et al.* 1996; Suzuki *et al.* 2000). In these infections, strong Th1 mediated immune responses are mounted against the parasite and in the absence of IL-10, enhanced mortality is experienced (Gazzinelli *et al.* 1996; Li *et al.* 1999). For example, IL-10 deficient *P.chabaudi* infected mice show elevated plasma levels of IFN-γ and TNF-α (Li *et al.* 1999). Blockade of IFN-γ in IL-10 deficient mice reduced morality and blockade of TNF-α significantly, but not completely, ameliorated immunopathology (Li *et al.* 1999; Li *et al.* 2003). IL-10 deficient *T.gondii* infected mice show higher levels of TNF-α, IL-12 and $CD4^+$ lymphocyte derived IFN-γ in response to infection (Gazzinelli *et al.* 1996). Depletion of $CD4^+$ cells protected these mice from immunopathology (Gazzinelli *et al.* 1996). Thus IL-10 is protective against excessive

Th1-based immune responses. The source of protective IL-10 in these infections has been shown to be the Th1 effector cells themselves (Jankovic *et al.* 2007; Freitas do Rosario *et al.* 2012). This demonstrates the concept that IL-10 is produced as a part of a self-regulating negative-feedback loop (O'Garra *et al.* 2007). IL-10 protection against immunopathology is also seen in infections with the parasite *Trypanosoma cruzi* (*T.cruzi*) (Hunter *et al.* 1997) and the bacterium *Helicobacter hepaticus* (*H.hepaticus*) (Kullberg *et al.* 1998). In *T.cruzi* infection, IL-10 deficiency correlated with enhanced IFN-γ derived from T cells and elevated IL-12 production, the neutralisation of which reduced mortality (Hunter *et al.* 1997). In *H.hepaticus* infection, immunopathology was associated with enhanced IFN-γ and TNF-α production in IL-10 deficient mice, and IL-12 or IFN-γ neutralisation resulted in reduced immunopathology (Kullberg *et al.* 1998).

A role for IL-10 has also been identified in the protection against inappropriate inflammatory responses to commensal microorganisms in the gut. This was originally demonstrated by the finding that IL-10 deficient mice develop colitis (Kuhn *et al.* 1993) associated with enhanced Th1 responses (Berg *et al.* 1996). It was later shown that this onset of colitis was dependent on the presence of commensal gut flora (Sellon *et al.* 1998). It has since been shown that T cells are an important source of protective IL-10 in this context (Roers *et al.* 2004). In humans, genetic studies have shown that IL-10 is strongly associated with the development of Ulcerative Colitis (Franke *et al.* 2008) and Crohn's disease (Franke *et al.* 2010). Further, mutations in genes encoding the IL-10 receptor chains (*Il10ra* and *Il10rb*) are associated with early-onset inflammatory bowel disease (Glocker *et al.* 2009), further implicating a role for IL-10 in the natural protection against these conditions.

IL-10 may also have a protective role in the setting of allergic asthma, which constitutes an inappropriate Th2 based response in association with IgE production and eosinophilia in response to an innocuous antigen such as pollen (Hawrylowicz *et al.* 2005). For example, a study which compared the cytokine levels in bronchial lavage fluid (BAL) from healthy and asthmatic individuals, found reduced levels of IL-10 in the BAL of asthma sufferers (Borish *et al.* 1996). Further, it has been postulated that the induction of IL-10 from T cells may be the mechanism of action for glucocorticoids which are given as therapy to asthma patients (O'Garra *et al.* 2008). In mouse models of allergic asthma, the intranasal administration of IL-10 during the challenge phase reduced the infiltration of inflammatory cells including neutrophils and eosinophils, into the bronchial tissues (Zuany-Amorim *et al.* 1995), supporting a protective role for IL-10.

1.3.2.2 *The inhibition of protective immune responses by IL-10*

Not every immune response has the potential to cause pathology in the absence of immunoregulatory mechanisms however, and there are several instances where anti-inflammatory mechanisms, such as the production of IL-10, actually hinder an otherwise protective response. For example, in the mouse model of *L.monocytogenes* infection, IL-10 deficiency enhanced resistance to infection without increasing tissue damage (Dai *et al.* 1997). Similarly, in the mouse model of *M.tuberculosis* infection, IL-10 deficient mice generated an elevated Th1 response and were more protected against infection with lower bacterial loads compared to WT (Redford *et al.* 2010). In *L.major* infection, IL-10 deficient mice were able to completely clear infection whereas WT mice developed a chronic, low grade infection (Belkaid *et al.* 2001; Belkaid *et al.*

2002). There is also evidence in humans that high levels of IL-10 correlate with the development of severe leishmaniasis, further supporting a detrimental role for IL-10 in the immune response against this pathogen (Nylen *et al.* 2007). IL-10 may additionally inhibit protective immune responses against viral infection, illustrated by the finding that blockade of the IL-10 receptor can improve the clearance of chronic LCMV infection, associated with an increased prevalence of virus-specific T cells (Brooks *et al.* 2006; Ejrnaes *et al.* 2006). In these instances, the induction of IL-10 by infection may be an immune evasion strategy by the pathogen to facilitate persistence within the host (Redford *et al.* 2011). The generation of a robust immune response is also desirable in the context of vaccination and conceivably, the production of IL-10 during vaccination may be detrimental in this process (O'Garra *et al.* 2008). In support of this concept, a recent study has shown that blockade of IL-10 signalling at the time of BCG vaccination can enhance vaccination efficacy, resulting in a reduction in the bacterial load after *M.tuberculosis* challenge (Pitt *et al.* 2012).

1.3.2.3 *The complex roles of IL-10 in autoimmune diseases and anti-tumour immune responses*

IL-10 has been implicated in both the protection against and promotion of autoimmune diseases, depending on the condition. Rheumatoid arthritis (RA) is an inflammatory condition affecting the joints in which TNF-α is an important pathological factor (Brennan *et al.* 1989). The neutralisation of endogenous IL-10 in cultures derived from human synovial tissue enhanced proinflammatory cytokine production, suggesting that IL-10 may be protective in this condition (Katsikis *et al.* 1994). In addition, treatment of rats with IL-10 in a collagen-induced model of arthritis was able to reduce the

frequency of arthritis onset, and the severity of already established disease (Persson *et al.* 1996). Experimental autoimmune encephalitis (EAE) is the animal model for multiple sclerosis (MS), an inflammatory condition of the central nervous system (Sospedra *et al.* 2005). IL-10 has been suggested to be protective against EAE by the finding that IL-10 deficient mice develop EAE with enhanced severity (Bettelli *et al.* 1998; Samoilova *et al.* 1998). In contrast, IL-10 has been suggested to promote systemic lupus erythematosis (SLE), an autoimmune condition affecting multiple organs and associated with the production of autoantibodies recognising nuclear components (Banchereau *et al.* 2006). The neutralisation of IL-10 in SLE prone NZB/W F1 mice reduces the onset of disease (Ishida *et al.* 1994) and in humans, IL-10 polymorphisms have been associated with the development of SLE although this may be dependent on the population (Beebe *et al.* 2002). Given the importance of autoantibodies in driving this condition, IL-10 may promote disease by having immunostimulatory effects on B cells (Rousset *et al.* 1992; Beebe *et al.* 2002).

Currently, the role of IL-10 in anti-tumour immune responses is unclear and studies so far have implied that the function of IL-10 may be dependent on the context (Mocellin *et al.* 2005). For example, it has been suggested that the induction of IL-10 by tumour associated macrophages can create an immunosuppressed environment that is permissive for tumour growth (Kim *et al.* 2005). However, it has also been proposed that the immunostimulatory activity of IL-10 on cytotoxic $CD8^+$ T cells may enhance anti-tumour immunity (Fujii *et al.* 2001). In addition, the heterogeneity of cancer as a disease may contribute to varying roles of IL-10 in anti-tumour immune responses (O'Garra *et al.* 2008).

Thus, the importance of IL-10 in the regulation of immune responses is verified by the multitude of immunological systems it has been shown to have roles in.

1.4 Macrophages and the immune response

We have discussed so far the importance of IL-10 and IL-12 in the immune response. Macrophages are an important source of these cytokines, and together with monocytes and DCs can collectively be referred to as the mononuclear phagocyte system. Mononuclear phagocytes, originally defined by their adherent and phagocytic capabilities, were initially thought to be derived from rapidly dividing bone marrow progenitors termed promonocytes (van Furth *et al.* 1968). Based on findings using peritoneal macrophages, it was put forward that bone marrow derived promonocytes entered the circulation as monocytes, which then migrated to the tissues to become macrophages (van Furth *et al.* 1968). It was also shown that this process can be promoted by an inflammatory stimulus (van Furth *et al.* 1968). Further study of lymphoid organ populations led to the discovery of DCs which had distinct properties and functions from other mononuclear phagocytes (Steinman *et al.* 1973; Steinman *et al.* 1974a; Steinman *et al.* 1974b). This began to give a concept of the mononuclear phagocyte heterogeneity that we are aware of today (Auffray *et al.* 2009b). The terms monocyte, macrophage and DC each encompass a diverse range of cells. In mice, blood monocytes can be divided into patrolling Gr1/Ly6C$^-$CX3CR1^{hi}, and inflammatory Gr1/Ly6C$^+$CX3CR1^{lo} subsets (Geissmann *et al.* 2003; Auffray *et al.* 2007; Serbina *et al.* 2008). DCs are a heterogeneous population including several subsets of mDC (or cDC) such as CD4$^-$CD8α$^-$, CD4$^-$CD8α$^+$ and CD4$^+$CD8α$^-$ DC, and pDCs (Hashimoto *et al.*

2011). Macrophages constitute many types of tissue resident phagocytes including lung alveolar macrophages, liver Kupffer cells and central nervous system microglial cells (Gordon *et al.* 2005). Due to a lack of high-fidelity subset specific surface markers, several factors must be taken into consideration when categorising mononuclear phagocytes including phenotype, function, anatomical location, and also developmental origin (Geissmann *et al.* 2010b). For example, a common macrophage/DC progenitor in the bone marrow has been shown to give rise to monocytes, several macrophage and cDC subsets, and pDC (Fogg *et al.* 2006; Auffray *et al.* 2009a). However, other tissue resident macrophages derive from embryonic precursor cells, independent of the rest of the mononuclear phagocytic system (Ginhoux *et al.* 2010; Schulz *et al.* 2012). A recent initiative to understand the relationship between mononuclear phagocytes using gene expression signatures has identified a 'macrophage core' signature derived from peritoneal macrophages, lung macrophages, microglia and splenic red-pulp macrophages (Gautier *et al.* 2012). Comparing this signature to that of other mononuclear phagocyte populations will further aid the classification of these cells (Gautier *et al.* 2012).

Although in reality a diverse population, macrophages have been linked to functions including the clearance of apoptotic cells and tissue repair (Gordon *et al.* 2005). Their expression of PRRs further makes them important in immuno-surveillance, cytokine production and antimicrobial effector mechanisms (Gordon *et al.* 2005). In addition to the tissue specific classifications, functional classifications such as that of M1 and M2, have also been made. M1 macrophages are considered to be 'classically' activated meaning that they are stimulated by LPS (or other TLR ligands) and IFN-γ (Mosser *et*

al. 2008a). They produce high levels of proinflammatory cytokines, including IL-12, and are thought to be important in immune defence, although may have pathogenic roles in autoimmune conditions (Mosser *et al.* 2008a). M2 macrophages, or alternatively activated macrophages, are stimulated by the Th2 associated cytokines IL-4 or IL-13 (Gordon *et al.* 2010). The expression of Arginase-1 is a key feature and they are suggested to have important roles in tissue repair (Gordon *et al.* 2010) and immune defence against helminths (Anthony *et al.* 2006). A further subset known as 'regulatory' macrophages has also been proposed. Originally characterised in the context of concomitant stimulation with immune complexes and proinflammatory stimuli such as LPS, these macrophages produce low levels of IL-12p40 and high levels of IL-10 (Gerber *et al.* 2001), imparting them with anti-inflammatory activity (Mosser *et al.* 2008a), although their exact identity and role *in vivo* is unclear.

The development of macrophages is largely dependent on the expression of the M-CSF receptor (Dai *et al.* 2002) and in the study of macrophage biology, several researchers have made use of M-CSF differentiated bone marrow derived macrophages (BMDM). The most similar *in vivo* counterpart to this *in vitro* derived cell type is currently unknown (Geissmann *et al.* 2010a) however, owing to the heterogeneity of macrophage populations, the findings made using these cells may not be applicable to every *in vivo* macrophage subtype. Nevertheless, due to the relative ease in generating large numbers of these cells and the homogeneity of the resulting population, the use of *in vitro* derived macrophages has been invaluable in the detailed studies of several innate immune processes such as the molecular regulation of cytokine production and the biology of inflammation (Geissmann *et al.* 2010a). Thus, murine BMDM remain a

mainstay of immunological research. Ultimately however, it will be important to relate the findings made in this cell type to macrophage or monocyte populations found *in vivo*, and in the human system.

1.5 The recognition of infection and signal transduction by PRRs

The induction of cytokine production by macrophages and DCs is largely dependent upon ligation of PRRs. These receptors mediate the recognition of infection by detecting the presence of conserved microbial motifs (Janeway 1989), known as pathogen associated molecular patterns (PAMPs) (Medzhitov 2007). Signalling through these PRRs also induces the production of chemokines and the up-regulation of co-stimulatory molecules, all of which are important for recruitment of other cells to the site of infection and activation of adaptive immune responses (Medzhitov 2007). There are several different families of PRR including Toll-like receptors (TLRs, Table 1.1), C-type lectin receptors, NOD-like receptors and RIG-I-like receptors, all of which mediate the recognition of different classes of PAMP and initiate distinct but overlapping signalling cascades upon activation (Takeuchi *et al.* 2010).

1.5.1 TLR-induced signal transduction

Originally identified in *Drosophila* (Hashimoto *et al.* 1988), TLRs were the first PRRs identified in mammals (Medzhitov *et al.* 1997), and have subsequently been the most studied PRR family. TLRs can be expressed either at the surface of the cell, or endosomally and each TLR binds a specific set of microbial ligands, inducing homo- or hetero-dimerisation, and signal transduction (Jin *et al.* 2008) (Table 1.1). TLRs 1, 2, 4,

5, and 6 are expressed on the cell surface and mainly recognise bacterial motifs. TLR 3, 7, and 9 are present on endosomal surfaces and mainly detect nucleic acids from viruses or internalised bacteria (Kumar *et al.* 2011). The above TLRs are conserved between mouse and human. Other TLRs however, such as TLR 8 and 10 have been shown to be non-functional in the murine system (Jurk *et al.* 2002; Hasan *et al.* 2005). Conversely, TLR11, 12 and 13 are not present in the human (Kawai *et al.* 2010) (Table 1.1).

TLRs are all type I transmembrane proteins and have a highly conserved cytoplasmic Toll/IL-1R homology (TIR) domain (Kawai *et al.* 2010). Upon ligand induced homotypic or heterotypic dimerisation, adaptor molecules Myeloid differentiation primary response gene (MyD88) or TIR domain-containing adaptor inducing IFN-β (TRIF) are recruited to initiate signal transduction (Medzhitov *et al.* 1998; Yamamoto *et al.* 2003a). All TLRs engage the MyD88-dependent pathway with the exception of TLR3, which exclusively recruits TRIF (Yamamoto *et al.* 2003a) (Figure 1.1). With use of the additional co-adaptors toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) (Horng *et al.* 2002) and TRIF-related adaptor molecule (TRAM) (Yamamoto *et al.* 2003b), TLR4 has the unique ability of being able to recruit MyD88 and TRIF, thereby activating both pathways (Figure 1.1). Of note, TLR4 induces the MyD88 pathway before the TRIF pathway, which becomes activated only after endocytosis of the receptor (Kagan *et al.* 2008). TLR2 has also been shown to utilise the co-adaptor TIRAP in the activation of the MyD88-dependent signalling pathway (Horng *et al.* 2002) (Figure 1.1). The signalling downstream of TLRs is highly complex, with both MyD88 and TRIF activating multiple unique and shared pathways which promote the production of pro- and anti-inflammatory cytokines (Figure 1.1).

1.5.1.1 *MyD88 dependent TLR signalling*

In macrophages and DCs, activation of the MyD88 signalling pathway initiates the formation of a signalling complex composed of MyD88, IRAK family kinases, namely IRAK4, IRAK1 and IRAK2, and the E3 ligase TNF receptor-associated factor 6 (TRAF6) (Kawai *et al.* 2010). This complex mediates the activation of transforming growth factor (TGF)-β activated kinase 1 (TAK1), a mitogen-activated protein kinase kinase kinase (MAP 3-kinase). TAK1 then activates the NF-κB pathway and the MAP kinase pathways which are crucial for the production of many cytokines (Bhoj *et al.* 2009)(Figure 1.1). In the case of TLR4, TRAF3 is also recruited to MyD88 and the subsequent degradation of TRAF3 is important for MAP kinase activation and proinflammatory cytokine production (Tseng *et al.* 2010).

TAK1 activates the NF-κB pathway through the activation of the IκB kinase (IKK) complex (Bhoj *et al.* 2009). The IKK complex induces degradation of IκB proteins which hold NF-κB transcription factor dimers, composed of combinations of RelA (p65), RelB, c-Rel, p50 and p52 subunits, in an inactive state (Hayden *et al.* 2008). Upon degradation of IκB proteins, NF-κB dimers are free to translocate to the nucleus and modulate gene expression (Hayden *et al.* 2008). The IKK complex also mediates the activation of tumour progression locus-2 (TPL-2), a MAP 3-kinase, through proteolysis of the inhibitory NF-κB precursor protein p105 (Gantke *et al.* 2011). Once activated, TPL-2 phosphorylates the MAP 2-kinases, MEK 1 and 2, which in turn activate the MAP kinases extracellular-signal-regulated kinase (ERK) 1 and 2 (from here referred to as ERK) (Dumitru *et al.* 2000). This TPL-2/ERK dependent pathway is

important in the regulation of several cytokines including TNF-α and IL-10 but also negatively regulates IL-12 and IFN-β (Dumitru *et al.* 2000; Banerjee *et al.* 2006; Kaiser *et al.* 2009). The activation of MAP kinases other than ERK, including p38α/β/γ/δ and the c-Jun N-terminal kinases 1/2/3 (JNK1/2/3) which are activated by the MAP-2 kinases MKK3/4/6 and MKK4/7, respectively, also occurs downstream of MyD88 signalling (Symons *et al.* 2006). The activation of MAP kinases has many downstream effects which modulate cytokine production including activation of the activator protein 1 (AP-1) transcription factor, composed of dimers from the Fos, Jun, activating transcription factor (ATF) and Jun dimerising partner (JDP) protein families (Karin *et al.* 1997).

In addition to the NF-κB and MAP kinase pathways, interferon regulatory factors (IRF) are also activated downstream of the MyD88 dependent pathway (Figure 1.1). IRF5 is induced downstream of MyD88/TRAF6 and is important for the production of several proinflammatory cytokines (Takaoka *et al.* 2005). IRF8 has also been shown to interact with TRAF6 and modulate inflammatory cytokine production in response to LPS (Zhao *et al.* 2006). IRF1 interacts directly with MyD88 (Negishi *et al.* 2006) and induces IL-12 downstream of TLR4 (Liu *et al.* 2003a), and type I IFN downstream of TLR9 in mDCs (Schmitz *et al.* 2007). In pDCs, MyD88 signalling downstream of TLR7 and 9 additionally leads to the recruitment of TRAF3 and TRAF6 (Honda *et al.* 2004; Oganesyan *et al.* 2006), which promote the activation of IRF7 and type I IFN production (Honda *et al.* 2005b) (Figure 1.1). Of note, the Myd88-dependent pathway is also activated downstream of the IL-1 receptor (Muzio *et al.* 1997).

1.5.1.2 *TRIF dependent TLR signalling*

The TRIF pathway is only activated downstream of TLR3 and TLR4 (Yamamoto *et al.* 2003a) (Figure 1.1). TRIF recruits TRAF6 (Sato *et al.* 2003), and with the additional recruitment of TNFR-associated death domain protein (TRADD), receptor interacting protein-1 (RIP-1) and the E3 ubiquitin ligase Pellino-1, activates TAK-1 (Kawai *et al.* 2010). Subsequently, NF-κB and MAP kinase pathways are activated and these induce cytokine production (Kawai *et al.* 2010) (Figure 1.1). TRIF also recruits TRAF3, which forms a signalling complex with the IKK proteins TANK-binding kinase 1 (TBK1) and IKK-ε (Fitzgerald *et al.* 2003; Hacker *et al.* 2006). Signalling through this complex leads to the activation of IRF3 which induces the production of type I IFN in TLR 3 and 4 stimulated APCs (Doyle *et al.* 2002) (Figure 1.1).

1.5.1.3 *PI(3)K/AKT/mTOR signalling induction by TLRs*

TLR ligation also activates the mammalian target of rapamycin (mTOR) pathway downstream of MyD88 and TRIF (Schmitz *et al.* 2008) (Figure 1.1). This occurs via activation of the Phosphatidylinositol 3-kinase (PI(3)K)/AKT pathway, in which PI(3)K becomes activated and recruits the kinase AKT. AKT is then activated by phosphorylation which leads to the downstream inhibition of tuberous sclerosis complex protein 1/2 (TSC2-TSC1), an inhibitor of mTOR complex 1 (mTORC1) (Weichhart *et al.* 2009). In macrophages, monocytes and mDCs, inhibition of mTORC1 modulates the production of pro- and anti-inflammatory cytokines, confirming its role in innate immune responses (Ohtani *et al.* 2008; Schmitz *et al.* 2008; Weichhart *et al.* 2008). In pDCs, mTOR activates IRF7 to mediate the production of type I IFN

downstream of TLR9 (Cao *et al.* 2008). An mTORC2 complex also exists, although the role it plays in signal transduction downstream of TLRs remains unclear (Weichhart *et al.* 2009).

1.5.2 Non-TLR PRR signal transduction

1.5.2.1 *CLRs*

Transmembrane bound C-type lectin receptors (CLRs) can also act as PRRs (Geijtenbeek *et al.* 2009). For example the CLR Dectin-1 (also known as CLEC7a), recognises mycobacterial motifs (Rothfuchs *et al.* 2007), fungal β-glucans (Reid *et al.* 2009) and the pure β-glucan, Curdlan (LeibundGut-Landmann *et al.* 2007). Downstream of Dectin-1, the MAP Kinase ERK is activated and this is dependent on spleen tyrosine kinase (SYK) (Slack *et al.* 2007). The MAP 3-kinase RAF1, nuclear factor of activated T-cells (NFAT), NF-κB and caspase recruitment domain 9 (CARD9) are also activated downstream of this receptor (Reid *et al.* 2009). Dectin-1 ligation alone can stimulate cytokine production in bone marrow derived DCs (BMDCs) but not BMDMs (Goodridge *et al.* 2009), illustrating the principle that signalling downstream of PRRs can be cell-type specific.

1.5.2.2 *NLRs*

NOD-like receptors (NLRs) are expressed in the cytoplasm of the cell. The NLR family includes NOD1, NOD2 and NLRP1-10 (Franchi *et al.* 2009). These receptors detect a range of ligands which include the muramyl dipeptide (MDP) motif of peptidoglycan present within most types of bacteria (recognised by NOD2), and bacterial and viral

RNA (recognised by NLRP3). Through the recruitment of the adaptor protein RIP2 (Inohara *et al.* 2000; Ogura *et al.* 2001), NOD1 and NOD2 signalling induces NF-κB and MAP kinase activation (Inohara *et al.* 2005). It has more recently been shown that NOD2 can activate IRF3, and hence type I IFN production in response to ssRNA (Sabbah *et al.* 2009). NLR family members can also induce the assembly of the inflammasome, the best characterised of which is the NLPR3 inflammasome (Schroder *et al.* 2010). The inflammasome is a multi-protein complex which mediates the activation of the enzyme caspase-1. Activated capsase-1 mediates the proteolytic cleavage and maturation of IL-1 family cytokines (e.g. IL-1β, IL-18) into their biologically active forms, making this process important for the induction of proinflammatory responses (Martinon *et al.* 2002).

1.5.2.3 *RLRs*

The RIG-I-like receptor (RLR) family includes retinoic acid inducible gene-1 (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2) (Pichlmair *et al.* 2007). Like the NLRs, these are cytosolic PRRs. Mitochondrial anti-viral signalling protein (MAVS, also known as IPS-1) is important for signalling downstream of RIG-1 and MDA-5 (Seth *et al.* 2005). Signalling through these receptors ultimately induces the activation of IRF3, IRF7 and NF-κB, and MAP kinases which promote the production of cytokines including type I IFN (Pichlmair *et al.* 2007). LGP-2 was thought to be a negative regulator of RIG-1 and MDA-5 activity, although more recent evidence suggests that LGP-2 may be important in the promotion of type I IFN production downstream of RIG-1 and MDA-5 (Satoh *et al.* 2010).

1.6 The molecular mechanisms of IL-10 regulation

The mechanisms of IL-10 regulation are diverse, owing to the fact that almost all cells of the immune system have been shown to make IL-10 (Saraiva *et al.* 2010). Within the innate immune system macrophages, mDCs but not pDCs are all sources of IL-10 (Boonstra *et al.* 2006; Kaiser *et al.* 2009). Within the adaptive immune system effector and regulatory $CD4^+$ T cells, cytotoxic $CD8^+$ T cells and B cells produce IL-10. Other cells including mast cells, eosinophils, neutrophils and NK cells have also been shown to produce IL-10, although the molecular mechanisms governing IL-10 production in these immune cells are still only superficially characterised (Saraiva *et al.* 2010).

1.6.1 The regulation of IL-10 production in macrophages and mDCs

In macrophages and mDCs, the induction of *Il10* expression and ultimate production of IL-10 protein, is dependent on stimulation with microbial products through PRRs (Saraiva *et al.* 2010). IL-10 production from macrophages and mDCs can be induced by several TLR ligands. In particular, it has been proposed that TLR2 is specialised in the production of IL-10 (Agrawal *et al.* 2003; Dillon *et al.* 2004), although IL-10 production also occurs downstream of TLR3, TLR4 and TLR9 (Boonstra *et al.* 2006; Kaiser *et al.* 2009). Importantly, the level of IL-10 produced by macrophages and DCs has been shown to differ. For example, CpG (TLR9) stimulated macrophages make the most IL-10, followed by mDCs, followed by pDCs which do not make any IL-10 in response to CpG, despite all these cell types expressing TLR9 in the mouse (Boonstra *et al.* 2006; Kaiser *et al.* 2009). In addition, TLR3 ligation can induce IL-10 production in macrophages, but not mDCs (Boonstra *et al.* 2006). A further example is the PRR

Dectin-1 which is a potent inducer of IL-10 in mDCs (Rogers *et al.* 2005; Slack *et al.* 2007), but does not induce cytokine production in macrophages (Goodridge *et al.* 2009). Collectively, these findings suggest that the intrinsic capacity of innate cell types to make IL-10 may not be the same.

1.6.1.1 *The induction of IL-10 by PRRs*

Upon TLR ligation, IL-10 production is dependent on the presence of the adaptor proteins MyD88 and/or TRIF, according to the TLR stimulated. For example, stimulation of macrophages or mDCs via TLR9, which depends on the MyD88 pathway, fails to induce any IL-10 in the absence of MyD88 (Boonstra *et al.* 2006). Likewise, stimulation of TLR3 in the absence of its only downstream adaptor TRIF, almost completely abrogates IL-10 production (Boonstra *et al.* 2006). TLR4 is able to activate both MyD88 and TRIF dependent pathways, and optimal induction of IL-10 by TLR4 has been shown to require both of these adaptors (Boonstra *et al.* 2006). Downstream of MyD88 and TRIF, the E3 ligase TRAF3 which is recruited by both TRIF and MyD88, has been reported to be important for IL-10 production in CpG, LPS and Poly I:C stimulated macrophages (Hacker *et al.* 2006). TRAF3 is also essential for the production of type I IFN production, but not proinflammatory cytokines such as IL-12 (Hacker *et al.* 2006). This report also showed that TRAF6, which is additionally recruited downstream of MyD88 and TRIF (Kawai *et al.* 2010), was not required for IL-10 production downstream of TLR4 (Hacker *et al.* 2006).

The activation and recruitment of adaptor proteins and TRAF3/6 collectively leads to the activation of MAP kinases, IRFs and the PI(3)K/AKT/mTOR pathway. The

activation of the MAP kinases ERK is critical for the positive regulation of IL-10 in macrophages and mDCs stimulated with TLR 2, 4 and 9 ligands (Yi *et al.* 2002; Dillon *et al.* 2004; Banerjee *et al.* 2006; Kaiser *et al.* 2009) (Figure 1.2). In addition, the differential capacity of macrophages, mDCs and pDCs to make IL-10 (Boonstra *et al.* 2006) may to correspond to the differential level of ERK activation in these cells (Kaiser *et al.* 2009). In keeping with this, the MAP 3-kinase TPL-2, the upstream regulator of ERK in this context (Dumitru *et al.* 2000), has been shown to positively regulate IL-10 in TLR4 and TLR9 stimulated macrophages and mDCs (Banerjee *et al.* 2006; Kaiser *et al.* 2009) (Figure 1.2). Downstream of ERK activation, the transcription factor c-Fos which can form part of the AP-1 transcription factor (Karin *et al.* 1997), may be important for the induction of *Il10* expression (Dillon *et al.* 2004; Kaiser *et al.* 2009). Indeed, an AP-1 consensus binding site has been shown within a conserved non-coding sequence downstream of the *Il10* gene (Wang *et al.* 2005) (Figure 1.2), and inhibition of Fos or Jun reduced IL-10 production in TLR2 stimulated human macrophages (Hu *et al.* 2006). ERK activation has further been shown to regulate the production of IL-10 by the phosphorylation of histone H3 at sites in the *Il10* promoter in macrophages stimulated with immune-complexes and LPS (Zhang *et al.* 2006). This activity was proposed to allow transcription factors to bind at the *Il10* locus and hence induce gene expression (Zhang *et al.* 2006). The induction of IL-10 by the CLR Dectin-1 in DCs requires the activation of spleen tyrosine kinase (SYK) and also the downstream activation of ERK, but is independent of c-Fos (Rogers *et al.* 2005; Slack *et al.* 2007). Activation of the MAP kinase p38 further positively regulates IL-10 in macrophages and mDCs (Foey *et al.* 1998; Yi *et al.* 2002; Jarnicki *et al.* 2008; Kim *et al.* 2008). Together with ERK activation, p38 has been shown to activate the downstream

kinases mitogen- and stress-activated protein kinase (MSK) 1 and 2 (Ananieva *et al.* 2008) (Figure 1.2). This promotes the binding of the transcription factors cAMP response element-binding (CREB) and ATF1 to the *Il10* gene which in turn positively regulate IL-10 (Ananieva *et al.* 2008) (Figure 1.2). The role of the third MAPK, JNK in the regulation of IL-10 is less well studied and although it has been reported that the inhibition of JNK reduces IL-10 production in TLR2 stimulated human macrophages (Hu *et al.* 2006), the inhibitor used in this study is known to be of poor specificity (Bain *et al.* 2007).

The PI(3)K/AKT/mTOR pathway has also been shown to positively regulate IL-10 downstream of TLR activation. Inhibition of the PI(3)K/AKT pathway was found to suppress TLR2 induced IL-10 production in human monocytes (Martin *et al.* 2003). In this study, the activation of ERK was proposed to be important in the regulation of IL-10 downstream of PI(3)K/AKT signalling (Martin *et al.* 2003). In LPS stimulated murine mDCs, the PI(3)K/AKT pathway has been shown to promote the production of IL-10 through the activation of mTOR (Ohtani *et al.* 2008)(Figure 1.2). This has also been shown in LPS activated human monocytes (Weichhart *et al.* 2008). A recent report has added further complexity to these regulatory networks by finding that p38 can activate mTOR independently of PI(3)K, proposing that this is one of the mechanisms whereby p38 can positively regulate IL-10 (Katholnig *et al.* 2013) (Figure 1.2). Glycogen synthase kinase 3 β (GSK3-β) is a constitutively active kinase which inhibits IL-10 production by modulating the DNA binding activity of the transcription factor CREB (Martin *et al.* 2005). This kinase is phosphorylated and inhibited by PI(3)K/AKT signalling downstream of TLR activation, providing a further mechanism of positive IL-

10 regulation by the PI(3)K/AKT pathway (Martin *et al.* 2005; Ohtani *et al.* 2008) (Figure 1.2).

Several lines of evidence propose that NF-κB may modulate the production of IL-10 in APCs. Firstly, upstream of the murine *Il10* promoter, a DNase I-hypersensitivity site (HSS-4.5), indicative of unstructured chromatin, has been identified in IL-10-producing macrophages and mDC and contains a putative conserved NF-κB binding site (Saraiva *et al.* 2005) (Figure 1.2). This study also showed that the NF-κB family member p65 bound to this site (Saraiva *et al.* 2005). Secondly, *Nfkb1* deficient macrophages have reduced IL-10 production (Banerjee *et al.* 2006; Cao *et al.* 2006). Although this is in part due to inhibition of the TPL-2/MEK/ERK pathway as one of the functions of NF-κB1 (p105) is to stabilise TPL-2 (Gantke *et al.* 2011), restoration of ERK signalling could not fully rescue IL-10 production suggesting an ERK-independent role for NF-κB1 (p105) in the regulation of IL-10 (Banerjee *et al.* 2006). Thirdly, p50 homodimers have been reported to form a complex with CREB-binding protein (CBP) and bind within the proximal *Il10* promoter in LPS stimulated macrophages (Cao *et al.* 2006) (Figure 1.2). Lastly, IKK2 deletion has been shown to reduce LPS induced IL-10 production from macrophages (Kanters *et al.* 2003). However, a caveat of this study is that IKK2 is upstream of TPL-2 (Gantke *et al.* 2011). Thus, the role of the NF-κB pathway independent of the TPL-2/ERK pathway cannot be determined in this system (Kanters *et al.* 2003). NF-κB has further been shown to play a role in the induction of IL-10 from splenic macrophages stimulated with dsRNA (Chakrabarti *et al.* 2008). NF-κB was found to bind the *Il10* promoter at a distal site (Figure 1.2), and this was shown to be dependent on the activation of protein kinase R (PKR) (Chakrabarti *et al.* 2008).

Several additional transcription factors have been reported to regulate IL-10 in macrophages and mDCs both through the identification of binding sites within the *Il10* promoter, and functional experiments (Figure 1.2). A c-MAF responsive element has been identified within the human *Il10* promoter and c-MAF was reported to enhance IL-10 production from human monocytes when stimulated with LPS in the presence of IL-4 (Cao *et al.* 2005). This study also showed a role for c-MAF in promoting IL-10 production from LPS stimulated fetal-liver derived macrophages in the presence and absence of IL-4 (Cao *et al.* 2005). A STAT motif has been identified approximately 120 bp upstream of the human *Il10* transcriptional start site and it was proposed that LPS induced STAT3 binding to this site to regulate IL-10 in human B cell and monocyte cell lines (Benkhart *et al.* 2000) (Figure 1.2). In LPS stimulated human monocytes, the induction of IL-10 by mTOR may be dependent on STAT3 (Weichhart *et al.* 2008) (Figure 1.2). An additional STAT motif has been identified further upstream in both human and mouse *Il10* promoters (Mosser *et al.* 2008b). In the murine macrophage cell line, RAW264.7, a specific protein 1 (SP1) binding site was identified within the *Il10* promoter and was important for trans-activation of the *Il10* gene (Brightbill *et al.* 2000). Simultaneously, another group identified this motif and reported that both SP1 and SP3 can bind to regulate *Il10* promoter activity (Tone *et al.* 2000) (Figure 1.2). A subsequent report identified an additional SP1 site much further upstream in a human monocytic cell line and it was shown that p38 activity downstream of LPS stimulation was required for SP1 activation (Ma *et al.* 2001). Of note, no role for ERK in the regulation of IL-10 was reported in this study, in contrast to several reports in primary murine cells (Yi *et al.* 2002; Dillon *et al.* 2004; Banerjee *et al.* 2006; Kaiser *et al.* 2009). The varying

reports on the requirement for specific promoter motifs or signalling pathways in the production of IL-10 may be a consequence of different experimental systems, highlighting the complexity of IL-10 regulation (Saraiva *et al.* 2010). CCAAT/enhancer-binding protein (C/EBP) binding sites have also been identified within the *Il10* promoter (Brenner *et al.* 2003) (Figure 1.2). It was further found that C/EBPβ and C/EBPδ binding at these sites can cooperate with SP1 binding for maximal transactivation of the *Il10* gene (Liu *et al.* 2003b). A role for the transcription factor pre-B cell leukaemia homeobox 1 (PBX1) and its co-factor PBX-regulating protein 1 (PREP1) has also been identified in the regulation of IL-10 production in macrophages downstream of p38, although this was in response to apoptotic cells and not LPS (Chung *et al.* 2007).

Thus, to date much is known about the regulation of PRR induced IL-10 in various types of innate cell, in particular in the context of LPS stimulation (Figure 1.2). However, there are still several aspects which are not clear. For example, the upstream pathways important for the activity of the various transcription factors proposed to regulate IL-10 are not fully understood. A comprehensive understanding of distinct and overlapping IL-10 inducing factors in different innate cell types is also lacking. In addition, as mentioned, some studies have shown that chromatin modifications at the *Il10* locus may be involved in the regulation of *Il10* gene expression (Saraiva *et al.* 2005; Zhang *et al.* 2006). However, which upstream signals induce these chromatin modifications and how they cooperate with transcription factor binding is only partially understood.

1.6.1.2 *The negative regulation of IL-10 in macrophages and DCs*

Several IRFs are activated downstream of PRR signalling and many of these are involved in the positive regulation of proinflammatory cytokine production (Kawai *et al.* 2010). However, the regulation of IL-10 provides an exception to this as in human LPS stimulated GM-CSF differentiated monocytes, IRF5 has been reported to directly negatively regulate IL-10 (Krausgruber *et al.* 2011). Other cellular mechanisms of IL-10 inhibition also exist. For example, the histone deacetylase 11 (HDAC11) negatively regulates IL-10 production in LPS stimulated murine macrophages and human DCs, potentially by affecting the binding of SP1 and STAT3 (Villagra *et al.* 2009).

Post-transcriptional mechanisms are additionally important in the negative regulation of IL-10 production. It has long been known that the 3'-untranslated region (3'UTR) of *Il10* mRNA mediates its destabilisation, thus negatively impacting on overall IL-10 production (Powell *et al.* 2000). More recently, a role for microRNAs and RNA binding proteins which bind the 3'UTR, has also become apparent. The microRNA hsa-miR-106a has been shown to target and degrade *Il10* mRNA in cell lines (Sharma *et al.* 2009). MicroRNAs can however have complex indirect effects on IL-10 regulation. For example, IL-10 is indirectly regulated by miR-21 which inhibits programmed cell death protein 4 (PDCD4), negative regulator of IL-10 production (Sheedy *et al.* 2010). Further negative post-transcriptional regulation of *Il10* mRNA is mediated by Tristetraprolin (TTP), an RNA binding protein that binds the AU-rich element (within the 3'UTR) of *Il10* mRNA and promotes degradation (Stoecklin *et al.* 2008). The inhibition of TTP is one mechanism whereby p38 activation positively regulates IL-10

production (Tudor *et al.* 2009). MicroRNA-4661 also interferes with TTP by blocking its binding at the 3'UTR and as a result, enhances IL-10 production (Ma *et al.* 2010).

1.6.1.3 *The regulation of IL-10 in macrophages and DCs by autocrine and paracrine factors*

Added complexity to the regulation of IL-10 is provided by autocrine and paracrine regulatory loops. Firstly, IL-10 can inhibit its own production by inducing the production of the dual phosphatase DUSP1 which negatively regulates p38 activation, and hence decreases IL-10 production (Hammer *et al.* 2005). IL-10 can also induce the production of TTP which destabilises *Il10* mRNA (Stoecklin *et al.* 2008; Gaba *et al.* 2012). Conversely, IL-10 can induce the expression of *Tpl2* (Lang *et al.* 2002), a positive regulator of IL-10 (Banerjee *et al.* 2006; Kaiser *et al.* 2009). The feed-forward self-regulatory loop of IL-10 has been shown to be dependent on STAT3 in human macrophages (Staples *et al.* 2007).

Endogenous and exogenous type I IFNs can further enhance IL-10 production from macrophages and DCs. This was first shown in human monocytes where high concentrations of IFN-α enhanced IL-10 production, provided the cells were also stimulated with LPS (Aman *et al.* 1996). Subsequently, type I IFN signalling was shown to be important for optimal IL-10 production in LPS stimulated macrophages (Chang *et al.* 2007a) and *M.tuberculosis* infected macrophages (Mayer-Barber *et al.* 2011). As TRAF3 is also required for the production of type I IFN in LPS stimulated phagocytes, this may explain the requirement for this molecule in the optimal production of IL-10 downstream of TLR4 (Hacker *et al.* 2006). Type I IFN signalling

has also been shown to promote IL-10 production in LPS stimulated murine DCs (Escors *et al.* 2008). A role for STAT1, a critical component of signal transduction downstream of the type I IFN receptor (Stark *et al.* 1998), has been identified in the type I IFN mediated promotion of IL-10 in LPS stimulated macrophages, although no role for STAT3 was found (Guarda *et al.* 2011). Type I IFN has also been shown to induce IL-10 through the activation of the PI(3)K/AKT pathway in human DCs (Wang *et al.* 2010). The activation of this pathway led to the subsequent inhibition of GSK3-β and activation of CREB (Wang *et al.* 2010). This report also negated a role for STAT3 in this process (Wang *et al.* 2010). Other studies have suggested that IRF-1 and STAT3 are recruited to the *Il10* promoter by IFN-α, although this was conducted in a human B cell line (Ziegler-Heitbrock *et al.* 2003). It has further been suggested that IL-27 is important for the optimal production of IL-10 in response to type I IFN in LPS stimulated murine macrophages (Iyer *et al.* 2010). In this report, it was proposed that IL-27 induced the direct binding of STAT1 and STAT3 to the *Il10* locus, thereby enhancing expression (Iyer *et al.* 2010). However, others have reported that murine macrophages are not responsive to IL-27 (Kalliolias *et al.* 2008). Thus, the role of IL-27 in the type I IFN mediated induction of IL-10 is currently unclear.

In contrast, IFN-γ has been shown to inhibit the production of IL-10 in TLR2 stimulated human macrophages (Hu *et al.* 2006). The mechanism of IFN-γ mediated IL-10 inhibition was reported to be through the inhibition of the PI(3)K-AKT pathway (Hu *et al.* 2006). This allowed GSK3-β to suppress AP-1 and CREB activity and hence IL-10 production. This study also found that IFN-γ inhibited IL-10 through suppression of MAP kinase activation (Hu *et al.* 2006).

1.6.2 The regulation of IL-10 in other cell types

IL-10 was initially characterised as a Th2 cytokine (Fiorentino *et al.* 1989) however, it is now known to be produced by all effector $CD4^+$ T cell subsets, and $CD8^+$ T cells (Saraiva *et al.* 2010). The mechanisms that induce IL-10 production in T cells are largely distinct from that of APCs and are still being characterised. This has been particularly difficult as factors required for subset differentiation are also thought to be required for IL-10 induction. For example, in Th2 cells, IL-4 which supports the differentiation of this subset, and the Th2 subset defining master regulator GATA3 are important for the production of IL-10 (Zhu *et al.* 2004; Shoemaker *et al.* 2006; Chang *et al.* 2007b). It is additionally known that GATA3 is able to modify the chromatin structure at the *Il10* locus, but itself is not sufficient to induce transcription (Shoemaker *et al.* 2006). This suggests that factors in addition to GATA3 are required to induce IL-10 production in this cell type. In Th1 cells, a strong TCR signal, IL-12, and its downstream signal transducer STAT4 are all required for the production of IL-10 (Saraiva *et al.* 2009). In Th17 cells, again, the factors critical in the differentiation of this subset i.e. TGF-β, IL-6 and its downstream signal transducer STAT3, are necessary for IL-10 production (Stumhofer *et al.* 2007). Negative regulatory mechanisms which suppress IL-10 production in T cells have also been identified. For example the transcriptional repressor ETS-1 inhibits IL-10 production in Th1 cells (Grenningloh *et al.* 2005). The level of IL-10 production from Th cells can further be modulated by autocrine and paracrine factors. A central example of this is IL-27, which can enhance IL-10 production from the different Th subsets in a number of disease models (Freitas do Rosario *et al.* 2012; Hunter *et al.* 2012). In $FOXP3^+$ Tregs which mediate their

suppressive activity at least in part through the production of IL-10 (Bacchetta *et al.* 2007; Maynard *et al.* 2008; Lloyd *et al.* 2009), TGF-β is thought to be an important IL-10 inducing factor (Maynard *et al.* 2007). Although the mechanisms of IL-10 induction in other cell types are even less well defined, in B cells, TLR ligation can stimulate IL-10 production (O'Garra *et al.* 1992; Mauri *et al.* 2012). Some factors which regulate IL-10 production in APCs do overlap with Th cells however. Similarly to APCs, ERK has been reported to be a requirement for IL-10 production in all Th subsets (Saraiva *et al.* 2009). A role for c-MAF, which has been shown to promote IL-10 in macrophages in the presence of IL-4 (Cao *et al.* 2005), has also been implicated in the induction of IL-10 in Th17 cells (Xu *et al.* 2009).

1.7 The molecular mechanisms of IL-12 regulation

The bioactive form of IL-12, IL-12p70, is composed of IL-12p40 and IL-12p35 subunits (Kobayashi *et al.* 1989). As mentioned previously, these subunits can additionally dimerise with other molecules to generate IL-23 (p40:p19 heterodimer) (Oppmann *et al.* 2000) and IL-35 (p35:Ebi3 heterodimer) (Collison *et al.* 2007; Niedbala *et al.* 2007). IL-12p40 and IL-12p35 are induced in monocytes, macrophages and DCs (including pDC), in response to microbial stimuli (D'Andrea *et al.* 1992; Macatonia *et al.* 1995; Jarrossay *et al.* 2001; Boonstra *et al.* 2006). IL-12p40 is mainly produced by cells that go on to make bioactive IL-12p70, whereas IL-12p35 mRNA can be detected in many immune cell types (Wolf *et al.* 1991; D'Andrea *et al.* 1992). IL-12p40 and IL-12p35 subunits must however be made by the same cell for IL-12p70 to be produced (Wolf *et al.* 1991). Thus, the regulation of IL-12 gains complexity due to the necessity to coordinate subunit production. IL-12p40 is made in excess and can be

secreted as both a monomer and a homodimer (Wolf *et al.* 1991). Of note, it has been reported that IL-12p40 homodimers can antagonise the activity of IL-12 *in vitro* and *in vivo* (Gillessen *et al.* 1995; Gately *et al.* 1996). More recently however, it has been suggested that IL-12p40 homodimers may facilitate DC migration and subsequent T cell activation in a model of *M.tuberculosis* infection (Khader *et al.* 2006). Thus, the function of IL-12p40 homodimers, if any, remains unclear. Conversely, there is no evidence that IL-12p35 is secreted unless dimerised with IL-12p40 (Wolf *et al.* 1991), and the production of IL-12p35 is considered to be the limiting factor in IL-12p70 production (Snijders *et al.* 1996).

1.7.1 The induction of IL-12 by PRRs

Among the PRRs, TLRs including TLR 2, 3, 4 and 9 have been described to induce the production of IL-12 in human and mouse phagocytes (Goriely *et al.* 2008). As is the case with IL-10, the level of IL-12 production depends on the subset of cell e.g. macrophage, mDC or pDC. This may be a reflection of differential TLR expression (Jarrossay *et al.* 2001), or different molecular mechanisms of cytokine regulation in the various cell types (Grumont *et al.* 2001). In addition, different TLRs may have a differential capacity to induce IL-12 production. For example, TLR2 has been reported to induce the bioactive form of IL-12 less efficiently than TLR4 in human immature DCs (Re *et al.* 2001). TLR2 also induces lower levels of IL-12p35 than TLR4 in human monocyte derived DCs, linked to differential TLR-adaptor use (Goriely *et al.* 2006). The study of IL-12 regulation has been also complicated by the fact that stimulation with a single TLR ligand often only inefficiently induces IL-12 production (Lyakh *et al.* 2008). Thus, several studies have been conducted in the presence of IFN-γ, a potent

inducer of IL-12p40 and IL-12p35, provided that cells are concomitantly stimulated with a TLR ligand (Hayes *et al.* 1995). In addition, stimulation of phagocytes with multiple TLRs, ideally targeting both Myd88 and TRIF dependent pathways, is considered important for optimal IL-12p70 production (Gautier *et al.* 2005; Napolitani *et al.* 2005; Ouyang *et al.* 2007).

Downstream of TLRs, IRFs are important positive regulators of IL-12p40 and IL-12p35 production (Figure 1.3). The activation of IRF1 has been shown to be required for the optimal production of IL-12p35, but not IL-12p40 in LPS and LPS/IFNγ stimulated macrophages (Liu *et al.* 2003a). In this study, LPS/IFN-γ treatment induced IRF1 binding to the *Il12a* (IL-12p35) locus in murine peritoneal macrophages and human blood-derived monocytes (Liu *et al.* 2003a). This contrasted with a previous report in which LPS/IFN-γ induced IL-12p40 was dramatically reduced in IRF1 deficient murine peritoneal macrophages (Taki *et al.* 1997), although the duration of IFN-γ treatment differed between these studies. IRF1 has been shown to interact with MyD88 to mediate the synergistic effect of CpG/IFN-γ treatment on IL-12p35 production in DCs (Negishi *et al.* 2006). IL-12p35 mRNA levels were also reduced in *Irf1*$^{-/-}$ DCs treated with CpG alone (Negishi *et al.* 2006), supporting a role for IRF1 in the regulation of IL-12p35 in the absence of IFN-γ. IRF1 may further cooperate with Sp1 sites (Liu *et al.* 2003a) which are present in the *Il12a* (IL-12p35) promoter and important for LPS/IFN-γ induced IL-12p35 production (Goriely *et al.* 2003). IRF3 is also involved in the regulation of IL-12p35 (Figure 1.3). In murine DCs stimulated with LPS, IRF3 deficiency led to a reduction in IL-12p35 mRNA expression and IL-12p70 production (Goriely *et al.* 2006). Further, IRF3 is recruited to the *Il12a* (IL-12p35) promoter in

TLR4 or TLR3 but not TLR2 stimulated human DCs, implying a role for IRF3 in IL-12p35 regulation downstream of the TRIF dependent pathway (Goriely *et al.* 2006).

In the context of IL-12p40, IRF5 has a positive regulatory role in TLR 3, 4, 5, 7 and 9 stimulated spleen-derived murine macrophages (Takaoka *et al.* 2005) (Figure 1.3). IRF5 is also required for the production of IL-12p40 in TLR9 stimulated murine mDC and pDC (Takaoka *et al.* 2005). More recently, IRF5 has been shown to promote both IL-12p40 and IL-12p35 mRNA expression in LPS stimulated GM-CSF differentiated human monocytes (Krausgruber *et al.* 2011). IRF5 may also be involved in mediating synergistic IL-12p40 production induced by stimulation with multiple TLRs (Ouyang *et al.* 2007). IRF8 has been associated with the induction of IL-12p40, but mainly in the context of IFN-γ co-stimulation (Figure 1.3). In LPS/IFN-γ stimulated RAW 264.7 cells, transfection of IRF8 led to *Il12b* (IL-12p40) promoter activation, potentially binding an ETS site in the *Il12b* promoter and synergising with IRF1 (Wang *et al.* 2000). It has additionally been reported that IRF8 interacts with TRAF6 in LPS/IFN-γ stimulated RAW 264.7 cells in a mechanism that promotes the production of IL-12p40 and other proinflammatory cytokines (Zhao *et al.* 2006). IRF8 may further be required for the induction of IL-12p35 in LPS and LPS/IFNγ stimulated peritoneal macrophages, again in synergy with IRF1 (Liu *et al.* 2004).

In addition to IRFs, NF-κB is an important positive regulator of IL-12 production downstream of TLRs (Figure 1.3). A Rel-binding element was identified within the *Il12b* (IL-12p40) promoter (Murphy *et al.* 1995), and upon LPS stimulation, c-Rel is required for IL-12p40 production in macrophages (Sanjabi *et al.* 2000; Grumont *et al.*

2001). A C/EBP site (Plevy *et al.* 1997) and an ETS consensus site, the latter of which binds PU.1 and ETS2 (Ma *et al.* 1997), also exist within the *Il12b* (IL-12p40) promoter (Figure 1.3). Binding at these sites functionally cooperates with c-Rel and, in the case of the ETS site, IRFs to activate the *Il12b* (IL-12p40) promoter (Ma *et al.* 1997; Plevy *et al.* 1997; Gri *et al.* 1998; Wang *et al.* 2000). In DCs, c-Rel containing complexes are specifically required for IL-12p35 production in response to LPS and other stimuli (Grumont *et al.* 2001), suggesting that c-Rel may regulate IL-12p40 and IL-12p35 differently in macrophages and DCs. Another study showed that overexpression of c-Rel in RAW264.7 cells enhanced IL-12p35 promoter activity in response to LPS or LPS/IFN-γ (Liu *et al.* 2003a).

MAP kinases are also activated down-stream of TLRs (Kawai *et al.* 2010). Of these, the MAP kinase JNK has been suggested to positively regulate IL-12p70 in TLR 2, 4 and 5 stimulated human monocyte derived DCs (Agrawal *et al.* 2003), however, the pharmacological inhibitor used in this study does not have optimal specificity (Bain *et al.* 2007). The same study also reported that the MAP kinase p38 promotes the production of IL-12p70 (Agrawal *et al.* 2003), in keeping with an earlier report showing that LPS stimulated macrophages derived from *Mkk3*$^{-/-}$ mice, which are unable to activate p38, produce reduced levels of IL-12p40 and IL-12p35 mRNA (Lu *et al.* 1999). More recently however, several studies have implied that p38 acts as a negative regulator of IL-12 (Jarnicki *et al.* 2008; Yang *et al.* 2010).

PRRs other than TLRs, such as the C-type lectin Dectin-1, have also been studied for their potential to induce IL-12 in DCs however, it was found that this receptor

preferentially induces IL-23, and only a small amount of IL-12p70 (LeibundGut-Landmann *et al.* 2007). In a related concept, unlike the IL-12p70 promoting effect of TLR/TLR synergy (Gautier *et al.* 2005; Napolitani *et al.* 2005; Ouyang *et al.* 2007), the combination of TLR2 and NOD2 ligands (which are present in pathogens such as *M.tuberculosis*) also preferentially induces IL-23 as opposed to IL-12p70 (Gerosa *et al.* 2008). Further, it has recently been shown that the combination of RLR and TLR activation, inhibits the production of IL-12p40 in a mechanism that involves the obstruction of IRF5 by IRF3 at the *Il12b* (IL-12p40) promoter, independently of type I IFN (Negishi *et al.* 2012).

As an additional step of regulation, once the IL-12p35 and IL-12p40 subunits have been transcribed and translated, further post-translational modifications are made (Carra *et al.* 2000). In the case of IL-12p40, the alterations are only minor, however, IL-12p35 undergoes significant glycosylation which has been proposed to be a determining factor in the amount of bioactive IL-12p70 produced by the cell (Carra *et al.* 2000).

1.7.2 The negative regulation of IL-12

As mentioned earlier, a key negative regulator of IL-12 in murine and human systems is the anti-inflammatory cytokine, IL-10 (D'Andrea *et al.* 1993; Hsieh *et al.* 1993). Endogenous IL-10 has been shown to regulate IL-12p70 production from both macrophages and mDCs, and exogenous IL-10 can regulate production of IL-12p70 from these cells and pDCs (Boonstra *et al.* 2006). The inhibition of IL-12 by IL-10 has been proposed to be in part at the level of transcription, with dampening of both IL-12p40 and IL-12p35 mRNA expression (Aste-Amezaga *et al.* 1998). Although the exact

mechanisms by which IL-10 inhibits IL-12 production remain incompletely understood, it has recently been shown that IL-10 induces the production of the transcription factor NFIL3 which binds upstream of the IL-12p40 promoter and suppresses transcription (Smith *et al.* 2011).

IL-12 production is also negatively regulated by activation of the MAP kinase ERK which occurs downstream of PRR signalling. This inhibitory activity of ERK is both dependent and independent of IL-10 (Yi *et al.* 2002; Agrawal *et al.* 2003; Dillon *et al.* 2004; Kaiser *et al.* 2009), and has been shown to involve the transcription factor c-Fos (Dillon *et al.* 2004). p38 activation can also negatively regulate IL-12 (Jarnicki *et al.* 2008), although as mentioned above, the positive regulation of IL-12 by p38 has also been reported (Lu *et al.* 1999; Agrawal *et al.* 2003). The inhibitory activity of p38 activation on IL-12 production is thought to be mainly dependent on the production of IL-10 (Jarnicki *et al.* 2008), although it has been shown that p38 can inhibit IL-12 production by destabilising IL-12p40 mRNA independently of IL-10 (Yang *et al.* 2010). The inconsistencies in the role of p38 activation in the regulation of IL-12 may reflect differences in cell type, cell density or level of stimulus used in each experimental system.

PI(3)K signalling also inhibits IL-12 production in TLR4, TLR2 and TLR9 stimulated DCs and this was suggested to be independent of IL-10 (Fukao *et al.* 2002). Others have shown the inhibition of IL-12 by PI(3)K signalling to be partially mediated through the inhibition of GSK3, a positive regulator of IL-12 (Ohtani *et al.* 2008). IL-12 is further negatively regulated by mTOR in TLR stimulated macrophages and DCs (Ohtani *et al.*

2008; Schmitz *et al.* 2008; Weichhart *et al.* 2008). Some findings suggest this is independent of IL-10 (Weichhart *et al.* 2008), whereas others imply that the up-regulation of IL-10 by mTOR is important for the inhibition of IL-12 (Ohtani *et al.* 2008).

1.7.3 The modulation of IL-12 production by T cell signals, and autocrine and paracrine factors

Other than through the effect of Th1 derived IFN-γ (Hayes *et al.* 1995), T cells are able influence the production of IL-12. Direct signalling from T cells through CD40/CD40L interaction has been shown to enhance IL-12p70 production in DCs (Cella *et al.* 1996), although *in vivo* this requires concomitant stimulation with microbial products (Schulz *et al.* 2000). Interestingly, it has also been reported that extended pre-treatment (20 h or more) with the Th2 cytokines IL-4 and IL-13 can promote the production of IL-12p70 from LPS or *Staphylococcus aureus* (*S.aureus*) stimulated PBMC although when added during stimulation, the presence of these cytokines conversely inhibited IL-12p70 production (D'Andrea *et al.* 1995).

Autocrine and paracrine type I IFN production has also been shown to regulate IL-12, however, the effects of type I IFN on IL-12 production have been difficult to dissect (Lyakh *et al.* 2008). For example, the addition of high levels of type I IFN have been shown to negatively regulate *S.aureus* induced IL-12 production independently of IL-10 in primary human monocytes (Byrnes *et al.* 2001). However, the stimulations in this study were done in the presence of IFN-γ, the activity of which is has been shown to be affected by type I IFN (Rayamajhi *et al.* 2010). In the context of LCMV infection, the

blockade of endogenous type I IFN has been shown to enhance IL-12 levels in the serum, and IL-12 production from splenocytes (Cousens *et al.* 1997). In contrast, endogenous type I IFN has been reported to be important for the optimal production of IL-12 in DCs that have been individually or co-stimulated with MyD88 and TRIF dependent TLR ligands (Gautier *et al.* 2005). Thus, the effect of type I IFN on IL-12 production appears to be dependent on the context.

1.8 The coordinate regulation of IL-10 and IL-12

The literature to date has reported several mechanisms of IL-10 and IL-12 regulation in macrophages and DCs. Some of these mechanisms are opposing, for example ERK positively regulates IL-10 whilst negatively regulating IL-12 (Yi *et al.* 2002; Agrawal *et al.* 2003; Dillon *et al.* 2004; Kaiser *et al.* 2009). Similarly, the activation of the PI(3)K pathway promotes IL-10 but inhibits IL-12 (Ohtani *et al.* 2008; Weichhart *et al.* 2008). However, some of these mechanisms have a similar effect on IL-10 and IL-12, for example NF-κB family transcription factors have been reported to promote both IL-10 and IL-12 production (Murphy *et al.* 1995; Saraiva *et al.* 2005; Zhang *et al.* 2006). This gives rise to extensive cross-regulation between the IL-10 and IL-12 regulating molecular networks. The dissection of these pathways is further complicated by the robust inhibition of IL-12 by IL-10 (D'Andrea *et al.* 1993; Murphy *et al.* 1994; Hsieh *et al.* 1995). The currently known interactions between the pathways that regulate IL-10 and IL-12 are summarised in Figure 1.4. IFN-γ, produced mainly by NK cells and T cells, promotes IL-12 production while inhibiting IL-10 production (Hayes *et al.* 1995; Hu *et al.* 2006) (Figure 1.4). Type I IFN, which can be produced by macrophages and DCs, conversely promotes IL-10 production but has unclear roles on the regulation of

IL-12 (Chang *et al.* 2007a; Lyakh *et al.* 2008). As type I IFN has an increasingly appreciated important role in immunoregulation (Trinchieri 2010), discussed in more detail below, a better understanding of how this cytokine affects the regulation of IL-10 and IL-12 may be relevant to many immunological processes.

1.9 The role of type I IFN in the immune response

Originally identified in the 1950s (Isaacs *et al.* 1957), the type I IFNs constitute a group of cytokines including 14 IFN-α's in mice (13 IFN-α's in human), and the singular IFN-β (Decker *et al.* 2005). Other type I IFNs e.g. IFN-ε, have also been described although their roles are only recently being characterised (Fung *et al.* 2013). Of the type I IFNs, a genetic evolutionary study has revealed that in humans, IFN-α 6, 8, 13 and 14 are highly conserved implying that they have non-redundant roles that are beneficial to the host (Manry *et al.* 2011). Type II IFN (IFN-γ) and type III IFNs (IL-28A, IL-28B and IL-29) also contribute the broader family of IFNs. The relationship between the functions of IFNs and how they may affect the activity of one another is an active area of research, particularly as several downstream signalling components are shared between the type I, II and III IFN receptors (Trinchieri 2010).

1.9.1 Cellular sources and targets of type I IFN

Type I IFNs are produced by a diverse range of cells within the body, including most immune cell types (Trinchieri 2010). All type I IFNs signal through a ubiquitously expressed common receptor, the type I IFN receptor, which is composed of two subunits, IFNAR1 and IFNAR2 (Platanias 2005). Despite the use of this shared receptor,

it is known that the various type I IFNs can mediate different biological effects on their target cells (van Boxel-Dezaire *et al.* 2006). How type I IFNs mediate distinct biological effects through the same receptor is still unclear, although a recent study has suggested a role for the relative stability of the type I IFN ligand/receptor complexes (Thomas *et al.* 2011). Downstream of the type I IFN receptor, several different signalling cascades are induced (Platanias 2005). The best described of these is the JAK/STAT pathway. The tyrosine kinases TYK2 and JAK1 are associated with IFNAR1 and IFNAR2 chains, respectively, and become activated upon ligand binding. These kinases then phosphorylate and activate STAT1 and STAT2, which interact with IRF9 to form a complex known as ISGF3 (Stark *et al.* 1998; Decker *et al.* 2005). This complex translocates to the nucleus of the cell where it binds DNA at IFN-stimulated response elements (ISRE), and regulates gene expression. Signalling through the type I IFN receptor also induces the formation of STAT1/STAT1 homodimers which bind at elements known as gamma activated sites (GAS) within DNA to regulate gene expression (Stark *et al.* 1998; Decker *et al.* 2005). This latter pathway also mediates signalling downstream of the IFN-γ receptor (Shuai *et al.* 1992). Other STATs, such as STAT3 and STAT5, can additionally be activated downstream of the type I IFN receptor (Platanias 2005). In addition to JAK/STAT signalling, MAP kinases are activated downstream of the type I IFN receptor including p38 (Uddin *et al.* 1999) (Li *et al.* 2004), and ERK2 (David *et al.* 1995), although the latter has been less well documented. The PI(3)K pathway can additionally be activated by type I IFN (Uddin *et al.* 1995), and this has wide-ranging effects including the regulation of gene expression, mRNA translation and pro- and anti-apoptotic effects (Platanias 2005).

1.9.2 The regulation of immune responses by type I IFN

Ultimately, signalling via the type I IFN receptor induces the expression of interferon inducible genes (ISGs). Type I IFN was first characterised in the context of anti-viral immunity (Isaacs *et al.* 1957) and indeed, several ISGs such as *Irf1*, *Ifitm3* and *Oasl*, have been shown to inhibit viral replication (Schoggins *et al.* 2011). In contrast, several reports have implicated a detrimental role for type I IFN in mouse models of intracellular bacterial infections including *L.monocytogenes* (Auerbuch *et al.* 2004; Carrero *et al.* 2004; O'Connell *et al.* 2004) and *M.tuberculosis* (Manca *et al.* 2005). The mechanisms of detrimental type I IFN during intracellular bacterial infection in the mouse model are not fully defined, although type I IFN mediated inhibition of protective IL-1 in the context of *M.tuberculosis* has been reported (Mayer-Barber *et al.* 2011). In tuberculosis patients, a type I and type II IFN related gene expression signature in the blood has been shown to correlate with the radiological extent of disease (Berry *et al.* 2010) although the role of type I IFN in humans is unclear. In the setting of *L.monocytogenes*, type I IFN has been shown to promote lymphocyte apoptosis leading to a dampening of the innate immune response, at least in part due to the induction of IL-10 production by phagocytic cells (Carrero *et al.* 2006). It has also been shown that type I IFN can mitigate responsiveness to IFN-γ in *L.monocytogenes* infected macrophages (Rayamajhi *et al.* 2010). These findings may further help to explain the relative resistance of type I IFN receptor deficient mice to this bacterium.

Aside from infectious diseases, type I IFN has been implicated in the pathogenesis of autoimmune diseases such as SLE where ISGs are overexpressed in active disease (Bennett *et al.* 2003), and type I IFN is considered to contribute to the breakdown of

peripheral tolerance (Banchereau *et al.* 2006). Conversely, type I IFN has been used as a therapy in the treatment of MS (Ann Marrie *et al.* 2006). Although the mechanism IFN therapy action is unclear, it is interesting that in patients treated with IFN-β, *Il10* mRNA levels are increased (Rudick *et al.* 1996) and as discussed earlier, IL-10 has been linked to the suppression of EAE, the animal model of MS (Bettelli *et al.* 1998; Samoilova *et al.* 1998). Type I IFN has relatively successfully been used in the treatment of some types of cancer e.g. hairy cell leukaemia and melanoma however, most likely owing to the diverse roles of type I IFN, this treatment can induce serious side-effects (Rizza *et al.* 2010).

1.9.3 The regulation of type I IFN production in macrophages and DCs

Innate cells including macrophages, mDCs and pDCs produce type I IFN in response to the recognition of microbial products by PRRs (Trinchieri 2010). In macrophages and mDCs, TLRs 3 and 4 induce type I IFN via the TRIF-dependent pathway (Yamamoto *et al.* 2003a). Downstream of this pathway, IRF3 phosphorylation and dimerization, in co-operation with NF-κB, induces expression of *Ifnb1* (encodes IFN-β) (Doyle *et al.* 2002). The *Ifnb1* gene itself contains consensus binding sites for IRFs and NF-κB transcription factors, in addition to AP-1 sites (Honda *et al.* 2005c), and there is evidence for co-operation between these factors in the induction of *Ifnb1* expression (Thanos *et al.* 1995). More recently, a cluster of κB binding sites was identified downstream of the *Ifnb1* gene that bind NF-κB complexes containing RelA, and are required for optimal *Ifnb1* expression downstream of LPS (Goh *et al.* 2010). The expression of *Ifna* genes depends on the induction of a positive feedback loop in which IFN-β signals through the type I IFN receptor and induces the expression of IRF7 which upon phosphorylation,

promotes the expression of *Ifna* genes (Marie *et al.* 1998). IFN-α4 may be an exception to this as it does not depend on STAT1 (and by proxy type I IFN signalling), for expression (Marie *et al.* 1998). In contrast, TLRs 7 and 9 induce the production of IFN-α and IFN-β by the activation of IRF7 through the MyD88 dependent pathway (Honda *et al.* 2005b) (Figure 1.1). TLR 7 and 9 mediated type I IFN production is best documented in pDCs which preferentially express these TLRs and IRF7, at least in humans, (Jarrossay *et al.* 2001; Kadowaki *et al.* 2001; Izaguirre *et al.* 2003) and may more efficiently mediate trafficking of ligands such as CpG to endosomal compartments, where IRF7 is activated (Honda *et al.* 2005a; Colonna 2007). In pDC, the PI(3)K/mTOR pathway contributes to the production of type I IFN via IRF7 activation downstream of TLR9 (Cao *et al.* 2008) (Figure 1.1). TLR9 has also been shown to induce type I IFN in mDC via MyD88 and IRF1 interactions (Schmitz *et al.* 2007) (Figure 1.1). Of note, although it is generally considered that TLR2 does not induce type I IFN (Toshchakov *et al.* 2002), viral ligands may be able to induce type I IFN through the activation of TLR2 in murine inflammatory monocytes (Barbalat *et al.* 2009). Other PRRs such as the RLR RIG-I, induce robust type I IFN production in response to viral nucleic acids through the activation of IRF3, IRF7, NF-κB and MAP kinases (Pichlmair *et al.* 2007). The NLRs NOD1 and NOD2 have also been shown to induce type I IFN in response to *Helicobacter pylori* (Watanabe *et al.* 2010) and *M.tuberculosis* infection (Pandey *et al.* 2009), respectively.

Similarly to other cytokines, negative regulatory mechanisms also exist which can attenuate the production of type I IFN. In TLR4 and TLR9 stimulated macrophages and mDCs, the TPL-2/ERK pathway has been shown to negatively regulate IFNβ

production (Kaiser *et al.* 2009; Yang *et al.* 2011a), and this may depend on the transcription factor c-Fos (Kaiser *et al.* 2009). In contrast however, this pathway promotes type I IFN production in pDC (Kaiser *et al.* 2009).

1.10 Using inbred strains of mice to study the immune response

The use of inbred mouse strains such as C57BL/6, BALB/c or 129, has underpinned many of the biological advances over the last century. Further, the development of gene targeting technology in the mouse (Evans *et al.* 1981), for which Mario Capecchi, Martin Evans and Oliver Smithies were awarded the 2007 Nobel Prize in Physiology or Medicine, revolutionised biological research by allowing investigators to genetically modify their mammalian model organism. The sequencing of the C57BL/6 mouse genome (Waterston *et al.* 2002) has been another major advance, by revealing a striking similarity between the human and mouse genomes and allowing investigators to experimentally link phenotypic traits to causative genetic components. Thus, the mouse model is often the organism of choice for immunologists.

1.10.1 The impact of genetic background: C57BL/6 and BALB/c mice

Arguably, the most commonly used mouse strain is C57BL/6. A large part of this is because the majority of genetically modified mice are made on a C57BL/6 background. This is because stable embryonic stem (ES) cells can most easily be derived from 129 mice (Ware *et al.* 2003), which share the MHC ($H\text{-}2^b$) haplotype with C57BL/6 mice. Due to their differing coat colours, the success of 129 ES cell injection into C57BL/6 blastocysts can be determined by chimerism in the pup coat colour (Seong *et al.* 2004).

Subsequent mutant mice are backcrossed onto the C57BL/6 genetic background which historically has been considered to have desirable breeding characteristics (Seong *et al.* 2004). This has meant that much immunological research is based on the C57BL/6 mouse. While useful in terms of being able to compare results across different studies, it is unclear if C57BL/6 mice are a good model of human disease (Seok *et al.* 2013), or if other strains may be more representative. Indeed, different mouse strains differ in their susceptibility to infection and have been frequently compared in an attempt to better understand mechanisms of susceptibility and resistance.

Two inbred mouse strains frequently used in comparative studies are C57BL/6 and BALB/c as these mice differ in their outcome of infection with several pathogens. For example, C57BL/6 mice are considered more resistant than BALB/c mice to *L.major* infection (Sacks *et al.* 2002). This has been previously correlated to a dominant Th1 response in C57BL/6 mice and a dominant Th2 response in BALB/c mice (Heinzel *et al.* 1989), although the mechanisms underlying this phenotype are complex with several genetic loci having been found to contribute to resistance (Sacks *et al.* 2002; Lipoldova *et al.* 2006). C57BL/6 mice are additionally more resistant than BALB/c mice to infection with *Listeria spps* (Mainou-Fowler *et al.* 1988) and *Mycobacterium avium* (Wakeham *et al.* 2000; Roque *et al.* 2007). BALB/c mice on the other hand have been shown to be more resistant than C57BL/6 mice to infections with *T.gondii* (Schluter *et al.* 1999) and *Helicobacter spps* (Mohammadi *et al.* 1996; Anderson *et al.* 2007). Although some immunological factors have been found to correlate with strain-dependent outcomes of infection, the mechanisms underlying these traits are complex and only partially understood. Differences in resistance and susceptibility are not only

in the context of infectious diseases however. In IL-10 deficient mice, which spontaneously develop enterocolitis dependent on the presence of gut flora (Sellon *et al.* 1998), mice on a C57BL/6 background were less susceptible to the onset of disease than mice on a BALB/c background (Berg *et al.* 1996). Similarly, in the *Tbet*$^{-/-}$*Rag*$^{-/-}$ mouse model of Ulcerative Colitis, mice on the C57BL/6 background are more resistant that those on the BALB/c background and this has been linked to the *Cdcs1* locus on chromosome 3 (Ermann *et al.* 2011). Thus, C57BL/6 and BALB/c mice have different immunological responses in a number of contexts and these differences, along with comparisons to other strains of mice, have been studied in order to better understand immunological processes.

1.10.2 *Burkholderia pseudomallei* as a model of C57BL/6 and BALB/c resistance and susceptibility.

An additional pathogen to which C57BL/6 and BALB/c mice have differing resistance and susceptibility to is *B.pseudomallei. B.pseudomallei* is an environmental Gram-negative bacterium and the causative agent of melioidosis (Wiersinga *et al.* 2006). Melioidosis is most prevalent in northern Australia and Southeast Asia, although cases of melioidosis are becoming more common in part due to the improvement of diagnostic techniques and increased global travel (Currie *et al.* 2008). Infection with *B.pseudomallei* can give rise to an array of clinical manifestations in humans. These include localised cutaneous disease after long-term asymptomatic infection (Ngauy *et al.* 2005), pneumonia, or bacteraemia which may progress to septic shock (Currie *et al.* 2010). Treatment of melioidosis requires prolonged antibiotic treatment with risk of

relapse at the end of treatment (Samuel *et al.* 2001) and the mortality rate is as high as 40% in northeast Thailand (Limmathurotsakul *et al.* 2010).

B.pseudomallei infection can be transmitted through soil or water and enters the host via the aerosol route or through skin abrasions (Wiersinga *et al.* 2006). As an intracellular bacterium, *B.pseudomallei* can invade and survive within host cells, including macrophages (Wiersinga *et al.* 2006). Several immunological responses have been shown to be activated by *B.pseudomallei* infection (Wiersinga *et al.* 2012). Studies investigating the innate recognition of *B.pseudomalli* in the murine system have shown that the bacterium can activate TLR2 and TLR4 *in vitro* (Wiersinga *et al.* 2007; West *et al.* 2008). *B.pseudomallei* mediated activation of TLR 2, 4 and 5 has also been shown in HEK293 cells expressing human and mouse TLRs (Wiersinga *et al.* 2007; Hii *et al.* 2008; West *et al.* 2008). Murine *in vivo* studies have suggested that the presence of TLR2 is detrimental to the host during *B.pseudomallei* infection with the 1026b strain, whereas TLR4 has no effect (Wiersinga *et al.* 2007). Importantly however, TLR activation of *B.pseudomallei* may depend on the bacterial strain. For example, LPS derived from the *B.pseudomallei* strain BP-1 strain has been reported to activate TLR4 (West *et al.* 2008) whereas LPS derived from the 1026b strain has been reported to activate TLR2 (Wiersinga *et al.* 2007). There is also evidence for the importance of TLRs in regulating immune responses to *B.pseudomallei* infection from human genetic studies as TLR4 and TLR5 variants have been associated with protection and disease (West *et al.* 2012; West *et al.* 2013). Additional studies have shown a critical role for MyD88 but not TRIF in protective immune responses to *B.pseudomallei* infection with the 1026b strain. This was correlated to a reduction in neutrophil influx into the lung of

intra-nasally infected *Myd88*$^{-/-}$ mice compared to WT (Wiersinga *et al.* 2008). Thus, the role of TLRs in *B.pseudomallei* infection is being elucidated. *B.pseudomallei* is further recognised by the NLRs NLRC4 and NLRP3, important for the production of IL-1 and IL-18 (Miao *et al.* 2010; Ceballos-Olvera *et al.* 2011). Cytokines that have been shown to be protective during *B.pseudomallei* infection include IL-12, IL-18 and IFN-γ as IFN-γ deficient, IL-12p35 deficient, and IL-18 deficient mice all rapidly succumb to infection (Haque *et al.* 2006; Ceballos-Olvera *et al.* 2011). Innate production of IFN-γ, driven by IL-12 and IL-18, appears to be important in the early containment of infection, however antigen specific $CD4^+$ T cells are ultimately required to control infection at later stages, with a less important role for B cells (Haque *et al.* 2006). Conversely, IL-1 may have a detrimental role during infection as IL-1 treated mice are more susceptible, and IL-1R deficient mice have enhanced resistance (Ceballos-Olvera *et al.* 2011).

Although progress has been made in understanding correlates of protection in response to this pathogen, the mechanisms mediating differential resistance in human populations is unknown. As the differing resistance of C57BL/6 and BALB/c mice has been suggested to represent the spectrum of disease caused by *B.pseudomallei* in humans (Leakey *et al.* 1998), C57BL/6 and BALB/c mice have been studied to gain insight into the factors which may contribute to differing outcomes of infection. An early comparative study showed that C57BL/6 mice are more resistant to *B.pseudomallei* than BALB/c mice (Leakey *et al.* 1998). BALB/c mice develop acute disease correlating with high bacteraemia and death within 96 hours (Leakey *et al.* 1998). C57BL/6 mice remain asymptomatic before developing chronic disease, although still succumb to infection 2-6 weeks post infection (Leakey *et al.* 1998). The difference in strain

resistance is most clear when mice are infected by the intravenous or intra-peritoneal route, although it can still be seen by the intranasal route (Tan *et al.* 2008; Titball *et al.* 2008). Differential resistance in C57BL/6 and BALB/c mice has been observed with several strains of *B.pseudomallei* including KHW, EB6103, K96243 and 576 (Tan *et al.* 2008; Titball *et al.* 2008).

Investigations into mechanisms underlying the differential resistance of C57BL/6 and BALB/c mice to *B.pseudomallei* have shown that C57BL/6 mice are able to better control infection than BALB/c mice within the first day, implicating a role for innate mechanisms in mediating differential resistance (Hoppe *et al.* 1999). In addition, an enhanced ability of C57BL/6 peritoneal exudate cells and BMDM to control bacterial replication compared to BALB/c has been reported (Leakey *et al.* 1998; Breitbach *et al.* 2006). Given that an essential role for macrophages in protection against disease has been shown in both C57BL/6 and BALB/c mice (Breitbach *et al.* 2006), this may imply that genetic differences at the level of the phagocyte contribute to resistance or susceptibility. However, a relatively higher induction of proinflammatory cytokines in BALB/c mice has also been correlated to the enhanced susceptibility of this strain (Ulett *et al.* 2000a; Ulett *et al.* 2000b; Liu *et al.* 2002; Tan *et al.* 2008). This suggests a potential contribution of immunopathology to the onset of acute disease in BALB/c mice. Thus, the mechanisms mediating the differential resistance of C57BL/6 and BALB/c mice to *B.pseudomallei* infection are still incompletely understood, although pathogen control and immunopathology may both contribute to the ultimate outcome of infection.

1.11 Project perspective: Using C57BL/6 and BALB/c mice to study the regulation of IL-10 and IL-12

Preliminary data from the O'Garra laboratory showed that in response stimulation with certain TLR ligands, macrophages from C57BL/6 mice produce high levels of IL-10, whereas macrophages from BALB/c mice produce low levels of IL-10. In addition, it was suggested that IL-12 production may be higher from BALB/c macrophages than from C57BL/6 macrophages. IL-10 and IL-12 are critical cytokines in regulating immune responses. We therefore expanded on these preliminary findings, using differential IL-10 and IL-12 production in C57BL/6 and BALB/c macrophages as a model to dissect the molecular mechanisms underlying the regulation of these cytokines. After a broad assessment of cytokine production in C57BL/6 and BALB/c macrophages stimulated with a range of TLR ligands, non-TLR PRR ligands and heat-killed bacteria, we focussed our studies on investigating differential cytokine production in response to LPS (TLR4), Pam3CSK4 (TLR2) and heat-killed *B.pseudomallei* (TLR2/4). Thus, in addition to investigating the mechanisms of IL-10 and IL-12 regulation, this study highlights core differences between the innate immune responses of C57BL/6 and BALB/c mice which could potentially contribute to the differential resistance of these mice to infection with *B.pseudomallei*, or other pathogens.

TLR (homo/hetero-dimer)	Location of expression	Ligand	Functional in human?	Functional in mouse?
TLR1:2	Surface	-Triacyl lipopeptides (bacterial cell wall) -Pam3CSK4 (synthetic)	Yes	Yes
TLR2:6 (CD36)	Surface	-Diacyl lipopeptides (bacterial cell wall)	Yes	Yes
TLR3	Endosome	-ssRNA/dsRNA (virus) -Poly I:C (synthetic)	Yes	Yes
TLR4 (LBP/CD14/ MD2)	Surface	- LPS (Gram-ve bacterial cell wall component)	Yes	Yes
TLR5	Surface	-Flagellin (flagellated bacteria)	Yes	Yes
TLR7	Endosome	-ssRNA (virus) -R848 (synthetic)	Yes	Yes
TLR8	Endosome	-ssRNA (virus) -R848 (synthetic)	Yes	No
TLR9	Endosome	-dsDNA (virus) -CpG DNA motif (bacteria) -hemozoin (*Plasmodium*)	Yes	Yes
TLR10	Endosome	Unknown	Yes	No
TLR11	Surface	-Profilin (*T.gondii*) -Uropathogenic bacteria	No	Yes
TLR12	(Surface)	-Profilin (*T.gondii*)	No	Yes
TLR13	Endosome	Unknown	No	Yes

Table 1.1 The TLR family in mouse and man.

TLR4 also interacts with LPS binding protein (LBP) which aids LPS binding to CD14. CD14 transfers LPS to TLR4 which forms a complex with myeloid differentiation factor 2 (MD2) to initiate signal transduction (Miyake 2006). CD36 acts as an accessory receptor for TLR2:6 (Miyake 2006). Adapted from (Kumar *et al.* 2011) with additional information from (Blasius *et al.* 2010) and (Koblansky *et al.* 2013).

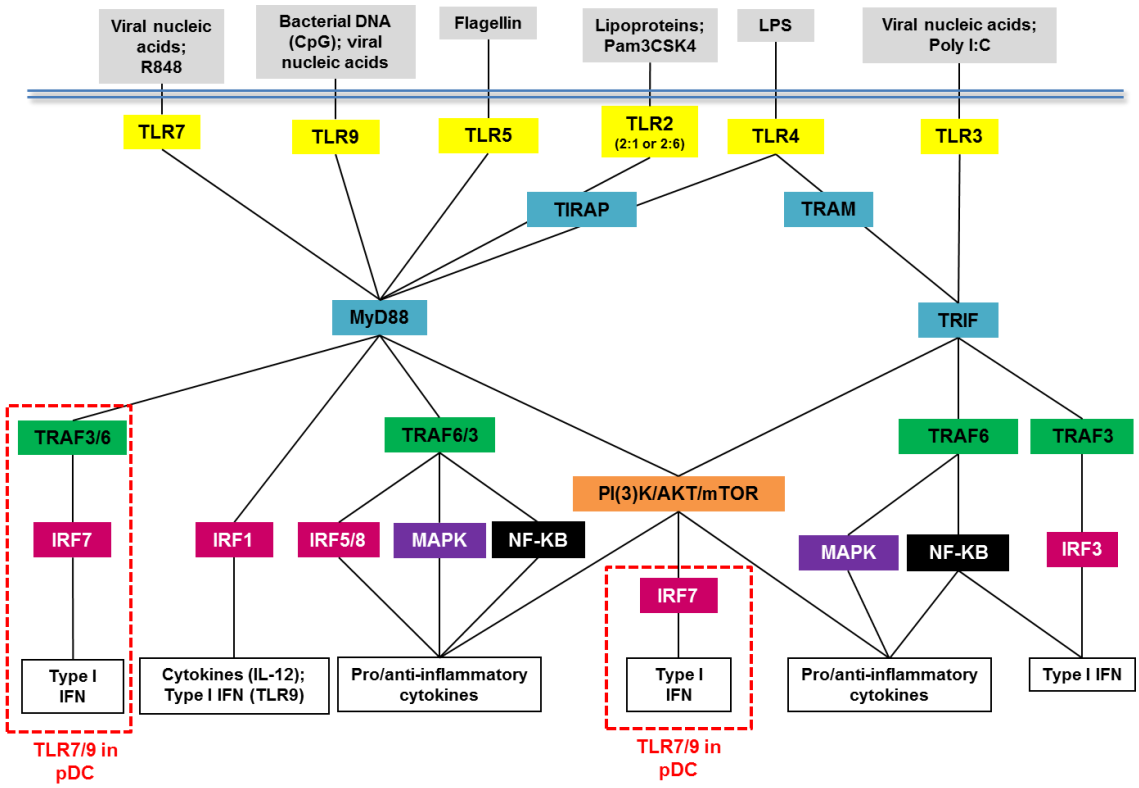


Figure 1.1 Summary of TLR signalling in macrophages and DCs.

Examples of TLR ligands are shown above each TLR in grey. Note the differential use of adaptors (dark blue) by each TLR and how each adaptor can activate overlapping and distinct pathways. pDC-specific pathways are highlighted in red. Adapted from (Kawai *et al.* 2010).

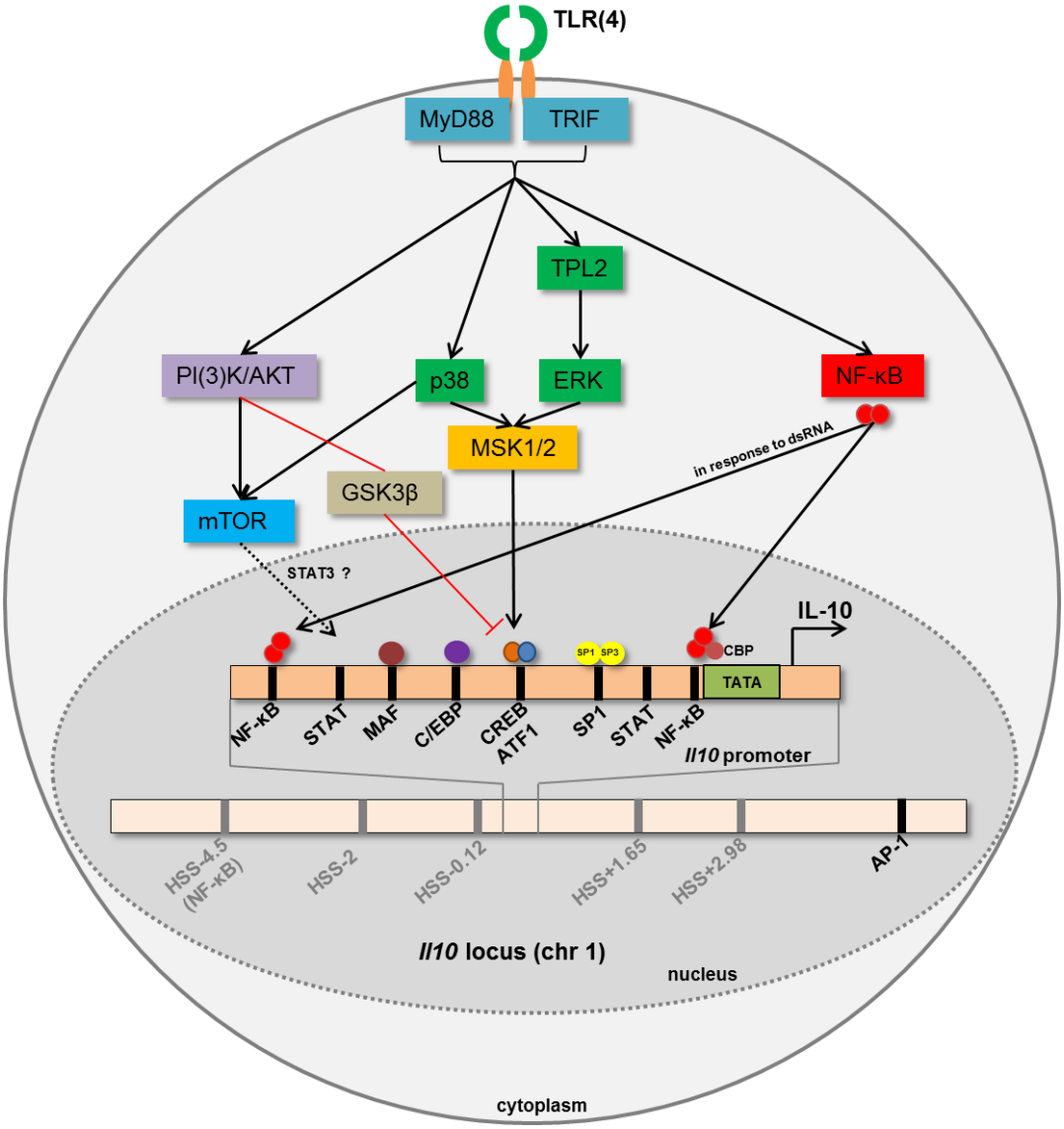


Figure 1.2 The induction of IL-10 by TLRs in macrophages, monocytes and DCs.
TLR4 signalling, given as an example here, depends on both MyD88 and TRIF adaptor proteins which collectively lead to the activation of the NF-κB pathway, MAP kinases (ERK, p38), the PI(3)K/AKT/mTOR pathway and IRFs (not shown here). Both MyD88 and TRIF are required for optimal IL-10 production. These signalling pathways lead to chromatin remodelling and the activation of transcription factors which bind at the *Il10* locus. DNase hypersensitivity sites (HSS) have been shown within the *Il10* locus of IL-10 producing APCs. HSS-4.5 contains an NF-κB binding site but the roles of the other HSS sites are unknown. A TATA box and several transcription factor binding motifs are present in the *Il10* promoter (denoted by black lines). Direct transcription factor binding has not yet been shown for all of them in APCs. If known, links between upstream signalling pathways and transcription factors are shown. Adapted from (Mosser *et al.* 2008b; Saraiva *et al.* 2010).

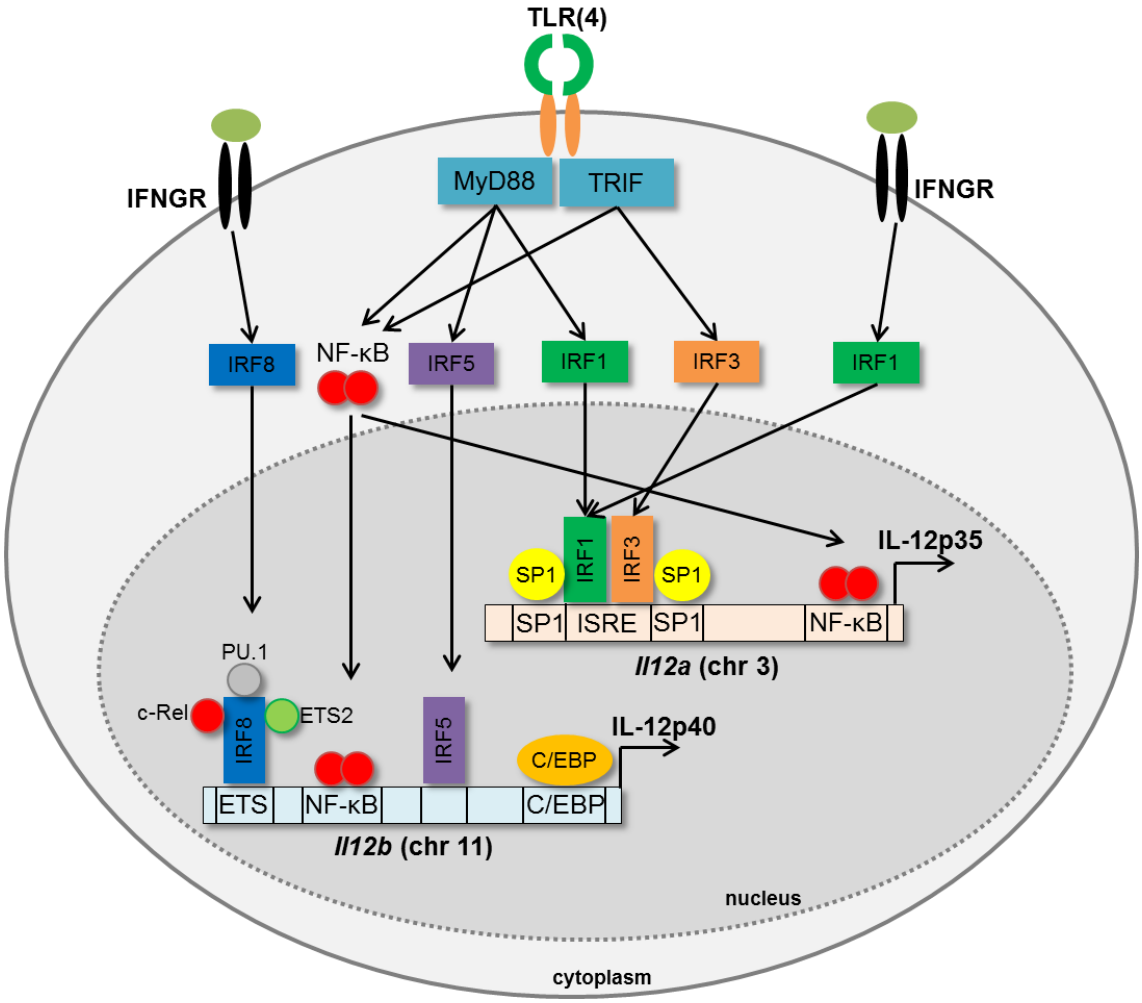


Figure 1.3 The induction of IL-12 by TLRs (and IFN-γ) in macrophages and DCs.
TLR4 signalling is shown here as an example as the majority of studies are conducted with this stimulus. Both MyD88 and TRIF contribute to IL-12 production in this context. In addition to the regulatory pathways shown, IRF5 may have a role in the induction of *Il12a*, and IRF8 may synergise with IRF1 for the induction of *Il12a*. Adapted from (Goriely *et al.* 2008).

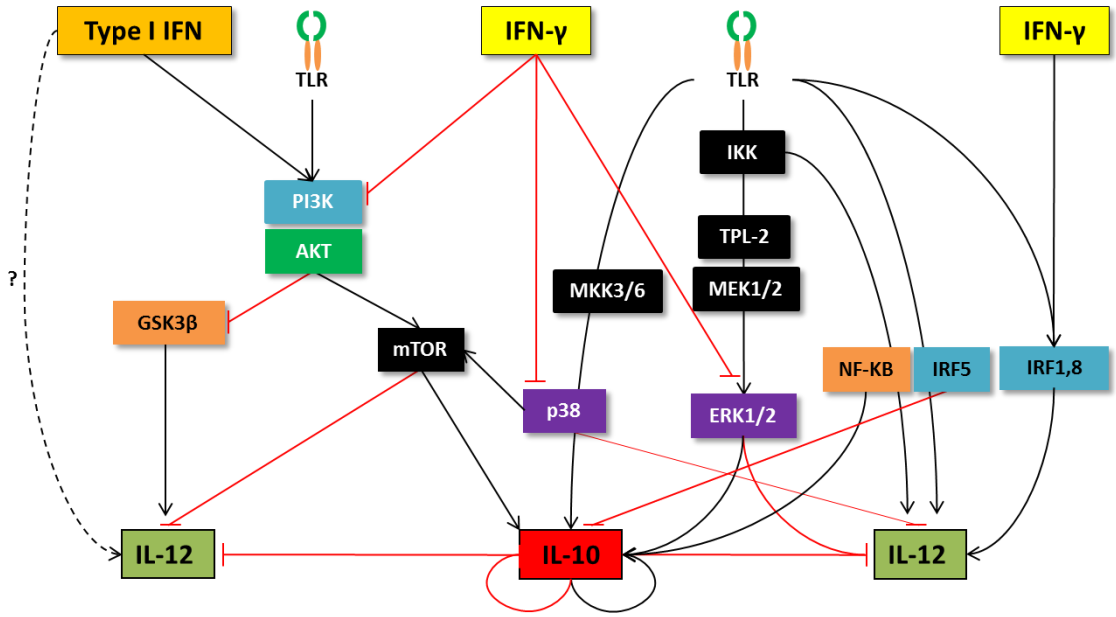


Figure 1.4 The co-ordinate regulation of IL-10 and IL-12 in macrophages and DCs. Downstream of TLR stimulation, the MAP kinases p38 and ERK positively regulate IL-10. NF-κB and the PI(3)K/AKT pathway also positively regulate IL-10. IL-12 is positively regulated by NF-κB and IRFs, of which IRF5 has also been shown to negatively regulate IL-10. IL-10 is a central inhibitor of IL-12. ERK, p38 and mTOR have all been shown to inhibit IL-12 dependent and independently of IL-10. These regulatory pathways can further be manipulated by the activity of type I IFN and IFN-γ. IFN-γ positively regulates IL-12 through the activation of IRFs, whereas IL-10 is negatively regulated through the inhibition of ERK, p38 and PI(3)K/AKT. Type I IFN positively regulates IL-10 and in DCs, this has been shown to be through the activation of PI(3)K/AKT pathway. It is not clear if there are other mechanisms of inducing IL-10 downstream of type I IFN, or if this mechanism is applicable in macrophages. Type I IFN can positively or negatively regulate IL-12, dependent on the cellular context.

Chapter 2. Materials and Methods

2.1 Mice

The following strains of mice were bred and maintained at the MRC National Institute for Medical Research (NIMR) under specific pathogen-free conditions in accordance with the Home Office, UK, Animal Scientific Procedures Act, 1986: C57BL/6 WT; BALB/c WT; 129S8 WT; C57BL/6 *Il10*$^{-/-}$; BALB/c *Il10*$^{-/-}$; C57BL/6 *Rag1*$^{-/-}$Mom; BALB/c *Rag2*$^{-/-}$. To generate F1 mice, C57BL/6 female mice were crossed to BALB/c male mice. The progeny of this breeding was intercrossed to generate C57BL/6 x BALB/c F2 mice. *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ breeding pairs, all on a C57BL/6 background, were provided by S. Akira (Osaka University, Osaka, Japan), C57BL/6 *Ifnar1*$^{-/-}$ breeders originated from B&K (Grimston, England), and C57BL/6 *Tccr*$^{-/-}$ (referred to as *Il27ra*$^{-/-}$ in text) breeders were provided by Genentech (San Francisco, USA). These mice were also bred and maintained at NIMR. 129S6SvEv-*Stat1*^{tm1Rds}, which have a disrupted *Stat1* gene, and 129S6SvEv control mice were purchased from Taconic (Taconic Farms Inc.). C57BL/6J and BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbour, USA). All mice used for *in vitro* experiments were female, between 8-16 weeks of age. All mice used for *in vivo* experiments were female, between 2-4 months of age and were age matched within experimental groups.

2.2 Reagents

2.2.1 Cell culture medium

cRPMI culture medium (RPMI 1640; 5% heat-inactivated FCS; 0.05 mM 2-Mercaptoethanol (Sigma); 10 mM HEPES buffer; 100 U/ml penicillin; 100 µg/ml

streptomycin; 2 mM L-glutamine; 1 mM sodium pyruvate) was used for all *in vitro* experiments. Unless stated, all components were purchased from BioWhittaker.

2.2.2 PRR ligands and heat-killed bacteria

Cells were treated with either cRPMI culture medium (control), *Salmonella Minnesota* LPS (Alexis), Pam3CSK4 (InvivoGen), heat-killed *B.pseudomallei* 576, heat-killed *B.pseudomallei* K9 (both from DSTL, Porton Down), heat-killed *Listeria monocytogenes* (provided by DNAX), CpG1668 (TriLink Biotech), R848 (InvivoGen), Poly I:C (InvivoGen) or Curdlan (WAKO). Dose and duration of stimulation are indicated in figure legends.

2.2.3 Recombinant cytokines and monoclonal antibodies

When indicated, cells were treated with recombinant murine IFN-β (PBL), IFN-γ (R&D) or IL-27 (R&D). Anti-IL-10 receptor (αIL-10R) monoclonal blocking antibody (1B1.3a, rat IgG1) and isotype control (GL113, rat IgG1) (O'Farrell *et al.* 1998) were gifts from DNAX (now Merck, USA) and were grown and purified by Harlan Laboratories (USA). Details of treatment are described in the relevant figure legends.

2.2.4 Inhibitors

PD184352 (MEK1 inhibitor) and SB203580 (p38 inhibitor) (Bain *et al.* 2007) were kind gifts from Sir Philip Cohen (University of Dundee). PD184352 was used at a final concentration of 1 μM and SB203580 was used at a final concentration of 2.5 μM.

Inhibitor stocks were diluted in DMSO, and DMSO vehicle controls are included in experiments. These doses were based on recommendations by Bain *et al.* for maximal efficiency with minimal off-target effects (Bain *et al.* 2007), and were confirmed by previous titrations in the O'Garra laboratory.

2.3 *In vitro* differentiation and stimulation of macrophages and DCs

2.3.1 Generation of bone marrow derived macrophages (BMDM)

Bone marrow (BM) was isolated from femurs and tibias by gentle flushing with cRPMI. BM cells were cultured (37^oC, 5% CO_2), in 90 mm petri dishes (Sterilin Ltd.) at $0.5x10^6$ cells/ml in cRPMI supplemented with 10% FCS and 20% L929-cell conditioned medium (LCCM), which contains M-CSF. LCCM was generated from the L929 cell line with the assistance of Jackie Wilson (Large Scale Laboratory Facility, NIMR). On day 4, cells were fed with 10 ml cRPMI, 10% FCS, 20% LCCM. On day 6, non-adherent cells were removed and adherent cells harvested by gentle flushing with cold PBS (GIBCO, Invitrogen). Cells were plated in flat bottomed 48-well tissue culture plates (Corning Inc.), 500 µl per well, at $1x10^6$ cells/ml (unless otherwise stated) in cRPMI. Cells were rested for 20 h, then stimulated by adding PRR ligands or heat-killed bacteria in a volume of 50 µl directly to rested cells. Pharmacological inhibitors or recombinant cytokines were added in a volume of 25 µl.

2.3.2 Generation of bone marrow derived DCs (BMDC)

BM was isolated as for BMDM. Red blood cells (RBC) were lysed with 0.83% ammonium-chloride. BM cells were cultured (37^oC, 5% CO_2), in flat bottomed 6-well

plates (Corning Inc.) at $1x10^6$ cells/ml in cRPMI, supplemented with 10 ng/ml GM-CSF (Schering-Plough). On days 2 and 4, non-adherent cells were removed and GM-CSF supplemented medium replaced. On day 6, non-adherent cells were harvested and re-plated in cell-culture petri-dishes (Corning Inc.) at $0.5x10^6$ cells/ml in GM-CSF supplemented medium for 16 h after which non-adherent cells were again harvested and plated in flat bottomed 48-well tissue culture plates (Corning Inc.), 500 µl per well, at $1x10^6$ cells/ml and stimulated immediately.

2.4 Quantification of cytokine production by ELISA

Supernatants were collected from cell cultures and cytokine concentrations measured by enzyme-linked immunosorbent assay (ELISA). Maxisorp 96-well plates (Nunc, Thermo Scientific) were used for the assay. Commercially available kits were used according to the manufacturer's instructions to quantify the concentration of IFN-β (PBL), IL-12p70 (eBioscience), TNF-α (eBioscience), IL-27 (eBioscience), and IL-1β (R&D). Matched-pair sandwich ELISAs were used to measure IL-10 and IL-12p40 concentrations. The assay details are summarised in Table 2.1. ELISA plates were read on a Safire^2 microplate reader (Tecan). Standard curve calculations and cytokine concentrations were determined using Magellan software (Tecan).

Table 2.1 Assay details for ELISA

Cytokine	Standard and starting concentration	Coating antibody	Detection (biotinylated) antibody	HRP-streptavidin	Developing substrate	Detection limit
IL-10	Recombinant IL-10 (R&D) 10 ng/ml	JES5-2A5 5 µg/ml (DNAX)	SXC-1 0.25 µg/ml (BD)	1 µg/ml (Jackson Immuno-Research)	TMB	50 pg/ml
IL-12p40	Recombinant IL-12p40 (R&D) 50 ng/ml	C15.6.7 5 µg/ml (DNAX)	C17.8 0.5 µg/ml (DNAX)	1 µg/ml (Jackson Immuno-Research)	ABTS	50 pg/ml
IL-12p70	10 ng/ml	(kit)	(kit)	(kit)	TMB	20 pg/ml
IFN-β	1000 pg/ml	(kit)	(kit)	(kit)	TMB	20 pg/ml
TNF-α	10 ng/ml	(kit)	(kit)	(kit)	TMB	20 pg/ml
IL-1β	10 ng/ml	(kit)	(kit)	(kit)	TMB	20 pg/ml
IL-27	10 ng/ml	(kit)	(kit)	(kit)	TMB	20 pg/ml

2.5 Western blotting analysis of cellular proteins

2.5.1 Protein extraction and quantification

For BMDM, cells were harvested on day 6 and rested in cRPMI 1% FCS (to lower background signal noise due to serum responses) for 20 h. Cells were stimulated as indicated, after which medium was removed and cells lysed with RIPA buffer composed of 50 mM Tris HCl (Sigma), pH 8; 150 mM NaCl (Sigma); 2 mM EDTA (Sigma); 2 mM sodium pyrophosphate (Sigma); 50 mM sodium fluoride (Sigma); 0.1% SDS (BioRad); 1% NP-40 (Fluka); 0.5% deoxycholate acid (Sigma); 100 mM vanadate (Sigma); complete EDTA-free protease inhibitor cocktail (Roche). For splenocytes, spleens were harvested and homogenised through a 70 µM sieve, RBC lysed with

0.83% ammonium chloride, and plated in 6-well tissue culture plates at $5x10^6$ cells/well in cRPMI, 1% FCS. Cells were rested for 5 h before treatment with recombinant cytokines. At the indicated times, cells were washed with PBS and lysed with RIPA buffer. Whole cell lysates were centrifuged for 10 min at 4°C and supernatant was collected. Protein concentration was measured using a reducing agent compatible Bicinchoninic Acid (BCA) Protein Assay Kit according to the manufacturer's instructions (Thermo scientific).

2.5.2 Protein separation and visualisation

Protein samples were denatured in SDS sample buffer (5 min, 95°C), and resolved by electrophoresis on a 12.5% SDS-polyacrylamide gel. 5-35 µg protein was loaded onto each gel, according to the protein being assayed and always consistent between experimental groups. Protein was transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked in 5% non-fat dried milk (Marvel) or bovine serum albumin (BSA) (Sigma) diluted in 0.05% PBS/Tween for 1 h at room temperature (RT) with agitation. Membranes were probed at 4°C overnight with primary antibodies anti-phospho-ERK1/2(T185-Y187) (Invitrogen); anti-ERK1/2 (Invitrogen); anti-phospho-p38(T180-Y182) (Cell signalling); anti-p-38 (Cell signalling); anti-phospho-STAT1(Y701) (Cell signalling); anti-STAT1 (Cell Signalling) or anti-Actin (Calbiochem) diluted in 5% milk or BSA (0.05% PBS/Tween). All primary antibodies were used at a dilution of 1:1000 with the exception of anti-Actin which was used at 1:5000. Membranes were rinsed in 0.05% PBS/Tween 4 times for 15 min each and incubated with goat anti-Rabbit IgG HRP-conjugated (Southern Biotech) or anti-mouse IgM HRP-conjugated secondary antibody (Calbiochem) diluted in 5%

milk (0.05% PBS/Tween) for 1 h at RT with agitation. Both secondary antibodies were used at a dilution of 1:2000. Membranes were rinsed again in 0.05% PBS/Tween as before. To visualise protein, membranes were incubated with ECL western blotting substrate (Thermo Scientific) for 1 min at RT and exposed to X-ray film (Kodak).

2.6 RNA isolation and purification

Supernatant was removed from stimulated cells at the indicated time-points. Cells were washed once in pre-warmed PBS. Cells were lysed immediately with RLT buffer (Qiagen) and lysates stored at -80^{o}C. RNA was isolated according to the manufacturer's instructions using an RNeasy Mini kit (Qiagen) with an on-column DNase digestion step to remove contaminating DNA (RNase-Free DNase kit, Qiagen). Purified RNA concentration was determined with a Nanodrop spectrophotometer (Nanodrop1000, Thermo Scientific).

2.7 cDNA preparation and real-time quantitative PCR (qPCR) analysis

cDNA was synthesised from purified RNA using a High Capacity cRNA Reverse Transcription Kit (Applied Biosystems). The reaction mixture is summarised in Table 2.2. The following PCR protocol was used to convert RNA to cDNA (Veriti Thermo Cycler, Applied Biosystems): Step 1 - 10 min 25^{o}C; Step 2 - 2 h 37 ^{o}C; Step 3 - 5 min 85 ^{o}C. This was followed by an RNA degradation step in which cDNA was incubated with RNase H (final concentration 0.03 U/µl, Invitrogen) at 37^{o}C for 30 min. cDNA was then diluted to 5 ng/µl in Nuclease-free H_2O (Promega).

Table 2.2 Reaction mixture for cDNA synthesis

Reagent	Volume	Final concentration	Source
Cellular RNA	10 µl	n/a	n/a
Reverse transcriptase buffer	2 µl	n/a	Applied Biosystems
dNTPs	0.8 µl	4 mM	Applied Biosystems
Random Primers	2 µl	n/a	Applied Biosystems
Multiscribe reverse transcriptase	1 µl	2.5 U/µl	Applied Biosystems
RNAsin (Ribonuclease inhibitor)	0.5 µl	1 U/µl	Promega
Nuclease-free H_2O	3.7 µl	n/a	Promega

qPCR was conducted using the TaqMan Assay system (Applied Biosystems). Reaction mixtures, summarised in Table 2.3, were made up in 96-well plates (optical reaction plates, Applied Biosystems), including a no-cDNA template control and a water only control to ensure reagents were not contaminated. The primer-probes used are summarised in Table 2.4.

Table 2.3 qPCR reaction mixture (per well)

Reagent	Volume	Final concentration	Source
Primer-probe	0.5 µl	900 nM	Applied Biosystems
TaqMan Universal Master Mix	5 µl	n/a	Applied Biosystems
cDNA	4.5 µl	2.25 ng/µl	n/a

Table 2.4 TaqMan primer-probes for qPCR

Target Gene	Applied Biosystems code
Il10	Mm00439616_m1
Il12a	Mm00434165_m1
Ifnb1	Mm00439552_s1
Tlr2	Mm00442346_m1
Tlr3	Mm00446577_g1
Tlr4	Mm0045273_m1
Hprt1	Mm00446968_m1

The following PCR protocol was used for quantitative PCR reactions (7900HT; Applied Biosystems): Step 1 - 2 min 50^{o}C; Step 2 - 10 min 95^{o}C; Step 3 - 15 seconds 95^{o}C; Step 4 - 1 min 60^{o}C. Steps 3-4 were repeated 40 times. Fluorescence was detected at step 4. The gene expression value, expressed in relative units (RU), for each sample was determined and normalised to the house-keeping gene *Hprt1* using the delta Ct (ΔCt) calculation: $\Delta Ct_{gene} = 1.8^{(Ct_{Hprt1}-Ct_{gene})} \times 100{,}000$.

2.7.1 Primer Design for the quantification of premature *Il10* mRNA

To quantify premature (unspliced) *Il10* mRNA transcripts, TaqMan primer/probe pairs were designed so that the forward (sense) primer and TaqMan probe annealed within an exonic sequence (in this case exon 3 of the 5 *Il10* exons), and the reverse (antisense) primer annealed within an intronic sequence (in this case intron 3). Primers were designed using Primer Express 2.0 software and were custom made by Applied Biosystems. Primer sequences are summarised in Table 2.5.

Table 2.5 Premature *Il10* mRNA primer probe sequences

Forward Primer	5'-AGCATGGCCCAGAAATCAAG-3'
TaqMan Probe	5'-CTCAGGATGCGGCTGA-3'
Reverse Primer	5'-AGAACGCATCTGCTACTCACACA-3'

2.8 Microarray analysis

Cells were stimulated with 10 ng/ml LPS or media (control) at $1x10^6$ cells/ml in a 48 well plate (500 µl cells/well) for 0.5, 1, 3, 5 and 8 h. RNA was isolated and purified as for qPCR analysis.

2.8.1 Verification of RNA quality

The quality of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). All RNA samples had a RIN (RNA integrity number) of 10, meaning that RNA was not degraded and of high quality (Schroeder *et al.* 2006). All RNA quality analysis was done by Dr Christine Graham (Division of Immunoregulation, NIMR, London).

2.8.2 Preparation of RNA for microarray analysis

RNA preparation for microarray was carried out using the Illumina®TotalPrep-96 RNA Amplification kit. In summary, 300 ng of total RNA was converted to cDNA by reverse transcription, and then purified. The cDNA was then transcribed *in vitro* to synthesise biotinylated antisense, or complementary, RNA molecules (cRNA). The synthesised cRNA then underwent a further purification step. RNA concentration was determined

using a Nandrop1000 (Thermo Scientific). All RNA amplification was done by Dr Christine Graham (Division of Immunoregulation, NIMR, London).

2.8.3 Microarray processing

1500 ng cRNA was loaded onto 6 sample Illumina BeadChip Arrays (MouseWG-6 v2). BeadChips were then incubated for 16-20 h in hybridisation chambers to allow sample hybridisation. The following day, BeadChips were put through a series of wash steps, blocked, treated with streptavidin Cy-3 to 'stain', washed, allowed to dry, and stored away from the light until scanned. BeadChips were scanned by an Illumina iSCAN array scanner. Intensity values were generated and background signal subtracted using BeadStudio software (Illumina). Microarray processing was done with the assistance of Dr Harsha Jani (Division of Systems Biology, NIMR, London).

2.8.4 Microarray data analysis

2.8.4.1 *Pre-analysis data processing, normalisation, fold change and statistical analyses*

Further analysis of microarray data was done using GeneSpring GX version 12.1 (Agilent Technologies). All data shown was subjected to the following data processing and normalisation steps which are standardly done to remove non-biological technical variation between samples or array chips (Quackenbush 2002). A lower threshold of signal intensity was set to 10, meaning that all expression values below this were set to 10. The expression values were then log transformed (base 2) and scaled to the 75th percentile of all samples. Following this, the expression value of each gene was

normalised to the median expression of that gene across all samples. After normalisation, all gene probes were filtered on 'Flags', a parameter which indicates the relative quality of a sample. Flags can be called as 'Present', 'Marginal', or 'Absent'. We retained gene probes that had 'Present' flag calls to a confidence level of $p<0.01$ in 100% of the samples within any one experimental condition. Further fold change filters and statistical analyses of data were carried out as described in Chapter 5, Figure 5.3.

2.8.4.2 *Clustering analyses*

Two types of clustering were used in our analyses to group genes based on their expression patterns - hierarchical and *k*-means clustering. Pearson Uncentered distance metric with average linkage was used in all cases unless specified. Hierarchical clustering constructs a dendogram in which the genes are represented in a relationship 'tree', and allows visualisation of all clustered genes within one heat map (Do *et al.* 2008). *k*-means clustering segregates genes with similar patterns into a user defined number of clusters (Do *et al.* 2008) and allows the visualisation of isolated clusters. All clustering analysis was carried out using GeneSpring software.

2.8.4.3 *Gene Ontology analysis*

The Gene Ontology (GO) project has developed a defined terminology with which biological functions and processes are described, and has used these to annotate genes with their respective functions (Harris *et al.* 2004). GO analysis, conducted using GeneSpring software, thus determines if a group of genes is significantly associated with any biological functions or processes within the GO database ($p<0.05$).

2.8.4.4 *Pathway and upstream regulator analyses by IPA*

Pathway and upstream regulator analyses were conducted using Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). Ingenuity relies on information that is present within a manually curated database, the Ingenuity Knowledge Base (Calvano *et al.* 2005). Significant association of gene lists to a biological pathway was determined by Fisher's Exact Test, with Benjimini-Hochberg multiple testing correction ($p<0.01$). Association of a gene list to an upstream transcriptional regulator was determined by a significant overlap with the mechanistic network of the regulator ($p<0.05$), and a significant activation *z*-score. The activation *z*-score is calculated based on the direction of gene expression, and how that correlates with the known activity of the transcriptional regulator. A value of less than -2 denotes a transcriptional regulator is inhibited. A value of more than 2 denotes a transcriptional regulator is activated (Ingenuity® Systems, www.ingenuity.com). In this analysis, gene expression levels were entered in the context of C57BL/6 *vs*. BALB/c or *vice versa*. Thus, the *z*-scores denoted a relative activation level between C57BL/6 and BALB/c macrophages.

2.8.4.5 *Transcription factor binding site enrichment analysis by PSCAN*

The online resource PSCAN (Zambelli *et al.* 2009) was used to determine the presence of significant transcription factor binding site (TFBS) motif enrichment within selected gene lists. If present, duplicate transcripts were removed from gene lists to eliminate bias and genes were entered into PSCAN in their RefSeq identifier format. Of note, PSCAN did not recognise RefSeq identifiers which had only predicted sequences,

(denoted by an XM annotation as opposed to an NM annotation in RefSeq identifier). This accounted for 15% of the genes being analysed. TFBS enrichment was assessed within the sequence region -950 to +50 relative to the transcriptional start site, described in more detail in Chapter 5, section 5.3.7.2. Enrichment was considered significant if $p<0.05$, with Bonferroni multiple testing correction applied.

2.8.4.6 *Quality control analysis of microarray samples*

Prior to the identification of significantly differentially expressed genes in C57BL/6 and BALB/c macrophages, a quality control analysis was carried out to ensure the integrity of the data. All samples from the experiment were hierarchically clustered according to condition using GeneSpring software. This groups samples based on their similarity, and therefore technical replicates with an experiment should cluster together. In our data set, the 0 h and media treated groups clustered together, but within their respective mouse strains (Figure 2.1 A). This suggested that 0 h cells and media control cells had similar transcriptional profiles, but that these were different between C57BL/6 and BALB/c macrophages. Note that one outlier sample, BALB/c 1 h media replicate 3, was removed from the analysis at this stage as it did not cluster with the other unstimulated samples. All LPS stimulated samples grouped within their technical triplicates (Figure 2.1 A), confirming the reliability of the experiment. We also noted that the samples from early stimulation time-points (0.5 and 1 h) clustered closely to the unstimulated samples within their respective strains (Figure 2.1 A) suggesting that the impact of differential basal C57BL/6 and BALB/c gene expression differences overpowers the effect of LPS stimulation at this stage. The 3, 5 and 8 h LPS stimulated samples however clustered away from the rest, but still separated according to strain (Figure 2.1

A). A similar observation was made when looking at the data by principle component analysis (PCA) plot, a technique which reduces the variability in the data to a small number of dimensions, in this case 3 (Figure 2.1 B). This clearly showed that most of the variation in the data was attributable to LPS stimulation. In this format however, the distinction between C57BL/6 and BALB/c macrophages was less apparent (Figure 2.1 B). As a final quality control step, we plotted the normalised intensity of *Il10*, *Ifnb1* and *Il12a* expression over time to confirm that the profile was consistent with our previous qPCR results (Figure 2.1 C).

2.8.4.7 *Comparison of basal gene expression in C57BL/6 and BALB/c macrophages*

Our initial analysis of gene expression in C57BL/6 and BALB/c macrophages showed that several genes were differentially expressed between the strains prior to stimulation (see Figure 5.2). We conducted a more formal analysis of these basal differences which revealed that 797 genes were differentially expressed at baseline (Figure 2.2 A). GO terms associated with these genes included ‘Immune system process’, ‘Antigen binding’ and ‘MHC protein complex’ (Figure 2.2 B). These differentially expressed genes may be relevant to the differential immune responses of C57BL/6 and BALB/c macrophages and were therefore included in later analyses.

2.9 *In vivo* experiments

All *in vivo* experiments were conducted with the assistance of Evangelos Stavropoulos (Division of Immunoregulation, NIMR).

2.9.1 LPS administration

Mice were administered with 150 µg LPS diluted in 300 µl PBS via the intra-peritoneal (i.p.) route. Control mice were administered with 300 µl PBS. Mice were sacrificed at 1, 3 and 6 h.

2.9.2 Spleen processing and RNA extraction

Spleens were dissected, immediately homogenised in 2 ml TRI Reagent (Ambion) and frozen at -80^{o}C in 1 ml aliquots for later processing. Total splenic RNA was isolated by adding 100 µl 1-bromo-3-chloropropane to 1 ml of spleen homogenate, vortexing and incubating at RT for 5 min, followed by centrifugation. The aqueous phase containing the RNA was collected and 200 µl ethanol added. RNA was then purified using RiboPure™ Kit (Ambion), according to the manufacturer's instructions. RNA was prepared for qPCR analysis as explained in Section 2.7.

2.9.3 Serum processing and cytokine quantification

Blood was collected from mice and allowed to coagulate at RT for 5 h or overnight at 4^{o}C. Samples were then centrifuged at 13,000 rpm at RT for 20 min. Serum was separated and stored at -80^{o}C for later protein analysis. IL-10 concentration in serum was determined using a Mouse Cytokine/Chemokine Magnetic Bead Panel (Milliplex MAP kit, Merk Millipore) according to the manufacturer's instructions. Samples were analysed using a Luminex 200™ analyser (Luminex). The range of detection for IL-10 was 10-9900 pg/ml.

2.10 Statistical analysis

Graphpad Prism software 5 was used to analyse data by one-way ANOVA with Tukey's post-hoc test. Microsoft Excel was used to analyse data by two-tailed Student's *t*-test. Statistical tests performed for each experiment and significance values are indicated in the figure legends.

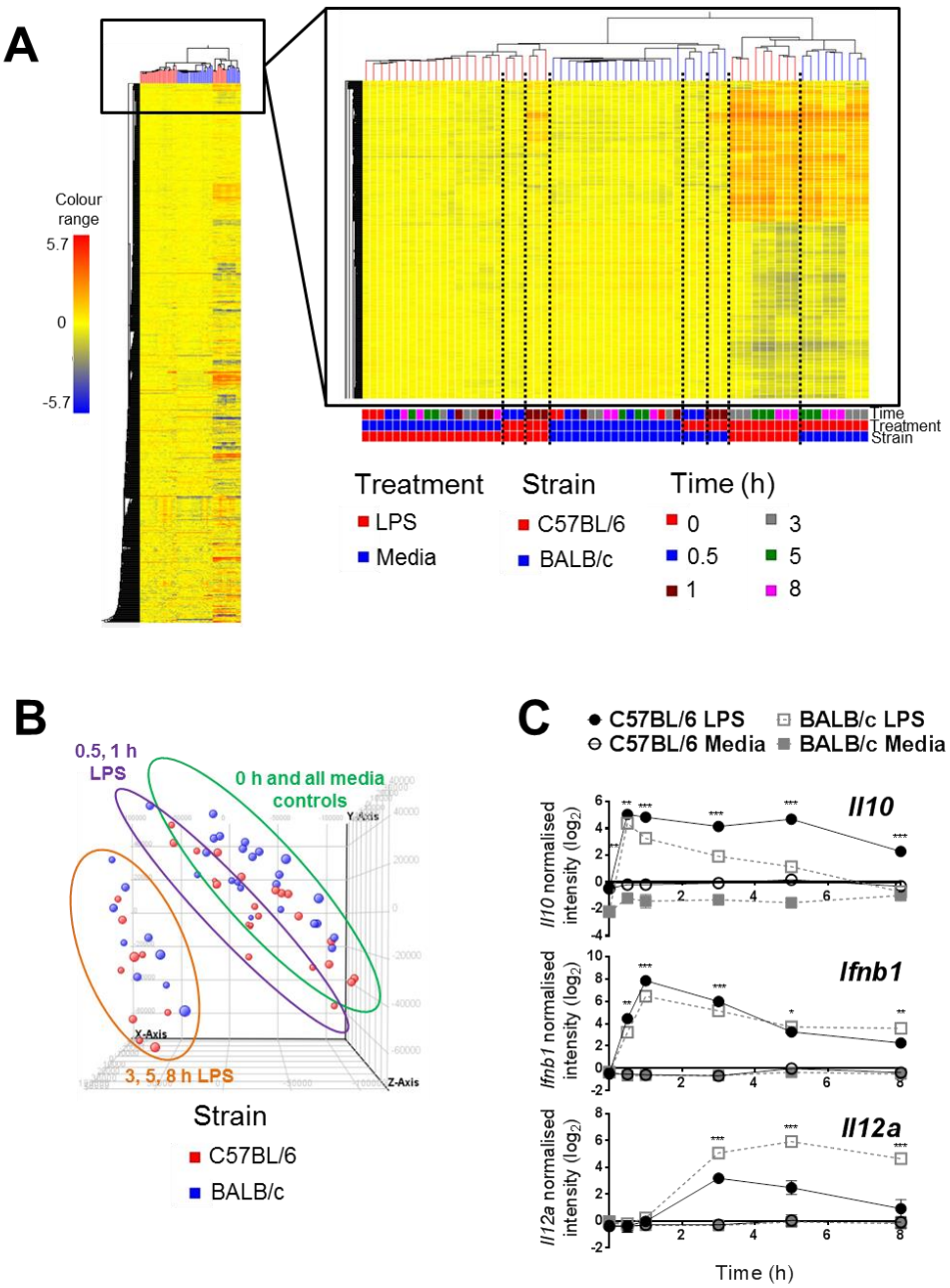


Figure 2.1 Quality control analysis of microarray samples.

(A) Data was normalised to the median of each gene and filtered on flags. Unsupervised hierarchical clustering on conditions (followed by entities) was carried out using the Differential distance metric with Wards linkage rule, recommended by GeneSpring for time series data. Colour range indicates normalised intensity. (B) PCA plot of samples analysed by microarray. (C) Plots of normalised intensity (log_2) of *Il10*, *Ifnb1* and *Il12a* mRNA expression as determined by microarray. Graphs show means of three samples ±SD. *p<0.05, **p<0.01, ***p<0.001 as determined by Student's *t*-test between C57BL/6 and BALB/c LPS stimulated samples only at each time point.

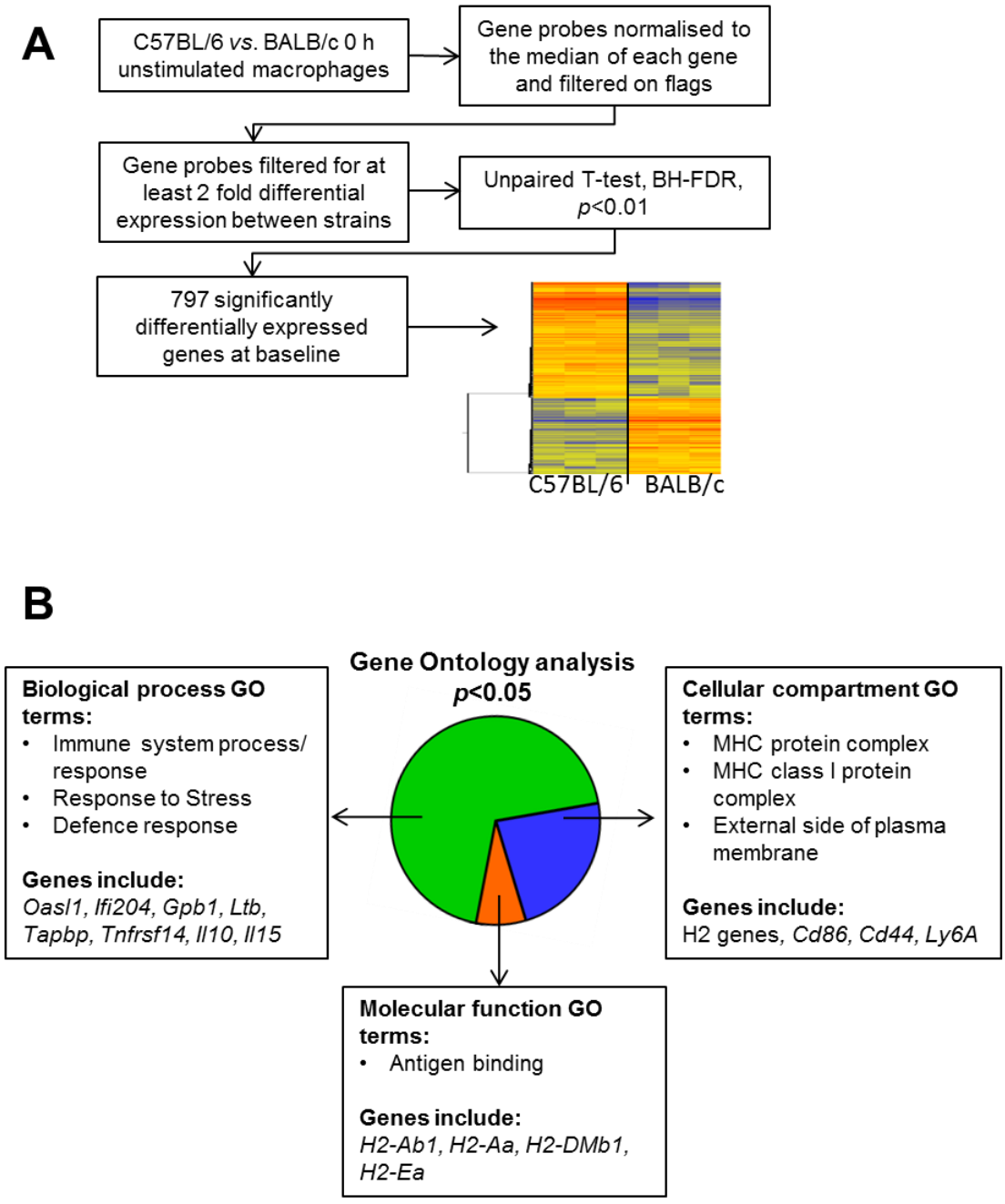


Figure 2.2 Genes differentially expressed at baseline in C57BL/6 and BALB/c macrophages associate with MHC protein and immune response GO terms.

(A) Analysis strategy of differential basal gene expression in unstimulated C57BL/6 and BALB/c macrophages. (B) GO analysis of differentially expressed genes in unstimulated C57BL/6 and BALB/c macrophages.

Chapter 3. The regulation of IL-10 in C57BL/6 and BALB/c macrophages

3.1 Background

Proinflammatory immune responses are critical in the defence against pathogens, however, excessive inflammation can cause immune-mediated damage to the host. IL-10 is an anti-inflammatory cytokine produced by almost all immune cell types, including macrophages and mDCs (Saraiva *et al.* 2010). IL-10 has an important role in dampening proinflammatory immune responses and is thought to act primarily at the level of the APC (Moore *et al.* 2001). The non-redundant role of IL-10 in the prevention of immune-mediated damage is illustrated by the finding that IL-10 deficient mice develop colitis in response to commensal gut flora (Kuhn *et al.* 1993; Sellon *et al.* 1998). However, the inappropriate production of IL-10 can limit the efficacy of immune responses and contribute to chronic infection (Belkaid *et al.* 2001; Ejrnaes *et al.* 2006; Redford *et al.* 2010). Hence, IL-10 is central in mediating the balance between immunopathology and chronic infection.

In macrophages and mDCs, the production of IL-10 is largely dependent on the stimulation of PRRs by microbial products (Saraiva *et al.* 2010). Of note, macrophages have been shown to be more potent producers of IL-10 than mDCs in response to TLR ligation (Boonstra *et al.* 2006; Kaiser *et al.* 2009), suggesting that not all cells have the same capacity to produce IL-10. Downstream of TLR ligation, the adaptor proteins MyD88 and/or TRIF initiate the activation of signalling pathways which regulate cytokine production (Kawai *et al.* 2010). The TPL-2/ERK pathway (Yi *et al.* 2002; Dillon *et al.* 2004; Banerjee *et al.* 2006; Kaiser *et al.* 2009), p38 MAPK (Yi *et al.* 2002; Jarnicki *et al.* 2008; Kim *et al.* 2008) and the PI(3)K/AKT/mTOR pathway (Ohtani *et al.* 2008; Weichhart *et al.* 2008) all have roles in the positive regulation of IL-10

downstream of TLR activation. Several transcription factors are associated with transactivation of the *Il10* gene in macrophages and mDCs such as NF-κB family members (Kanters *et al.* 2003; Saraiva *et al.* 2005; Banerjee *et al.* 2006; Cao *et al.* 2006), c-Fos (Agrawal *et al.* 2003; Dillon *et al.* 2004; Kaiser *et al.* 2009), CREB and ATF1 (Ananieva *et al.* 2008), c-Maf (Cao *et al.* 2005), C/EBP-β and δ (Brenner *et al.* 2003; Liu *et al.* 2003b; Csoka *et al.* 2007), SP1, SP3 (Brightbill *et al.* 2000; Tone *et al.* 2000) and STAT3 (Benkhart *et al.* 2000). In contrast, IRF5 has been identified as a negative regulator of IL-10 in human monocytes (Krausgruber *et al.* 2011). Stimulus induced chromatin modifications have also been reported in macrophages at the *Il10* locus and may contribute to *Il10* gene expression (Saraiva *et al.* 2005; Zhang *et al.* 2006). Post-transcriptional regulation of IL-10 can be mediated by the activity of microRNAs which can dampen or enhance IL-10 production, depending on their target and mode of action (Sharma *et al.* 2009; Ma *et al.* 2010; Sheedy *et al.* 2010). Additionally, the mRNA binding protein TTP post-transcriptionally inhibits IL-10 production by destabilising *Il10* mRNA (Stoecklin *et al.* 2008; Tudor *et al.* 2009).

In addition to direct TLR signals, autocrine and paracrine factors present in the extracellular environment further modulate IL-10 production. For example, IFN-γ dampens IL-10 production in macrophages through the inhibition of the PI(3)K pathway and MAP kinases (Hu *et al.* 2006). Conversely, type I IFN can enhance IL-10 production in murine macrophages and human monocytes (Aman *et al.* 1996; Chang *et al.* 2007a; Teles *et al.* 2013). In murine macrophages, this has been proposed to require IL-27 (Iyer *et al.* 2010). However, the role of IL-27 in the regulation of innate IL-10 production is unclear as another study found that murine macrophages did not respond

to IL-27 and in human monocytes, IL-27 actually inhibited IL-10 production (Kalliolias *et al.* 2008).

Thus, the regulation of IL-10 is determined by the integration of several molecular pathways. Further, due to the combinatorial nature of IL-10 regulation, the precise mechanisms governing the IL-10 production in response to different TLR ligands or more complex stimuli such as an intact bacterium, remain incompletely understood.

3.2 Investigating the regulation of IL-10 in C57BL/6 and BALB/c macrophages: Study Aims

Preliminary data from our laboratory suggested that C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages in response to TLR stimulation. With the intention of using this strain difference as a tool to investigate the molecular mechanisms of IL-10 regulation, we aimed to answer the following questions:

1. In response to which TLR and non-TLR PRR stimuli do C57BL/6 and BALB/c macrophages differentially regulate IL-10, and can this be recapitulated with heat-killed bacteria?
2. What is the mechanism of differential IL-10 production in C57BL/6 and BALB/c macrophages?
3. Can differential IL-10 production in C57BL/6 and BALB/c macrophages be translated into an *in vivo* setting?

3.3 Results

3.3.1 Investigation into TLR ligand and heat-killed bacteria induced IL-10 production in C57BL/6 and BALB/c macrophages

3.3.1.1 *C57BL/6 and BALB/c macrophages produce distinct levels of IL-10 when stimulated with TLR ligands or heat-killed bacteria*

We chose to stimulate C57BL/6 and BALB/c macrophages with ligands for TLRs 2, 3 and 4 as each has been previously reported to induce IL-10 from macrophages (Boonstra *et al.* 2006; Hu *et al.* 2006). In addition, this choice of TLR encompassed cell surface expressed TLRs (TLR 2 and 4) and an endosomally expressed TLR (TLR 3) (Akira *et al.* 2004). C57BL/6 and BALB/c BMDM were stimulated for 24 h with titrated concentrations of the TLR ligands LPS (TLR4), Pam3CSK4 (TLR2) and Poly I:C (TLR3) (Figure 3.1 A). In all three conditions, as the stimulus dose increased, IL-10 production generally increased in both C57BL/6 and BALB/c macrophages. However at all doses of stimulus where IL-10 was substantially induced, IL-10 levels were higher in C57BL/6 macrophages (Figure 3.1 A).

We also investigated differential IL-10 production from C57BL/6 and BALB/c macrophages in response to the TLR9 ligand CpG1668 which is also known to induce IL-10 in innate cells (Boonstra *et al.* 2006), and the TLR7 ligand R848 which is less well studied in the context of IL-10 regulation. Although IL-10 production was robustly induced in response to both stimuli, we found that the difference in relative IL-10 production from C57BL/6 and BALB/c macrophages was not consistent between experiments (Appendix Figure 7.1). We were unable to identify the source of this inter-

experiment variation, and it could not be explained by the batch of CpG1668 or R848, but possibly could be due to changes in batches of media or FCS. It was thus decided to discontinue the use of these TLR ligands in this study. C57BL/6 and BALB/c macrophages were also stimulated with the non-TLR PRR ligands MDP (NOD2 ligand) and Curdlan (Dectin-1 ligand). Neither of these ligands induced detectable levels of IL-10 in either C57BL/6 or BALB/c macrophages (data not shown), in agreement with published findings (Franchi *et al.* 2009; Goodridge *et al.* 2009).

Purified PRR ligands stimulate only one receptor, whereas upon infection with a bacterium or virus, innate cells are presented with a host of PRR ligands that are incorporated into the microbe. Thus, to investigate if differential IL-10 production by C57BL/6 and BALB/c macrophages could be recapitulated with whole bacteria, we extended our study to analyse IL-10 production in response to titrated doses of heat-killed *B.pseudomallei* (HkBps) and heat-killed *L.monocytogenes* (HkLm). Both of these bacteria are intracellular pathogens, however *L.monocytogenes* is a Gram-positive bacterium (Hamon *et al.* 2006), whereas *B.pseudomallei* is a Gram-negative bacterium (Wiersinga *et al.* 2006). C57BL/6 and BALB/c macrophages were stimulated with two isolates of HkBps, 576 and K9 (Figure 3.1 B). IL-10 production was induced in C57BL/6 and BALB/c macrophages in response to both isolates of HkBps, and as seen with TLR ligands, C57BL/6 macrophages consistently produced more IL-10 than BALB/c macrophages (Figure 3.1 B). Similarly, in response to HkLm (Figure 3.1 C), IL-10 production was induced in both strains, but was significantly higher in C57BL/6 macrophages (Figure 3.1 C). These results confirmed that C57BL/6 and BALB/c

macrophages have a differential capacity to produce IL-10 in response to TLR ligands and heat-killed bacteria.

3.3.1.2 *TLR2, TLR4, MyD88 and TRIF contribute to HkBps induced IL-10 production*

As *B.pseudomallei* has been reported to activate both TLR2 and TLR4 (Wiersinga *et al.* 2007; Hii *et al.* 2008; West *et al.* 2008), we decided to focus our study on differential IL-10 production induced by LPS (TLR4), Pam3CSK4 (TLR2) and HkBps. This would allow us to investigate the mechanisms governing differential IL-10 production in response to a biologically relevant, combined TLR2 and TLR4 stimulus, and the individual TLR2 and TLR4 ligands themselves. Further, C57BL/6 mice are more resistant to *B.pseudomallei* infection than BALB/c mice, and this difference has been proposed to represent the spectrum of disease in humans (Leakey *et al.* 1998; Hoppe *et al.* 1999; Titball *et al.* 2008). However, the mechanisms underlying this differential susceptibility are currently incompletely understood. Including HkBps in this study therefore provided an opportunity to further explore strain-dependent immunological responses to *B.pseudomallei* which could potentially be relevant to broader mechanisms of disease with this pathogen.

We elected to use HkBps576, as this isolate induced relatively higher levels of IL-10 than HkBpsK9 (Figure 3.1B). However, previous studies examining TLR activation by *B.pseudomallei* have used BP-1 (West *et al.* 2008), KHW (Hii *et al.* 2008) and 1026b (Wiersinga *et al.* 2007) isolates. In addition, HkBps576 (from here on referred to as HkBps) has been reported to have an atypical LPS structure (Anuntagool *et al.* 2000).

We therefore sought to confirm the contribution of TLR2 and TLR4 to HkBps induced IL-10 production in our system using TLR deficient macrophages. We also assessed the dependence of HkBps induced IL-10 on the TLR adaptor proteins MyD88 and TRIF as the former is essential for TLR2 signalling, whereas both contribute to TLR4 signalling (Kawai *et al.* 2010). Macrophages were generated from WT, *Tlr4*$^{-/-}$, *Tlr2*$^{-/-}$, *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ mice (all on C57BL/6 background) and stimulated with LPS, Pam3CSK4 or HkBps over a time-course up to 24 h (Figure 3.2). LPS induced IL-10 production peaked at 6 h in WT macrophages, and as expected was abrogated in *Tlr4*$^{-/-}$, but not *Tlr2*$^{-/-}$ macrophages. LPS induced IL-10 was not detectable in the absence of MyD88 and was severely reduced in the absence of TRIF, only being detected at a low level at 24 h (Figure 3.2). This is in agreement with previous findings that both MyD88 and TRIF are required for IL-10 production downstream of TLR4 (Boonstra *et al.* 2006). Pam3CSK4 induced IL-10 increased up to 24 h and also as anticipated, was abrogated in the absence of TLR2 and MyD88, but not substantially affected in the absence of TRIF or TLR4 (Figure 3.2). HkBps induced IL-10 production peaked at 12-24 h in WT macrophages (Figure 3.2). In *Tlr2*$^{-/-}$ macrophages, IL-10 production was decreased at 6, 12 and 24 h relative to WT macrophages during HkBps stimulation. IL-10 production was also decreased in HkBps stimulated *Tlr4*$^{-/-}$ macrophages relative to WT, and this decrease was considerably more pronounced than in *Tlr2*$^{-/-}$ macrophages (Figure 3.2). Therefore both TLR2 and TLR4 contribute to HkBps induced IL-10, although TLR4 appears to play a more dominant role. *Trif*$^{-/-}$ macrophages stimulated with HkBps produced similarly reduced levels of IL-10 to *Tlr4*$^{-/-}$ macrophages (Figure 3.2), in keeping with a dominant role for TLR4, and stressing the importance of the TRIF pathway downstream of this receptor. No IL-10 was induced by HkBps in the absence

of MyD88 (Figure 3.2), suggesting that IL-10 production in response to HkBps is completely dependent on TLR signalling. These data confirm that in our system, TLR2 and TLR4 significantly contribute to the production of IL-10 in response to HkBps.

3.3.2 Investigation into the mechanisms of differential IL-10 production in LPS, Pam3CSK4 and HkBps stimulated C57BL/6 and BALB/c macrophages.

3.3.2.1 *C57BL/6 macrophages produce higher levels of IL-10 compared to BALB/c macrophages throughout the response to LPS, Pam3CSK4 and HkBps.*

To investigate differential production of IL-10 in response to LPS, Pam3CSK4 and HkBps in more detail, C57BL/6 and BALB/c macrophages were stimulated with optimal doses of these ligands over a time-course up to 24 h (Figure 3.3). C57BL/6 macrophages produced significantly higher levels of IL-10 than BALB/c macrophages throughout the response to all three stimuli (Figure 3.3). The deficiency in IL-10 production from BALB/c macrophages is therefore consistent across the duration of the response, and not a reflection of either delayed induction of IL-10, or premature termination of IL-10 production in macrophages from this strain.

3.3.2.2 *C57BL/6 mDCs do not produce higher levels of IL-10 than BALB/c mDCs when stimulated with LPS or Pam3CSK4.*

In addition to macrophages, DCs are an important component of the innate immune response. To determine if higher IL-10 production was also seen in C57BL/6 TLR2 or TLR4 activated mDCs, C57BL/6 and BALB/c BMDC were stimulated for 24 h with optimal doses of LPS or Pam3CSK4 (Figure 3.4 A). IL-10 production from mDCs was similar between the strains when stimulated with LPS (Figure 3.4 A). In response to

Pam3CSK4 however, IL-10 production was significantly higher in BALB/c mDCs compared to C57BL/6 mDCs (Figure 3.4 A). Thus, there is a difference in IL-10 production from C57BL/6 and BALB/c mDC, but the trends seen are distinct from those between macrophages derived from C57BL/6 and BALB/c mice. This implies that the mechanisms of TLR induced IL-10 regulation are not completely conserved in macrophages and mDCs. We also stimulated C57BL/6 and BALB/c mDCs with Curdlan, a ligand for the non-TLR PRR Dectin -1 (Figure 3.4 B). In keeping with previous reports (Rogers *et al.* 2005; Slack *et al.* 2007), IL-10 was robustly induced by Curdlan (Figure 3.4 B). However, IL-10 production was substantially higher in C57BL/6 mDCs than BALB/c mDCs in response to this stimulus (Figure 3.4 B), in keeping with our observations in TLR stimulated macrophages.

3.3.2.3 *The level of* Tlr *mRNA expression does not correlate with the level of IL-10 production in C57BL/6 and BALB/c macrophages.*

We have observed that C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages in response to LPS, Pam3CSK4 and HkBps across all doses of stimulus and all time points assayed (Figure 3.1 and 3.3). To determine if differential IL-10 production was simply a result of differential TLR expression between the strains, we investigated the expression level of *Tlr* mRNA in C57BL/6 and BALB/c macrophages. *Tlr2* mRNA expression was slightly lower in BALB/c macrophages compared to C57BL/6 (Figure 3.5 A). In contrast, there was no significant difference in *Tlr4* mRNA expression between the strains (Figure 3.5 A). As we also previously observed higher IL-10 production in TLR3 stimulated C57BL/6 macrophages (Figure 3.1 A), we additionally determined the relative expression of *Tlr3* mRNA and again

found no difference between the strains (Figure 3.5 B). Thus, there was no clear correlation between the level of *Tlr* mRNA expression and the level of IL-10 production in C57BL/6 and BALB/c macrophages. This suggests that *Tlr* expression does not govern the level of IL-10 production in macrophages from these two strains of mice.

3.3.2.4 *ERK1/2 and p38 are central regulators of IL-10, but are similarly activated in TLR2 and TLR4 stimulated C57BL/6 and BALB/c macrophages.*

Several pathways and molecules are known to regulate IL-10 production downstream of TLR2 and TLR4 (Saraiva *et al.* 2010). Among these, the MAP kinases ERK1/2 (referred to here as ERK) and p38 have been reported to be key regulators of TLR induced IL-10, as inhibition of ERK or p38 activation substantially reduces IL-10 production in C57BL/6 macrophages and mDC (Dillon *et al.* 2004; Banerjee *et al.* 2006; Jarnicki *et al.* 2008; Kim *et al.* 2008; Kaiser *et al.* 2009). To establish the importance of these factors in the regulation of IL-10 downstream of TLR2 and TLR4 in both C57BL/6 and BALB/c macrophages, we treated cells with PD184352, a pharmacological inhibitor of MEK1, the MAP 2-kinase which phosphorylates and activates ERK, and SB203580, a pharmacological inhibitor of p38 (Bain *et al.* 2007). Cells were pre-treated with individual inhibitors or combined inhibitors (or with DMSO as a vehicle control) for 1 hour. Cells were then stimulated with LPS or Pam3CSK4 for 24 h (Figure 3.6). DMSO did not affect IL-10 production in either C57BL/6 or BALB/c macrophages (Figure 3.6 A and B). In LPS stimulated macrophages, individual MEK1/ERK or p38 inhibition decreased IL-10 production in both strains (Figure 3.6 A). Of note, in C57BL/6 macrophages the requirement for ERK and p38 for IL-10 production was comparable, whereas in BALB/c macrophages the requirement for p38

seemed dominant over the requirement for ERK in response to LPS (Figure 3.6 A). Dual inhibition of ERK and p38 had an additive effect in LPS stimulated macrophages and resulted in very low levels of IL-10 production in C57BL/6 macrophages, and undetectable levels of IL-10 production in BALB/c macrophages (Figure 3.6 A). In Pam3CSK4 stimulated cells, individual MEK1/ERK or p38 inhibition reduced IL-10 production in both strains and in BALB/c macrophages, was sufficient to completely abrogate IL-10 production (Figure 3.6 B). In contrast to LPS stimulation, dual MEK1/ERK and p38 inhibition completely abolished IL-10 production in Pam3CSK4 stimulated C57BL/6 and BALB/c macrophages (Figure 3.6 B). These data confirm the central roles of ERK and p38 in C57BL/6 and BALB/c macrophages as regulators of IL-10 downstream of TLR2 and TLR4.

We then considered that differential activation of these MAP kinases may be responsible for the distinct levels of IL-10 produced by TLR2 and TLR4 stimulated C57BL/6 and BALB/c macrophages. To investigate this further, we assessed the phosphorylation (activation) of ERK and p38 in C57BL/6 and BALB/c macrophages at 0, 7.5, 15, 30 and 60 min post LPS or Pam3CSK4 stimulation (Figure 3.7). In LPS stimulated cells, ERK phosphorylation was clearly detectable from 7.5 min and peaked at 15 min, but was similar in C57BL/6 and BALB/c macrophages (Figure 3.7). In Pam3CSK4 stimulated cells, ERK phosphorylation had slower kinetics, peaking at 30 min post-stimulation but again was similar between the strains (Figure 3.7). p38 phosphorylation was detectable by 7.5 min in both LPS and Pam3CSK4 stimulated cells and was similar in C57BL/6 and BALB/c macrophages (Figure 3.7). Total levels of ERK and p38 were likewise consistent in C57BL/6 and BALB/c macrophages (Figure

3.7). These data suggest that low IL-10 production by TLR2 or TLR4 stimulated BALB/c macrophages is not due to differential activation of ERK or p38 within the first hour of stimulation.

3.3.2.5 *LPS, Pam3CSK4 and HkBps differentially induce* Il10 *transcription in C57BL/6 and BALB/c macrophages.*

In order to gain further insight into the mechanisms mediating differential IL-10 production in C57BL/6 and BALB/c macrophages, *Il10* mRNA levels were analysed over a detailed time-course in LPS, Pam3CSK4 and HkBps stimulated cells (Figure 3.8). *Il10* mRNA initially peaked at 0.5-1 h in LPS stimulated C57BL/6 macrophages and then declined. This was followed by a pronounced second peak of *Il10* mRNA at 4 h post LPS stimulation, from which point the *Il10* mRNA again declined (Figure 3.8). In clear contrast, LPS stimulated BALB/c macrophages only had one small *Il10* mRNA peak at 0.5-1 h post-stimulation and no second *Il10* mRNA peak (Figure 3.8). In Pam3CSK4 stimulated cells, *Il10* mRNA expression peaked at 0.5 h in both C57BL/6 and BALB/c macrophages and rapidly declined thereafter with no second *Il10* mRNA peak in either strain. Although the overall profiles of *Il10* mRNA expression were similar in Pam3CSK4 stimulated C57BL/6 and BALB/c macrophages, at each time point C57BL/6 macrophages expressed higher levels of *Il10* mRNA (Figure 3.8). In addition, whereas *Il10* mRNA in Pam3CSK4 stimulated BALB/c macrophages returned to baseline by 4 h, it appeared sustained above baseline in C57BL/6 macrophages up to 12 h (Figure 3.8). In HkBps stimulated cells, C57BL/6 macrophages expressed an initial *Il10* mRNA peak at 0.5 h followed by a second and more pronounced peak at 4 h. However, as was seen in LPS stimulation, HkBps stimulated BALB/c macrophages, had

only one smaller initial *Il10* mRNA peak at 0.5 h and no second *Il10* mRNA peak (Figure 3.8).

The differences in C57BL/6 and BALB/c *Il10* mRNA production observed (Figure 3.8) are likely to underlie the differential IL-10 protein production by these cells. The contrasting profiles of *Il10* mRNA expression observed in LPS and Pam3CSK4 stimulated macrophages however, suggest that distinct mechanisms underlie differential C57BL/6 and BALB/c IL-10 production in response to these stimuli. As TLR4 has a larger contribution to HkBps induced IL-10 than TLR2 (see Figure 3.2), and the profile of HkBps induced *Il10* mRNA is similar to that seen in LPS stimulated cells (Figure 3.8), it is possible that common or overlapping mechanism(s) mediate differential IL-10 production of C57BL/6 and BALB/c macrophages in response to LPS and HkBps.

3.3.2.6 *LPS and HkBps stimulated C57BL/6 macrophages express higher levels of* Ifnb1 *mRNA and IFN-β protein than BALB/c macrophages.*

Microarray data generated by another member of the O'Garra laboratory prior to this investigation, suggested that C57BL/6 macrophages may induce higher levels of type I IFN than BALB/c macrophages in response to LPS at early time-points (Wu, O'Garra, unpublished). We were interested in this observation as it has been reported previously that type I IFN positively regulates IL-10 in LPS stimulated macrophages (Chang *et al.* 2007a) and parallel work in the O'Garra laboratory showed that this was also the case in *M.tuberculosis* infected macrophages (Ewbank, McNab, O'Garra unpublished). In order to determine if there was differential production of type I IFN in LPS, Pam3CSK4 or HkBps stimulated C57BL/6 and BALB/c macrophages, the expression of *Ifnb1* mRNA

was determined by qPCR in these cells (Figure 3.9 A). Upon LPS stimulation, *Ifnb1* mRNA expression peaked at 1 h in both C57BL/6 and BALB/c macrophages. However, C57BL/6 macrophages expressed significantly higher levels of *Ifnb1* mRNA than BALB/c macrophages (Figure 3.9 A). No substantial increase in *Ifnb1* mRNA was seen in Pam3CSK4 stimulated C57BL/6 or BALB/c cells (Figure 3.9 A). This is in keeping with reports that Pam3CSK4 does not induce type I IFN due to its inability to activate the TRIF pathway (Toshchakov *et al.* 2002). In HkBps stimulated macrophages, *Ifnb1* mRNA was induced in both C57BL/6 and BALB/c macrophages, with maximal expression at 1 h. As with LPS stimulation, HkBps induced *Ifnb1* mRNA expression was significantly higher in C57BL/6 macrophages than BALB/c macrophages (Figure 3.9 A). IFN-β protein production was also quantified (Figure 3.9 B). Although the overall levels were quite low, the trends of IFN-β protein production largely followed that of *Ifnb1* mRNA. When detectable, IFN-β protein was higher in C57BL/6 macrophages compared to BALB/c macrophages stimulated with LPS or HkBps (Figure 3.9 B). IFN-β production from Pam3CSK4 stimulated cells was generally below the limit of detection and not consistently different between the strains (Figure 3.9 B).

3.3.2.7 *Type I IFN signalling mediates the second peak of* Il10 *mRNA and contributes to higher levels of IL-10 protein production in LPS and HkBps stimulated C57BL/6 macrophages.*

We observed that LPS and HkBps stimulated C57BL/6 macrophages produce more IL-10 than BALB/c macrophages; express two peaks of *Il10* mRNA whereas BALB/c macrophages only have one; and produce more *Ifnb1* mRNA and IFN-β protein than BALB/c macrophages. We therefore hypothesised that type I IFN may be responsible

for inducing the second wave of *Il10* mRNA in C57BL/6 macrophages, and hence higher levels of IL-10 protein production. This would be in keeping with a positive role for type I IFN in the regulation of IL-10 (Chang *et al.* 2007a). Further, this would be consistent with both C57BL/6 and BALB/c macrophages expressing very low levels of *Ifnb1* mRNA and IFN-β protein upon Pam3CSK4 stimulation (see Figure 3.9), and consequently only one early peak of *Il10* mRNA (see Figure 3.8), and consistently lower levels of IL-10 protein (see Figure 3.3).

To determine if type I IFN was inducing the second peak of *Il10* mRNA and hence higher levels of IL-10 in LPS and HkBps but not Pam3CSK4 stimulated C57BL/6 macrophages, C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages (which lack a functional type I IFN receptor), were stimulated with LPS, Pam3CSK4 and HkBps over a time-course. Levels of *Il10* mRNA expression and IL-10 protein production were then determined (Figure 3.10). Strikingly, while the first peak of *Il10* mRNA was mostly unaffected by the absence of type I IFN signalling, the second peak was completely abrogated in LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages (Figure 3.10 A). In Pam3CSK4 stimulated cells, *Il10* mRNA was slightly lower at 3, 4 and 6 h, then elevated at 12 h in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 WT macrophages, although in general was not substantially affected (Figure 3.10 A). In HkBps stimulated macrophages, the first peak of *Il10* mRNA was unaffected in C57BL/6 *Ifnar1*$^{-/-}$ macrophages, but similarly to LPS stimulation, the second *Il10* mRNA peak was abrogated in the absence of type I IFN signalling (Figure 3.10 A). Analysis of IL-10 protein levels showed that in both LPS and HkBps stimulated cells, the level of IL-10 produced by C57BL/6 *Ifnar1*$^{-/-}$ macrophages was reduced relative to

C57BL/6, and was now comparable to the levels produced by BALB/c macrophages (Figure 3.10 B). This was despite C57BL/6 *Ifnar1*$^{-/-}$ macrophages expressing a stronger initial peak of *Il10* mRNA than BALB/c macrophages (Figure 3.10 A). Unexpectedly, in Pam3CSK4 stimulated cells the level of IL-10 produced by C57BL/6 *Ifnar1*$^{-/-}$ macrophages was enhanced relative to C57BL/6 macrophages (Figure 3.10 B).

Thus, the second peak of *Il10* mRNA in LPS and HkBps stimulated C57BL/6 macrophages is completely dependent on endogenous type I IFN signalling. Further, loss of the second *Il10* mRNA peak translates to a significant reduction in IL-10 production, to a level similar to that observed in BALB/c macrophages. This does not occur in Pam3CSK4 stimulated cells where *Il10* mRNA is only marginally affected, and IL-10 protein production actually increases in the absence of type I IFN signalling. Thus, the presence of type I IFN appears to be critical for the higher level of IL-10 production in C57BL/6 macrophages compared to BALB/c macrophages in response to LPS and HkBps, but not Pam3CSK4.

3.3.2.8 *Treatment with exogenous IFN-β enhances IL-10 production in both C57BL/6 and BALB/c macrophages.*

We next considered that if a reduced level of type I IFN is responsible for the lower IL-10 production in LPS and HKBps stimulated BALB/c macrophages, then the addition of type I IFN may rescue IL-10 production in BALB/c macrophages. To assess whether we could enhance IL-10 production by the addition of type I IFN, we treated C57BL/6 and BALB/c macrophages with 2 or 20 ng/ml IFN-β for 2 h prior to stimulation with LPS or HkBps for 24 h (Figure 3.11). We chose a 2 h pre-incubation period as this

provided an enhanced effect of IFN-β relative to IFN-β addition at the time of stimulation (data not shown). Treatment of unstimulated C57BL/6 or BALB/c cells with IFN-β did not induce IL-10 production (Figure 3.11). In LPS stimulated cells, the addition of IFN-β enhanced IL-10 production in both C57BL/6 and BALB/c macrophages (Figure 3.11). Therefore BALB/c macrophages are able to elevate their production of IL-10 in response to IFN-β, provided that the cells are also TLR stimulated. Once treated with IFN-β, the levels of LPS induced IL-10 were similar in C57BL/6 and BALB/c macrophages, although still marginally higher in C57BL/6 macrophages (Figure 3.11). In HkBps stimulated cells, the addition of IFN-β again enhanced IL-10 in C57BL/6 and BALB/c macrophages, but under all conditions, the levels of IL-10 remained significantly higher in C57BL/6 macrophages (Figure 3.11). Thus, the addition of IFN-β enhanced IL-10 production in LPS and HkBps stimulated macrophages from both strains, but did not fully rescue IL-10 production in BALB/c macrophages to the level of the C57BL/6.

3.3.3 Investigation into the mechanisms of type I IFN mediated regulation of IL-10 in C57BL/6 and BALB/c macrophages.

3.3.3.1 *The second peak of* Il10 *mRNA in C57BL/6 macrophages is likely to be mediated by a transcriptional mechanism*

We sought to more fully understand how type I IFN induces the second peak of *Il10* mRNA and thus higher levels of IL-10 protein in C57BL/6 macrophages. Specifically, we wanted to confirm that the second peak of *Il10* mRNA was the result of active transcription from the *Il10* gene. The level of unspliced premature mRNA is considered a better correlate of *de novo* transcription than the mature mRNA level which is a

combination of *de novo* transcription and mRNA degradation (Guhaniyogi *et al.* 2001). We therefore designed qPCR primers to amplify a section of the *Il10* mRNA transcript containing an intronic sequence, making it possible to quantify the unspliced premature *Il10* mRNA transcript (see Materials and Methods). C57BL/6 and BALB/c macrophages were stimulated with LPS over a time-course and mature and premature *Il10* mRNA levels were quantified (Figure 3.12). In C57BL/6 macrophages, the premature *Il10* transcript level peaked at 15 min to 1 h and again at 4 h post stimulation, from which point it then declined (Figure 3.12). In general, expression of the precursor *Il10* transcript had similar kinetics to the mature *Il10* transcript but as expected, shifted slightly earlier in time (Figure 3.12). In BALB/c macrophages, a similar trend was seen where the precursor *Il10* transcript followed similar kinetics to the mature *Il10* mRNA, with only one initial peak that was shifted slightly earlier in time (Figure 3.12). This data supports the hypothesis that the type I IFN dependent second peak of mature *Il10* mRNA in C57BL/6 macrophages is driven by active transcription from the *Il10* gene.

3.3.3.2 *IL-27 is not required for type I IFN mediated enhancement of IL-10 production in macrophages.*

We show that type I IFN signalling is central in driving the higher levels of IL-10 in LPS and HkBps stimulated C57BL/6 macrophages compared to BALB/c macrophages (see Figure 3.10). It has been reported that in LPS stimulated BMDM, type I IFN induces IL-27, which then in turn is required for the optimal enhancement of IL-10 by type I IFN (Iyer *et al.* 2010). However, in contrast, others have shown that murine macrophages are unresponsive to IL-27 (Kalliolias *et al.* 2008), suggesting that this may not be a requirement for type I IFN to enhance IL-10 production. We sought to

determine if the type I IFN mediated enhancement of IL-10 production that we observe is dependent on IL-27. Further, as IFN-β treatment did not fully rescue IL-10 production in BALB/c macrophages (Figure 3.11), we considered the possibility that BALB/c macrophages are lacking an additional factor required for maximal induction of IL-10 by type I IFN, a candidate for which could be IL-27.

To begin to address these questions, C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages were stimulated with LPS or HkBps and IL-27 production was quantified (Figure 3.13 A). IL-27 was strongly induced in C57BL/6 and BALB/c macrophages and in response to LPS, IL-27 production was slightly higher in BALB/c macrophages. In LPS and HkBps stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages, IL-27 production was drastically reduced (Figure 3.13 A). This is in agreement with previous studies showing a role for type I IFN in the promotion of IL-27 (Molle *et al.* 2010). In keeping with this, the addition of IFN-β significantly increased IL-27 production in both C57BL/6 and BALB/c LPS stimulated macrophages (Figure 3.13 B). Thus, LPS and HkBps stimulated BALB/c macrophages are not deficient in their production of IL-27, and LPS stimulated BALB/c macrophages are able to enhance IL-27 production upon IFN-β treatment.

To determine if IL-27 is able modulate IL-10 production in C57BL/6 or BALB/c macrophages, cells were treated with 2 or 20 ng/ml IL-27 either 2 h prior to stimulation, or at the time of stimulation with LPS (Figure 3.14). As IL-27 production was largely dependent on type I IFN signalling (see Figure 3.13 A), we also included C57BL/6 *Ifnar1*$^{-/-}$ macrophages as these cells have lower endogenous IL-27, and so the effects of

IL-27 treatment may be more exaggerated. However, IL-27 treatment had no effect on IL-10 production in C57BL/6, BALB/c or C57BL/6 *Ifnar1*$^{-/-}$ macrophages (Figure 3.14), suggesting that IL-27 does not modulate IL-10 production in this cell type under any conditions tested.

To assess the possible dependence of IL-10 production on endogenous IL-27, we compared IL-10 production from LPS and HkBps stimulated macrophages from C57BL/6, BALB/c, C57BL/6 *Ifnar1*$^{-/-}$ and C57BL/6 *Il27ra*$^{-/-}$ (which lack a functional IL-27 receptor) mice over a time-course (Figure 3.15 A). No significant difference in IL-10 production was found between C57BL/6 and C57BL/6 *Il27ra*$^{-/-}$ macrophages, and both produced more IL-10 than BALB/c or C57BL/6 *Ifnar1*$^{-/-}$ macrophages (Figure 3.15 A). To directly investigate if IL-27 was required for type I IFN to enhance IL-10 production, C57BL/6 and C57BL/6 *Il27ra*$^{-/-}$ macrophages were treated with IFN-β and stimulated with LPS (Figure 3.15 B). The enhancement of IL-10 by IFN-β treatment was unimpaired in C57BL/6 *Il27ra*$^{-/-}$ macrophages, demonstrating that this mechanism was independent of IL-27 (Figure 3.15 B). As this data contradicted a recent report (Iyer *et al.* 2010), we confirmed the absence of a functional IL-27 receptor in our C57BL/6 *Il27ra*$^{-/-}$ mice and the viability of our recombinant IL-27 preparation. C57BL/6 and C57BL/6 *Il27ra*$^{-/-}$ splenocytes were treated with IL-27 or as a positive control, IFN-γ, for 10 and 30 min. The phosphorylation of STAT1 on Tyr-701 was determined as both the IL-27 receptor and IFN-γ receptor are known to phosphorylate STAT1 on this site (Platanias 2005; Hall *et al.* 2012) (Figure 3.15 C). IL-27 treatment induced robust STAT1 phosphorylation in C57BL/6 but not C57BL/6 *Il27ra*$^{-/-}$ splenocytes. However, STAT1 phosphorylation was triggered by IFN-γ in both C57BL/6 and C57BL/6 *Il27ra*$^{-/-}$

splenocytes (Figure 3.15 C). This confirms that the C57BL/6 *Il27ra*$^{-/-}$ mice used here have a specific deficiency in IL-27 receptor signalling, and that our recombinant IL-27 preparation is functional. Together, these data demonstrate that although IL-27 is induced in C57BL/6 and BALB/c macrophages in a predominantly type I IFN dependent manner, IL-27 is not involved in the type I IFN mediated enhancement of IL-10 production in LPS or HkBps stimulated macrophages.

3.3.3.3 *STAT-1 has an important role in the type I IFN mediated induction of IL-10.*

Signalling via the type I IFN receptor activates several pathways, the most well studied of which is the JAK-STAT pathway (Platanias 2005). Downstream of the type I IFN receptor, JAK phosphorylation of STAT molecules induces formation of STAT1-STAT2 heterodimers which in turn bind IRF9 to form the ISGF3 complex. This complex binds genes containing ISREs and mediates their transcription (Stark *et al.* 1998). STAT1-STAT1 homodimers are also formed downstream of the type I IFN receptor and these regulate transcription of genes containing GAS elements. These pathways are important for the induction of IFN-regulated genes (Stark *et al.* 1998). STAT1 has also been previously linked to the induction of IL-10 by type I IFN (Guarda *et al.* 2011) and there are putative STAT binding sites within the *Il10* locus (Benkhart *et al.* 2000; Mosser *et al.* 2008b).

We therefore investigated the role of STAT-1 in the regulation of IL-10 by type I IFN in our system by using STAT1 deficient mice. The only STAT1 deficient mice available to us were 129 *Stat1*^{tm1Rds} mice (129S6/SvEv genetic background) which have a disruption in the *Stat1* gene. 129 WT and 129 *Stat1*^{tm1Rds} macrophages were stimulated with LPS

for 24 h (Figure 3.16 A). No significant difference in IL-10 production was observed between WT and STAT1 deficient mice, however, the overall amount of IL-10 production was very low (Figure 3.16 A). Indeed, when we had previously compared LPS induced IL-10 production from C57BL/6, BALB/c and 129S8 macrophages, we found that similar to BALB/c, 129S8 macrophages are low IL-10 producers (Figure 3.16 B). Although this experiment used 129 mice on the S8 background and the 129 *Stat1*^{tm1Rds} mice are on an S6/SvEv background, this suggested to us that macrophages derived from the 129 strain may be low IL-10 producers. Therefore 129 macrophages may be unlikely to induce a type I IFN dependent second peak of *Il10* mRNA, and consequently a lack of type I IFN signalling, or STAT1, may not affect IL-10 production in 129 macrophages. Thus, to assess the role of STAT1 in type I IFN mediated regulation of IL-10 using 129 *Stat1*^{tm1Rds} mice, IFN-β was added to 129 WT and 129 *Stat1*^{tm1Rds} macrophages 2 or 12 h prior to LPS stimulation, and IL-10 production was quantified (Figure 3.16 C). The addition of IFN-β 2 or 12 h prior to LPS stimulation, enhanced IL-10 production in both 129 WT and 129 *Stat1*^{tm1Rds} macrophages (Figure 3.16 C). Importantly however, the relative increase in IL-10 production in response to IFN-β treatment was greatly reduced in 129 *Stat1*^{tm1Rds} macrophages relative to WT, particularly after a 12 h IFN-β pre-incubation (Figure 3.16 C). This suggests that STAT1 is an important component of the mechanism by which type I IFN regulates IL-10. The finding that 129 *Stat1*^{tm1Rds} macrophages are able to enhance IL-10 production after IFN-β treatment however (Figure 3.16 C), suggests that STAT1 independent mechanisms may also be present.

3.3.3.4 *STAT-1 is phosphorylated in LPS stimulated C57BL/6 and BALB/c macrophages but not C57BL/6* **Ifnar1**$^{-/-}$ *macrophages.*

Our data (Figure 3.16 C), and a previously published report (Guarda *et al.* 2011) suggest that type I IFN largely depends on STAT1 to promote IL-10 production. We postulated that the relatively low levels of IFN-β produced by BALB/c macrophages (see Figure 3.9) may not sufficiently activate STAT-1 and that this may be linked to the absence of a type I IFN dependent second peak of *Il10* mRNA in this strain (see Figure 3.8). To investigate this, we assessed STAT-1 phosphorylation in LPS stimulated C57BL/6 and BALB/c macrophages at 1, 2, 4 and 6 h post-stimulation. We also included C57BL/6 *Ifnar1*$^{-/-}$ macrophages in the experiment to be able to confirm that any STAT-1 phosphorylation present in WT macrophages was dependent on type I IFN signalling. In C57BL/6 macrophages, STAT-1 phosphorylation was strongest at 2 h but was still detectable at 4 and 6 h (Figure 3.17). In BALB/c macrophages, STAT1 phosphorylation was again seen at 2, 4 and 6 h and was not markedly different compared to C57BL/6 macrophages (Figure 3.17). No STAT1 phosphorylation was detected in C57BL/6 *Ifnar1*$^{-/-}$ macrophages, confirming that type I IFN signalling was responsible for the STAT1 activation in C57BL/6 WT cells (Figure 3.17). Of note, total STAT1 levels were greatly reduced in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 or BALB/c macrophages, in keeping with a reported role for basal levels of type I IFN signalling in the maintenance of STAT1 expression (Gough *et al.* 2010). In contrast, the level of total STAT-1 appeared consistent in C57BL/6 and BALB/c macrophages. Thus, the low level of IFN-β produced by LPS stimulated BALB/c macrophages appears sufficient to phosphorylate STAT1 to a level comparable to C57BL/6 macrophages. This suggests that a deficiency in STAT1 phosphorylation, at

least at Tyr-701, is not responsible for the lack of a second peak of *Il10* mRNA in BALB/c macrophages.

3.3.4 Investigation into the genetic factors regulating differential IL-10 production in LPS, Pam3CSK4 and HkBps stimulated C57BL/6 and BALB/c macrophages.

Our investigation so far has provided significant insight into the mechanisms that regulate differential IL-10 production in C57BL/6 and BALB/c macrophages. We were further interested in understanding the genetic factors which contribute to this phenotype. A commonly used approach to gain an understanding of the genetic elements that control a phenotype, is the genetic cross (Flint *et al.* 2012). In this study design, mice derived from two different inbred strains are crossed to produce an F1 generation. The F1 generation is then intercrossed to produce a genetically diverse F2 generation. Phenotyping and genotyping of the F2 generation can then detect areas of the genome that are significantly associated with a given phenotype (Flint *et al.* 2012). Thus, as a complimentary approach to investigating the mechanisms underlying differential IL-10 production in LPS, Pam3CSK4 and HkBps stimulated C57BL/6 and BALB/c macrophages, we analysed IL-10 production from C57BL/6 x BALB/c F1 and F2 macrophages.

3.3.4.1 *C57BL/6 x BALB/c F1 macrophages produce intermediate levels of IL-10*

F1 mice were derived from C57BL/6 females and BALB/c males. C57BL/6, BALB/c and C57BL/6 x BALB/c F1 macrophages (derived from 7 individual F1 mice) were stimulated with LPS for 6 h, Pam3CSK4 for 24 h and HkBps for 24 h (Figure 3.18).

These time points were chosen to represent peak IL-10 production with the respective stimuli, based on previous experiments (see Figure 3.3). In response to each stimulus, F1 macrophages produced levels of IL-10 that were almost exactly intermediate between C57BL/6 and BALB/c macrophages (Figure 3.18). By definition, the F1 population is identical as each mouse is heterozygous at every loci for C57BL/6 and BALB/c alleles. Thus, as expected, macrophages derived from different F1 mice produced similar amounts of IL-10 (Figure 3.18). This intermediate F1 phenotype suggests that the genetic factor(s) contributing to the level of IL-10 production are not dominant in either C57BL/6 or BALB/c mice. This could be the result of one co-dominant locus, or the result of many interacting genes contributing to IL-10 production.

3.3.4.2 *Preliminary analysis of LPS, Pam3CSK4 and HkBps stimulated C57BL/6 x BALB/c F2 macrophages shows segregation of the genetic factors controlling IL-10 production.*

Unlike the F1 population, the F2 population is genetically diverse due to the heterozygosity of the F1 parents. Thus, the genetic factor(s) which control IL-10 production will segregate within the population. The type of segregation observed can provide information about the nature of the trait-determining genetic factors. For example, if a trait is controlled by one gene or a small number of linked genes, a phenotypic ratio of 1 C57BL/6 (homozygous): 2 F1 (heterozygous): 1 BALB/c (homozygous) would be predicted. If several genetic loci determine the level of IL-10 production, the F2 generation may have a spectrum of IL-10 production. We began by analysing IL-10 production from a relatively small number of C57BL/6 x BALB/c F2 mice. Macrophages derived from five C57BL/6, five BALB/c, and fourteen C57BL/6 x

BALB/c F2 macrophages (labelled A-N) were stimulated with LPS for 6 h, Pam3CSK4 for 24 h and HkBps for 24 h (Figure 3.19). In response to all three stimuli, the F2 population displayed diverse levels of IL-10 production. Some F2 macrophages e.g. A and B (orange boxes), produced consistently low levels of IL-10 in response to all three stimuli (Figure 3.19). However, other F2 macrophages e.g. F and H (green boxes), produced low levels of IL-10 in response to LPS or HkBps, but even more IL-10 than C57BL/6 mice in response to Pam3CSK4 (Figure 3.19). This suggests that different genes may control the level of IL-10 production in response to LPS and HkBps compared to Pam3CSK4 as they are segregating separately in the F2 population (Figure 3.19). This would fit with our previous data, suggesting that a common type I IFN signalling dependent mechanism underlies the level of IL-10 production in both LPS and HkBps stimulated cells, but not Pam3CSK4 stimulated cells (see Figure 3.10). With this conservative number of F2 mice it is difficult to clearly determine the segregation pattern of IL-10 production, however, given that several mice had an IL-10 expression level that was distinct from C57BL/6, BALB/c and what would be predicted from the F1 population (see Figure 3.18), the regulation of IL-10 production in C57BL/6 and BALB/c macrophages may be controlled by several distinct genetic loci.

3.3.5 Investigation into IL-10 production in C57BL/6 and BALB/c mice *in vivo*.

3.3.5.1 *LPS treatment induces higher* Il10 *and* Ifnb1 *mRNA expression in the spleen of C57BL/6* Rag1$^{-/-}$ *mice compared to BALB/c* Rag2$^{-/-}$ *mice*

The majority of experiments so far in this investigation have been focussed on macrophages stimulated *in vitro*. We next investigated if we could translate our key observations into an *in vivo* setting. To do this, we treated C57BL/6 *Rag1*$^{-/-}$ and BALB/c

Rag2$^{-/-}$ mice by i.p. injection with 150 µg LPS (or the equivalent volume of PBS as a control) for 1, 3 and 6 h (Figure 3.20). Although the duration of these experiments is short and the adaptive immune response is unlikely to be activated within this time-frame, RAG1 or RAG2 deficient mice which lack T and B cells were used as peritoneal B cells are known to produce IL-10 in response to LPS (O'Garra *et al.* 1992) and may therefore confound our results. At the chosen time-points, spleens were collected for the analysis of *Il10* and *Ifnb1* mRNA expression, and serum was collected for IL-10 protein analysis (Figure 3.20). PBS treated mice expressed low levels of splenic *Il10* mRNA, and *Ifnb1* mRNA and serum IL-10 was undetectable (Figure 3.20, top). LPS treatment elevated *Il10* mRNA expression at 1, 3 and 6 h in the spleens of both C57BL/6 *Rag1*$^{-/-}$ and BALB/c *Rag2*$^{-/-}$mice compared to the PBS controls (Figure 3.20, bottom). At each time point, *Il10* mRNA expression was higher in spleens of C57BL/6 *Rag1*$^{-/-}$ mice (Figure 3.20). LPS treatment also elevated expression of *Ifnb1* in the spleen, predominantly at 1 h post-injection. Similarly to *Il10* mRNA, *Ifnb1* mRNA was more highly expressed in C57BL/6 *Rag1*$^{-/-}$ than BALB/c *Rag2*$^{-/-}$mice (Figure 3.20). The level of IL-10 protein in the serum of LPS treated mice was at its highest at 1 h post-injection, but was highly variable and not significantly different between the strains at this time-point (Figure 3.20). IL-10 levels were lower but still present in the serum at 3 h post-injection and at this time were significantly higher in C57BL/6 *Rag1*$^{-/-}$ mice (Figure 3.20). By 6 h, serum IL-10 levels were almost back to baseline in both C57BL/6 *Rag1*$^{-/-}$ and BALB/c *Rag2*$^{-/-}$mice (Figure 3.20). Thus, although the differences in IL-10 serum levels were not profound in this *in vivo* model, the splenic mRNA data was concordant with our *in vitro* macrophage results with higher levels of *Il10* mRNA and *Ifnb1* mRNA expression in C57BL/6 compared to BALB/c mice.

3.4 Discussion

IL-10 is an anti-inflammatory cytokine with an important role in limiting immune mediated damage to the host (Moore *et al.* 2001). Macrophages are potent producers of IL-10 in response to TLR ligation (Saraiva *et al.* 2010). However, owing to the complex nature of IL-10 regulation, a full understanding of the combined factors which regulate this cytokine in response to different stimuli is incomplete. To further our understanding of IL-10 regulation, we compared IL-10 production from C57BL/6 and BALB/c macrophages stimulated with purified PRR ligands and heat-killed bacteria. We observed that C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages when stimulated with LPS (TLR4), Pam3CSK4 (TLR2), or heat-killed *B.pseudomallei* (TLR2/4), and focussed our studies on these three stimuli. Our data shows that a type I IFN dependent but IL-27 independent mechanism regulates differential IL-10 production in LPS and heat-killed *B.pseudomallei,* but not Pam3CSK4 stimulated C57BL/6 and BALB/c macrophages.

3.4.1 Mechanisms of differential IL-10 production in C57BL/6 and BALB/c macrophages

Our initial studies into the mechanisms underlying differential IL-10 production in TLR2, TLR4 and TLR2/4 stimulated C57BL/6 and BALB/c macrophages demonstrated no clear correlation between *Tlr* mRNA expression and the level of IL-10 production. Although we did not assess TLR expression at the protein level, a recent report showed that TLR4 surface expression is equivalent in C57BL/6 and BALB/c BMDM and resident peritoneal macrophages (Tsukamoto *et al.* 2013). Thus, we reason that

differential TLR expression is unlikely to be a driving force behind strain-dependent IL-10 production in macrophages. We additionally compared ERK and p38 activation within the first hour of TLR2 and TLR4 stimulation in C57BL/6 and BALB/c macrophages as these MAP kinases are important for the positive regulation of IL-10 downstream of TLRs (Foey *et al.* 1998; Yi *et al.* 2002; Dillon *et al.* 2004; Kaiser *et al.* 2009). However, we found no evidence for differential activation of these signalling molecules in C57BL/6 and BALB/c macrophages within this early time-frame.

We went on to study the transcriptional profile of *Il10* mRNA in LPS, Pam3CSK4 and heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages. Our detailed kinetics of *Il10* mRNA expression revealed that C57BL/6 macrophages express two peaks of *Il10* mRNA when stimulated with LPS or heat-killed *B.pseudomallei*, whereas BALB/c macrophages only express one. To our knowledge, this biphasic regulation of *Il10* gene expression in C57BL/6 macrophages has not previously been described. The further finding that C57BL/6 macrophages produce higher levels of IFN-β than BALB/c macrophages when stimulated with LPS or heat-killed *B.pseudomallei*, lead us to investigate the role of type I IFN in strain-dependent IL-10 production. We found that the second peak of *Il10* mRNA in LPS or heat-killed *B.pseudomallei* C57BL/6 macrophages was dependent on type I IFN signalling. Further, the abrogation of the second *Il10* mRNA peak resulted in a significant reduction in IL-10 protein levels in these cells. Thus, type I IFN is central in mediating differential IL-10 production in LPS and heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages. These findings are in keeping with previous reports that endogenous type I IFN is important for the production of IL-10 in LPS stimulated C57BL/6 macrophages (Chang

et al. 2007a) and that the TRIF pathway, which induces type I IFN downstream of TLR4 (Yamamoto *et al.* 2003a), contributes to IL-10 production (Boonstra *et al.* 2006). However, our novel findings indicate that this regulatory loop is absent in BALB/c macrophages. Despite complete abrogation of the second peak of *Il10* mRNA in C57BL/6 *Ifnar1*$^{-/-}$ macrophages, the first *Il10* mRNA peak at 30 min was largely unaffected by the absence of type I IFN signalling. This suggested that type I IFN is important for the maintenance of IL-10 production, in agreement with the recent findings of Pattison *et al.*, where the absence of type I IFN only affected *Il10* mRNA expression at later time points (Pattison *et al.* 2012). This may also suggest that type I IFN produced in response to stimulation, as opposed to basal type I IFN production which has been found to have widespread cellular effects (Gough *et al.* 2012), may be most important for the induction of the second peak of *Il10* mRNA. This could be formally tested with the use of a blocking antibody against the type I IFN receptor during stimulation, although a complete abrogation of type I IFN mediated signalling would have to be confirmed.

We postulated that in LPS and heat-killed *B.pseudomallei* stimulated BALB/c macrophages, the lower level of IFN-β production in response to stimulation may be responsible for the lack of secondary *Il10* transcription in this strain. Differential production of type I IFN in C57BL/6 and BALB/c mice has been shown in the context of Newcastle disease virus infection where it was associated with variant alleles at the *If-1* locus on chromosome 3 (De Maeyer-Guignard *et al.* 1986). However, differential type I IFN production has only been previously implied in LPS stimulated macrophages by a delayed profile of IFN-inducible genes in BALB/c macrophages relative to

C57BL/6 (Wells *et al.* 2003). Our observation that the addition of IFN-β enhanced IL-10 production in both C57BL/6 and BALB/c macrophages, demonstrated that BALB/c macrophages do have the ability to regulate IL-10 in response to type I IFN and thus lower type I IFN induction by LPS and HkBps may contribute to lower IL-10 production. We did not however consistently observe a complete rescue of IL-10 production from BALB/c macrophages through the addition of IFN-β, even at high dose. There are several possible explanations for this. Firstly, it could be that together with differential type I IFN production, there is a difference in responsiveness to type I IFN in C57BL/6 and BALB/c macrophages. If this is the case, the addition of type I IFN alone would not be sufficient to rescue IL-10 production in BALB/c macrophages. Differential type I IFN responsiveness of C57BL/6 and BALB/c mice has been previously reported in context of the IRF2 deficient skin psoriasis model (Arakura *et al.* 2007). IRF2 deficient C57BL/6 mice spontaneously develop psoriasis associated with heightened responsiveness to type I IFN (Hida *et al.* 2000). BALB/c IRF2 deficient mice however do not develop disease, and this was attributed to lower type I IFN responsiveness in this strain (Arakura *et al.* 2007). More detailed investigations into the activation of type I IFN mediated signal transduction in C57BL/6 and BALB/c macrophages in our system would help to clarify this issue. Secondly, the induction of the second peak of *Il10* mRNA in C57BL/6 macrophages may be the result of a combined IFN-β and IFN-α mediated signal, as all type I IFNs signal through the same receptor (Stark *et al.* 1998). Thus, the addition of IFN-β alone may not fully recapitulate the type I IFN requirement for secondary *Il10* mRNA expression. We did attempt to quantify IFN-α2 and IFN-α5 production in LPS and heat-killed *B.pseudomallei* C57BL/6 and BALB/c macrophages as these IFN-α's had been previously detected by

microarray in LPS stimulated C57BL/6 and BALB/c macrophages (Wu, O'Garra, unpublished). However, we found that neither were reliably detectable by qPCR in either strain. This suggests that IFN-α may not play a role in our system, although it is possible that IFN-α production is below the limit of detection by qPCR, or that IFN-α's other than 2 and 5 are induced in response to these stimuli. Lastly, the timing of endogenous type I IFN production may be important in the downstream regulation of *Il10* expression. For example, factors activated or induced downstream of the type I IFN receptor may be required to synergise with factors activated by TLR signalling. As we added IFN-β 2 h prior to stimulation, it is possible that this does not fully replicate the signalling events induced by endogenous type I IFN. Nevertheless, the complete lack of a second *Il10* mRNA peak in C57BL/6 *Ifnar1*$^{-/-}$ macrophages and subsequent reduction in IL-10 protein production to the level of the BALB/c, demonstrates the importance of type I IFN in driving the higher levels of IL-10 production in C57BL/6 macrophages in response to LPS and heat-killed *B.pseudomallei*.

It is generally accepted that TLR2 does not induce type I IFN in macrophages due to the lack of TRIF pathway activation (Doyle *et al.* 2002; Toshchakov *et al.* 2002). More recently however, this view has been challenged by a report that TLR2 can directly induce type I IFN in response to Pam3CSK4 in macrophages via a Myd88/IRF1/IRF7 dependent pathway (Dietrich *et al.* 2010). Other studies have shown that TLR2 activation can induce type I IFN in bone marrow cells, but only in response to vaccinia virus, not Pam3CSK4 (Barbalat *et al.* 2009). Thus, the induction of type I IFN by bacterial TLR2 ligands remains controversial and may vary according to the cell type. We observed that relative to LPS or heat-killed *B.pseudomallei* stimulation, the level of

IFN-β produced by Pam3CSK4 stimulated cells was negligible. Our findings are therefore more in agreement with the widespread view that the capacity of Pam3CSK4 to induce type I IFN is limited (Doyle *et al.* 2002; Toshchakov *et al.* 2002). This may explain the different kinetics of IL-10 protein production in Pam3CSK4 stimulated compared to LPS and heat-killed *B.pseudomallei* stimulated macrophages, and the lack of a second *Il10* mRNA peak in C57BL/6 cells. Surprisingly, in Pam3CSK4 stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages, despite *Il10* mRNA only being modestly affected by the absence of type I IFN, IL-10 protein levels were elevated compared to C57BL/6 WT at 24 h. Of note, there was only a difference in IL-10 protein production in C57BL/6 WT and C57BL/6 *Ifnar1*$^{-/-}$ macrophages after 12 h, suggesting a delayed effect of the absence of type I IFN signalling. Thus, in the context of TLR2 stimulation, an extremely low level of induced type I IFN may antagonise the production of IL-10, potentially by a post-transcriptional mechanism. Alternatively, the absence of basal type I IFN may be influencing IL-10 production in response to this stimulus. A role for induced or basal type I IFN in the regulation of Pam3CSK4 induced IL-10 production has not previously been described and warrants further investigation. However, the inability to clearly distinguish differential type I IFN production in Pam3CSK4 stimulated C57BL/6 and BALB/c macrophages and the enhancement of IL-10 production in the absence of type I IFN in response to this stimulus, do not support a role for type I IFN in mediating differential IL-10 production in Pam3CSK4 stimulated C57BL/6 and BALB/c macrophages. As the magnitude of peak *Il10* mRNA at 30 min was consistently lower in Pam3CSK4 stimulated BALB/c macrophages compared to C57BL/6 macrophages, a potential mechanism could include enhanced transcription from the *Il10* gene in C57BL/6 macrophages as a result of differential TLR signalling,

(independent of p38 or ERK). However, given that the mRNA dynamics at later time points appear to determine downstream IL-10 protein levels in LPS and heat-killed *B.pseudomallei* stimulated macrophages, the maintenance of *Il10* mRNA above baseline in Pam3CSK4 stimulated C57BL/6 macrophages but not BALB/c, may also be relevant to differential IL-10 production. Further investigation is required to determine if the prolonged *Il10* mRNA levels observed in C57BL/6 macrophages are a consequence of continued expression, or enhanced stability of *Il10* mRNA in this strain.

In our initial analysis, we also observed higher IL-10 production in C57BL/6 macrophages compared to BALB/c macrophages when stimulated with Poly I:C and heat-killed *L.monocytogenes*. In view of our finding that type I IFN mediates differential IL-10 production in response to TLR4 stimulation, we postulate that type I IFN may also have a role in the differential IL-10 production downstream of Poly I:C, which itself is able to induce type I IFN in macrophages (Doyle *et al.* 2002). The innate recognition of *L.monocytogenes* has previously been associated with TLR2, with a less important role for TLR4 (Seki *et al.* 2002; Torres *et al.* 2004). It may be that differential IL-10 production in response to heat-killed *L.monocytogenes* is mediated by a similar mechanism as Pam3CKS4 stimulated cells, although as *L.monocytogenes* also stimulates additional PRRs such as TLR5 (Hayashi *et al.* 2001) and NOD2 (Leber *et al.* 2008), other factors may also contribute.

3.4.2 The mechanisms of type I IFN mediated IL-10 regulation in LPS and heat-killed *B.pseudomallei* stimulated cells

The observation that type I IFN signalling induces an increase in *Il10* mRNA levels manifested by a second peak in C57BL/6 macrophages, strongly suggested that type I IFN promotes IL-10 production through a transcriptional mechanism. This was confirmed by the quantification of premature *Il10* mRNA, which also had a second peak of *Il10* mRNA from 4-6 h post LPS stimulation in C57BL/6 macrophages and is in agreement with previous studies which have also suggested a transcriptional mechanism of *Il10* regulation by type I IFN (Ziegler-Heitbrock *et al.* 2003; Wang *et al.* 2010). However, an additional stabilising effect of type I IFN on *Il10* mRNA, which has not been previously studied, cannot be discarded at this stage and we are currently investigating this possibility.

IL-27 is a cytokine with pleiotropic functions (Hall *et al.* 2012; Vignali *et al.* 2012). In the context of immunoregulatory roles, it is established that IL-27 enhances IL-10 production from various effector T cell subsets (Awasthi *et al.* 2007; Fitzgerald *et al.* 2007; Stumhofer *et al.* 2007; Batten *et al.* 2008; Anderson *et al.* 2009; Freitas do Rosario *et al.* 2012). In keeping with previous reports (Molle *et al.* 2010), we observed that IL-27 was positively regulated by type I IFN. Interestingly, although BALB/c macrophages express low levels of type I IFN, their production of IL-27 was not impaired. This could be because only a small amount of type I IFN is required for IL-27 production or that in BALB/c macrophages, IL-27 is induced by an additional signal. Although the effects of IL-27 on innate cell populations are less clearly defined, it has been reported that in LPS stimulated macrophages, type I IFN requires the induction of

IL-27 to optimally enhance IL-10 production (Iyer *et al.* 2010). The results of our study contradict that of Iyer *et al.* as have we found that IL-10 production from C57BL/6 macrophages in response LPS and heat-killed *B.pseudomallei* was unaffected by the absence of IL-27 signalling. We also show that treatment of cells with IL-27 failed to modulate IL-10 production in media or LPS treated C57BL/6, BALB/c or C57BL/6 *Ifnar1*$^{-/-}$ macrophages. Additionally, the enhancement of IL-10 by IFN-β treatment was independent of IL-27 signalling. Thus, we have no evidence to suggest that IL-27 regulates IL-10 production in macrophages. A potentially relevant difference between our study and that of Iyer *et al*. is that our investigations of type I IFN mediated regulation of IL-10 have mainly centred around IFN-β, whereas that of Iyer *et al.* focussed on IFN-α (Iyer *et al.* 2010). As type I IFNs are known to mediate differential effects despite signalling through the same receptor (van Boxel-Dezaire *et al.* 2006; Thomas *et al.* 2011), it is conceivable that the requirements of IFN-β and IFN-α for the induction of IL-10 may be different, the former being independent of IL-27 but the latter requiring IL-27. Nevertheless, our findings are in keeping with those of an earlier study showing that resting murine macrophages are unresponsive to IL-27 (Kalliolias *et al.* 2008). This study from Kalliolias *et al.* further showed a negative impact of IL-27 on IL-10 production in human monocytes (Kalliolias *et al.* 2008). Thus, the regulation of IL-10 by IL-27 in monocytes and macrophages is disputed in the literature and our study may help to resolve this issue.

In our investigation into the involvement of STAT1 in type I IFN mediated regulation of IL-10, we observed similar phosphorylation of STAT1 at Tyr-701 in LPS stimulated C57BL/6 and BALB/c macrophages. STAT1 Tyr-701 phosphorylation was dependent

on type I IFN in C57BL/6 macrophages. Therefore, despite a reduced level of type I IFN production in BALB/c macrophages, STAT1 Tyr-701 phosphorylation was not substantially impaired. This may imply that responses to type I IFN other than the phosphorylation of STAT1 at Tyr701 are lacking in BALB/c macrophages. Of note, the STAT1-α isoform is also phosphorylated by type I IFN signalling at Ser727 and this modification has been reported to alter STAT1 transcriptional activity (Bancerek *et al.* 2013). In light of this, it would be interesting to additionally compare STAT1 Ser727 phosphorylation in C57BL/6 and BALB/c macrophages stimulated with LPS and/or treated with type I IFN.

Our finding that LPS stimulated STAT1 deficient 129 macrophages had a greatly reduced enhancement of IL-10 upon IFN-β treatment, supports an important role for STAT1 in this process. However, the effects of type I IFN on IL-10 production were not completely abrogated in the absence of STAT1, suggesting the presence of STAT1 independent pathways of IL-10 regulation by type I IFN. This is in contrast to a previous study using STAT1 deficient C57BL/6 macrophages which showed that the enhancement of IL-10 by IFN-β and IFN-α addition to LPS stimulated cells was completely dependent on STAT1 (Guarda *et al.* 2011). The differences between this study and ours could be due to the genetic background of the mouse, or that we quantified IL-10 at 24 h, whereas Guarda *et al.* quantified IL-10 at 4 h. STAT1 independent pathways of IL-10 regulation in response to type I IFN may include the PI(3)K/AKT pathway which has been shown to mediate the type I IFN dependent regulation of IL-10 in human DCs (Wang *et al.* 2010). Activation of the MAP kinases p38 (Uddin *et al.* 1999; Li *et al.* 2004) and potentially ERK2 (David *et al.* 1995) has

also been reported to occur downstream of the type I IFN receptor. As positive regulators of IL-10 (Foey *et al.* 1998; Yi *et al.* 2002; Dillon *et al.* 2004), it is possible that p38 and ERK contribute to the regulation of IL-10 by type I IFN. However, in this investigation we noted that upon the inhibition of p38 and MEK1/ERK activation in C57BL/6 macrophages, IL-10 production was completely abrogated in Pam3CSK4 but not LPS stimulated cells. This difference may reflect the presence of a p38 and ERK independent IL-10 inducing pathway that is activated downstream of LPS, but not Pam3CSK4, a candidate for which could be type I IFN.

3.4.3 Genetic factors underlying differential IL-10 production in C57BL/6 and BALB/c macrophages

We generated C57BL/6 x BALB/c F1 and F2 mice to gain insight into the genetic variation that contributes to differential IL-10 production in C57BL/6 and BALB/c macrophages. The F1 population displayed an intermediate phenotype between the parental strains. This result may be indicative of genetic factors that have incomplete dominance, or potentially a gene dosage effect. As an example, if the level of type I IFN production determines the level of IL-10 production in LPS and heat-killed *B.pseudomallei* stimulated macrophages, an intermediate production of type I IFN from F1 macrophages could give rise to an intermediate level of IL-10. In this respect, it would be interesting to determine the level of *Ifnb1* expression and IFN-β production in F1 macrophages stimulated with LPS or heat-killed *B.pseudomallei*. The F1 macrophages analysed in this study were derived from a cross of C57BL/6 female and BALB/c male mice. In future studies, it will also be important to assess F1 macrophages derived from the reciprocal cross (BALB/c female, C57BL/6 male) to exclude any

parental genetic effects. Although macrophages derived from only fourteen F2 mice were analysed, our preliminary findings suggest that the F2 population may produce a spectrum of IL-10 levels. Thus, it may be that the several genetic loci determine the ultimate level of IL-10, meaning that differential IL-10 production may be a complex genetic trait. Continuation of analysis of F2 mice to add to this dataset would help to clarify this issue. Of note, genetic intercross studies which go on to identify associated genetic loci by statistical linkage analysis, typically require in the region of at least 100+ F2 mice in order to sufficiently power the study (Flint *et al.* 2012). This has been outside of the scope of the current work, but may form a basis for future investigations.

3.4.4 Differential IL-10 and type I IFN production in C57BL/6 and BALB/c macrophages in the context of bacterial infection

C57BL/6 mice are more resistant than BALB/c mice to *B.pseudomallei* infection (Leakey *et al.* 1998). The mechanism of this strain difference, and if it involves differing resistance to the pathogen or immunopathology is unclear. We showed that heat-killed *B.pseudomallei* induces IL-10 in macrophages through both TLR2 and TLR4, however the dependence of heat-killed induced *B.pseudomallei* IL-10 production on type I IFN and the more severe decrease in IL-10 production from *Tlr4*$^{-/-}$ macrophages relative to *Tlr2*$^{-/-}$, implies that TLR4 is dominant for the production of this cytokine. We observed higher IL-10 production in heat-killed *B.pseudomallei* stimulated C57BL/6 macrophages compared to BALB/c. Comparative studies of C57BL/6 and BALB/c mice infected with *B.pseudomallei* by the intra-nasal route have shown higher levels of *Il10* mRNA in the lungs and livers of C57BL/6 mice (Liu *et al.* 2002). A later study of C57BL/6 and BALB/c mice infected by the aerosol route,

reported the presence of IL-10 in the lungs and spleens of C57BL/6 mice, but not BALB/c mice (Tan *et al.* 2008). These findings are congruent with our results and suggest that our findings of differential IL-10 production *in vitro*, may translate to an *in vivo B.pseudomallei* infection model, although the *in vivo* sources of IL-10 have not been confirmed in these studies.

The significance of IL-10 production in *B.pseudomallei* infection is currently unclear and there are no published findings on the outcome of *B.pseudomallei* infection in IL-10 deficient mice. However, some reports have implicated a role for uncontrolled inflammation in the greater susceptibility of BALB/c mice. For example, higher expression of liver proinflammatory cytokine mRNA, including that of IL-6, TNF-α, IL-1β and IFN-γ has been observed in BALB/c mice (Ulett *et al.* 2000a; Ulett *et al.* 2000b). In an aerosol infection study, BALB/c mice were found to produce higher levels of IL-6, TNF-α and IFN-γ protein compared to C57BL/6 mice in the lung and the spleen (Tan *et al.* 2008). As IL-10 is an inhibitor of proinflammatory cytokine production (Moore *et al.* 2001), it is possible that type I IFN driven IL-10 may have a protective role in reducing immune mediated pathology in C57BL/6 mice. Importantly however, type I IFN has many immuno-modulatory effects (Trinchieri 2010), and differential type I IFN production in C57BL/6 and BALB/c macrophages may influence the outcome of infection with *B.pseudomallei*, independently of IL-10. Of note, a differential capacity of C57BL/6 and BALB/c phagocytes to contain bacterial replication has also been implicated as a mechanism for strain-dependent resistance to *B.pseudomallei* infection (Leakey *et al.* 1998; Breitbach *et al.* 2006). Further, BALB/c mice critically depend on iNOS to control bacterial replication, whereas in C57BL/6

mice, the absence of iNOS improved the course of disease (Breitbach *et al.* 2011). Thus, the full mechanisms leading to resistance in C57BL/6 mice and susceptibility in BALB/c mice may be multifaceted.

3.4.5 IL-10 and type I IFN production in C57BL/6 and BALB/c mice *in vivo*

We finally extended our study to assess LPS induced IL-10 and type I IFN production from RAG deficient C57BL/6 and BALB/c mice *in vivo*. The splenic mRNA data correlated well with our previous *in vitro* findings that *Il10* and *Ifnb1* mRNA production were higher in the C57BL/6. Although our study was over too short a time-frame to assess the survival of LPS administered mice, BALB/c mice have been described to be more sensitive to endotoxin challenge than C57BL/6 mice (Yang *et al.* 2011b). Further, IL-10 is known to be protective in animal models of septic shock (Berg *et al.* 1995) and non-T cells have been suggested to be an important source of protective IL-10 in this model (Roers *et al.* 2004). These reports may be congruent with our mRNA data, however, we did not find a substantial difference in IL-10 production in the serum of RAG deficient C57BL/6 and BALB/c mice. Of relevance, our earlier findings indicated that LPS and Pam3CSK4 stimulated C57BL/6 and BALB/c mDCs do not differentially produce IL-10 in the way that macrophages do, suggesting that mechanisms of strain-dependent IL-10 production are not conserved amongst macrophages and DCs. This may not be surprising as it has been previously proposed that macrophages and DCs differ in their level of IL-10 production, potentially linked to a lower activation of ERK in DCs compared to macrophages (Kaiser *et al.* 2009). Thus, it is possible that the serum IL-10 level is too general a read out for the macrophage-specific phenotype we are studying. A more directed approach of assessing IL-10 production from cells *ex vivo*,

such monocytes or tissue resident macrophages, may help us begin to assess if C57BL/6 and BALB/c cells that have not been differentiated *in vitro* also differ in their level of IL-10 production.

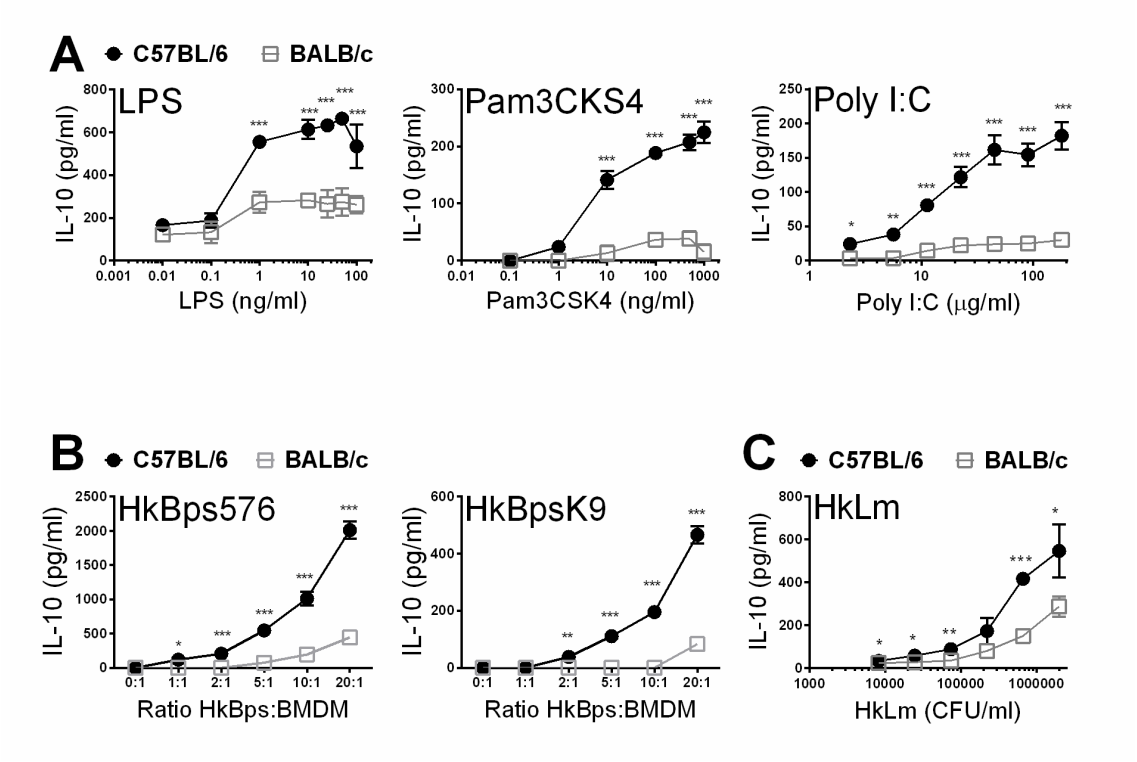


Figure 3.1 C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages when stimulated with LPS, Pam3CSK4, Poly I:C, HkBps or HkLm. BMDM were generated from C57BL/6 and BALB/c mice. Cells were stimulated for 24 h with (A) LPS, Pam3CSK4, or Poly I:C and (B) heat-killed *B.pseudomallei* (HkBps, 576 or K9 isolates) or (C) heat-killed *L.monocytogenes* (HkLm) at the indicated doses. IL-10 protein in supernatants was quantified by ELISA (detection limit 50 pg/ml). Graphs show means ± SD of three cultures. *p<0.05, **p<0.01, ***p<0.001 as determined by Student's t-test at each dose. Representative of two independent experiments.

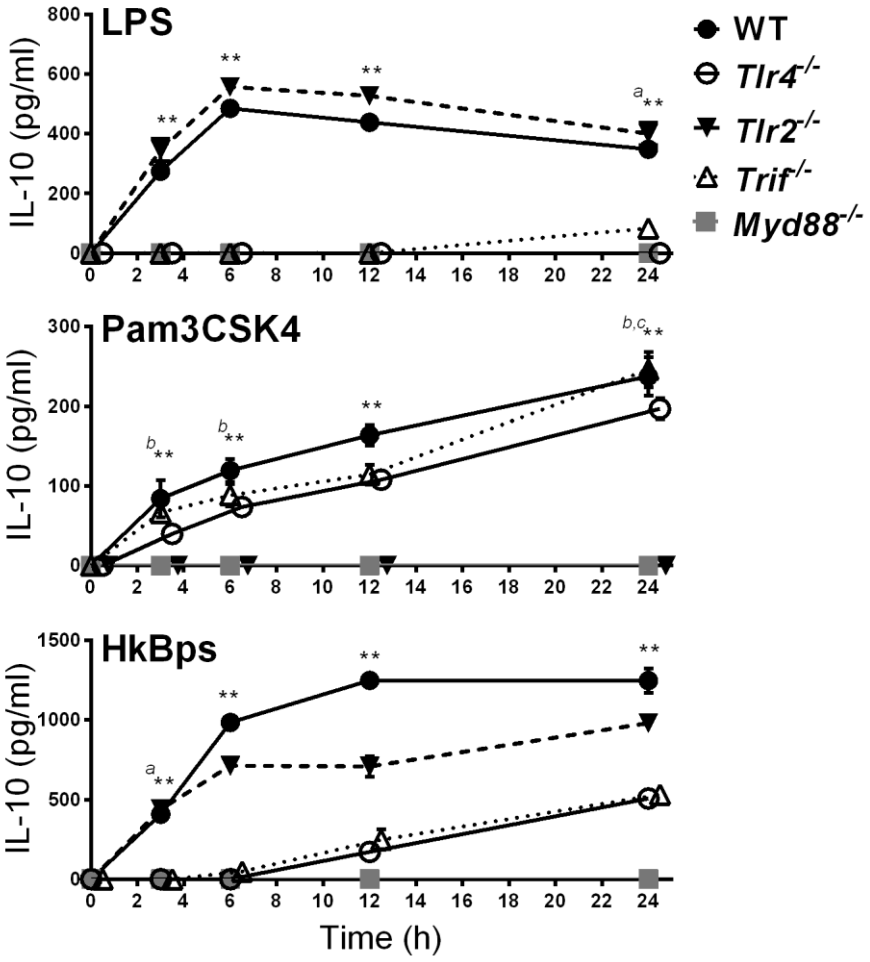


Figure 3.2 TLR4, TLR2, TRIF and MyD88 contribute to IL-10 production in HkBps stimulated macrophages.

BMDM were generated from WT, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ mice, all on a C57BL/6 background. Cells were stimulated with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or HkBps (10 HkBps: 1 BMDM) for the indicated times. IL-10 protein in cell-free supernatants was measured by ELISA (detection limit 50 pg/ml). Graphs show means of three cultures ±SD. Significance (**p<0.01) represents each individual strain compared to C57BL/6 with the following exceptions: [a] *Tlr2*$^{-/-}$; [b] *Trif*$^{-/-}$; [c] *Tlr4*$^{-/-}$, as determined by one-way ANOVA for each time point. Representative of two to three independent experiments.

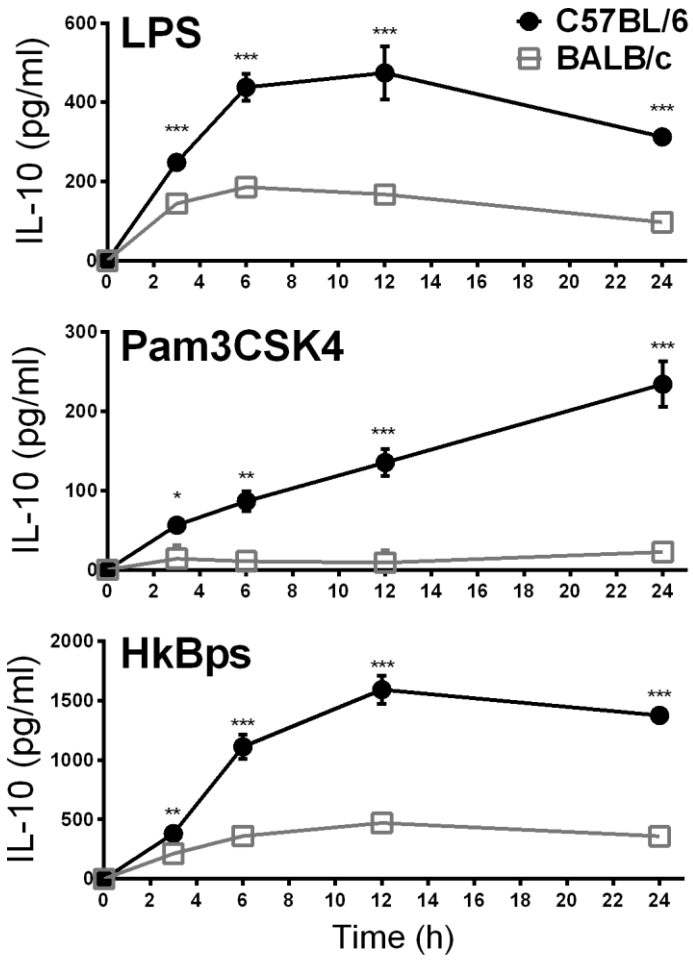


Figure 3.3 LPS, Pam3CSK4 and HkBps stimulated C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages at all time-points during the response.

C57BL/6 and BALB/c BMDM were stimulated with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or HkBps (10 HkBps:1 BMDM) for the indicated times. IL-10 protein was measured in supernatants by ELISA (detection limit 50 pg/ml). Graphs show means ±SD of three cultures. *p<0.05, **p<0.01, ***p<0.001 as determined by Student's t-test at each time point. Representative of six independent experiments.

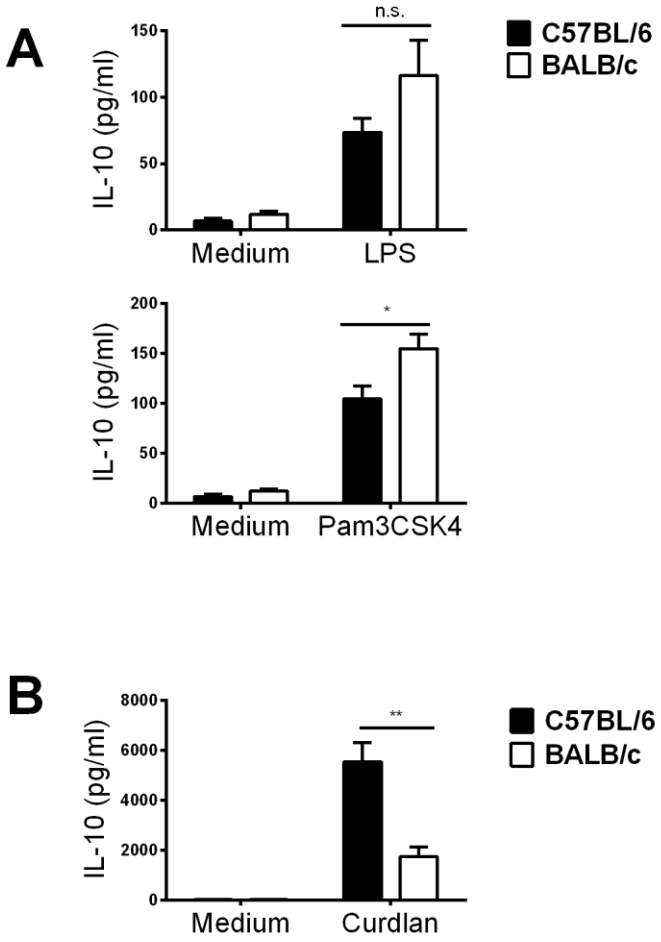


Figure 3.4 C57BL/6 DCs produce higher levels of IL-10 than BALB/c DCs when stimulated with Curdlan, but not LPS or Pam3CSK4.

GM-CSF differentiated BMDC were generated from C57BL/6 and BALB/c mice. Cells were stimulated with (A) LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or (B) Curdlan (200 µg/ml) for 24 h. IL-10 protein in cell free supernatants was measured by ELISA (detection limit 50 pg/ml). Graphs show means ± SD of three cultures. *$p < 0.05$; **$p < 0.01$ as determined Student's *t*-test. Representative of four or more independent experiments.

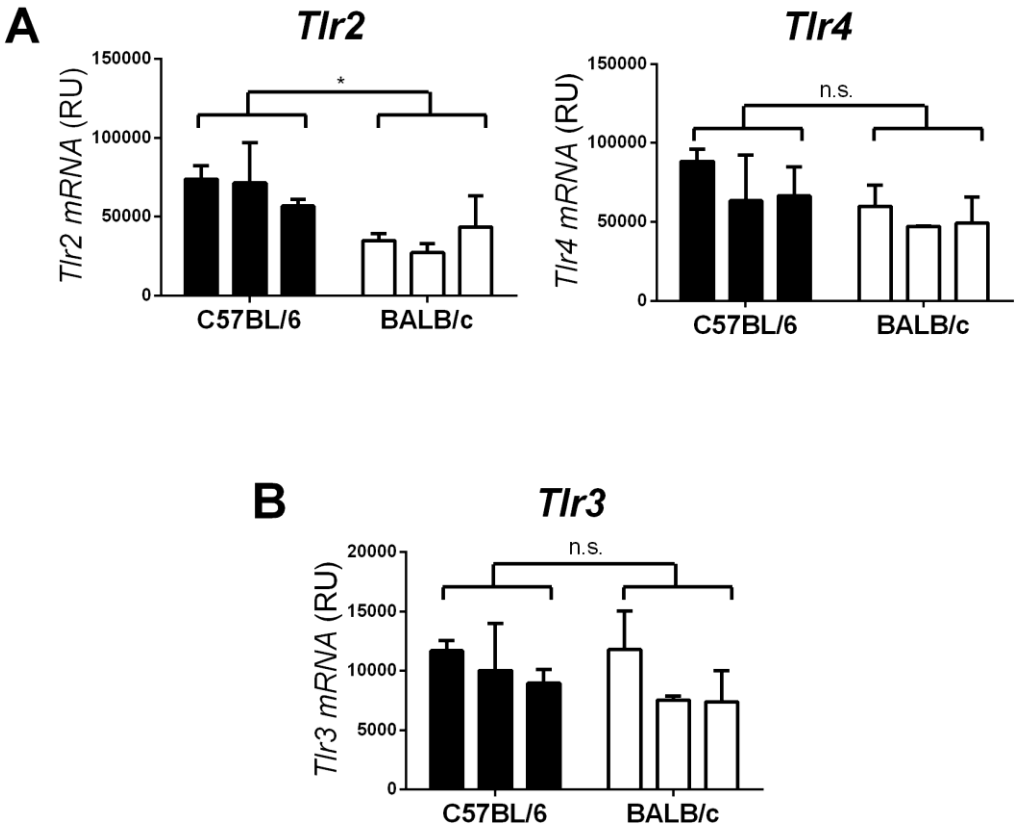


Figure 3.5 The level of *Tlr* mRNA expression in C57BL/6 and BALB/c macrophages does not correlate with IL-10 production.

BMDM were generated from C57BL/6 and BALB/c mice. Total RNA was harvested and isolated from unstimulated cells. *Tlr2*, *Tlr4* (A), and *Tlr3* (B) transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. C57BL/6 and BALB/c cells were cultured on three separate occasions, each represented by one bar. Bars represent mean of triplicate cultures ± SD. *$p<0.05$ as determined by Student's *t*-test.

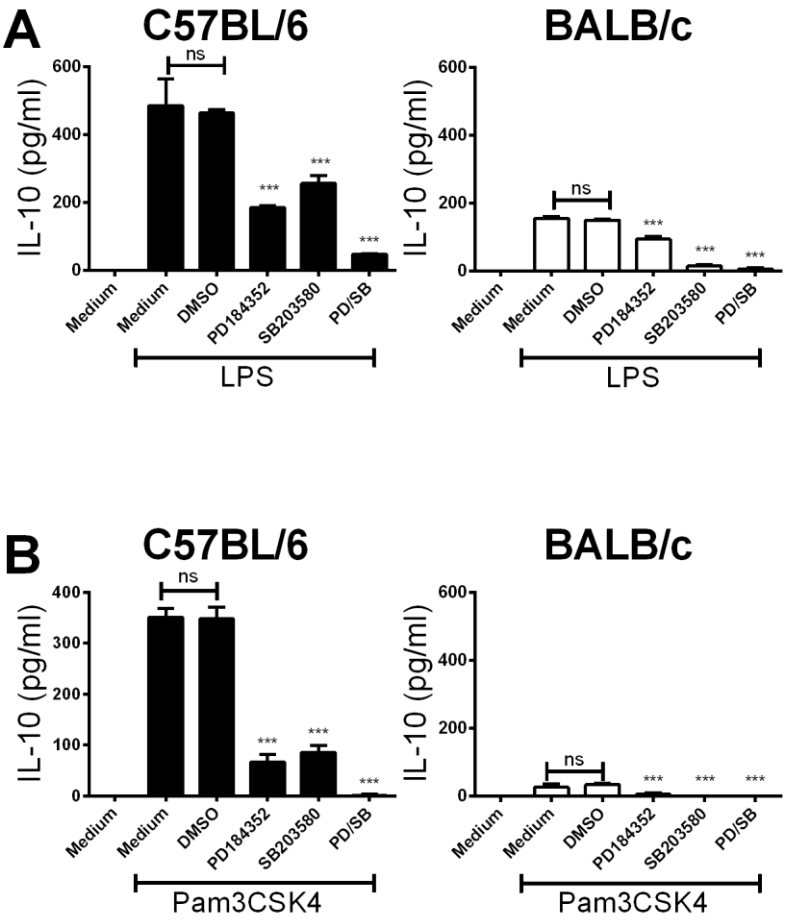


Figure 3.6 ERK and p38 are important regulators of TLR2 and TLR4 induced IL-10 in C57BL/6 and BALB/c macrophages.

BMDM were generated from C57BL/6 (black bars) and BALB/c (white bars) mice and stimulated for 24 hours with (A) LPS (10 ng/ml) or (B) Pam3CSK4 (200 ng/ml) in the presence or absence of the MEK1 (ERK1/2) inhibitor PD184352, p38 inhibitor SB203580 or DMSO (vehicle control). Inhibitors were added 1 hour prior to stimulation. IL-10 protein in supernatants was measured by ELISA (detection limit 50 pg/ml). Graphs show means ± SD of three cultures. ***p<0.001 relative to DMSO control as determined by one-way ANOVA. Representative of three or more (LPS) or two (Pam3CSK4) independent experiments.

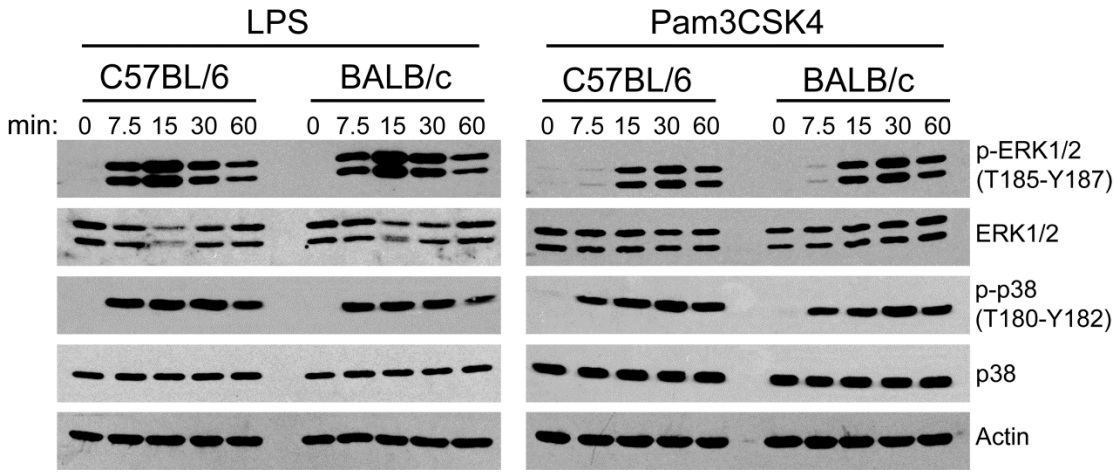


Figure 3.7 LPS and Pam3CSK4 induced phosphorylation of ERK1/2 and p38 is similar in C57BL/6 and BALB/c macrophages.

BMDM were generated from C57BL/6 and BALB/c mice. Cells were stimulated with LPS (10 ng/ml) or Pam3CSK4 (200 ng/ml) for 0, 7.5, 15, 30 or 60 min. Whole cell protein extracts were generated and analysed by Western blot for total and phospho-ERK1/2, and total and phospho-p38. Actin was used as a loading control on each membrane and representative actin blots are shown. Representative of one experiment.

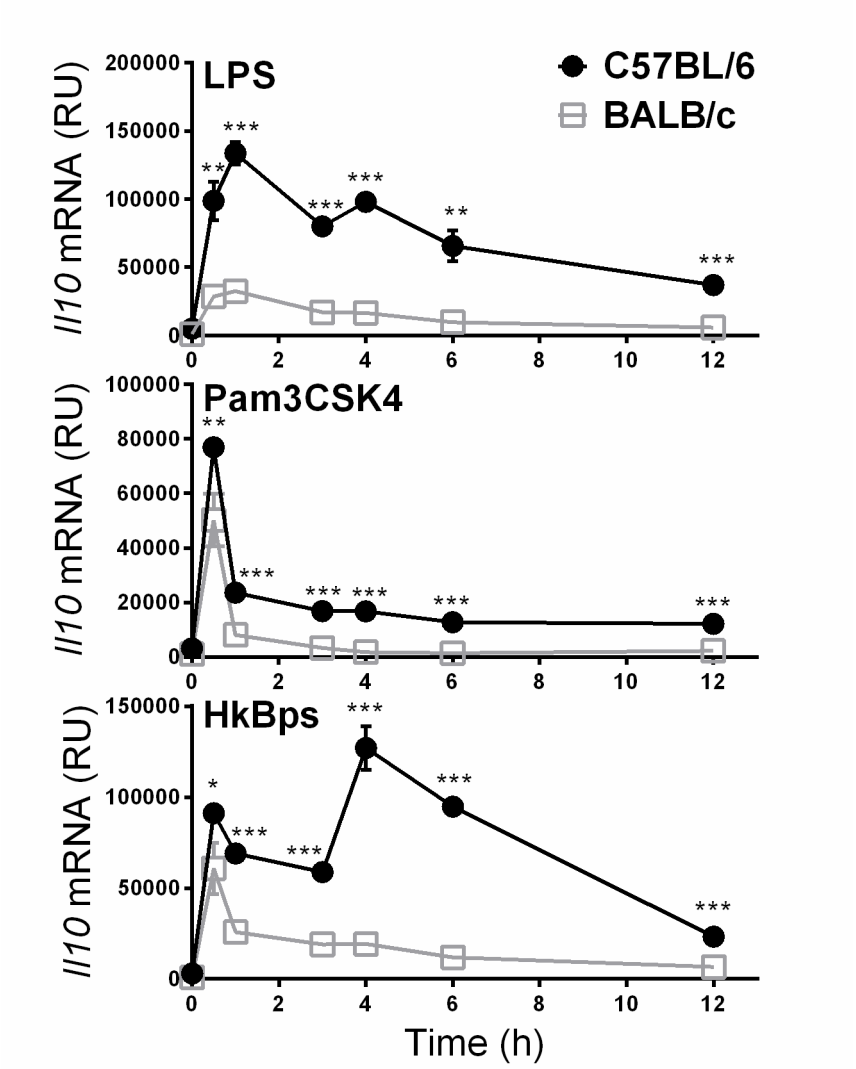


Figure 3.8 *Il10* mRNA expression differs in C57BL/6 and BALB/c macrophages stimulated with LPS, Pam3CSK4 and HkBps.

C57BL/6 and BALB/c BMDM were stimulated with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or HkBps (10 HKBps: 1 BMDM) for the indicated times. Total RNA was harvested and isolated. *Il10* transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. Graphs show means ± SD of three cultures. **p*<0.05, ***p*<0.01, ****p*<0.001 as determined by Student's *t*-test at each time point. Representative of four independent experiments.

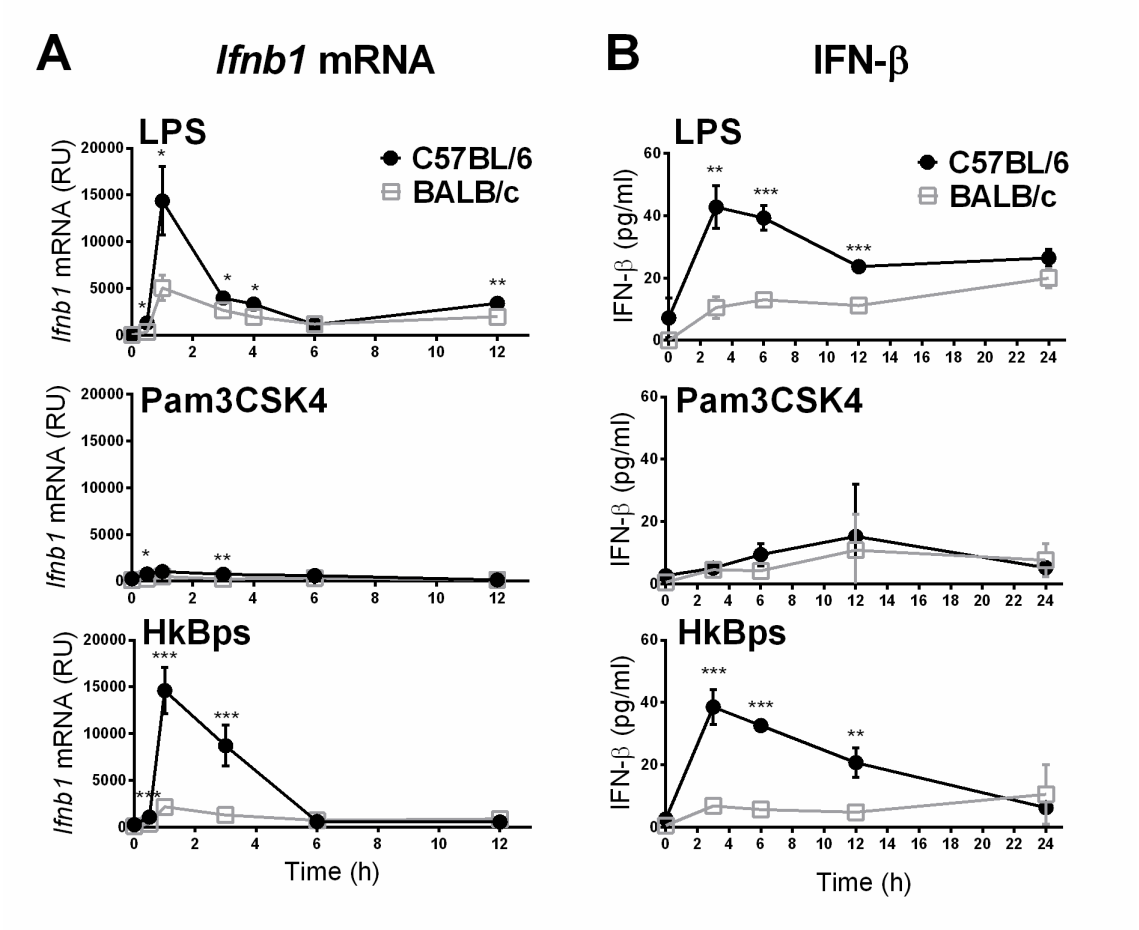


Figure 3.9 LPS and HkBps stimulated C57BL/6 macrophages produce higher levels of *Ifnb1* mRNA and IFN-β protein than BALB/c macrophages.

C57BL/6 and BALB/c BMDM were stimulated for the indicated times with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or HkBps (10 HkBps: 1 BMDM). (A) Total RNA was harvested and isolated. *Ifnb1* transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. (B) IFN-β protein levels in supernatants were determined by ELISA (detection limit 20 pg/ml). Graphs show means ± SD of three cultures. *p<0.05, **p<0.01, ***p<0.001 as determined by Student's t-test at each time point. Representative of at least three independent experiments.

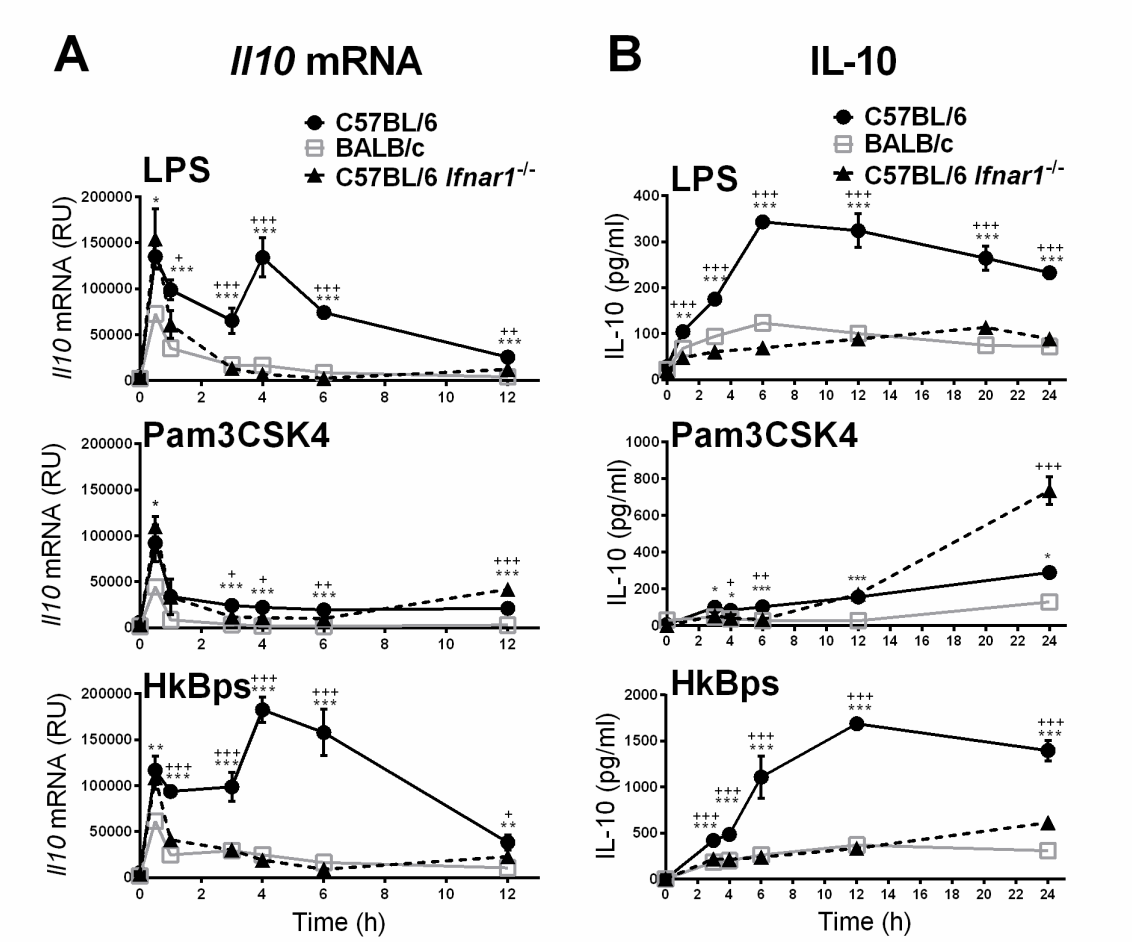


Figure 3.10 The second peak of *Il10* mRNA in LPS and HkBps stimulated C57BL/6 macrophages is dependent on type I IFN signalling, and in the absence of type I IFN signalling, C57BL/6 IL-10 production is similar to BALB/c.

C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDM were stimulated for the indicated times with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or HkBps (10 HkBps: 1 BMDM). (A) Total RNA was harvested and isolated. *Il10* transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. (B) Supernatants were collected at the indicated times and IL-10 was measured by ELISA (detection limit 50 pg/ml). (A and B) Graphs show means of three cultures ±SD. Statistics show significance of C57BL/6 *vs.* BALB/c (*p<0.05 **p<0.01, ***p<0.001) or C57BL/6 *vs.* C57BL/6 *Ifnar1*$^{-/-}$ (^{+}p<0.05, ^{++}p<0.01, ^{+++}p<0.001) as determined by one-way ANOVA for each time point. Representative of two (A) or four (B) independent experiments.

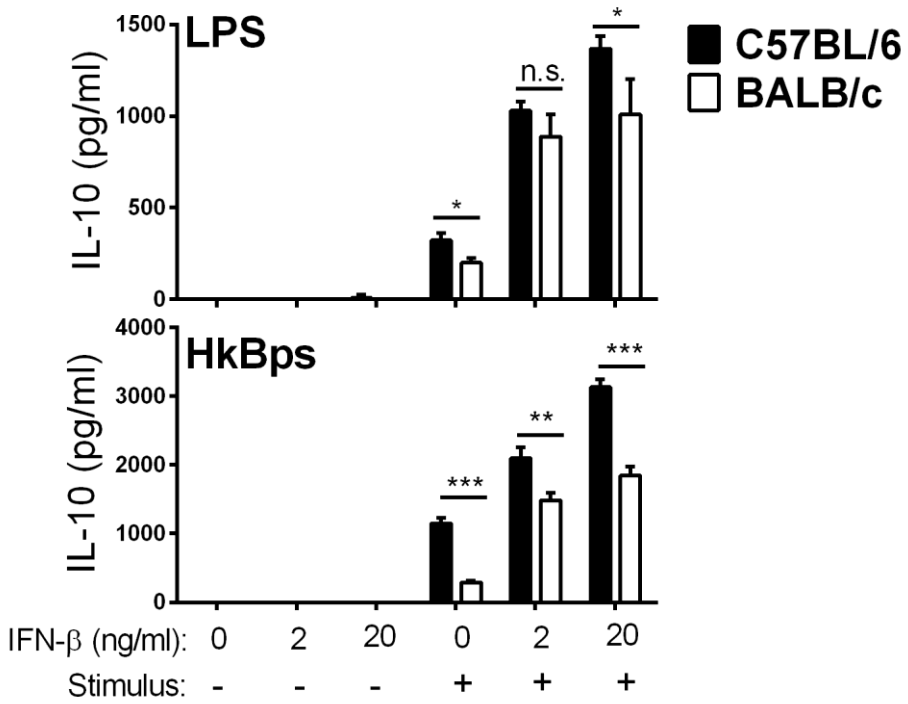


Figure 3.11 Exogenous IFN-β enhances IL-10 production in C57BL/6 and BALB/c macrophages.

C57BL/6 and BALB/c BMDM were treated with 2 or 20 ng/ml IFN-β for 2 h prior to 24 h stimulation with LPS (10 ng/ml) or HkBps (10 HkBps: 1 BMDM). IL-10 protein in supernatants was measured by ELISA (detection limit 50 pg/ml). Graphs show means ±SD of three cultures. *p<0.05, **p<0.01, ***p<0.001 as determined by Student's t-test. Representative of three or more independent experiments.

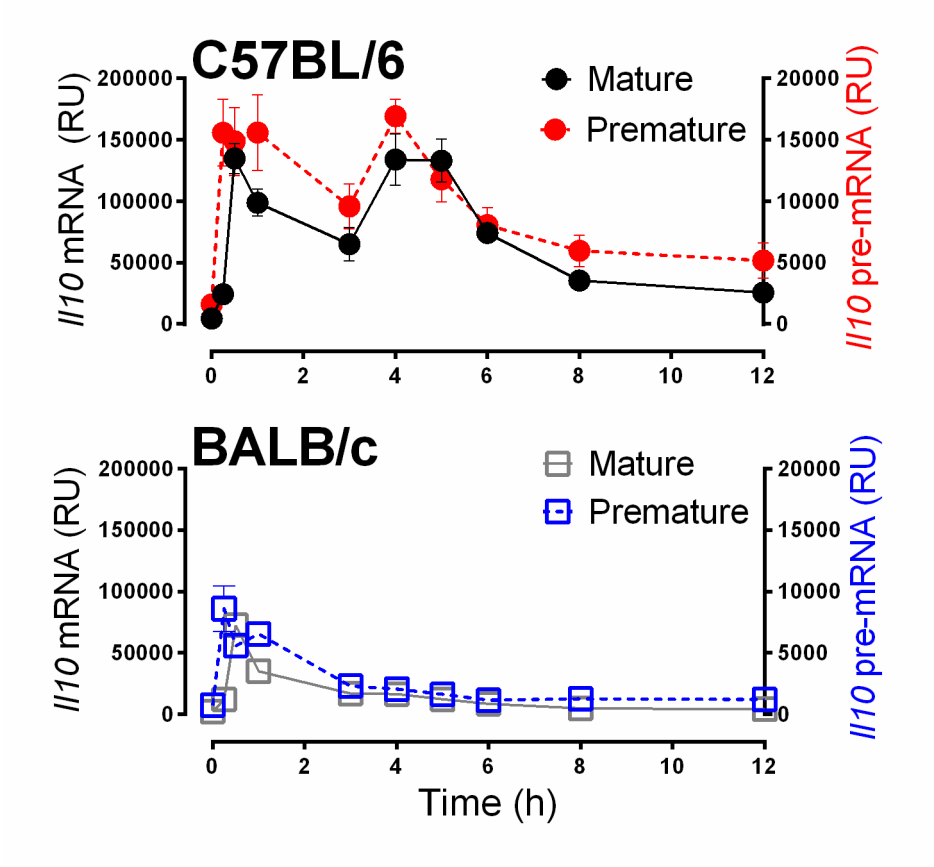


Figure 3.12 Premature *Il10* mRNA follows similar dynamics to mature *Il10* mRNA in LPS stimulated C57BL/6 and BALB/c macrophages.

C57BL/6 (top) and BALB/c (bottom) BMDM were stimulated with LPS (10 ng/ml) for the indicated times. Total RNA was harvested and isolated. Mature and premature *Il10* transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. Graphs show means ± SD of three cultures. Representative of three similar independent experiments.

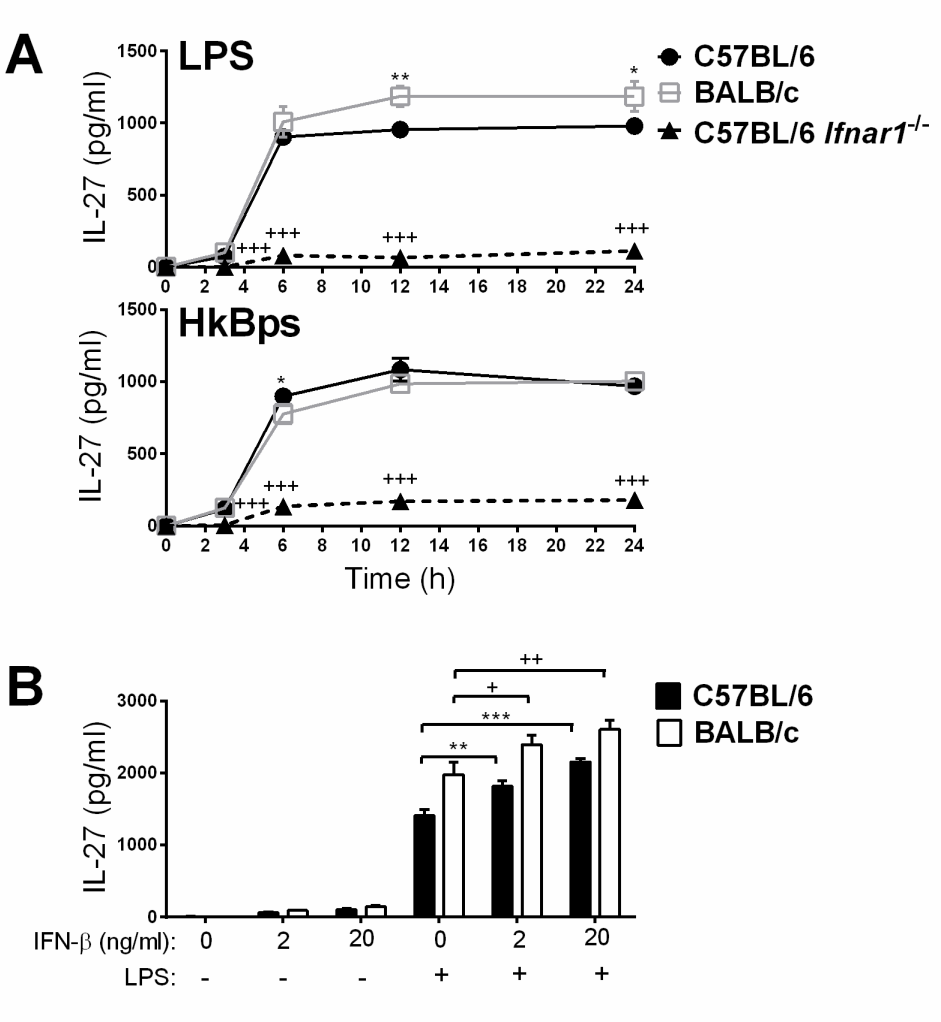


Figure 3.13 IL-27 is induced by LPS and HkBps stimulated C57BL/6 and BALB/c macrophages and is positively regulated by type I IFN.

(A) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages were stimulated for the indicated times with LPS (10 ng/ml) or HkBps (10 HkBps: 1 BMDM). Statistics show significance of C57BL/6 *vs.* BALB/c (**p*<0.05, ***p*<0.01) or C57BL/6 *vs.* C57BL/6 *Ifnar1*$^{-/-}$ (^{+++}p<0.001) as determined by one-way ANOVA for each time point. (B) C57BL/6 and BALB/c BMDM were stimulated for 24 h with LPS (10 ng/ml) in the presence or absence of IFN-β. Statistics compare LPS alone to LPS with 2 or 20 ng/ml IFN-β for C57BL/6 (***p*<0.01; ****p*<0.001) or BALB/c (^{+}p<0.05; ^{++}p<0.01) BMDM as determined by one-way ANOVA. (A and B) IL-27 protein was measured in supernatants by ELISA (detection limit 20 pg/ml). Graphs show means ±SD of three cultures. Representative of four independent experiments.

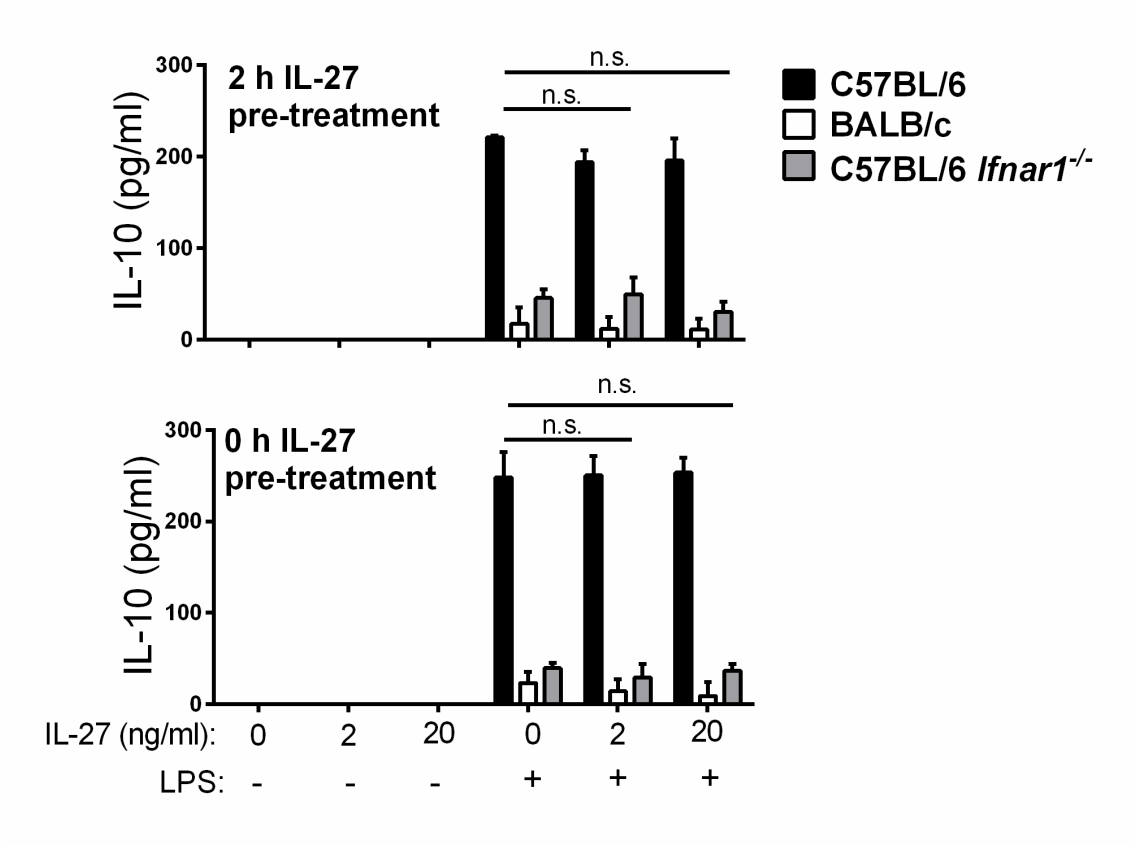


Figure 3.14 The addition of IL-27 does not modulate IL-10 production in C57BL/6, BALB/c or C57BL/6 *Ifnar1*$^{-/-}$ macrophages.

IL-27 was added 2 h prior to stimulation (top) or at the time of stimulation (bottom) to C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDM. Cells were stimulated with LPS for 24 h. IL-10 protein levels in supernatants were measured by ELISA (limit of 50 pg/ml). Graphs show means of three cultures ±SD. Statistics compare LPS alone to LPS with 2 or 20 ng/ml IL-27 for C57BL/6, BALB/c or C57BL/6 *Ifnar1*$^{-/-}$ BMDM. Representative of two independent experiments.

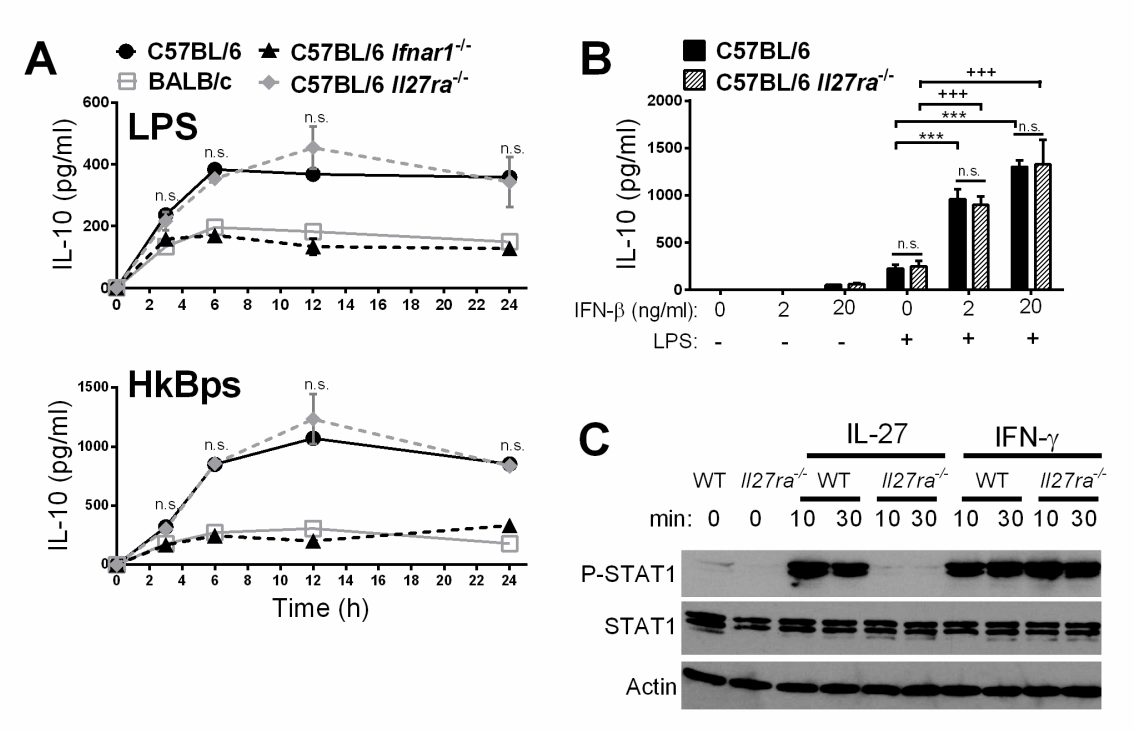


Figure 3.15 IL-10 production is not affected by the absence of IL-27 signalling.
(A) C57BL/6, BALB/c, C57BL/6 *Ifnar1*$^{-/-}$ and C57BL/6 *Il27ra*$^{-/-}$ BMDM were stimulated with LPS (10 ng/ml) or HkBps (500 HkBps: 1 BMDM). Statistics were determined by one-way ANOVA and show significance of C57BL/6 *vs.* C57BL/6 *Il27ra*$^{-/-}$ for each time point. This comparison is representative of three independent experiments. (B) C57BL/6 and C57BL/6 *Il27ra*$^{-/-}$ BMDM were stimulated for 24 h with LPS in the presence or absence of 2 h pre-incubation with IFN-β. Statistics compare LPS alone to LPS with 2 or 20 ng/ml IFN-β for C57BL/6 (***p<0.001) or C57BL/6 *Il27ra*$^{-/-}$ (^{+++}p<0.001) BMDM as determined by one-way ANOVA. Direct C57BL/6 *vs.* C57BL/6 *Il27ra*$^{-/-}$ comparisons for each condition were determined by Student's *t*-test. Representative of three independent experiments. (A and B) IL-10 protein levels in supernatants were measured by ELISA (detection limit 50 pg/ml). Graphs show means of three cultures ±SD. (C) Splenocytes from C57BL/6 WT and C57BL/6 *Il27ra*$^{-/-}$ mice were stimulated with 50 ng/ml IL-27 or 10 ng/ml IFN-γ for 10 or 30 min. Whole cell extracts were generated and analysed by Western blot for total and phospho-STAT1 (Tyr701). Actin was probed for on each membrane as a loading control, a representative Actin blot is shown. Representative of two independent experiments.

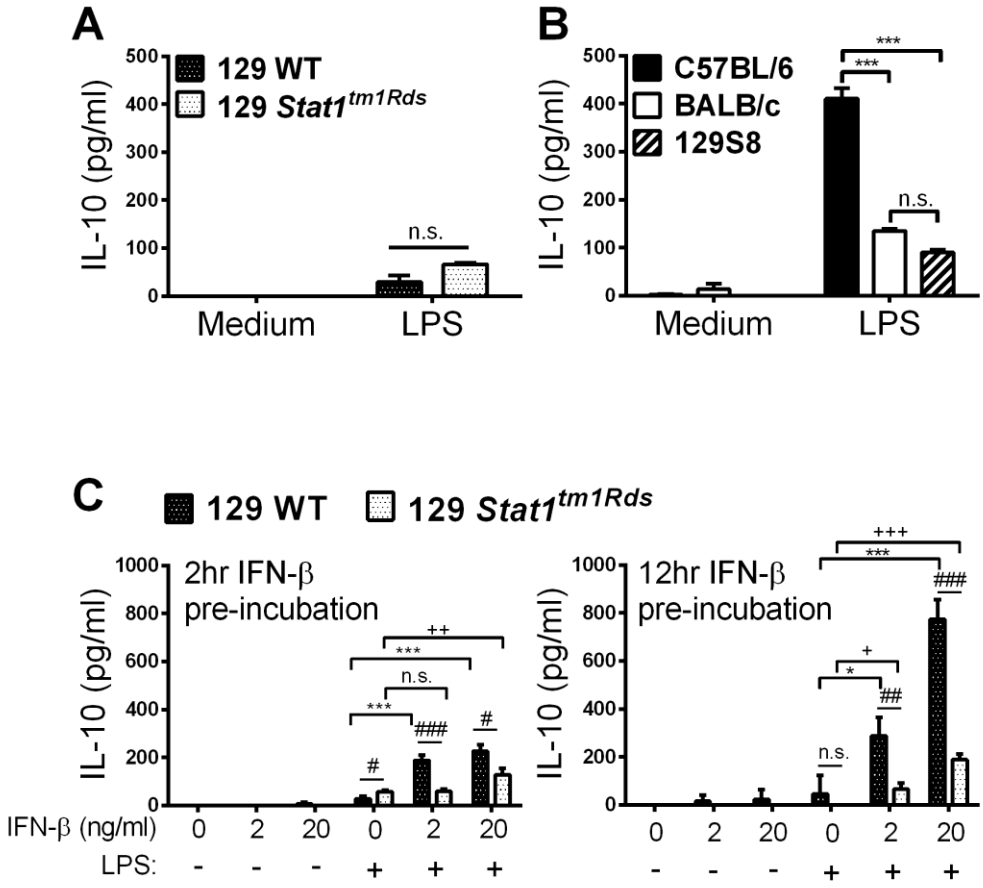


Figure 3.16 The enhancement of IL-10 by IFN-β is predominantly STAT1 dependent.

(A) 129 WT and 129 *Stat1*^{tm1Rds} (both 129S6/SvEv) BMDM were stimulated with LPS (10 ng/ml) for 24 h. (B) C57BL/6, BALB/c and 129 (129S8) BMDM were stimulated with LPS (10 ng/ml) for 24 h. (C) 129 WT and 129 *Stat1*^{tm1Rds} BMDM were stimulated with LPS (10 ng/ml) for 24 h in the presence or absence of 2 or 20 ng/ml IFN-β (2 or 12 h pre-incubation). IL-10 levels in supernatants were measured by ELISA (detection limit 50 pg/ml). Graphs show means ± SD of three cultures. Statistics show (A) n.s. as determined by Student's *t*-test; (B) ***p<0.001 as determined by one-way ANOVA; (C) *p<0.05, ***p<0.001 for WT, or ^{+}p<0.05, ^{++}p<0.01, ^{+++}p<0.001 for *Stat1*^{tm1Rds} BMDM, of LPS alone compared to LPS with 2 or 20 ng/ml IFN-β, as determined by one-way ANOVA. Direct WT and *Stat1*^{tm1Rds} comparison for each condition were determined by Student's *t*-test ($^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001). Representative of two (A, C) or three (B) independent experiments.

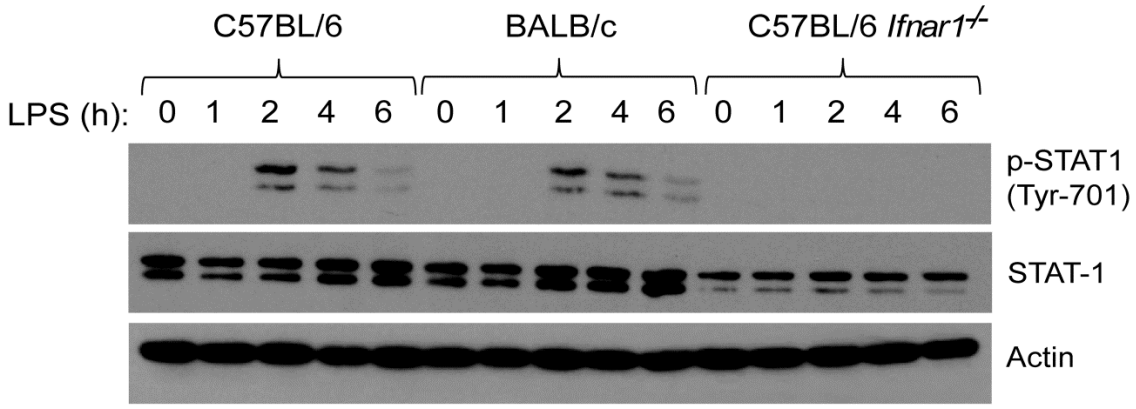


Figure 3.17 STAT-1 is phosphorylated in C57BL/6 and BALB/c macrophages stimulated with LPS and is dependent on type I IFN signalling.

BMDM were generated from C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ mice. Cells were stimulated with LPS (10 ng/ml) for 1, 2, 4 and 6 h. Whole cell protein extracts were generated and analysed by Western blot for total and phospho-STAT1. Actin was used as a loading control on each membrane and a representative actin blot is shown. Representative of two independent experiments.

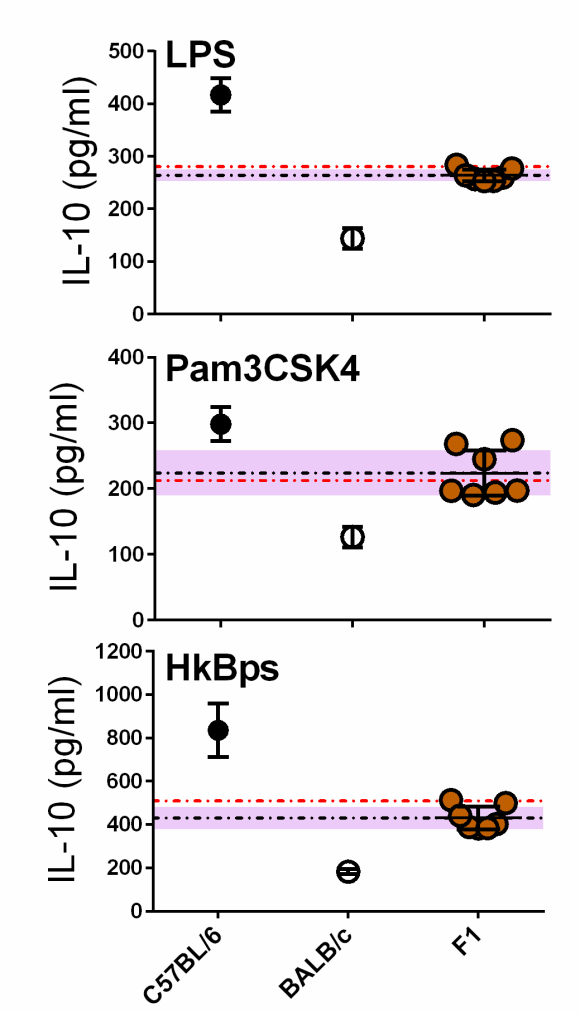


Figure 3.18 C57BL/6 x BALB/c F1 macrophages produce intermediate levels of IL-10.

BMDM were generated from C57BL/6, BALB/c and 7 individual C57BL/6 x BALB/c F1 mice. Cells were stimulated with LPS (10 ng/ml) for 6 h, Pam3CSK4 (200 ng/ml) for 24 h or HkBps (10 HkBps:1 BMDM) for 24 h. IL-10 levels in supernatants were quantified by ELISA (detection limit 50 pg/ml). For C57BL/6 and BALB/c data, graphs show means ± SD of three cultures. For F1 data, each point represents IL-10 levels from macrophages derived from an individual F1 mouse (mean of three cultures). The F1 error bars represent the 95% confidence interval of the F1 population, also represented by pink shading. The black dotted line represents the mean of the F1 population. The red dotted line represents half way between C57BL/6 and BALB/c IL-10 levels. Representative of one experiment.

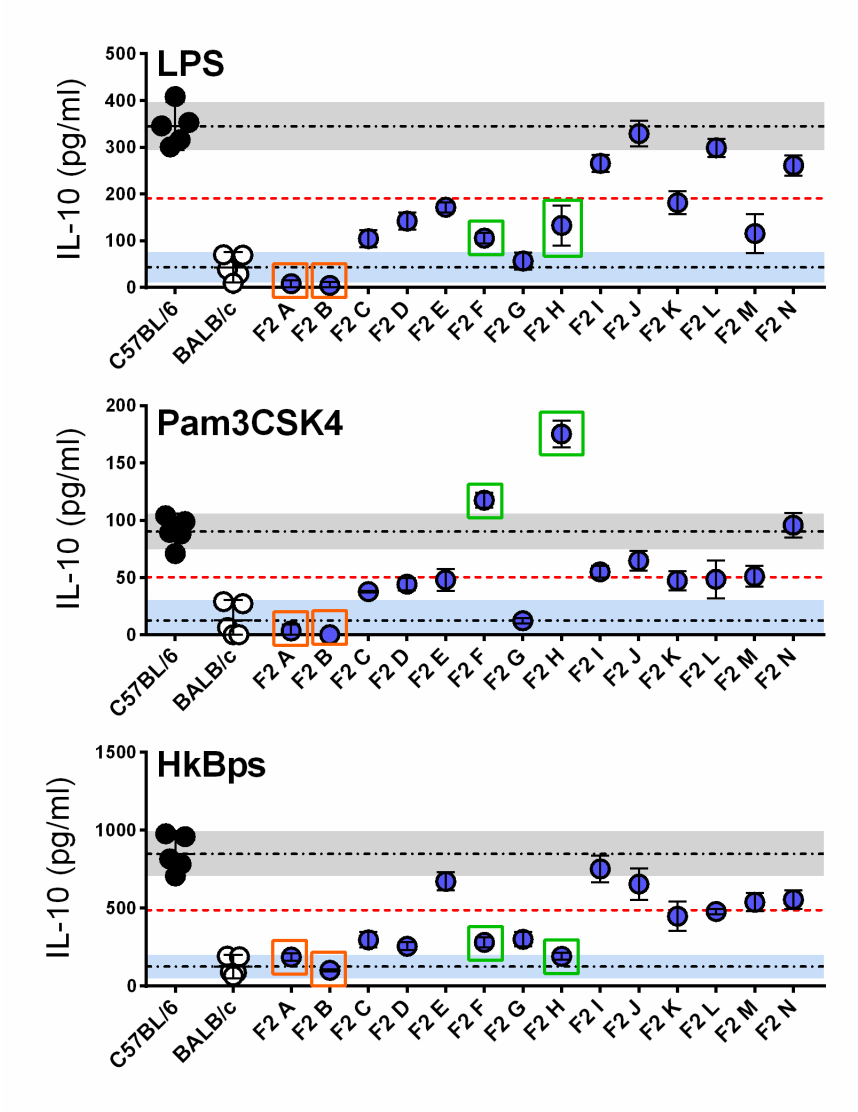


Figure 3.19 C57BL/6 x BALB/c F2 macrophages show segregation in the level of IL-10 production.

BMDM were generated from C57BL/6 (n=5), BALB/c (n=5) and C57BL/6 x BALB/c F2 mice (n=14, labelled A-N). Cells were stimulated with LPS (10 ng/ml) for 6 h, Pam3CSK4 (200 ng/ml) for 24 h, or HkBps (500 HkBps:1 BMDM) for 24 h. IL-10 levels in supernatants were quantified by ELISA (detection limit 50 pg/ml). Each point represents IL-10 levels from macrophages derived from an individual mouse (mean of three cultures). The C57BL/6 and BALB/c error bars represent the 95% confidence interval of the populations also represented by grey (C57BL/6) and blue (BALB/c) shading with population means represented by black dotted lines. Red dotted lines represents half way between C57BL/6 and BALB/c IL-10 levels. For F2 data, graphs show means ±SD of triplicate cultures. Data pooled from two experiments.

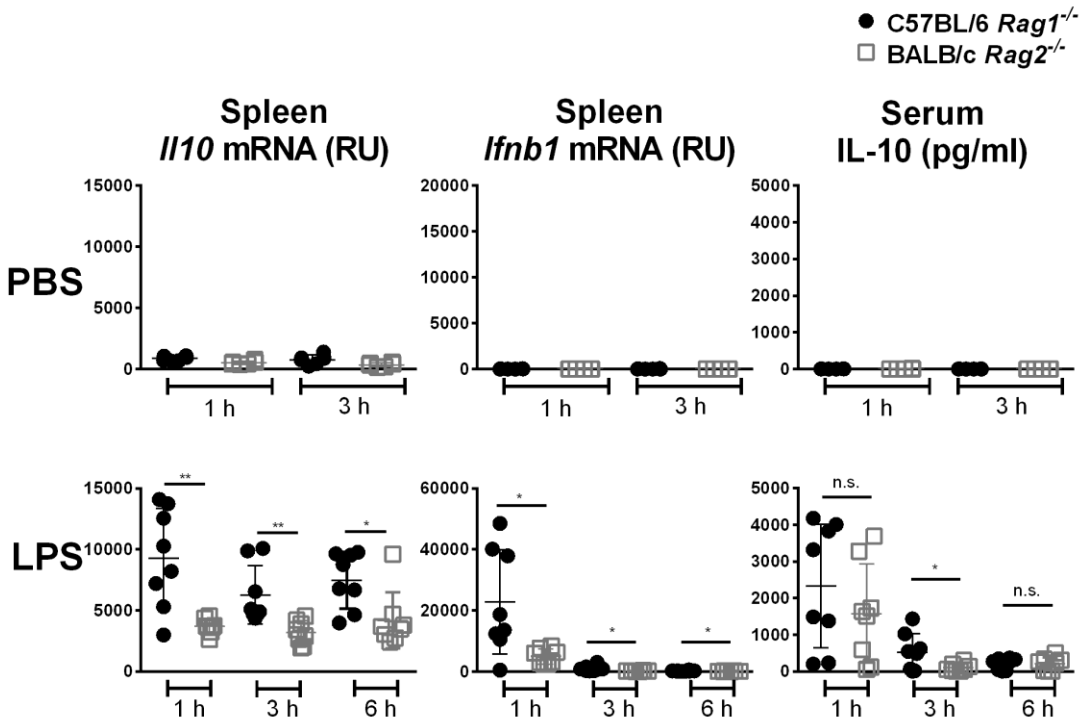


Figure 3.20 LPS treatment induces higher levels of *Il10* and *Ifnb1* mRNA in C57BL/6 compared to BALB/c mice *in vivo*.

C57BL/6 *Rag1*$^{-/-}$ and BALB/c *Rag2*$^{-/-}$ mice were injected i.p. with 150 µg LPS or the equivalent volume of PBS. At the indicated times mice were culled. Spleens were harvested, immediately homogenised in tri-reagent and total RNA was isolated. *Il10* and *Ifnb1* transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. IL-10 levels in the serum were quantified by cytometric bead array (detection limit 10 pg/ml). Graphs show individual mice ± SD (n=6 for PBS group, n=8 for LPS group, data pooled from two experiments). Statistics on LPS treated groups were determined by Student's *t*-test at each time point (**p*<0.05, ***p*<0.001).

Chapter 4. The Regulation of IL-12 in C57BL/6 and BALB/c macrophages

4.1 Background

IL-12 is a proinflammatory cytokine, predominantly made by monocytes, macrophages and DCs in response to microbial products (D'Andrea *et al.* 1992; Macatonia *et al.* 1995; Trinchieri 2003). IL-12 has a central role in promoting immune responses by inducing the differentiation of naïve $CD4^+$ T cells into IFN-γ producing Th1 cells (Hsieh *et al.* 1993; Manetti *et al.* 1993; Macatonia *et al.* 1995). IFN-γ is a potent activator of macrophage antimicrobial mechanisms (Gordon *et al.* 2005), and additionally enhances proinflammatory cytokine production from these cells (Hayes *et al.* 1995). Thus, phagocyte derived IL-12 induces the production of IFN-γ from Th1 cells, which then further activates phagocytic cells, forming a positive feedback loop. IL-12 also has the capacity to induce IFN-γ production from NK cells (Kobayashi *et al.* 1989; Chan *et al.* 1991) and enhance the cytotoxic activities of NK and $CD8^+$ T cells (Gately *et al.* 1994). Through these mechanisms, IL-12 is critical in the generation of protective immune responses particularly against intracellular bacterial and parasitic pathogens including *L.major*, *T.gondii*, *L.monocytogenes*, *M.tuberculosis* and *B.pseudomaelli* (Sypek *et al.* 1993; Gazzinelli *et al.* 1994; Tripp *et al.* 1994; Cooper *et al.* 2002; Haque *et al.* 2006).

IL-12 is a heterodimeric cytokine formed of two subunits, p40 and p35, which generate the active form of IL-12, IL-12p70 (Kobayashi *et al.* 1989). p40 and p35 can also dimerise with other subunits to form the cytokines IL-23 (p19:p40 heterodimer) (Oppmann *et al.* 2000) and IL-35 (Ebi3:p35 heterodimer) (Collison *et al.* 2007; Niedbala *et al.* 2007). Downstream of TLRs, IRF1 and IRF3 have been shown to be involved in the induction of IL-12p35 (Liu *et al.* 2003a; Goriely *et al.* 2006; Negishi *et*

al. 2006). In the case of IL-12p40, IRF5 and IRF8 have been more strongly implicated (Wang *et al.* 2000; Takaoka *et al.* 2005; Zhao *et al.* 2006; Ouyang *et al.* 2007), although roles in the regulation of IL-12p35 have also been described (Liu *et al.* 2004; Krausgruber *et al.* 2011). NF-κB family members, particularly c-Rel, are also important for the regulation of IL-12p40 and IL-12p35 in macrophages and DCs, respectively (Sanjabi *et al.* 2000; Grumont *et al.* 2001). IL-12p35 is further regulated at the post-translational level which plays a role in determining the final level of bioactive IL-12p70 produced by the cell (Carra *et al.* 2000).

Importantly, due to its powerful proinflammatory activity, IL-12 is subject to several negative regulatory mechanisms, without which the host may experience severe immunopathology (Gazzinelli *et al.* 1996; Hunter *et al.* 1997; Kullberg *et al.* 1998). IL-10 is one of the best described inhibitors of IL-12 (D'Andrea *et al.* 1993; Hsieh *et al.* 1993; Trinchieri 2003). The molecular mechanisms of how IL-10 suppresses IL-12 production are not fully known, although they are thought to be at least in part at the level of transcription (Aste-Amezaga *et al.* 1998). IL-10 however is not the only negative regulator of IL-12, with IL-10 independent roles for the MAP kinase ERK (Dillon *et al.* 2004; Kaiser *et al.* 2009), and the PI(3)K pathway (Ohtani *et al.* 2008), also having been described. The MAP kinase p38 has been reported to be an additional negative regulator of IL-12 downstream of TLR activation dependent and independent of IL-10 (Jarnicki *et al.* 2008; Yang *et al.* 2010). This is however in contrast to earlier studies which implicated a role for p38 in the positive regulation of IL-12 in DCs (Lu *et al.* 1999; Agrawal *et al.* 2003).

In addition to the positive regulatory effect of IFN-γ (Hayes *et al.* 1995), IL-12 production can be modulated by other cytokines such as type I IFN. The precise role of type I IFN in the regulation of IL-12 production however, remains incompletely understood as some studies have shown a positive role for type I IFN in the regulation of IL-12 (Gautier *et al.* 2005), whereas others have shown a negative role for type I IFN in the regulation of IL-12 (Cousens *et al.* 1997; Byrnes *et al.* 2001).

Thus, the regulation of IL-12 downstream of TLR activation is highly complex due to the multiple factors which collectively influence IL-12 production, and the need to coordinate IL-12p40 and IL-12p35 expression. Additionally, the mechanisms of IL-12 regulation by other cytokines such as IL-10 which inhibits IL-12, and type I IFN which has context specific effects on IL-12, are incompletely understood.

4.2 Investigating the regulation of IL-12 in C57BL/6 and BALB/c macrophages: Study Aims

We have found that IL-10 is differentially regulated in LPS (TLR4), Pam3CSK4 (TLR2) and heat-killed *B.pseudomallei* (TLR2/4) stimulated C57BL/6 and BALB/c macrophages (Chapter 3). IL-10 was more highly expressed in C57BL/6 macrophages, and in the context of LPS and HkBps, this was dependent on type I IFN signalling, but independent of IL-27. Preliminary data from the O'Garra laboratory suggested that in addition to IL-10, C57BL/6 and BALB/c macrophages may have a differential capacity to produce IL-12 in response to TLR ligands. Thus, due to the important role of IL-10 in the negative regulation of IL-12, and the uncertain role of type I IFN in the regulation of IL-12, we aimed to answer the following questions:

1. Does IL-12 production differ in LPS, Pam3CSK4 or HkBps stimulated C57BL/6 and BALB/c macrophages?
2. If so, is differential IL-12 production a down-stream consequence of differential IL-10 and/or type I IFN production, or due to other mechanism(s)?
3. Are additional proinflammatory cytokines such as TNF-α and IL-1β also differentially regulated in C57BL/6 and BALB/c macrophages?

4.3 Results

4.3.1 IL-12p40 production is not consistently different between C57BL/6 and BALB/c macrophages, and IL-12p70 production is difficult to detect.

In order to determine if C57BL/6 and BALB/c macrophages differentially express IL-12 in response to LPS, Pam3CSK4 or HkBps, IL-12p40 protein production was quantified over a time-course of stimulation (Figure 4.1 A). In LPS stimulated cells, IL-12p40 was generally similar between the strains with the exception of the 24 h time-point when IL-12p40 production was higher in BALB/c macrophages (Figure 4.1 A). In Pam3CSK4 stimulated cells, IL-12p40 production was similar between the strains over the 24 h stimulation period albeit slightly elevated at 6 and 12 h in C57BL/6 macrophages (Figure 4.1 A). IL-12p40 was strongly induced by HkBps but in contrast to the other stimulations, at 12 and 24 h IL-12p40 production was significantly higher in BALB/c compared to C57BL/6 macrophages (Figure 4.1 A). IL-12p40 is only one chain of the IL-12p70 heterodimer (Kobayashi *et al.* 1989), and also heterodimerises with p19 to form IL-23 (Oppmann *et al.* 2000). Thus, in order to more accurately determine IL-12 production, IL-12p70 levels were also quantified (Figure 4.1 B). In LPS and Pam3CSK4 stimulated cells, IL-12p70 protein production was not detectable at any time-point in either strain (Figure 4.1 B). In HkBps stimulated cells however, IL-12p70 production was detectable in BALB/c, but not C57BL/6 macrophages (Figure 4.1 B). This data suggested that BALB/c macrophages have a higher capacity to produce IL-12p70 than C57BL/6 macrophages in response to HkBps, although the trend of IL-12p70 production in response to LPS or Pam3CSK4 was unclear at this time.

4.3.2 LPS, Pam3CSK4 and HkBps induce higher levels of *Il12a* mRNA expression in BALB/c macrophages.

As we were able to detect IL-12p70 production in HkBps stimulated macrophages (Figure 4.1), we postulated that LPS and Pam3CSK4 stimulated macrophages may be able to produce IL-12p70, but that the levels were too low to be detected by ELISA. To gain insight into whether LPS and Pam3CSK4 stimulated macrophages had the potential to produce IL-12p70, *Il12a* mRNA expression (encodes IL-12p35), was quantified over a time-course in LPS, Pam3CSK4 and HkBps stimulated C57BL/6 and BALB/c macrophages (Figure 4.2). Under all three stimulation conditions, *Il12a* mRNA expression was induced in both C57BL/6 and BALB/c macrophages (Figure 4.2). However, at each time-point, BALB/c macrophages expressed significantly higher levels of *Il12a* mRNA than C57BL/6 macrophages (Figure 4.2). C57BL/6 and BALB/c macrophages therefore express both subunits of IL-12p70 in response to LPS and Pam3CSK4, suggesting that IL-12p70 may be induced at a low level in response to these stimuli. Further, the consistently higher level of *Il12a* mRNA expression in BALB/c macrophages suggested that if we were able to detect LPS or Pam3CSK4 induced IL-12p70, it may be higher in BALB/c macrophages.

4.3.3 Higher levels of IL-12p70 in BALB/c macrophages are revealed at low cell density, and are independent of IL-10 in LPS and HkBps stimulated macrophages.

As part of an *in vitro* experimental optimisation process, we carried out a cell density titration of C57BL/6 and BALB/c macrophages from $1x10^6$ cells/ml (the standard cell density which has been used in this study), to $0.25x10^6$ cells/ml, and stimulated the cells

with LPS, Pam3CSK4 and HkBps for 24 h (Figure 4.3 A). Somewhat unexpectedly, in all three stimulation conditions, we observed that as the cell density decreased, IL-12p70 production increased (Figure 4.3 A). This cell density dependent effect was most pronounced in BALB/c macrophages which produced higher levels of IL-12p70 than C57BL/6 macrophages at all cell densities tested (Figure 4.3 A). From this data, we were able to make two conclusions. Firstly, a cell density dependent factor inhibits the production of IL-12p70 in macrophages. Secondly, BALB/c macrophages produce higher levels of IL-12p70 than C57BL/6 macrophages in response to LPS, Pam3CSK4 and HkBps.

We had previously established that C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages (Chapter 3). As IL-10 is a negative regulator of IL-12 (D'Andrea *et al.* 1993; Hsieh *et al.* 1993), we hypothesised that the higher level of IL-10 production in C57BL/6 macrophages could be responsible for their lower level of IL-12p70 production. Further, we postulated that the autocrine regulatory activity of IL-10 may be more effective at high cell density. In this way, IL-10 could be responsible for the cell density dependent effect on IL-12p70 levels. To test these hypotheses, we performed a cell density titration of C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ macrophages. Cells were stimulated with LPS, Pam3CSK4 or HkBps for 24 h (Figure 4.3 B). In the absence of IL-10, overall IL-12p70 levels greatly increased in C57BL/6 and BALB/c macrophages, supporting an important role for IL-10 in the negative regulation of IL-12 (Figure 4.3 B). We also found that in the absence of IL-10, the cell density dependent effect was reversed, with the levels of IL-12p70 generally increasing as the cell density increased (Figure 4.3 B). This suggested that IL-10 was indeed responsible for the

inhibitory cell density dependent effect on IL-12p70. Despite the overall increase in IL-12p70 production however, BALB/c *Il10*$^{-/-}$ macrophages still produced significantly higher levels of IL-12p70 than C57BL/6 *Il10*$^{-/-}$ macrophages when stimulated with LPS or HkBps (Figure 4.3 B). Thus, differential IL-12p70 production in LPS and HkBps stimulated C57BL/6 and BALB/c macrophages remains present in the absence of IL-10. In contrast, in Pam3CSK4 stimulated cells, IL-12p70 production was equivalent in C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ macrophages (Figure 4.3 B), suggesting that differential IL-12p70 production in response to this stimulus is solely a consequence of differential IL-10 production.

4.3.4 IL-12p70 production in LPS or HkBps stimulated C57BL/6 macrophages is not affected by endogenous type I IFN.

In addition to IL-10, we had also previously established that C57BL/6 macrophages produce higher levels of IFN-β than BALB/c macrophages in response to LPS and HkBps (see Figure 3.9). Type I IFN has been reported to modulate IL-12 production in human and murine innate cell types (Byrnes *et al.* 2001; Gautier *et al.* 2005). However, whether type I IFN promotes or inhibits IL-12 production appears to depend on the context (Lyakh *et al.* 2008). We sought to determine if type I IFN, other than through the promotion of IL-10, was affecting IL-12p70 production in C57BL/6 macrophages. To test this, we investigated the production of LPS and HkBps induced IL-12p70 production in C57BL/6 *Ifnar1*$^{-/-}$ treated with an antibody which blocks signalling from the IL-10 receptor (α-IL-10R) (Figure 4.4). We compared this IL-12p70 production to that of C57BL/6 and BALB/c WT and *Il10*$^{-/-}$ macrophages. C57BL/6 macrophages treated with α-IL-10R were also included to ensure that blockade of the IL-10 receptor

had a similar impact on IL-12p70 production as genetic *Il10* deletion (Figure 4.4). This experiment was carried out at low cell density to optimise for IL-12p70 production. As previously demonstrated (see Figure 4.3 A), LPS and HkBps stimulated C57BL/6 macrophages produced barely detectable levels of IL-12p70 (Figure 4.4). Treatment with α-IL-10R or genetic ablation of *Il10*, increased IL-12p70 production by a similar amount in C57BL/6 macrophages (Figure 4.4). C57BL/6 *Ifnar1*$^{-/-}$ macrophages produced similar levels of IL-12p70 to C57BL/6 macrophages in response to LPS or HkBps (Figure 4.4). Additional blockade of IL-10 receptor signalling did enhance IL-12p70 production in LPS and HkBps stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages, but not beyond the levels observed in C57BL/6 *Il10*$^{-/-}$ macrophages (Figure 4.4). Treatment with the isotype control antibody for the α-IL-10R had very little impact on IL-12p70 production from C57BL/6 or C57BL/6 *Ifnar1*$^{-/-}$ macrophages (Figure 4.4). Finally, although the levels of IL-12p70 produced by C57BL/6+α-IL-10R, C57BL/6 *Il10*$^{-/-}$ and C57BL/6 *Ifnar1*$^{-/-}$+α-IL-10R macrophages were now similar to those of BALB/c WT, BALB/c *Il10*$^{-/-}$ macrophages produced markedly higher levels of IL-12p70 than all other groups in response to both LPS and HkBps (Figure 4.4). Thus, IL-12p70 production in C57BL/6 *Il10*$^{-/-}$ macrophages remains lower than in BALB/c *Il10*$^{-/-}$ macrophages, even in the absence of type I IFN signalling, suggesting that autocrine type I IFN signalling does not appear to affect IL-12p70 production in this system.

4.3.5 BALB/c macrophages produce higher levels of TNF-α and IL-1β in response to LPS, Pam3CSK4 and HkBps.

We sought to investigate if this trend of higher proinflammatory cytokine production was specific to IL-12p70, or was common across other proinflammatory cytokines such

as TNF-α and IL-1β. C57BL/6 and BALB/c macrophages were stimulated with LPS, Pam3CSK4 and HkBps over a time-course and TNF-α and IL-1β production were quantified (Figure 4.5). Unlike IL-12p70, both cytokines were readily detectable at normal cell density ($1x10^6$ cells/ml) (Figure 4.5). In LPS stimulated macrophages, TNF-α production peaked by 6 h post-stimulation and was consistently higher in BALB/c than C57BL/6 macrophages up to 24 h (Figure 4.5 A). In Pam3CSK4 and HkBps stimulated cells, TNF-α production peaked at 12 h post-stimulation, and again was consistently higher in BALB/c than C57BL/6 macrophages (Figure 4.5 A). Similarly, IL-1β production was consistently higher in BALB/c macrophages relative to C57BL/6 macrophages under all three stimulation conditions (Figure 4.5 B).

4.3.6 IL-10 accounts for differential proinflammatory cytokine production in response to Pam3CSK4 but not LPS or HkBps.

As IL-10 can inhibit the production of proinflammatory cytokines from macrophages (Fiorentino *et al.* 1991a), we wanted to determine to what extent IL-10 was responsible for the differential TNF-α and IL-1β production in LPS, Pam3CSK4 and HkBps stimulated C57BL/6 and BALB/c macrophages. C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ macrophages were stimulated with LPS, Pam3CSK4 or HkBps for 24 h and the production of these cytokines was quantified (Figure 4.6). We observed that LPS, Pam3CSK4 and HkBps induced TNF-α production was equivalent in C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ macrophages (Figure 4.6 A). In contrast, IL-1β production remained higher in BALB/c *Il10*$^{-/-}$ macrophages than C57BL/6 *Il10*$^{-/-}$ macrophages in response to LPS and HkBps (Figure 4.6 B). In Pam3CSK4 stimulated cells however, C57BL/6 and BALB/c IL-1β production was equivalent in the absence of IL-10 (Figure 4.6 B). Thus,

differential TNF-α production is a consequence of differential IL-10 production in response to all three stimuli whereas reminiscent to IL-12p70 (Figure 4.3 B), the differential production of IL-1β is only dependent on IL-10 in Pam3CSK4 stimulated macrophages.

4.3.7 Type I IFN may contribute to differential IL-1β production in LPS and HkBps stimulated C57BL/6 and BALB/c macrophages.

Differential IL-10 production in LPS and HkBps stimulated C57BL/6 and BALB/c macrophages did not account for differential IL-1β production (Figure 4.6 B). However, type I IFN has been reported to inhibit IL-1β production in the context of LPS stimulation (Guarda *et al.* 2011) and *M.tuberculosis* infection (Mayer-Barber *et al.* 2011). This prompted us to assess if type I IFN was affecting IL-1β production in our system. C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages were stimulated with LPS or HkBps over a time-course, and IL-1β production was quantified (Figure 4.7). In LPS stimulated cells, IL-1β production was enhanced in C57BL/6 *Ifnar1*$^{-/-}$ macrophages relative to C57BL/6 WT, and was overall similar to the levels produced by BALB/c macrophages (Figure 4.7). In HkBps stimulated macrophages, IL-1β was again elevated in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 WT, but remained slightly lower than the level of IL-1β produced by BALB/c macrophages. This data shows that type I IFN can inhibit IL-1β production in our system. Thus, type I IFN may be contribute to differential IL-1β production in LPS or HkBps stimulated C57BL/6 and BALB/c macrophages.

4.4 Discussion

IL-12 is a proinflammatory cytokine with important roles in driving the differentiation of IFN-γ producing Th1 cells and IFN-γ production from NK cells (Trinchieri 2003). Our previous work identified that C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages when stimulated with LPS, Pam3CSK4 and heat-killed *B.pseudomalli*. In the context of LPS and heat-killed *B.pseudomallei*, this was dependent on type I IFN signalling. Our investigation into relative IL-12 production from these macrophages revealed that IL-12p70, the biologically active form of IL-12, is more highly expressed in BALB/c macrophages. Differential IL-12p70 production was dependent on IL-10 in the context of Pam3CSK4 stimulation. However, LPS and heat-killed *B.pseudomallei* stimulated C57BL/6 macrophages continued to show reduced levels of IL-12p70 production compared BALB/c macrophages in the absence of IL-10 and this was not further affected by autocrine type I IFN. We additionally investigated TNF-α and IL-1β production in these cells and found they were more highly expressed in BALB/c macrophages. In all cases, differential TNF-α production was dependent on IL-10. Differential IL-1β production was only dependent on IL-10 in the context of Pam3CSK4 stimulation. Collectively, these results show that in comparison to BALB/c macrophages, C57BL/6 macrophages have a striking inability to produce substantial levels of IL-12p70 and potentially IL-1β, particularly in response to TLR4 stimulation.

4.4.1 Differential IL-12 production in C57BL/6 and BALB/c macrophages and the effect of cell density.

In our initial experiments, conducted with cells at a density of $1x10^6$ cells/ml, we were unable to detect IL-12p70 production in response to LPS or Pam3CSK4 in either C57BL/6 or BALB/c macrophages. Difficulty in detecting IL-12p70 production from macrophages or DCs stimulated with single TLR ligands is well documented (Trinchieri 2003). It is also reported that the stimulation of DCs with multiple TLR ligands, particularly combinations targeting both MyD88 and TRIF dependent pathways, enhances the production of IL-12p70 (Gautier *et al.* 2005). In keeping with this, we observed that *B.pseudomallei*, which activates TLR2 (MyD88), TLR4 (Myd88/TRIF) and potentially other TLR ligands, induced a higher level of IL-12p70 than TLR2 and TLR4 stimulation alone. However, even under these conditions, IL-12p70 production was only detectable in BALB/c macrophages. Further experiments revealed that IL-12 production can be enhanced in both C57BL/6 and BALB/c macrophages by reducing the cell culture density. We additionally found that the inhibitory cell density dependent effect required the presence of IL-10, potentially attributable to a reduced production of IL-10 at low cell density, although this will require further clarification. Nevertheless, these findings highlight the sensitivity of IL-12 regulatory mechanisms and how they can be significantly altered by cell culture conditions. Regardless, throughout these experiments, in LPS and heat-killed *B.pseudomallei* stimulated cells, IL-12p70 production was higher in BALB/c macrophages compared to C57BL/6, even in the absence of IL-10. We have therefore identified two potential causes for the historical difficulty in detecting IL-12p70 production from TLR stimulated murine macrophages and DCs *in vitro*, i) the density of the cell culture (although this may be difficult to

assess as many publications omit this information), and ii) the majority of investigators use cells derived from C57BL/6 mice.

4.4.2 Mechanisms of differential IL-12 production in C57BL/6 and BALB/c macrophages.

The finding that differential IL-12p70 production was independent of IL-10 in TLR4 stimulated cells but dependent on IL-10 in TLR2 stimulated cells, suggests that the MyD88-independent signalling pathway, TRIF, may be contributing to elevated levels of IL-12p70 production in LPS and heat-killed *B.pseudomallei* stimulated BALB/c macrophages. In TLR3/4 stimulated macrophages, the TRIF pathway is essential for the induction of type I IFN (Yamamoto *et al.* 2003a) which has been shown to both inhibit (Cousens *et al.* 1997; Byrnes *et al.* 2001) and promote (Gautier *et al.* 2005) IL-12 in different contexts. In our system however, endogenous type I IFN did not affect IL-12p70 production in the presence or absence of IL-10, as shown by the similar level of IL-12p70 production from C57BL/6 *Il10*$^{-/-}$ macrophages and C57BL/6 *Ifnar1*$^{-/-}$ macrophages treated with a blocking α-IL-10R antibody. Given the substantial decrease in IL-10 production in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 WT macrophages that we previously observed, we were surprised to find that the absence of type I IFN signalling had no effect on IL-12p70 regulation. This may suggest that only a small amount of IL-10 is required to maximally inhibit IL-12 production in C57BL/6 macrophages. This may also be the case in BALB/c macrophages given the dramatic increase in IL-12p70 production upon the removal of IL-10, despite a relatively small amount of IL-10 being produced in BALB/c WT cells.

Although clearly independent of endogenous type I IFN, we still postulate that the mechanisms underlying differential IL-12p70 production in LPS and heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages may be due to the TRIF signalling pathway for two reasons. Firstly, the earlier observation that type I IFN is differentially expressed in C57BL/6 and BALB/c macrophages is indicative of differences in TRIF pathway activity. Secondly, as IL-12p40 production does not consistently correlate with IL-12p70 production in our experiments, and IL-12p35 production is generally considered the limiting factor IL-12p70 production (Snijders *et al.* 1996), we hypothesise that strain differences in *Il12a* (IL-12p35) expression may underlie differential IL-12p70 production. Further, the TRIF pathway, via the activation of IRF3, has been reported to regulate *Il12a* expression in LPS stimulated murine DC (Goriely *et al.* 2006). Thus, collectively our data may indicate differential activity of the TRIF pathway and potentially IRF3 in TLR4 stimulated C57BL/6 and BALB/c macrophages. Analysis of IL-12p70 production from TLR3 stimulated C57BL/6 and BALB/c macrophages which recruit only TRIF (Yamamoto *et al.* 2003a) may help to further establish if this is the case. Of note, if present, differences in this pathway may be complex as TRIF positively regulates *Ifnb1* and *Il12a*, which are oppositely expressed in C57BL/6 and BALB/c macrophages.

4.4.3 Mechanisms of differential TNF and IL-1β production in C57BL/6 and BALB/c macrophages

The absence of differential TNF-α and IL-1β production in Pam3CSK4 stimulated IL-10 deficient C57BL/6 and BALB/c macrophages further indicated that the potential to induce proinflammatory cytokines downstream of TLR2 was equivalent in C57BL/6

and BALB/c macrophages, provided that IL-10 is absent. In LPS and heat-killed *B.pseudomallei* stimulated cells, TNF-α production was also equivalent in C57BL/6 and BALB/c macrophages in the absence of IL-10. Thus, the positive regulatory signals that govern TNF-α production may be similarly activated in C57BL/6 and BALB/c macrophages. However, the regulation of TNF-α is complex involving transcriptional mechanisms, post-transcriptional mechanisms and processing of pre-TNF-α protein at the cell surface (Raabe *et al.* 1998; Dumitru *et al.* 2000; Rousseau *et al.* 2008). Thus, it is difficult to determine at this stage if all of these processes are happening equivalently in C57BL/6 and BALB/c macrophages in the absence of IL-10, or if different steps are happening at different rates, ultimately leading to a similar level of TNF-α protein production.

IL-1β remained differentially expressed in LPS and heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages in the absence of IL-10. In contrast to what we observed with IL-12p70 however, IL-1β production was enhanced in C57BL/6 *Ifnar1*$^{-/-}$ macrophages relative to C57BL/6 WT. Type I IFN may therefore have a role in mediating the differential production of IL-1β in LPS and heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages. The negative regulation of IL-1β by type I IFN in the context of LPS stimulated and *M.tuberculosis* infected macrophages has been reported to be partially dependent on IL-10 (Guarda *et al.* 2011; Mayer-Barber *et al.* 2011). Thus, the increase in IL-1β production that we observe in C57BL/6 *Ifnar1*$^{-/-}$ macrophages is likely to be at least in part due to a decrease in IL-10 production in the absence of type I IFN signalling. Further experiments are required to determine if type I IFN inhibits IL-1β through IL-10 dependent and/or independent mechanisms in our

system. Additionally, a direct comparison between IL-1β production in BALB/c *Il10*$^{-/-}$ macrophages and C57BL/6 *Ifnar1*$^{-/-}$ cells in which IL-10 signalling has been blocked, will ultimately determine if IL-10 and type I IFN are solely responsible for differential IL-1β production in LPS and heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages.

4.4.4 Differential proinflammatory cytokine production in C57BL/6 and BALB/c macrophages in the context of infection and inflammatory diseases

Our central observation that BALB/c macrophages produce higher levels of IL-12 than C57BL/6 macrophages may initially seem at odds with the C57BL/6 Th1 and BALB/c Th2 dogma that has been associated with *L.major* infection (Sacks *et al.* 2002). In this infection model, the non-healing phenotype of BALB/c mice can be ameliorated with the administration of IL-12 (Heinzel *et al.* 1993; Sypek *et al.* 1993), suggesting that BALB/c mice may be defective in their IL-12 production. Importantly, DCs and not macrophages, as we have studied here, have been suggested to be the important source of IL-12 in this infection model (von Stebut *et al.* 2000). However, even taking this into account, studies have shown that the mechanisms underlying the development of Th1 or Th2 responses against this pathogen are complex. For example, Langerhans cell-like DCs derived from C57BL/6 and BALB/c mice and incubated with *L.major in vitro* induced a higher level of IL-12p70 production from BALB/c DCs compared to C57BL/6 DCs in the presence and absence of IFN-γ (von Stebut *et al.* 2000). Genetic differences affecting the T cell compartment may also contribute to the development of Th1 or Th2 responses in susceptible and resistant strains (Hsieh *et al.* 1995). Our findings are therefore not necessarily in disagreement with the C57BL/6 and BALB/c

differences associated with *L.major* infection. In the case of *B.pseudomallei* infection which is more closely linked to our study, no clear Th1/Th2 cytokine profile is seen in C57BL/6 and BALB/c mice (Ulett *et al.* 2000a), and higher proinflammatory cytokine production in BALB/c mice has been associated with the enhanced disease of this strain (Tan *et al.* 2008). Our C57BL/6 and BALB/c *in vitro* macrophage cytokine profiles findings may therefore be in keeping with the broader cytokine profiles of *B.pseudomallei* infected C57BL/6 and BALB/c mice (Ulett *et al.* 2000b; Ulett *et al.* 2000a; Tan *et al.* 2008). Finally, IL-10 deficient BALB/c mice are more susceptible to enterocolitis than IL-10 deficient C57BL/6 mice (Berg *et al.* 1996). As this disease is associated with elevated production of IL-1α, TNFα, IL-6 and IFN-γ, which is often induced by IL-12 (Berg *et al.* 1996; Trinchieri 2003), this observation may be in keeping with our findings that BALB/c macrophages have a higher capacity to produce proinflammatory cytokines than C57BL/6 macrophages in the absence of IL-10. Thus, the relative deficiency in IL-12p70 and potentially IL-1β production from C57BL/6 macrophages compared to BALB/c macrophages that we observe, even in the absence of IL-10, may have consequences for downstream immune responses in the setting of infectious and inflammatory diseases.

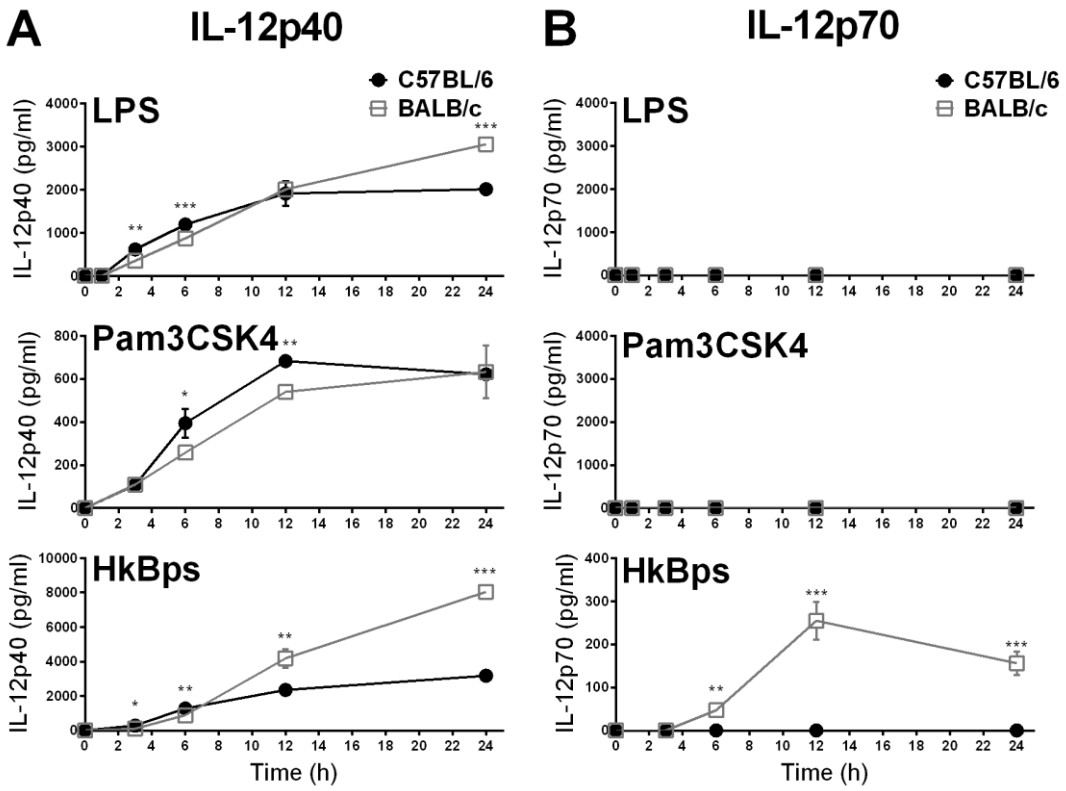


Figure 4.1 Kinetics of IL-12p40 and IL-12p70 production in C57BL/6 and BALB/c macrophages.

BMDM were generated from C57BL/6 and BALB/c mice and stimulated for the indicated times with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml), or HkBps (10 HkBps: 1 BMDM). IL-12p40 (A) and IL-12p70 protein (B) were quantified by ELISA (IL-12p40 detection limit 50 pg/ml; IL-12p70 detection limit 20 pg/ml). Graphs show means ± SD of three cultures. *p<0.05, **p<0.01, ***p<0.001 as determined by Student's t-test at each time point. Representative of at least three independent experiments.

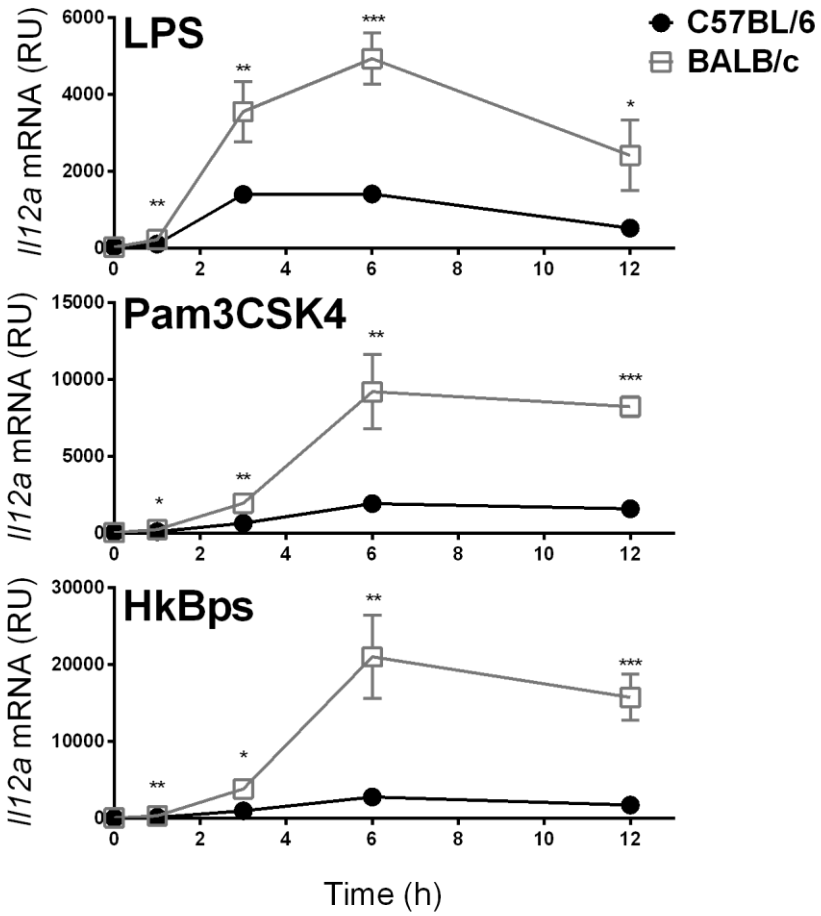


Figure 4.2 *Il12a* mRNA is more highly expressed in BALB/c macrophages than C57BL/6 macrophages.

BMDM were generated from C57BL/6 and BALB/c mice and stimulated for the indicated times with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml), or HkBps (10 HkBps: 1 BMDM). Total RNA was harvested and isolated. *Il12a* transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. Graphs show means ± SD of three cultures. **p*<0.05, ***p*<0.01, ****p*<0.001 as determined by Student's *t*-test at each time point. Representative of three independent experiments.

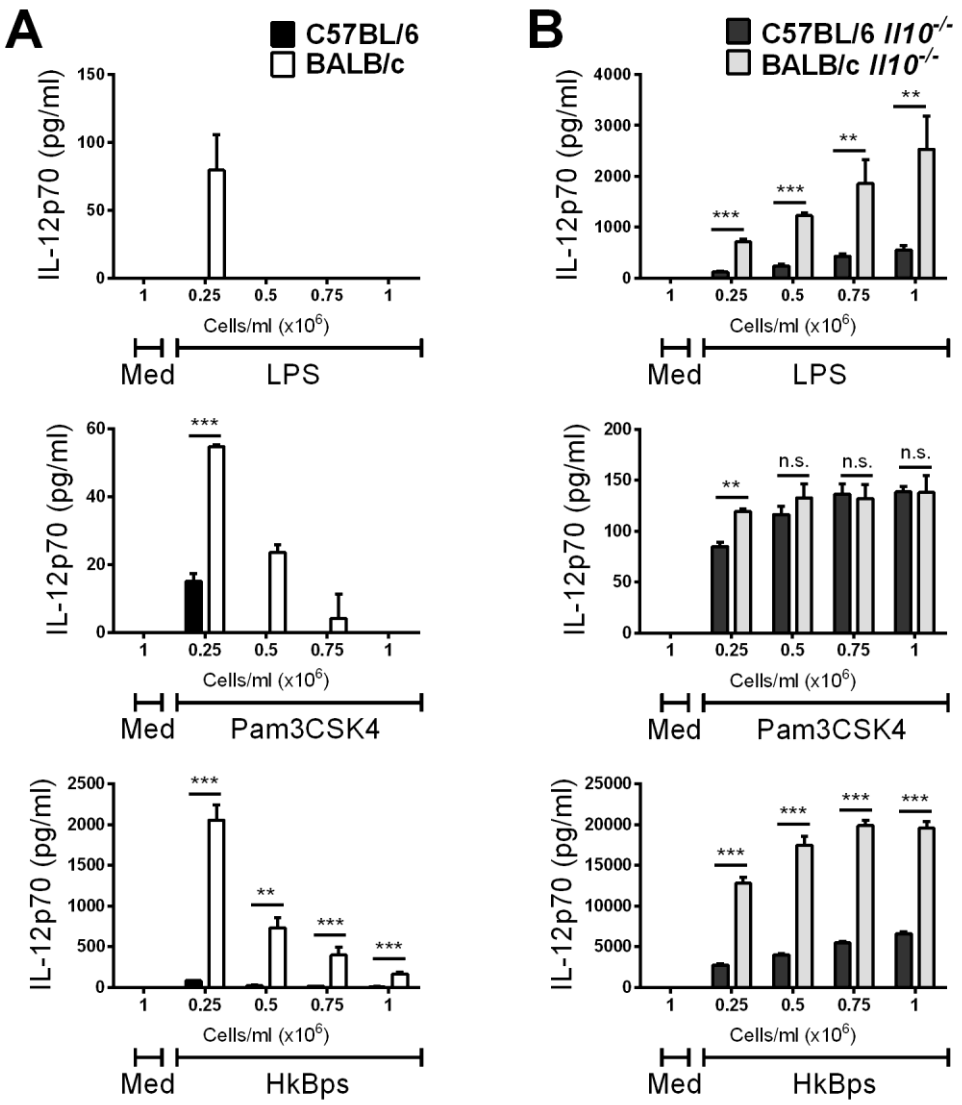


Figure 4.3 Higher IL-12p70 production in BALB/c macrophages is revealed at low cell density due to negative autocrine regulation by IL-10 and in the absence of IL-10, C57BL/6 IL-12p70 production is rescued in Pam3CSK4 stimulated cells.

BMDM were generated from C57BL/6 and BALB/c mice and plated at the indicated cell densities. Cells were stimulated for 24 h with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml), or HkBps (10 HkBps: 1 BMDM). IL-12p70 production was quantified by ELISA (detection limit 20 pg/ml). Graphs show means ± SD of three cultures. $^{**}p<0.01$, $^{***}p<0.001$ as determined by Student's *t*-test at each cell density. Representative of at least three (LPS, Pam3CSK4) or two (HkBps) independent experiments.

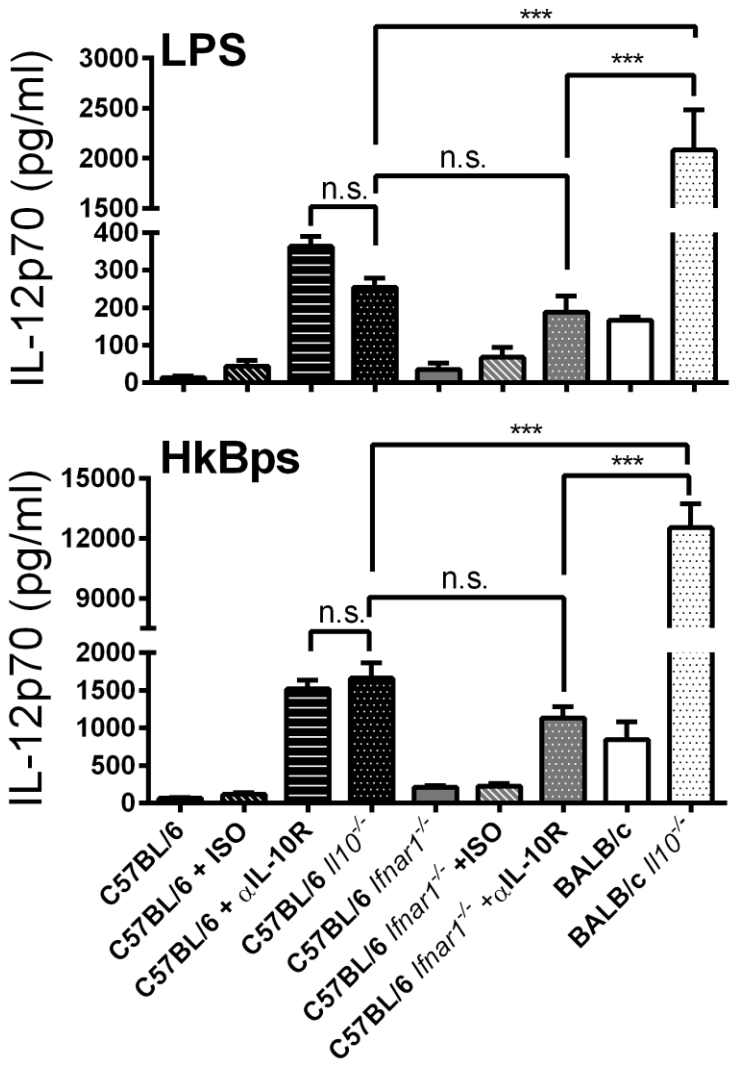


Figure 4.4 The absence of type I IFN signalling or combined absence of type I IFN and IL-10 signalling, does not rescue IL-12p70 production in LPS or HkBps stimulated C57BL/6 macrophages.

BMDM were generated from C57BL/6, BALB/c, C57BL/6 *Il10*$^{-/-}$, BALB/c *Il10*$^{-/-}$, and C57BL/6 *Ifnar1*$^{-/-}$ mice. Cells were plated at 0.25x10^{6} cells/ml. Where indicated, cells were treated with α-IL-10R (10 µg/ml) or isotype control antibody (10 µg/ml) at the time of stimulation. Cells were stimulated with LPS (10 ng/ml) or HkBps (10 HkBps: 1 BMDM) for 24 h. IL-12p70 protein was quantified by ELISA (detection limit 20 pg/ml). Graphs show means ± SD of three cultures. ***p<0.001 as determined by one-way ANOVA. Only selected statistical comparisons are shown for clarity. Representative of two independent experiments.

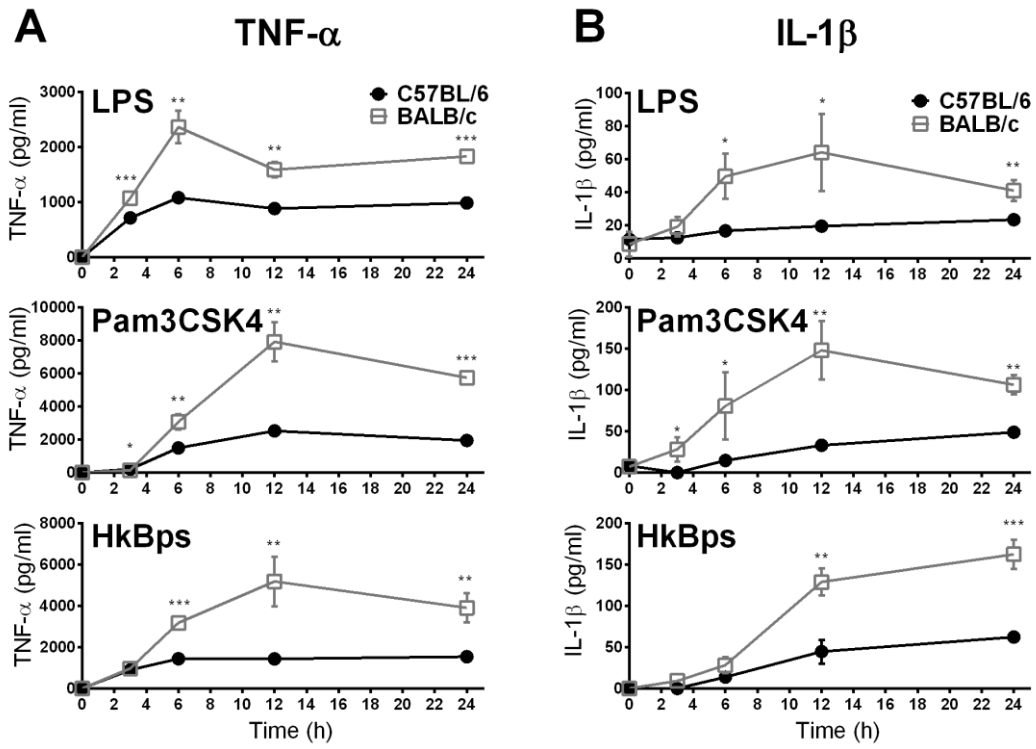


Figure 4.5 The production of TNF-α and IL-1β is higher in LPS, Pam3CSK4 and HkBps stimulated BALB/c macrophages compared to C57BL/6 macrophages.
C57BL/6 and BALB/c BMDM were stimulated with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or HkBps (10 HkBps: 1 BMDM) for the indicated times. TNF-α (A) and IL-1β (B) levels were quantified by ELISA (detection limits 20 pg/ml). Graphs show means of three cultures ±SD. **p*<0.05, ***p*<0.01, ****p*<0.001 as determined by Student's *t*-test at each time point. Representative of at least three independent experiments.

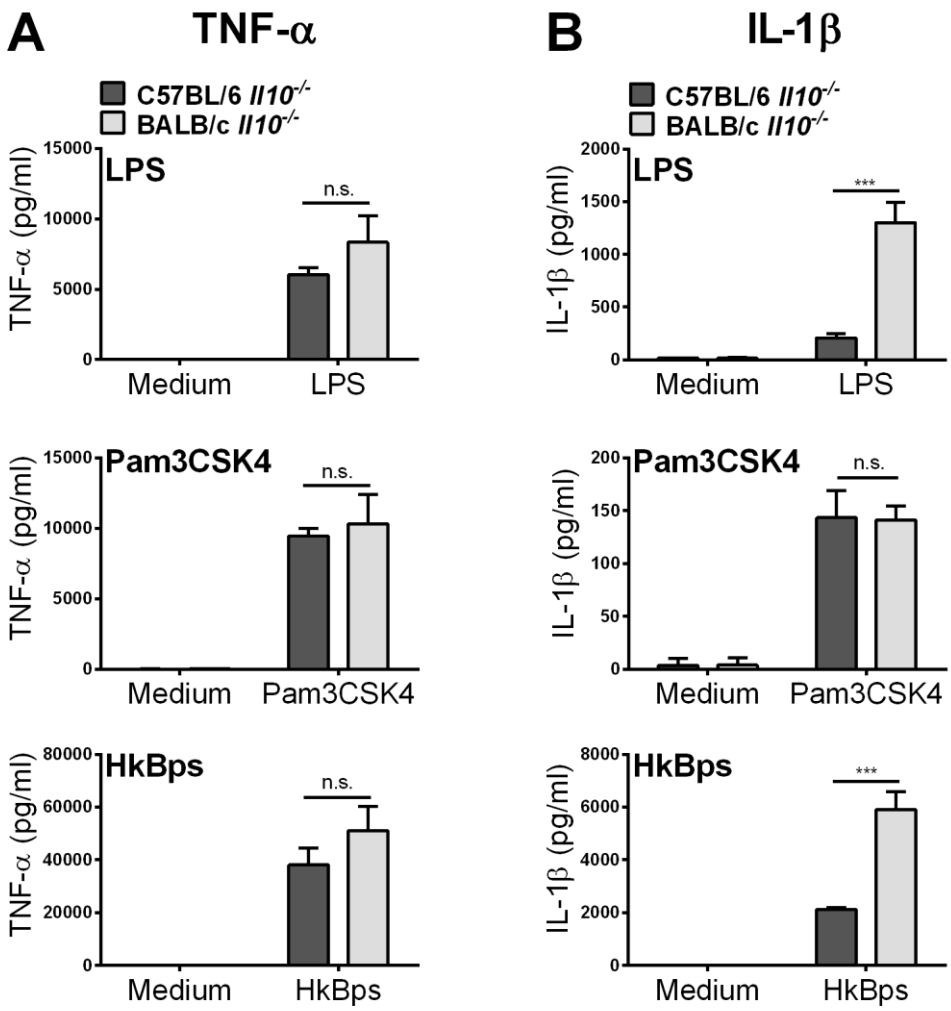


Figure 4.6 IL-10 accounts for the differential proinflammatory cytokine production in Pam3CSK4 stimulated C57BL/6 and BALB/c cells, but not LPS or HkBps stimulated cells.

BMDM were generated from C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ mice. Cells were stimulated with LPS (10 ng/ml), Pam3CSK4 (200 ng/ml) or HkBps (10 HkBps: 1 BMDM) for 24 h. Cytokines were quantified by ELISA (detection limits 20 pg/ml). Graphs show means ± SD of triplicate cultures. ***p<0.001 as determined by Student's *t*-test. Representative of at least two independent experiments.

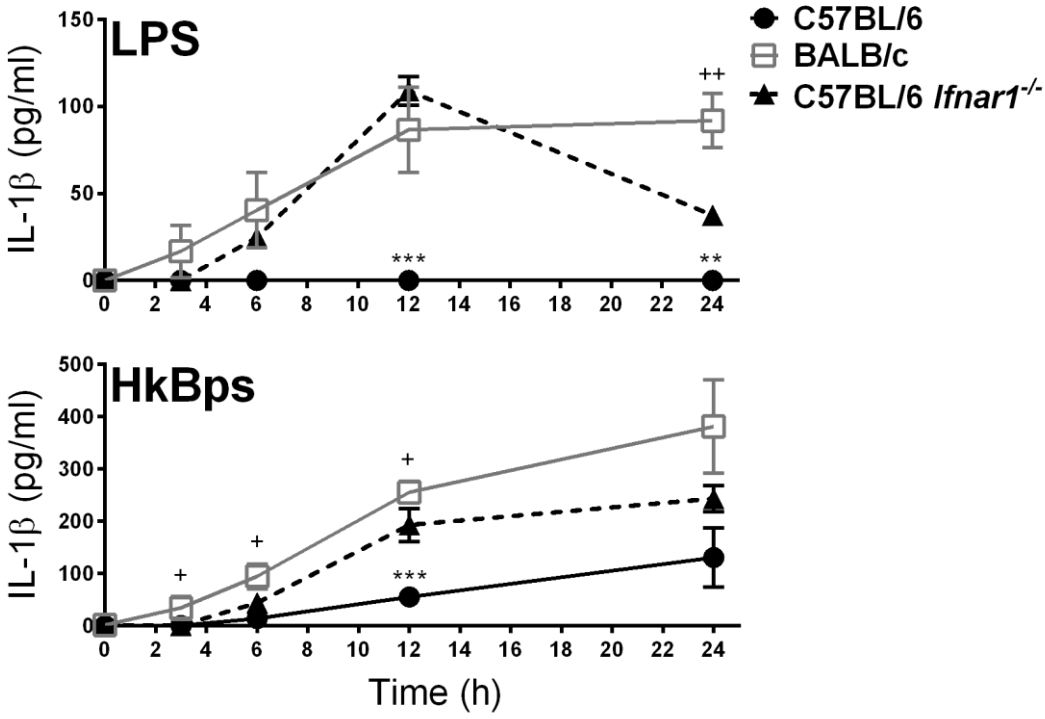


Figure 4.7Abrogation of type I IFN signalling enhances IL-1β production in LPS and HkBps stimulated C57BL/6 macrophages.

C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDM were stimulated with LPS (10 ng/ml) or HkBps (500 HkBps: 1 BMDM) for the indicated times. IL-1β levels in supernatants were quantified by ELISA (detection limit 20 pg/ml). Graphs show means of three cultures ±SD. Statistics were determined by one-way ANOVA at each time-point and show C57BL/6 *vs*. C57BL/6 *Ifnar1*$^{-/-}$ (**p<0.01, ***p<0.001) or BALB/c *vs*. C57BL/6 *Ifnar1*$^{-/-}$ (^{+}p<0.05, ^{++}p<0.01). Representative of three independent experiments.

Chapter 5. Microarray Analysis of the LPS induced Transcriptional Response in C57BL/6 and BALB/c Macrophages

5.1 Background

In this investigation, we have found that IL-10 and IL-12 are reciprocally expressed in C57BL/6 and BALB/c macrophages when stimulated with LPS. C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages, and this is dependent on type I IFN signalling. Conversely, BALB/c macrophages produce higher levels of IL-12. This is maintained in the absence of IL-10 and is not affected by endogenous type I IFN. LPS stimulated BALB/c macrophages additionally produce higher levels of TNF-α and IL-1β compared C57BL/6 macrophages. Differential TNF-α production is predominantly mediated by IL-10. In contrast, differential IL-1β production remains present in the absence of IL-10, although there may be a role for type I IFN in mediating this phenotype. Thus, there are several outstanding questions regarding the mechanisms of differential cytokine production in TLR4 stimulated C57BL/6 and BALB/c macrophages. For example, what is the molecular mechanism of type I IFN mediated IL-10 regulation in C57BL/6 macrophages? Additionally, as we have found that LPS stimulated C57BL/6 macrophages produce higher levels of IFN-β than BALB/c macrophages, and hence hypothesise that this contributes to differential IL-10 production, what is the mechanism of differential IFN-β production? Finally, if not accounted for by IL-10 or type I IFN, what are the factors governing the higher levels of IL-12 production in LPS stimulated BALB/c macrophages?

To begin to answer these questions, we have taken a microarray approach to study the differences in global gene expression of LPS stimulated C57BL/6 and BALB/c macrophages. Microarray technology can be used as a high throughput method of expression profiling to simultaneously quantify the mRNA levels derived from

thousands of genes within a given sample (Lockhart *et al.* 2000). The Illumina BeadArray technology which we have used here, relies on the generation of thousands oligonucleotide probes which are complementary to specific mRNAs. These probes are immobilised onto beads which are placed onto an array chip (Kuhn *et al.* 2004). Purified RNA from the sample of interest is converted to cRNA, fluorescently labelled, and hybridised to the chip. The intensity of hybridisation, determined by fluorescence, can then be used as a measure of RNA abundance and thus relative gene expression (Lockhart *et al.* 2000; Kuhn *et al.* 2004). Microarray technology has been used to assess global gene expression in a number of different studies. For example, blood transcriptional profiling of patients suffering from autoimmune or infectious diseases has enabled the identification of gene-signatures associated with health and disease (Bennett *et al.* 2003; Berry *et al.* 2010; Pascual *et al.* 2010). On a cellular level, microarray technology has been used to understand the dynamic changes in gene expression induced by a stimulus (Gilchrist *et al.* 2006; Nilsson *et al.* 2006; Elkon *et al.* 2007; Ramsey *et al.* 2008), or the specific gene expression profiles of different cell types, for example tissue-specific macrophage subsets (Gautier *et al.* 2012). Additionally, in a concept similar to this study, genome wide transcriptome analyses have been used to better understand biological differences between C57BL/6 and BALB/c mice in the context of susceptibility to asthma (Kelada *et al.* 2011) and *L.major* infection (Ehrchen *et al.* 2010). Microarray expression profiling is therefore a powerful tool which can be used to understand global gene expression differences between populations or conditions. Further, this data can be used as part of a wider analysis to understand the biological networks which may give rise to specific profiles of gene expression (Amit *et al.* 2009; Zak *et al.* 2009).

5.2 Investigating the LPS induced transcriptional response of C57BL/6 and BALB/c macrophages: Study aims

By analysing the transcriptional profiles of LPS stimulated C57BL/6 and BALB/c macrophages, we aim to address the following questions:

1. Which genes, other than *Il10*, *Ifnb1* and *Il12a*, are differentially expressed in LPS stimulated C57BL/6 and BALB/c macrophages?
2. Using this expression data, can we identify biological pathways or regulatory networks that may differ in LPS stimulated C57BL/6 and BALB/c macrophages?
3. If found, could these biological pathways or regulatory networks be involved in the differential regulation of *Il10*, *Ifnb1* and *Il12a* in these cells?

Of note, we observe similar C57BL/6 and BALB/c cytokine profiles in LPS and heat-killed *B.pseudomallei* stimulated macrophages. Hence, findings made in this analysis have the potential to be relevant in the context of heat-killed *B.pseudomallei* stimulation.

5.3 Results

5.3.1 Experimental design

We elected to carry out microarray analysis of C57BL/6 and BALB/c LPS stimulated macrophages at 0, 0.5, 1, 3, 5 and 8 h post-stimulation. This was to ensure that we would be able to assess the regulation of genes which have different kinetics of expression. For example, *Il10* mRNA has a complex pattern of expression in LPS stimulated C57BL/6 macrophages, with one peak at 0.5-1 h and another a 4-6 h post stimulation (Figure 5.1 A). *Ifnb1* mRNA however only has one sharp peak at 1 h post stimulation, whereas *Il12a* mRNA expression has slower kinetics with gradually increasing levels up to 6 h, followed by a decline (Figure 5.1 A). Thus, the selection of these time-points, indicated by the red dotted lines in Figure 5.1 A, provided the breadth required to capture early, late and dynamic transcriptional changes. In addition to a 0 h condition, we included media treated control cells at each time-point to ensure that the transcriptional responses we observed were LPS specific and not due to duration of cell culture alone. All experimental groups of C57BL/6 and BALB/c macrophages included in the microarray analysis are summarised in Figure 5.1 B. Once the microarray was carried out, a quality control analysis was conducted to ensure the robustness of the experiment. This is described in more detail in Materials and Methods (see Chapter 2).

5.3.2 LPS induces a dynamic transcriptional response in C57BL/6 and BALB/c macrophages

Prior to analysing differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages, we assessed the overall transcriptional profiles in macrophages from both

strains. Gene probes were normalised to the median of each gene across all samples to remove non-biological variation, then filtered on flags (an attribute denoting the quality of any given gene probe). Genes were visualised in a heat map (Figure 5.2). Cells that had been treated with media for 0.5-8 h did not appear different from unstimulated (0 h) macrophages (Figure 5.2). This is confirmed in more detail in the quality control analysis (see Chapter 2, Figure 2.1). In LPS stimulated cells, clusters of genes were up- and down-regulated in response to LPS and even on this broad scale, kinetic differences in LPS induced gene expression were apparent (Figure 5.2). For example, some genes were already strongly up-regulated by 0.5-1 h of LPS stimulation (Group 1, Figure 5.2). Several of these genes encoded regulators of cytokine production e.g. *Irf1, Rel*, *Atf3* or cytokines e.g. *Il10*, *Tnf*, *Il1a* and *Il1b* (Group 1, Figure 5.2). In keeping with this, Gene Ontology (GO) analysis associated this group of genes with GO terms that included 'regulation of cytokine production' and 'regulation of cytokine biosynthetic process' (Group 1, Figure 5.2). Other genes were predominantly up-regulated from 3-8 h post-stimulation (Group 2, Figure 5.2) and included cytokines such as *Il12a* and *Il6*, and type I IFN-inducible genes such as *Mx1*, *Ifit2*, *Oasl2* and *Pml* (Sadler *et al.* 2008). In contrast, there was a small group of genes that only had a very short window of expression at 1 h, and these included transcription factors such as *Irf4*, *Maff*, *Myc*, *Egr1*, *Egr2* and *Egr3* (Group 3, Figure 5.2). Down-regulation of genes occurred mostly from 3 h (Groups 4 and 5, Figure 5.2). GO analysis identified these genes to be associated with biological processes such as 'cell cycle', 'mitosis', 'cellular metabolic process' and 'response to DNA damage' (Groups 4 and 5, Figure 5.2). We also observed that there was a notable level of differential gene expression in unstimulated C57BL/6 and BALB/c macrophages, some of which was maintained in stimulated cells (Figure 5.2, strain-

dependent basal expression). Analysis of these genes, described in more detail in Materials and Methods (see Chapter 2 Figure 2.2), showed that several of these genes encoded MHC molecules, but other immune related genes e.g. *Gpb1* and *Il10* were also present, highlighting the importance of the inclusion of these genes in later analyses.

5.3.3 Identification of significantly differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages

We next determined which genes were differentially expressed in LPS stimulated C57BL/6 and BALB/c macrophages. To identify these genes, the expression data was analysed on a per time-point basis, outlined in Figure 5.3 A. The decision was made to analyse time-points individually to enable us to determine when genes were differentially expressed, as this cannot be done with a combined time-point analysis using GeneSpring software. Genes were again normalised to the median of each gene and filtered on flags. To narrow the analysis to LPS responsive genes, gene probes were filtered to select for those that were at least 2-fold differentially expressed from the media control within each strain. A 2-way ANOVA was then conducted specifying that genes must pass a cut-off of $p<0.01$ after a Benjamini-Hochberg multiple testing correction (Figure 5.3 A). From the 2-way ANOVA results, we identified three groups of genes. Firstly, genes that significantly changed their expression in response to LPS, but were not different between the strains (Figure 5.3 B far left graph, Δ). This group represented the majority of genes suggesting that the LPS induced transcriptional responses of C57BL/6 and BALB/c macrophages are actually quite similar. Secondly, we identified genes that did not significantly change in response to LPS, but were differentially expressed between the strains (Figure 5.3 B far right graph, Ψ). This group

represented the vast minority of genes, ranging from only 42-82 gene probes across each time point. Lastly, we identified our genes of interest which fulfilled two criteria, i) significantly differentially expressed between C57BL/6 and BALB/c macrophages, and ii) significantly modulated by LPS treatment (Figure 5.3 B middle graph, *). This final group contained 101, 141, 867, 1251 and 1109 gene probes at 0.5, 1, 3, 5 and 8 h of LPS stimulation, respectively. Of note, this analysis successfully identified the differential expression of *Il10* (significantly higher in C57BL/6 macrophages at all time-points), *Ifnb1* (significantly higher in C57BL/6 macrophages at 1 h) and *Il12a* (significantly higher in BALB/c macrophages at 3, 5 and 8 h), in keeping with our previous data and supporting the validity of this approach.

In order to better understand the nature of differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages, differentially expressed genes of interest (Figure 5.3 B middle graph, *) were separated into those which were up-regulated compared to media control in both strains; down-regulated compared to media control in both strains; or bi-directionally regulated according to strain (i.e. up-regulated in one strain and down regulated in the other, compared to the media control) (Figure 5.3 C). We found that in C57BL/6 and BALB/c macrophages, the directionality of gene expression compared to media control was the same for the majority of differentially regulated genes (Figure 5.3 C). This indicated that primarily quantitative differences in gene expression account for the differential transcriptional profiles of LPS stimulated C57BL/6 and BALB/c macrophages.

5.3.4 C57BL/6 macrophages may have a stronger LPS induced transcriptional response than BALB/c macrophages

In order to further understand differences in the transcriptional responses of LPS stimulated C57BL/6 and BALB/c macrophages, differentially expressed up-, down-, or bi-directionally regulated genes at each time-point (see Figure 5.3 C) were further segregated into their relative C57BL/6 *versus* BALB/c expression level (Figure 5.4). This analysis clearly showed that of the differentially expressed up-regulated genes, the majority were more highly expressed in C57BL/6 macrophages (Figure 5.4, left). This trend was observed at every time-point, suggesting that on a global scale, C57BL/6 macrophages may have an enhanced response to LPS compared to BALB/c macrophages. Very few differentially expressed genes were bi-directionally regulated in LPS stimulated C57BL/6 and BALB/c macrophages, and their relative expression levels were overall evenly distributed between C57BL/6 and BALB/c macrophages (Figure 5.4, middle). Few differentially expressed down-regulated transcripts were identified at 0.5 and 1 h (Figure 5.4, right), in keeping with our earlier observation that LPS induced gene repression is more prominent from 3 h (see Figure 5.2). Indeed, from 3 h, many genes were down-regulated and of these, the majority appeared more down-regulated in C57BL/6 macrophages compared to BALB/c macrophages (Figure 5.4, right). Collectively, these observations suggest that C57BL/6 macrophages may have an overall stronger or more efficient transcriptional response to LPS than BALB/c macrophages.

5.3.5 *k*-means clustering of differentially expressed genes identifies the groups of genes that most clearly distinguish LPS induced C57BL/6 and BALB/c transcriptional profiles

Across the different time-points of this analysis, we identified hundreds of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages (see Figure 5.3 B*). We thus aimed to distinguish the genes that had the most profound expression differences between C57BL/6 and BALB/c LPS stimulated macrophages. To do this, we separated the differentially expressed genes at each time-point into 6 clusters using *k*-means, a method of clustering that groups genes with similar transcriptional profiles into a user-defined number of clusters (Do *et al.* 2008). This analysis generated 30 clusters in total, each termed C0-C5 for each time-point (Figure 5.5). We observed all 30 clusters and selected 13 in which the gene expression profiles were considered to be substantially different between C57BL/6 and BALB/c macrophages (Figure 5.5, clusters outlined in blue). We performed GO analysis on each of these selected clusters to identify related functions and also manually searched for any genes of interest. The results of these analyses are discussed below.

5.3.5.1 *Clusters with higher expression in LPS stimulated C57BL/6 macrophages*

From these 13 selected clusters, 9 had profiles of higher expression in C57BL/6 macrophages compared to BALB/c macrophages – 0.5 h C3 and C4; 1 h C1 and C5; 3 h C1 and C3; 5 h C0 and C3; and 8 h C0 (Figure 5.5, shown separated in Figure 5.6). Genes within each of these clusters are listed in Appendix Tables 7.1-7.9.

0.5 h C3 and C4: 0.5 h C3 and C4 both contained genes that were basally more highly expressed in C57BL/6 macrophages, and were up-regulated in response to LPS but more so in C57BL/6 than BALB/c macrophages (Figure 5.6). Neither cluster associated with any GO terms. C3 included the IFN-inducible gene *Ifi205* (Ludlow *et al.* 2005), the transcription factor *Atf4*, the p38 responsive gene *Cish* (Kim *et al.* 2008) and the gene encoding vascular endothelial factor A, *Vegfa* (Figure 5.6). 0.5 h C4 included the IFN-inducible gene *Cxcl10* (Thomas *et al.* 2006), again *Ifi205* (often there are several probes specific to one gene), and *Tnfrsf5*, which encodes CD40 (Figure 5.6).

1 h C1 and C5: 1 h C1 contained genes which were more highly expressed at baseline in C57BL/6 macrophages and up-regulated in response to LPS more notably in C57BL/6 compared to BALB/c macrophages (Figure 5.6). 1 h C1 did not associate with any GO terms, but contained the chemokine genes *Ccl5* and *Cxcl2*, the IFN-inducible GTPase *Gbp2* (guanylate binding protein 2) (Vestal 2005) and *Dusp16*, a dual specificity phosphatase, involved in the negative regulation of MAP kinases (Finch *et al.* 2012) (Figure 5.6). 1 h C5 was composed of genes which had less marked differential basal expression, but were clearly more highly expressed in C57BL/6 macrophages upon LPS stimulation. 1 h C5 did not associate with any GO terms but included *Cxcl10* and *Il10* in addition to the TLR signalling adaptor *Myd88* (Kawai *et al.* 2010) (Figure 5.6). The transcription factor *Bcl3* was also present within this cluster.

3 h C1 and C3: The genes within 3 h C1 were more highly expressed at baseline in C57BL/6 macrophages and appeared to be up-regulated by LPS in both strains, although more strongly in C57BL/6 macrophages (Figure 5.6). 3 h C1 associated with

the GO terms 'MHC protein complex' and 'MHC class I protein complex' due to the presence of *H2-Ab1*, *H2-D4* and *H2-T10* within the cluster (Figure 5.6). This cluster additionally contained the TPL-2/ERK induced transcriptional regulator *Egr1* (Waterfield *et al.* 2003), the MAP kinase pathway related genes *Map3k6* and *Dusp16*, and the NF-κB family member *Nfkb2* (encodes p105 (Hayden *et al.* 2008)) (Figure 5.6). Compared to 3 h C1, the basal expression of genes within 3 h C3 was more similar in C57BL/6 and BALB/c macrophages. Upon stimulation, these genes were strongly induced in C57BL/6 macrophages but only weakly in BALB/c macrophages (Figure 5.6). 3 h C3 did not associate with any GO terms but included the transcription factors *Bcl6* (a transcriptional repressor (Dent *et al.* 2002)), *Irf2*, *Nfil3* and *Stat4* (Figure 5.6). *Nfil3* is a target of IL-10 mediated signalling (Smith *et al.* 2011) and thus, at this time-point differential gene expression may be contributed to by autocrine IL-10 signalling. Two negative regulators of TLR signalling, *Trim30* (a member of the tripartite motif containing (TRIM) family of proteins), and *Irak3* were also present in 3 h C1 and C3, respectively (Kobayashi *et al.* 2002; Kawai *et al.* 2011; McNab *et al.* 2011)(Figure 5.6).

5 h C0 and C3: 5 h C0 contained genes that were clearly more highly expressed in C57BL/6 macrophages both at baseline and upon LPS stimulation, and associated with the GO terms 'MHC protein complex' and 'MHC class I protein complex' again due to the presence of *H2* genes (Figure 5.6). Genes of particular interest in 5 h C0 were largely consistent with earlier clusters, with the exception of the IFN-inducible TRIM protein *Trim56* (Kawai *et al.* 2011) (Figure 5.6). The genes within 5 h C3 collectively varied less in their basal expression in C57BL/6 and BALB/c macrophages. These genes were up-regulated by LPS in both strains, but were more highly expressed in LPS

stimulated C57BL/6 macrophages (Figure 5.6). 5 h C3 associated with five GO terms – 'inflammatory response', 'immune response', 'defence response', 'immune system process' and 'response to wounding' (Figure 5.6). Many IFN-inducible genes were present in 5 h C3 such as *Mx1*, *Cxcl10*, *Pml*, *Ifit3* and *Oas3* (Sadler *et al.* 2008; Schoggins *et al.* 2011) (Figure 5.6). This time-point constitutes the type I IFN dependent second peak of *Il10* mRNA, and *Il10* was also present in this cluster. However, although not initially selected for this analysis, we noted that 5 h C4 (see Figure 5.5), contained IFN-inducible genes such as *Mx2*, *Ifitm1* and *Oasl1* (Sadler *et al.* 2008) which were more highly expressed in BALB/c macrophages. Thus, despite C57BL/6 macrophages producing higher levels of IFN-β than BALB/c macrophages, not all IFN-inducible genes are more highly expressed in C57BL/6 macrophages. The C57BL/6^{hi} genes of 5 h C3 also included *Map3k8* (TPL-2) which positively regulates IL-10 but negatively regulate IL-12 and IFN-β (Agrawal *et al.* 2003; Kaiser *et al.* 2009) and *Zfp36* (TTP), an RNA binding protein which has been shown to target and degrade *Il10* (Stoecklin *et al.* 2008) but also negatively regulates IL-12 and other proinflammatory cytokines (Gaba *et al.* 2012)(Figure 5.6). The NF-κB pathway related gene *Ikbkb* (IKK-2/β) and the gene encoding IKK-ε, *Ikbke*, which is involved in the TRIF-dependent signalling pathway (Kawai *et al.* 2010), were also more highly expressed in C57BL/6 macrophages and present within 5 h C3 (Figure 5.6).

8 h C0: Finally, one cluster was selected at the 8 h time-point, C0, which contained genes that were generally more highly expressed in C57BL/6 macrophages at baseline, and upon LPS stimulation (Figure 5.6). This cluster did not associate with any GO

terms, but included *Il10, Ifi205, Egr1, Atf4, Trim30, Trim56, Nfkb2* and *Dusp16*, largely consistent with earlier clusters.

5.3.5.2 *Clusters with higher expression in LPS stimulated BALB/c macrophages*

The four remaining clusters selected by this approach had higher expression profiles in BALB/c macrophages – 1 h C4; 3 h C4; 5 h C2; 8 h C3 (Figure 5.5, shown separated in Figure 5.7). Genes within each of these clusters are listed in Appendix Tables 7.10-7.13.

1 h C4: 1 h C4 contained genes which were overall more highly expressed in BALB/c macrophages, but not consistently up-regulated by LPS at this time-point. 1 h C4 did not associated with any GO terms. Of interest, this cluster contained the IFN-inducible GTPase *Gpb1* (Vestal 2005) again indicating that not all IFN-inducible genes are more highly expressed in C57BL/6 macrophages. The histone lysine demethylase, *Jmjd2a*, which has been associated with transcriptional activation and repression (Marmorstein *et al.* 2009) was also more highly expressed in BALB/c macrophages and within this cluster (Figure 5.7).

3 h C4: 3 h C4 contained genes that were up-regulated by LPS in both strains, but were more highly expressed BALB/c macrophages under all conditions. 3 h C4 did not associated with any GO terms but contained *Gbp1* and *Jmjd2a*, which were also present in 1 h C4, and the additional type I IFN-inducible *Ifi202b* (Figure 5.7).

5 h C2: 5 h C2 had a similar expression profile to 3 h C4, but associated with the GO terms ‘Immune response’ and ‘Immune system process’ (Figure 5.7). In addition to the

genes mentioned above in 1 h C4 and 3 h C4, the matrix metalloproteinase (MMP) *Mmp9* was present within 5 h C2. Of note, MMPs are associated with the breakdown of extracellular matrix but are also reported to cleave and modulate the activity of cytokines (Van Lint *et al.* 2007). Of particular interest for this study, MMP-9 has been reported to cleave and inactivate IFN-β (Nelissen *et al.* 2003) which may be in keeping with its higher expression in BALB/c macrophages. *Ltb* (lymphotoxin-β) was also present within this cluster of BALB/c^{hi} genes (Figure 5.7).

8 h C3: The expression profile of 8 h C3 was similar to the previous BALB/c^{hi} clusters and as 5 h C2, associated with the GO terms 'Immune response' and 'Immune system process' (Figure 5.7). Genes of interest within this cluster were consistent with 5 h C3, with the additional inclusion of *Il12a* (Figure 5.7), further corroborating our observation that *Il12a* is more highly expressed in LPS stimulated BALB/c macrophages. At this stage we additionally noted that amongst these selected BALB/c clusters (1 h C4; 3 h C4; 5 h C2; 8 h C3), there was much overlap of gene content indicating that several of these genes (e.g. *Gbp1*, *Jmjd2a*) were consistently more highly expressed in BALB/c macrophages throughout the response.

In summary, this *k*-means analysis has highlighted the differential regulation of several potentially interesting genes in LPS stimulated C57BL/6 and BALB/c macrophages. Although the GO analysis was not highly informative, we noted the differential expression of some genes which may influence differential cytokine production in C57BL/6 and BALB/c macrophages. These included regulators of TLR signalling (e.g. *Myd88*, *Map3k8* (TPL-2), *Ikbkb*, *Ikbke*, *Irak3*, *Trim30* – all C57BL/6^{hi}), regulators of

chromatin modification (*Jmjd2a* – BALB/c^{hi}), and transcription factors (e.g. *Egr1*, *Bcl3*, *Atf4* – C57BL/6^{hi}). This analysis further brought attention to the differential expression of IFN-inducible genes which depending on the gene, were more highly expressed in either C57BL/6 or BALB/c macrophages.

5.3.6 Ingenuity pathway analysis of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages.

In order to additionally establish links between differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages and relevant biological pathways, we used Ingenuity Pathway Analysis (IPA) software. IPA is a web-based analysis programme in which genes can be analysed for associations with pathways defined within a curated database known as the Ingenuity Knowledge Base. Taking each time-point individually, we analysed the lists of differentially expressed genes of interest (see Figure 5.3 B*), for associated pathways. The rational for assessing each time-point individually, was that the transcriptional response to LPS is regulated in distinct temporal waves (Smale 2012), and thus different signalling pathways may be operating at different times (Elkon *et al.* 2007). Of note, IPA determines if a group of genes is associated with a biological pathway based on the number of genes that overlap with the pathway, not the relative expression level of those genes. In addition, any given pathway may up-regulate certain genes and down-regulate others. Thus, per time-point gene lists encompassing both LPS up- and down-regulated genes were analysed as a whole. The top 5 pathways significantly associated with differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages at each time-point (p<0.01) are represented in Figure 5.8. Within each gene list, some genes were more highly

expressed in C57BL/6 macrophages and others in BALB/c macrophages. This is represented by the red (C57BL/6) or green (BALB/c) shading of the bars. The clear area of the bars denotes genes that were present within a defined IPA pathway, but not present in the uploaded list of differentially expressed genes (Figure 5.8). Genes within our dataset that were associated with each pathway by IPA are listed in Appendix tables 7.14-7.18.

Pathways identified at several time-points included 'TREM1 signalling', 'Communication between innate and adaptive cells', 'Role of macrophages, fibroblasts and endothelial cells in Rheumatoid Arthritis', 'DC maturation' and 'Type I diabetes mellitus signalling' (Figure 5.8). At 0.5 and 1 h, 'TREM1 signalling' was the top associated pathway, however, relatively few genes from our dataset (6 genes at 0.5 h, 8 genes at 1 h) were associated with this pathway (see Appendix Tables 7.14 and 7.15). Further, these 'TREM1 signalling' associated genes, which included *Il10*, *Myd88, Ccl2*, *Ccl7*, *Cxcl3* and *Tnf* (all C57Bl/6^{hi}), represent genes that could be associated with many molecular pathways. Of note, the higher expression of *Tnf* mRNA in C57BL/6 macrophages was interesting as we had previously observed TNF-α protein production to be higher in BALB/c macrophages. This may imply a post-transcriptional mechanism preventing high levels of TNF-α protein production in C57BL/6 macrophages. The other pathways which appeared at several time-points were quite broad and as such, did not provide substantial insight into the mechanisms of differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages.

Pathways identified at one time-point included 'Allograft rejection signalling' (0.5 h), 'Glucocorticoid receptor signalling' (0.5 h), 'Granulocyte/Agranulocyte adhesion and diapedesis' (1 h), 'Role of hypercytokinemia/chemokinemia in the pathogenesis of influenza' (1 h), 'NF-κB signalling' (3 h) and 'Hepatic fibrosis/stellate cell activation' (8 h) (Figure 5.8). Of these, we considered the most relevant to be NF-κB signalling at the 3 h time-point. 24 genes from the 3 h dataset were associated with this pathway, and the majority were more highly expressed in C57BL/6 macrophages including *Tbk1*, *Irak3*, *Nfkb2*, *Tank* and *Ikbkb* (see Appendix Table 7.16). Thus, the results of this analysis suggested that there may be differential NF-κB signalling at 3 h in LPS stimulated C57BL/6 and BALB/c macrophages and that this may play a part in differential gene expression between the strains.

5.3.7 Identification of candidate transcription factors that may be responsible for differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages

To further our insight into the differential molecular networks that could be operating in C57BL/6 and BALB/c macrophages, we went on to do a complimentary analysis that was focussed on identifying transcription factors that may be upstream of the differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages. Towards this aim, we took three approaches: i) identification of putative transcriptional regulators upstream of differentially expressed genes by IPA (*in silico*); ii) assessment of predicted transcription factor binding motif enrichment in differentially expressed genes by PSCAN (*in silico*) and iii) analysis of differential transcription factor mRNA expression in LPS stimulated C57BL/6 and BALB/c macrophages (experimental data).

As a data validation technique, we overlapped the results from these three approaches, each explained in more detail below, with the hypothesis that if a transcriptional regulator is identified by more than one analysis strategy, it has a higher likelihood of being involved in mediating differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages.

5.3.7.1 *Identification of upstream transcriptional regulators associated with differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages by IPA*

IPA software has an 'upstream regulator' application in which it can be predicted if groups of genes are significantly associated with particular upstream transcription factors. Taking each time-point individually as we did for the *k*-means clustering and pathway analysis, we used this tool to analyse the lists of differentially expressed genes of interest (see Figure 5.3 B*). Further, using the relative C57BL/6 *versus* BALB/c expression levels of each gene, IPA was able assign an activation *z*-score (described in more detail in Materials and Methods) to predict if a given transcriptional regulator is likely to be more active in one strain over the other (Figure 5.9). Selected transcriptional regulators identified are discussed below.

NF-κB: NF-κB (complex) was predicted to be more activated in LPS stimulated C57BL/6 macrophages at 0.5 h, 1 h, 3 h and 5 h, with the highest activation score in C57BL/6 macrophages at 0.5, 1 and 3 h (Figure 5.9). This suggests that differential NF-κB signalling may be a dominant factor in mediating distinct profiles of gene expression in C57BL/6 and BALB/c macrophages at these time-points. This is in keeping with our

our IPA pathway analysis (see Section 5.3.6) which also suggested that differentially expressed genes at 3 h may be associated with NF-κB signalling.

STATs: STAT1 was predicted to be preferentially activated in C57BL/6 macrophages and this was most prominent at 3 and 5 h (Figure 5.9). This could be a consequence of differential gene expression due to autocrine type I IFN as based on our previous data, this is when we would predict autocrine type I IFN signalling to be at its height. STAT3 was additionally predicted to be more active in C57BL/6 macrophages at 3 and 8 h (Figure 5.9). This may be a consequence of autocrine IL-10 signalling which we predict to be enhanced in C57BL/6 compared to BALB/c macrophages. STAT4 had the highest C57BL/6 activation score at 8 h (Figure 5.9). This transcription factor is generally associated with IL-12 signalling (Bacon *et al.* 1995b) which we predict would be less strong in C57BL/6 macrophages, if they express the IL-12 receptor at all. It is therefore possible that other factors may be inducing STAT4 activity in this system.

IRFs: Several IRFs were associated with differentially expressed genes at 1 and 3 h. The majority were predicted to be preferentially activated in C57BL/6 macrophages including IRF 1, 8, 7, 3 and 5 of which IRF 1, 3 and 7 are associated in the literature with the production of type I IFN or responses to type I IFN (Ziegler-Heitbrock *et al.* 2003; Platanias 2005; Trinchieri 2010). In contrast, IRF2, which has been reported to antagonise type I IFN responsiveness (Hida *et al.* 2000), was the only IRF predicted to be preferentially activated in BALB/c macrophages (Figure 5.9).

ERK associated transcriptional regulators: The TPL-2/ERK MAP kinase pathway is activated downstream of TLR4 (Gantke *et al.* 2011). Interestingly, several ERK regulated transcription factors such as ELK1, AP1, EGR1 and CREB (Waterfield *et al.* 2003; Chastel *et al.* 2004; Ananieva *et al.* 2008), were predicted to be more active in C57BL/6 macrophages, particularly at 3 h (Figure 5.9). In contrast, at 1 h, the AP-1 family member FOSL1 (Karin *et al.* 1997) was predicted to be more active in BALB/c macrophages.

Other transcriptional regulators: SP1, which has roles in the regulation of both IL-10 and IL-12 (Tone *et al.* 2000; Goriely *et al.* 2003) was predicted to be preferentially activated in C57BL/6 macrophages at 0.5 h, however at 5 h was the top preferentially activated transcription factor in BALB/c macrophages (Figure 5.9). The transcriptional repressor BCL6 (Dent *et al.* 2002) was also predicted to be preferentially activated in BALB/c macrophages at 1, 5 and 8 h post-LPS stimulation (Figure 5.9). Additionally, ETS1 which has been shown to negatively regulate IL-10 in T cells (Lee *et al.* 2012) was predicted to be more active in BALB/c macrophages at 5 h (Figure 5.9).

5.3.7.2 *Transcription factor binding motif enrichment analysis of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages*

An alternative approach to identifying transcription factor candidates that regulate groups of genes is transcription factor binding site (TFBS) enrichment analysis. This approach makes the assumption that co-expressed genes are regulated by common factors, which can be revealed by the presence of TFBS sequence motifs within those genes. TFBS analysis has been used by several groups to identify transcription factors

responsible for the temporal regulation of gene clusters in PRR stimulated macrophages (Nilsson *et al.* 2006; Ramsey *et al.* 2008). We have used the web based software PSCAN (Zambelli *et al.* 2009) to identify TFBS which may be over-represented amongst the genes that are differentially expressed in LPS stimulated C57BL/6 and BALB/c macrophages. This programme scans the gene sequences and compares them against known TFBS within publically available, or user defined databases (Zambelli *et al.* 2009). We elected to scan the gene sequences between -950 and +50 bp relative to the transcriptional start site. This was because within the PSCAN programme, only five scanning ranges were possible and this choice allowed us to go a reasonable distance upstream while retaining a small area within the gene, where TFBS are often found (Zambelli *et al.* 2009). We used the open-access JASPAR database as the source of TFBS motifs (Sandelin *et al.* 2004). As before, we analysed the differentially expressed genes of interest identified in Figure 5.3 B* on a per-time-point basis.

At 0.5 h, no significant enrichment of TFBS was found within the genes that were differentially expressed in LPS stimulated C57BL/6 and BALB/c macrophages, although NF-κB was the top result (data not shown). At 1 h, TFBS enrichment for NF-κB and RELA was found within differentially expressed genes (Figure 5.10). Significant enrichment for NF-κB TFBS was also identified at the 3, 5 and 8 h time-points, along with NFKB1 TFBS enrichment (Figure 5.10). This provides further evidence for the involvement of NF-κB signalling in mediating the differential transcriptional profiles of LPS stimulated C57BL/6 and BALB/c macrophages. At 3, 5 and 8 h, there was substantial overlap between the TFBS enrichment results. The M2 macrophage associated transcription factor KLF4 (Liao *et al.* 2011) had the most

significant *p* value for enrichment at these time-points (Figure 5.10). TFBS enrichment for the transcription factors SP1, EGR1, IRF1 and the insulator protein CTCF, which has been implicated in the organisation of chromatin structure (Phillips *et al.* 2009), was also identified at the 3, 5 and 8 h time-points (Figure 5.10). Additionally, TFBS enrichment for HIF-1α/ARNT and ARNT/AHR complexes were identified at 3, 5 and 8 h. Very few TFBS motifs were enriched at only one time-point but included PAX5, USF1 and NHLH1 at the 5 h time-point, and SPIB at the 8 h time-point (Figure 5.10).

5.3.7.3 *Differential transcription factor mRNA expression in LPS stimulated C57BL/6 and BALB/c macrophages.*

As our final approach to identifying transcription factor candidates responsible for differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages, we used our microarray expression data to determine which transcription factors were differentially expressed at the mRNA level in these cells. Although it is known that some transcription factors are regulated at the post-transcriptional level leading to rapid expression of primary response genes in LPS stimulated macrophages, other transcription factors are transcriptionally induced after stimulation and these may modulate the expression of secondary response genes (Medzhitov *et al.* 2009; Smale 2012). Thus, the expression of transcription factor mRNA may give some indication of differential transcription factor activity, particularly at later time-points. A comprehensive list of murine transcription factors was generated using GO annotation from Affymetrix (another microarray platform) and Illumina databases (Gabrysova, O'Garra unpublished resource), and was used to interrogate the differentially expressed genes of interest (Figure 5.3 B*). A total of 88 transcription factors were found to be

differentially expressed in C57BL/6 and BALB/c macrophages in response to LPS at at least one time-point. These genes were hierarchically clustered according to expression, and based on the resulting dendogram, split into 11 clusters, C1-C11 (Figure 5.11). All genes in each cluster are listed in Appendix table 7.19.

C1 contained a mixture of genes that were more highly expressed in either C57BL/6 or BALB/c macrophages, and although statistically differentially expressed, their overall expression profiles were quite similar (Figure 5.11). Genes in C2 were up-regulated by LPS mainly from 1 h, were clearly more highly expressed in LPS stimulated C57BL/6 macrophages, and contained some genes that were previously noted in the *k*-means clustering such as *Irf2*, *Bcl6* and *Bcl3* (Figure 5.11). Genes in C3 were up-regulated by LPS from 0.5 h, were more highly expressed in C57BL/6 macrophages and included *Nfil3* and *Egr1* which had also been previously highlighted in the *k*-means clustering. C4 contained genes that were up-regulated by LPS from 3 h, and the majority, including *Mycbp*, were more highly expressed in BALB/c macrophages. The genes in C5 were also up-regulated in response to LPS in both strains, but substantially more highly expressed in C57BL/6 macrophages (at baseline and upon stimulation), and included genes such as *Atf4*, *Nfkb2*, *Stat4* and the interferon-inducible *Ifi204* (Doyle *et al.* 2002) (Figure 5.11). C6 contained *Bach2*, *Btbd11* and *Ddef1* and these genes were more highly expressed at baseline in C57BL/6 macrophages, but down-regulated in response to LPS in both strains (Figure 5.11). C7 contained genes including *Ctnnb1* (encodes β-catenin) and *Tgfb1i4* (encodes EGR5), that were again differentially expressed in unstimulated cells, but in this case were subtly up-regulated in response to LPS with overall higher expression in BALB/c macrophages (Figure 5.11). C8 and 9 contained

transcription factors that were differentially down regulated in response to LPS in both C57BL/6 and BALB/c macrophages and included genes such as *E2f1* and *Tsc22d3* (Figure 5.11, see Appendix Table 7.19). Genes within C10 were up-regulated transiently by LPS, more highly expressed in C57BL/6 macrophages and included the AP-1 transcription factors *Jun* and *Fos* (Figure 5.11). C11, similarly to C6, contained transcription factors that were very clearly differentially expressed at baseline in C57BL/6 and BALB/c macrophages, but were down-regulated upon LPS stimulation with overall higher expression in BALB/c macrophages and included the ETS family transcription factors *Etv1*, *Etv5* and *Elk3* (Figure 5.11).

5.3.7.4 *Transcription factor candidates mediating differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages identified by comparison of IPA, PSCAN and transcription factor mRNA expression.*

Overlapping the results from the three approaches taken – upstream regulator analysis by IPA; TFBS motif enrichment analysis by PSCAN; and differential expression of transcription factor genes, generated a list of 16 potential transcription factor candidates (Table 5.1). Based on the complied information in Table 5.1, EGR1 and NF-κB are the strongest candidates for regulating the C57BL/6 specific transcriptional profile as the data gathered on these regulators from the three analysis strategies was largely congruent. For example, EGR1 was predicted to be more active in C57BL/6 macrophages by IPA at 0.5 and 3 h; differentially expressed genes were enriched for the EGR1 TFBS at 3, 5 and 8 h; and *Egr1* was more highly expressed in C57BL/6 macrophages. Similar overall findings were made for NF-κB related transcription factors (Table 5.1). AP-1 (FOS/JUN), HIF-1α, IRF1, IRF5, STAT3 and STAT4 were all

identified by two approaches and the data indicates that these regulators may also be more active in C57BL/6 compared to BALB/c macrophages (Table 5.1). Although down-regulated in response to LPS, the data also suggested that E2F1 may be more active in C57BL/6 macrophages (Table 5.1). MYC was the strongest candidate for regulating the BALB/c specific transcriptional profile as it was predicted to be more activated in BALB/c macrophages by IPA at 5 h; differentially expressed genes were enriched for the MYC TFBS at 5 and 8 h; and *Mycbp* (MYC-binding protein, also known as AMY-1) which has been reported to enhance the transcriptional activity of MYC (Taira *et al.* 1998; Sakamuro *et al.* 1999), was more highly expressed in BALB/c macrophages (Table 5.1). The data also suggested that FOXM1, a transcription factor linked to cell cycle progression (Kalin *et al.* 2011), was more active in BALB/c macrophages, although this was down-regulated in response to LPS. SP1 is an interesting candidate as at 0.5 h it was predicted to be more activated in C57BL/6 macrophages, whereas at 5 h, a time-point at which SP1 TFBS enrichment was also found, it was predicted to be more active in BALB/c macrophages.

The compiled data gathered on other transcriptional regulators however was less congruent. For example, BCL6 was identified by IPA to be more activated in BALB/c macrophages, but its expression was higher in C57BL/6 macrophages. Similar discrepancies were found with HDAC, IRF2, and TSC22D3. Although this does not completely negate the relevance of these transcription factors as many are regulated at the post-transcriptional level, it does not suggest consistent evidence for their involvement in differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages.

5.4 Discussion

Our lab based studies (Chapter 3 and 4) have revealed higher production of IL-10 but lower production of IL-12 in LPS stimulated C57BL/6 macrophages compared to BALB/c macrophages. We further found that type I IFN signalling was responsible for differential IL-10 production, but that differences in IL-12 production were not fully accounted for by differential IL-10 production or type I IFN. In order to better understand the transcriptional networks that differ in LPS stimulated C57BL/6 and BALB/c macrophages and thus gain further insight into the potential mechanisms underlying differential cytokine production in these cells, we conducted genome wide expression profiling by microarray over a time-course of LPS stimulation.

5.4.1 The overall transcriptional profiles of LPS stimulated C57BL/6 and BALB/c macrophages

In our initial observations, we noted that in both C57BL/6 and BALB/c macrophages, genes were up- and down-regulated in response to LPS with kinetics that differed amongst specific sets of genes. These observations are in keeping with other studies which also show the temporal regulation of gene expression in LPS stimulated macrophages (Gilchrist *et al.* 2006; Nilsson *et al.* 2006). Further, our functional classification of these genes agreed with previous reports. For example, an early increase in expression of transcription factors as we observed, was also seen in the study of Gilchrist *et al.* and these factors may contribute to the expression of secondary LPS response genes (Gilchrist *et al.* 2006; Elkon *et al.* 2007; Smale 2012). In addition, the association of down-regulated genes with cell cycle related processes is in keeping

with previous microarray studies of LPS stimulated macrophages, and has been proposed to reflect the anti-proliferative activity of LPS (Nilsson *et al.* 2006; Ramsey *et al.* 2008).

5.4.2 Basal gene expression in C57BL/6 and BALB/c macrophages

Early in our analysis, we noted clear differences in the gene expression of C57BL/6 and BALB/c unstimulated macrophages. Our analysis of these basal differences revealed that several immune related genes were differentially expressed prior to stimulation. In the current investigation, we have primarily focussed on differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages. We did however find that *Il10* was among the differentially expressed basal genes, and was more highly expressed in C57BL/6 macrophages. Of note, our *k*-means clustering analysis placed *Il10* in gene clusters with only subtle differences in basal expression at 1 and 5 h, suggesting that this difference may not be extensive. Nevertheless, in unstimulated macrophages, it has been reported that there are DNase hypersensitivity sites within the *Il10* locus, indicative of chromatin remodelling in the basal state (Saraiva *et al.* 2005). It has also been reported that in resting macrophages, active histone modifications are associated with certain 'rapid' response genes, to facilitate their immediate expression upon stimulation (Ramirez-Carrozzi *et al.* 2009). Thus, *Il10* may be a gene that is 'poised' for expression and the higher level of basal *Il10* expression in C57BL/6 compared to BALB/c macrophages may be indicative of a different resting chromatin state. In this respect, it may be interesting to analyse active and repressive histone marks or DNase hypersensitivity sites at the *Il10* locus in unstimulated C57BL/6 and BALB/c macrophages. Of note, in contrast to what we observe in LPS stimulated macrophages, a

study analysing the effect of type I IFN on gene expression in unstimulated C57BL/6 macrophages found that basal *Il10* gene expression was not affected in the absence of the type I IFN receptor (Fleetwood *et al.* 2009). Further, we have no evidence in this study that *Ifnb1* is differentially expressed at baseline in C57BL/6 and BALB/c macrophages. This suggests that differential basal *Il10* expression, and perhaps other basal differences, may not necessarily be dependent on type I IFN. However, these basal gene expression differences warrant more in depth investigation as they could provide further information on existing differences between C57BL/6 and BALB/c macrophages which may affect LPS induced gene expression.

5.4.3 Differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages

Upon identification of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages, we noted that the majority of LPS responsive genes were similarly expressed between the strains. In addition, of the genes that were differentially expressed, the vast majority had a similar directionally of regulation in response to LPS. This suggested that the LPS induced transcriptional responses of C57BL/6 and BALB/c macrophages were quite similar, with quantitative differences accounting for the majority of differential gene expression. *Il10*, *Ifnb1* and *Il12a* were amongst the differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages, further validating the differential expression of these genes that we had previously observed by qPCR. In addition, we observed a stronger overall transcriptional response of both up- and down-regulated genes in C57BL/6 macrophages compared to BALB/c macrophages. In keeping with this observation, a previous microarray study analysing

LPS induced gene expression in C57BL/6, BALB/c, DBA/2J, C3H/ARC and C3H/HeJ macrophages, also found that fewer transcripts were more than 2 fold up-regulated by LPS in BALB/c macrophages compared to C57BL/6 macrophages (Wells *et al.* 2003). Thus, reduced transcriptional activity in BALB/c macrophages may be a generalised phenomenon and could indicate a state of relative repression in BALB/c macrophages, or the reduced activation of regulatory pathways.

Our earlier experiments (Chapter 3) had shown that LPS stimulated C57BL/6 macrophages produce higher levels of IFN-β than BALB/c macrophages. In keeping with this, in our analysis of C57BL/6^{hi} and BALB/c^{hi} gene clusters by *k*-means, we observed that the IFN-inducible genes *Mx1*, *Pml*, *Oas3* and *Ifit3* (Sadler *et al.* 2008) were more highly expressed in C57BL/6 macrophages at 5 h of LPS stimulation. However, we were surprised to find that other IFN-inducible genes such as *Mx2*, *Ifitm1* and *Oasl1* (Sadler *et al.* 2008) were more highly expressed in BALB/c macrophages at this time-point. We also observed differential expression of the IFN-inducible genes *Cxcl10* (C57BL/6^{hi}) and *Gbp1* (BALB/c^{hi}) from 0.5 h post LPS stimulation. Taken together, these observations suggest a complex relationship between the level of type I IFN produced by the cell, and downstream expression of IFN-inducible genes. It is possible that a high level of type I IFN preferentially induces one set of genes (i.e. those more highly expressed in C57BL/6 macrophages) whereas a lower level of type I IFN preferentially induces another (i.e. those expressed more highly in BALB/c macrophages), although to our knowledge this has not yet been reported. Alternatively, potential differences in how C57BL/6 and BALB/c macrophages are responding to type I IFN could be dictating the relative expression of type I IFN responsive genes.

However, the differential expression of these genes may also be a consequence of other genetic differences in C57BL/6 and BALB/c macrophages. In order to further understand the type I IFN dependence of differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages, it would be informative to conduct a microarray analysis of LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages to compare to C57BL/6 and BALB/c macrophages over this detailed time-course. This would allow an assessment of which genes truly depend on autocrine type I IFN signalling for their expression, and may therefore help to identify the genes which are differentially regulated in C57BL/6 and BALB/c macrophages due to type I IFN or other genetic differences.

Our investigation into differential gene expression by *k*-means also highlighted the higher expression of *Bcl3*, *Irak3* and *Trim30* in C57BL/6 macrophages. This is of interest as the products of these genes have been shown to regulate TLR-induced cytokine production. For example, BCL3 (B-cell CLL/lymphoma 3) has been reported to promote IL-10 and inhibit IL-1β in LPS stimulated macrophages (Wessells *et al.* 2004), and this fits with the profile of cytokine production that we observe in C57BL/6 and BALB/c LPS stimulated macrophages. *Irak3* encodes IRAK-M which has been shown disrupt interactions between IRAK1 and IRAK4 downstream of MyD88, inhibiting proinflammatory cytokine production (Kobayashi *et al.* 2002). *Trim30* has been associated with negative regulation of the NF-κB pathway downstream of TLR4 (Kawai *et al.* 2011; McNab *et al.* 2011). The higher expression of *Irak3* and *Trim30* expression in C57BL/6 macrophages could therefore potentially be involved in the differential cytokine production that we observe in LPS stimulated C57BL/6 and BALB/c macrophages.

5.4.4 Putative transcriptional regulators involved in the differential gene expression of LPS stimulated C57BL/6 and BALB/c macrophages

We focussed the latter part of our analysis on using unbiased approaches to identify transcriptional regulators that may be differentially activated in LPS stimulated C57BL/6 and BALB/c macrophages. Towards this aim, we used IPA upstream regulator analysis, transcription factor binding site (TFBS) motif enrichment analysis and compared the expression level of genes encoding transcription factors in C57BL/6 and BALB/c macrophages. Importantly, each of these methods has different strengths and weaknesses. The IPA upstream regulator analysis depended on information derived from the Ingenuity Knowledge Base. This information is manually curated and is compiled from over 200,000 publications on human, mouse and rat experimental systems (Calvano *et al.* 2005). Thus, although a highly inclusive approach, it may identify factors that are not relevant to our biological system. The TFBS enrichment analysis is powerful as it focuses on information within the genes themselves however, it will only identify transcription factor involvement if those factors directly bind to the DNA within the specified search region, in this case -950 to +50 relative to the transcriptional start site. Thus, if a transcriptional regulator has an indirect effect or its binding site is very distal, it will be missed. Additionally, transcription factors with a small number of target genes may not be identified by this method (Elkon *et al.* 2007). Analysis of transcription factor mRNA expression uses the expression data in its purest form, without bioinformatic extrapolations. However, the activity of many transcription factors is regulated at the post-transcriptional level and it is not completely clear how the level of transcription factor expression affects their function (Smale 2012). Thus, differential transcription factor mRNA expression, may or may not correlate to

differential transcription factor activity. Aware of these limitations, our approach of overlapping the results from each method aimed to gather several lines of evidence for the involvement of any given transcriptional regulator in regulating the distinct transcriptional profiles of LPS stimulated C57BL/6 and BALB/c macrophages. This combined analysis resulted in the identification of several potential transcription factor candidates, some of which may be involved in the differential regulation of IL-10, IL-12 and type I IFN.

One of the strongest candidates generated by the above approach was NF-κB as it was identified by all three analysis methods at the majority of time-points. In support of this, our earlier IPA pathway analysis associated differentially expressed genes with NF-κB signalling at 3 h and our *k*-means analysis highlighted the higher expression of NF-κB pathway related genes such as *Ikbkb* and *Nfkb2* in C57BL/6 macrophages. The NF-κB pathway is well known to regulate LPS induced transcriptional responses in macrophages and DCs (Medzhitov *et al.* 2009). It is therefore plausible that this pathway may be involved in mediating the differential cytokine expression we observe in C57BL/6 and BALB/c macrophages. Further, NF-κB transcription factors have been strongly implicated in the positive regulation of IL-10 (Saraiva *et al.* 2005; Zhang *et al.* 2006), IL-12 (Murphy *et al.* 1995; Plevy *et al.* 1997; Sanjabi *et al.* 2000; Grumont *et al.* 2001) and type I IFN (Honda *et al.* 2005c). Overall, our analysis has predicted that the NF-κB pathway may be more active in C57BL/6 macrophages. Thus, enhanced or differential NF-κB signalling could theoretically contribute to elevated IL-10 and/or type I IFN production in C57BL/6 compared to BALB/c macrophages.

An additional candidate identified by all three analysis methods was EGR1 (early growth response-1). EGR1 is a zinc finger transcription factor, previously reported to promote the differentiation of macrophages (Nguyen *et al.* 1993). Downstream of LPS stimulation, the expression of *Egr1* is induced by the TPL-2/ERK pathway (Waterfield *et al.* 2003). In the context of IL-10 regulation, EGR1 has been shown to induce the expression of the microRNA hsa-mir-106a which destabilises *Il10* mRNA (Sharma *et al.* 2009). Based on our information, EGR1 is currently predicted to be more activated in C57BL/6 macrophages which produce more IL-10 and therefore is not in keeping with the findings of Sharma *et al*. (Sharma *et al.* 2009). However, there may be as of yet unidentified roles for EGR1 in the regulation of IL-10, IL-12 or type I IFN and thus the role of this transcription factor in mediating differential cytokine production in C57BL/6 and BALB/c macrophages warrants further investigation. Of note, ELK-1, AP-1 and CREB were also predicted to be more activated in LPS stimulated C57BL/6 macrophages compared to BALB/c macrophages by IPA (and *Fos*/*Jun* expression in the case of AP-1). Each of these factors can also be induced by the MAP kinase ERK (Karin *et al.* 1997; Tsai *et al.* 2000), which itself along with AP-1 and CREB have been directly linked to the positive regulation of IL-10 (Agrawal *et al.* 2003; Dillon *et al.* 2004; Ananieva *et al.* 2008; Kaiser *et al.* 2009). Also interesting in this context was that *Nfkb1*, a negative regulator of the TPL-2/ERK pathway (Gantke *et al.* 2011), was more highly expressed in BALB/c macrophages, and *Map3k8* which encodes TPL-2, was more highly expressed in C57BL/6 macrophages. Collectively, these findings may provide evidence for the enhanced activation of ERK in LPS stimulated C57BL/6 macrophages. However, our previous data comparing ERK phosphorylation in LPS stimulated C57BL/6 and BALB/c macrophages did not provide evidence for this (see

Chapter 3, Figure 3.8). It could be that that potential strain differences in this pathway are not at the level of ERK phosphorylation or, as we assessed ERK phosphorylation only within the first hour of LPS stimulation, differences may be at later time-points.

MYC (also known as c-MYC) was the final transcriptional regulator identified by all three analysis approaches. MYC is strongly linked to the biology of cancer, most likely due to its central role in regulating cell proliferation and apoptosis (Dang 2012). In serum stimulated fibroblasts, MYC target genes have broadly been associated with RNA processing functions, DNA replication and ribosome biogenesis (Perna *et al.* 2012). In macrophages, MYC has been associated with M-CSF induced cell cycle entry (Roussel 1997) and *c-myc* mRNA is induced upon LPS stimulation (Introna *et al.* 1986). MYC was predicted to be more active in BALB/c macrophages. This may be interesting in the context that the NF-κB pathway is predicted to be more active in C57BL/6 macrophages, and MYC has been shown to inhibit LPS induced NF-κB signalling in MYC-transformed murine B cell lines, although the effect of MYC on cytokine production was not reported (Klapproth *et al.* 2009). Thus, preferential activation of MYC in BALB/c cells could be a reflection of cell-cycle progression, but could potentially have additional consequences for cytokine production.

In addition to the three main candidates discussed above, several other transcriptional regulators which we know from the literature to be important in the regulation of IL-10, IFN-β and IL-12 were highlighted during our combined analyses. For example, IRF1 and STAT3 were predicted to be more active in C57BL/6 macrophages. These transcription factors have been implicated in the enhancement of IL-10 by type I IFN

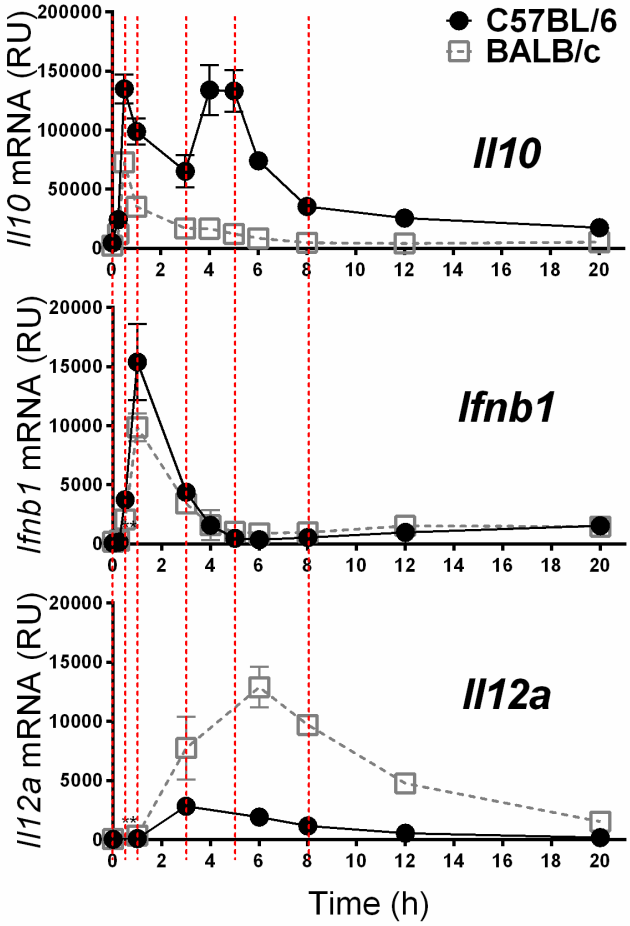
(Ziegler-Heitbrock *et al.* 2003) and are therefore of extremely high interest as they may have a role in the induction of the second peak of *Il10* mRNA that we observe in C57BL/6 macrophages. IRF5 was also predicted to be more active in C57BL/6 macrophages. However, IRF5 has been reported to inhibit IL-10 while promoting IL-12 (Krausgruber *et al.* 2011), the opposite cytokine profile to what we observe in C57BL/6 macrophages, so is unlikely to be causal to our findings. SP1 was predicted to be more activated in BALB/c macrophages at 5 h, a time-point when SP1 TFBS enrichment was also found. This transcription factor is known to be important in the regulation of *Il12a* (IL-12p35) expression (Goriely *et al.* 2003), in keeping with higher *Il12a* gene expression in BALB/c macrophages, although SP1 has also been shown to positively regulate *Il10* gene expression (Tone *et al.* 2000). *Bcl6* was more highly expressed in C57BL/6 macrophages, however, it was predicted to be more active in BALB/c macrophages at 1, 5 and 8 h. This may be interesting in the context that BCL-6 is considered a transcriptional repressor (Dent *et al.* 2002) and as already discussed, we observed an overall more muted induction of generalised gene expression in BALB/c macrophages compared to C57BL/6 macrophages. Our data also indicate that HIF-1α (Hypoxia-inducible factor 1 α), an IFN-inducible gene (Der *et al.* 1998), may be involved in mediating differential gene expression in C57BL/6 and BALB/c macrophages. The role of HIF-1α in the regulation of IL-10, IL-12 and type I IFN is not clear, although this transcription factor has recently been shown to regulate IL-1β in LPS stimulated macrophages (Tannahill *et al.* 2013). HIF-1α may therefore be an interesting novel candidate for follow-up studies on differential cytokine production in C57BL/6 and BALB/c macrophages.

Finally, although we were most interested in transcription factors identified by more than two analysis strategies, KLF4 (Kruppel-like factor 4) and AHR (Aryl hydrocarbon receptor) although only identified by TFBS enrichment analysis, may also be of interest as they have been associated with IL-10 and IL-12 regulation. For example, KLF4 has been linked to the positive regulation of IL-10 in response to TLR ligands in RAW264.7 macrophages (Liu *et al.* 2007). AHR has been shown to inhibit IL-6, TNF-α and IL-12p40 but promote IL-10 in LPS stimulated macrophages (Kimura *et al.* 2009). Further, AHR has been implicated in the transactivation of the *Il10* gene in Tr1 cells, in synergy with c-MAF (Apetoh *et al.* 2010). The activity of these transcription factors in C57BL/6 and BALB/c macrophages may therefore be interesting in the context of our study.

In summary, our microarray analysis has revealed complex patterns of differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages which extend far beyond *Il10*, *Ifnb1* and *Il12a*. We observed differential gene expression in resting and LPS stimulated C57BL/6 and BALB/c macrophages. This included the differential expression of IFN-inducible genes in LPS stimulated cells, some of which were more highly expressed in C57BL/6 macrophages, and others in BALB/c macrophages. In addition, several lines of evidence suggested that NF-κB signalling may have a role in mediating differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages. Our findings also implicated a role for EGR1, and potentially other ERK regulated transcription factors in mediating strain dependent gene expression. In addition, other valid candidates such as MYC, BCL3, BCL6, STAT3, IRF1, SP1, HIF-1α, KLF4 and AHR were identified throughout the various approaches taken to mine

this microarray data. Thus, this analysis has provided several potential leads for the continued investigation into the underlying mechanisms of differential IL-10 and IL-12 production in C57BL/6 and BALB/c macrophages.

A



B

Time-point (h)	Untreated	Media	LPS
0	x		
0.5		x	x
1		x	x
3		x	x
5		x	x
8		x	x

Figure 5.1 Experimental design for microarray analysis of differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages.

C57BL/6 and BALB/c BMDM were stimulated with LPS (10 ng/ml) for the indicated times. Total RNA was harvested and isolated. *Il10*, *Ifnb1* and *Il12a* transcript levels were determined by qPCR and normalised to *Hprt1* mRNA. Graphs show mean ± SD of three cultures. Red lines indicate time-points chosen for microarray analysis and are summarised in (B). All experimental groups were done in triplicate.

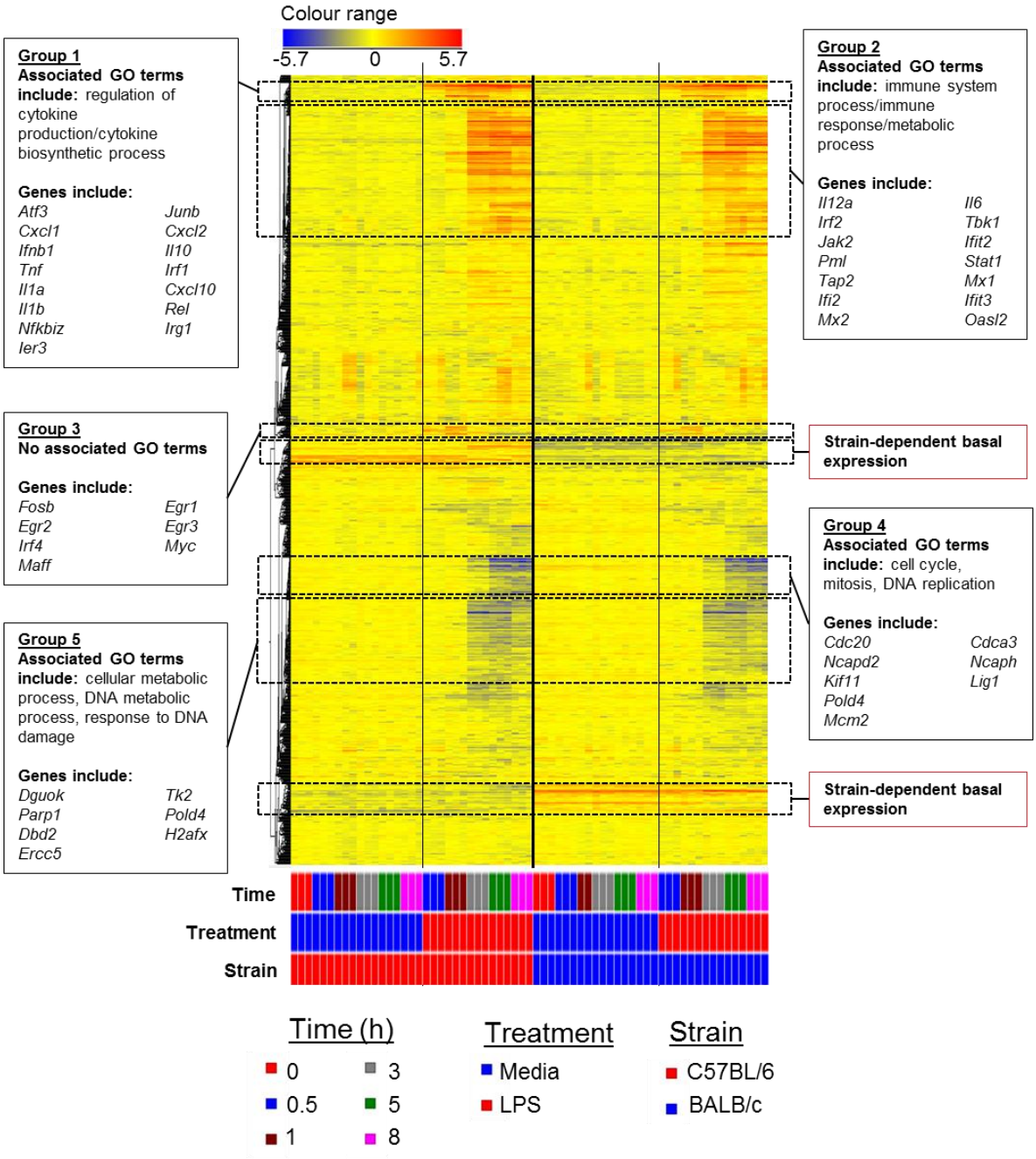


Figure 5.2 LPS induces dynamic transcriptional changes in C57BL/6 and BALB/c macrophages.

Gene probes were normalised to the median of each gene and filtered on flags. Samples were ordered according to experimental condition and gene probes hierarchically clustered according to expression. Groups of genes outlined were analysed for associated GO terms. Select GO terms (p<0.05) and example genes related to those terms (where applicable), are shown in the figure. Colour range denotes normalised intensity of expression.

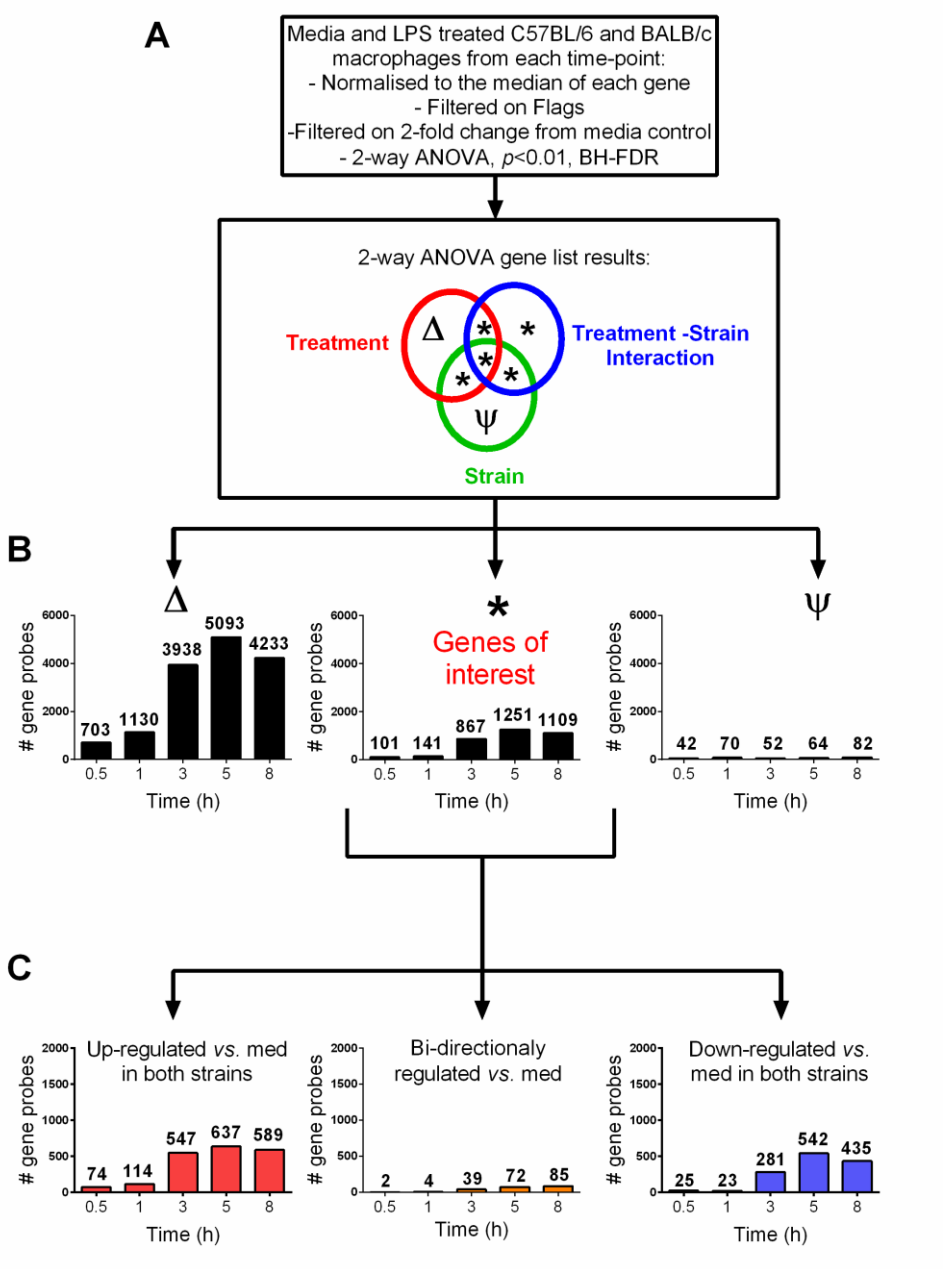


Figure 5.3 Identification of significantly differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages.

(A) Analysis strategy of gene expression in media and LPS treated C57BL/6 and BALB/c macrophages at each time-point. (B) Numerical representation of results from analysis strategy in (A). Δ, * and Ψ symbols correlate between (A) and (B). (C) Break-down of significantly differentially expressed genes (*) into up-regulated compared to media in both strains (red), bi-directionally regulated compared to media according to the strain of mouse (orange), or down-regulated compared to media in both strains (blue). BH-FDR, Benjamini-Hochberg False Detection Rate; med, media.

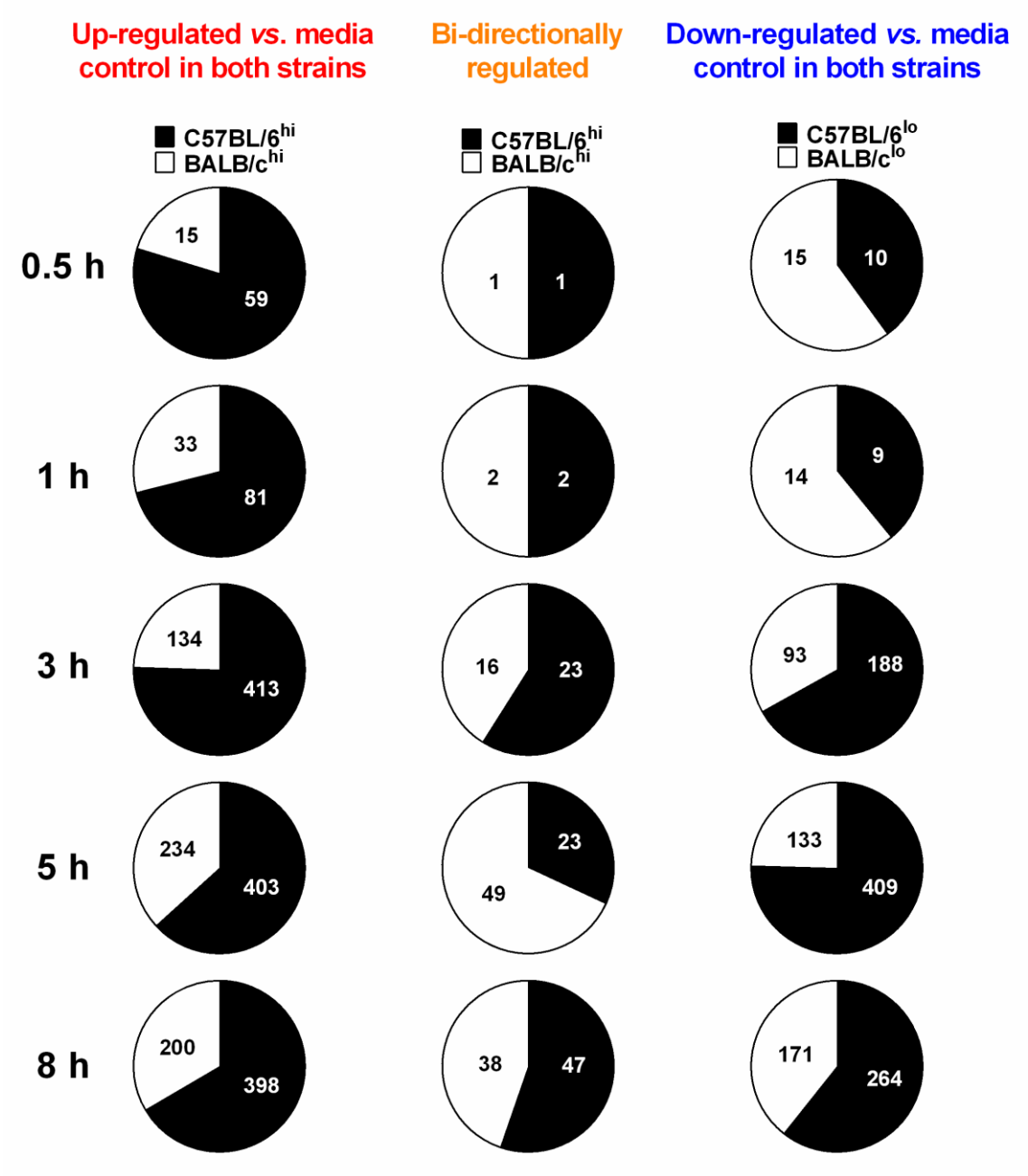


Figure 5.4 C57BL/6 macrophages may have a stronger transcriptional response to LPS than BALB/c macrophages.

Differentially expressed genes in C57BL/6 and BALB/c LPS stimulated macrophages which were up-, bi-directionally or down-regulated compared to media control at each time-point, were separated according to their relative C57BL/6 *vs.* BALB/c expression level. For genes up- and bi-directionally regulated, this is expressed as C57BL/6^{hi} or BALB/c^{hi}. For genes that were down-regulated compared to media control, this is expressed as C57BL/6^{lo} or BALB/c^{lo}. The number of gene probes within each segment is represented in the figure.

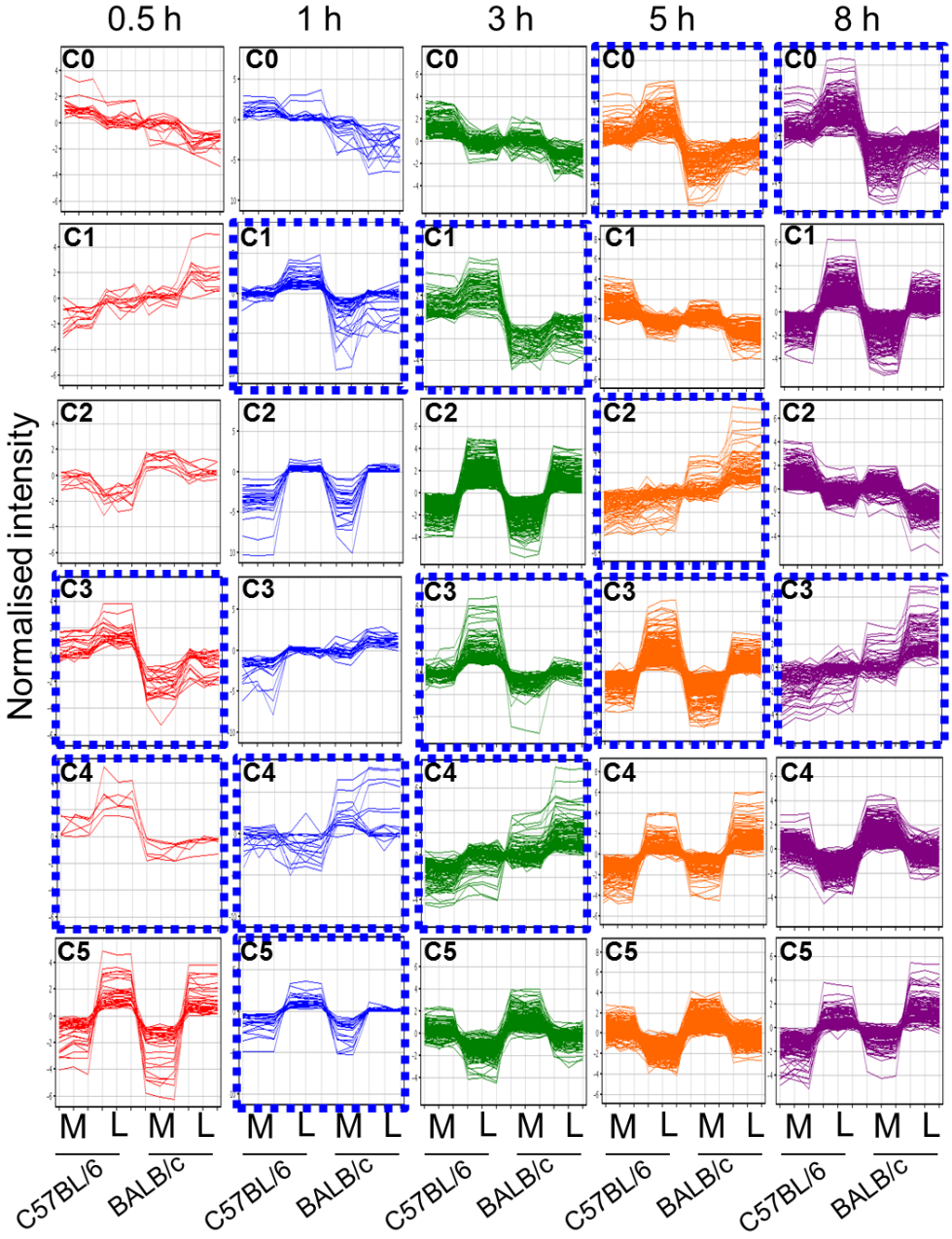


Figure 5.5 *k*-means clustering of differentially expressed genes at each time-point identifies the groups of genes that most clearly distinguish C57BL/6 and BALB/c transcriptional profiles.

Differentially expressed genes of interest identified at each time-point in the strategy outlined in Figure 5.3 B (*), were each separated into 6 clusters (C0-C5) by *k*-means clustering. Clusters considered the most differentially expressed between C57BL/6 and BALB/c LPS stimulated macrophages are outlined in blue. Genes in these clusters are further analysed in Figures 5.6-5.7. M, media; L, LPS.

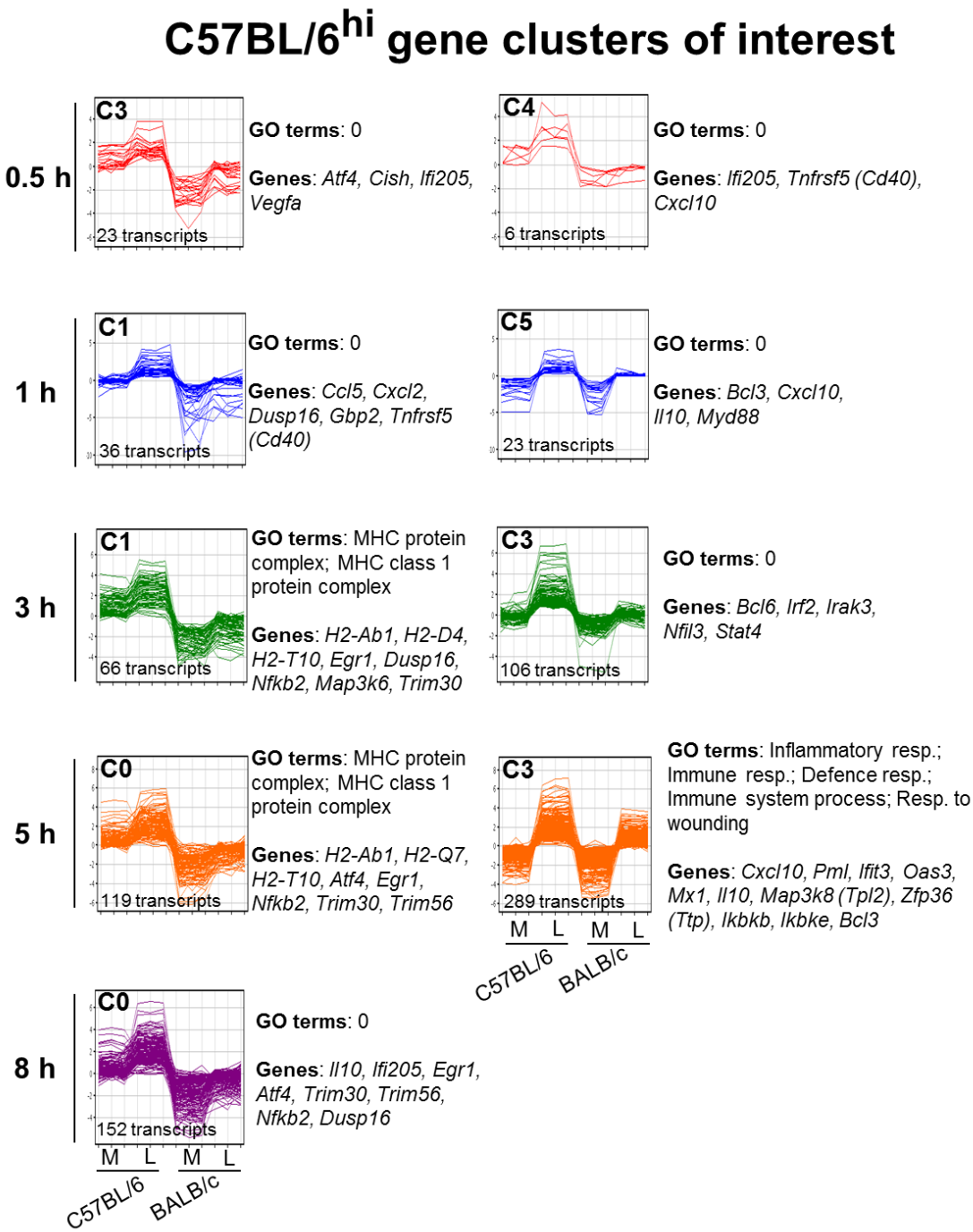


Figure 5.6 C57BL/6^{hi} *k*-means clusters of interest.

Gene clusters of interest identified in Figure 5.5 which had higher expression in LPS stimulated C57BL/6 macrophages compared to BALB/c macrophages are shown. Associated GO terms (p<0.05) and select genes from each cluster are listed. All genes within each cluster are listed in Appendix tables 7.1-9. M, media; L, LPS.

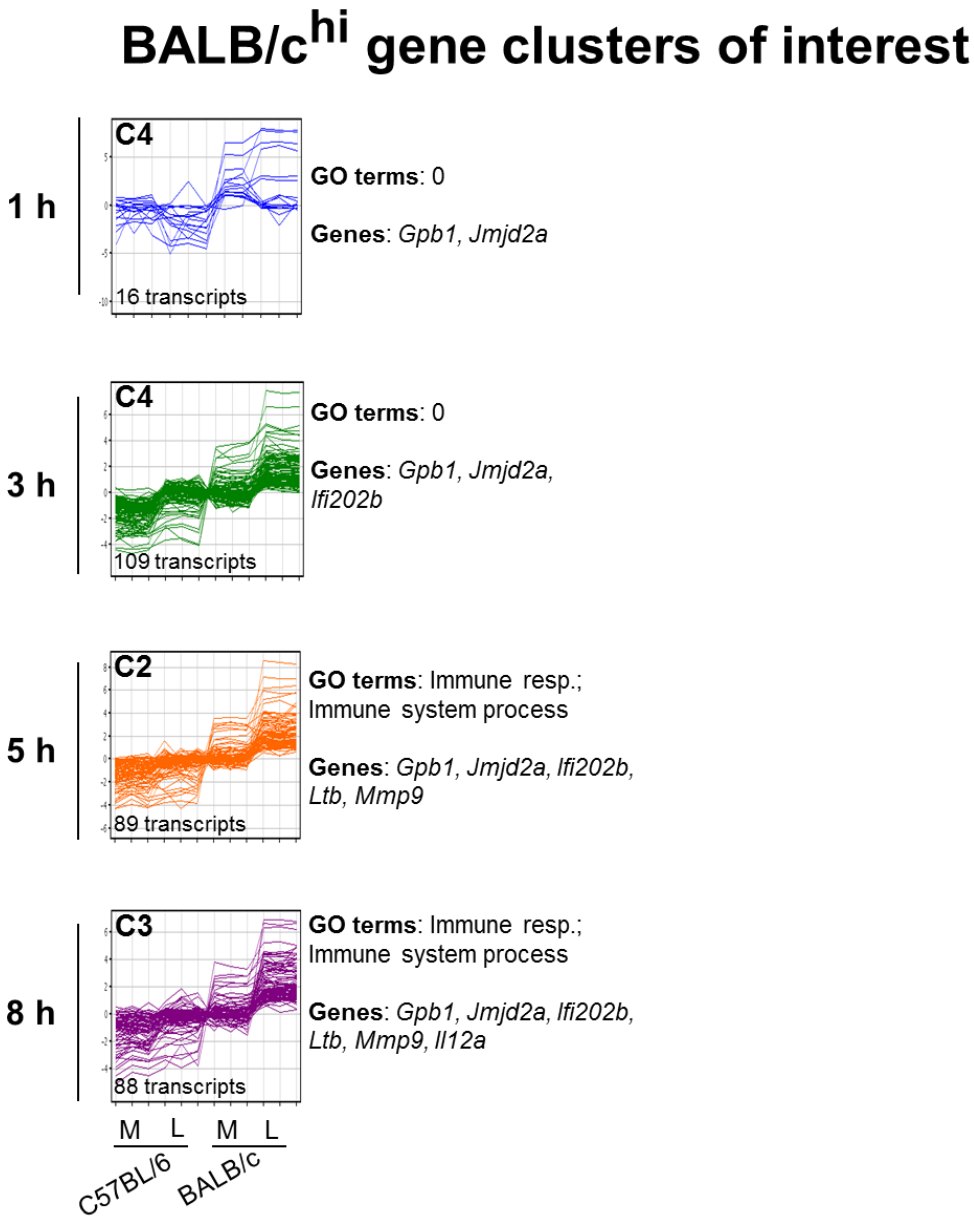


Figure 5.7 BALB/c^{hi} *k*-means clusters of interest.

Gene clusters of interest identified in Figure 5.5 which had higher expression in LPS stimulated BALB/c macrophages compared to C57BL/6 macrophages are shown. Associated GO terms (p<0.05) and select genes from each cluster are listed. All genes within each cluster are listed in Appendix tables 7.10-13. M, media; L, LPS.

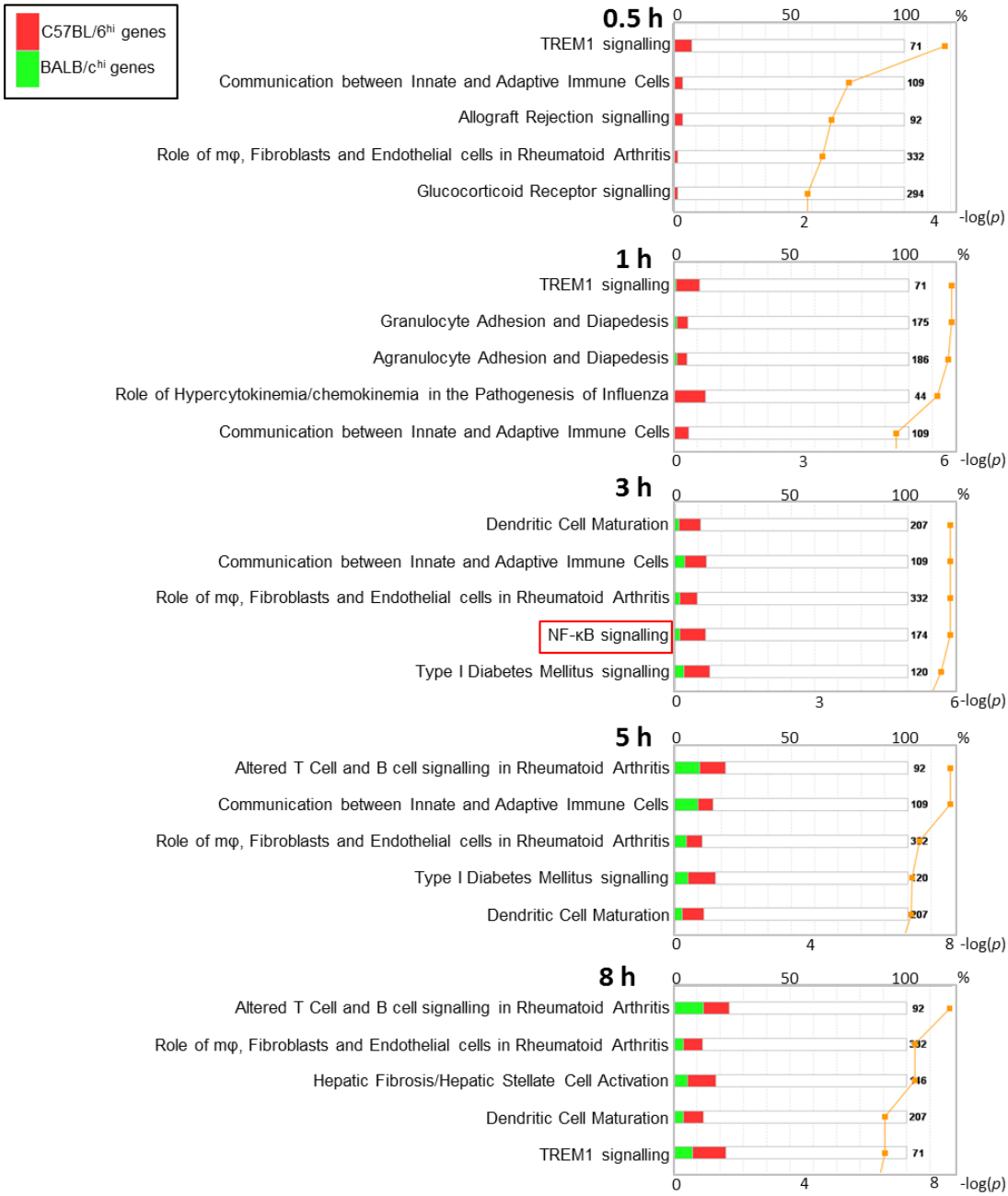


Figure 5.8 IPA pathway analyses of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages.

Differentially expressed genes of interest identified at each time-point in the strategy outlined in Figure 5.3 B (*) were analysed by IPA to assess if they were associated with any known biological pathways. The top 5 pathways associated with differentially expressed genes at each time-point are shown. Top axis represents the percentage overlap between input genes and genes in the pathway. Bottom axis denotes Benjamini-Hochberg corrected –log(*p*), represented by the gold line, for pathway association. All pathways shown pass a cut off of $p<0.01$. Pathway associated genes within our dataset are listed in Appendix Tables 7.14-7.18.

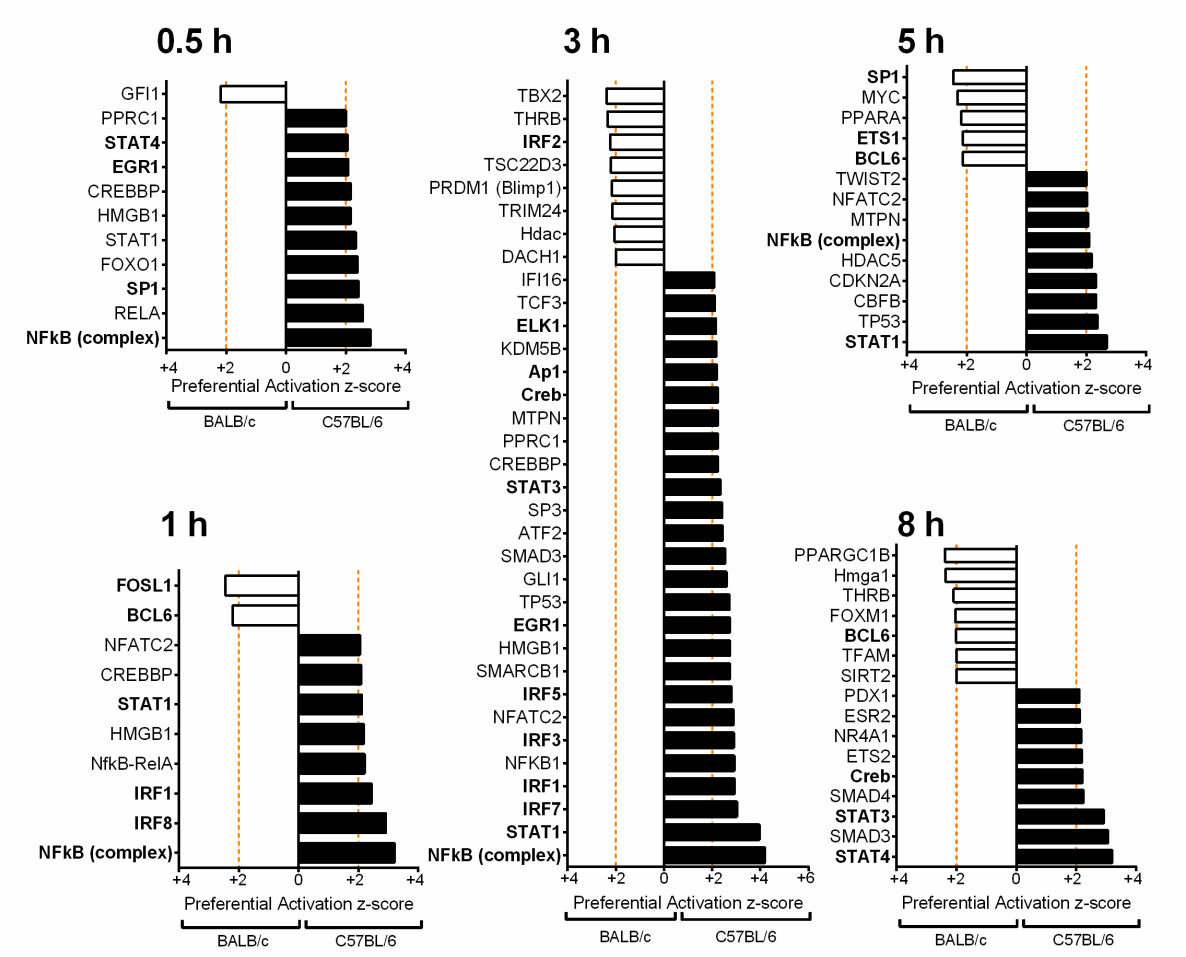


Figure 5.9 IPA transcriptional regulators associated with the differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages.

Differentially expressed genes of interest identified in the strategy outlined in Figure 5.3 B (*) were analysed by IPA in order to identify associated upstream transcriptional regulators. Using the relative C57BL/6 *vs.* BALB/c expression level of each transcript, the programme generated an activation *z*-score which predicted if a transcription factor was likely to be more active in one strain over the other (denoted as 'preferential' activation). Black bars represent C57BL/6 preferential activation, open bars represent BALB/c preferential activation. Transcriptional regulators shown were selected based on significant association with the gene list ($p<0.05$) and significant activation z-score ($z>2$). Bolded transcription factors are discussed in the text.

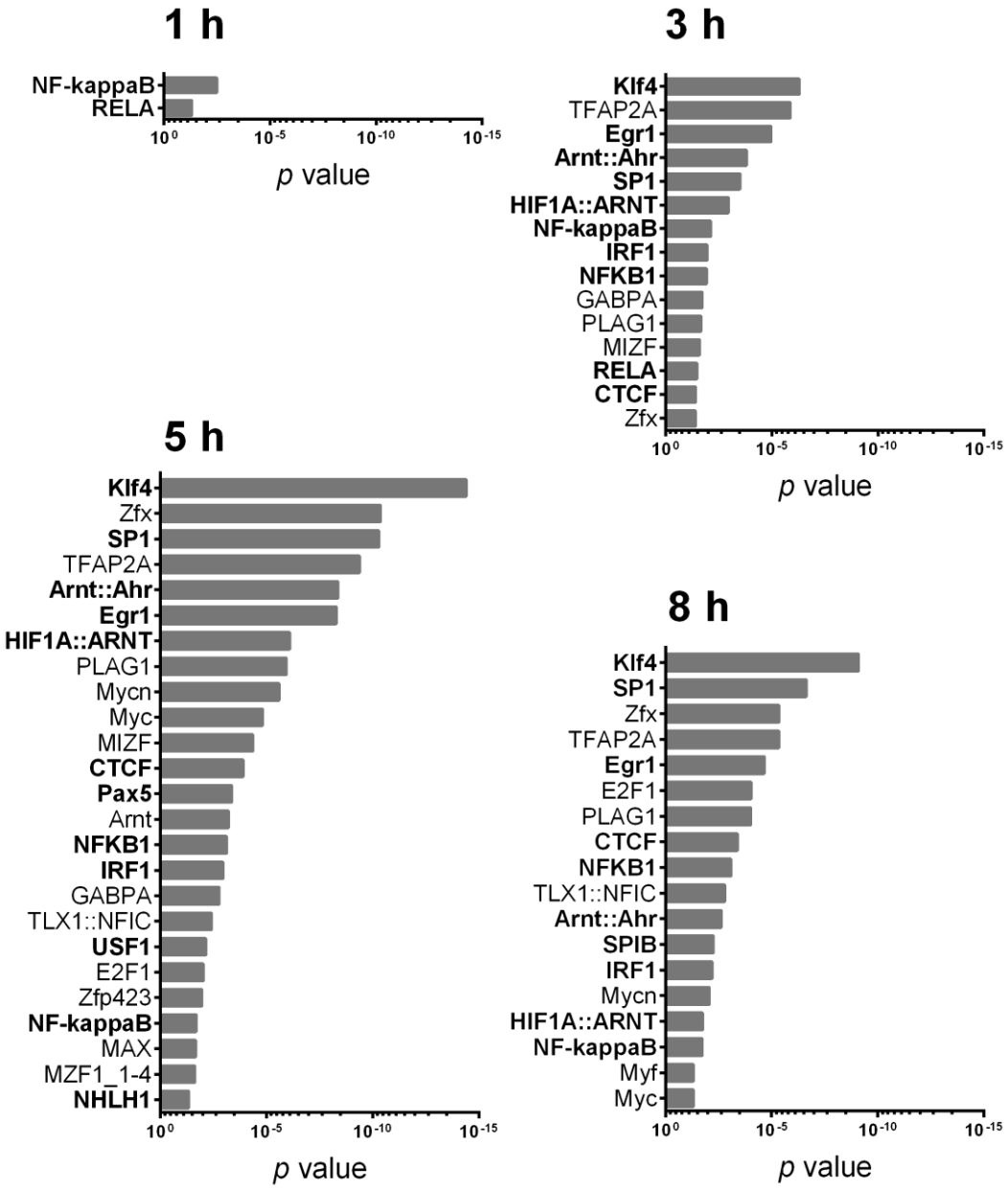


Figure 5.10 PSCAN transcription factor binding site (TFBS) enrichment analysis of differentially expressed genes in LPS stimulated C57BL/6 and BALB/c macrophages.

Differentially expressed genes of interest identified at each time-point in the strategy outlined in Figure 5.3 B (*) were analysed for enrichment of TFBS motifs using PSCAN software (Zambelli *et al.* 2009). Transcription factors with significantly enriched motifs (Bonferroni corrected $p<0.05$) are shown. No significant enrichment of TFBS was found at 0.5 h post-stimulation. Bolded transcription factors are discussed in the text.

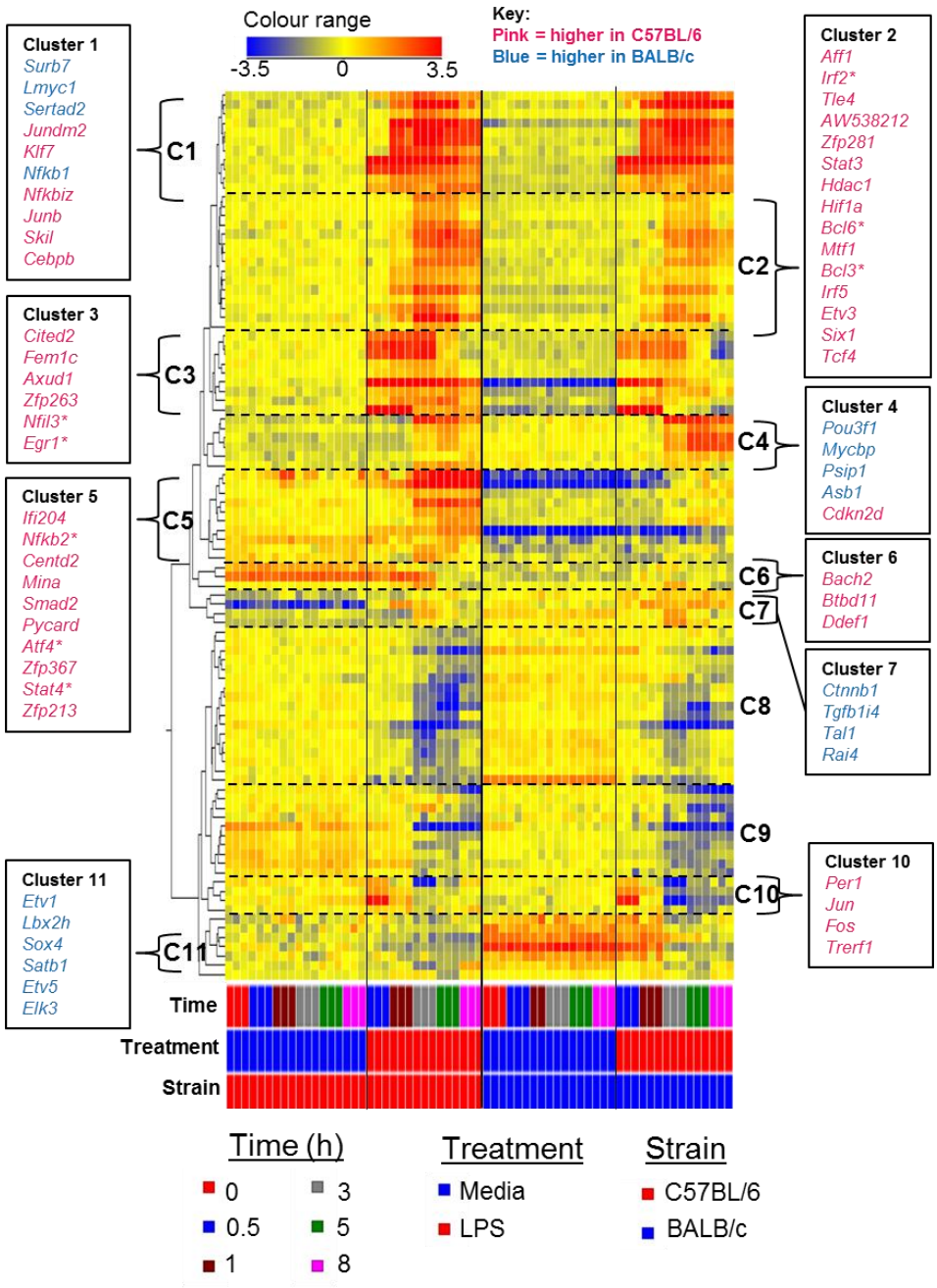


Figure 5.11 Differential expression of transcription factor genes in LPS stimulated C57BL/6 and BALB/c macrophages.

Genes encoding transcription factors were selected from the lists of differentially expressed genes of interest identified in the strategy outlined in Figure 5.3 B (*). Heat map shows hierarchical clustering of the 88 transcription factor genes identified. Transcription factor genes were separated into clusters C1-C11 based on the hierarchical dendogram. Genes present in selected clusters are shown, asterisk denotes gene was previously noted in the *k*-means analysis. All transcription factor genes in each cluster are listed in Appendix Table 7.19. Colour range denotes normalised intensity of expression.

C57BL/6 preferential activation (IPA)/higher expression

BALB/c preferential activation (IPA)/higher expression

	Identified by IPA (*in silico*)		Identified by PSCAN (*in silico*)		Gene expression data, C57BL/6 *vs.* BALB/c	
Candidate Transcription factor (or complex)	**TF or complex component**	**Time-point**	**TF or complex component**	**Time-point**	**Gene**	**effect of LPS**
AP-1	AP-1	3 h			*Fos*	up
					Jun	up
BCL-6	BCL6	1, 5, 8 h			*Bcl6*	up
E2-F1			E2f1	5, 8 h	*E2f1*	down
EGR1	EGR1	0.5, 3 h	EGR1	3, 5, 8 h	*Egr1*	up
FOXM1	FOXM1	8 h			*Foxm1*	down
HDAC	HDAC	3 h			*Hdac1*	up
HIF-1α			HIF1A::ARNT	3, 5, 8 h	*Hif1a*	up
IRF1	IRF1	1, 3 h	IRF1	3, 5, 8 h		
IRF2	IRF2	3 h			*Irf2*	up
IRF5	IRF5	3 h			*Irf5*	up
MYC	MYC	5 h	MYC	5, 8 h	*(Mycbp)*	up
NF-κB family	NFκB complex	0.5, 1, 3, 5 h	NF-κB	1, 3, 5, 8 h		
	RELA	0.5, 1 h	RELA	1, 3 h		
	NFKB1	3 h	NFKB1	3, 5, 8 h	*Nfkb1*	up
					NfKb2	up
SP-1	SP1	0.5 h				
	SP1	5 h	SP1	3, 5, 8 h		
STAT3	STAT3	3, 8 h			*Stat3*	up
STAT4	STAT4	0.5, 8 h			*Stat4*	up
TSC22D3	TSC22D3	3 h			*Tsc22d3*	down

Table 5.1 Candidate transcriptional regulators mediating differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages.

Transcriptional regulators are listed if they were identified by at least two approaches at a given time-point. Note that no strain preference can be identified by the PSCAN analysis. For reference, all time-points at which a particular transcriptional regulator was identified by IPA or PSCAN are noted. Relative transcription factor expression in stimulated cells is derived from information at all time-points. TF, transcription factor.

Chapter 6. Summary and Future Perspectives

6.1 Summary

Over the course of this investigation, we have analysed differential IL-10 and IL-12 production in C57BL/6 and BALB/c macrophages. In doing so, we sought to elucidate the molecular mechanisms underlying these strain differences, with the aim of better understanding the regulation of IL-10 and IL-12 in PRR stimulated macrophages. We began by analysing IL-10 production in response to several TLR-dependent and TLR-independent PRR stimuli in addition to heat-killed bacteria. We subsequently narrowed our study to investigate cytokine production in response to LPS (TLR4), Pam3CSK4 (TLR2) and heat-killed *B.pseudomallei* (TLR2/4).

In the context of LPS and heat-killed *B.pseudomallei* stimulation, we found that C57BL/6 macrophages produced higher levels of IL-10 than BALB/c macrophages. Further, C57BL/6 macrophages induced two temporally distinct waves of *Il10* transcription, whereas BALB/c macrophages induced only one. Additional studies revealed that the second wave of *Il10* transcription, observed only in C57BL/6 macrophages, was dependent on type I IFN signalling and was directly linked to the higher level of IL-10 production in these cells. Further, in contrast to a previous report (Iyer *et al.* 2010), the type I IFN mediated up-regulation of IL-10 was not dependent on IL-27, which itself had no ability to regulate IL-10 in our system. Our investigation into differential proinflammatory cytokine production revealed that in contrast to IL-10 and type I IFN, the production of IL-12p70 was higher in BALB/c macrophages in response to these stimuli. Differential IL-12p70 production in C57BL/6 and BALB/c macrophages was still apparent in the absence of IL-10 and not further affected by the abrogation of type I IFN signalling in C57BL/6 macrophages. We also found that TNF-

α and IL-1β were produced at a higher level in LPS and heat-killed *B.pseudomallei* stimulated BALB/c macrophages. Differential TNF-α production but not IL-1β production was dependent on IL-10. However, there may be a role for type I IFN in the differential production of IL-1β. Genome wide expression profiling of LPS stimulated C57BL/6 and BALB/c macrophages by microarray confirmed the differential expression of *Il10*, *Ifnb1* and *Il12a* and revealed complex differences in gene expression between LPS stimulated C57BL/6 and BALB/c macrophages over time. Subsequent bioinformatic analyses of this data predicted that there may be roles for the transcription factors NF-κB and EGR1 amongst other candidates including MYC, STAT3, IRF1 and potentially ERK-regulated transcription factors, in mediating the differential gene expression in LPS stimulated C57BL/6 and BALB/c macrophages.

Our investigation of cytokine production in C57BL/6 and BALB/c Pam3CSK4 stimulated macrophages yielded quite different results. C57BL/6 macrophages still produced higher levels of IL-10 than BALB/c macrophages. However, only one wave of *Il10* transcription was induced in either strain and differential IL-10 production was independent of type I IFN. Again, similarly to what we observed in LPS and heat-killed *B.pseudomallei* stimulated macrophages, the production of IL-12p70, TNF-α and IL-1β was higher in Pam3CSK4 stimulated BALB/c macrophages. However, differential production of all of these cytokines was completely dependent on IL-10. This suggested that unlike TLR4 stimulation, TLR2 stimulation has a similar capacity to induce proinflammatory cytokines in C57BL/6 and BALB/c macrophages, provided that IL-10 is absent. This highlights the contrasting mechanisms of cytokine regulation

downstream of different TLRs which may be attributed to their differential use of the adaptor proteins MyD88 and TRIF (Kawai *et al.* 2010).

6.2 Future perspectives

6.2.1 Continuing to use C57BL/6 and BALB/c macrophages to study the molecular regulation of IL-10 and IL-12

6.2.1.1 *Further investigation into the mechanisms of type I IFN dependent regulation of IL-10*

Our data shows that the previously reported type I IFN mediated autocrine regulatory loop promoting IL-10 production in TLR4 stimulated C57BL/6 macrophages (Chang *et al.* 2007a), is absent BALB/c macrophages. Other investigators studying how type I IFN regulates IL-10 and indeed other cytokines, have made use of C57BL/6 and C57BL/6 *Ifnar1*$^{-/-}$ macrophages (Chang *et al.* 2007a; Guarda *et al.* 2011; Mayer-Barber *et al.* 2011; Pattison *et al.* 2012). However, we postulate that C57BL/6 and C57BL/6 *Ifnar1*$^{-/-}$ macrophages present two extremes of a scale – robust type I IFN production and responsiveness (C57BL/6) *versus* a complete inability to detect type I IFN (C57BL/6 *Ifnar1*$^{-/-}$). Thus, BALB/c macrophages may provide a valuable intermediate scenario where some type I IFN is made and indeed signals through the type I IFN receptor, but *Il10* transcription is not induced. For example, our studies and others (Guarda *et al.* 2011) support an important role for STAT1 in the regulation of IL-10 by type I IFN. However, we observe notable STAT1 Tyr-701 phosphorylation in BALB/c macrophages suggesting that this signalling event alone is not adequate for the induction of *Il10* transcription by type I IFN. In future studies we will continue to

investigate other differences between type I IFN induced signalling in C57BL/6 and BALB/c macrophages which may impact on differential IL-10 production. Central candidates for this analysis will be the p38, ERK and PI(3)K pathways, as these are activated by type I IFN signalling (Platanias 2005) and have been shown to positively regulate IL-10 (Agrawal *et al.* 2003; Dillon *et al.* 2004; Jarnicki *et al.* 2008; Kim *et al.* 2008; Ohtani *et al.* 2008; Weichhart *et al.* 2008; Kaiser *et al.* 2009). Further, the PI(3)K pathway has previously been associated with the regulation of IL-10 by type I IFN in DCs (Wang *et al.* 2010). These experiments, by using the comparison of C57BL/6 and BALB/c macrophages, may give us a more detailed insight into the exact signalling requirements for type I IFN mediated IL-10 regulation in macrophages.

Additionally, in our system for the first time, we are able to temporally separate the early TLR induced *Il10* mRNA induction and later type I IFN dependent *Il10* mRNA induction. It would therefore be interesting as a complimentary approach to use pharmacological inhibitors to assess the relative importance of p38 activation, ERK activation and PI(3)K signalling for the early and late transcription of *Il10* in C57BL/6 macrophages. Such an analysis may elucidate the common and unique signalling pathways required for each phase of *Il10* expression in TLR4 stimulated macrophages. Other studies have proposed that type I IFN treatment recruits STAT1, STAT3 and IRF1 to the *Il10* locus (Ziegler-Heitbrock *et al.* 2003; Iyer *et al.* 2010). Comparison of the recruitment of these molecules and potentially other transcription factors to the *Il10* locus in LPS stimulated C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ over a time-course of stimulation would build on these findings, and greatly enhance our understanding of what factors may be required at different stages to induce the expression of *Il10*.

6.2.1.2 *Independent validation of microarray analysis and understanding the C57BL/6 and BALB/c genetic differences that are dependent on type I IFN.*

From the results of our microarray analysis, we have identified several potential transcriptional regulators that may be responsible for the global differential gene expression and potentially differential *Il10, Il12a* and *Ifnb1* expression in LPS stimulated C57BL/6 and BALB/c macrophages. However, before moving forward, our analysis would be greatly strengthened by independent experimental validation. One option is to conduct another microarray analysis, potentially on a different array platform such as Affymetrix. However, a more robust way to validate our current data would be to analyse the transcriptional profiles of LPS stimulated C57BL/6 and BALB/c macrophages by RNA sequencing (RNA-seq). RNA-seq has the benefit over microarray of a larger dynamic range of expression level detection and has no reliance on predefined oligonucleotide probes (Zak *et al.* 2009). This latter point may be particularly relevant in our study as polymorphisms within mRNA sequences from genetically distinct mouse strains have the potential to give rise to artefacts when only using oligonucleotide hybridization techniques (Bottomly *et al.* 2011). Therefore, if genes that we have identified as differentially expressed by microarray are also differentially expressed by RNA-seq, we can be confident that these findings are robust. The subsequent bioinformatic analyses used to identify pathways or transcriptional regulators involved in mediating differential gene expression in C57BL/6 and BALB/c macrophages would additionally be strengthened by this validation. Once this has been carried out, functional analysis of transcription factor candidates by assessment of activation, localisation to the nucleus, or recruitment to the *Il10, Il12a* or *Ifnb1* loci would be necessary to determine their roles in regulating the differential production of

these cytokines in C57BL/6 and BALB/c macrophages. Importantly however, the inclusion of C57BL/6 *Ifnar1*$^{-/-}$ macrophages in this future transcriptional profiling, would allow us to more formally assess which gene expression differences in C57BL/6 and BALB/c macrophages are attributable to type I IFN, or are a consequence of other genetic differences. This may provide a better understanding of overall gene expression and cytokine regulation in LPS stimulated macrophages. In the context of IL-12, transcriptional profiling of IL-10 deficient C57BL/6 and BALB/c macrophages, in which differential IL-12 production is greatly exaggerated, may help to further understand the mechanisms governing the differential production of this cytokine.

6.2.1.3 *Studies towards understanding the genetic variation underlying differential IL-10 and IL-12 production in C57BL/6 and BALB/c macrophages.*

Our preliminary data on the production of IL-10 in LPS, Pam3CSK4 and heat-killed *B.pseudomallei* stimulated C57BL/6 x BALB/c F1 and F2 macrophages, indicated that several genes may be contributing to the differential production of IL-10 in C57BL/6 and BALB/c macrophages. We will be continuing this study by quantifying *Ifnb1* and *Il12a* expression from F1 and F2 macrophages as this information may provide insight into the types of genetic variation that contribute to the differential production of these additional cytokines. At this stage, we do not have the capacity to follow through with a full genetic intercross study which would lead to the mapping of genetic loci that determine the level of IL-10, IL-12 and potentially type I IFN production in C57BL/6 and BALB/c macrophages, although this may be revisited in the future.

A recent study from the group of Dr David Adams at the Sanger Institute has re-sequenced the genomes of 17 inbred strains of mice, including C57BL/6J and BALB/cJ (Keane *et al.* 2011). This study identified 3,920,925 single nucleotide polymorphisms (SNPs), 831,193 indels (deletions or insertions) and 25,702 structural variants (e.g. translocations, inversions or copy number variants) which differentiated the BALB/cJ and C57BL/6J genomes (Keane *et al.* 2011). Thus, the full extent of variation in the genomes of C57BL/6 and BALB/c mice is complex. We have confirmed that the phenotype of higher IL-10 production in C57BL/6 compared to BALB/c macrophages is conserved in macrophages derived from C57BL/6J and BALB/cJ mice used in the study of Keane *et al*. (Appendix Figure 7.2). Data on the expression of *Ifnb1* and *Il12a* is pending. In future work, we plan to use this available sequence data to compare genetic regions of interest in C57BL/6 and BALB/c genomes, such as the *Il10*, *Ifnb1* and *Il12a* loci. Although in light of our previous data, we are aware that polymorphisms in single genes are unlikely to fully explain the differential cytokine production in C57BL/6 and BALB/c macrophages, this will reveal if there is genetic variation in important regulatory regions of these genes, which may provide useful information for the continuation of this project.

6.2.2 Extending differential cytokine production in C57BL/6 and BALB/c macrophages to models of *B.pseudomallei* infection

Aside from using C57BL/6 and BALB/c macrophages purely as a tool to investigate the molecular mechanisms of IL-10 and IL-12 regulation, the differential cytokine production that we observe may have wider relevance as these mice differ in their resistance and susceptibility to several pathogens. Thus, differential innate cytokine

production may have implications for the capacity of C57BL/6 and BALB/c mice to clear certain infections. One of the stimuli that we focussed on in this investigation was heat-killed *B.pseudomallei*. C57BL/6 mice are more resistant to live infection with this pathogen than BALB/c mice and this has been proposed to represent the spectrum of disease seen in humans (Leakey *et al.* 1998). However, the underlying mechanisms behind this strain difference are not well understood (Titball *et al.* 2008). In addition, while it is clear that IL-12 production is critical for protective immune responses against *B.pseudomallei* infection (Haque *et al.* 2006), the roles of IL-10 and type I IFN are unclear and we postulate that these cytokines may be of importance in regulating over-exuberant immune responses in this context. Future studies which we have already initiated in collaboration with Dr Gregory Bancroft (London School of Hygiene and Tropical Medicine), will investigate if similar profiles of cytokine production that we observe in heat-killed *B.pseudomallei* stimulated C57BL/6 and BALB/c macrophages also occur upon live *B.pseudomallei* infection. Preliminary data from this study has been promising, showing that C57BL/6 macrophages produce higher levels of IL-10 than BALB/c macrophages upon infection with this bacterium and this is indeed dependent on type I IFN signalling (N.Patel, G.Bancroft, unpublished data). Additional investigation into IL-10, IL-12 and type I IFN production in C57BL/6 and BALB/c *ex vivo* cells such as bone marrow monocytes or tissue resident macrophages may further help to determine if the differential cytokine production that we observe in BMDM may have an impact upon *in vivo* infection. Of note, as *B.pseudomallei* infection often presents as pneumonia (Currie *et al.* 2010), assessment of differential cytokine production in heat-killed *B.pseudomallei* stimulated or live *B.pseudomallei* infected C57BL/6 and BALB/c alveolar macrophages may be of particular relevance. Thus, the

results of these initial studies will begin to elucidate if the findings that we have made in this investigation are applicable to the more complex setting of live *B.pseudomallei* infection of C57BL/6 and BALB/c macrophages, or indeed mice.

6.3 Concluding remarks

We have shown that comparing the responses of macrophages derived from different inbred strains of mice can be a fruitful approach to furthering our knowledge of cytokine regulation. The central findings of this study highlight the importance of autocrine type I IFN in the regulation of IL-10 in response to TLR4 dependent stimuli. In a broader context, this work is in agreement with an increasingly appreciated role for type I IFN in immune responses against bacterial infections including *L.monocytogenes* (Auerbuch *et al.* 2004; Carrero *et al.* 2004; O'Connell *et al.* 2004), *M.tuberculosis* (Manca *et al.* 2005; Berry *et al.* 2010; Mayer-Barber *et al.* 2011) and now potentially *B.pseudomallei*. Importantly however, our data shows that the presence of this type I IFN mediated autocrine loop, critical for the maintenance of IL-10 production in TLR4 stimulated macrophages, is dependent on the genetic background of the host. Thus, this regulatory loop is functional in C57BL/6 macrophages but not BALB/c macrophages and based on the findings in this investigation, may also be non-functional in 129 macrophages. In addition, we have revealed that the production of IL-12p70, an important cytokine in the induction of Th1 mediated immune responses (Trinchieri 2003), is severely attenuated in C57BL/6 compared to BALB/c macrophages. In light of our findings and given that the majority of immunological studies are carried out using C57BL/6 mice, we raise the question: how has this affected scientific conclusions that have been made in the literature so far? Finally, this study additionally contributes to

the on-going debate: which inbred mouse strain most reflects the human condition? Most likely, the answer to this latter question depends on the immunological system being studied, and the human population it is being compared to.

Chapter 7. Appendix

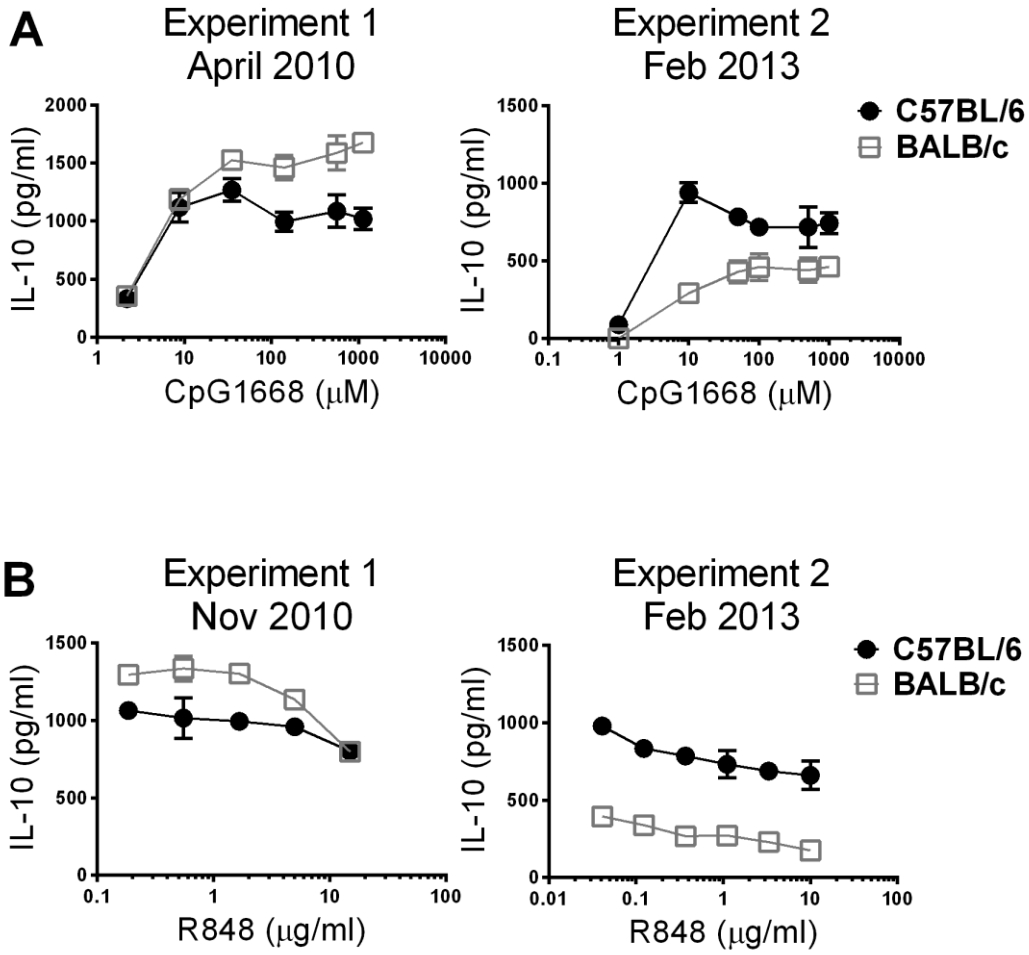


Figure 7.1 The levels of IL-10 production are variable in CpG1668 and R848 stimulated C57BL/6 and BALB/c macrophages.

BMDM were generated from C57BL/6 and BALB/c mice. Cells were stimulated for 24 hours with (A) CpG1668 and (B) R848 at the indicated doses. IL-10 protein in supernatants was quantified by ELISA (detection limit 50 pg/ml). Graphs show means ± SD of individual triplicate cultures. Repeat experiments of titrations are shown. Additional experiments were conducted using 50 µM CpG1668 and 1 µg/ml R848, also with variable results.

Table 7.1 0.5 h, *k*-means C3 (C57BL/6^{hi}). Refers to Figure 5.6.

4631422O05Rik
5430435G22Rik
Atf4
BC043118
Ccng2
Cd52
Cish
E030040J22Rik
F730045P10Rik
Fgfr1op2
Fndc7
Gdf15
H2-M2
Hist1h4a
Ifi205
Malt1
Picalm
Rassf3
Rhoe
Rpl21
Rps3a
Stx11
Vegfa

Table 7.1 Genes present within 0.5 h C3 generated by *k*-means clustering are shown.

Table 7.2 0.5 h, *k*-means C4 (C57BL/6^{hi}). Refers to Figure 5.6.

Cxcl10
Fmo3
Ifi205
LOC240921
Tnfrsf5
Ybx3

Table 7.2 Genes present within 0.5 h C4 generated by *k*-means clustering are shown.

Table 7.3 1 h, *k*-means C1 (C57BL/6^{hi}). Refers to Figure 5.6.

1190002H23Rik
2500002E12Rik
2810408M09Rik
5730537H01Rik
9030216K14Rik
9130604K18Rik
A530060O05Rik
AA467197
C5r1
Ccl5
Cxcl2
Dusp16
F730031O20Rik
F730045P10Rik
Fbxo34
Gbp2
Gdf15
H2-Ab1
Ifi205
Ifi205
Map3k6
Mefv
Npn3
Odc1
Osbpl3
Osbpl3
Pde1b
Pilra
Slamf7
Slc30a7
Stx11
Tmem171
Tnfrsf5
Tnfrsf6
Trim36
Trim36

Table 7.3 Genes present within 1 h C1 generated by *k*-means clustering are shown.

Table 7.4 1 h, *k*-means C5 (C57BL/6^{hi}). Refers to Figure 5.6.

1700047I17Rik
Axud1
Bcl3
C430002D13Rik
Ccl12
Cited2
Coq10b
Cxcl10
Cxcl16
Etv3
H2-M2
Idb2
Il10
Il15
Myd88
Nab1
Npn3
Peli1
Plat
Rhoe
Sh3bgrl2
U2af1-rs2
Ybx3

Table 7.4 Genes present within 1 h C5 generated by *k*-means clustering are shown.

Table 7.5 3 h, *k*-means C1 (C57BL/6^{hi}). Refers to Figure 5.6.

1200015F23Rik	*Dusp16*	*Sgk3*
2310015N21Rik	*E030040L08Rik*	*Siat4a*
2810021O14Rik	*E130207H16Rik*	*Slc13a3*
4933432P15Rik	*EG433016*	*Snx2*
9030216K14Rik	*EG630499*	*Snx2*
9030625A04Rik	*Egr1*	*Tens1*
9630007E23Rik	*Egr3*	*Tens1*
A130065C13Rik	*F730045P10Rik*	*Tjp2*
A130072J07	*Glrx1*	*Trim30*
A630085K21	*Gus-s*	*Trim34*
A830089I03Rik	*H2-Ab1*	*Trim34*
Aoah	*H2-D4*	*Zfp213*
Aytl1	*H2-T10*	
Bat5	*H2-T10*	
BC003314	*Ifi204*	
BC050811	*LOC226690*	
Bcdo2	*LOC240921*	
Brdt	*LOC382190*	
C5r1	*Map3k6*	
Casp9	*Map3k6*	
Ccng2	*Nedd4l*	
Chd9	*Nfkb2*	
Clcn7	*Parp3*	
Cpne8	*Parp8*	
Ctsc	*Pop4*	
Ctsc	*Psmb9*	
Ctsc	*Rsad1*	

Table 7.5 Genes present within 3 h C1 generated by *k*-means clustering are shown.

Table 7.6 3 h, *k*-means C3 (C57BL/6^{hi}). Refers to Figure 5.6.

1100001A21Rik	*B230334I05Rik*	*Ifi205*	*Six1*
1200007D18Rik	*BC049354*	*Ikbkb*	*Slamf8*
1810044J04Rik	*Bcl6*	*Irak3*	*Snx2*
1810054D07Rik	*Bzw2*	*Irf2*	*Stat4*
2010004N17Rik	*C130023K05Rik*	*Klhl5*	*Stk2*
2010005O13Rik	*Cd164*	*Leng9*	*Tapbp*
2010012C16Rik	*Cd244*	*LOC244882*	*Tbc1d13*
2310046K10Rik	*Cd86*	*LOC381329*	*Tcf4*
2410025L10Rik	*Cd86*	*March1*	*Tm9sf4*
2410039M03Rik	*Cd86*	*Mina*	*Tmem171*
3110050N22Rik	*Centd2*	*Morc3*	*Tnfrsf6*
4921513D23Rik	*Chd9*	*Mrpl3*	*Ybx3*
4930563C06Rik	*Chd9*	*Msi2h*	*Zfp212*
5730508B09Rik	*Cpeb4*	*Nfil3*	
8030462N17Rik	*Dusp16*	*Nup98*	
8430438D04Rik	*EG622976*	*Ocil*	
9130604K18Rik	*Ep400*	*Ogfrl1*	
9830148G24Rik	*F7*	*Ogfrl1*	
9930022N03Rik	*Fcrl3*	*Pilra*	
A030007L17Rik	*Frag1*	*Pilra*	
A130072J07	*Gadd45g*	*Pilrb*	
A530023O14Rik	*Gvin1*	*Plat*	
A530083B17Rik	*H2-Ab1*	*Plekhf2*	
A930008A22Rik	*H2-T17*	*Plod2*	
Adora3	*H2-T22*	*Pnp*	
Aggf1	*H60*	*Pxn*	
AI929863	*Homer1*	*scl000868.1_2*	
Alas1	*I830077J02Rik*	*Seh1l*	
Apobec3	*Ifi205*	*Sema4a*	
Arf6	*Ifi205*	*Sh3kbp1*	
Asrij	*Ifi205*	*Sipa1l2*	

Table 7.6 Genes present within 3 h C3 generated by *k*-means clustering are shown.

Table 7.7 5 h, *k*-means C0 (C57BL/6^{hi}). Refers to Figure 5.6.

AI838661	*Bahd1*	*H2-K1*	*Psmd8*
1110001A05Rik	*Bat5*	*H2-Q7*	*Rapgef5*
1200015F23Rik	*BC002236*	*H2-T10*	*Rnf34*
1700123O20Rik	*BC003314*	*H2-T10*	*Rnf34*
2010300G19Rik	*BC035954*	*H2-T22*	*Rnps1*
2200001I15Rik	*Bcdo2*	*Hpcal1*	*Rsad1*
2310015N21Rik	*C430002D13Rik*	*Iqgap2*	*Sc4mol*
2410039M03Rik	*C5r1*	*LOC226690*	*Scoc*
2500002E12Rik	*Ccng2*	*LOC240921*	*Sfrs2*
2610029J22Rik	*Cd52*	*LOC382190*	*Sgk3*
2810021O14Rik	*Chi3l3*	*LOC383308*	*Slc12a9*
4631422C13Rik	*Clcn7*	*LOC386270*	*Slc13a3*
4732429D16Rik	*Cpne8*	*LOC545056*	*Slc13a3*
4933439C20Rik	*Ctsc*	*Ly78*	*Snx2*
6430527G18Rik	*Ctsc*	*Manbal*	*Snx2*
9030216K14Rik	*Ctsc*	*Mina*	*Srpr*
9030625A04Rik	*Dppa3*	*Mpeg1*	*Stat4*
9430077D24Rik	*Dusp16*	*Ndufb10*	*Tmem171*
9830148G24Rik	*E130207H16Rik*	*Nfkb2*	*Tnfrsf6*
9930027N05Rik	*EG630499*	*Odc1*	*Tomm22*
A130072J07	*Egr1*	*Osbp*	*Trim30*
A130072J07	*Eme2*	*Parp3*	*Trim34*
AI316802	*Epb4.1l2*	*Parp8*	*Trim56*
Aoah	*F730045P10Rik*	*Pde1b*	*Tspan32*
Apobec3	*Fgfbp3*	*Pde1b*	*Ush2a*
Aps	*Fmo3*	*Pgls*	*Zfp367*
Atf4	*Gadd45g*	*Pnp*	
Atp2c1	*Glrx1*	*Pop4*	
Atp2c1	*H2-Aa*	*Psmb5*	
Atp6v1d	*H2-Ab1*	*Psmb9*	
Aytl1	*H2afj*	*Psmc4*	

Table 7.7 Genes present within 5 h C0 generated by *k*-means clustering are shown.

Table 7.8 5 h, *k*-means C3 (C57BL/6^{hi}). Refers to Figure 5.6.

1190003J15Rik	*Aftph*	*Cited2*	*Hdac1*	*LOC215088*
1300002F13Rik	*AI429613*	*Cndp2*	*Hdc*	*LOC215405*
1300017K07Rik	*AI451557*	*Cpeb4*	*I830077J02Rik*	*LOC381329*
1700047I17Rik	*Aif1*	*Cxcl10*	*Ier3*	*LOC384475*
1700048E23Rik	*Aif1*	*Cxcl10*	*Ifi204*	*Loh11cr2a*
1810044J04Rik	*Aim1*	*Cxcl2*	*Ifi205*	*Lox*
2010005H15Rik	*Alas1*	*Cxcl9*	*Ifi205*	*Lrrc25*
2210019E14Rik	*Armc8*	*Cyfip1*	*Ifi205*	*Ly96*
2210037E17Rik	*Armc8*	*D3Ucla1*	*Ifi205*	*Malt1*
2310046K10Rik	*Asrij*	*Daam1*	*Ifi205*	*Map2k4*
2410025L10Rik	*Axud1*	*Dab2*	*Ifi205*	*Map2k4*
2610027C15Rik	*Bambi-ps1*	*Dgkh*	*Ifit3*	*Map3k8*
2700024D06Rik	*BC019206*	*Dock11*	*Ifit3*	*Map4k3*
2810021O14Rik	*BC023823*	*Dscr1*	*Ifngr2*	*Mapkbp1*
2810405I11Rik	*BC049354*	*Dusp1*	*Ifngr2*	*March1*
2810407C02Rik	*BC049975*	*Dusp16*	*Ifngr2*	*Marco*
2810423A18Rik	*BC050811*	*Dusp16*	*Ift172*	*Mefv*
3110050N22Rik	*Bcl3*	*Dusp2*	*Igsf9*	*Mrpl3*
4921513D23Rik	*Bcl6*	*Dync1i2*	*Ii*	*Mx1*
4931440N07Rik	*Bfar*	*Ep400*	*Ikbkb*	*Myadm*
4933426M11Rik	*Bzw2*	*Etv3*	*Ikbke*	*Myo1g*
4933432P15Rik	*C030027K23Rik*	*Fcgr1*	*Il10*	*Nfil3*
5031414D18Rik	*C130032J12Rik*	*Fcgr2b*	*Il13ra1*	*Nsmaf*
5730537H01Rik	*Card4*	*Fcgr2b*	*Il15*	*Nupr1*
5730596K20Rik	*Carhsp1*	*Fndc7*	*Il15*	*Oas3*
6030446I19Rik	*Casp8*	*Frag1*	*Il15*	*Ogfrl1*
6230400I06Rik	*Ccl12*	*Frag1*	*Il19*	*Ogfrl1*
9030611K07Rik	*Ccl5*	*Furin*	*Iqsec2*	*Osbpl3*
9630037P07Rik	*Ccl8*	*Fzd7*	*Irak3*	*Osbpl3*
9930022N03Rik	*Ccnd2*	*G430091H17Rik*	*Irf2*	*Pdk3*
A030007L17Rik	*Ccrn4l*	*Gadd45g*	*Irg1*	*Peli1*
A530023O14Rik	*Cd164*	*Gbp2*	*Irg1*	*Pik3ap1*
A530023O14Rik	*Cd180*	*Gbp2*	*Itpkb*	*Pik3cd*
A530023O14Rik	*Cd244*	*Gcnt2*	*Itpr1*	*Pilra*
A530026H04Rik	*Cd44*	*Gda*	*Junb*	*Pilra*
A530060O05Rik	*Cd52*	*Ggta1*	*Kcnn4*	*Pilra*
A630077B13Rik	*Cd86*	*Gna-rs1*	*Kctd12*	*Pilrb*
AA467197	*Cd86*	*Gpr73*	*Khdrbs1*	*Pira11*
Adora2b	*Cd86*	*Gpr84*	*Khdrbs1*	*Pirb*
Adora3	*Cebpb*	*Gvin1*	*Klhl5*	*Pkn2*
Adprh	*Centd2*	*H2-M2*	*Lcn2*	*Plat*
Aff1	*Chst11*	*H2-T17*	*Leng9*	*Plekhf2*

Table 7.8 continued

Plekhg2	*Sgcb*	*Tapbp*	*Zyx*
Pml	*Sgk3*	*Tbc1d13*	
Pml	*Sh3bgrl2*	*Tes*	
Pnp	*Sh3kbp1*	*Tle3*	
Poldip3	*Siat4a*	*Tlk2*	
Ppfibp1	*Sipa1l1*	*Tlr7*	
Ppp4r1	*Slamf7*	*Tnfrsf1a*	
Prm1	*Slamf8*	*Tnfrsf1a*	
Psat1	*Slamf9*	*Tnfrsf1a*	
Psd4	*Slc28a2*	*Tnfrsf5*	
Ptk2b	*Slc30a7*	*Tnip1*	
Pycard	*Slc44a1*	*Tor3a*	
Rab5a	*Slc7a11*	*Trim21*	
Rac3	*Slco3a1*	*Trim36*	
Rad9	*Slco3a1*	*Trim36*	
Rap2c	*Slfn1*	*Tspyl3*	
Reps1	*Smad2*	*Ubc*	
Rin2	*Snx2*	*Ubtd1*	
Riok3	*Spata13*	*Upp1*	
Rnf34	*Spata5l1*	*Usp18*	
Rps6ka3	*Stard5*	*Ybx3*	
scl0002617.1_582	*Stard5*	*Zc3h12a*	
scl000868.1_2	*Stx11*	*Zc3h7a*	
Sema4a	*Stx11*	*Zfp281*	
Sema4d	*Stx6*	*Zfp36*	
Sertad1	*Tagln2*	*Zmynd15*	

Table 7.8 Genes present within 5 h C3 generated by *k*-means clustering are shown.

Table 7.9 8 h *k*-means C0 (C57BL/6^{hi}). Refers to Figure 5.6.

AI838661	*Arg1*	*H2-K1*	*Pik3ap1*	*Tomm22*
0610009K11Rik	*Asrij*	*H2-Q7*	*Pilrb*	*Trim30*
1110067L22Rik	*Atf4*	*H2-T10*	*Pirb*	*Trim56*
1190003J15Rik	*Axud1*	*H2-T10*	*Plekhf2*	*Tspan32*
1200015F23Rik	*Bahd1*	*H2-T22*	*Pnp*	*Ubc*
1700093E07Rik	*Bat5*	*H60*	*Procr*	*Ubg*
1700123O20Rik	*BC026370*	*Hpcal1*	*Psmb5*	*Ush2a*
1700123O20Rik	*BC063749*	*I830077J02Rik*	*Psmb9*	*Ybx3*
1810044J04Rik	*Bzrap1*	*Ifi204*	*Psmd8*	
1810054D07Rik	*C430002D13Rik*	*Ifi205*	*Pycard*	
2010005H15Rik	*C5r1*	*Ifi205*	*Reps1*	
2010300G19Rik	*C79267*	*Ifi205*	*Rnf144*	
2310010B21Rik	*Ccrn4l*	*Il10*	*Rnf34*	
2310015N21Rik	*Cd52*	*Il6st*	*Rnf41*	
2410039M03Rik	*Chst11*	*Iqgap2*	*Rnps1*	
2500002E12Rik	*Csf2rb1*	*Klhl5*	*Rsad1*	
2610029J22Rik	*Ctsc*	*Leng9*	*Sc4mol*	
2810021O14Rik	*Ctsc*	*LOC209372*	*scl000868.1_2*	
4930583H14Rik	*Ctsc*	*LOC226690*	*Sdc4*	
4933432P15Rik	*Cyp4f18*	*LOC240921*	*Sfrs2*	
4933439C20Rik	*D130067I07Rik*	*LOC381329*	*Sgk3*	
9030216K14Rik	*D4Ertd432e*	*LOC545056*	*Siat4a*	
9030625A04Rik	*Dusp16*	*Lrp4*	*Sipa1l1*	
9830148G24Rik	*EG433016*	*Ly78*	*Slc13a3*	
A030007L17Rik	*EG630499*	*Manbal*	*Slc13a3*	
A130072J07	*Egr1*	*Map3k6*	*Slc16a6*	
A230021I18Rik	*F730045P10Rik*	*March1*	*Snx2*	
A530023O14Rik	*Flot1*	*Mina*	*Srpr*	
A530023O14Rik	*Flot1*	*Mrps10*	*Ssbp4*	
A630086H07Rik	*Frag1*	*Nfkb2*	*Stard5*	
Abi3	*Ggta1*	*Olfm1*	*Tagln2*	
Adora3	*Glrx1*	*Parp3*	*Tgfbi*	
AI316802	*H2-Ab1*	*Parp8*	*Tgfbi*	
Alas1	*H2-Ab1*	*Pde1b*	*Tmem171*	
Aoah	*H2-DMb1*	*Pde1b*	*Tnfrsf1a*	
Apobec3	*H2-DMb2*	*Pgpep1*	*Tnfrsf6*	

Table 7.9 Genes present within 8 h C0 generated by *k*-means clustering are shown.

Table 7.10 1 h *k*-means C4 (BALB/c^{hi}). Refers to Figure 5.7.

1110068E08Rik
1700060H10Rik
2810012L14Rik
6030458C11Rik
6030470M02Rik
Cxcr4
D630046D15Rik
Eno2
Gbp1
Gstt2
H2-Q5
Jmjd2a
Plau
Raet1c
Rhov
Zfp30

Table 7.10 Genes present within 1 h C4 generated by *k*-means clustering are shown.

Table 7.11 3 h *k*-means C4 (BALB/c^{hi}). Refers to Figure 5.7.

0610037M15Rik	*Dnm1l*	*LOC380781*	*Stfa1*
1600021P15Rik	*Dtx4*	*Mcm10*	*Stk38l*
1700052K11Rik	*Dtx4*	*Mid1*	*Stx3*
2410127E16Rik	*Dvl1*	*Mid1*	*Stx3*
2810457M08Rik	*E130012A19Rik*	*Mki67*	*Syncrip*
6330444G18Rik	*Emilin2*	*Nat5*	*Tal1*
9930022F21Rik	*Eno2*	*Pi4k2b*	*Tgfb1i4*
A330066M24Rik	*Eps15-rs*	*Pign*	*Timd4*
Adam17	*F2rl2*	*Ppfibp1*	*Tlr6*
Aim2	*Fabp3*	*Ppic*	*Ube2e2*
Arl6ip5	*Gbp1*	*Prnp*	
Atp10d	*Gpc1*	*Psip1*	
B430201G11Rik	*Gpr23*	*Psip1*	
B930075F07	*Gpr31c*	*Ptafr*	
Bat2	*Grap*	*Ptafr*	
BC002216	*H2-Ea*	*Pvrl3*	
BC018462	*H2-Q5*	*Raet1b*	
C530043G21Rik	*H2-Q5*	*Raet1b*	
C78339	*H2-Q6*	*Raet1c*	
Card15	*H2-Q7*	*Rai14*	
Ccdc93	*H2-Q8*	*Rog*	
Ccl24	*Htr1f*	*Rras2*	
Ciapin1	*Ifi202b*	*scl0002855.1_1056*	
Ciapin1	*Ifi202b*	*Sepw1*	
Cldn23	*IGHV14S3*	*Serpina3h*	
Cnot7	*Igsf3*	*Slc11a2*	
Cpne8	*Il1rap*	*Slc25a25*	
Crnkl1	*Inppl1*	*Slc7a5*	
Ctsl	*Inppl1*	*Slc7a8*	
D5Ertd593e	*Inppl1*	*Smpdl3b*	
D6Mm5e	*Jmjd2a*	*Srrm2*	
Daf1	*Klrk1*	*Stau2*	
Dgat2	*LOC270152*	*Stfa1*	

Table 7.11 Genes present within 3 h C4 generated by *k*-means clustering are shown.

Table 7.12 5 h *k*-means C2 (BALB/c^{hi}). Refers to Figure 5.7.

0610037M15Rik	*Gbp1*	*Pdgfrl*
1700029F09Rik	*Grap*	*Pip5k1a*
1810018P12Rik	*H28*	*Ppfibp1*
2010305C02Rik	*H2afz*	*Ppic*
2410003B16Rik	*H2-Ea*	*Ppm1l*
2810022L02Rik	*H2-Q5*	*Raet1c*
9530076I17Rik	*H2-Q5*	*Rnase6*
Akap2	*H2-Q6*	*Ryk*
Arl6ip5	*H2-Q7*	*scavenger receptor*
Arl6ip5	*H2-Q8*	*scl0002855.1_1056*
Asb1	*Hak*	*Serpina3h*
BC022593	*Hal*	*Shf*
BC027373	*Htr1f*	*Slco4a1*
C530043G21Rik	*Ick*	*Slpi*
Ccdc93	*Ick*	*Stau2*
Ccl17	*Ifi202b*	*Stau2*
Ciapin1	*Ifi202b*	*Stfa1*
Ciapin1	*IGHV14S3*	*Stfa3*
Cnot7	*IGHV1S52*	*Tbc1d9*
Cox6a2	*Igsf3*	*Timd4*
Csprs	*Jmjd2a*	*Tnfrsf14*
Ctse	*Klrk1*	*Wdfy1*
Cxcl13	*LOC381105*	*Wdfy1*
D330023A14Rik	*LOC384528*	
D330037A14Rik	*LOC384607*	
Dcn	*LOC385019*	
Dtx4	*Ltb*	
Dusp4	*Mid1*	
Dusp4	*Mid1*	
Eef1e1	*Mmp9*	
Eno2	*Nsdhl*	
Epb4.9	*Nsdhl*	
F2rl2	*Palld*	

Table 7.12 Genes present within 5 h C2 generated by *k*-means clustering are shown.

Table 7.13 8 h *k*-means C3 (BALB/c^{hi}). Refers to Figure 5.7.

0610010E21Rik	*Eno2*	*Psip1*
0610037M15Rik	*F2r*	*Psip1*
1810018P12Rik	*F2rl2*	*Rab38*
2310014H19Rik	*Fads2*	*Rims3*
2410003B16Rik	*Gbp1*	*Rin3*
2410003B16Rik	*Grap*	*Rog*
2600001B17Rik	*Gypc*	*Rras2*
2810022L02Rik	*H28*	*Scd1*
2810022L02Rik	*H2-Ea*	*Sephs2*
4732458O05Rik	*H2-K1*	*Serpina3h*
A2bp1	*H2-Q5*	*Slc1a2*
A630072M18Rik	*H2-Q5*	*Stau2*
Adamts4	*H2-Q6*	*Stim2*
AI591476	*H2-Q7*	*Stk38l*
Aim2	*H2-Q8*	*Tbc1d9*
Arhgap22	*Htr1f*	*Tgfb1i4*
Arl6ip5	*Ick*	*Timd4*
Arl6ip5	*Ick*	*Txndc7*
Aven	*Ifi202b*	*Ube1x*
B430201G11Rik	*Ifi202b*	*Wdfy1*
BC022593	*IGHV14S3*	
C530043G21Rik	*IGHV1S52*	
Chst10	*Il12a*	
Csf1	*Jmjd2a*	
Csprs	*Klrk1*	
Cx3cl1	*Lmyc1*	
Cxcl13	*LOC386068*	
D430030K24Rik	*Ltb*	
Dcn	*Mid1*	
Dtx4	*Mid1*	
Dtx4	*Mmp9*	
Dusp4	*Nrbf1*	
E130012A19Rik	*Pi4k2b*	
Eef1e1	*Ppap2b*	

Table 7.13 Genes present within 8 h C3 generated by *k*-means clustering are shown.

Table 7.14 0.5 h differentially expressed genes associated with IPA pathways. Refers to Figure 5.8.

Pathway	**Associated genes C57BL/6^{hi}**	**Associated genes BALB/c^{hi}**
TREM1 Signalling	*Tlr2, Cxcl3, Ccl7, Cd40, Il10, Tnf*	
Communication between Innate and Adaptive Immune Cells	*Cxcl10, Tlr2, Cd40, Il10, Tnf*	
Allograft Rejection Signalling	*Cd40, H2-M2, Il10, Tnf*	
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	*Vegfa, Tlr2, Il10, Atf4, Cebpb, Tnf, Traf1*	*Ctnnb1*
Glucocorticoid Receptor Signalling	*Cxcl3, Il10, Cdkn1a, Cebpb, Tnf, Tsc22d3*	*Adrb2*

Table 7.14 Differentially expressed genes of interest in LPS stimulated C57BL/6 and BALB/c macrophages identified at 0.5 h in the strategy outlined in Figure 5.3 B (*) were analysed by IPA to assess association with known biological pathways. The top 5 significant pathways are shown. Genes from the input list that are associated with those pathways and their relative C57BL/6 *versus* BALB/c expression are shown.

Table 7.15 1 h differentially expressed genes associated with IPA pathways. Refers to Figure 5.8.

Pathway	**Associated genes C57BL/6^{hi}**	**Associated genes BALB/c^{hi}**
TREM1 Signaling	*Cxcl3, Ccl2, Ccl7, Cd40, Il10, Myd88, Tnf*	*Nod2*
Granulocyte Adhesion and Diapedesis	*Cxcl10, Cxcl3, Cxcl16, C5ar1, Ccl2, Ccl7, Ccl5, Tnf*	*Cxcr4, Ccl24, Cxcl2*
Agranulocyte Adhesion and Diapedesis	*Cxcl10, Cxcl3, Cxcl16, C5ar1, Ccl2, Ccl7, Ccl5, Tnf*	*Cxcr4, Ccl24, Cxcl2*
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	*Cxcl10, Ccl2, Il15, Ifnb1, Ccl5, Tnf*	
Communication between Innate and Adaptive Immune Cells	*Cxcl10, Cd40, Il10, Il15, Ifnb1, Ccl5, Tnf*	

Table 7.15 Differentially expressed genes of interest in LPS stimulated C57BL/6 and BALB/c macrophages identified at 1 h in the strategy outlined in Figure 5.3 B (*) were analysed by IPA to assess association with known biological pathways. The top 5 significant pathways are shown. Genes from the input list that are associated with those pathways and their relative C57BL/6 *versus* BALB/c expression are shown.

Table 7.16 3 h differentially expressed genes associated with IPA pathways. Refers to Figure 5.8.

Pathway	Associated genes C57BL/6^{hi}	Associated genes BALB/c^{hi}
Dendritic Cell Maturation	*Map2k4, Plcb2, Tnfrsf1a, Myd88, Il10, Il15, Ikbke, Nfkb2, HLA-DQB1, Fcgr2b, Stat4, Tlr2, Ikbkb, Cd40, Il1rn, Pik3cg, Cd86, Tnfrsf1b, Fcgr3a*	*Il12a, HLA-DRA, HLA-B, Tab1*
Communication between Innate and Adaptive Immune Cells	*Il10, Il15, Ccl5, Cxcl10, Tlr2, Ccl4, Cc40, Tlr5, Il1rn, Cd86*	*Il12a, Cd8b, HLA-DRA, Tlr6, HLA-B*
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	*Map2k4, Il6st, Tcf4, Plcb2, Mmp13, Ccl5, Vegfa, Ikbkb, C5ar1, Ccl2, Pik3cg, Tnfrsf1b, Fcgr3a, Tnfrsf1a, Il10, Myd88, Il15, Ikbke, Irak3, Tlr2, Fos, Ripkl, Tlr5, Il1rn, Fzd7*	*Mras, Ctnnb1, Il1rap, Src, Dvl1, Pdgfb, Rras2, Tlr6*
NF-κB Signalling	*Azi2, Tnfrsf1a, Myd88, Tbk1, Irak3, Nfkb2, Malt1, Tank, Tlr2, Ikbkb, Ripk1, Tlr5, Cd40, Il1rn, Pik3cg, Map3k8, Eif2ak2, Casp8, Tnfrsf1b*	*Rras2, Tlr6, Mras, Tab1*
Type I Diabetes Mellitus Signalling	*Map2k4, Tnfrsf1a, Myd88, Ikbke, Nfkb2, HLA-DQB1, Fas, Ikbkb, Casp9, Ripk1, Cd86, Casp8, Tnfrsf1b*	*Il12a, Ica1, HLA-DRA, HLA-B, Il1rap*

Table 7.16 Differentially expressed genes of interest in LPS stimulated C57BL/6 and BALB/c macrophages identified at 3 h in the strategy outlined in Figure 5.3 B (*) were analysed by IPA to assess association with known biological pathways. The top 5 significant pathways are shown. Genes from the input list that are associated with those pathways and their relative C57BL/6 *versus* BALB/c expression are shown.

Table 7.17 5 h differentially expressed genes associated with IPA pathways. Refers to Figure 5.8.

Pathway	Associated genes C57BL/6^{hi}	Associated genes BALB/c^{hi}
Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	*Il10, Il15, HLA-DQA1, Nfkb2, HLA-DQB1, Fas, Tlr4, Cd40, Tlr7, Cd86*	*Il1a, Il12a, Ltb, Cd28, Cxcl13, HLA-DRA, Tlr6, Tlr13, Il1b, Tnfsf13b*
Communication between Innate and Adaptive Immune Cells	*Il10, Il15, Cxcl10, Tlr4, Cd40, Tlr7, Cd86*	*Il1a, Il12a, Ccl5, Cd8b, Cd28, Tlr6, HLA-DRA, HLA-B, Tlr13, Il1b, Tnfsf13b*
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	*Map2k4, Plcb2, Fcgr1a, Ccnd1, Ikbkb, Traf3ip2, Jun, C5ar1, Ccl2, Pik3cg, Tlr7, Atf4, Tnfrsf1a, Il10, Daam1, Il15, Ikbke, Cebpb, Irak3, Tlr4, Fos, Pik3cd, Fzd7*	*Il1a, Ltb, Ccl5, Vegfb, Mras, Pik3r2, Ctnnb1, Tnfsf13b, Adamts4, Src, Vcam1, Pdgfb, Tlr6, Tlr13, Il1b, Ryk, Camk2g*
Type I Diabetes Mellitus Signalling	*Map2k4, Tnfrsf1a, Ifngr2, HLA-DQA1, Apaf1, Ikbke, Ifngr1, Nfkb2, HLA-DQB1, Fas, Ikbkb, Casp9, Cd86, Casp8*	*Il12a, Ica1, Cd28, HLA-DRA, HLA-B, Il1b, Socs5*
Dendritic Cell Maturation	*Map2k4, Plcb2, HLA-DQA1, HLA-DQB1, Fcgr2b, Fcgr1a, Ikbkb, Pik3cg, Atf4, Il10, Tnfrsf1a, Il15, Ikbke, Nfkb2, Stat4, Tlr4, Cd40, Cd86, Pik3cd*	*Il1a, Il12a, Ltb, HLA-DRA, HLA-B, Pik3r2, Il1b*

Table 7.17 Differentially expressed genes of interest in LPS stimulated C57BL/6 and BALB/c macrophages identified at 5 h in the strategy outlined in Figure 5.3 B (*) were analysed by IPA to assess association with known biological pathways. The top 5 significant pathways are shown. Genes from the input list that are associated with those pathways and their relative C57BL/6 *versus* BALB/c expression are shown.

Table 7.18 8 h differentially expressed genes associated with IPA pathways. Refers to Figure 5.8.

Pathway	Associated genes C57BL/6^{hi}	Associated genes BALB/c^{hi}
Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	*Map3k14, Il10, HLA-DMB, Nfkb2, HLA-DQB1, Fas, Tlr4, Il1rn, Cd86, Tlr13*	*Il1a, Il12a, Il15, Ltb, Nfkb1, Tlr2, Tlr5, Cxcl13, Cd40, Csf1, HLA-DRA, Tlr6*
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	*Map2k4, Il6st, Plcb2, Pdgfa, Mmp13, Ccl5, Fcgr1a, Ccnd1, C5ar1, Ccl2, Atf4, Tnfrsf1b, Fcgr3a, Map3k14, Tnfrsf1a, Il10, Ikbke, Cebpb, Stat3, Irak3, Pdgfb, Il16, Tlr4, Fos, Il1rn, Tlr13, Pik3cd*	*Il1a, Ltb, Nfkb1, Prkd3, Il1rap, Traf1, Adamts4, Src, Vcam1, Il15, Tlr2, Rras2, Tlr5, Csf1, Tlr6*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	*Ccr5, Pdgfa, Mmp13, Ccl5, Fas, Cxcl3, Ccl2, Timp1, Tnfrsf1b, Smad2, Il10, Tnfrsf1a, Ifngr2, Ifngr1, Nfkb2, Ifnar2, Pdgfb, Tlr4*	*Igfbp4, Il1a, Nfkb1, Il1rap, Timp2, Vcam1, Cd40, Csf1, Mmp9*
Dendritic Cell Maturation	*Map2k4, Plcb2, HLA-DMB, HLA-DQB1, Fcgr2b, Fcgr1a, Atf4, Tnfrsf1b, Fcgr3a, Map3k14, Il10, Tnfrsf1a, Ikbke, Nfkb2, Tlr4, Il1rn, Cd86, Pik3cd*	*Il1a, Il12a, Ltb, Nfkb1, HLA-DRA, HLA-B, Il15, Tlr2, Cd40*
TREM1 Signalling	*Il10, Stat3, Nfkb2, Fcgr2b, Cxcl3, Tlr4, Ccl2, Ccl7, Cd86, Tlr13*	*Nfkb1, Tlr2, Nod2, Cd40, Tlr5, Tlr6*

Table 7.18 Differentially expressed genes of interest in LPS stimulated C57BL/6 and BALB/c macrophages identified at 8 h in the strategy outlined in Figure 5.3 B (*) were analysed by IPA to assess association with known biological pathways. The top 5 significant pathways are shown. Genes from the input list that are associated with those pathways and their relative C57BL/6 *versus* BALB/c expression are shown.

Table 7.19 Transcription factors differentially expressed in LPS stimulated C57BL/6 and BALB/c macrophages. Refers to Figure 5.11.

Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Surb7	*Aff1*	*Cited2*	*Pou3f1*	*Ifi204*	*Bach2*
Lmyc1	*Irf2*	*Fem1c*	*Mycbp*	*Nfkb2*	*Btbd11*
Sertad2	*Tle4*	*Axud1*	*Psip1*	*Centd2*	*Ddef1*
Jundm2	*AW538212*	*Zfp263*	*Asb1*	*Mina*	
Klf7	*Zfp281*	*Nfil3*	*Cdkn2d*	*Smad2*	
Nfkb1	*Stat3*	*Egr1*		*Pycard*	
Nfkbiz	*Hdac1*			*Atf4*	
Junb	*Hif1a*			*Zfp367*	
Skil	*Bcl6*			*Stat4*	
Cebpb	*Mtf1*			*Zfp213*	
	Bcl3				
	Irf5				
	Etv3				
	Six1				
	Tcf4				

Cluster 7	Cluster 8	Cluster 9	Cluster 10	Cluster 11
Ctnnb1	*Pou6f1*	*Pparg*	*Per1*	*Etv1*
Tgfb1i4	*Zfhx2*	*Hlx1*	*Jun*	*Lbx2h*
Tal1	*Tcf19*	*Bach1*	*Fos*	*Sox4*
Rai4	*Mrpl28*	*Smarca2*	*Trerf1*	*Satb1*
	Gcdh	*E2f2*		*Etv5*
	Foxm1	*Gtf3a*		*Elk3*
	E2f6	*Ing4*		
	Gtf2i	*Rxrb*		
	E2f1	*Gtf3c1*		
	Tsc22d3	*Ppp1r12c*		
	Irf2bp1			
	Pbx2			
	Hes6			
	Tdrd3			
	OTTMUSG000 00000421			

Table 7.19 Transcription factors that are differentially expressed in LPS stimulated C57BL/6 and BALB/c macrophages at at least one time-point (divided into 11 clusters) are shown. Pink denotes overall higher expression in C57BL/6 macrophages. Blue denotes overall higher expression in BALB/c macrophages.

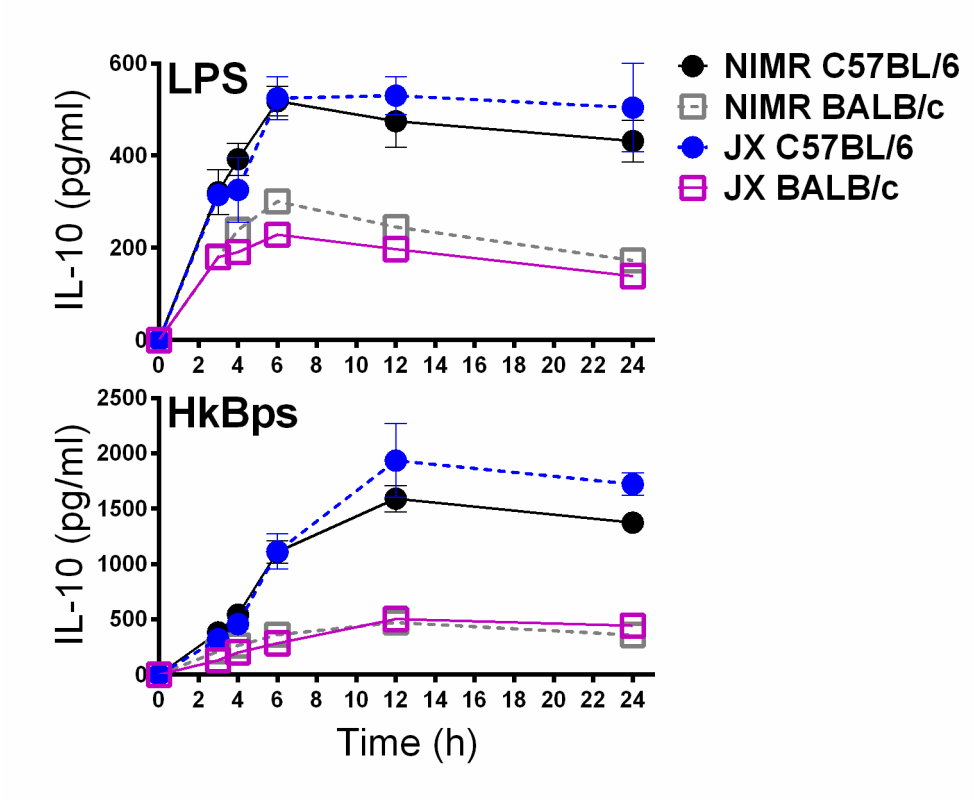


Figure 7.2 Differential IL-10 production is consistent in C57BL/6 and BALB/c macrophages derived from mice bred at NIMR or Jackson Laboratories.

BMDM were generated from C57BL/6 and BALB/c mice bred either at NIMR or Jackson Laboratories (JX). BMDM were stimulated over a time-course with LPS (10 ng/ml), or HkBps (10 HkBps: 1 BMDM). IL-10 protein in supernatants was quantified by ELISA (detection limit 50 pg/ml). Graphs show means ± SD of individual triplicate cultures. Representative of one experiment.

Reference List

Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. Van Dyke and B. Pulendran (2003). Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J Immunol 171 10:4984-4989.

Akira, S. and K. Takeda (2004). Toll-like receptor signalling. Nat Rev Immunol 4 7:499-511.

Alcais, A., C. Fieschi, L. Abel and J. L. Casanova (2005). Tuberculosis in children and adults: two distinct genetic diseases. J Exp Med 202 12:1617-1621.

Aman, M. J., T. Tretter, I. Eisenbeis, G. Bug, T. Decker, W. E. Aulitzky, H. Tilg, C. Huber and C. Peschel (1996). Interferon-alpha stimulates production of interleukin-10 in activated CD4+ T cells and monocytes. Blood 87 11:4731-4736.

Amit, I., M. Garber, N. Chevrier, A. P. Leite, Y. Donner, T. Eisenhaure, M. Guttman, J. K. Grenier, W. Li, O. Zuk, L. A. Schubert, B. Birditt, T. Shay, A. Goren, X. Zhang, Z. Smith, R. Deering, R. C. McDonald, M. Cabili, B. E. Bernstein, J. L. Rinn, A. Meissner, D. E. Root, N. Hacohen and A. Regev (2009). Unbiased reconstruction of a mammalian transcriptional network mediating pathogen responses. Science 326 5950:257-263.

Ananieva, O., J. Darragh, C. Johansen, J. M. Carr, J. McIlrath, J. M. Park, A. Wingate, C. E. Monk, R. Toth, S. G. Santos, L. Iversen and J. S. Arthur (2008). The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. Nat Immunol 9 9:1028-1036.

Anderson, A. E., M. L. Worku, W. Khamri, K. B. Bamford, M. M. Walker and M. R. Thursz (2007). TLR9 polymorphisms determine murine lymphocyte responses to Helicobacter: results from a genome-wide scan. Eur J Immunol 37 6:1548-1561.

Anderson, C. F., J. S. Stumhofer, C. A. Hunter and D. Sacks (2009). IL-27 regulates IL-10 and IL-17 from CD4+ cells in nonhealing Leishmania major infection. J Immunol 183 7:4619-4627.

Ann Marrie, R. and R. A. Rudick (2006). Drug Insight: interferon treatment in multiple sclerosis. Nat Clin Pract Neurol 2 1:34-44.

Anthony, R. M., J. F. Urban, Jr., F. Alem, H. A. Hamed, C. T. Rozo, J. L. Boucher, N. Van Rooijen and W. C. Gause (2006). Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nat Med 12 8:955-960.

Anuntagool, N., P. Aramsri, T. Panichakul, V. R. Wuthiekanun, R. Kinoshita, N. J. White and S. Sirisinha (2000). Antigenic heterogeneity of lipopolysaccharide among Burkholderia pseudomallei clinical isolates. Southeast Asian J Trop Med Public Health 31 Suppl 1146-152.

Apetoh, L., F. J. Quintana, C. Pot, N. Joller, S. Xiao, D. Kumar, E. J. Burns, D. H. Sherr, H. L. Weiner and V. K. Kuchroo (2010). The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. Nat Immunol 11 9:854-861.

Arakura, F., S. Hida, E. Ichikawa, C. Yajima, S. Nakajima, T. Saida and S. Taki (2007). Genetic control directed toward spontaneous IFN-alpha/IFN-beta responses and downstream IFN-gamma expression influences the pathogenesis of a murine psoriasis-like skin disease. J Immunol 179 5:3249-3257.

Aste-Amezaga, M., X. Ma, A. Sartori and G. Trinchieri (1998). Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. J Immunol 160 12:5936-5944.

Auerbuch, V., D. G. Brockstedt, N. Meyer-Morse, M. O'Riordan and D. A. Portnoy (2004). Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. J Exp Med 200 4:527-533.

Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau and F. Geissmann (2007). Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 317 5838:666-670.

Auffray, C., D. K. Fogg, E. Narni-Mancinelli, B. Senechal, C. Trouillet, N. Saederup, J. Leemput, K. Bigot, L. Campisi, M. Abitbol, T. Molina, I. Charo, D. A. Hume, A. Cumano, G. Lauvau and F. Geissmann (2009a). CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation. J Exp Med 206 3:595-606.

Auffray, C., M. H. Sieweke and F. Geissmann (2009b). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol 27669-692.

Awasthi, A., Y. Carrier, J. P. Peron, E. Bettelli, M. Kamanaka, R. A. Flavell, V. K. Kuchroo, M. Oukka and H. L. Weiner (2007). A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol 8 12:1380-1389.

Bacchetta, R., E. Gambineri and M. G. Roncarolo (2007). Role of regulatory T cells and FOXP3 in human diseases. J Allergy Clin Immunol 120 2:227-235; quiz 236-227.

Bacon, C. M., D. W. McVicar, J. R. Ortaldo, R. C. Rees, J. J. O'Shea and J. A. Johnston (1995a). Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. J Exp Med 181 1:399-404.

Bacon, C. M., E. F. Petricoin, 3rd, J. R. Ortaldo, R. C. Rees, A. C. Larner, J. A. Johnston and J. J. O'Shea (1995b). Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. Proc Natl Acad Sci U S A 92 16:7307-7311.

Bain, J., L. Plater, M. Elliott, N. Shpiro, C. J. Hastie, H. McLauchlan, I. Klevernic, J. S. Arthur, D. R. Alessi and P. Cohen (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J 408 3:297-315.

Bancerek, J., Z. C. Poss, I. Steinparzer, V. Sedlyarov, T. Pfaffenwimmer, I. Mikulic, L. Dolken, B. Strobl, M. Muller, D. J. Taatjes and P. Kovarik (2013). CDK8 kinase phosphorylates transcription factor STAT1 to selectively regulate the interferon response. Immunity 38 2:250-262.

Banchereau, J. and V. Pascual (2006). Type I interferon in systemic lupus erythematosus and other autoimmune diseases. Immunity 25 3:383-392.

Banchereau, J. and R. M. Steinman (1998). Dendritic cells and the control of immunity. Nature 392 6673:245-252.

Banerjee, A., R. Gugasyan, M. McMahon and S. Gerondakis (2006). Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. Proc Natl Acad Sci U S A 103 9:3274-3279.

Barbalat, R., L. Lau, R. M. Locksley and G. M. Barton (2009). Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol 10 11:1200-1207.

Batten, M., N. M. Kljavin, J. Li, M. J. Walter, F. J. de Sauvage and N. Ghilardi (2008). Cutting edge: IL-27 is a potent inducer of IL-10 but not FoxP3 in murine T cells. J Immunol 180 5:2752-2756.

Beebe, A. M., D. J. Cua and R. de Waal Malefyt (2002). The role of interleukin-10 in autoimmune disease: systemic lupus erythematosus (SLE) and multiple sclerosis (MS). Cytokine Growth Factor Rev 13 4-5:403-412.

Belkaid, Y., K. F. Hoffmann, S. Mendez, S. Kamhawi, M. C. Udey, T. A. Wynn and D. L. Sacks (2001). The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med 194 10:1497-1506.

Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach and D. L. Sacks (2002). CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature 420 6915:502-507.

Benkhart, E. M., M. Siedlar, A. Wedel, T. Werner and H. W. Ziegler-Heitbrock (2000). Role of Stat3 in lipopolysaccharide-induced IL-10 gene expression. J Immunol 165 3:1612-1617.

Bennett, L., A. K. Palucka, E. Arce, V. Cantrell, J. Borvak, J. Banchereau and V. Pascual (2003). Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med 197 6:711-723.

Berg, D. J., N. Davidson, R. Kuhn, W. Muller, S. Menon, G. Holland, L. Thompson-Snipes, M. W. Leach and D. Rennick (1996). Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest 98 4:1010-1020.

Berg, D. J., R. Kuhn, K. Rajewsky, W. Muller, S. Menon, N. Davidson, G. Grunig and D. Rennick (1995). Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. J Clin Invest 96 5:2339-2347.

Berry, M. P., C. M. Graham, F. W. McNab, Z. Xu, S. A. Bloch, T. Oni, K. A. Wilkinson, R. Banchereau, J. Skinner, R. J. Wilkinson, C. Quinn, D. Blankenship, R. Dhawan, J. J. Cush, A. Mejias, O. Ramilo, O. M. Kon, V. Pascual, J. Banchereau, D. Chaussabel and A. O'Garra (2010). An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 466 7309:973-977.

Bettelli, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel and V. K. Kuchroo (1998). IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. J Immunol 161 7:3299-3306.

Bhoj, V. G. and Z. J. Chen (2009). Ubiquitylation in innate and adaptive immunity. Nature 458 7237:430-437.

Blasius, A. L. and B. Beutler (2010). Intracellular toll-like receptors. Immunity 32 3:305-315.

Boonstra, A., R. Rajsbaum, M. Holman, R. Marques, C. Asselin-Paturel, J. P. Pereira, E. E. Bates, S. Akira, P. Vieira, Y. J. Liu, G. Trinchieri and A. O'Garra (2006). Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol 177 11:7551-7558.

Borish, L., A. Aarons, J. Rumbyrt, P. Cvietusa, J. Negri and S. Wenzel (1996). Interleukin-10 regulation in normal subjects and patients with asthma. J Allergy Clin Immunol 97 6:1288-1296.

Bottomly, D., N. A. Walter, J. E. Hunter, P. Darakjian, S. Kawane, K. J. Buck, R. P. Searles, M. Mooney, S. K. McWeeney and R. Hitzemann (2011). Evaluating gene expression in C57BL/6J and DBA/2J mouse striatum using RNA-Seq and microarrays. PLoS One 6 3:e17820.

Breitbach, K., S. Klocke, T. Tschernig, N. van Rooijen, U. Baumann and I. Steinmetz (2006). Role of inducible nitric oxide synthase and NADPH oxidase in early control of Burkholderia pseudomallei infection in mice. Infect Immun 74 11:6300-6309.

Breitbach, K., P. Wongprompitak and I. Steinmetz (2011). Distinct roles for nitric oxide in resistant C57BL/6 and susceptible BALB/c mice to control Burkholderia pseudomallei infection. BMC Immunol 1220.

Brennan, F. M., D. Chantry, A. Jackson, R. Maini and M. Feldmann (1989). Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. Lancet 2 8657:244-247.

Brenner, S., S. Prosch, K. Schenke-Layland, U. Riese, U. Gausmann and C. Platzer (2003). cAMP-induced Interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. J Biol Chem 278 8:5597-5604.

Brightbill, H. D., S. E. Plevy, R. L. Modlin and S. T. Smale (2000). A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. J Immunol 164 4:1940-1951.

Brooks, D. G., M. J. Trifilo, K. H. Edelmann, L. Teyton, D. B. McGavern and M. B. Oldstone (2006). Interleukin-10 determines viral clearance or persistence in vivo. Nat Med 12 11:1301-1309.

Byrnes, A. A., X. Ma, P. Cuomo, K. Park, L. Wahl, S. F. Wolf, H. Zhou, G. Trinchieri and C. L. Karp (2001). Type I interferons and IL-12: convergence and cross-regulation among mediators of cellular immunity. Eur J Immunol 31 7:2026-2034.

Calvano, S. E., W. Xiao, D. R. Richards, R. M. Felciano, H. V. Baker, R. J. Cho, R. O. Chen, B. H. Brownstein, J. P. Cobb, S. K. Tschoeke, C. Miller-Graziano, L. L. Moldawer, M. N. Mindrinos, R. W. Davis, R. G. Tompkins and S. F. Lowry (2005). A network-based analysis of systemic inflammation in humans. Nature 437 7061:1032-1037.

Cao, S., J. Liu, L. Song and X. Ma (2005). The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. J Immunol 174 6:3484-3492.

Cao, S., X. Zhang, J. P. Edwards and D. M. Mosser (2006). NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. J Biol Chem 281 36:26041-26050.

Cao, W., S. Manicassamy, H. Tang, S. P. Kasturi, A. Pirani, N. Murthy and B. Pulendran (2008). Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. Nat Immunol 9 10:1157-1164.

Carra, G., F. Gerosa and G. Trinchieri (2000). Biosynthesis and posttranslational regulation of human IL-12. J Immunol 164 9:4752-4761.

Carrero, J. A., B. Calderon and E. R. Unanue (2004). Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection. J Exp Med 200 4:535-540.

Carrero, J. A., B. Calderon and E. R. Unanue (2006). Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes. J Exp Med 203 4:933-940.

Ceballos-Olvera, I., M. Sahoo, M. A. Miller, L. Del Barrio and F. Re (2011). Inflammasome-dependent pyroptosis and IL-18 protect against Burkholderia pseudomallei lung infection while IL-1beta is deleterious. PLoS Pathog 7 12:e1002452.

Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia and G. Alber (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med 184 2:747-752.

Chakrabarti, A., A. J. Sadler, N. Kar, H. A. Young, R. H. Silverman and B. R. Williams (2008). Protein kinase R-dependent regulation of interleukin-10 in response to double-stranded RNA. J Biol Chem 283 37:25132-25139.

Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark and G. Trinchieri (1991). Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J Exp Med 173 4:869-879.

Chang, E. Y., B. Guo, S. E. Doyle and G. Cheng (2007a). Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production. J Immunol 178 11:6705-6709.

Chang, H. D., C. Helbig, L. Tykocinski, S. Kreher, J. Koeck, U. Niesner and A. Radbruch (2007b). Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. Eur J Immunol 37 3:807-817.

Chastel, C., J. Jiricny and R. Jaussi (2004). Activation of stress-responsive promoters by ionizing radiation for deployment in targeted gene therapy. DNA Repair (Amst) 3 3:201-215.

Chaudhry, A., R. M. Samstein, P. Treuting, Y. Liang, M. C. Pils, J. M. Heinrich, R. S. Jack, F. T. Wunderlich, J. C. Bruning, W. Muller and A. Y. Rudensky (2011). Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 34 4:566-578.

Chen, W. F. and A. Zlotnik (1991). IL-10: a novel cytotoxic T cell differentiation factor. J Immunol 147 2:528-534.

Chung, E. Y., J. Liu, Y. Homma, Y. Zhang, A. Brendolan, M. Saggese, J. Han, R. Silverstein, L. Selleri and X. Ma (2007). Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. Immunity 27 6:952-964.

Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg and D. A. Vignali (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 450 7169:566-569.

Colombo, M. P. and G. Trinchieri (2002). Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev 13 2:155-168.

Colonna, M. (2007). TLR pathways and IFN-regulatory factors: to each its own. Eur J Immunol 37 2:306-309.

Colonna, M., G. Trinchieri and Y. J. Liu (2004). Plasmacytoid dendritic cells in immunity. Nat Immunol 5 12:1219-1226.

Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell and I. M. Orme (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 178 6:2243-2247.

Cooper, A. M., A. Kipnis, J. Turner, J. Magram, J. Ferrante and I. M. Orme (2002). Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. J Immunol 168 3:1322-1327.

Cousens, L. P., J. S. Orange, H. C. Su and C. A. Biron (1997). Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. Proc Natl Acad Sci U S A 94 2:634-639.

Cousens, L. P., R. Peterson, S. Hsu, A. Dorner, J. D. Altman, R. Ahmed and C. A. Biron (1999). Two roads diverged: interferon alpha/beta- and interleukin 12-mediated pathways in promoting T cell interferon gamma responses during viral infection. J Exp Med 189 8:1315-1328.

Csoka, B., Z. H. Nemeth, L. Virag, P. Gergely, S. J. Leibovich, P. Pacher, C. X. Sun, M. R. Blackburn, E. S. Vizi, E. A. Deitch and G. Hasko (2007). A2A adenosine receptors and C/EBPbeta are crucially required for IL-10 production by macrophages exposed to Escherichia coli. Blood 110 7:2685-2695.

Currie, B. J., D. A. Dance and A. C. Cheng (2008). The global distribution of Burkholderia pseudomallei and melioidosis: an update. Trans R Soc Trop Med Hyg 102 Suppl 1S1-4.

Currie, B. J., L. Ward and A. C. Cheng (2010). The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year Darwin prospective study. PLoS Negl Trop Dis 4 11:e900.

D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin and G. Trinchieri (1993). Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178 3:1041-1048.

D'Andrea, A., X. Ma, M. Aste-Amezaga, C. Paganin and G. Trinchieri (1995). Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production. J Exp Med 181 2:537-546.

D'Andrea, A., M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young, E. Nickbarg, R. Chizzonite, S. F. Wolf and G. Trinchieri (1992). Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J Exp Med 176 5:1387-1398.

Dai, W. J., G. Kohler and F. Brombacher (1997). Both innate and acquired immunity to Listeria monocytogenes infection are increased in IL-10-deficient mice. Journal of Immunology 158 5:2259-2267.

Dai, X. M., G. R. Ryan, A. J. Hapel, M. G. Dominguez, R. G. Russell, S. Kapp, V. Sylvestre and E. R. Stanley (2002). Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood 99 1:111-120.

Dang, C. V. (2012). MYC on the path to cancer. Cell 149 1:22-35.

David, M., E. Petricoin, 3rd, C. Benjamin, R. Pine, M. J. Weber and A. C. Larner (1995). Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. Science 269 5231:1721-1723.

De Kock, I., C. Van Daele and J. Poelaert (2010). Sepsis and septic shock: pathophysiological and cardiovascular background as basis for therapy. Acta Clin Belg 65 5:323-329.

De Maeyer-Guignard, J., F. Dandoy, D. W. Bailey and E. De Maeyer (1986). Interferon structural genes do not participate in quantitative regulation of interferon production by If loci as shown in C57BL/6 mice that are congenic with BALB/c mice at the alpha interferon gene cluster. J Virol 58 3:743-747.

Decker, T., M. Muller and S. Stockinger (2005). The yin and yang of type I interferon activity in bacterial infection. Nat Rev Immunol 5 9:675-687.

Dent, A. L., F. H. Vasanwala and L. M. Toney (2002). Regulation of gene expression by the proto-oncogene BCL-6. Crit Rev Oncol Hematol 41 1:1-9.

Der, S. D., A. Zhou, B. R. Williams and R. H. Silverman (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A 95 26:15623-15628.

Desai, B. B., P. M. Quinn, A. G. Wolitzky, P. K. Mongini, R. Chizzonite and M. K. Gately (1992). IL-12 receptor. II. Distribution and regulation of receptor expression. J Immunol 148 10:3125-3132.

Dietrich, N., S. Lienenklaus, S. Weiss and N. O. Gekara (2010). Murine toll-like receptor 2 activation induces type I interferon responses from endolysosomal compartments. PLoS One 5 4:e10250.

Dillon, S., A. Agrawal, T. Van Dyke, G. Landreth, L. McCauley, A. Koh, C. Maliszewski, S. Akira and B. Pulendran (2004). A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. J Immunol 172 8:4733-4743.

Do, J. H. and D. K. Choi (2008). Clustering approaches to identifying gene expression patterns from DNA microarray data. Mol Cells 25 2:279-288.

Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin and G. Cheng (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. Immunity 17 3:251-263.

Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias and P. N. Tsichlis (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103 7:1071-1083.

Ehrchen, J. M., K. Roebrock, D. Foell, N. Nippe, E. von Stebut, J. M. Weiss, N. A. Munck, D. Viemann, G. Varga, C. Muller-Tidow, H. J. Schuberth, J. Roth and C. Sunderkotter (2010). Keratinocytes determine Th1 immunity during early experimental leishmaniasis. PLoS Pathog 6 4:e1000871.

Ejrnaes, M., C. M. Filippi, M. M. Martinic, E. M. Ling, L. M. Togher, S. Crotty and M. G. von Herrath (2006). Resolution of a chronic viral infection after interleukin-10 receptor blockade. J Exp Med 203 11:2461-2472.

El Kasmi, K. C., A. M. Smith, L. Williams, G. Neale, A. D. Panopoulos, S. S. Watowich, H. Hacker, B. M. Foxwell and P. J. Murray (2007). Cutting edge: A transcriptional repressor and corepressor induced by the STAT3-regulated anti-inflammatory signaling pathway. J Immunol 179 11:7215-7219.

Elkon, R., C. Linhart, Y. Halperin, Y. Shiloh and R. Shamir (2007). Functional genomic delineation of TLR-induced transcriptional networks. BMC Genomics 8394.

Ermann, J., W. S. Garrett, J. Kuchroo, K. Rourida, J. N. Glickman, A. Bleich and L. H. Glimcher (2011). Severity of innate immune-mediated colitis is controlled by the cytokine deficiency-induced colitis susceptibility-1 (Cdcs1) locus. Proc Natl Acad Sci U S A 108 17:7137-7141.

Escors, D., L. Lopes, R. Lin, J. Hiscott, S. Akira, R. J. Davis and M. K. Collins (2008). Targeting dendritic cell signaling to regulate the response to immunization. Blood 111 6:3050-3061.

Evans, M. J. and M. H. Kaufman (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature 292 5819:154-156.

Finbloom, D. S. and K. D. Winestock (1995). IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. J Immunol 155 3:1079-1090.

Finch, A. R., C. J. Caunt, R. M. Perrett, K. Tsaneva-Atanasova and C. A. McArdle (2012). Dual specificity phosphatases 10 and 16 are positive regulators of EGF-stimulated ERK activity: indirect regulation of ERK signals by JNK/p38 selective MAPK phosphatases. Cell Signal 24 5:1002-1011.

Fiorentino, D. F., M. W. Bond and T. R. Mosmann (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170 6:2081-2095.

Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard and A. O'Garra (1991a). IL-10 inhibits cytokine production by activated macrophages. J Immunol 147 11:3815-3822.

Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore and A. O'Garra (1991b). IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 146 10:3444-3451.

Fitzgerald, D. C., G. X. Zhang, M. El-Behi, Z. Fonseca-Kelly, H. Li, S. Yu, C. J. Saris, B. Gran, B. Ciric and A. Rostami (2007). Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. Nat Immunol 8 12:1372-1379.

Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao and T. Maniatis (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 4 5:491-496.

Fleetwood, A. J., H. Dinh, A. D. Cook, P. J. Hertzog and J. A. Hamilton (2009). GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. J Leukoc Biol 86 2:411-421.

Flint, J. and E. Eskin (2012). Genome-wide association studies in mice. Nat Rev Genet 13 11:807-817.

Flynn, J. L. and J. Chan (2001). Immunology of tuberculosis. Annu Rev Immunol 1993-129.

Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart and B. R. Bloom (1993). An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med 178 6:2249-2254.

Foey, A. D., S. L. Parry, L. M. Williams, M. Feldmann, B. M. Foxwell and F. M. Brennan (1998). Regulation of monocyte IL-10 synthesis by endogenous IL-1 and TNF-alpha: role of the p38 and p42/44 mitogen-activated protein kinases. J Immunol 160 2:920-928.

Fogg, D. K., C. Sibon, C. Miled, S. Jung, P. Aucouturier, D. R. Littman, A. Cumano and F. Geissmann (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. Science 311 5757:83-87.

Franchi, L., N. Warner, K. Viani and G. Nunez (2009). Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev 227 1:106-128.

Franke, A., T. Balschun, T. H. Karlsen, J. Sventoraityte, S. Nikolaus, G. Mayr, F. S. Domingues, M. Albrecht, M. Nothnagel, D. Ellinghaus, C. Sina, C. M. Onnie, R. K. Weersma, P. C. Stokkers, C. Wijmenga, M. Gazouli, D. Strachan, W. L. McArdle, S. Vermeire, P. Rutgeerts, P. Rosenstiel, M. Krawczak, M. H. Vatn, C. G. Mathew and S. Schreiber (2008). Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. Nat Genet 40 11:1319-1323.

Franke, A., D. P. McGovern, J. C. Barrett, K. Wang, G. L. Radford-Smith, T. Ahmad, C. W. Lees, T. Balschun, J. Lee, R. Roberts, C. A. Anderson, J. C. Bis, S. Bumpstead, D. Ellinghaus, E. M. Festen, M. Georges, T. Green, T. Haritunians, L. Jostins, A. Latiano, C. G. Mathew, G. W. Montgomery, N. J. Prescott, S. Raychaudhuri, J. I. Rotter, P. Schumm, Y. Sharma, L. A. Simms, K. D. Taylor, D. Whiteman, C. Wijmenga, R. N. Baldassano, M. Barclay, T. M. Bayless, S. Brand, C. Buning, A. Cohen, J. F. Colombel, M. Cottone, L. Stronati, T. Denson, M. De Vos, R. D'Inca, M. Dubinsky, C. Edwards, T. Florin, D. Franchimont, R. Gearry, J. Glas, A. Van Gossum, S. L. Guthery, J. Halfvarson, H. W. Verspaget, J. P. Hugot, A. Karban, D. Laukens, I. Lawrance, M. Lemann, A. Levine, C. Libioulle, E. Louis, C. Mowat, W. Newman, J. Panes, A.

Phillips, D. D. Proctor, M. Regueiro, R. Russell, P. Rutgeerts, J. Sanderson, M. Sans, F. Seibold, A. H. Steinhart, P. C. Stokkers, L. Torkvist, G. Kullak-Ublick, D. Wilson, T. Walters, S. R. Targan, S. R. Brant, J. D. Rioux, M. D'Amato, R. K. Weersma, S. Kugathasan, A. M. Griffiths, J. C. Mansfield, S. Vermeire, R. H. Duerr, M. S. Silverberg, J. Satsangi, S. Schreiber, J. H. Cho, V. Annese, H. Hakonarson, M. J. Daly and M. Parkes (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 42 12:1118-1125.

Freitas do Rosario, A. P., T. Lamb, P. Spence, R. Stephens, A. Lang, A. Roers, W. Muller, A. O'Garra and J. Langhorne (2012). IL-27 promotes IL-10 production by effector Th1 CD4+ T cells: a critical mechanism for protection from severe immunopathology during malaria infection. J Immunol 188 3:1178-1190.

Fujii, S., K. Shimizu, T. Shimizu and M. T. Lotze (2001). Interleukin-10 promotes the maintenance of antitumor CD8(+) T-cell effector function in situ. Blood 98 7:2143-2151.

Fukao, T., M. Tanabe, Y. Terauchi, T. Ota, S. Matsuda, T. Asano, T. Kadowaki, T. Takeuchi and S. Koyasu (2002). PI3K-mediated negative feedback regulation of IL-12 production in DCs. Nat Immunol 3 9:875-881.

Fung, K. Y., N. E. Mangan, H. Cumming, J. C. Horvat, J. R. Mayall, S. A. Stifter, N. De Weerd, L. C. Roisman, J. Rossjohn, S. A. Robertson, J. E. Schjenken, B. Parker, C. E. Gargett, H. P. Nguyen, D. J. Carr, P. M. Hansbro and P. J. Hertzog (2013). Interferon-epsilon protects the female reproductive tract from viral and bacterial infection. Science 339 6123:1088-1092.

Gaba, A., S. I. Grivennikov, M. V. Do, D. J. Stumpo, P. J. Blackshear and M. Karin (2012). Cutting Edge: IL-10-Mediated Tristetraprolin Induction Is Part of a Feedback Loop That Controls Macrophage STAT3 Activation and Cytokine Production. J Immunol.

Gantke, T., S. Sriskantharajah and S. C. Ley (2011). Regulation and function of TPL-2, an IkappaB kinase-regulated MAP kinase kinase kinase. Cell Res 21 1:131-145.

Garcia-Sastre, A. and C. A. Biron (2006). Type 1 interferons and the virus-host relationship: a lesson in detente. Science 312 5775:879-882.

Gately, M. K., D. M. Carvajal, S. E. Connaughton, S. Gillessen, R. R. Warrier, K. D. Kolinsky, V. L. Wilkinson, C. M. Dwyer, G. F. Higgins, Jr., F. J. Podlaski, D. A. Faherty, P. C. Familletti, A. S. Stern and D. H. Presky (1996). Interleukin-12 antagonist activity of mouse interleukin-12 p40 homodimer in vitro and in vivo. Ann N Y Acad Sci 7951-12.

Gately, M. K., R. R. Warrier, S. Honasoge, D. M. Carvajal, D. A. Faherty, S. E. Connaughton, T. D. Anderson, U. Sarmiento, B. R. Hubbard and M. Murphy (1994). Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-gamma in vivo. Int Immunol 6 1:157-167.

Gautier, E. L., T. Shay, J. Miller, M. Greter, C. Jakubzick, S. Ivanov, J. Helft, A. Chow, K. G. Elpek, S. Gordonov, A. R. Mazloom, A. Ma'ayan, W. J. Chua, T. H. Hansen, S. J. Turley, M. Merad and G. J. Randolph (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nat Immunol 13 11:1118-1128.

Gautier, G., M. Humbert, F. Deauvieau, M. Scuiller, J. Hiscott, E. E. Bates, G. Trinchieri, C. Caux and P. Garrone (2005). A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. J Exp Med 201 9:1435-1446.

Gazzinelli, R., Y. Xu, S. Hieny, A. Cheever and A. Sher (1992). Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii. J Immunol 149 1:175-180.

Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf and A. Sher (1993). Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc Natl Acad Sci U S A 90 13:6115-6119.

Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri and A. Sher (1994). Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with Toxoplasma gondii. J Immunol 153 6:2533-2543.

Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri and A. Sher (1996). In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol 157 2:798-805.

Geijtenbeek, T. B. and S. I. Gringhuis (2009). Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol 9 7:465-479.

Geissmann, F., S. Gordon, D. A. Hume, A. M. Mowat and G. J. Randolph (2010a). Unravelling mononuclear phagocyte heterogeneity. Nat Rev Immunol 10 6:453-460.

Geissmann, F., S. Jung and D. R. Littman (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 19 1:71-82.

Geissmann, F., M. G. Manz, S. Jung, M. H. Sieweke, M. Merad and K. Ley (2010b). Development of monocytes, macrophages, and dendritic cells. Science 327 5966:656-661.

Gerber, J. S. and D. M. Mosser (2001). Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. J Immunol 166 11:6861-6868.

Gerosa, F., B. Baldani-Guerra, L. A. Lyakh, G. Batoni, S. Esin, R. T. Winkler-Pickett, M. R. Consolaro, M. De Marchi, D. Giachino, A. Robbiano, M. Astegiano, A. Sambataro, R. A. Kastelein, G. Carra and G. Trinchieri (2008). Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. J Exp Med 205 6:1447-1461.

Gilchrist, M., V. Thorsson, B. Li, A. G. Rust, M. Korb, J. C. Roach, K. Kennedy, T. Hai, H. Bolouri and A. Aderem (2006). Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature 441 7090:173-178.

Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, U. Gubler, D. H. Presky, A. S. Stern and M. K. Gately (1995). Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur J Immunol 25 1:200-206.

Ginhoux, F., M. Greter, M. Leboeuf, S. Nandi, P. See, S. Gokhan, M. F. Mehler, S. J. Conway, L. G. Ng, E. R. Stanley, I. M. Samokhvalov and M. Merad (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330 6005:841-845.

Glocker, E. O., D. Kotlarz, K. Boztug, E. M. Gertz, A. A. Schaffer, F. Noyan, M. Perro, J. Diestelhorst, A. Allroth, D. Murugan, N. Hatscher, D. Pfeifer, K. W. Sykora, M. Sauer, H. Kreipe, M. Lacher, R. Nustede, C. Woellner, U. Baumann, U. Salzer, S. Koletzko, N. Shah, A. W. Segal, A. Sauerbrey, S. Buderus, S. B. Snapper, B. Grimbacher and C. Klein (2009). Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 361 21:2033-2045.

Goh, F. G., S. J. Thomson, T. Krausgruber, A. Lanfrancotti, R. R. Copley and I. A. Udalova (2010). Beyond the enhanceosome: cluster of novel kappaB sites downstream of the human IFN-beta gene is essential for lipopolysaccharide-induced gene activation. Blood 116 25:5580-5588.

Goodridge, H. S., T. Shimada, A. J. Wolf, Y. M. Hsu, C. A. Becker, X. Lin and D. M. Underhill (2009). Differential use of CARD9 by dectin-1 in macrophages and dendritic cells. J Immunol 182 2:1146-1154.

Gordon, S. and F. O. Martinez (2010). Alternative activation of macrophages: mechanism and functions. Immunity 32 5:593-604.

Gordon, S. and P. R. Taylor (2005). Monocyte and macrophage heterogeneity. Nat Rev Immunol 5 12:953-964.

Goriely, S., D. Demonte, S. Nizet, D. De Wit, F. Willems, M. Goldman and C. Van Lint (2003). Human IL-12(p35) gene activation involves selective remodeling of a single nucleosome within a region of the promoter containing critical Sp1-binding sites. Blood 101 12:4894-4902.

Goriely, S., C. Molle, M. Nguyen, V. Albarani, N. O. Haddou, R. Lin, D. De Wit, V. Flamand, F. Willems and M. Goldman (2006). Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. Blood 107 3:1078-1084.

Goriely, S., M. F. Neurath and M. Goldman (2008). How microorganisms tip the balance between interleukin-12 family members. Nat Rev Immunol 8 1:81-86.

Gough, D. J., N. L. Messina, C. J. Clarke, R. W. Johnstone and D. E. Levy (2012). Constitutive type I interferon modulates homeostatic balance through tonic signaling. Immunity 36 2:166-174.

Gough, D. J., N. L. Messina, L. Hii, J. A. Gould, K. Sabapathy, A. P. Robertson, J. A. Trapani, D. E. Levy, P. J. Hertzog, C. J. Clarke and R. W. Johnstone (2010). Functional crosstalk between type I and II interferon through the regulated expression of STAT1. PLoS Biol 8 4:e1000361.

Grenningloh, R., B. Y. Kang and I. C. Ho (2005). Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. J Exp Med 201 4:615-626.

Gri, G., D. Savio, G. Trinchieri and X. Ma (1998). Synergistic regulation of the human interleukin-12 p40 promoter by NFkappaB and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. J Biol Chem 273 11:6431-6438.

Grumont, R., H. Hochrein, M. O'Keeffe, R. Gugasyan, C. White, I. Caminschi, W. Cook and S. Gerondakis (2001). c-Rel regulates interleukin 12 p70 expression in CD8(+) dendritic cells by specifically inducing p35 gene transcription. J Exp Med 194 8:1021-1032.

Guarda, G., M. Braun, F. Staehli, A. Tardivel, C. Mattmann, I. Forster, M. Farlik, T. Decker, R. A. Du Pasquier, P. Romero and J. Tschopp (2011). Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity 34 2:213-223.

Guhaniyogi, J. and G. Brewer (2001). Regulation of mRNA stability in mammalian cells. Gene 265 1-2:11-23.

Hacker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L. C. Hsu, G. G. Wang, M. P. Kamps, E. Raz, H. Wagner, G. Hacker, M. Mann and M. Karin (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 439 7073:204-207.

Hall, A. O., J. S. Silver and C. A. Hunter (2012). The immunobiology of IL-27. Adv Immunol 1151-44.

Hammer, M., J. Mages, H. Dietrich, F. Schmitz, F. Striebel, P. J. Murray, H. Wagner and R. Lang (2005). Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. Eur J Immunol 35 10:2991-3001.

Hamon, M., H. Bierne and P. Cossart (2006). Listeria monocytogenes: a multifaceted model. Nat Rev Microbiol 4 6:423-434.

Haque, A., A. Easton, D. Smith, A. O'Garra, N. Van Rooijen, G. Lertmemongkolchai, R. W. Titball and G. J. Bancroft (2006). Role of T cells in innate and adaptive immunity against murine Burkholderia pseudomallei infection. J Infect Dis 193 3:370-379.

Harris, M. A., J. Clark, A. Ireland, J. Lomax, M. Ashburner, R. Foulger, K. Eilbeck, S. Lewis, B. Marshall, C. Mungall, J. Richter, G. M. Rubin, J. A. Blake, C. Bult, M. Dolan, H. Drabkin, J. T. Eppig, D. P. Hill, L. Ni, M. Ringwald, R. Balakrishnan, J. M. Cherry, K. R. Christie, M. C. Costanzo, S. S. Dwight, S. Engel, D. G. Fisk, J. E. Hirschman, E. L. Hong, R. S. Nash, A. Sethuraman, C. L. Theesfeld, D. Botstein, K. Dolinski, B. Feierbach, T. Berardini, S. Mundodi, S. Y. Rhee, R. Apweiler, D. Barrell, E. Camon, E. Dimmer, V. Lee, R. Chisholm, P. Gaudet, W. Kibbe, R. Kishore, E. M. Schwarz, P. Sternberg, M. Gwinn, L. Hannick, J. Wortman, M. Berriman, V. Wood, N. de la Cruz, P. Tonellato, P. Jaiswal, T. Seigfried and R. White (2004). The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res 32 Database issue:D258-261.

Harty, J. T., A. R. Tvinnereim and D. W. White (2000). CD8+ T cell effector mechanisms in resistance to infection. Annu Rev Immunol 18275-308.

Hasan, U., C. Chaffois, C. Gaillard, V. Saulnier, E. Merck, S. Tancredi, C. Guiet, F. Briere, J. Vlach, S. Lebecque, G. Trinchieri and E. E. Bates (2005). Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol 174 5:2942-2950.

Hashimoto, C., K. L. Hudson and K. V. Anderson (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52 2:269-279.

Hashimoto, D., J. Miller and M. Merad (2011). Dendritic cell and macrophage heterogeneity in vivo. Immunity 35 3:323-335.

Hawrylowicz, C. M. and A. O'Garra (2005). Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol 5 4:271-283.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill and A. Aderem (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410 6832:1099-1103.

Hayden, M. S. and S. Ghosh (2008). Shared principles in NF-kappaB signaling. Cell 132 3:344-362.

Hayes, M. P., J. Wang and M. A. Norcross (1995). Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. Blood 86 2:646-650.

Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman and R. M. Locksley (1989). Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. J Exp Med 169 1:59-72.

Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser and M. K. Gately (1993). Recombinant interleukin 12 cures mice infected with Leishmania major. J Exp Med 177 5:1505-1509.

Hida, S., K. Ogasawara, K. Sato, M. Abe, H. Takayanagi, T. Yokochi, T. Sato, S. Hirose, T. Shirai, S. Taki and T. Taniguchi (2000). CD8(+) T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-alpha/beta signaling. Immunity 13 5:643-655.

Hii, C. S., G. W. Sun, J. W. Goh, J. Lu, M. P. Stevens and Y. H. Gan (2008). Interleukin-8 induction by Burkholderia pseudomallei can occur without Toll-like receptor signaling but requires a functional type III secretion system. J Infect Dis 197 11:1537-1547.

Honda, K., Y. Ohba, H. Yanai, H. Negishi, T. Mizutani, A. Takaoka, C. Taya and T. Taniguchi (2005a). Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature 434 7036:1035-1040.

Honda, K., H. Yanai, T. Mizutani, H. Negishi, N. Shimada, N. Suzuki, Y. Ohba, A. Takaoka, W. C. Yeh and T. Taniguchi (2004). Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. Proc Natl Acad Sci U S A 101 43:15416-15421.

Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida and T. Taniguchi (2005b). IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 434 7034:772-777.

Honda, K., H. Yanai, A. Takaoka and T. Taniguchi (2005c). Regulation of the type I IFN induction: a current view. Int Immunol 17 11:1367-1378.

Hoppe, I., B. Brenneke, M. Rohde, A. Kreft, S. Haussler, A. Reganzerowski and I. Steinmetz (1999). Characterization of a murine model of melioidosis: comparison of different strains of mice. Infect Immun 67 6:2891-2900.

Horng, T., G. M. Barton, R. A. Flavell and R. Medzhitov (2002). The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature 420 6913:329-333.

Howard, M., T. Muchamuel, S. Andrade and S. Menon (1993). Interleukin 10 protects mice from lethal endotoxemia. J Exp Med 177 4:1205-1208.

Hsieh, C. S., S. E. Macatonia, A. O'Garra and K. M. Murphy (1995). T cell genetic background determines default T helper phenotype development in vitro. J Exp Med 181 2:713-721.

Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra and K. M. Murphy (1993). Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science 260 5107:547-549.

Hsu, D. H., R. de Waal Malefyt, D. F. Fiorentino, M. N. Dang, P. Vieira, J. de Vries, H. Spits, T. R. Mosmann and K. W. Moore (1990). Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. Science 250 4982:830-832.

Hu, X., P. K. Paik, J. Chen, A. Yarilina, L. Kockeritz, T. T. Lu, J. R. Woodgett and L. B. Ivashkiv (2006). IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins. Immunity 24 5:563-574.

Huber, S., N. Gagliani, E. Esplugues, W. O'Connor, Jr., F. J. Huber, A. Chaudhry, M. Kamanaka, Y. Kobayashi, C. J. Booth, A. Y. Rudensky, M. G. Roncarolo, M. Battaglia and R. A. Flavell (2011). Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity 34 4:554-565.

Hunter, C. A., L. A. Ellis-Neyes, T. Slifer, S. Kanaly, G. Grunig, M. Fort, D. Rennick and F. G. Araujo (1997). IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. J Immunol 158 7:3311-3316.

Hunter, C. A. and R. Kastelein (2012). Interleukin-27: balancing protective and pathological immunity. Immunity 37 6:960-969.

Hutchins, A. P., S. Poulain and D. Miranda-Saavedra (2012). Genome-wide analysis of STAT3 binding in vivo predicts effectors of the anti-inflammatory response in macrophages. Blood 119 13:e110-119.

Inohara, Chamaillard, C. McDonald and G. Nunez (2005). NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu Rev Biochem 74355-383.

Inohara, N., T. Koseki, J. Lin, L. del Peso, P. C. Lucas, F. F. Chen, Y. Ogura and G. Nunez (2000). An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem 275 36:27823-27831.

Introna, M., T. A. Hamilton, R. E. Kaufman, D. O. Adams and R. C. Bast, Jr. (1986). Treatment of murine peritoneal macrophages with bacterial lipopolysaccharide alters expression of c-fos and c-myc oncogenes. J Immunol 137 8:2711-2715.

Isaacs, A. and J. Lindenmann (1957). Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 147 927:258-267.

Ishida, H., T. Muchamuel, S. Sakaguchi, S. Andrade, S. Menon and M. Howard (1994). Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice. J Exp Med 179 1:305-310.

Ivanov, II, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua and D. R. Littman (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126 6:1121-1133.

Iyer, S. S., A. A. Ghaffari and G. Cheng (2010). Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. J Immunol 185 11:6599-6607.

Izaguirre, A., B. J. Barnes, S. Amrute, W. S. Yeow, N. Megjugorac, J. Dai, D. Feng, E. Chung, P. M. Pitha and P. Fitzgerald-Bocarsly (2003). Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. J Leukoc Biol 74 6:1125-1138.

Jacobson, N. G., S. J. Szabo, R. M. Weber-Nordt, Z. Zhong, R. D. Schreiber, J. E. Darnell, Jr. and K. M. Murphy (1995). Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. J Exp Med 181 5:1755-1762.

Janeway, C. A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 54 Pt 11-13.

Jankovic, D., M. C. Kullberg, C. G. Feng, R. S. Goldszmid, C. M. Collazo, M. Wilson, T. A. Wynn, M. Kamanaka, R. A. Flavell and A. Sher (2007). Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. J Exp Med 204 2:273-283.

Jarnicki, A. G., H. Conroy, C. Brereton, G. Donnelly, D. Toomey, K. Walsh, C. Sweeney, O. Leavy, J. Fletcher, E. C. Lavelle, P. Dunne and K. H. Mills (2008). Attenuating regulatory T cell induction by TLR agonists through inhibition of p38 MAPK signaling in dendritic cells enhances their efficacy as vaccine adjuvants and cancer immunotherapeutics. J Immunol 180 6:3797-3806.

Jarrossay, D., G. Napolitani, M. Colonna, F. Sallusto and A. Lanzavecchia (2001). Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 31 11:3388-3393.

Jin, M. S. and J. O. Lee (2008). Structures of the toll-like receptor family and its ligand complexes. Immunity 29 2:182-191.

Jurk, M., F. Heil, J. Vollmer, C. Schetter, A. M. Krieg, H. Wagner, G. Lipford and S. Bauer (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat Immunol 3 6:499.

Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan and Y. J. Liu (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 194 6:863-869.

Kagan, J. C., T. Su, T. Horng, A. Chow, S. Akira and R. Medzhitov (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nat Immunol 9 4:361-368.

Kaiser, F., D. Cook, S. Papoutsopoulou, R. Rajsbaum, X. Wu, H. T. Yang, S. Grant, P. Ricciardi-Castagnoli, P. N. Tsichlis, S. C. Ley and A. O'Garra (2009). TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. J Exp Med 206 9:1863-1871.

Kalin, T. V., V. Ustiyan and V. V. Kalinichenko (2011). Multiple faces of FoxM1 transcription factor: lessons from transgenic mouse models. Cell Cycle 10 3:396-405.

Kalliolias, G. D. and L. B. Ivashkiv (2008). IL-27 activates human monocytes via STAT1 and suppresses IL-10 production but the inflammatory functions of IL-27 are abrogated by TLRs and p38. J Immunol 180 9:6325-6333.

Kanters, E., M. Pasparakis, M. J. Gijbels, M. N. Vergouwe, I. Partouns-Hendriks, R. J. Fijneman, B. E. Clausen, I. Forster, M. M. Kockx, K. Rajewsky, G. Kraal, M. H. Hofker and M. P. de Winther (2003). Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 112 8:1176-1185.

Kaplan, M. H., Y. L. Sun, T. Hoey and M. J. Grusby (1996). Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature 382 6587:174-177.

Karin, M., Z. Liu and E. Zandi (1997). AP-1 function and regulation. Curr Opin Cell Biol 9 2:240-246.

Katholnig, K., C. C. Kaltenecker, H. Hayakawa, M. Rosner, C. Lassnig, G. J. Zlabinger, M. Gaestel, M. Muller, M. Hengstschlager, W. H. Horl, J. M. Park, M. D. Saemann and T. Weichhart (2013). p38alpha Senses Environmental Stress To Control Innate Immune Responses via Mechanistic Target of Rapamycin. J Immunol.

Katsikis, P. D., C. Q. Chu, F. M. Brennan, R. N. Maini and M. Feldmann (1994). Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med 179 5:1517-1527.

Kawai, T. and S. Akira (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11 5:373-384.

Kawai, T. and S. Akira (2011). Regulation of innate immune signalling pathways by the tripartite motif (TRIM) family proteins. EMBO Mol Med 3 9:513-527.

Keane, T. M., L. Goodstadt, P. Danecek, M. A. White, K. Wong, B. Yalcin, A. Heger, A. Agam, G. Slater, M. Goodson, N. A. Furlotte, E. Eskin, C. Nellaker, H. Whitley, J. Cleak, D. Janowitz, P. Hernandez-Pliego, A. Edwards, T. G. Belgard, P. L. Oliver, R. E. McIntyre, A. Bhomra, J. Nicod, X. Gan, W. Yuan, L. van der Weyden, C. A. Steward, S. Bala, J. Stalker, R. Mott, R. Durbin, I. J. Jackson, A. Czechanski, J. A. Guerra-Assuncao, L. R. Donahue, L. G. Reinholdt, B. A. Payseur, C. P. Ponting, E. Birney, J. Flint and D. J. Adams (2011). Mouse genomic variation and its effect on phenotypes and gene regulation. Nature 477 7364:289-294.

Kelada, S. N., M. S. Wilson, U. Tavarez, K. Kubalanza, B. Borate, G. S. Whitehead, S. Maruoka, M. G. Roy, M. Olive, D. E. Carpenter, D. M. Brass, T. A. Wynn, D. N. Cook, C. M. Evans, D. A. Schwartz and F. S. Collins (2011). Strain-dependent genomic factors affect allergen-induced airway hyperresponsiveness in mice. Am J Respir Cell Mol Biol 45 4:817-824.

Khader, S. A., S. Partida-Sanchez, G. Bell, D. M. Jelley-Gibbs, S. Swain, J. E. Pearl, N. Ghilardi, F. J. Desauvage, F. E. Lund and A. M. Cooper (2006). Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. J Exp Med 203 7:1805-1815.

Kim, C., Y. Sano, K. Todorova, B. A. Carlson, L. Arpa, A. Celada, T. Lawrence, K. Otsu, J. L. Brissette, J. S. Arthur and J. M. Park (2008). The kinase p38 alpha serves cell type-specific inflammatory functions in skin injury and coordinates pro- and anti-inflammatory gene expression. Nat Immunol 9 9:1019-1027.

Kim, R., M. Emi and K. Tanabe (2005). Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. Cancer Biol Ther 4 9:924-933.

Kimura, A., T. Naka, T. Nakahama, I. Chinen, K. Masuda, K. Nohara, Y. Fujii-Kuriyama and T. Kishimoto (2009). Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. J Exp Med 206 9:2027-2035.

Klapproth, K., S. Sander, D. Marinkovic, B. Baumann and T. Wirth (2009). The IKK2/NF-{kappa}B pathway suppresses MYC-induced lymphomagenesis. Blood 114 12:2448-2458.

Kobayashi, K., L. D. Hernandez, J. E. Galan, C. A. Janeway, Jr., R. Medzhitov and R. A. Flavell (2002). IRAK-M is a negative regulator of Toll-like receptor signaling. Cell 110 2:191-202.

Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia and G. Trinchieri (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 170 3:827-845.

Koblansky, A. A., D. Jankovic, H. Oh, S. Hieny, W. Sungnak, R. Mathur, M. S. Hayden, S. Akira, A. Sher and S. Ghosh (2013). Recognition of profilin by Toll-like receptor 12 is critical for host resistance to Toxoplasma gondii. Immunity 38 1:119-130.

Kotenko, S. V., C. D. Krause, L. S. Izotova, B. P. Pollack, W. Wu and S. Pestka (1997). Identification and functional characterization of a second chain of the interleukin-10 receptor complex. EMBO J 16 19:5894-5903.

Krausgruber, T., K. Blazek, T. Smallie, S. Alzabin, H. Lockstone, N. Sahgal, T. Hussell, M. Feldmann and I. A. Udalova (2011). IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. Nat Immunol 12 3:231-238.

Kuhn, K., S. C. Baker, E. Chudin, M. H. Lieu, S. Oeser, H. Bennett, P. Rigault, D. Barker, T. K. McDaniel and M. S. Chee (2004). A novel, high-performance random array platform for quantitative gene expression profiling. Genome Res 14 11:2347-2356.

Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky and W. Muller (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell 75 2:263-274.

Kullberg, M. C., J. M. Ward, P. L. Gorelick, P. Caspar, S. Hieny, A. Cheever, D. Jankovic and A. Sher (1998). Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. Infect Immun 66 11:5157-5166.

Kumar, H., T. Kawai and S. Akira (2011). Pathogen recognition by the innate immune system. Int Rev Immunol 30 1:16-34.

Lang, R., D. Patel, J. J. Morris, R. L. Rutschman and P. J. Murray (2002). Shaping gene expression in activated and resting primary macrophages by IL-10. J Immunol 169 5:2253-2263.

Leakey, A. K., G. C. Ulett and R. G. Hirst (1998). BALB/c and C57Bl/6 mice infected with virulent Burkholderia pseudomallei provide contrasting animal models for the acute and chronic forms of human melioidosis. Microbial Pathogenesis 24 5:269-275.

Leber, J. H., G. T. Crimmins, S. Raghavan, N. P. Meyer-Morse, J. S. Cox and D. A. Portnoy (2008). Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen. PLoS Pathog 4 1:e6.

Lee, C. G., H. K. Kwon, A. Sahoo, W. Hwang, J. S. So, J. S. Hwang, C. S. Chae, G. C. Kim, J. E. Kim, H. S. So, E. S. Hwang, R. Grenningloh, I. C. Ho and S. H. Im (2012). Interaction of Ets-1 with HDAC1 represses IL-10 expression in Th1 cells. J Immunol 188 5:2244-2253.

LeibundGut-Landmann, S., O. Gross, M. J. Robinson, F. Osorio, E. C. Slack, S. V. Tsoni, E. Schweighoffer, V. Tybulewicz, G. D. Brown, J. Ruland and C. Reis e Sousa (2007). Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol 8 6:630-638.

Leonard, W. J. and J. J. O'Shea (1998). Jaks and STATs: biological implications. Annu Rev Immunol 16293-322.

Li, C., I. Corraliza and J. Langhorne (1999). A defect in interleukin-10 leads to enhanced malarial disease in Plasmodium chabaudi chabaudi infection in mice. Infect Immun 67 9:4435-4442.

Li, C., L. A. Sanni, F. Omer, E. Riley and J. Langhorne (2003). Pathology of Plasmodium chabaudi chabaudi infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor beta antibodies. Infect Immun 71 9:4850-4856.

Li, Y., A. Sassano, B. Majchrzak, D. K. Deb, D. E. Levy, M. Gaestel, A. R. Nebreda, E. N. Fish and L. C. Platanias (2004). Role of p38alpha Map kinase in Type I interferon signaling. J Biol Chem 279 2:970-979.

Liao, X., N. Sharma, F. Kapadia, G. Zhou, Y. Lu, H. Hong, K. Paruchuri, G. H. Mahabeleshwar, E. Dalmas, N. Venteclef, C. A. Flask, J. Kim, B. W. Doreian, K. Q. Lu, K. H. Kaestner, A. Hamik, K. Clement and M. K. Jain (2011). Kruppel-like factor 4 regulates macrophage polarization. J Clin Invest 121 7:2736-2749.

Limmathurotsakul, D., S. Wongratanacheewin, N. Teerawattanasook, G. Wongsuvan, S. Chaisuksant, P. Chetchotisakd, W. Chaowagul, N. P. Day and S. J. Peacock (2010). Increasing incidence of human melioidosis in Northeast Thailand. Am J Trop Med Hyg 82 6:1113-1117.

Lipoldova, M. and P. Demant (2006). Genetic susceptibility to infectious disease: lessons from mouse models of leishmaniasis. Nat Rev Genet 7 4:294-305.

Liu, B., G. C. Koo, E. H. Yap, K. L. Chua and Y. H. Gan (2002). Model of differential susceptibility to mucosal Burkholderia pseudomallei infection. Infect Immun 70 2:504-511.

Liu, J., S. Cao, L. M. Herman and X. Ma (2003a). Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. J Exp Med 198 8:1265-1276.

Liu, J., X. Guan, T. Tamura, K. Ozato and X. Ma (2004). Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein. J Biol Chem 279 53:55609-55617.

Liu, J., H. Zhang, Y. Liu, K. Wang, Y. Feng, M. Liu and X. Xiao (2007). KLF4 regulates the expression of interleukin-10 in RAW264.7 macrophages. Biochem Biophys Res Commun 362 3:575-581.

Liu, Y., S. H. Wei, A. S. Ho, R. de Waal Malefyt and K. W. Moore (1994). Expression cloning and characterization of a human IL-10 receptor. J Immunol 152 4:1821-1829.

Liu, Y. W., H. P. Tseng, L. C. Chen, B. K. Chen and W. C. Chang (2003b). Functional cooperation of simian virus 40 promoter factor 1 and CCAAT/enhancer-binding protein beta and delta in lipopolysaccharide-induced gene activation of IL-10 in mouse macrophages. J Immunol 171 2:821-828.

Lloyd, C. M. and C. M. Hawrylowicz (2009). Regulatory T cells in asthma. Immunity 31 3:438-449.

Lockhart, D. J. and E. A. Winzeler (2000). Genomics, gene expression and DNA arrays. Nature 405 6788:827-836.

Lu, H. T., D. D. Yang, M. Wysk, E. Gatti, I. Mellman, R. J. Davis and R. A. Flavell (1999). Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. EMBO J 18 7:1845-1857.

Ludlow, L. E., R. W. Johnstone and C. J. Clarke (2005). The HIN-200 family: more than interferon-inducible genes? Exp Cell Res 308 1:1-17.

Lyakh, L., G. Trinchieri, L. Provezza, G. Carra and F. Gerosa (2008). Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. Immunol Rev 226112-131.

Ma, F., X. Liu, D. Li, P. Wang, N. Li, L. Lu and X. Cao (2010). MicroRNA-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing RNA-binding protein tristetraprolin-mediated IL-10 mRNA degradation. J Immunol 184 11:6053-6059.

Ma, W., W. Lim, K. Gee, S. Aucoin, D. Nandan, M. Kozlowski, F. Diaz-Mitoma and A. Kumar (2001). The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. J Biol Chem 276 17:13664-13674.

Ma, X., M. Neurath, G. Gri and G. Trinchieri (1997). Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. J Biol Chem 272 16:10389-10395.

Macatonia, S. E., T. M. Doherty, S. C. Knight and A. O'Garra (1993). Differential effect of IL-10 on dendritic cell-induced T cell proliferation and IFN-gamma production. J Immunol 150 9:3755-3765.

Macatonia, S. E., N. A. Hosken, M. Litton, P. Vieira, C. S. Hsieh, J. A. Culpepper, M. Wysocka, G. Trinchieri, K. M. Murphy and A. O'Garra (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol 154 10:5071-5079.

Mainou-Fowler, T., A. P. MacGowan and R. Postlethwaite (1988). Virulence of Listeria spp.: course of infection in resistant and susceptible mice. J Med Microbiol 27 2:131-140.

Maizels, R. M., J. P. Hewitson and K. A. Smith (2012). Susceptibility and immunity to helminth parasites. Curr Opin Immunol 24 4:459-466.

Manca, C., L. Tsenova, S. Freeman, A. K. Barczak, M. Tovey, P. J. Murray, C. Barry and G. Kaplan (2005). Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. J Interferon Cytokine Res 25 11:694-701.

Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinni, E. Maggi, G. Trinchieri and S. Romagnani (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1

(Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med 177 4:1199-1204.

Manry, J., G. Laval, E. Patin, S. Fornarino, Y. Itan, M. Fumagalli, M. Sironi, M. Tichit, C. Bouchier, J. L. Casanova, L. B. Barreiro and L. Quintana-Murci (2011). Evolutionary genetic dissection of human interferons. J Exp Med 208 13:2747-2759.

Marie, I., J. E. Durbin and D. E. Levy (1998). Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. EMBO J 17 22:6660-6669.

Marmorstein, R. and R. C. Trievel (2009). Histone modifying enzymes: structures, mechanisms, and specificities. Biochim Biophys Acta 1789 1:58-68.

Martin, M., K. Rehani, R. S. Jope and S. M. Michalek (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol 6 8:777-784.

Martin, M., R. E. Schifferle, N. Cuesta, S. N. Vogel, J. Katz and S. M. Michalek (2003). Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. J Immunol 171 2:717-725.

Martinon, F., K. Burns and J. Tschopp (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10 2:417-426.

Mauri, C. and A. Bosma (2012). Immune regulatory function of B cells. Annu Rev Immunol 30221-241.

Mayer-Barber, K. D., B. B. Andrade, D. L. Barber, S. Hieny, C. G. Feng, P. Caspar, S. Oland, S. Gordon and A. Sher (2011). Innate and adaptive interferons suppress IL-1alpha and IL-1beta production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection. Immunity 35 6:1023-1034.

Maynard, C. L., L. E. Harrington, K. M. Janowski, J. R. Oliver, C. L. Zindl, A. Y. Rudensky and C. T. Weaver (2007). Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. Nat Immunol 8 9:931-941.

Maynard, C. L. and C. T. Weaver (2008). Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. Immunol Rev 226219-233.

McNab, F. W., R. Rajsbaum, J. P. Stoye and A. O'Garra (2011). Tripartite-motif proteins and innate immune regulation. Curr Opin Immunol 23 1:46-56.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. Nature 449 7164:819-826.

Medzhitov, R. and T. Horng (2009). Transcriptional control of the inflammatory response. Nat Rev Immunol 9 10:692-703.

Medzhitov, R., P. Preston-Hurlburt and C. A. Janeway, Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388 6640:394-397.

Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh and C. A. Janeway, Jr. (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell 2 2:253-258.

Miao, E. A., D. P. Mao, N. Yudkovsky, R. Bonneau, C. G. Lorang, S. E. Warren, I. A. Leaf and A. Aderem (2010). Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. Proc Natl Acad Sci U S A 107 7:3076-3080.

Miyake, K. (2006). Roles for accessory molecules in microbial recognition by Toll-like receptors. J Endotoxin Res 12 4:195-204.

Mocellin, S., F. M. Marincola and H. A. Young (2005). Interleukin-10 and the immune response against cancer: a counterpoint. J Leukoc Biol 78 5:1043-1051.

Mohammadi, M., R. Redline, J. Nedrud and S. Czinn (1996). Role of the host in pathogenesis of Helicobacter-associated gastritis: H. felis infection of inbred and congenic mouse strains. Infect Immun 64 1:238-245.

Molle, C., M. Goldman and S. Goriely (2010). Critical role of the IFN-stimulated gene factor 3 complex in TLR-mediated IL-27p28 gene expression revealing a two-step activation process. J Immunol 184 4:1784-1792.

Monteiro, J. M., C. Harvey and G. Trinchieri (1998). Role of interleukin-12 in primary influenza virus infection. J Virol 72 6:4825-4831.

Moore, K. W., R. de Waal Malefyt, R. L. Coffman and A. O'Garra (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19683-765.

Moore, K. W., P. Vieira, D. F. Fiorentino, M. L. Trounstine, T. A. Khan and T. R. Mosmann (1990). Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science 248 4960:1230-1234.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin and R. L. Coffman (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136 7:2348-2357.

Mosser, D. M. and J. P. Edwards (2008a). Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8 12:958-969.

Mosser, D. M. and X. Zhang (2008b). Interleukin-10: new perspectives on an old cytokine. Immunol Rev 226205-218.

Murai, M., O. Turovskaya, G. Kim, R. Madan, C. L. Karp, H. Cheroutre and M. Kronenberg (2009). Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 10 11:1178-1184.

Murphy, E. E., G. Terres, S. E. Macatonia, C. S. Hsieh, J. Mattson, L. Lanier, M. Wysocka, G. Trinchieri, K. Murphy and A. O'Garra (1994). B7 and interleukin 12 cooperate for proliferation and interferon gamma production by mouse T helper clones that are unresponsive to B7 costimulation. J Exp Med 180 1:223-231.

Murphy, K. M., W. Ouyang, J. D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian and T. L. Murphy (2000). Signaling and transcription in T helper development. Annu Rev Immunol 18451-494.

Murphy, T. L., M. G. Cleveland, P. Kulesza, J. Magram and K. M. Murphy (1995). Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. Mol Cell Biol 15 10:5258-5267.

Murray, P. J. (2005). The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. Proc Natl Acad Sci U S A 102 24:8686-8691.

Murray, P. J. (2006). Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response. Curr Opin Pharmacol 6 4:379-386.

Muzio, M., J. Ni, P. Feng and V. M. Dixit (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science 278 5343:1612-1615.

Napolitani, G., A. Rinaldi, F. Bertoni, F. Sallusto and A. Lanzavecchia (2005). Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 6 8:769-776.

Negishi, H., Y. Fujita, H. Yanai, S. Sakaguchi, X. Ouyang, M. Shinohara, H. Takayanagi, Y. Ohba, T. Taniguchi and K. Honda (2006). Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. Proc Natl Acad Sci U S A 103 41:15136-15141.

Negishi, H., H. Yanai, A. Nakajima, R. Koshiba, K. Atarashi, A. Matsuda, K. Matsuki, S. Miki, T. Doi, A. Aderem, J. Nishio, S. T. Smale, K. Honda and T. Taniguchi (2012). Cross-interference of RLR and TLR signaling pathways modulates antibacterial T cell responses. Nat Immunol.

Nelissen, I., E. Martens, P. E. Van den Steen, P. Proost, I. Ronsse and G. Opdenakker (2003). Gelatinase B/matrix metalloproteinase-9 cleaves interferon-beta and is a target for immunotherapy. Brain 126 Pt 6:1371-1381.

Ngauy, V., Y. Lemeshev, L. Sadkowski and G. Crawford (2005). Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II. J Clin Microbiol 43 2:970-972.

Nguyen, H. Q., B. Hoffman-Liebermann and D. A. Liebermann (1993). The zinc finger transcription factor Egr-1 is essential for and restricts differentiation along the macrophage lineage. Cell 72 2:197-209.

Niedbala, W., X. Q. Wei, B. Cai, A. J. Hueber, B. P. Leung, I. B. McInnes and F. Y. Liew (2007). IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. Eur J Immunol 37 11:3021-3029.

Nilsson, R., V. B. Bajic, H. Suzuki, D. di Bernardo, J. Bjorkegren, S. Katayama, J. F. Reid, M. J. Sweet, M. Gariboldi, P. Carninci, Y. Hayashizaki, D. A. Hume, J. Tegner and T. Ravasi (2006). Transcriptional network dynamics in macrophage activation. Genomics 88 2:133-142.

Nylen, S. and D. Sacks (2007). Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 28 9:378-384.

O'Connell, R. M., S. K. Saha, S. A. Vaidya, K. W. Bruhn, G. A. Miranda, B. Zarnegar, A. K. Perry, B. O. Nguyen, T. F. Lane, T. Taniguchi, J. F. Miller and G. Cheng (2004). Type I interferon production enhances susceptibility to Listeria monocytogenes infection. J Exp Med 200 4:437-445.

O'Farrell, A. M., Y. Liu, K. W. Moore and A. L. Mui (1998). IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. EMBO J 17 4:1006-1018.

O'Garra, A., F. J. Barrat, A. G. Castro, A. Vicari and C. Hawrylowicz (2008). Strategies for use of IL-10 or its antagonists in human disease. Immunol Rev 223114-131.

O'Garra, A., R. Chang, N. Go, R. Hastings, G. Haughton and M. Howard (1992). Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. Eur J Immunol 22 3:711-717.

O'Garra, A., P. S. Redford, F. W. McNab, C. I. Bloom, R. J. Wilkinson and M. P. Berry (2013). The immune response in tuberculosis. Annu Rev Immunol 31475-527.

O'Garra, A. and P. Vieira (2007). T(H)1 cells control themselves by producing interleukin-10. Nat Rev Immunol 7 6:425-428.

O'Quinn, D. B., M. T. Palmer, Y. K. Lee and C. T. Weaver (2008). Emergence of the Th17 pathway and its role in host defense. Adv Immunol 99115-163.

Oganesyan, G., S. K. Saha, B. Guo, J. Q. He, A. Shahangian, B. Zarnegar, A. Perry and G. Cheng (2006). Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature 439 7073:208-211.

Ogura, Y., N. Inohara, A. Benito, F. F. Chen, S. Yamaoka and G. Nunez (2001). Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. J Biol Chem 276 7:4812-4818.

Ohtani, M., S. Nagai, S. Kondo, S. Mizuno, K. Nakamura, M. Tanabe, T. Takeuchi, S. Matsuda and S. Koyasu (2008). Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells. Blood 112 3:635-643.

Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, F. Zonin, E. Vaisberg, T. Churakova, M. Liu, D. Gorman, J. Wagner, S. Zurawski, Y. Liu, J. S. Abrams, K. W. Moore, D. Rennick, R. de Waal-Malefyt, C. Hannum, J. F. Bazan and R. A. Kastelein (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13 5:715-725.

Orange, J. S., B. Wang, C. Terhorst and C. A. Biron (1995). Requirement for natural killer cell-produced interferon gamma in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. J Exp Med 182 4:1045-1056.

Ouyang, W., S. Rutz, N. K. Crellin, P. A. Valdez and S. G. Hymowitz (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol 2971-109.

Ouyang, X., H. Negishi, R. Takeda, Y. Fujita, T. Taniguchi and K. Honda (2007). Cooperation between MyD88 and TRIF pathways in TLR synergy via IRF5 activation. Biochem Biophys Res Commun 354 4:1045-1051.

Pamer, E. G. (2004). Immune responses to Listeria monocytogenes. Nat Rev Immunol 4 10:812-823.

Pandey, A. K., Y. Yang, Z. Jiang, S. M. Fortune, F. Coulombe, M. A. Behr, K. A. Fitzgerald, C. M. Sassetti and M. A. Kelliher (2009). NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. PLoS Pathog 5 7:e1000500.

Park, A. Y., B. D. Hondowicz and P. Scott (2000). IL-12 is required to maintain a Th1 response during Leishmania major infection. J Immunol 165 2:896-902.

Pascual, V., D. Chaussabel and J. Banchereau (2010). A genomic approach to human autoimmune diseases. Annu Rev Immunol 28535-571.

Pattison, M. J., K. F. Mackenzie and J. S. Arthur (2012). Inhibition of JAKs in Macrophages Increases Lipopolysaccharide-Induced Cytokine Production by Blocking IL-10-Mediated Feedback. J Immunol 189 6:2784-2792.

Perna, D., G. Faga, A. Verrecchia, M. M. Gorski, I. Barozzi, V. Narang, J. Khng, K. C. Lim, W. K. Sung, R. Sanges, E. Stupka, T. Oskarsson, A. Trumpp, C. L. Wei, H. Muller and B. Amati (2012). Genome-wide mapping of Myc binding and gene regulation in serum-stimulated fibroblasts. Oncogene 31 13:1695-1709.

Persson, S., A. Mikulowska, S. Narula, A. O'Garra and R. Holmdahl (1996). Interleukin-10 suppresses the development of collagen type II-induced arthritis and ameliorates sustained arthritis in rats. Scand J Immunol 44 6:607-614.

Pflanz, S., J. C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W. M. Blumenschein, J. D. Mattson, J. L. Wagner, W. To, S. Zurawski, T. K. McClanahan, D. M. Gorman, J. F. Bazan, R. de Waal Malefyt, D. Rennick and R. A. Kastelein (2002). IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity 16 6:779-790.

Phillips, J. E. and V. G. Corces (2009). CTCF: master weaver of the genome. Cell 137 7:1194-1211.

Pichlmair, A. and C. Reis e Sousa (2007). Innate recognition of viruses. Immunity 27 3:370-383.

Pitt, J. M., E. Stavropoulos, P. S. Redford, A. M. Beebe, G. J. Bancroft, D. B. Young and A. O'Garra (2012). Blockade of IL-10 Signaling during Bacillus Calmette-Guerin Vaccination Enhances and Sustains Th1, Th17, and Innate Lymphoid IFN-gamma and IL-17 Responses and Increases Protection to Mycobacterium tuberculosis Infection. J Immunol 189 8:4079-4087.

Platanias, L. C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 5 5:375-386.

Plevy, S. E., J. H. Gemberling, S. Hsu, A. J. Dorner and S. T. Smale (1997). Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. Mol Cell Biol 17 8:4572-4588.

Powell, M. J., S. A. Thompson, Y. Tone, H. Waldmann and M. Tone (2000). Posttranscriptional regulation of IL-10 gene expression through sequences in the 3'-untranslated region. J Immunol 165 1:292-296.

Presky, D. H., H. Yang, L. J. Minetti, A. O. Chua, N. Nabavi, C. Y. Wu, M. K. Gately and U. Gubler (1996). A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. Proc Natl Acad Sci U S A 93 24:14002-14007.

Quackenbush, J. (2002). Microarray data normalization and transformation. Nat Genet 32 Suppl496-501.

Raabe, T., M. Bukrinsky and R. A. Currie (1998). Relative contribution of transcription and translation to the induction of tumor necrosis factor-alpha by lipopolysaccharide. J Biol Chem 273 2:974-980.

Ramirez-Carrozzi, V. R., D. Braas, D. M. Bhatt, C. S. Cheng, C. Hong, K. R. Doty, J. C. Black, A. Hoffmann, M. Carey and S. T. Smale (2009). A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell 138 1:114-128.

Ramsey, S. A., S. L. Klemm, D. E. Zak, K. A. Kennedy, V. Thorsson, B. Li, M. Gilchrist, E. S. Gold, C. D. Johnson, V. Litvak, G. Navarro, J. C. Roach, C. M. Rosenberger, A. G. Rust, N. Yudkovsky, A. Aderem and I. Shmulevich (2008). Uncovering a macrophage transcriptional program by integrating evidence from motif scanning and expression dynamics. PLoS Comput Biol 4 3:e1000021.

Rayamajhi, M., J. Humann, K. Penheiter, K. Andreasen and L. L. Lenz (2010). Induction of IFN-alphabeta enables Listeria monocytogenes to suppress macrophage activation by IFN-gamma. J Exp Med 207 2:327-337.

Re, F. and J. L. Strominger (2001). Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. J Biol Chem 276 40:37692-37699.

Redford, P. S., A. Boonstra, S. Read, J. Pitt, C. Graham, E. Stavropoulos, G. J. Bancroft and A. O'Garra (2010). Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. Eur J Immunol 40 8:2200-2210.

Redford, P. S., P. J. Murray and A. O'Garra (2011). The role of IL-10 in immune regulation during M. tuberculosis infection. Mucosal Immunol 4 3:261-270.

Reid, D. M., N. A. Gow and G. D. Brown (2009). Pattern recognition: recent insights from Dectin-1. Curr Opin Immunol 21 1:30-37.

Rizza, P., F. Moretti and F. Belardelli (2010). Recent advances on the immunomodulatory effects of IFN-alpha: implications for cancer immunotherapy and autoimmunity. Autoimmunity 43 3:204-209.

Roers, A., L. Siewe, E. Strittmatter, M. Deckert, D. Schluter, W. Stenzel, A. D. Gruber, T. Krieg, K. Rajewsky and W. Muller (2004). T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. J Exp Med 200 10:1289-1297.

Rogers, N. C., E. C. Slack, A. D. Edwards, M. A. Nolte, O. Schulz, E. Schweighoffer, D. L. Williams, S. Gordon, V. L. Tybulewicz, G. D. Brown and C. Reis e Sousa (2005). Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity 22 4:507-517.

Roque, S., C. Nobrega, R. Appelberg and M. Correia-Neves (2007). IL-10 underlies distinct susceptibility of BALB/c and C57BL/6 mice to Mycobacterium avium infection and influences efficacy of antibiotic therapy. J Immunol 178 12:8028-8035.

Rossato, M., G. Curtale, N. Tamassia, M. Castellucci, L. Mori, S. Gasperini, B. Mariotti, M. De Luca, M. Mirolo, M. A. Cassatella, M. Locati and F. Bazzoni (2012). IL-10-induced microRNA-187 negatively regulates TNF-alpha, IL-6, and IL-12p40 production in TLR4-stimulated monocytes. Proc Natl Acad Sci U S A 109 45:E3101-3110.

Rothfuchs, A. G., A. Bafica, C. G. Feng, J. G. Egen, D. L. Williams, G. D. Brown and A. Sher (2007). Dectin-1 interaction with Mycobacterium tuberculosis leads to enhanced IL-12p40 production by splenic dendritic cells. J Immunol 179 6:3463-3471.

Rousseau, S., M. Papoutsopoulou, A. Symons, D. Cook, J. M. Lucocq, A. R. Prescott, A. O'Garra, S. C. Ley and P. Cohen (2008). TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. J Cell Sci 121 Pt 2:149-154.

Roussel, M. F. (1997). Regulation of cell cycle entry and G1 progression by CSF-1. Mol Reprod Dev 46 1:11-18.

Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore and J. Banchereau (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc Natl Acad Sci U S A 89 5:1890-1893.

Rudensky, A. Y. (2011). Regulatory T cells and Foxp3. Immunol Rev 241 1:260-268.

Rudick, R. A., R. M. Ransohoff, R. Peppler, S. VanderBrug Medendorp, P. Lehmann and J. Alam (1996). Interferon beta induces interleukin-10 expression: relevance to multiple sclerosis. Ann Neurol 40 4:618-627.

Sabbah, A., T. H. Chang, R. Harnack, V. Frohlich, K. Tominaga, P. H. Dube, Y. Xiang and S. Bose (2009). Activation of innate immune antiviral responses by Nod2. Nat Immunol 10 10:1073-1080.

Sacks, D. and N. Noben-Trauth (2002). The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol 2 11:845-858.

Sadler, A. J. and B. R. Williams (2008). Interferon-inducible antiviral effectors. Nat Rev Immunol 8 7:559-568.

Sakamuro, D. and G. C. Prendergast (1999). New Myc-interacting proteins: a second Myc network emerges. Oncogene 18 19:2942-2954.

Samoilova, E. B., J. L. Horton and Y. Chen (1998). Acceleration of experimental autoimmune encephalomyelitis in interleukin-10-deficient mice: roles of interleukin-10 in disease progression and recovery. Cell Immunol 188 2:118-124.

Samuel, M. and T. Y. Ti (2001). Interventions for treating melioidosis. Cochrane Database Syst Rev 2:CD001263.

Sandelin, A., W. Alkema, P. Engstrom, W. W. Wasserman and B. Lenhard (2004). JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic Acids Res 32 Database issue:D91-94.

Sanjabi, S., A. Hoffmann, H. C. Liou, D. Baltimore and S. T. Smale (2000). Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. Proc Natl Acad Sci U S A 97 23:12705-12710.

Saraiva, M., J. R. Christensen, A. V. Tsytsykova, A. E. Goldfeld, S. C. Ley, D. Kioussis and A. O'Garra (2005). Identification of a macrophage-specific chromatin signature in the IL-10 locus. J Immunol 175 2:1041-1046.

Saraiva, M., J. R. Christensen, M. Veldhoen, T. L. Murphy, K. M. Murphy and A. O'Garra (2009). Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. Immunity 31 2:209-219.

Saraiva, M. and A. O'Garra (2010). The regulation of IL-10 production by immune cells. Nat Rev Immunol 10 3:170-181.

Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda and S. Akira (2003). Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol 171 8:4304-4310.

Satoh, T., H. Kato, Y. Kumagai, M. Yoneyama, S. Sato, K. Matsushita, T. Tsujimura, T. Fujita, S. Akira and O. Takeuchi (2010). LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. Proc Natl Acad Sci U S A 107 4:1512-1517.

Schluter, D., M. Deckert-Schluter, E. Lorenz, T. Meyer, M. Rollinghoff and C. Bogdan (1999). Inhibition of inducible nitric oxide synthase exacerbates chronic cerebral toxoplasmosis in Toxoplasma gondii-susceptible C57BL/6 mice but does not reactivate the latent disease in T. gondii-resistant BALB/c mice. J Immunol 162 6:3512-3518.

Schmitz, F., A. Heit, S. Dreher, K. Eisenacher, J. Mages, T. Haas, A. Krug, K. P. Janssen, C. J. Kirschning and H. Wagner (2008). Mammalian target of rapamycin (mTOR) orchestrates the defense program of innate immune cells. Eur J Immunol 38 11:2981-2992.

Schmitz, F., A. Heit, S. Guggemoos, A. Krug, J. Mages, M. Schiemann, H. Adler, I. Drexler, T. Haas, R. Lang and H. Wagner (2007). Interferon-regulatory-factor 1 controls Toll-like receptor 9-mediated IFN-beta production in myeloid dendritic cells. Eur J Immunol 37 2:315-327.

Schoggins, J. W., S. J. Wilson, M. Panis, M. Y. Murphy, C. T. Jones, P. Bieniasz and C. M. Rice (2011). A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 472 7344:481-485.

Schroder, K. and J. Tschopp (2010). The inflammasomes. Cell 140 6:821-832.

Schroeder, A., O. Mueller, S. Stocker, R. Salowsky, M. Leiber, M. Gassmann, S. Lightfoot, W. Menzel, M. Granzow and T. Ragg (2006). The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 73.

Schulz, C., E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S. E. Jacobsen, J. W. Pollard, J. Frampton, K. J. Liu and F. Geissmann (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science 336 6077:86-90.

Schulz, O., A. D. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher and C. Reis e Sousa (2000). CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. Immunity 13 4:453-462.

Seki, E., H. Tsutsui, N. M. Tsuji, N. Hayashi, K. Adachi, H. Nakano, S. Futatsugi-Yumikura, O. Takeuchi, K. Hoshino, S. Akira, J. Fujimoto and K. Nakanishi (2002). Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice. J Immunol 169 7:3863-3868.

Sellon, R. K., S. Tonkonogy, M. Schultz, L. A. Dieleman, W. Grenther, E. Balish, D. M. Rennick and R. B. Sartor (1998). Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun 66 11:5224-5231.

Seok, J., H. S. Warren, A. G. Cuenca, M. N. Mindrinos, H. V. Baker, W. Xu, D. R. Richards, G. P. McDonald-Smith, H. Gao, L. Hennessy, C. C. Finnerty, C. M. Lopez, S. Honari, E. E. Moore, J. P. Minei, J. Cuschieri, P. E. Bankey, J. L. Johnson, J. Sperry, A. B. Nathens, T. R. Billiar, M. A. West, M. G. Jeschke, M. B. Klein, R. L. Gamelli, N. S. Gibran, B. H. Brownstein, C. Miller-Graziano, S. E. Calvano, P. H. Mason, J. P. Cobb, L. G. Rahme, S. F. Lowry, R. V. Maier, L. L. Moldawer, D. N. Herndon, R. W. Davis, W. Xiao and R. G. Tompkins (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A 110 9:3507-3512.

Seong, E., T. L. Saunders, C. L. Stewart and M. Burmeister (2004). To knockout in 129 or in C57BL/6: that is the question. Trends Genet 20 2:59-62.

Serbina, N. V., T. Jia, T. M. Hohl and E. G. Pamer (2008). Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol 26421-452.

Seth, R. B., L. Sun, C. K. Ea and Z. J. Chen (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell 122 5:669-682.

Sharma, A., M. Kumar, J. Aich, M. Hariharan, S. K. Brahmachari, A. Agrawal and B. Ghosh (2009). Posttranscriptional regulation of interleukin-10 expression by hsa-miR-106a. Proc Natl Acad Sci U S A 106 14:5761-5766.

Sheedy, F. J., E. Palsson-McDermott, E. J. Hennessy, C. Martin, J. J. O'Leary, Q. Ruan, D. S. Johnson, Y. Chen and L. A. O'Neill (2010). Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. Nat Immunol 11 2:141-147.

Sher, A. and R. L. Coffman (1992). Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu Rev Immunol 10385-409.

Shoemaker, J., M. Saraiva and A. O'Garra (2006). GATA-3 directly remodels the IL-10 locus independently of IL-4 in CD4+ T cells. J Immunol 176 6:3470-3479.

Shuai, K., C. Schindler, V. R. Prezioso and J. E. Darnell, Jr. (1992). Activation of transcription by IFN-gamma: tyrosine phosphorylation of a 91-kD DNA binding protein. Science 258 5089:1808-1812.

Slack, E. C., M. J. Robinson, P. Hernanz-Falcon, G. D. Brown, D. L. Williams, E. Schweighoffer, V. L. Tybulewicz and C. Reis e Sousa (2007). Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan. Eur J Immunol 37 6:1600-1612.

Slobedman, B., P. A. Barry, J. V. Spencer, S. Avdic and A. Abendroth (2009). Virus-encoded homologs of cellular interleukin-10 and their control of host immune function. J Virol 83 19:9618-9629.

Smale, S. T. (2012). Transcriptional regulation in the innate immune system. Curr Opin Immunol 24 1:51-57.

Smallie, T., G. Ricchetti, N. J. Horwood, M. Feldmann, A. R. Clark and L. M. Williams (2010). IL-10 inhibits transcription elongation of the human TNF gene in primary macrophages. J Exp Med 207 10:2081-2088.

Smith, A. M., J. E. Qualls, K. O'Brien, L. Balouzian, P. F. Johnson, S. Schultz-Cherry, S. T. Smale and P. J. Murray (2011). A distal enhancer in Il12b is the target of transcriptional repression by the STAT3 pathway and requires the basic leucine zipper (B-ZIP) protein NFIL3. J Biol Chem 286 26:23582-23590.

Snijders, A., C. M. Hilkens, T. C. van der Pouw Kraan, M. Engel, L. A. Aarden and M. L. Kapsenberg (1996). Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. J Immunol 156 3:1207-1212.

Sospedra, M. and R. Martin (2005). Immunology of multiple sclerosis. Annu Rev Immunol 23683-747.

Staples, K. J., T. Smallie, L. M. Williams, A. Foey, B. Burke, B. M. Foxwell and L. Ziegler-Heitbrock (2007). IL-10 induces IL-10 in primary human monocyte-derived macrophages via the transcription factor Stat3. J Immunol 178 8:4779-4785.

Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman and R. D. Schreiber (1998). How cells respond to interferons. Annu Rev Biochem 67227-264.

Steinman, R. M. (2007). Dendritic cells: understanding immunogenicity. Eur J Immunol 37 Suppl 1S53-60.

Steinman, R. M. and Z. A. Cohn (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 137 5:1142-1162.

Steinman, R. M. and Z. A. Cohn (1974a). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J Exp Med 139 2:380-397.

Steinman, R. M., D. S. Lustig and Z. A. Cohn (1974b). Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo. J Exp Med 139 6:1431-1445.

Stoecklin, G., S. A. Tenenbaum, T. Mayo, S. V. Chittur, A. D. George, T. E. Baroni, P. J. Blackshear and P. Anderson (2008). Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. J Biol Chem 283 17:11689-11699.

Stumhofer, J. S., J. S. Silver, A. Laurence, P. M. Porrett, T. H. Harris, L. A. Turka, M. Ernst, C. J. Saris, J. J. O'Shea and C. A. Hunter (2007). Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. Nat Immunol 8 12:1363-1371.

Suzuki, Y., A. Sher, G. Yap, D. Park, L. E. Neyer, O. Liesenfeld, M. Fort, H. Kang and E. Gufwoli (2000). IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with Toxoplasma gondii. J Immunol 164 10:5375-5382.

Symons, A., S. Beinke and S. C. Ley (2006). MAP kinase kinase kinases and innate immunity. Trends Immunol 27 1:40-48.

Sypek, J. P., C. L. Chung, S. E. Mayor, J. M. Subramanyam, S. J. Goldman, D. S. Sieburth, S. F. Wolf and R. G. Schaub (1993). Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J Exp Med 177 6:1797-1802.

Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman and L. H. Glimcher (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100 6:655-669.

Taira, T., J. Maeda, T. Onishi, H. Kitaura, S. Yoshida, H. Kato, M. Ikeda, K. Tamai, S. M. Iguchi-Ariga and H. Ariga (1998). AMY-1, a novel C-MYC binding protein that stimulates transcription activity of C-MYC. Genes Cells 3 8:549-565.

Takaoka, A., H. Yanai, S. Kondo, G. Duncan, H. Negishi, T. Mizutani, S. Kano, K. Honda, Y. Ohba, T. W. Mak and T. Taniguchi (2005). Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 434 7030:243-249.

Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster and S. Akira (1999). Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity 10 1:39-49.

Takeuchi, O. and S. Akira (2010). Pattern recognition receptors and inflammation. Cell 140 6:805-820.

Taki, S., T. Sato, K. Ogasawara, T. Fukuda, M. Sato, S. Hida, G. Suzuki, M. Mitsuyama, E. H. Shin, S. Kojima, T. Taniguchi and Y. Asano (1997). Multistage regulation of Th1-type immune responses by the transcription factor IRF-1. Immunity 6 6:673-679.

Tan, G. Y., Y. Liu, S. P. Sivalingam, S. H. Sim, D. Wang, J. C. Paucod, Y. Gauthier and E. E. Ooi (2008). Burkholderia pseudomallei aerosol infection results in differential inflammatory responses in BALB/c and C57Bl/6 mice. J Med Microbiol 57 Pt 4:508-515.

Tannahill, G. M., A. M. Curtis, J. Adamik, E. M. Palsson-McDermott, A. F. McGettrick, G. Goel, C. Frezza, N. J. Bernard, B. Kelly, N. H. Foley, L. Zheng, A. Gardet, Z. Tong, S. S. Jany, S. C. Corr, M. Haneklaus, B. E. Caffrey, K. Pierce, S. Walmsley, F. C. Beasley, E. Cummins, V. Nizet, M. Whyte, C. T. Taylor, H. Lin, S. L. Masters, E. Gottlieb, V. P. Kelly, C. Clish, P. E. Auron, R. J. Xavier and L. A. O'Neill (2013). Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. Nature 496 7444:238-242.

Teles, R. M., T. G. Graeber, S. R. Krutzik, D. Montoya, M. Schenk, D. J. Lee, E. Komisopoulou, K. Kelly-Scumpia, R. Chun, S. S. Iyer, E. N. Sarno, T. H. Rea, M. Hewison, J. S. Adams, S. J. Popper, D. A. Relman, S. Stenger, B. R. Bloom, G. Cheng and R. L. Modlin (2013). Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. Science 339 6126:1448-1453.

Thanos, D. and T. Maniatis (1995). Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell 83 7:1091-1100.

Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld and J. N. Ihle (1996). Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature 382 6587:171-174.

Thomas, C., I. Moraga, D. Levin, P. O. Krutzik, Y. Podoplelova, A. Trejo, C. Lee, G. Yarden, S. E. Vleck, J. S. Glenn, G. P. Nolan, J. Piehler, G. Schreiber and K. C. Garcia (2011). Structural linkage between ligand discrimination and receptor activation by type I interferons. Cell 146 4:621-632.

Thomas, K. E., C. L. Galligan, R. D. Newman, E. N. Fish and S. N. Vogel (2006). Contribution of interferon-beta to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. J Biol Chem 281 41:31119-31130.

Titball, R. W., P. Russell, J. Cuccui, A. Easton, A. Haque, T. Atkins, M. Sarkar-Tyson, V. Harley, B. Wren and G. J. Bancroft (2008). Burkholderia pseudomallei: animal models of infection. Trans R Soc Trop Med Hyg 102 Suppl 1S111-116.

Tone, M., M. J. Powell, Y. Tone, S. A. Thompson and H. Waldmann (2000). IL-10 gene expression is controlled by the transcription factors Sp1 and Sp3. J Immunol 165 1:286-291.

Torres, D., M. Barrier, F. Bihl, V. J. Quesniaux, I. Maillet, S. Akira, B. Ryffel and F. Erard (2004). Toll-like receptor 2 is required for optimal control of Listeria monocytogenes infection. Infect Immun 72 4:2131-2139.

Toshchakov, V., B. W. Jones, P. Y. Perera, K. Thomas, M. J. Cody, S. Zhang, B. R. Williams, J. Major, T. A. Hamilton, M. J. Fenton and S. N. Vogel (2002). TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol 3 4:392-398.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3 2:133-146.

Trinchieri, G. (2010). Type I interferon: friend or foe? J Exp Med 207 10:2053-2063.

Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling and E. R. Unanue (1994). Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-gamma. J Immunol 152 4:1883-1887.

Tripp, C. S., S. F. Wolf and E. R. Unanue (1993). Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proc Natl Acad Sci U S A 90 8:3725-3729.

Tsai, E. Y., J. V. Falvo, A. V. Tsytsykova, A. K. Barczak, A. M. Reimold, L. H. Glimcher, M. J. Fenton, D. C. Gordon, I. F. Dunn and A. E. Goldfeld (2000). A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. Mol Cell Biol 20 16:6084-6094.

Tseng, P. H., A. Matsuzawa, W. Zhang, T. Mino, D. A. Vignali and M. Karin (2010). Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. Nat Immunol 11 1:70-75.

Tsukamoto, H., K. Fukudome, S. Takao, N. Tsuneyoshi, S. Ohta, Y. Nagai, H. Ihara, K. Miyake, Y. Ikeda and M. Kimoto (2013). Reduced Surface Expression of TLR4 by a V254I Point Mutation Accounts for the Low Lipopolysaccharide Responder Phenotype of BALB/c B Cells. J Immunol 190 1:195-204.

Tudor, C., F. P. Marchese, E. Hitti, A. Aubareda, L. Rawlinson, M. Gaestel, P. J. Blackshear, A. R. Clark, J. Saklatvala and J. L. Dean (2009). The p38 MAPK pathway inhibits tristetraprolin-directed decay of interleukin-10 and pro-inflammatory mediator mRNAs in murine macrophages. FEBS Lett 583 12:1933-1938.

Uddin, S., B. Majchrzak, J. Woodson, P. Arunkumar, Y. Alsayed, R. Pine, P. R. Young, E. N. Fish and L. C. Platanias (1999). Activation of the p38 mitogen-activated protein kinase by type I interferons. J Biol Chem 274 42:30127-30131.

Uddin, S., L. Yenush, X. J. Sun, M. E. Sweet, M. F. White and L. C. Platanias (1995). Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3'-kinase. J Biol Chem 270 27:15938-15941.

Ulett, G. C., N. Ketheesan and R. G. Hirst (2000a). Cytokine gene expression in innately susceptible BALB/c mice and relatively resistant C57BL/6 mice during infection with virulent Burkholderia pseudomallei. Infect Immun 68 4:2034-2042.

Ulett, G. C., N. Ketheesan and R. G. Hirst (2000b). Proinflammatory cytokine mRNA responses in experimental Burkholderia pseudomallei infection in mice. Acta Trop 74 2-3:229-234.

van Boxel-Dezaire, A. H., M. R. Rani and G. R. Stark (2006). Complex modulation of cell type-specific signaling in response to type I interferons. Immunity 25 3:361-372.

van Furth, R. and Z. A. Cohn (1968). The origin and kinetics of mononuclear phagocytes. J Exp Med 128 3:415-435.

Van Lint, P. and C. Libert (2007). Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. J Leukoc Biol 82 6:1375-1381.

Varesio, L., E. Blasi, G. B. Thurman, J. E. Talmadge, R. H. Wiltrout and R. B. Herberman (1984). Potent activation of mouse macrophages by recombinant interferon-gamma. Cancer Res 44 10:4465-4469.

Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley and B. Stockinger (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24 2:179-189.

Vestal, D. J. (2005). The guanylate-binding proteins (GBPs): proinflammatory cytokine-induced members of the dynamin superfamily with unique GTPase activity. J Interferon Cytokine Res 25 8:435-443.

Vieira, P., R. de Waal-Malefyt, M. N. Dang, K. E. Johnson, R. Kastelein, D. F. Fiorentino, J. E. deVries, M. G. Roncarolo, T. R. Mosmann and K. W. Moore (1991). Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. Proc Natl Acad Sci U S A 88 4:1172-1176.

Vignali, D. A. and V. K. Kuchroo (2012). IL-12 family cytokines: immunological playmakers. Nat Immunol 13 8:722-728.

Villagra, A., F. Cheng, H. W. Wang, I. Suarez, M. Glozak, M. Maurin, D. Nguyen, K. L. Wright, P. W. Atadja, K. Bhalla, J. Pinilla-Ibarz, E. Seto and E. M. Sotomayor (2009). The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. Nat Immunol 10 1:92-100.

von Stebut, E., Y. Belkaid, B. V. Nguyen, M. Cushing, D. L. Sacks and M. C. Udey (2000). Leishmania major-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis. Eur J Immunol 30 12:3498-3506.

Wakeham, J., J. Wang and Z. Xing (2000). Genetically determined disparate innate and adaptive cell-mediated immune responses to pulmonary Mycobacterium bovis BCG infection in C57BL/6 and BALB/c mice. Infect Immun 68 12:6946-6953.

Wang, H., J. Brown, C. A. Garcia, Y. Tang, M. R. Benakanakere, T. Greenway, P. Alard, D. F. Kinane and M. Martin (2010). The Role of Glycogen Synthase Kinase 3 in Regulating IFN-{beta}-Mediated IL-10 Production. J Immunol 186 2:675-684.

Wang, I. M., C. Contursi, A. Masumi, X. Ma, G. Trinchieri and K. Ozato (2000). An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. J Immunol 165 1:271-279.

Wang, Z. Y., H. Sato, S. Kusam, S. Sehra, L. M. Toney and A. L. Dent (2005). Regulation of IL-10 gene expression in Th2 cells by Jun proteins. J Immunol 174 4:2098-2105.

Ware, C. B., L. A. Siverts, A. M. Nelson, J. F. Morton and W. C. Ladiges (2003). Utility of a C57BL/6 ES line versus 129 ES lines for targeted mutations in mice. Transgenic Res 12 6:743-746.

Watanabe, T., N. Asano, S. Fichtner-Feigl, P. L. Gorelick, Y. Tsuji, Y. Matsumoto, T. Chiba, I. J. Fuss, A. Kitani and W. Strober (2010). NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. J Clin Invest 120 5:1645-1662.

Waterfield, M. R., M. Zhang, L. P. Norman and S. C. Sun (2003). NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. Mol Cell 11 3:685-694.

Waterston, R. H., K. Lindblad-Toh, E. Birney, J. Rogers, J. F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S. E. Antonarakis, J. Attwood, R. Baertsch, J. Bailey, K. Barlow, S. Beck, E. Berry, B. Birren, T. Bloom, P. Bork, M. Botcherby, N. Bray, M. R. Brent, D. G. Brown, S. D. Brown, C. Bult, J. Burton, J. Butler, R. D. Campbell, P. Carninci, S. Cawley, F. Chiaromonte, A. T. Chinwalla, D. M. Church, M. Clamp, C. Clee, F. S. Collins, L. L. Cook, R. R. Copley, A. Coulson, O. Couronne, J. Cuff, V. Curwen, T. Cutts, M. Daly, R. David, J. Davies, K. D. Delehaunty, J. Deri, E. T. Dermitzakis, C. Dewey, N. J. Dickens, M. Diekhans, S. Dodge, I. Dubchak, D. M. Dunn, S. R. Eddy, L. Elnitski, R. D. Emes, P. Eswara, E. Eyras, A. Felsenfeld, G. A. Fewell, P. Flicek, K. Foley, W. N. Frankel, L. A. Fulton, R. S. Fulton, T. S. Furey, D. Gage, R. A. Gibbs, G. Glusman, S. Gnerre, N. Goldman, L. Goodstadt, D. Grafham, T. A. Graves, E. D. Green, S. Gregory, R. Guigo, M. Guyer, R. C. Hardison, D. Haussler, Y. Hayashizaki, L. W. Hillier, A. Hinrichs, W. Hlavina, T. Holzer, F. Hsu, A. Hua, T. Hubbard, A. Hunt, I. Jackson, D. B. Jaffe, L. S. Johnson, M. Jones, T. A. Jones, A. Joy, M. Kamal, E. K. Karlsson, D. Karolchik, A. Kasprzyk, J. Kawai, E. Keibler, C. Kells, W. J. Kent, A. Kirby, D. L. Kolbe, I. Korf, R. S. Kucherlapati, E. J. Kulbokas, D. Kulp, T. Landers, J. P. Leger, S. Leonard, I. Letunic, R. Levine, J. Li, M. Li, C. Lloyd, S. Lucas, B. Ma, D. R. Maglott, E. R. Mardis, L. Matthews, E. Mauceli, J. H. Mayer, M. McCarthy, W. R. McCombie, S. McLaren, K. McLay, J. D. McPherson, J. Meldrim, B. Meredith, J. P. Mesirov, W. Miller, T. L. Miner, E. Mongin, K. T. Montgomery, M. Morgan, R. Mott, J. C. Mullikin, D. M. Muzny, W. E. Nash, J. O. Nelson, M. N. Nhan, R. Nicol, Z. Ning, C. Nusbaum, M. J. O'Connor, Y. Okazaki, K. Oliver, E. Overton-Larty, L. Pachter, G. Parra, K. H. Pepin, J. Peterson, P. Pevzner, R. Plumb, C. S. Pohl, A. Poliakov, T. C. Ponce, C. P. Ponting, S. Potter, M. Quail, A. Reymond, B. A. Roe, K. M. Roskin, E. M. Rubin, A. G. Rust, R. Santos, V. Sapojnikov, B. Schultz, J. Schultz, M. S. Schwartz, S. Schwartz, C. Scott, S. Seaman, S. Searle, T. Sharpe, A. Sheridan, R. Shownkeen, S. Sims, J. B. Singer, G. Slater, A. Smit, D. R. Smith, B. Spencer, A. Stabenau, N. Stange-Thomann, C. Sugnet, M. Suyama, G. Tesler, J. Thompson, D. Torrents, E. Trevaskis, J. Tromp, C. Ucla, A. Ureta-Vidal, J. P. Vinson, A. C. Von Niederhausern, C. M. Wade, M. Wall, R. J. Weber, R. B. Weiss, M. C. Wendl, A. P. West, K. Wetterstrand, R. Wheeler, S. Whelan, J. Wierzbowski, D. Willey, S. Williams, R. K. Wilson, E. Winter, K. C. Worley, D. Wyman, S. Yang, S. P. Yang, E. M. Zdobnov, M. C. Zody and E. S. Lander (2002). Initial sequencing and comparative analysis of the mouse genome. Nature 420 6915:520-562.

Weichhart, T., G. Costantino, M. Poglitsch, M. Rosner, M. Zeyda, K. M. Stuhlmeier, T. Kolbe, T. M. Stulnig, W. H. Horl, M. Hengstschlager, M. Muller and M. D. Saemann (2008). The TSC-mTOR signaling pathway regulates the innate inflammatory response. Immunity 29 4:565-577.

Weichhart, T. and M. D. Saemann (2009). The multiple facets of mTOR in immunity. Trends Immunol 30 5:218-226.

Wells, C. A., T. Ravasi, G. J. Faulkner, P. Carninci, Y. Okazaki, Y. Hayashizaki, M. Sweet, B. J. Wainwright and D. A. Hume (2003). Genetic control of the innate immune response. BMC Immunol 45.

Wessells, J., M. Baer, H. A. Young, E. Claudio, K. Brown, U. Siebenlist and P. F. Johnson (2004). BCL-3 and NF-kappaB p50 attenuate lipopolysaccharide-induced inflammatory responses in macrophages. J Biol Chem 279 48:49995-50003.

West, T. E., N. Chantratita, W. Chierakul, D. Limmathurotsakul, V. Wuthiekanun, N. D. Myers, M. J. Emond, M. M. Wurfel, T. R. Hawn, S. J. Peacock and S. J. Skerrett (2013). Impaired TLR5 Functionality Is Associated with Survival in Melioidosis. J Immunol.

West, T. E., W. Chierakul, N. Chantratita, D. Limmathurotsakul, V. Wuthiekanun, M. J. Emond, T. R. Hawn, S. J. Peacock and S. J. Skerrett (2012). Toll-like receptor 4 region genetic variants are associated with susceptibility to melioidosis. Genes Immun 13 1:38-46.

West, T. E., R. K. Ernst, M. J. Jansson-Hutson and S. J. Skerrett (2008). Activation of Toll-like receptors by Burkholderia pseudomallei. BMC Immunol 946.

Wiersinga, W. J., B. J. Currie and S. J. Peacock (2012). Melioidosis. N Engl J Med 367 11:1035-1044.

Wiersinga, W. J., T. van der Poll, N. J. White, N. P. Day and S. J. Peacock (2006). Melioidosis: insights into the pathogenicity of Burkholderia pseudomallei. Nat Rev Microbiol 4 4:272-282.

Wiersinga, W. J., C. W. Wieland, M. C. Dessing, N. Chantratita, A. C. Cheng, D. Limmathurotsakul, W. Chierakul, M. Leendertse, S. Florquin, A. F. de Vos, N. White, A. M. Dondorp, N. P. Day, S. J. Peacock and T. van der Poll (2007). Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by Burkholderia pseudomallei (Melioidosis). PLoS Med 4 7:e248.

Wiersinga, W. J., C. W. Wieland, J. J. Roelofs and T. van der Poll (2008). MyD88 dependent signaling contributes to protective host defense against Burkholderia pseudomallei. PLoS One 3 10:e3494.

Williams, L., L. Bradley, A. Smith and B. Foxwell (2004). Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. J Immunol 172 1:567-576.

Wolf, S. F., P. A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, K. Kelleher, S. H. Herrmann, S. C. Clark, L. Azzoni, S. H. Chan, G. Trinchieri and B. Perussia (1991). Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J Immunol 146 9:3074-3081.

Xu, J., Y. Yang, G. Qiu, G. Lal, Z. Wu, D. E. Levy, J. C. Ochando, J. S. Bromberg and Y. Ding (2009). c-Maf regulates IL-10 expression during Th17 polarization. J Immunol 182 10:6226-6236.

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda and S. Akira (2003a). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301 5633:640-643.

Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda and S. Akira (2003b). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol 4 11:1144-1150.

Yang, H. T., Y. Wang, X. Zhao, E. Demissie, S. Papoutsopoulou, A. Mambole, A. O'Garra, M. F. Tomczak, S. E. Erdman, J. G. Fox, S. C. Ley and B. H. Horwitz (2011a). NF-kappaB1 inhibits TLR-induced IFN-beta production in macrophages through TPL-2-dependent ERK activation. J Immunol 186 4:1989-1996.

Yang, I. V., S. Alper, B. Lackford, H. Rutledge, L. A. Warg, L. H. Burch and D. A. Schwartz (2011b). Novel regulators of the systemic response to lipopolysaccharide. Am J Respir Cell Mol Biol 45 2:393-402.

Yang, Z., X. Zhang, P. A. Darrah and D. M. Mosser (2010). The regulation of Th1 responses by the p38 MAPK. J Immunol 185 10:6205-6213.

Yap, G., M. Pesin and A. Sher (2000). Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii. J Immunol 165 2:628-631.

Yi, A. K., J. G. Yoon, S. J. Yeo, S. C. Hong, B. K. English and A. M. Krieg (2002). Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. J Immunol 168 9:4711-4720.

Zak, D. E. and A. Aderem (2009). Systems biology of innate immunity. Immunol Rev 227 1:264-282.

Zambelli, F., G. Pesole and G. Pavesi (2009). Pscan: finding over-represented transcription factor binding site motifs in sequences from co-regulated or co-expressed genes. Nucleic Acids Res 37 Web Server issue:W247-252.

Zhang, X., J. P. Edwards and D. M. Mosser (2006). Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. J Immunol 177 2:1282-1288.

Zhao, J., H. J. Kong, H. Li, B. Huang, M. Yang, C. Zhu, M. Bogunovic, F. Zheng, L. Mayer, K. Ozato, J. Unkeless and H. Xiong (2006). IRF-8/interferon (IFN) consensus sequence-binding protein is involved in Toll-like receptor (TLR) signaling and contributes to the cross-talk between TLR and IFN-gamma signaling pathways. J Biol Chem 281 15:10073-10080.

Zheng, W. and R. A. Flavell (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 89 4:587-596.

Zhu, J., B. Min, J. Hu-Li, C. J. Watson, A. Grinberg, Q. Wang, N. Killeen, J. F. Urban, Jr., L. Guo and W. E. Paul (2004). Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. Nat Immunol 5 11:1157-1165.

Ziegler-Heitbrock, L., M. Lotzerich, A. Schaefer, T. Werner, M. Frankenberger and E. Benkhart (2003). IFN-alpha induces the human IL-10 gene by recruiting both IFN regulatory factor 1 and Stat3. J Immunol 171 1:285-290.

Zuany-Amorim, C., S. Haile, D. Leduc, C. Dumarey, M. Huerre, B. B. Vargaftig and M. Pretolani (1995). Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. J Clin Invest 95 6:2644-2651.