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The role of infection and inflammation in a mouse model of preterm labour

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ABSTRACT

Increasing evidence highlights that term labour is an inflammatory event associated with increased production of pro-inflammatory mediators and leukocyte influx into the intrauterine tissues. Preterm labour (PTL), defined as labour before 37 weeks gestation, is a major clinical problem, and preterm birth is the leading cause of neonatal mortality and morbidity worldwide. The causes of PTL are poorly understood, but intrauterine infection and inflammation have been shown to be important factors. Therefore, there is growing interest in the hypothesis that preterm labour may occur as a result of the premature activation of the inflammatory pathways normally initiated with labour at term, either idiopathically, or in response to a pathological intrauterine infection.

The aim of this thesis was to use a mouse model of infection-induced PTL to: characterise the local inflammatory and immune response to an intrauterine infection; investigate the potential of anti-inflammatory agents to delay delivery of pups and to improve their survival; and to investigate the role of specific immune cell populations in infection-induced preterm labour.

To characterise the inflammatory and immune response to intrauterine infection, CD1 mice received an intrauterine injection of PBS vehicle or increasing doses of bacterial-derived lipopolysaccharide (LPS) on day 17 of gestation. Time to delivery, and the number of live born pups were determined. Intrauterine administration of increasing doses of LPS dose-dependently induced preterm labour and reduced the proportion of live born pups. Analysis of tissues harvested six hours post-surgery demonstrated that in response to intrauterine LPS administration, there was increased expression of inflammatory cytokines and chemokines within the utero-placental tissues, amniotic fluid and maternal serum; and an influx of neutrophils into the decidua, compared to mice receiving PBS.

Given these results, the potential of anti-inflammatory agents to delay LPS-induced preterm delivery and improve pup survival was then investigated using the same mouse model. Prior to intrauterine LPS administration, mice were pre-treated with epi-lipoxin, BML-111 (a stable lipoxin analogue), or IL-10. Time to delivery was unaffected by pre-treatment with the anti-inflammatory agents, however epi-lipoxin

significantly increased the proportion of live born pups in mice delivering preterm, compared to mice receiving only LPS.

To further investigate the role of immune cells in infection-induced PTL, antibody-based depletion strategies were used to selectively deplete specific immune cell populations to determine whether they played a causative role in LPS-induced preterm delivery. Despite successful depletion of macrophages or neutrophils, it was not found to significantly affect LPS-induced preterm delivery, suggesting these immune cells are not required for the induction of preterm labour in response to intrauterine infection. However, it is likely that they contribute to the intrauterine inflammatory response as depletion resulted in altered inflammatory signalling within the intrauterine tissues.

Collectively, this work has demonstrated that the presence of intrauterine bacterial LPS, as a surrogate model of infection, induces a robust inflammatory and immune response within the utero-placental tissues that involves the increased production of inflammatory mediators and the influx of immune cells into the decidua, which ultimately leads to PTL. Whilst the anti-inflammatory treatments tested here did not delay LPS-induced PTL, epi-lipoxin attenuated LPS-induced mortality in pups born preterm, suggesting this anti-inflammatory agent may be useful in protecting the fetus from the adverse effects of infection-induced preterm birth. Using models such as the one described here, are vital to improving our understanding of the events regulating the induction of PTL and will ultimately aid the search for novel therapeutic options for the treatment of PTL.

DECLARATION

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of the work described in this thesis has been previously accepted for, or is currently being submitted in candidature for another degree.

Chapter 3

I acknowledge the assistance of Lucy Lecky-Thompson, a medical student, who helped perform some of the qRT-PCR analysis of the fetal brains and ELISA analysis of the amniotic fluid samples.

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PUBLICATIONS, PRESENTATIONS AND POSTERS

PUBLICATIONS

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ORAL PRESENTATIONS

Can lipoxin administration prevent LPS-induced preterm labour in a mouse model? The 2nd Edinburgh Perinatal Festival, Edinburgh May 2012

- *Best oral presentation*

Characterisation of the inflammatory response in a mouse model of LPS-induced preterm labour. SRF 2012 Annual Conference, Edinburgh July 2012.

- *Shortlisted for student prize*

POSTERS

Investigating the anti-inflammatory agents epi-lipoxin and IL-10 in a mouse model of infection-induced preterm labour. SGI 2013 Annual Conference, Orlando March 2013.

Investigating the role of neutrophils in a mouse model of infection-induced preterm labour. SGI 2013 Annual Conference, Orlando March 2013.

CONTENTS

ABSTRACT II

DECLARATION IV

ACKNOWLEDGEMENTS V

PUBLICATIONS, PRESENTATIONS AND POSTERS VI

CONTENTS VII

LIST OF FIGURES XIII

LIST OF TABLES XVIII

ABBREVIATIONS XIX

CHAPTER 1 - LITERATURE REVIEW 1

1.1 INTRODUCTION 2

1.2 LABOUR: AN INFLAMMATORY EVENT 3
1.2.1 Immune cells and parturition 3
1.2.1.1 Neutrophils *4*
1.2.1.2 Macrophages *5*
1.2.2 Inflammatory mediator production and parturition 6
1.2.2.1 Cytokines and chemokines *6*
1.2.2.2 Prostaglandins *7*
1.2.2.3 MMPs *8*
1.2.3 Role of NF-κB activation in parturition 9
1.2.4 Steroid hormones and parturition 10
1.2.4.1 Progesterone *10*
1.2.4.2 Estrogen *11*
1.2.5 Initiation of the inflammatory response at term 12

1.3 PRETERM LABOUR 13
1.3.1 The incidence and consequences of preterm labour 13
1.3.2 Causes of preterm labour 14
1.3.3 Intrauterine infection as a major cause of preterm labour 16
1.3.3.1 Molecular mechanisms of infection-induced preterm labour: role of toll-like receptors *17*
1.3.4 Current therapeutic options for the treatment of preterm labour 20
1.3.4.1 Tocolytics *20*
1.3.4.2 Antibiotics *21*
1.3.4.3 Progesterone *21*
1.3.5 Novel therapeutic options to treat preterm labour: the potential of anti-inflammatory agents 23
1.3.5.1 Blocking IL-1β and TNF-α *26*
1.3.5.2 NF-κB inhibitors *28*

1.3.5.3 15d-PGJ$_2$ *30*
1.3.5.4 IL-10 *31*
1.3.5.5 Lipoxins *34*
1.3.6 Animal models of preterm labour 38

1.4 SUMMARY 42

1.5 HYPOTHESIS AND AIMS 43

CHAPTER 2 - GENERAL METHODS 44

2.1 MOUSE MODEL OF PRETERM LABOUR 45
2.1.1 Time to delivery 47
2.1.2 Proportion of live born pups 47
2.1.3 Tissue collection 47
2.1.3.1 Uterus *47*
2.1.3.2 Fetal membranes *48*
2.1.3.3 Placenta *48*
2.1.3.4 Fetal brains *48*
2.1.3.5 Amniotic fluid *48*
2.1.3.6 Serum *48*

2.2 RNA EXTRACTION AND REVERSE TRANSCRIPTION 49
2.2.1 RNA extraction 49
2.2.1.1 RNA extraction from utero-placental tissues *49*
2.2.1.2 RNA extraction from fetal brains *50*
2.2.1.3 RNA quantification *50*
2.2.2 Reverse transcription 50

2.3 QUANTITATIVE REAL-TIME PCR 51
2.3.1 Primers and probes 52
2.3.2 Method 54
2.3.3 Analysis 55

2.4 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) 55
2.4.1 Sandwich ELISAs 56
2.4.1.1 TNF-α ELISA *56*
2.4.1.2 IL-1β ELISA *56*
2.4.1.3 IL-6 ELISA *57*
2.4.1.4 IL-10 ELISA *57*
2.4.2 Competitive binding ELISA 57
2.4.2.1 Progesterone ELISA *57*

2.5 IMMUNOHISTOCHEMISTRY 58
2.5.1 Paraffin embedding and slide cutting 58
2.5.2 Staining protocol 58
2.5.3 Quantification of positively stained cells 60

2.6 WESTERN BLOTTING 60
2.6.1 Protein Extraction 61
2.6.1.1 Protein Quantification *61*
2.6.2 Method 61
2.6.3 Analysis 62

2.7 FLOW CYTOMETRY **64**
2.7.1 Sample preparation 64
2.7.2 Flow cytometric analysis 64
2.8 STATISTICAL ANALYSIS **66**
CHAPTER 3 - CHARACTERISATION OF THE INFLAMMATORY AND IMMUNE RESPONSE IN A MOUSE MODEL OF INFECTION-INDUCED PTL **67**
3.1 INTRODUCTION **68**
3.2 METHODS **69**
3.2.1 Mouse model of PTL 69
3.2.2 Tissue collection 69
3.2.3 qRT-PCR 69
3.2.4 ELISA 70
3.2.5 Western Blotting 70
3.2.6 Immunohistochemistry 70
3.2.7 Statistical analysis 70
3.3 RESULTS **72**
3.3.1 Intrauterine administration of LPS induced PTL in a dose-dependent manner 72
3.3.1.1 Time to delivery *72*
3.3.1.2 Proportion of live born pups *74*
3.3.2 Expression of inflammatory mediators in the utero-placental tissues in response to intrauterine LPS 76
3.3.2.1 Inflammatory gene expression in the uterus *76*
3.3.2.2 Inflammatory gene expression in the fetal membranes *80*
3.3.2.3 Inflammatory gene expression in the placenta *84*
3.3.3 TNF-α protein levels in utero-placental tissues following LPS treatment. 87
3.3.3.1 Uterine TNF-α protein production *87*
3.3.3.2 Fetal membrane TNF-α protein production *89*
3.3.3.3 Placental TNF-α protein production *91*
3.3.4 Immune cell influx in response to intrauterine LPS administration 93
3.3.4.1 Macrophage marker expression in the utero-placental tissues *93*
3.3.4.2 Macrophage localisation in the uterus *95*
3.3.4.3 Neutrophil marker expression in the utero-placental tissues *98*
3.3.4.4 Neutrophil localisation in the uterus *100*
3.3.5 Inflammatory markers in the amniotic fluid 103
3.3.6 Serum markers in response to intrauterine LPS administration 105
3.3.7 Gene expression in the fetal brain following intrauterine LPS administration 107
3.4 DISCUSSION **109**
CHAPTER 4 - INVESTIGATING THE USE OF ANTI-INFLAMMATORY AGENTS TO DELAY PRETERM LABOUR AND IMPROVE PUP SURVIVAL IN A MOUSE MODEL **127**
4.1 INTRODUCTION **128**
4.2 METHODS **130**
4.2.1 Mouse model of PTL 130
4.2.1.1 Epi-lipoxin experiments *130*
4.2.1.2 BML-111 experiments *130*

4.2.1.3 IL-10 experiments *131*
4.2.2 Tissue collection 131
4.2.3 qRT-PCR 132
4.2.4 ELISA 132
4.2.5 Statistical analysis 132

4.3 RESULTS **133**
4.3.1 Expression of the lipoxin receptor, Fpr2, in utero-placental tissues following intrauterine LPS administration 133
4.3.2 Effect of pre-treatment with epi-lipoxin on time to delivery and proportion of live born pups 135
4.3.2.1 Time to delivery *135*
4.3.2.2 Proportion of live born pups *138*
4.3.3 Expression of inflammatory mediators in the utero-placental tissues following intra-peritoneal pre-treatment with epi-lipoxin 140
4.3.3.1 Inflammatory gene expression in the uterus *140*
4.3.3.2 Inflammatory gene expression in the fetal membranes *145*
4.3.3.3 Inflammatory gene expression in the placenta *150*
4.3.4 Effect of pre-treatment with epi-lipoxin on amniotic fluid cytokine levels 154
4.3.5 Expression of inflammatory mediators in the utero-placental tissues following intrauterine administration of epi-lipoxin 156
4.3.5.1 Inflammatory gene expression in the uterus *156*
4.3.5.2 Inflammatory gene expression in the fetal membranes *161*
4.3.5.3 Inflammatory gene expression in the placenta *165*
4.3.6 Effect of intrauterine epi-lipoxin administration on amniotic fluid cytokine levels 169
4.3.7 Effect of pre-treatment with BML-111 on time to delivery and the proportion of live born pups 171
4.3.7.1 Time to delivery *171*
4.3.7.2 Proportion of live born pups *174*
4.3.8 Effect of pre-treatment with IL-10 on time to delivery and the proportion of live pups born 176
4.3.8.1 Time to delivery *176*
4.3.8.2 Proportion of live born pups *178*

4.4 DISCUSSION **180**

CHAPTER 5 - INVESTIGATING THE ROLE OF IMMUNE CELLS IN A MOUSE MODEL OF INFECTION-INDUCED PRETERM LABOUR **195**

5.1 INTRODUCTION **196**

5.2 METHODS **197**
5.2.1 Mouse model of PTL 197
5.2.1.1 Anti-Gr-1 experiments *197*
5.2.1.2 Anti-Ly-6G experiments *197*
5.2.1.3 Anti-F4/80 experiments *197*
5.2.2 Tissue collection 198
5.2.2.1 Anti-Gr-1 experiments *198*
5.2.2.2 Anti-Ly-6G experiments *198*
5.2.2.3 Anti-F4/80 experiments *198*
5.2.3 Flow cytometry 198
5.2.4 Immunohistochemistry 199

5.2.5 qRT-PCR 199
5.2.6 ELISA 199
5.2.7 Statistical analysis 199

5.3 RESULTS 201
5.3.1 Administration of anti-Gr-1 to deplete neutrophils on day 16 of gestation 201
5.3.1.1 Depletion of circulating neutrophils 201
5.3.1.2 Effect of neutrophil depletion using anti-Gr-1 on LPS-induced neutrophil recruitment to the uterus 203
5.3.2 Effect of neutrophil depletion using anti-Gr-1 on LPS-induced preterm labour 205
5.3.2.1 Time to delivery 205
5.3.2.2 Proportion of live born pups 205
5.3.3 LPS-induced expression of inflammatory mediators in the utero-placental tissues following neutrophil depletion with anti-Gr-1 207
5.3.3.1 Inflammatory gene expression in the uterus 207
5.3.3.2 Inflammatory gene expression in the fetal membranes 210
5.3.3.3 Inflammatory gene expression in the placenta 213
5.3.4 Effect of anti-Gr-1 administration on LPS-induced serum TNF-α levels 216
5.3.5 Administration of anti-Ly-6G to deplete neutrophils on day 16 of gestation 218
5.3.5.1 Depletion of circulating neutrophils 218
5.3.5.2 Effect of neutrophil depletion with anti-Ly-6G on LPS-induced neutrophil recruitment to the uterus 220
5.3.6 Effect of neutrophil depletion using anti-Ly-6G on LPS-induced preterm labour 222
5.3.6.1 Time to delivery 222
5.3.6.2 Proportion of live born pups 222
5.3.7 LPS-induced expression of inflammatory mediators in the utero-placental tissues following neutrophil depletion with anti-Ly-6G 224
5.3.7.1 Inflammatory gene expression in the uterus 224
5.3.7.2 Inflammatory gene expression in the fetal membranes 227
5.3.7.3 Inflammatory gene expression in the placenta 230
5.3.8 Effect of anti-Ly-6G treatment on LPS-induced serum TNF-α levels 233
5.3.9 Administration of anti-F4/80 to deplete macrophages on day 17 of gestation 235
5.3.10 Effect of anti-F4/80 treatment on LPS-induced preterm labour 238
5.3.10.1 Time to delivery 238
5.3.10.2 Proportion of live born pups 238
5.3.11 LPS-induced expression of inflammatory mediators in the utero-placental tissues following treatment with anti-F4/80 240
5.3.11.1 Inflammatory gene expression in the uterus 240
5.3.11.2 Inflammatory gene expression in the fetal membranes 243
5.3.11.3 Inflammatory gene expression in the placenta 246
5.3.12 Effect of anti-F4/80 treatment on LPS-induced serum TNF-α levels 249

5.4 DISCUSSION 251

CHAPTER 6 - GENERAL DISCUSSION 260

6.1 SUMMARY OF FINDINGS 261

6.2 MOUSE MODEL OF PRETERM LABOUR 262

6.3 ROLE OF IMMUNE CELLS IN PRETERM LABOUR 266

6.4 NOVEL TREATMENT OPTIONS FOR PRETERM LABOUR: WILL ANTI-INFLAMMATORY DRUGS BE THE ANSWER? **267**

6.5 CONCLUSIONS **269**

REFERENCES **271**

APPENDICES **302**

Appendix 1 - Materials **303**

Appendix 2 – Publications **309**

LIST OF FIGURES

CHAPTER 1 – LITERATURE REVIEW

FIGURE 1.1 - INFLAMMATORY EVENTS ASSOCIATED WITH TERM LABOUR AND INFECTION-INDUCED PRETERM LABOUR. **15**

FIGURE 1.2 – OVERVIEW OF NF-κB ACTIVATION IN RESPONSE TO LPS SIGNALLING VIA TLR-4. **19**

FIGURE 1.3 - SCHEMATIC REPRESENTATION OF THE THREE PATHWAYS OF LIPOXIN BIOSYNTHESIS. **35**

CHAPTER 2 - GENERAL METHODS

FIGURE 2.1 - MOUSE MODEL OF INFECTION-INDUCED PRETERM LABOUR. **46**

FIGURE 2.2 - *COX-2* AND *β-ACTIN* PRIMER AND PROBE VALIDATION. **54**

FIGURE 2.3 - REPRESENTATIVE FLOW CYTOMETRY PLOTS TO MEASURE CIRCULATING NEUTROPHILS IN BLOOD. **65**

CHAPTER 3 - CHARACTERISATION OF THE INFLAMMATORY AND IMMUNE RESPONSE IN A MOUSE MODEL OF INFECTION-INDUCED PTL

FIGURE 3.1 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON TIME TO DELIVERY. **73**

FIGURE 3.2 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON THE PROPORTION OF LIVE BORN PUPS. **75**

FIGURE 3.3 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON INFLAMMATORY GENE EXPRESSION IN THE UTERUS. **78**

FIGURE 3.4 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON INFLAMMATORY CHEMOKINE EXPRESSION IN THE UTERUS. **79**

FIGURE 3.5 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON INFLAMMATORY GENE EXPRESSION IN THE FETAL MEMBRANES. **82**

FIGURE 3.6 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON INFLAMMATORY CHEMOKINE EXPRESSION IN THE FETAL MEMBRANES. **83**

FIGURE 3.7 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON INFLAMMATORY GENE EXPRESSION IN THE PLACENTA. **85**

FIGURE 3.8 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON INFLAMMATORY CHEMOKINE EXPRESSION IN THE PLACENTA. **86**

FIGURE 3.9 - UTERINE TNF-α PRODUCTION. **88**
FIGURE 3.10 - FETAL MEMBRANE TNF-α PRODUCTION. **90**
FIGURE 3.11 - PLACENTAL TNF-α PRODUCTION. **92**
FIGURE 3.12 - EXPRESSION OF *EMR1* MRNA IN THE UTERUS, PLACENTA AND FETAL MEMBRANES. **94**
FIGURE 3.13 - MACROPHAGE LOCALISATION IN THE UTERUS. **96**
FIGURE 3.14 - QUANTIFICATION OF F4/80 POSITIVE CELLS IN THE UTERUS FOLLOWING SURGERY. **97**
FIGURE 3.15 - EXPRESSION OF *NGP* MRNA IN THE UTERUS, FETAL MEMBRANES AND PLACENTA. **99**
FIGURE 3.16 - NEUTROPHIL LOCALISATION IN THE UTERUS. **101**
FIGURE 3.17 - QUANTIFICATION OF GR-1 POSITIVE CELLS IN THE UTERUS FOLLOWING SURGERY. **102**
FIGURE 3.18 - INFLAMMATORY MARKERS IN THE AMNIOTIC FLUID. **104**
FIGURE 3.19 - SERUM MARKERS IN RESPONSE TO INTRAUTERINE LPS ADMINISTRATION. **106**
FIGURE 3.20 - GENE EXPRESSION IN THE FETAL BRAINS FOLLOWING INTRAUTERINE LPS ADMINISTRATION. **108**

CHAPTER 4 - INVESTIGATING THE USE OF ANTI-INFLAMMATORY AGENTS TO DELAY PRETERM LABOUR AND IMPROVE PUP SURVIVAL IN A MOUSE MODEL

FIGURE 4.1 - EXPRESSION OF *FPR2* MRNA IN UTERUS, FETAL MEMBRANES AND PLACENTA. **134**
FIGURE 4.2 - EFFECT OF EPI-LIPOXIN PRE-TREATMENT ON TIME TO DELIVERY. **137**
FIGURE 4.3 - EFFECT OF PRE-TREATMENT WITH EPI-LIPOXIN ON THE PROPORTION OF LIVE BORN PUPS. **139**
FIGURE 4.4 - EFFECT OF I.P. EPI-LIPOXIN PRE-TREATMENT ON INFLAMMATORY GENE EXPRESSION IN THE UTERUS. **143**
FIGURE 4.5 - EFFECT OF I.P. EPI-LIPOXIN TREATMENT ON INFLAMMATORY CHEMOKINE EXPRESSION IN THE UTERUS. **144**
FIGURE 4.6 - EFFECT OF I.P. EPI-LIPOXIN PRE-TREATMENT ON INFLAMMATORY GENE EXPRESSION IN THE FETAL MEMBRANES. **148**
FIGURE 4.7 - EFFECT OF I.P. EPI-LIPOXIN TREATMENT ON INFLAMMATORY CHEMOKINE EXPRESSION IN THE FETAL MEMBRANES. **149**
FIGURE 4.8 - EFFECT OF I.P. EPI-LIPOXIN PRE-TREATMENT ON INFLAMMATORY GENE EXPRESSION IN THE PLACENTA. **152**

FIGURE 4.9 - EFFECT OF I.P. EPI-LIPOXIN TREATMENT ON INFLAMMATORY CHEMOKINE EXPRESSION IN THE PLACENTA. **153**
FIGURE 4.10 - EFFECT OF PRE-TREATMENT WITH EPI-LIPOXIN ON INFLAMMATORY CYTOKINE PRODUCTION IN THE AMNIOTIC FLUID. **155**
FIGURE 4.11 - EFFECT OF CO-TREATMENT WITH EPI-LIPOXIN ON INFLAMMATORY GENE EXPRESSION IN THE UTERUS. **159**
FIGURE 4.12 - EFFECT OF CO-TREATMENT WITH EPI-LIPOXIN ON CHEMOKINE EXPRESSION IN THE UTERUS. **160**
FIGURE 4.13 - EFFECT OF CO-TREATMENT WITH EPI-LIPOXIN ON INFLAMMATORY GENE EXPRESSION IN THE FETAL MEMBRANES. **163**
FIGURE 4.14 - EFFECT OF CO-TREATMENT WITH EPI-LIPOXIN ON CHEMOKINE EXPRESSION IN THE FETAL MEMBRANES. **164**
FIGURE 4.15 - EFFECT OF CO-TREATMENT WITH EPI-LIPOXIN ON INFLAMMATORY GENE EXPRESSION IN THE PLACENTA. **167**
FIGURE 4.16 - EFFECT OF CO-TREATMENT WITH EPI-LIPOXIN ON CHEMOKINE EXPRESSION IN THE PLACENTA. 168
FIGURE 4.17 - EFFECT OF CO-TREATMENT WITH EPI-LIPOXIN ON INFLAMMATORY CYTOKINE PRODUCTION IN THE AMNIOTIC FLUID. **170**
FIGURE 4.18 - EFFECT OF PRE-TREATMENT WITH BML-111 ON LPS-INDUCED TIME TO DELIVERY. **173**
FIGURE 4.19 - EFFECT OF PRE-TREATMENT WITH BML-111 ON THE PROPORTION OF LIVE BORN PUPS. **175**
FIGURE 4.20 - EFFECT OF PRE-TREATMENT WITH IL-10 ON LPS-INDUCED TIME TO DELIVERY. **177**
FIGURE 4.21 - EFFECT OF PRE-TREATMENT WITH IL-10 ON THE PROPORTION OF LIVE BORN PUPS. **179**

CHAPTER 5 - INVESTIGATING THE ROLE OF IMMUNE CELLS IN A MOUSE MODEL OF INFECTION-INDUCED PRETERM LABOUR

FIGURE 5.1- EFFECT OF ANTI-GR-1 ADMINISTRATION ON CIRCULATING BLOOD NEUTROPHILS. **202**
FIGURE 5.2 – EFFECT OF ANTI-GR-1 TREATMENT ON NEUTROPHIL LOCALISATION IN THE UTERUS. **204**
FIGURE 5.3 – EFFECT OF NEUTROPHIL DEPLETION WITH ANTI-GR-1 ON LPS-INDUCED PRETERM DELIVERY AND THE PROPORTION OF LIVE BORN PUPS. **206**
FIGURE 5.4 – EFFECT OF ANTI-GR-1 TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE UTERUS. **208**

FIGURE 5.5 – EFFECT OF ANTI-GR-1 TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE UTERUS. **209**

FIGURE 5.6 – EFFECT OF ANTI-GR-1 TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE FETAL MEMBRANES. **211**

FIGURE 5.7 - EFFECT OF ANTI-GR-1 TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE FETAL MEMBRANES. **212**

FIGURE 5.8 – EFFECT OF ANTI-GR-1 TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE PLACENTA. **214**

FIGURE 5.9 - EFFECT OF ANTI-GR-1 TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE PLACENTA. **215**

FIGURE 5.10 – EFFECT OF ANTI-GR-1 TREATMENT ON SERUM TNF-α LEVELS. **217**

FIGURE 5.11 - EFFECT OF ANTI-LY-6G ADMINISTRATION ON CIRCULATING BLOOD NEUTROPHILS. **219**

FIGURE 5.12 - EFFECT OF ANTI-LY-6G TREATMENT ON NEUTROPHIL LOCALISATION IN THE UTERUS. **221**

FIGURE 5.13 - EFFECT OF NEUTROPHIL DEPLETION WITH ANTI-LY-6G ON LPS-INDUCED PRETERM DELIVERY AND THE PROPORTION OF LIVE BORN PUPS. **223**

FIGURE 5.14 - EFFECT OF ANTI-LY-6G TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE UTERUS. **225**

FIGURE 5.15 - EFFECT OF ANTI-LY-6G TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE UTERUS. **226**

FIGURE 5.16 - EFFECT OF ANTI-LY-6G TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE FETAL MEMBRANES. **228**

FIGURE 5.17 - EFFECT OF ANTI-LY-6G TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE FETAL MEMBRANES. **229**

FIGURE 5.18 - EFFECT OF ANTI-LY-6G TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE PLACENTA. **231**

FIGURE 5.19 - EFFECT OF ANTI-LY-6G TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE PLACENTA. **232**

FIGURE 5.20 - EFFECT OF ANTI-LY-6G TREATMENT ON SERUM TNF-α LEVELS. **234**

FIGURE 5.21 - EFFECT OF ANTI-F4/80 TREATMENT ON MACROPHAGE POPULATIONS IN THE SPLEEN, LIVER, LUNG AND CERVIX. **236**

FIGURE 5.22 - EFFECT OF ANTI-F4/80 TREATMENT ON MACROPHAGE LOCALISATION IN THE UTERUS. **237**

FIGURE 5.23 - EFFECT OF ANTI-F4/80 TREATMENT ON LPS-INDUCED PRETERM DELIVERY AND THE PROPORTION OF LIVE BORN PUPS. **239**

FIGURE 5.24 - EFFECT OF ANTI-F4/80 TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE UTERUS. **241**

FIGURE 5.25 - EFFECT OF ANTI-F4/80 TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE UTERUS. **242**
FIGURE 5.26 - EFFECT OF ANTI-F4/80 TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE FETAL MEMBRANES. **244**
FIGURE 5.27 - EFFECT OF ANTI-F4/80 TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE FETAL MEMBRANES. **245**
FIGURE 5.28 - EFFECT OF ANTI-F4/80 TREATMENT ON LPS-INDUCED INFLAMMATORY GENE EXPRESSION IN THE PLACENTA. **247**
FIGURE 5.29 - EFFECT OF ANTI-F4/80 TREATMENT ON LPS-INDUCED INFLAMMATORY CHEMOKINE EXPRESSION IN THE PLACENTA. **248**
FIGURE 5.30 - EFFECT OF ANTI-F4/80 TREATMENT ON SERUM TNF-α LEVELS. **250**

CHAPTER 6 - GENERAL DISCUSSION

FIGURE 6.1 - IMAGE OF ULTRASOUND-GUIDED INTRAUTERINE INJECTION. **265**

LIST OF TABLES

CHAPTER 2 - GENERAL METHODS

TABLE 2.1 - REVERSE TRANSCRIPTION MASTERMIX **51**

TABLE 2.2 - TAQMAN® GENE EXPRESSION ASSAY IDS USED IN QRT-PCR EXPERIMENTS. **52**

TABLE 2.3 - PRIMER AND PROBE SEQUENCES DESIGNED USING PRIMER EXPRESS SOFTWARE. **53**

TABLE 2.4 - QRT-PCR REACTION MIX. **55**

TABLE 2.5 - ANTIBODY INFORMATION FOR SPECIFIC ANTIBODIES USED IN IMMUNOHISTOCHEMISTRY EXPERIMENTS. **59**

TABLE 2.6 - SOLUTIONS USED FOR TISSUE FIXATION AND IMMUNOHISTOCHEMISTRY EXPERIMENTS. **60**

TABLE 2.7 - SOLUTIONS USED FOR PROTEIN EXTRACTION AND WESTERN BLOTTING EXPERIMENTS. **63**

CHAPTER 3 - CHARACTERISATION OF THE INFLAMMATORY AND IMMUNE RESPONSE IN A MOUSE MODEL OF INFECTION-INDUCED PTL

TABLE 3.1 - EFFECT OF INTRAUTERINE LPS ADMINISTRATION ON PRETERM DELIVERY RATE. **73**

CHAPTER 4 - INVESTIGATING THE USE OF ANTI-INFLAMMATORY AGENTS TO DELAY PRETERM LABOUR AND IMPROVE PUP SURVIVAL IN A MOUSE MODEL

TABLE 4.1 - EFFECT OF EPI-LIPOXIN PRE-TREATMENT ON LPS-INDUCED PRETERM DELIVERY. **137**

TABLE 4.2 - EFFECT OF PRE-TREATMENT WITH BML-111 ON PRETERM DELIVERY RATE. 173

TABLE 4.3 - EFFECT OF PRE-TREATMENT WITH IL-10 ON PRETERM DELIVERY RATE. **177**

ABBREVIATIONS

15-HPGD	15-hydroxyprostaglandin dehydrogenase
15d-PGJ$_2$	15-deoxy-D^{12,14}-prostaglandin-J$_2$
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
BCP	Bromochloropropane
CAP	contraction-associated protein
CCL	Chemokine (C-C) motif ligand
cDNA	complementary DNA
COX	cyclo-oxygenase
CREB	cAMP response element-binding protein
CRTH2	Chemoattractant receptor-homologous molecule expression on TH2 cells
Ct	Cycle threshold
CXCL	Chemokine (C-X-C) motif ligand
DAB	3,3'diaminaobenzidine
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme linked immunosorbent assay
EMR1	EGF-like module-containing mucin-like hormone receptor-like 1
ER	Estrogen Receptor
FIRS	Fetal inflammatory response syndrome
FRP2/ALX	Formyl peptide receptor 2/lipoxin A4 receptor
GFAP	Glial fibrillary acidic protein
HRP	Horseradish peroxidase
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
JNK	c-Jun N-terminal kinase
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MIP-2	Macrophage inflammatory protein-2
MMP	Matrix metalloproteinase

MPA	Medroxyprogesterone acetate
MTAP2	Microtubule-associated protein 2
MyD88	Myeloid differentiation primary response gene 88
NAC	N-acetylcysteine
NBF	Neutral buffered formalin
NF-κB	Nuclear factor kappa B
NGP	Neutrophilic granule protein
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate-buffered saline
PG	Prostaglandin
PPAR	Peroxisome proliferator-activated receptor
PPROM	Preterm premature rupture of membranes
PR	Progesterone receptor
PTGS	Prostaglandin-endoperoxide synthase
PTL	Preterm labour
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase
SEM	Standard error of the mean
SSZ	Sulfasalazine
STAT	Signal transducer and activator of transcription
TACE	TNF-alpha converting enzyme
TBS	Tris buffered saline
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TMB	3,3'5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter-inducing interferon beta

Chapter 1 - Literature review

1.1 INTRODUCTION

Although the exact mechanisms controlling the onset of parturition in humans remain incompletely understood, there is now strong evidence that labour is an inflammatory process associated with increased production of inflammatory cytokines and leukocyte influx into the utero-placental tissues. The timing of the initiation of this inflammatory cascade must be tightly regulated as the premature activation of these events can result in preterm labour. In contrast, delayed activation results in post-term pregnancy and increased perinatal mortality. Preterm labour (defined as labour prior to 37 weeks gestation) is a major clinical problem worldwide and is the leading cause of neonatal mortality and morbidity. Although the causes of preterm labour are poorly understood, intrauterine infection and inflammation are important causal factors in many cases. This chapter introduces the concept that labour is an inflammatory event and discusses the role of intrauterine infection as a cause of preterm labour. The ineffectiveness of currently available treatments for preterm labour is discussed and the potential use of anti-inflammatory agents as novel therapeutic agents is then considered.

1.2 LABOUR: AN INFLAMMATORY EVENT

The physiological process of parturition involves three main processes: cervical ripening and dilation, rupture of the fetal membranes, and the development of synchronous myometrial contractions, resulting in successful delivery of the fetus. It is clear that the timing of the initiation of labour must be tightly controlled, as the premature onset of labour can result in preterm birth (delivery prior to 37 weeks gestation), which is the leading cause of neonatal mortality and morbidity worldwide (Goldenberg *et al.* 2008). However, despite intensive research over recent decades, the molecular mechanisms responsible for regulating the initiation of labour remain relatively poorly understood. Over recent years, strong evidence has emerged that the process of parturition shares many characteristics with a classical inflammatory response, including leukocyte influx and increased production of pro-inflammatory mediators in the intrauterine tissues, leading to the now well-accepted concept that labour is an inflammatory event.

1.2.1 Immune cells and parturition

Some of the earliest evidence associating labour and inflammation came from work examining the process of cervical ripening more than 30 years ago, where it was shown that cervical ripening was characterised by an influx of neutrophils into the cervical stroma (Junqueira *et al.* 1980, Liggins 1981). Since these early studies, more recent work has confirmed that both neutrophils and macrophages infiltrate the cervix either just prior to, or at the onset of labour (Bokström *et al.* 1997, Osman *et al.* 2003). A similar leukocyte influx during labour at term has also been reported in the myometrium, fetal membranes and decidua (Thomson *et al.* 1999, Keski-Nisula *et al.* 2000, Osman *et al.* 2003, Osman *et al.* 2006, Gomez-Lopez *et al.* 2009, Hamilton *et al.* 2012). Additionally, Yuan *et al.* also reported increased activation of peripheral blood leukocytes at onset of labour (Yuan *et al.* 2009).

The recruitment of leukocytes from peripheral blood into tissues involves two main processes, the production of chemotactic signals, and increased expression of cell adhesion molecules. Chemokines are a subfamily of cytokines which are involved in directing leukocyte migration to the site of tissue injury/inflammation and promoting their activation (Simon *et al.* 1998). Leukocytes are recruited to the intrauterine

tissues in response to chemotactic signals released by the gestational tissues (Gomez-Lopez *et al.* 2009, Gomez-Lopez *et al.* 2010). Cell adhesion molecules are proteins expressed on the cell surface of circulating immune cells and the vascular endothelium, which are involved in the recruitment, adhesion and migration of leukocytes from the circulation into target tissues during an inflammatory response (Bevilacqua 1993). During labour, increased expression of several cell adhesion molecules, such as intercellular cell adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule (PECAM), vascular cell adhesion molecule (VCAM) and e-selectin has been reported in the cervix, myometrium and fetal membranes (Thomson *et al.* 1999, Ledingham *et al.* 2001, Osman *et al.* 2004), suggesting that these molecules are involved in regulating the labour-associated immune cell influx into the utero-placental tissues.

These infiltrating leukocytes contribute to the local intrauterine inflammatory environment by producing a number of pro-inflammatory mediators (Roh *et al.* 2000, Helmig *et al.* 2002, Young *et al.* 2002), which can stimulate labour progression (these will be discussed in greater detail in the next section). Although neutrophils and macrophages are the predominant immune cells infiltrating the intrauterine tissues in association with labour, there are other immune cell populations present at the maternal-fetal interface which could contribute to the labour-associated inflammatory response, including T cells, uterine NK cells and mast cells (Gomez-Lopez *et al.* 2010).

1.2.1.1 Neutrophils

Neutrophils are one of three leukocyte subtypes collectively known as granulocytes (which also include eosinophils and basophils) characterised by a distinct multi-lobed nucleus and numerous granules (there are at least four types of granules in human neutrophils). They are synthesised continuously from myeloid precursor cells in the bone marrow and are usually released into the circulation as mature terminally differentiated cells. They are the most abundant population of leukocytes in circulating human blood (approximately 70% of total circulating leukocytes are neutrophils) (Leitch *et al.* 2008). Neutrophils play important roles in acute inflammation and are usually the first leukocytes to be recruited to an inflammatory site. In response to an infection, they have multiple effector mechanisms to neutralise and eliminate pathogens, these include: phagocytosis, degranulation and release of antibacterial

proteins (e.g. defensins and cathespins); production of reactive oxygen species (e.g. O_2^-); and formation of neutrophil extracellular traps (NETS), which can immobilise and kill pathogens extracellularly. Additionally, neutrophils are an important source of pro-inflammatory mediators, (e.g. leukotriene B_4), cytokines (e.g. IL-1), chemokines (e.g. IL-8) and matrix metalloproteinases (MMPs, particularly MMP-9) (Kolaczkowska and Kubes 2013). Many of these factors can exacerbate the inflammatory response by causing the recruitment, activation and increased longevity of neutrophils (Leitch *et al.* 2008, Fox *et al.* 2010) and other inflammatory cells, including macrophages (Nathan 2006).

1.2.1.2 Macrophages

Macrophages, which are usually derived from circulating monocytes, are found in almost all tissues of the body, where they have important roles in a diverse number of processes, including development, homeostasis, repair and immune responses (Wynn *et al.* 2013). Macrophages can be divided based on the tissue in which they are located in the body, for example lung macrophages are termed alveolar macrophages, macrophages in the bone are termed osteoblasts, and macrophages found in the liver are called Kupffer cells. These tissue-resident macrophages are involved in maintaining normal tissue homeostasis by constantly monitoring for signs of tissue damage and phagocytosing dead or dying cells (Murray and Wynn 2011). Macrophages can have important pro- and anti-inflammatory actions. In response to a pathogen, macrophages phagocytose the invading organism and release a number of pro-inflammatory mediators (e.g. TNF-α and IL-1β) that can activate other immune cells and drive the inflammatory response; these pro-inflammatory activated macrophages have been previously classified as M1 macrophages. However, macrophages are also important in driving the resolution of inflammation and tissue repair, by producing anti-inflammatory cytokines (e.g. IL-10) and growth factors (e.g. TGF-β) and removing apoptotic cells from the tissue, these have been classically termed M2 macrophages (Murray and Wynn 2011). Interestingly, specialised 'resolution-phase macrophages' have been identified in mice, which generally have an M2 phenotype, but which also express classical M1 markers, such as COX-2, alongside other markers which promote the resolution of inflammation (Bystrom *et al.* 2008, Stables *et al.* 2011).

1.2.2 Inflammatory mediator production and parturition

Further evidence that labour is an inflammatory event comes from data showing that co-incident with the onset of parturition and the influx of immune cells into the utero-placental tissues, there is also increased production of inflammatory mediators in these tissues which are proposed to have multiple actions in stimulating cervical ripening and dilation, fetal membrane rupture and myometrial contractility, the three fundamental processes of labour. The inflammatory mediators that play important roles in the onset and progression of parturition include cytokines and chemokines, prostaglandins, and MMPs.

1.2.2.1 Cytokines and chemokines

Cytokines are a family of small glycoprotein signalling molecules that are produced by both immune and non-immune cells in the body and act locally to regulate multiple biological processes (Saito 2000). Most focus is on their role in regulating inflammatory/immune responses; indeed there is strong evidence that cytokines are important in regulating many reproductive processes associated with inflammation, particularly during pregnancy. As previously described, chemokines are a sub-family of cytokines which have specific chemoattractant activities. Chemokines mediate their effects via binding to specific G-protein-coupled receptors that are expressed on both immune cells and many other cell types in the body. Cytokines work together in complex networks, where they can work synergistically together, or antagonise the actions of other cytokines. Two other unique properties of cytokines include pleiotropism, that is they can act on multiple target cells to produce different responses; and functional redundancy, where multiple cytokines can act on the same cell to produce the same effect (Paulesu *et al.* 2010). The roles of a number of chemokines and cytokines in pregnancy and parturition have been well reviewed (Orsi and Tribe 2008, Gomez-Lopez *et al.* 2010, Paulesu *et al.* 2010).

The onset of labour is associated with increased expression of pro-inflammatory cytokines and chemokines, including the pro-inflammatory cytokines, tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and the chemokine, IL-8 in the cervix, decidua, fetal membranes myometrium and placenta (Elliott *et al.* 2000, Sennstrom *et*

al. 2000, Young *et al.* 2002, Osman *et al.* 2003). Additionally, recent microarray analysis comparing gene expression in myometrium, cervix and fetal membranes obtained from labouring and non-labouring women at term, confirmed that labour is associated with a core inflammatory response in these tissues, with chemokines being some of the most highly up-regulated genes associated with labour (Haddad *et al.* 2006, Bollapragada *et al.* 2009). Increased production of inflammatory mediators, including IL-6, TNF-α, chemokine (C-C motif) ligand 2 (CCL2, also known as MCP-1), and chemokine (C-X-C motif) ligand 5 (CXCL5, also known as ENA-78), has also been described in the amniotic fluid of women in spontaneous labour at term, compared with non-labouring women (Olah *et al.* 1996, Maymon *et al.* 1999, Esplin *et al.* 2003, Keelan *et al.* 2004).

These cytokines and chemokines can further perpetuate the local inflammatory environment by further promoting leukocyte recruitment, activating inflammatory signalling pathways, such as the NF-κB pathway, and by inducing the production of other inflammatory mediators which can stimulate the processes of fetal membrane rupture, cervical ripening and dilation, and myometrial contractions. *In vitro,* both IL-1β and TNF-α increase expression of the proteases, MMPs (So *et al.* 1992, Arechavaleta-Velasco *et al.* 2002), while decreasing expression of their inhibitors, tissue inhibitor of metalloproteinases (TIMP) (So *et al.* 1992). The expression of MMPs and TIMPs in the cervix is similarly affected by pro-inflammatory cytokines *in vitro* (Watari *et al.* 1999, Yoshida *et al.* 2002). IL-1β and TNF-α can also stimulate myometrial contractility by increasing calcium entry and concentration in myometrial smooth muscle cells (Tribe *et al.* 2003, Barata *et al.* 2004) and by up-regulating the production and activity of prostaglandins in the myometrium, by increasing expression of cyclooxygenase (COX)-2, and their receptors (Pollard and Mitchell 1996, Rauk and Chiao 2000, Olson *et al.* 2003, Sooranna *et al.* 2006, Duggan *et al.* 2007).

1.2.2.2 Prostaglandins

Prostaglandins are another class of important inflammatory mediators involved in parturition. They are arachidonic acid-derived lipid mediators that are produced by most cell types in the body. Phospholipase enzymes release arachidonic acid from the phospholipid cell membrane, allowing it to be converted to the intermediate prostaglandin, prostaglandin endoperoxide H_2 (PGH_2) by the actions of the

prostaglandin endoperoxidase synthase (PTGS) enzymes 1 and 2 (more commonly known as COX-1 and COX-2). COX-1 is constitutively expressed in tissues throughout the body, whereas COX-2 expression is up-regulated by cytokines and growth factors. This intermediate prostaglandin is then converted by the action of specific prostaglandin synthases into active prostaglandins, such as PGE_2, $PGF_{2\alpha}$ and PGD_2. Prostaglandins are produced and act locally, and are rapidly metabolised by the enzyme 15-hydroxyprostaglandin dehydrogenase (15-HPGD), which breaks them down into inactive 15-keto metabolites (Olson 2003).

The expression of COX-2 increases with the onset of spontaneous labour at term in the fetal membranes, decidua and myometrium (Hirst *et al.* 1995, Slater *et al.* 1995, Hirst *et al.* 1998, Slater *et al.* 1999); and the production of PGE_2 and $PGF_{2\alpha}$ from the amnion, myometrium, decidua and amniotic fluid is increased during spontaneous term labour (Willman and Collins 1976, Bennett *et al.* 1993, Erkinheimo *et al.* 2000, Lee *et al.* 2008). In addition, increased expression of the specific PGE_2 and $PGF_{2\alpha}$ receptors has also been reported during term labour in the fetal membranes and decidua (Unlugedik *et al.* 2010). Several studies have demonstrated that prostaglandins can simulate myometrial contractions (Dyal and Crankshaw 1988, Senior *et al.* 1993), fetal membrane rupture (McLaren *et al.* 2000, Keelan *et al.* 2001) and cervical ripening (Fletcher *et al.* 1993, Kelly *et al.* 2009).

1.2.2.3 MMPs

MMPs are a family of zinc-dependent protease enzymes that are involved in degradation of extracellular matrix proteins. MMPs affect a wide range of biological processes, including tissue morphogenesis, wound repair and inflammation. They are secreted by a wide range of tissues and their production can be induced by cytokines and growth factors. The production of MMPs is tightly regulated by the production of their endogenous inhibitor proteins, TIMPs (Sternlicht and Werb 2001). Given their well-defined role in tissue breakdown and remodelling, MMPs play important roles in the processes of cervical ripening and fetal membrane rupture. Several studies have reported increased MMP expression, particularly MMP-2 and MMP-9, in association with spontaneous labour at term in the myometrium, fetal membranes, placenta and cervix (Stygar *et al.* 2002, Xu *et al.* 2002, Yoshida *et al.* 2002, Choi *et al.* 2007); co-incident with decreased TIMP expression (Riley *et al.* 1999).

1.2.3 Role of NF-κB activation in parturition

Central to the dramatic increase in pro-inflammatory mediator production with the onset of parturition, is the transcription factor, nuclear factor-κB (NF-κB). NF-κB is a key transcription factor involved in regulating the transcription of a wide number of genes, but is classically linked to inflammation and immune responses (Hayden and Ghosh 2004). There are five different transcription factors involved in the NF-κB pathway, these are p50, p52, p65, c-REL and RELB; these transcription factors interact with one another to form either homo- or hetero-dimers which can bind to specific κB sequences on target genes to regulate their transcription. Normally, in unstimulated cells, NF-κB is held inactive in the cell cytoplasm by the actions of its endogenous inhibitor proteins, the IκB proteins. In response to an inflammatory stimulus, such as IL-1β, TNF-α, or bacterial lipopolysaccharide (LPS), the IκB inhibitor protein is phosphorylated by the actions of the IκB kinase enzymes, usually IKKβ, leading to subsequent ubiquitination and proteosomal degradation of the IκB inhibitor protein, thus freeing the NFκB dimer to translocate to the nucleus of the cell where it can bind to specific NF-κB DNA binding sites to regulate the transcription of target genes (Hayden and Ghosh 2004).

Two recent reviews have discussed the role of NF-κB in regulating the inflammatory events surrounding the onset of human parturition (Lindstrom and Bennett 2005, Lappas and Rice 2007). There are conflicting reports regarding changes in NF-κB activity in the intrauterine tissues in association with labour. There have been a number of studies which have reported increased NF-κB activity and nuclear localisation of p65 in amnion (Allport *et al.* 2001, Lee *et al.* 2003, Lim *et al.* 2012), decidua (Yan *et al.* 2002, Vora *et al.* 2010) and myometrium (Condon *et al.* 2006, Vora *et al.* 2010) collected from women in labour, compared to non-labouring women; however, other studies have not observed any significant changes in NF-κB activity when comparing amnion and myometrial tissues obtained from women pre- and post-labour (Yan *et al.* 2002, Vora *et al.* 2010, Khanjani *et al.* 2011), although nuclear p65 staining was observed, suggesting activation of NF-κB prior to labour onset. There is considerable *in vitro* evidence that demonstrates a role for NF-κB in the regulation of several key pro-inflammatory genes associated with labour. Blocking NF-κB activity has been reported to: inhibit inflammatory cytokine expression, including IL-8, IL-6 and TNF-α in amnion and cervical epithelial cells (Elliott *et al.* 2001) and amnion and

chorio-decidua tissue (Lappas *et al.* 2003); inhibit COX-2 expression and prostaglandin production in amnion and myometrial cells (Lindstrom and Bennett 2005); and decrease MMP activity in amnion and chorio-decidual tissues (Lappas *et al.* 2003) Additionally, a recent study using a cDNA microarray to examine NF-κB-regulated genes in myocytes obtained from women at term demonstrated that NF-κB activation up-regulated the expression of a number of genes related to inflammation and immunity in pregnant myometrium (Khanjani *et al.* 2011). Collectively these studies demonstrate an important role for NF-κB in regulating the production of inflammatory mediators within the utero-placental tissues in association with parturition.

1.2.4 Steroid hormones and parturition

1.2.4.1 Progesterone

Progesterone is the main steroid hormone produced throughout pregnancy in humans, initially by the corpus luteum, and then by the placenta, and plays a crucial role in maintaining uterine quiescence throughout gestation. Evidence that progesterone is required for the maintenance of pregnancy comes from studies which demonstrate that removal of the corpus luteum or administration of progesterone receptor (PR) antagonists during the first trimester of pregnancy led to pregnancy loss (Csapo and Pulkkinen 1978, Peyron *et al.* 1993). It is proposed that progesterone maintains uterine quiescence during pregnancy via anti-inflammatory actions, which will be discussed in greater detail later. The exact mechanisms by which progesterone maintains uterine quiescence are still being investigated, but are thought to include: inhibiting the expression of contraction-associated proteins (CAPs), such as connexin-43 and oxytocin receptors; inhibiting prostaglandin production by inhibiting COX-2 expression; and inhibiting inflammatory signalling, via blocking of NF-κB activity (Loudon *et al.* 2003, Renthal *et al.* 2010, Mesiano *et al.* 2011, Tan *et al.* 2012).

In most species, labour is preceded by a sharp drop in circulating progesterone levels, which is not observed in humans, suggesting that a decline in progesterone levels is not required for the onset of labour in humans. However, as administration of the PR antagonist, mifepristone (or RU486) can be used to ripen the cervix and increase the responsiveness of the uterus to contractile agents (Chwalisz *et al.* 1991, Frydman *et al.*

1992, Elliott *et al.* 1998), this suggests that some form of progesterone withdrawal, locally within the uterus, is important to the initiation of parturition. This has led to the hypothesis that in humans at term there is a functional progesterone withdrawal, which results in a local reduction in responsiveness of the uterus to the actions of progesterone. The mechanisms by which this functional progesterone withdrawal is proposed to come about include: changes in PR isoform ratios in the myometrium, where there is increased expression of the inhibitory PR-A isoform, resulting in an increase in the PR-A/PR-B ratio (Pieber *et al.* 2001, Mesiano *et al.* 2002, Merlino *et al.* 2007); increased local progesterone metabolism (Mitchell and Wong 1993, Williams *et al.* 2012); negative interactions between NF-κB and the PR (Allport *et al.* 2001); and a decline in the expression of PR co-activators, cAMP-response element-binding protein (CREB), and steroid receptor co-activators, which may impair PR function (Condon *et al.* 2003).

1.2.4.2 Estrogen

The role of estrogens in regulating the inflammatory cascade associated with parturition is less clear. Estrogen can stimulate myometrial contractility by increasing the expression of CAPs, such as COX-2, connexin-43 and oxytocin receptor (Pinto *et al.* 1966, Petrocelli and Lye 1993, Mesiano *et al.* 2002). Estrogen is produced throughout gestation, with circulating estrogen levels gradually increasing from mid-gestation until birth, but there is no evidence of increased circulating estrogen in association with the initiation of labour in women (Tulchinsky *et al.* 1972, Boroditsky *et al.* 1978); however, it has been proposed that similar to the functional progesterone withdrawal, parturition involves functional estrogen activation (Mesiano and Welsh 2007). Estrogen receptor α (ERα) expression is increased in labouring myometrium, compared to non-labouring, co-incident with decreased PR-B expression, suggesting that during pregnancy progesterone attenuates the actions of estrogen by inhibiting ERα expression, and at the time of functional progesterone withdrawal, increased ERα expression allows estrogen to act on the uterus to stimulate contractions (Mesiano *et al.* 2002).

1.2.5 Initiation of the inflammatory response at term

Therefore, whilst there is now strong evidence that labour is an inflammatory event, the mechanisms responsible for initiating the inflammatory cascade normally at term remain unclear. Mechanical stretch of the uterus, which occurs at the end of gestation, is one mechanism that has been proposed to initiate labour. *In vitro* studies have demonstrated that myometrial cells respond to stretch by increasing chemokine expression and expression of COX-2 via activation of both the NF-κB and MAPK signalling pathways (Loudon *et al.* 2004, Sooranna *et al.* 2004, Hua *et al.* 2012); and similar effects of stretch have been reported in amnion epithelial cells *in vitro* (Mohan *et al.* 2007). The effect of stretch on myometrial chemokine production has also been confirmed *in vivo,* where Shynlova *et al.,* reported that in a unilateral pregnant rat model, expression of *Ccl2* was much greater in uterine tissue taken from the pregnant uterine horn, which had been stretched as the implanted fetuses grew through gestation, compared to the empty, non-pregnant horn (Shynlova *et al.* 2008).

Another hypothesis to explain how the inflammatory cascade of labour is normally initiated involves the production of a signal from the maturing fetus that triggers the onset of parturition. Surfactant, a protein produced by the maturing fetal lung, is proposed to be an important fetal trigger for labour. Condon *et al.,* reported that in a mouse model, surfactant protein secretion into the amniotic fluid by the maturing fetal lung from day 17 of gestation onwards, was associated with increased IL-1β expression in amniotic fluid macrophages and NF-κB activation in the uterus; and showed that injection of surfactant protein A into the amniotic fluid of mice on day 15 of gestation induced preterm labour (Condon *et al.* 2004). Additionally, amniotic fluid-derived surfactant protein has been reported to increase PGE_2 release from cultured amnion discs *in vitro*, suggesting that surfactant can be a source of arachidonic acid for prostaglandin synthesis, which may also initiate labour in humans (Lopez Bernal *et al.* 1988). However, the importance of surfactant in inducing labour has been questioned, as over-expression of surfactant protein in mice was not found to induce labour (Salminen *et al.* 2011); and other studies have questioned the role of surfactant in inducing labour in humans (Lee *et al.* 2010).

Interestingly, a recent study investigating whether mice deficient in surfactant proteins had delayed onset of parturition highlighted the complexity in understanding the mechanisms responsible for initiating labour. Montalbano *et al.,* reported that mice

deficient in surfactant proteins A and D delivered normally during their first pregnancies, but demonstrated a significant delay in delivery in their second pregnancies, suggesting that the initiation of labour is likely to be a multifactorial process, which may also be affected by parity (Montalbano *et al.* 2013).

1.3 PRETERM LABOUR

1.3.1 The incidence and consequences of preterm labour

Preterm labour, defined as the initiation of labour prior to 37 weeks of gestation, is a major clinical problem which is estimated to affect between 5 and 18% of pregnancies annually across the world, resulting in around 15 million preterm births each year (March of Dimes 2012). In Scotland, in 2011, around 7.3% of all births were preterm (ISD Scotland 2012). The incidence of preterm birth in recent decades has remained relatively unchanged, with some studies actually demonstrating an increase in the rate of preterm birth in many developed countries including the USA and the UK (Norman *et al.* 2009, Blencowe *et al.* 2012). Despite advances in the medical care of preterm infants, preterm birth remains the leading cause of neonatal mortality and morbidity worldwide, and has been estimated to account for up to 75% of neonatal deaths (Goldenberg *et al.* 2008), with more than one million babies dying each year due to the complications of their premature birth (March of Dimes 2012). Indeed, although a greater number of premature infants now survive, with 77% of babies born at 26 weeks of gestation in the UK now leaving hospital (Costeloe *et al.* 2012), premature birth is also associated with an increased risk of both short-term morbidities and long-term disabilities, including cerebral palsy, bronchopulmonary dysplasia, retinopathy of prematurity and learning difficulties (Saigal and Doyle 2008).

In addition to the huge emotional burden premature birth can place on families, the economic burden that premature birth places on public sector resources is vast. Taking into consideration not only the immediate costs of caring for a newborn preterm infant, but also the continuing costs of dealing with the long-term morbidities associated with premature birth until the age of 18, Mangham *et al.* estimate that the total annual cost of preterm birth to the public sector in England and Wales was £2.946 billion (at 2006 prices)(Mangham *et al.* 2009). This translates into an additional incremental cost of

£22,885 throughout childhood required for every preterm child surviving to 18 years of age, compared with costs associated with the care of babies born at term (Mangham *et al.* 2009).

1.3.2 Causes of preterm labour

Preterm labour can be categorised into three groups. The first is medically indicated preterm labour, where labour is induced or the baby is delivered prematurely by caesarean section to reduce the risk of significant harm to either the mother or fetus (or both); this accounts for 30-35% of preterm births. The second category of preterm labour occurs following preterm premature rupture of the membranes (PPROM), which accounts for 25-30% of preterm births. The third, and largest, category of preterm labour is spontaneous preterm labour, which accounts for 40-45% of all preterm births (Goldenberg *et al.* 2008). Whilst preterm births have been categorised in this manner for many years, interestingly, a recent paper has suggested that a more comprehensive classification system is required to better define the variable complex phenotypes of preterm birth. Villar *et al.* suggest that preterm birth classification should take into account maternal and fetal conditions, placental pathology, whether there are any signs of the initiation of parturition, and the pathway to delivery (Villar *et al.* 2012).

The causes of spontaneous preterm labour are poorly understood and in the majority of cases, the underlying reasons why a woman has delivered prematurely are unknown. Intensive research into this area has suggested a number of pathological processes that are associated with preterm birth including, uterine overdistention, cervical deficiency, abnormal allograft reaction, utero-placental ischemia, endocrine disorders and an allergic reaction (Romero *et al.* 2006). However, to date, the only factor for which a firm causal link as been proven is the presence of intrauterine infection and/or inflammation (Goldenberg *et al.* 2000, Romero *et al.* 2006). Therefore, there is growing interest in the hypothesis that in many cases, preterm labour may occur because of the premature activation of the same inflammatory pathways normally initiated with labour at term, either idiopathically, or in response to a pathological intrauterine infection. The inflammatory events proposed to be involved in the initiation of physiological term labour and pathological infection-induced preterm labour are summarised in Figure 1.1.

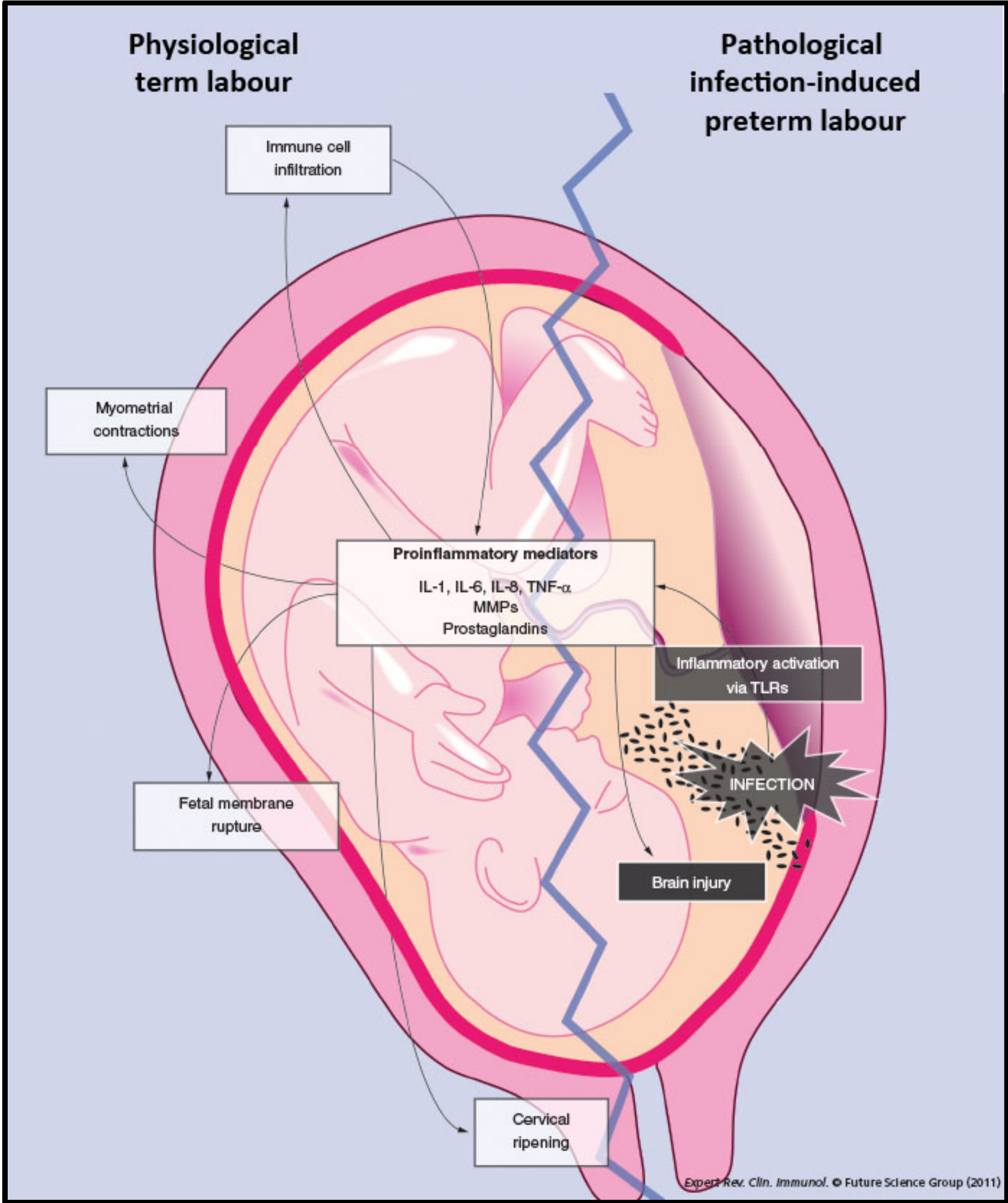

Figure 1.1 - Inflammatory events associated with term labour and infection-induced preterm labour. The inflammatory cascade associated with term labour is shown on the left, and the events of infection-induced preterm labour on the right. In response to an intrauterine infection, which is recognised via TLRs present at the maternal-fetal interface, a similar inflammatory cascade to that proposed to occur during normal physiological term labour is prematurely initiated, resulting in preterm birth. Adapted from Rinaldi *et al.,* (2011).

1.3.3 Intrauterine infection as a major cause of preterm labour

Up to 40% of preterm deliveries are associated with the presence of an intrauterine infection (Agrawal and Hirsch 2012), with early preterm deliveries (i.e. before 30 weeks gestation), being more strongly associated with infection (Mueller-Heubach *et al.* 1990, Lahra and Jeffery 2004). A diverse range of bacteria is associated with preterm labour, including *Ureaplasma urealyticum, Mycoplamsma hominis*, *Gardnerella vaginalis, Escherichia coli,* and group B streptococci (Goldenberg *et al.* 2000). The most common route that microorganisms are thought to gain access to the usually sterile amniotic cavity is via ascension from the vagina and cervix, although other proposed routes include: haematogenous dissemination through the placenta; retrograde invasion from the abdominal cavity via the fallopian tubes; and accidental contamination at the time of invasive obstetric procedures such as amniocentesis (Goldenberg *et al.* 2008).

Supporting evidence that intrauterine infection causes preterm labour includes data showing that women delivering preterm have: a higher incidence of positive amniotic fluid cultures (Romero *et al.* 1989), a higher incidence of chorioamnionitis (infection of the fetal membranes) (Mueller-Heubach *et al.* 1990), and higher levels of pro-inflammatory mediators in their amniotic fluid, including IL-1β, TNF-α, IL-6 and MMP-8, compared to women delivering at term (Yoon *et al.* 2001, Thomakos *et al.* 2010, Marconi *et al.* 2011). Additionally, studies in animal models demonstrate that the intrauterine or systemic administration of bacteria, or bacterial products, effectively induces preterm labour in mice (Hirsch and Muhle 2002, Elovitz *et al.* 2003, Wang and Hirsch 2003, Pirianov *et al.* 2009, Sykes *et al.* 2013), rats (Bennett *et al.* 2000, Terrone *et al.* 2001), rabbits (McDuffie *et al.* 1992, Davies *et al.* 2000) and rhesus monkeys (Gravett *et al.* 1994, Adams Waldorf *et al.* 2008). Interestingly, administration of either IL-1β or TNF-α to pregnant animals has also been shown to induce preterm labour (Romero *et al.* 1991, Silver *et al.* 1994, Sadowsky *et al.* 2003, Yoshimura and Hirsch 2005, Sadowsky *et al.* 2006); highlighting that the inflammatory response to intrauterine infection is likely to be key to the induction of preterm labour, as even in the absence of infection pro-inflammatory mediators can induce preterm labour.

The presence of an intrauterine infection is also associated with a number of adverse effects on the fetus. Exposure of the fetus to an intrauterine infection can result in the development of a systemic inflammatory response, known as the fetal inflammatory response syndrome (FIRS), which can result in damage to multiple organs, including

the heart, lungs, eyes and kidneys (Gomez *et al.* 1998, Gotsch *et al.* 2007). In particular, perinatal brain damage, a major consequence of preterm birth, has been strongly linked to the presence of intrauterine inflammation and infection (Galinsky *et al.* 2013). Indeed Shatrov *et al*. reported in a recent meta-analysis study that the presence of histological chorioamnionitis was associated with an 80% increase in the risk of development of cerebral palsy (Shatrov *et al.* 2010).

1.3.3.1 Molecular mechanisms of infection-induced preterm labour: role of toll-like receptors

The mechanism by which intrauterine infection is proposed to induce preterm labour is via Toll-like receptor (TLR) recognition of bacterial products resulting in the activation of an innate immune response. TLRs are a family of pattern recognition receptors which play a key role in activating the innate immune response to pathogens by recognising pathogen-associated molecular patterns expressed by microbes. To date 10 TLRs and their respective ligands have been identified in humans, including: TLR-4, which is involved in binding LPS, a cell wall component of Gram-negative bacteria; TLR-2 which is involved in the recognition of Gram-positive bacterial components including peptidoglycan and lipoteichoic acid; TLR-3 which is involved in co-ordinating the immune response to viruses by binding viral double-stranded RNA; and TLR-9 which is required for the recognition of specific unmethylated CpG motifs present in bacterial DNA (Takeda and Akira 2001, Koga *et al.* 2009).

Upon ligand binding, TLR-signalling is mediated via either the myeloid differentiation factor 88 (MyD88)-dependent pathway or MyD88-independent pathway. In the MyD88-dependent pathway, ligand binding to the TLR results in recruitment of the adapter protein MyD88 which in turn recruits and activates other intracellular signalling molecules, IL-1 receptor-associated kinases (IRAK), and TNF receptor associated factor 6 (TRAF-6), which ultimately results in activation of two important inflammatory signalling pathways, the JNK and NF-κB signalling pathways. In the MyD88-independent pathway, TLR signalling is mediated via the adaptor protein Toll/IL-1 receptor domain-containing adapter-inducing interferon-β (TRIF) which can in turn result in activation of the NF-κB signalling pathway, and is also involved in inducing transcription of type-1 interferons via activation of the transcription factor interferon regulatory factor 3 (IRF3). The MyD88-dependent pathway is the common

pathway of TLR signalling for all TLRs, while TLR-3 and TLR-4 have also been shown to be capable of signalling via the MyD88-independent pathway (Takeda and Akira 2001, Koga *et al.* 2009). An overview of the LPS-TLR-4 signalling pathway resulting in NF-κB activation is given in Figure 1.2.

At the maternal-fetal interface TLR expression has been reported in the decidua (Canavan and Simhan 2007, Krikun *et al.* 2007, Schatz *et al.* 2012), myometrium (Youssef *et al.* 2009, Thota *et al.* 2013), placenta (Holmlund *et al.* 2002, Kumazaki *et al.* 2004, Pineda *et al.* 2011, Chatterjee *et al.* 2012), and fetal membranes (Kim *et al.* 2004, Choi *et al.* 2012, Abrahams *et al.* 2013). Some of these studies reported increased expression of TLR-2 and TLR-4 in term labouring samples, compared with non-labouring (Kim *et al.* 2004, Youssef *et al.* 2009); and TLR expression was also increased in placenta and fetal membranes obtained from women with evidence of chorioamnionitis (Kim *et al.* 2004, Kumazaki *et al.* 2004), and in response to bacterial treatment of fetal membrane explants *in vitro* (Abrahams *et al.* 2013, Thota *et al.* 2013).

Animal models have confirmed the role of TLR-4 in infection-induced preterm labour, where studies using TLR-4 deficient mice demonstrated that TLR-4 signalling is required for LPS- or *E.coli*-induced preterm delivery (Elovitz *et al.* 2003, Wang and Hirsch 2003). Additionally, blocking TLR-4 signalling inhibited uterine contractions and amniotic pro-inflammatory mediator production in a model of LPS-induced preterm labour in rhesus monkeys (Adams Waldorf *et al.* 2008); and reduced the rate of preterm delivery and improved pup survival in a mouse model of LPS-induced preterm labour (Li *et al.* 2010).

Therefore, taken together this evidence suggests that TLR signalling in response to an intrauterine infection is likely to activate inflammatory signalling pathways, such as NF-κB, inducing the premature activation of the inflammatory cascade proposed to occur at term, resulting in preterm labour.

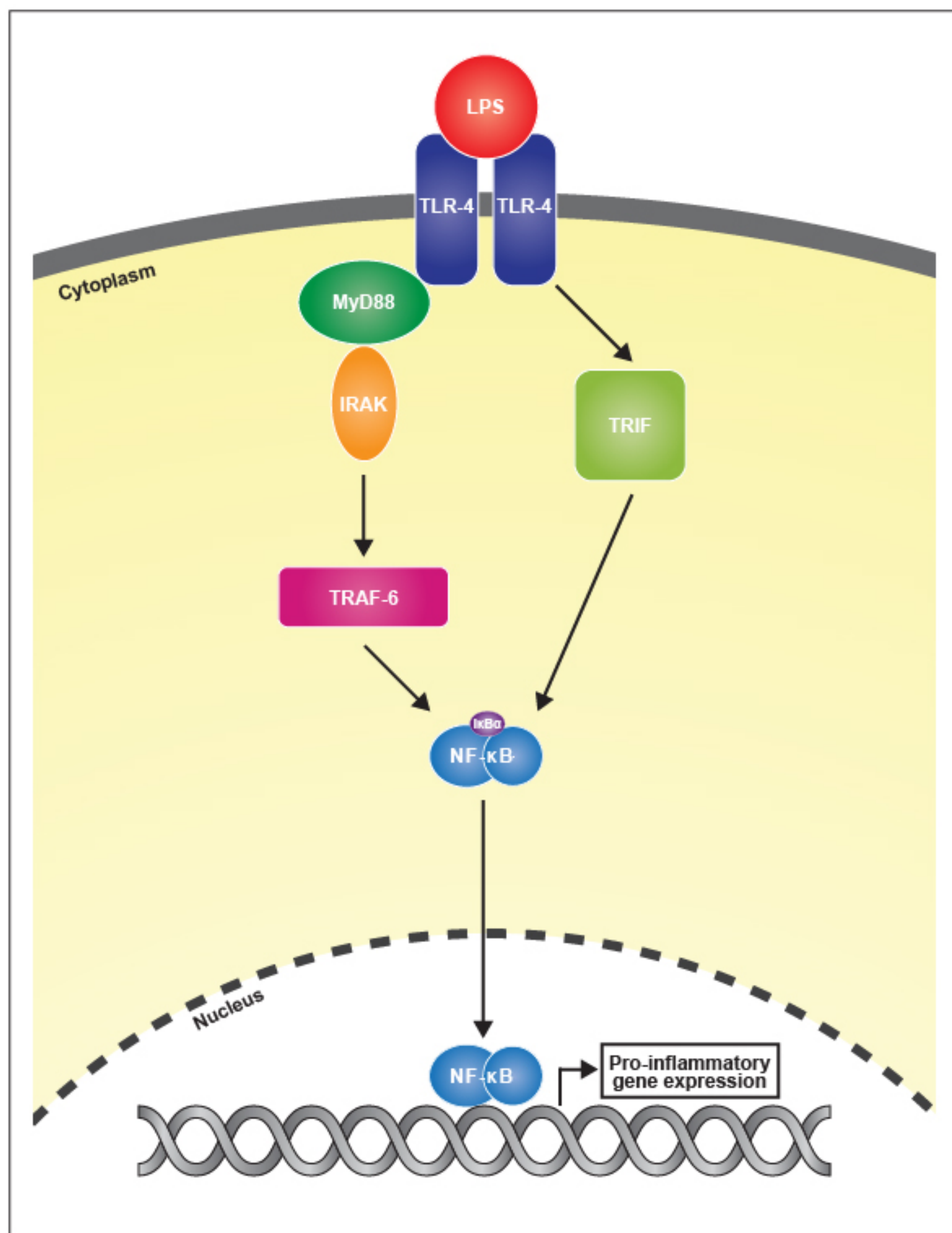

Figure 1.2 – Overview of NF-κB activation in response to LPS signalling via TLR-4. LPS binding to TLR-4 can induce an inflammatory response via NF-κB activation which can occur as a result of a MyD88-dependent or MyD88-independent pathway. In the MyD88-dependent pathway, LPS binding to TLR-4 results in MyD88 recruitment which in turn recruits and activates other intracellular signalling molecules including IRAK and TRAF-6, leading to the release of NF-κB molecules from their inhibitor protein in the cytoplasm, allowing NF-κB to translocate to the nucleus where it can bind to target genes and promote pro-inflammatory gene expression. In the MyD88-independent pathway, NF-κB activation can occur through activation of the intracellular signalling molecule, TRIF.

1.3.4 Current therapeutic options for the treatment of preterm labour

Despite considerable medical advances over recent decades resulting in more premature babies surviving, preterm birth remains the single biggest cause of neonatal mortality worldwide and the second leading cause of death in children under five years of age (March of Dimes 2012). A major reason why preterm birth remains such a huge clinical problem is because current treatment options are limited and largely ineffective at improving neonatal outcome, which is the ultimate goal of preterm birth prevention. The following section briefly discusses the currently available therapeutic options to treat preterm labour.

1.3.4.1 Tocolytics

Current treatments for preterm labour focus on using tocolytic drugs to stop myometrial contractions. Drugs used for this purpose include calcium channel blockers, β-mimetics and nitric oxide donors, which act to directly inhibit myometrial smooth muscle contractions; and oxytocin receptor antagonists and non-steroidal anti-inflammatory agents (NSAIDs), which target the production and action of two pro-contractile agents, prostaglandins and oxytocin (Hubinont and Debieve 2011). Whilst a number of these commonly used tocolytic agents have been shown to delay preterm labour for 48 hours up to 7 days (King *et al.* 2003, Smith *et al.* 2009, Haas *et al.* 2012), there is little evidence that these drugs actually improve neonatal outcome. Indeed, the latest guidelines published by the Royal College of Obstetricians and Gynaecologists on the topic of tocolysis for women in preterm labour state that "there is no clear evidence that tocolytic drugs improve outcome and therefore it is reasonable not to use them" (RCOG 2011); and a recent review article questions the continued use of tocolytics in clinical practice today (Kenyon and Peebles 2011). The limited effectiveness of tocolytic drugs in delaying preterm delivery rates and improving neonatal outcome, is likely due to the fact that these drugs do not target the underlying cause of preterm labour, instead focussing on the inhibition of myometrial contractions, which are an end-point in the inflammatory cascade of labour, therefore it is possible that at this point in the inflammatory cascade, the physiological process of labour may be hard to reverse.

1.3.4.2 Antibiotics

Given the strong link between intrauterine infection and preterm labour, the use of broad-spectrum antibiotic therapy for the treatment of spontaneous preterm labour was trialled in a large randomised multicentre trial. Whilst initial results of the ORACLE I trial appeared promising, where erythromycin treatment was associated with prolongation of pregnancy and a reduction in neonatal morbidities in women with PPROM, compared to the placebo group (Kenyon *et al.* 2001); in the ORACLE II trial, women who were in spontaneous preterm labour with intact membranes showed no benefit of antibiotic treatment (Kenyon *et al.* 2001). Furthermore, follow-up of the babies treated with antibiotics in ORACLE I and II at 7 years of age revealed the short-term benefits observed in the initial ORACLE I study were no longer apparent, with no difference in terms of the rates of functional impairment between children treated with erythromycin and those that were not (Kenyon *et al.* 2008); and of more concern, a greater proportion of children in the ORACLE II trial whose mothers received erythromycin treatment had functional impairment compared with the placebo group; and antibiotic treatment was also associated with an increased risk of cerebral palsy (Kenyon *et al.* 2008). These findings highlight the importance of long-term follow-up studies to fully investigate whether delaying preterm delivery results in improved neonatal outcome. Particularly in the context of intrauterine infection, delaying delivery could result in prolonged exposure of the fetus to a hostile inflammatory environment, which may ultimately lead to adverse, rather than improved, outcomes.

1.3.4.3 Progesterone

The use of progesterone treatment to delay preterm delivery has been considered for many decades, with the first report investigating progesterone administration for the treatment of threatened preterm labour published in 1960 (Fuchs and Stakemann 1960), however it is only in the last decade, following the publication of two randomised controlled trials (da Fonseca *et al.* 2003, Meis *et al.* 2003), that there has been renewed interest in investigating the potential of progesterone as a therapeutic option to treat preterm labour. As already discussed, progesterone plays a key role in maintaining uterine quiescence throughout pregnancy and progesterone withdrawal, or reduced local action of progesterone, is thought to be an important regulator of the initiation of parturition; alongside evidence that progesterone receptor antagonists

(such as mifepristone, RU486) can induce the onset of labour and cervical ripening in women and animal models (Clark *et al.* 2006, Hapangama and Neilson 2009), progesterone treatment seems to be a logical option in the treatment of preterm labour.

There are a number of clinical trials still on-going evaluating the effectiveness of different progesterone derivatives, dosage regimes and optimal routes of administration, but to date progesterone supplementation has been reported to reduce preterm delivery rates in high-risk women, that is those with a previous preterm delivery or with a short cervix (Mackenzie *et al.* 2006, Dodd *et al.* 2008, Conde-Agudelo *et al.* 2013, Schmouder *et al.* 2013). However in contrast, O'Brien *et al.,* reported that although progesterone treatment was associated with less cervical shortening, compared to the placebo group in a randomised double blind placebo controlled trial, this did not significantly affect the rate of recurrent preterm birth (O'Brien *et al.* 2007, O'Brien *et al.* 2009). Additionally, progesterone prophylaxis does not appear to be effective in reducing preterm birth rates in women with multiple pregnancies (Briery *et al.* 2009, Norman *et al.* 2009, Combs *et al.* 2010, Wood *et al.* 2012); or in those with PPROM (Briery *et al.* 2011), highlighting that whilst progesterone is a promising new treatment option, it only appears to be effective in a specific sub-group of high-risk women with a singleton pregnancy. Furthermore, although progesterone treatment has delayed delivery in some cases, there is still little convincing evidence that progesterone prophylaxis ultimately improves neonatal outcome (Smith *et al.* 2009, Dodd and Crowther 2010). Results of the OPPTIMUM trial, a UK-based randomised double blind placebo controlled trial examining the effect of progesterone prophylaxis on neonatal outcome at 2 years of age, will provide more information regarding the benefits of progesterone treatment.

Therefore, clinicians face a difficult task when attempting to treat preterm labour, they have few treatment options to choose from, and of those that are available, there is insufficient evidence that they actually improve neonatal outcome. The limited effectiveness of the currently available treatments is likely due to poor understanding of the varied underlying mechanisms causing preterm labour and the fact that most focus on blocking myometrial contractions, an end-point in the inflammatory cascade proposed to initiate labour normally. The limited number of treatment options is in part due to under-investment by the pharmaceutical industry in the development of new drugs to treat pregnancy-related disorders. Due to the risks associated with administering drugs to pregnant women, where there are possible fetal side-effects to

consider, long-term follow-up studies are required to confirm any adverse effects on the children; and the fact that pregnant women represent a relatively small target market, where pregnancy and pregnancy-related pathologies are considered 'short-term', there is very little interest from the pharmaceutical industry to develop new drugs to treat pregnancy-related disorders. Indeed, a recent study highlighted that only 17 drugs were currently under development for treating obstetric-related problems, compared with 660 drugs being investigated for cardiovascular diseases; and in the last 20 years, only one new class of drug (oxytocin receptor antagonists) has been licensed to treat pregnancy-related conditions in Europe (Fisk and Atun 2008).

Preterm labour is a multi-factorial condition, and as demonstrated in the case of progesterone treatment, one treatment is not going to be applicable or effective for each woman. Improving our understanding of the underlying causes of preterm labour and improved identification of 'high-risk' women will hopefully lead to novel and more targeted treatments. As mentioned earlier, a further complication in attempting to stop preterm delivery is that in the presence of an intrauterine infection, delaying delivery could potentially leave the fetus in a hostile inflammatory environment, which may ultimately cause more harm. Therefore, if the primary aim of therapeutics for preterm labour is to improve outcome for the baby, then novel treatment options must focus not only on blocking myometrial contractions, but also resolve the inflammatory environment of the uterus to limit damage to the fetus. Taking these factors into consideration, and the hypothesis that many cases of preterm labour occur as a result of the premature activation of the inflammatory cascade proposed to occur normally at term, there is now growing interest in examining the potential of anti-inflammatory agents as a novel treatment option for preterm labour.

1.3.5 Novel therapeutic options to treat preterm labour: the potential of anti-inflammatory agents

Anti-inflammatory agents are used to treat a vast number of inflammation-associated pathologies, including rheumatoid arthritis, inflammatory bowel diseases, airway inflammation, cardiovascular disease and some cancers; and in recent years investigating the potential of anti-inflammatory agents as exciting novel therapeutic options to treat preterm labour has become a major focus of premature birth research.

Interestingly, support for the hypothesis that anti-inflammatory agents may be useful novel therapeutic agents comes from two treatments already in use for preterm labour, NSAIDs and progesterone, both of which are proposed to mediate their effects by blocking inflammatory signalling.

Indomethacin is a non-selective COX inhibitor and is the most common NSAID used as a tocolytic agent in the treatment of preterm labour. The first reported use of indomethacin as a tocolytic drug was in 1974 (Zuckerman *et al.* 1974), and subsequent studies reported a promising reduction in preterm birth rates following the use of indomethacin, compared to placebo (Niebyl *et al.* 1980, Panter *et al.* 1999) and compared to other tocolytic drugs (King *et al.* 2005). However, these trials have been small, and the data is not conclusive. Furthermore, there are safety concerns associated with the use of indomethacin, as it has been associated with adverse effects on the fetus, including premature closure of the ductus arteriosus and necrotizing enterocolitis (Norton *et al.* 1993, Koren *et al.* 2006). Although a recent systematic review assessing the safety of indomethacin as a tocolytic agent did not find increased risk of adverse neonatal outcomes, again this study emphasised that the small number of randomised trials with limited statistical power that have been carried out to date do not allow firm conclusions to be made on the safety of indomethacin as a tocolytic agent (Loe *et al.* 2005). As previously discussed, prostaglandins are key regulators of myometrial contractility, therefore inhibition of COX enzymes using NSAIDs resulting in inhibition of prostaglandin synthesis, is thought to be the key mechanism by which NSAID treatment reduces preterm labour. However, there is also some evidence that they can act directly to inhibit calcium channel currents in human myometrial cells (Sawdy *et al.* 1998), and they may also be capable of inhibiting NF-κB activity, as has been shown in other systems (Yin *et al.* 1998, Loudon *et al.* 2003). Therefore, although the use of indomethacin demonstrates some success of using anti-inflammatory agents to delay preterm delivery, the risk of adverse neonatal outcomes associated with its use suggest that continued research is required into more suitable alternatives.

As discussed above, progesterone is currently being intensively investigated as a promising treatment option for preterm labour. Interestingly, although it has been used clinically for a number of years, the mechanisms by which progesterone delays preterm delivery are relatively poorly understood. Animal models and *in vitro* studies are now being used to elucidate the underlying mechanisms of progesterone action,

many of which appear to be related to anti-inflammatory actions of progesterone in the utero-placental tissues. Using a mouse model of LPS-induced preterm labour, Elovitz *et al.* demonstrated that pre-treatment with the progestin medroxyprogesterone acetate (MPA), delayed preterm delivery. MPA treatment significantly reduced the LPS-induced expression of several inflammatory genes which are normally up-regulated with labour, *Cox-2, Il-1β* and *Tnf-α* in the uterus and cervix, and connexin-43 mRNA expression in the uterus; and MPA treatment inhibited cervical ripening (Elovitz and Wang 2004). Using the same model, they also reported that MPA decreased the LPS-induced expression of *Tlr-2* and *Tlr-4* in the cervix and placenta (Elovitz and Mrinalini 2005), highlighting that in the presence of an intrauterine infection, MPA may reduce the rate of preterm birth by decreasing inflammatory mediator production, reducing expression of contraction-associated genes and inhibiting cervical ripening. These anti-inflammatory actions of MPA on utero-placental inflammation and cervical ripening have been confirmed in subsequent studies using the same animal model (Elovitz and Mrinalini 2006, Elovitz and Gonzalez 2008, Xu *et al.* 2008, Yellon *et al.* 2009).

MPA has also been reported to have anti-inflammatory actions *in vitro* where MPA treatment of human myometrial samples significantly suppressed LPS-induced production of IL-1β, IL-6 and IL-8 (Youssef *et al.* 2009). A recent study by Norman *et al,* reported that prolonged progesterone administration *in vivo* to women from 24-34 weeks of pregnancy resulted in decreased myometrial expression of connexin proteins, and down-regulated expression of CD11b on neutrophils, thus altering the ability of the myometrium to efficiently contract, and possibly attenuating neutrophil recruitment to the uterus (Norman *et al.* 2011). Additionally, progesterone treatment has also been shown to significantly attenuate IL-1β–induced IL-8 and PGE_2 production and COX-2 expression in lower segment fibroblast cells and amnion cells collected from pre-labour biopsies at term (Loudon *et al.* 2003). These anti-inflammatory actions of progesterone may involve negative interactions between the progesterone receptor and NF-κB, which have been previously reported in other cell systems (Kalkhoven *et al.* 1996), and in human amnion cells *in vitro* (Allport *et al.* 2001). Interestingly, there is also some evidence that progesterone treatment may be capable of modulating FIRS, where progesterone pre-treatment has been reported to decrease IL-6 production in feto-placental arteries (Shields *et al.* 2005, Gotkin *et al.* 2006) and in fetal mononuclear cells *in vitro* (Schwartz *et al.* 2009); this would be of particular importance in the context of intrauterine infection. Whilst further work is required to elucidate fully how

progesterone treatment delays preterm delivery, taken together these studies highlight, anti-inflammatory actions appear to be important underlying mechanisms of progesterone action in the utero-placental tissues, suggesting that progesterone can be considered as an anti-inflammatory agent in the context of preterm labour.

Therefore, although indomethacin and progesterone are two treatments with strong anti-inflammatory actions, neither treatment have yet made a significant impact on improving neonatal outcome. So, as previously mentioned, given the current lack of effective treatment options to delay preterm delivery and improve neonatal outcome, there has been intensive research over the last decade examining a large number of anti-inflammatory agents as novel therapeutic options. The reason why these drugs are thought to have greater potential to treat preterm delivery is that they focus on targeting the underlying cause of the premature initiation of labour (the inflammatory cascade), rather than the end-point (myometrial contractions). The use of animal models has been invaluable in investigating the potential of anti-inflammatory treatments to delay delivery *in vivo*, and many promising treatments options have been proposed. These can be broadly categorised into two groups: the administration of agents that aim to directly block or antagonise pro-inflammatory signalling; and the administration of endogenous anti-inflammatory agents. Evidence demonstrating the potential of anti-inflammatory mediators as novel therapeutic options for the treatment of preterm labour has recently been comprehensively reviewed (Keelan 2011, Rinaldi *et al.* 2011, MacIntyre *et al.* 2012). The following section will briefly detail a number of the anti-inflammatory interventions investigated to date, with particular focus on two endogenous anti-inflammatory agents, IL-10 and lipoxins.

1.3.5.1 Blocking IL-1β and TNF-α

Given the evidence that expression of both IL-1β and TNF-α is increased in gestational tissues and amniotic fluid at both term and preterm labour (Maymon *et al.* 1999, Young *et al.* 2002, Osman *et al.* 2003, Marconi *et al.* 2011); and administration of either cytokine can induce preterm labour in animal models (Romero *et al.* 1991, Silver *et al.* 1994, Yoshimura and Hirsch 2005, Sadowsky *et al.* 2006), studies have investigated whether blocking IL-1β or TNF-α signalling can attenuate inflammatory signalling in gestational tissues *in vitro,* and preterm delivery rates *in vivo.*

Anti-TNF-α therapy has been used clinically as a therapeutic option to treat a number of chronic inflammatory conditions, including inflammatory bowel disease and rheumatoid arthritis (Ali *et al.* 2013). Studies in animal models have reported that blocking TNF-α, either using anti-TNF-α antibodies, soluble TNF receptors, or pentoxifylline (an inhibitor of TNF-α synthesis), improved LPS-mediated adverse pregnancy outcomes in mice, including fetal resorption, intrauterine growth restriction and fetal mortality (Gendron *et al.* 1990, Silver *et al.* 1994, Xu *et al.* 2006). In the context of preterm labour there have been conflicting reports on the effectiveness of anti-TNF-α treatment to delay delivery. Blocking TNF-α action using a soluble TNF-α receptor Fc fusion protein did not prevent LPS-induced preterm delivery in a mouse model (Fidel *et al.* 1997); whereas more recently, pre-treatment with an anti-TNF-α antibody has been reported to significantly decrease the number of LPS-induced preterm deliveries, improve pup survival and decrease the expression of several pro-inflammatory mediators in the uterus (Holmgren *et al.* 2008).

Similarly, blocking IL-1β signalling has also been used therapeutically in the treatment of inflammatory conditions and the endogenous IL-1 receptor antagonist (IL-1Ra) is clinically approved for the treatment of rheumatoid arthritis (Dinarello 2011). Early studies by Romero *et al.* demonstrated that IL-1Ra decreased IL-1β-induced PGE_2 production in amnion and chorion explant culture *in vitro* (Romero *et al.* 1992); and prevented IL-1-induced preterm birth in a mouse model (Romero and Tartakovsky 1992). However, subsequent studies have proved less successful, where IL-1Ra administration did not prevent LPS-induced preterm labour in a mouse model (Fidel *et al.* 1997); LPS-induced preterm delivery rates were unaffected in transgenic mice overexpressing IL-1Ra (Yoshimura and Hirsch 2005); and similarly, infection-induced preterm delivery rates were unaffected in IL-1β and IL-1 receptor knockout mice (Reznikov *et al.* 1999, Hirsch *et al.* 2002), suggesting that IL-1β signalling is not required for the induction of preterm labour in response to intrauterine infection. Interestingly, the rate of infection-induced preterm delivery in double knockout mice lacking both the type 1 receptors for IL-1 and TNF was found to be significantly reduced compared to the wild-type control mice; and the knockout mice also had significantly lower myometrial expression of COX-2 (Hirsch *et al.* 2006). These studies highlight the complexity of the cytokine interactions associated with preterm labour, and suggest that due to the redundancy of the cytokine network, simply attempting to

block one cytokine is unlikely to be a useful therapeutic strategy in the treatment of preterm labour.

1.3.5.2 NF-κB inhibitors

As discussed earlier, the NF-κB signalling pathway has a key role in regulating the inflammatory response associated with the onset of parturition, therefore making it an attractive therapeutic target in the search for novel treatments for inflammation-associated preterm labour. To date, more than 700 NF-κB inhibitors have been described which target the signalling pathway at various points (Gupta *et al.* 2010); and NF-κB inhibitors have been investigated as novel therapeutic options in the treatment of rheumatoid arthritis (Okamoto 2006), inflammatory lung diseases (Decramer *et al.* 2005, Demedts *et al.* 2005) and inflammatory bowel disease (Egan and Sandborn 1998, Atreya *et al.* 2008). The potential of a number of different NF-κB inhibitors to control inflammatory signalling in human gestational tissues and to delay delivery in animal models has been investigated; this section shall discuss the evidence regarding *N*-acetylcysteine (NAC) and sulfasalazine (SSZ) as potential novel therapeutic options to treat inflammation-induced preterm labour.

NAC is an antioxidant and anti-inflammatory mediator involved in regulating oxidative stress by increasing glutathione levels (Millea 2009). NAC treatment has been shown to inhibit nuclear translocation of NF-κB in a number of cell systems (Origuchi *et al.* 2000, Martinez-Losa *et al.* 2007). NAC has been used therapeutically in the treatment of a variety of inflammation-related pathologies, including chronic obstructive pulmonary disease (Decramer *et al.* 2005), influenza (De Flora *et al.* 1997), and pulmonary fibrosis (Demedts *et al.* 2005); and has been investigated as a treatment for a number of reproductive pathologies including polycystic ovarian syndrome (Rizk *et al.* 2005), recurrent pregnancy loss (Amin *et al.* 2008) and pre-eclampsia (Rumiris *et al.* 2006). *In vitro,* NAC treatment of human fetal membranes inhibited NF-κB DNA-binding activity, which subsequently attenuated the LPS-induced pro-inflammatory cytokine and prostaglandin release and MMP-9 activity in the fetal membranes (Lappas *et al.* 2003). In animal models *in vivo,* NAC pre-treatment delayed LPS-induced preterm labour and improved pup survival (Buhimschi *et al.* 2003, Chang *et al.* 2011); attenuated the LPS-induced inflammatory response in fetal plasma and myometrium

(Beloosesky *et al.* 2006, Chang *et al.* 2011); and protected the fetal brain from inflammation-induced injury (Chang *et al.* 2011). These studies highlight the potential of NAC to not only delay preterm delivery, but also suggest it may be useful in protecting the fetus from infection-associated brain injury. Interestingly, a recently published study reported that administration of NAC (in conjunction with metronidazole and 17-hydroxyprogestrone caproate) to women with a history of preterm labour and bacterial vaginosis improved outcome. Gestational age at delivery, and fetal weight were both significantly increased in the NAC treatment group, compared to the placebo group, and there were also significantly fewer neonatal deaths in the NAC group (Shahin *et al.* 2009). Although this was a relatively small study, this is the first clinical study to demonstrate an effect of NAC in delaying preterm labour, suggesting it could be a useful treatment option to use along-side other agents, such as progesterone.

Sulfasalazine is a 5-aminosalicylic acid drug that has been used for many years as an anti-inflammatory treatment for inflammatory bowel disease (Egan and Sandborn 1998) and rheumatoid arthritis (Box and Pullar 1997). Sulfasalazine was first identified as a potent and specific NF-κB inhibitor in 1998 (Wahl *et al.* 1998), and has subsequently been shown to act by directly inhibiting the IKK kinases, IKKα and IKKβ (Weber *et al.* 2000). The anti-inflammatory actions of SSZ have been demonstrated in human gestational tissues *in vitro*, where SSZ treatment inhibited NF-κB translocation to the nucleus and subsequently attenuated LPS-induced pro-inflammatory cytokine and prostaglandin release in placenta, amnion and choriodecidua explants (Lappas *et al.* 2002, Keelan *et al.* 2009). In addition SSZ treatment inhibited IL-8 production by endocervical epithelial cells in response to a variety of bacterial pathogens (Peltier *et al.* 2009). In a mouse model of preterm labour, SSZ pre-treatment significantly reduced the rate of *E.coli*-induced preterm labour and was associated with a modest (non-significant) increase in pup survival, compared with mice receiving *E.coli* alone (Nath *et al.* 2010).

Taken together, these studies demonstrate the potential of drugs such as NAC and SSZ to target the NF-κB signalling pathway as a potential novel therapeutic strategy in the treatment of preterm labour.

1.3.5.3 15d-PGJ$_2$

15-deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2) is a metabolite of PGD_2 that has been shown to have potent anti-inflammatory actions in a variety of cell systems (Scher and Pillinger 2005). 15d-PGJ_2 is produced in the later phase of an inflammatory response *in vivo* and is therefore postulated to play an important role in the resolution of inflammatory responses (Gilroy *et al.* 1999), as it has been shown to stimulate granulocyte apoptosis (Ward *et al.* 2002) and promote macrophage clearance from the site of inflammation (Rajakariar *et al.* 2007). The mechanisms underlying 15d-PGJ_2 action are not clear. 15d-PGJ_2 has been reported to act via a prostaglandin D receptor, chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2) (Monneret *et al.* 2002); and it has been proposed that 15d-PGJ_2 may exert its actions via interactions with intracellular targets, such as the transcription factors, peroxisome proliferator-activated receptors (PPARs) and NF-κB (Scher and Pillinger 2005). A major anti-inflammatory mechanism of 15d-PGJ_2 action is through inhibition of NF-κB, either directly by inhibiting IKKβ activity (Rossi *et al.* 2000); or indirectly via activation of PPAR-γ, which has been shown to antagonise the activity of transcription factors involved in pro-inflammatory gene transcription, such as NF-κB (Ricote *et al.* 1998). Research into the use of 15d-PGJ_2 as an anti-inflammatory therapeutic agent is still at a relatively early stage, but several *in vivo* studies have now reported that 15d-PGJ_2 administration was associated with reduced disease severity in animal models of a number of inflammation-associated pathologies, including chronic lung injury (Wang *et al.* 2009) and arthritis (Kawahito *et al.* 2000).

During pregnancy, PGD_2 is produced by the placenta and decidua (Mitchell *et al.* 1982, Norwitz *et al.* 1992) and both PGD_2 and 15d-PGJ_2 have been detected in the amniotic fluid of women at term (Helliwell *et al.* 2006). The anti-inflammatory actions of 15d-PGJ_2 have been demonstrated in human gestational tissue culture *in vitro*, where 15d-PGJ_2 treatment has been reported to down-regulate inflammatory gene expression, PGE_2 release, and inhibit NF-κB in human amnion, myometrial and trophoblast cells, and amnion-derived WISH cells (Ackerman *et al.* 2005, Berry *et al.* 2005, Lindstrom and Bennett 2005, Helliwell *et al.* 2006); and in placenta, amnion and choriodecidua explants (Lappas *et al.* 2006). Additionally, a recent study demonstrated that 15d-PGJ_2 treatment down-regulated production of IFN-γ, TNF-α and reduced NF-κB activation in peripheral blood mononuclear cells obtained from pregnant women (Sykes *et al.* 2012).

In a mouse model of LPS-induced preterm labour, Pirianov *et al.* recently demonstrated that treatment with 15d-PGJ_2 significantly delayed preterm delivery and improved pup survival rates. 15d-PGJ_2 treatment was associated with: decreased IKKβ activity in the myometrium and fetal brain; inhibition of LPS-induced myometrial pro-inflammatory cytokine production; and inhibition of cPLA2 and COX-2 expression in the myometrium, suggesting a negative feedback mechanism on prostaglandin synthesis (Pirianov *et al.* 2009). In a follow-up study, Sykes *et al.* recently reported that these anti-inflammatory actions of 15d-PGJ_2 in the mouse model of LPS-induced preterm labour do not appear to be mediated via CRTH2 (Sykes *et al.* 2013)

Overall, the *in vitro* and *in vivo* evidence to date suggests that 15d-PGJ_2 could be an exciting novel therapeutic agent to treat inflammation-induced preterm labour which ultimately may improve neonatal outcome by protecting the fetal brain from inflammation-induced damage. Further research is required to elucidate the underlying mechanisms of 15d-PGJ_2 actions in the gestational tissues and fully investigate its potential as a therapeutic option.

1.3.5.4 IL-10

IL-10 was initially discovered in 1989 as a novel product of mouse Th2 cells which suppressed inflammatory cytokine production from Th1 cells, and was originally named 'cytokine synthesis inhibitory factor' (Fiorentino *et al.* 1989). Subsequent studies confirmed these inhibitory actions of IL-10, and it is now considered as the classical anti-inflammatory cytokine. IL-10 is primarily produced by monocytes, macrophages and some T-cell subsets *in vivo*, but other immune cells including dendritic cells, B-cells, NK cells, mast cells and neutrophils have also been reported to produce IL-10 (Sabat *et al.* 2010). Monocytes and macrophages are proposed to be the main target cells of immuno-modulatory IL-10 signalling, although IL-10 has also been shown to act on other immune cell populations, including T cells, B cells, NK cells and neutrophils via activation of its specific IL-10 receptor (Sabat *et al.* 2010). The key actions of IL-10 on monocytes and macrophages include: inhibition of pro-inflammatory cytokine production (de Waal Malefyt *et al.* 1991, Fiorentino *et al.* 1991); promoting the release of anti-inflammatory mediators such as IL-1Ra (Jenkins *et al.* 1994); and enhancing the phagocytic ability of monocytes/macrophages (Buchwald *et al.* 1999). Upon IL-10 binding to the IL-10 receptor, the anti-inflammatory actions of

IL-10 are largely mediated through the activation of the signal transducer and activator of transcription (STAT) transcription factors, particularly STAT3. The activated STAT transcription factors translocate to the cell nucleus and bind to STAT-binding elements in the promoter regions of target genes, such as the suppressor of cytokine signalling 3 (SOCS3), which can act to inhibit further cytokine synthesis (Sabat *et al.* 2010). IL-10 signalling has also been shown to inhibit NF-κB activation in a number of different cell systems (Wang *et al.* 1995, Yoshidome *et al.* 1999, Al-Ashy *et al.* 2006). Given the potent anti-inflammatory actions of IL-10, it has been investigated clinically as a novel therapeutic option in the treatment of a number of inflammation-associated pathologies including Crohn's disease (Buruiana *et al.* 2010), psoriasis (Kimball *et al.* 2002) and chronic hepatitis C infection (Nelson *et al.* 2000); however the results of these trials have been mixed, suggesting that further studies are required to fully elucidate the therapeutic potential of IL-10 therapy (Asadullah *et al.* 2003).

Although much research has focussed on identifying the pro-inflammatory mediators associated with the onset of parturition, both at term and preterm, over recent years there has been a growing interest in understanding how these pro-inflammatory signals may be regulated *in vivo*, and what role IL-10 may play in controlling the inflammation surrounding parturition. The important role of IL-10 signalling in pregnancy has been recently reviewed (Thaxton and Sharma 2010, Kalkunte *et al.* 2011). During pregnancy, IL-10 expression and secretion has been reported in placental villous cytotrophoblasts, decidua, chorion, uterine NK cells and decidual macrophages (Roth *et al.* 1996, Trautman *et al.* 1997, Denison *et al.* 1998, Hanna *et al.* 2000, Lidstrom *et al.* 2003); and IL-10 has been detected in amniotic fluid (Greig *et al.* 1995, Dudley *et al.* 1997, Gotsch *et al.* 2008). There are conflicting reports of changes in IL-10 expression through pregnancy and with the onset of labour. Placental IL-10 expression was found to be higher in first and second trimester placental tissue, compared to term placenta (Hanna *et al.* 2000); and IL-10 production from choriodecidual tissue was reported to be decreased with labour, compared with tissue obtained before labour (Simpson *et al.* 1998). Whilst in the amniotic fluid some studies have reported increased amniotic fluid IL-10 in the third trimester, compared to earlier stages of gestation (Greig *et al.* 1995); others have reported no change across gestation (Dudley *et al.* 1997, Gotsch *et al.* 2008). Increased amniotic fluid IL-10 has also been reported in association with spontaneous term and preterm labour (Greig *et al.* 1995, Gotsch *et al.* 2008). IL-10 deficiency has been associated with a number of adverse

pregnancy outcomes including, pre-eclampsia, recurrent spontaneous abortions and preterm labour (Hill *et al.* 1995, Hennessy *et al.* 1999, Makhseed *et al.* 2003); highlighting that IL-10 plays a key role in maintaining the balance between pro-and anti-inflammatory signalling throughout pregnancy.

Evidence that IL-10 can regulate inflammatory signalling in the gestational tissues comes from *in vitro* studies which have demonstrated that treatment with IL-10 attenuated the LPS-induced production of IL-6, TNF-α, IL-1β, MMPs and PGE_2 in the fetal membranes (Fortunato *et al.* 1996, Brown *et al.* 2000, Fortunato *et al.* 2001); inhibited LPS-induced TNF-α and IL-1β expression in choriodecidual explants (Sato *et al.* 2003); and reduced IL-1β-induced COX-2 and PGE_2 and basal MMP-9 production in cultured placental trophoblasts (Pomini *et al.* 1999, Roth and Fisher 1999).

In addition to these *in vitro* studies, animal models have been utilised to further investigate the effect of IL-10 administration on preterm labour. Using *Il-10* knockout mice, Robertson *et al.* demonstrated that IL-10 played an essential role in response to intrauterine infection (Robertson *et al.* 2006). The *Il-10* knockout mice required a 10-fold lower dose of LPS to induce 50% fetal loss compared with wild-type controls, and LPS-treatment induced greater pro-inflammatory cytokine production in the serum, uterus and placenta, compared to the wild-type animals; these differences between the *Il-10* knockout mice and wild-type mice were attenuated by the administration of recombinant IL-10. Interestingly, the administration of exogenous IL-10 to both wild-type and *Il-10* knockout animals significantly reduced the number of LPS-induced fetal losses and down-regulated inflammatory cytokine synthesis (Robertson *et al.* 2006). In a rat model of LPS-induced preterm labour, administration of IL-10, either at the same time as LPS, or 24 hours later, significantly delayed the time to delivery and improved pup survival rates (Terrone *et al.* 2001). Also using a similar rat model, IL-10 was found to inhibit *E.coli*-induced white matter damage in rat pup brains by inhibiting microglial activation (Rodts-Palenik *et al.* 2004, Pang *et al.* 2005). Additionally, in a non-human primate model, treatment with IL-10 attenuated IL-1β-induced uterine contractions, reduced the levels of TNF-α and prostaglandins in the amniotic fluid and reduced leukocyte influx into the amniotic fluid (Sadowsky *et al.* 2003).

Collectively, these studies highlight the potential of IL-10 as a novel anti-inflammatory treatment for preterm labour, which can block the inflammatory signalling cascade

associated with the initiation of parturition, and more importantly could protect the fetal brain from the adverse effects of intrauterine infection and/or inflammation.

1.3.5.5 Lipoxins

To prevent chronic inflammation, which can often lead to tissue injury, acute inflammation must be halted once the original stimulus or infection has been neutralised. This phase is referred to as the resolution of inflammation, and was previously believed to be a passive process, which occurred as pro-inflammatory signals gradually fizzled out. However, in recent decades it has become increasingly clear that the resolution of an inflammatory response is a highly co-ordinated, active process involving the production of mediators with specific pro-resolution and anti-inflammatory actions (Gilroy *et al.* 2004, Serhan *et al.* 2008). Lipoxins were the first family of lipid mediators recognised to have dual-acting anti-inflammatory and pro-resolution actions. Lipoxins, which are trihydroxy tetraene-containing eicosanoids, were first identified in 1984 as novel derivatives of arachidonic acid that were proposed to be involved in regulating specific cellular responses in neutrophils (Serhan *et al.* 1984, Serhan *et al.* 1984). Subsequent studies have identified a number of anti-inflammatory and pro-resolution actions lipoxins have on a range of cell types

Lipoxins are derived from arachidonic acid via the action of their specific lipoxygenase enzymes, 5-lipoxygenase (5-LOX), 12-lipoxygenase (12-LOX) and 15-lipoxygenase (15-LOX). In humans two main pathways of lipoxin synthesis have been identified, both of which involve transcellular interactions between leukocytes and other cell types. The first pathway, which was identified in blood vessels, involves platelet-leukocyte interactions, where arachidonic acid is oxygenated by 5-LOX in leukocytes resulting in the production of leukotriene A_4, which can then be rapidly converted by the actions of 12-LOX in platelets into lipoxin A_4 and lipoxin B_4. The second pathway, identified in mucosal tissues, involves epithelial cell-leukocyte interactions, where arachidonic acid is oxygenated by 15-LOX in epithelial cells, resulting in the formation of the intermediate lipid 15S-HETE, which can then be rapidly converted to lipoxin A_4 and lipoxin B_4 by 5-LOX in leukocytes. A third pathway of lipoxin occurs in the presence of aspirin, which results in the production of aspirin-triggered or 15-epi-lipoxins, which are epimers of native lipoxin. In the presence of aspirin, COX-2 becomes irreversibly acetylated, and drives arachidonic acid down the lipoxin synthesis pathway, rather

than the prostaglandin synthesis pathway by producing 15R-HETE, this intermediate can then be rapidly converted by 5-LOX in activated leukocytes to 15-epi-lipoxin A_4 and 15-epi-lipoxin B_4 (Chiang *et al.* 2005). Epi-lipoxins have many of the same anti-inflammatory and pro-resolution actions as lipoxins and it has been proposed that the generation of epi-lipoxins in the presence aspirin may underlie many of its therapeutic actions (Claria and Serhan 1995, Chiang *et al.* 2005). The lipoxin synthesis pathways are summarised in Figure 1.3.

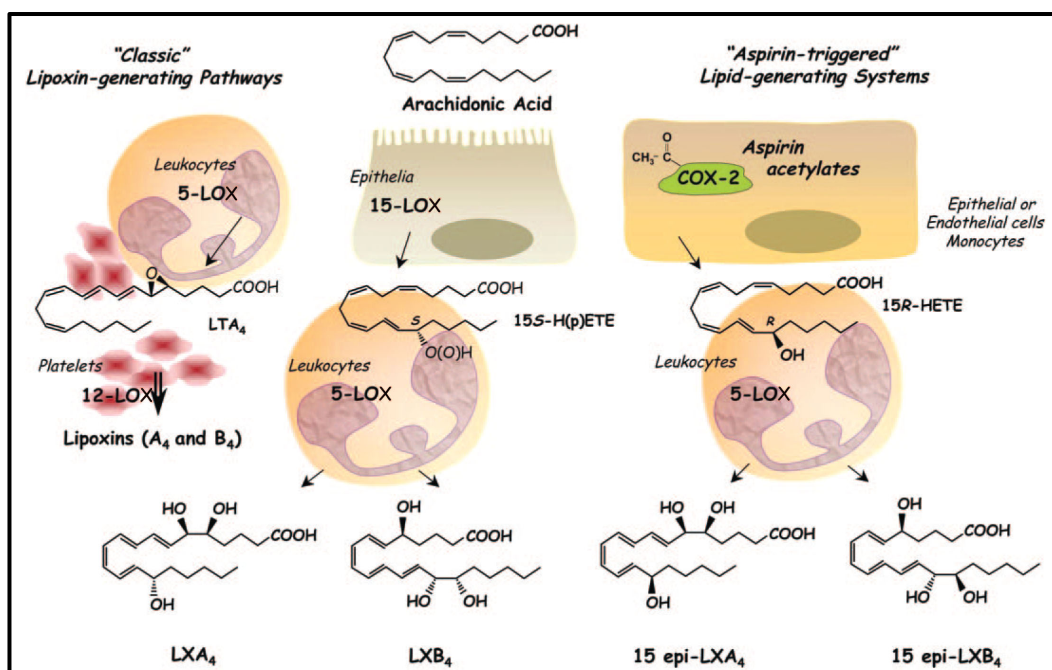

Figure 1.3 - Schematic representation of the three pathways of lipoxin biosynthesis. Lipoxins are synthesised from arachidonic acid via the actions of specific lipoxygenase enzymes, 5-LOX, 12-LOX and 15-LOX, during cell-cell interactions. A third pathway of lipoxin synthesis occurs in the presence of aspirin where COX-2 becomes acetylated and drives arachidonic acid synthesis down the lipoxin-generating pathway, rather than the prostaglandin pathway, resulting in the production of aspirin-triggered, or 15-epi-lipoxins. Adapted from Chiang *et al.,* 2006.

The actions of lipoxin A_4 are mediated via binding to a specific G-protein coupled receptor, FPR2/ALX, which is a member of the formyl peptide receptor family (Ye *et al.* 2009); although there is evidence that lipoxin A_4 can bind to other receptors including

the cysteinyl leukotriene receptors (Badr *et al.* 1989, McMahon *et al.* 2000) and nuclear aryl carbon receptor (Schaldach *et al.* 1999, Machado *et al.* 2006, McBerry *et al.* 2012), the anti-inflammatory and pro-resolution actions of lipoxins acting via FPR2/ALX are the best characterised. FPR2/ALX is expressed on a wide number of cell types in the body, including monocytes, macrophages, neutrophils, lymphocytes, microglial cells, epithelial cells and fibroblasts in a variety of tissues (Migeotte *et al.* 2006). During the course of an inflammatory response, binding of lipoxin A_4 to FPR2/ALX elicits cell specific actions to promote resolution. Lipoxins have been shown to inhibit neutrophil activation, adhesion and chemotaxis (Lee *et al.* 1989, Colgan *et al.* 1993, Papayianni *et al.* 1996, Filep *et al.* 2005), while increasing neutrophil apoptosis (El Kebir *et al.* 2007); in contrast lipoxins have been reported to stimulate monocyte adhesion and migration (Maddox and Serhan 1996, Maddox *et al.* 1997), inhibit monocyte apoptosis (Simoes *et al.* 2010), and stimulate the non-phlogistic phagocytosis of apoptotic neutrophils by macrophages (Godson *et al.* 2000). Additionally, lipoxins have also been reported to down-regulate the production of a number of pro-inflammatory mediators, such as IL-1β, TNF-α IL-6, IL-8 and MMPs in synovial fibroblasts (Sodin-Semrl *et al.* 2000), endothelial cells (Wu *et al.* 2008), astrocytes (Decker *et al.* 2009) and intestinal epithelial cells *in vitro* (Kure *et al.* 2009). The mechanisms by which lipoxins are thought to regulate inflammatory mediator production have been reported to include inhibition of the NF-κB pathway (Gewirtz *et al.* 2002, Wu *et al.* 2008, Kure *et al.* 2009, Wang *et al.* 2011); activation of PPAR-γ (Sobrado *et al.* 2009); and activation of STAT3, resulting in increased SOCS3 expression (Li *et al.* 2011).

In vivo, lipoxin administration has been associated with decreased pro-inflammatory mediator production and reduced disease severity in animal models of a number of inflammation-associated pathologies including asthma (Levy *et al.* 2002, El Kebir *et al.* 2009), arthritis (Conte *et al.* 2010), Alzheimer's disease (Medeiros *et al.* 2013) and inflammatory bowel disease (Gewirtz *et al.* 2002), among others. These studies demonstrate the therapeutic potential of lipoxins as a novel treatment for inflammation-associated pathologies.

The role of lipoxins in regulating inflammatory events in the reproductive tissues has been less well explored. Given that many reproductive events, including ovulation, menstruation, implantation and the initiation labour, are associated with inflammation (Jabbour *et al.* 2009), it is possible that lipoxins are important mediators in controlling

these inflammatory events and driving the resolution and repair of these tissues following inflammation. Evidence demonstrating the potential role of lipoxins in regulating these inflammatory reproductive events has been recently reviewed (Hutchinson *et al.* 2011). Several studies have examined the role of lipoxins in regulating inflammatory signalling in the endometrium. Macdonald *et al.* reported that FPR2/ALX expression is increased in glandular epithelial cells and stromal cells in the menstrual endometrium and first-trimester decidua; and showed the lipoxin A_4 suppressed phorbol myristate acetate-induced expression of IL-6 and IL-8 in human endometrial and decidual tissue explants (Macdonald *et al.* 2011). Lipoxins have also been investigated therapeutically in animal models of endometriosis (Chen *et al.* 2010, Xu *et al.* 2012). Additionally, lipoxins have recently been identified as novel ERα modulators (Russell *et al.* 2011), and Xiong *et al.* reported that lipoxin A_4 blocked embryo implantation in a mouse model by controlling ERα activity (Xiong *et al.* 2013).

In gestational tissues, lipoxygenase enzyme expression has been detected. Expression of 5-LOX has been reported throughout pregnancy in the human amnion and chorio-decidua tissue, with increased 5-LOX mRNA and protein in chorio-decidual tissue collected after labour at term, compared with before labour (Brown *et al.* 1999); and decreased 15-LOX has been reported in myometrial smooth muscle cells at term and during labour (Lei and Rao 1992). Additionally, 5-LOX and 12-LOX have also been detected in baboon myometrium, cervix, chorion and decidua at term (Smith *et al.* 2001). Taken together, these studies demonstrate that the utero-placental tissues are capable of lipoxin synthesis, suggesting lipoxins may play a role in regulating the inflammatory events surrounding parturition. Indeed, a recent study from our laboratory has provided further evidence that lipoxins may be important mediators in parturition. Maldonado-Perez *et al.* reported that circulating serum lipoxin levels were elevated in pregnant women, compared with non-pregnant women, and FPR2/ALX expression was increased in myometrial tissue obtained from women during term labour, compared to non-labouring women (Maldonado-Perez *et al.* 2010). Additionally, the same study demonstrated that lipoxin A_4 is capable of regulating inflammatory signalling in myometrium, where pre-treatment with lipoxin A_4 was able to significantly attenuate LPS-induced IL-6 and IL-8 expression in cultured myometrial explants *in vitro* (Maldonado-Perez *et al.* 2010).

Collectively, these preliminary data showing lipoxins can regulate inflammation in myometrial tissue obtained at term, alongside evidence from animal models demonstrating lipoxins are useful in treating other inflammation-associated pathologies, suggest that lipoxins could be an exciting novel therapeutic option in the treatment of inflammation-induced preterm labour. Lipoxins have the potential to be more useful than simple anti-inflammatory agents alone due to their ability to promote the resolution of inflammation, suggesting that rather than simply stopping the inflammatory cascade associated with labour, they could also act to resolve the hostile inflammatory intrauterine environment, thereby limiting inflammation-induced neonatal injury. Indeed studies have demonstrated the potential for lipoxins to regulate inflammatory signalling in a human astrocytoma cell line *in vitro* (Decker *et al.* 2009) and also in a rat model of brain injury *in vivo* (Ye *et al.* 2010), therefore lipoxins may be able to protect the fetal brain from inflammation-induced injury.

1.3.6 Animal models of preterm labour

Animal models have been extensively used in the field of premature birth research, both to investigate the underlying mechanisms surrounding the onset of preterm labour and to test interventions to stop preterm delivery. The pros and cons of the various animal models of preterm labour have been extensively reviewed (Elovitz and Mrinalini 2004, Mitchell and Taggart 2009, Ratajczak *et al.* 2010). Obviously due to species differences, animal models can never fully recapitulate the clinical scenario of preterm labour in women, but given the practical and ethical issues surrounding the collection of tissues from pregnant women and the concerns about testing novel therapies in pregnant women, animal models have been invaluable in providing insight into the molecular events surrounding the onset of labour and providing proof of principle of therapeutic strategies. Several species have been used as models of preterm labour, including sheep, rodents and non-human primates. The advantages and disadvantages of each model will be briefly discussed.

Sheep have been primarily used to study the mechanisms surrounding the onset of parturition at term, and the use of this animal model demonstrated a role for the fetus in regulating the timing of parturition. Work by Liggins *et al.* more than 40 years ago identified a role for the fetal hypothalamic-pituitary axis in triggering the onset of parturition (Liggins 1968, Liggins *et al.* 1973). As the fetus matures during late

gestation, there is increased production of adrenocorticotropic hormone (ACTH) in the fetal circulation, which stimulates the fetal adrenal gland to produce increased levels of cortisol. This cortisol acts to promote maturation of the fetal lungs and stimulates progesterone metabolism by the placenta, resulting in a drop in progesterone levels and an increase in estrogen levels, which leads to the onset of parturition (Mitchell and Taggart 2009). The importance of ACTH and cortisol in initiating parturition in sheep was demonstrated by studies showing infusion of either of these hormones successfully induced preterm labour (Liggins 1968). The main advantage of the sheep as a model to study human parturition is the large size, which enables surgical manipulation of both the fetus and the mother, allowing for longitudinal sampling of both across gestation. Additionally, there are a number of similarities between human and sheep pregnancy which include: the placenta being the source of progesterone throughout most of pregnancy; a small number of fetuses; and the relatively long gestational period, which is more similar to humans than other models. However, compared to other models, sheep are relatively expensive and take longer to breed. Furthermore, unlike in humans, the onset of parturition is dependent upon systemic progesterone withdrawal; and the role of ACTH and cortisol in triggering the onset of parturition appears not to apply to humans, as glucocorticoids do not induce labour in women (Elovitz and Mrinalini 2004, Mitchell and Taggart 2009, Ratajczak *et al.* 2010). Nevertheless, the sheep provides a useful model system to study the fetal response to intrauterine infection and the events surrounding fetal maturation (Schlafer *et al.* 1994, Kallapur *et al.* 2001, Grigsby *et al.* 2003, Westover *et al.* 2012).

Non-human primates have also been used in premature birth research. Intra-amniotic injection of bacteria or bacterial products such as LPS, and the pro-inflammatory cytokines IL-1β or TNF-α have been shown to induce preterm labour in rhesus monkeys (Gravett *et al.* 1994, Sadowsky *et al.* 2006), thus confirming the role of intrauterine infection and/or inflammation in the induction of preterm labour. The non-human primate model has also been used to test therapeutic interventions to delay preterm delivery, including TLR-4 antagonists (Adams Waldorf *et al.* 2008), IL-10 (Sadowsky *et al.* 2003) and oxytocin receptor antagonists (Reinheimer 2007). The main advantage of the non-human primate as a model for studying preterm labour is that it shares many similarities with human pregnancy, including: similar reproductive physiology; no requirement for systemic progesterone withdrawal; similar gestational length; and similar number of fetuses. Additionally, like the sheep, the non-human

primate model is amenable to chronic instrumentation and surgical interventions, thus allowing for longitudinal sampling of both mother and fetus and monitoring of myometrial contractions (Elovitz and Mrinalini 2004, Mitchell and Taggart 2009). However, the high cost of using non-human primates and the ethical considerations associated with their use has limited the widespread use of non-human primate models in the study of preterm labour.

Another commonly used animal model in the field of parturition research is the rodent model. Both mice and rats have been used, with mice being the most commonly used animal model in this field. The administration of both bacterial products and the pro-inflammatory cytokines IL-1β or TNF-α have been found to induce preterm labour in mice (Romero *et al.* 1991, Silver *et al.* 1994, Elovitz *et al.* 2003, Yoshimura and Hirsch 2005), making the mouse a useful model with which to study the mechanisms surrounding infection-induced preterm labour and to test novel therapeutic interventions for the treatment of preterm labour. Indeed, the mouse has been extensively used to test a number of therapeutic agents, as detailed earlier. Using the mouse as a model of preterm labour has several advantages over the other models already described, these include: a short gestational period; ease of breeding; their immune system has been relatively well characterised; and they can be genetically manipulated such that specific genes can be either knocked out or over-expressed to examine the role of particular genes in preterm labour (Hirsch and Wang 2005); thus making them a useful cost-effective model for the study of preterm labour. However, there are clearly physiological differences between human and mouse pregnancy which limit the usefulness of the mouse as a model of preterm labour, including: multiple fetuses, very short gestational period compared to the human; and dependence on systemic progesterone withdrawal for the initiation of term labour (Elovitz and Mrinalini 2004). Despite these physiological differences the requirement for progesterone withdrawal for the onset of preterm labour in the context of intrauterine infection has recently been questioned (Hirsch and Muhle 2002, Murphy *et al.* 2009, Gonzalez *et al.* 2011); suggesting that the mouse may be more relevant to study in this context, rather than in spontaneous term labour.

The guinea pig has also been recently proposed as a more suitable model for parturition research for a number of reasons, including: they do not require systemic progesterone withdrawal prior to the onset of parturition; they have a longer

gestational period compared with other rodents, which allows more opportunity for longitudinal sampling; and the guinea pig fetus born at term is more developmentally competent compared to other rodent species, making it more similar to the human (Mitchell and Taggart 2009). However, although the guinea pig could prove to be a more applicable model for future studies of preterm labour, the genome of the guinea pig is currently less well established than that of the mouse, which is limiting its widespread use as model of preterm labour.

Aside from the choice of which animal model to use to study preterm labour, in models of infection-induced preterm labour, consideration must also be given to the route of administration of the infectious agent, as each route of administration can model a different clinical scenario in women. Systemic administration of bacterial products via intra-peritoneal (i.p.) injection has been carried out in a number of animal models (Fidel *et al.* 1994, Schlafer *et al.* 1994, Kaga *et al.* 1996, Robertson *et al.* 2006); however, systemic infection is rare in women in preterm labour, limiting the applicability of i.p. administration as a model of infection-induced preterm labour (Elovitz and Mrinalini 2004). Therefore, since the majority of women with infection-associated preterm labour actually present with a subclinical, local intrauterine infection, the local administration of bacterial products is perhaps more appropriate. This has been carried out in a number of ways including intra-amniotic injection (Gravett *et al.* 1994), intrauterine administration (Elovitz *et al.* 2003), intracervical injection (Reznikov *et al.* 1999) and intravaginal administration (Gonzalez *et al.* 2011). Elovitz *et al.* have discussed the pros and cons of each method (Elovitz and Mrinalini 2004)*;* it is clear that, whichever method is chosen, a model of a localised infection within the uterus is more applicable and more relevant to the scenario in women.

Therefore, although there are considerable inter-species variations meaning that discoveries in animal models are not always easily translatable to humans, given the considerable practical and ethical limitations surrounding the collection of gestation tissues throughout pregnancy in humans, animal models continue to be an invaluable resource to this field of research. They have provided a greater understanding of the molecular mechanisms responsible for the onset of parturition both at term and preterm, and have been useful tools with which to investigate potential novel therapeutic interventions.

1.4 SUMMARY

This chapter has discussed the evidence that in humans the onset of labour is associated with a core inflammatory response at the maternal-fetal interface culminating in fetal membrane rupture, cervical ripening and dilation and myometrial contractions, which results in delivery of the fetus. The inappropriately timed initiation of these inflammatory pathways, often in response to a pathological intrauterine infection, can result in preterm birth. Despite decades of research, preterm birth remains the leading cause of neonatal mortality and morbidity worldwide and the currently available treatments have made little impact on reducing the incidence of preterm labour and there is limited evidence that they improve neonatal outcome.

Given the causal link between intrauterine infection and/or inflammation and preterm labour, there is growing interest in examining whether using anti-inflammatory treatments may prove more successful. Animal models have proved invaluable in this research, as they have provided a powerful tool to understand the mechanisms underlying infection-induced preterm labour and therefore have helped in the identification of appropriate targets for novel treatment options. Anti-inflammatory agents have the potential to be more effective than the currently used tocolytic drugs, as rather than simply targeting myometrial contractions, which are likely the physical endpoint of a complex biological inflammatory cascade, anti-inflammatory agents can target the underlying mechanisms occurring much earlier in the cascade and hopefully halt its progress before contractions are induced. Furthermore, anti-inflammatory agents which can also act to promote the resolution of inflammation, such as lipoxins, may have the added benefit of being capable of resolving the inflammatory *in utero* environment, thereby limiting exposure of the fetus to harmful inflammatory signals, which are associated with adverse outcomes.

Therefore, as preterm birth continues to affect millions of babies worldwide each year causing significant neonatal death and long-term morbidities, research must continue into improving our understanding of the multiple causes to enable the identification of novel treatment options which will be effective in delaying preterm labour and ultimately improving the long-term health prospects of the baby.

1.5 HYPOTHESIS AND AIMS

The work presented in this thesis was based on the hypothesis that preterm labour occurs as a result of a local intrauterine infection which stimulates an inflammatory and immune response in the utero-placental tissues, and that using anti-inflammatory treatments or directly targeting immune cells would successfully delay delivery and improve neonatal outcome. Therefore, the specific aims of the work presented here were to use a mouse model of infection-induced preterm labour to:

- Characterise the local inflammatory and immune response to a bacterial intrauterine infection.
- Investigate the potential of anti-inflammatory agents to delay delivery and improve pup survival.
- Investigate the role of specific immune cell populations in infection-induced preterm labour.

Chapter 2 - General methods

Details for all the materials and reagents used are given in Appendix 1.

2.1 MOUSE MODEL OF PRETERM LABOUR

All animal experiments were conducted under a UK Home Office licence in line with the Animals Scientific Procedure Act (1986). Timed-pregnant CD1 mice were obtained from Charles River Laboratories on day 9-11 of gestation (the day vaginal plug was found was designated day 1 of gestation), and were allowed to acclimatise for a minimum of 6 days prior to surgery.

On day 17 (D17) of gestation mice were anaesthetised with isoflurane (5% for induction, 2.5% for maintenance) in oxygen, and a mini-laparotomy procedure was performed, exposing the two uterine horns (Figure 2.1A). The number of viable fetuses in each horn was recorded. Using a 33-gauge Hamilton syringe, the mice then received an intrauterine injection of either LPS (from *E.Coli* O111:B4) or PBS in a 25μl volume between the first and second anterior fetuses directly into the uterine lumen of the horn with the most viable fetuses. Care was taken not to inject into the amniotic cavity (Figure 2.1B). The uterine horns were then carefully returned to the peritoneal cavity (Figure 2.1C) and the abdominal wall incision was then closed with a running stitch using 5/0 sutures, and the skin was closed using interrupted double and single stitches with 4/0 sutures (Figure 2.1D). Immediately following surgery mice received a subcutaneous injection of vetergesic analgesia, at a dose of 0.03mg/ml in 60μl. Mice were kept at 30°C while they recovered from surgery, before being transferred to individual cages for monitoring.

Mice with fewer than seven viable fetuses at the time of intrauterine injection were excluded, because this was considered an abnormal pregnancy for CD1 mice due to the small number of fetuses and early experiments indicated that these mice did not respond to treatment normally. Following delivery mice were culled by cervical dislocation.

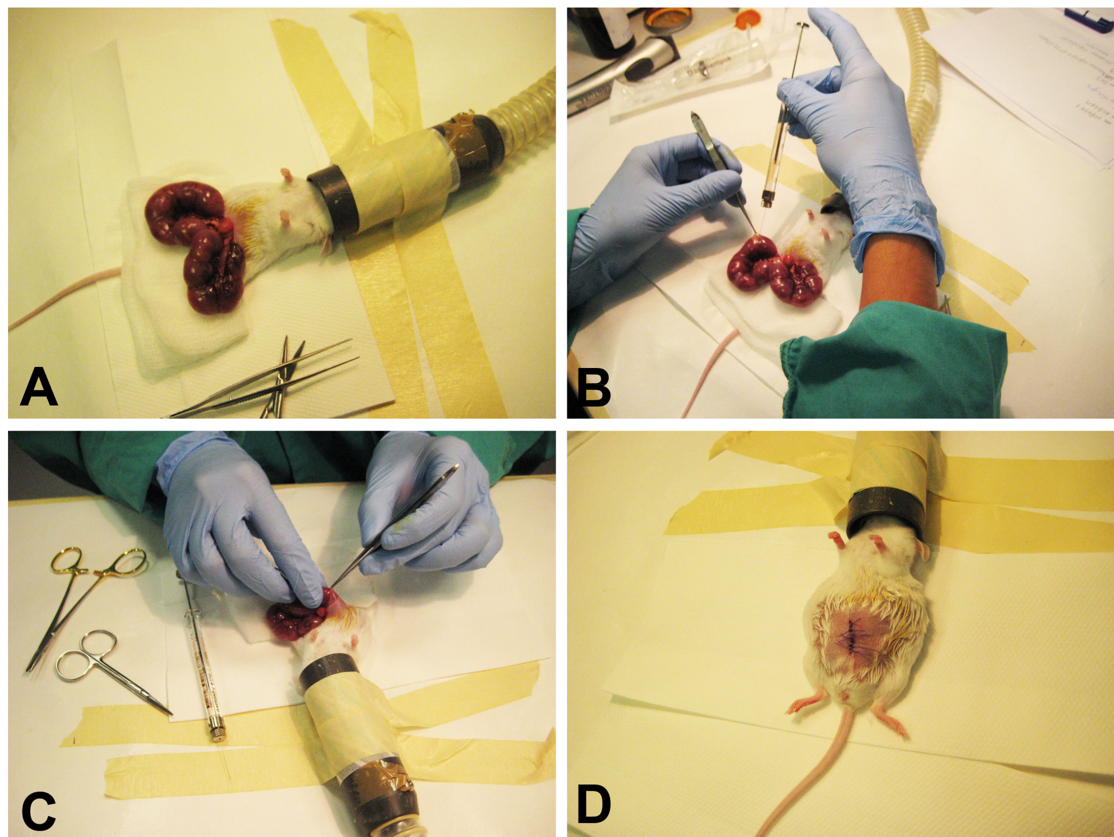

Figure 2.1 - Mouse model of infection-induced preterm labour. (A) CD-1 mice were anaesthetised on D17 of gestation and a mini laparotomy performed to expose the uterine horns. **(B)** Mice received an intrauterine injection of either PBS or LPS between the first and second anterior fetuses directly into the uterine lumen of the horn with the most viable fetuses. **(C)** The uterine horns were carefully returned to the peritoneal cavity. **(D)** The abdominal wall and skin wounds were then sutured.

2.1.1 Time to delivery

Following surgery, individual cameras were attached to the end of each cage and the mice were recorded using a digital video recorder. Time to delivery was recorded as the number of hours from the time of intrauterine injection, to delivery of the first pup. Preterm delivery was defined as delivery of the first pup within 36 hours of intrauterine injection, with the preterm delivery rate calculated by dividing the number of mice that delivered within 36 hours by the total number of mice in each treatment group.

2.1.2 Proportion of live born pups

Following delivery, the number of live pups present in the cage was recorded. The proportion of live pups per dam was then calculated by taking the number of live pups delivered and dividing this by the number of pups recorded at the time of intrauterine injection. The proportion of live pups was determined within 24 hours of delivery. Any live pups still within the uterine horns at this time were not considered as live born pups as they had not been through labour.

2.1.3 Tissue collection

Mice were culled by lethal exposure to CO_2 6 hours post-surgery and the utero-placental tissues were collected and fixed in three separate ways: RNA*later*® for RNA extraction; frozen on dry ice for protein extraction; and fixed in 4% NBF for histological analysis. RNA*later*® samples were stored overnight at 4°C and then transferred to an -80°C freezer. Samples frozen in dry ice were stored immediately at -80°C. Samples collected in 4% NBF were left for 24 hours and then transferred to 70% ethanol prior to embedding in paraffin blocks. Additionally fetal brains were collected in RNAlater®. Amniotic fluid and maternal serum were also collected for analysis.

2.1.3.1 Uterus

Samples of uterine horn were collected from three separate sites within the uterus: from the top of the injected uterine horn; the lower part of the uterine horn, close to the cervix; and from the top of the un-injected uterine horn. One piece of uterus collected

from each site was fixed in RNAlater®, frozen on dry ice and fixed in 4% NBF, resulting in a total of nine pieces of uterus taken from each mouse.

2.1.3.2 Fetal membranes

Fetal membranes were dissected free from the placenta and collected from nine separate gestational sacs. Fetal membranes from the three pups surrounding the site of injection were collected in RNAlater®. The following three sets of fetal membranes from adjacent pups were frozen in dry ice. The final three sets of fetal membranes were collected into NBF.

2.1.3.3 Placenta

Placental tissue was collected from six separate gestational sacs. Placentas from the three pups surrounding the site of injection were collected and halved, with half stored in RNAlater®, and half frozen in dry ice. The placentas from the adjacent three pups were stored in NBF.

2.1.3.4 Fetal brains

Fetal brains were collected from the three pups surrounding the site of intrauterine injection. Whole brains were dissected free from the skull and fixed in RNAlater®.

2.1.3.5 Amniotic fluid

Amniotic fluid was collected from each gestational sac and pooled for each horn, resulting in two amniotic fluid samples per mouse. Amniotic fluid was kept on ice, centrifuged at 8000 *g* for 10 minutes at 4°C and aliquoted in 0.5ml volumes for storage at -80°C.

2.1.3.6 Serum

Immediately after mice were culled, blood was collected from the vena cava using a 25-gauge needle and 1ml syringe. Blood samples were allowed to clot for 1-2 hours at room temperature, prior to being centrifuged at 2000 *g* for 20 minutes. Serum was then aliquoted in 25μl volumes and stored at -80°C.

2.2 RNA EXTRACTION AND REVERSE TRANSCRIPTION

2.2.1 RNA extraction

Two similar methods of RNA extraction were used, depending on the tissue the RNA was to be extracted from. Both methods were based on homogenisation of tissue in a phenol-guanidine thyiocyanate solution, followed by isolation of the RNA using RNeasy® mini columns. The spin columns contain a silica-based membrane that can selectively bind the RNA and the RNA is then eluted in nuclease-free water.

2.2.1.1 RNA extraction from utero-placental tissues

Utero-placental tissue samples stored in RNA*later*® were thawed and transferred to a 2ml Eppendorf tube containing 1ml TRI Reagent® and a stainless steel bead. Samples were then homogenised using a tissue lyser for two 3 minutes cycles at 25 Hz and incubated at room temperature for 15 minutes. Following this, samples were centrifuged at 8000*g* for 10 minutes in order to remove any insoluble material. The resulting supernatant was transferred to phase-lock tubes and to precipitate the RNA, 200µl bromochloropropane (BCP) added to each tube and the samples were left to incubate for 15 minutes before being centrifuged at maximum speed for 15 minutes at 4°C to isolate the RNA in the aqueous phase. The aqueous phase was transferred to a fresh 1.5ml Eppendorf tube and an equal volume of 70% ethanol was added to each sample and mixed by pipetting.

To isolate the RNA, samples were then added to the RNeasy mini spin columns and manufacturer's guidelines were followed. Briefly, columns were centrifuged for 15 seconds at 8000*g* at room temperature. The resultant flow through was discarded, 350µl of RW1 buffer was then added to each column and they were centrifuged again. On column DNA digestion was then performed by the addition of 80µl DNase 1 solution directly onto the column membrane. Samples were incubated for 15 minutes at room temperature. The columns were then washed with 350µl RW1 buffer. This was followed by a further 2 washes with 500µl of RPE buffer. To ensure no carry over of ethanol, the columns were placed in clean collection tubes and centrifuged at 12000*g* for 1 minute. Finally, RNA was eluted in 30-50µl of nuclease-free water. The RNA was stored at -80°C.

2.2.1.2 RNA extraction from fetal brains

A slightly different protocol was used to extract RNA from fetal brains due to the fact that brain tissue is rich in fat, therefore the protocol in the RNeasy® Lipid Kit was followed. Fetal brains stored in RNA*later*® were thawed and transferred to a 2ml Eppendorf tube containing 1ml QIAzol® Lysis Reagent and a stainless steel bead. Samples were then homogenised using a tissue lyser for two 3 minute cycles at 25 Hz and incubated at room temperature for 5 minutes. 200µl of chloroform was added to each sample to precipitate the RNA and the samples were incubated at room temperature for 2-3 minutes before being centrifuged at 12000*g* for 15 minutes at 4°C. The aqueous phase was transferred to a fresh 1.5ml Eppendorf tube and an equal volume of 70% ethanol was added to each sample and mixed by pipetting.

Samples were then added to the RNeasy® mini columns and the protocol followed, as detailed above.

2.2.1.3 RNA quantification

The RNA concentration was calculated by measuring the absorbance of the RNA sample at 260 and 280nm on the Nanodrop ND-1000. The 260:280 absorbance ratio was used as an indicator of RNA purity, with ratios of 1.8-2.1 considered acceptable.

2.2.2 Reverse transcription

300ng of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit. A 2X mastermix containing RT buffer, dNTPs, random primers, RNase inhibitor and reverse transcriptase, was prepared for each sample, see Table 2.1. 10µl of the mastermix was mixed with 10µl of RNA diluted to 30ng/µl in RNase-free water. Samples were incubated for 10 minutes at 25°C, followed by 120 minutes at 37°C and 5 minutes at 85°C. Two controls were included in each cDNA experiment: RT negative (template RNA but no reverse transcriptase) and RT H_20 (including reverse transcriptase, but with nuclease free water instead of template RNA). The resultant cDNA was stored at -20°C for use in quantitative real-time PCR reactions, as detailed below.

RT-PCR mastermix component	Volume per sample (µl)
10x RT Buffer	2
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2
Multiscribe Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease-free H_20	3.2

Table 2.1 - Reverse transcription mastermix

2.3 QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR (qRT-PCR) was carried out to quantify the expression of specific genes. The addition of a fluorescently labelled probe, in addition to specific forward and reverse primers, allows for quantification of the abundance of a target gene, relative to the levels of a housekeeping gene. The probe is labelled with a fluorescent reporter dye at the 5'-end and a quencher dye at the 3'-end. The probe anneals to the target DNA sequence between the forward and reverse primers. When the probe is intact and annealed to the DNA sequence, very little fluorescence is emitted due to the close proximity to the quencher. During amplification of the target sequence, the 5' to 3' exonuclease activity of the enzyme Taq polymerase cleaves the probe, resulting in the fluorescent reporter dye being released from the quencher, thus fluorescent light is then emitted from the reporter dye upon excitation with a laser. The amount of fluorescence measured is directly proportional to the amount of template present in the PCR.

The use of different reporter dyes allows the expression of both target and endogenous control genes to be measured in each sample at the same time. Expression of the gene, *β-actin* was found to be consistent during late pregnancy and between different treatment groups, therefore this was used as the endogenous control gene in all experiments. The amount of target gene measured was normalised to the amount of *β-actin* present.

2.3.1 Primers and probes

The expression of the majority of genes was measured using pre-designed Taqman® gene expression assays from Applied Biosystems, which are optimised for use in qRT-PCR. The gene names and corresponding assay IDs are given in Table 2.2.

Gene	Taqman® Gene expression Assay ID
15-Hpgd	Mm00515121_m1
Ccl2	Mm00441242_m1
Cxcl1	Mm04207460_m1
Cxcl2	Mm00436450_m1
Cxcl5	Mm00436451_g1
Emr1	Mm00802529_m1
Fpr2	Mm00484464_s1
Gfap	Mm01253033_m1
Il-1β	Mm00434228_m1
Il-10	Mm00439614_m1
Mtap2	Mm00485231_m1
Ngp	Mm01250218_m1
TNF-α	Mm99999068_m1

Table 2.2 - Taqman® gene expression assay IDs used in qRT-PCR experiments.

Primers and probes for *β-actin, Cox-2* and *Il-6* were designed using Primer Express Software Version 3 (Applied Biosystems). To avoid amplification of genomic DNA, probes were designed to span an exon-exon junction. The specificity of the designed primers was then checked using BLAST (Basic Local Alignment Tool). The sequences for the primers and probes are given in Table 2.3.

Gene	Primer/Probe	Sequence
β-actin	Forward	5’-GCTTCTTTGCAGCTCCTTCGT-3’
	Reverse	5’-GCGCAGCGATATCGTCATC-3’
	Probe	5’-CACCCGCCACCAGTTCGCCAT-3’
Cox-2	Forward	5’-GCTTCGGGAGCACAACAG-3’
	Reverse	5’-TGGTTTGGAATAGTTGCTC-3’
	Probe	5’-TGTGCGACATACTCAAGCA-3’
Il-6	Forward	5’-CCACGGCCTTCCCTACTTC-3’
	Reverse	5’-TGCACAACTCTTTTCTCATTCCA-3’
	Probe	5’-TCACAGAGGATACCACTCCCAACAGACCTG-3’

Table 2.3 - Primer and probe sequences designed using Primer Express Software.

The designed primer and probe sets were validated to ensure that the endogenous control gene and target gene had similar amplification efficiencies, as is required when using the comparative Ct method of analysis (discussed below). Serially diluted cDNA samples were used to determine the amplification efficiencies of each gene, both in singleplex and multiplex reactions (i.e. target gene with the endogenous control gene in the same well). The amplification efficiency of each gene was calculated by plotting the Ct against the log input of RNA (ng), to give a semi-log regression line. The slope of the line indicates the efficiency of the reaction, with a slope value of around -3.3 indicating 100% amplification efficiency. Plotting the ΔCT value (Ct of target gene – Ct of endogenous control gene) against the log input of RNA and calculating the slope of the semi-log regression line was also used to check the amplification efficiencies of the genes would not be affected by running in multiplex. In this case, the absolute value of the slope was to be <0.1. Representative data from a validation experiment are shown in Figure 2.2.

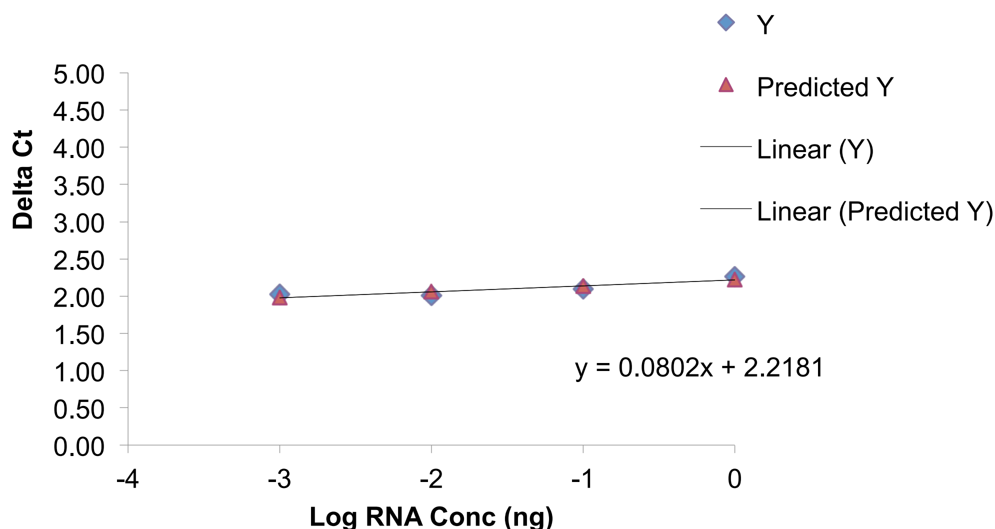

Figure 2.2 - *Cox-2* and *β-actin* primer and probe validation. Log RNA input plotted against ΔCt value. Absolute value of slope <0.1.

2.3.2 Method

Details of the reaction mix prepared for each duplicate sample are given in Table 2.4, including the final concentration of each component. Either target gene primers and probes or a Taqman® gene expression assay were used in each reaction, depending on the gene of interest, as detailed in Table 2.2. and Table 2.3. above. Reactions were made up to 30μl with nuclease-free water. In addition to the two controls made during reverse transcription, RT negative and RT H_20, a negative control was included which consisted of reaction mixture plus nuclease-free water in place of cDNA. On each plate a calibrator sample was also included, this was cDNA from an untreated tissue, which expressed the gene of interest. Calibrators used are detailed in the relevant results chapters. All qRT-PCR plates were run on the ABI 7900HT machine.

qRT-PCR Mix component	Volume (μl)	Final Concentration
2X Taqman® mastermix	15	1X
β-actin forward and reverse primers	0.72	60nM
β-actin probe	1.2	200nM
Target gene forward and reverse primers	0.72	300nM
Target gene probe	1.2	200nM
20x Taqman® Gene Expression Assay	1.5	900nM (primers) 250nM (probe)
cDNA	1.5	-

Table 2.4 - qRT-PCR reaction mix.

2.3.3 Analysis

Analysis of qRT-PCR data was carried out using the comparative Ct method. In this method of analysis, after normalising Ct values to the endogenous control gene, all samples are compared to a control or calibrator sample, resulting in the calculation of the fold change in expression of the target gene relative to the control sample, using the formula $2^{-\Delta\Delta Ct}$. The Ct value is the cycle at which expression of the gene crosses a threshold; ΔCt is the Ct of target gene – Ct of *β-actin*; and ΔΔCt is the difference between the ΔCt of the sample and that of the calibrator or control.

Each sample is representative of one dam, where RNA was extracted from three pieces of tissue per dam (i.e., three pieces of uterus, three fetal membranes, three placentas or three fetal brains per dam) and analysed by qRT-PCR and the average ΔΔCt value of the three pieces of tissue was then used for calculation of the fold change.

2.4 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISAs were used to quantify the amount of particular proteins in serum and amniotic fluid samples.

2.4.1 Sandwich ELISAs

In a sandwich ELISA, plates are pre-coated with a monoclonal antibody that is specific to the protein of interest. Standards, samples and controls are then added to the plate and any protein of interest present in the sample will be bound by the antibody. The plate is washed to remove any unbound proteins, prior to the addition of a secondary antibody, specific to a different epitope on the protein of interest. This second antibody is conjugated to the enzyme horseradish peroxidase (HRP). A further wash is carried out to remove any unbound antibody-enzyme conjugates and a substrate solution of hydrogen peroxide and tetramethylbenzidine (TMB) is added to detect peroxidase activity. The enzyme reaction results in a colour change which can be measured by spectrophotometry. The intensity of colour measured is proportional to the amount of the specific protein of interest in the sample. The standard curve can then be used to extrapolate protein concentration.

All sandwich ELISA plates were read at 450nm with wavelength correction at 570nm using a spectrophotometer and Softmax Pro software.

2.4.1.1 TNF-α ELISA

A Quantikine® ELISA kit was used for the quantification of TNF-α in amniotic fluid and serum. The standard curve allowed for detection of 700 to 10.9 pg/ml of TNF-α. 50μl of assay diluent was added to each well, followed by 50μl of standards and samples and the plate was then incubated for 2 hours at room temperature. All samples were assayed neat. The plate was then washed four times with wash buffer and 100μl of TNF-α conjugate (polyclonal antibody against mouse TNF-α conjugated to horseradish peroxidase) added to each well. The plate was incubated for 2 hours at room temperature. Following a further wash, 100μl of substrate solution was added to each well and the plate incubated at room temperature for 30 minutes. 100μl of stop solution was then added to quench the reaction and the plate read as described above.

2.4.1.2 IL-1β ELISA

As with TNF-α, a Quantikine® ELISA kit was used for the quantification of IL-1β in amniotic fluid. The standard curve allowed for detection of 800 to 12.5pg/ml of IL-1β. All samples were assayed neat.

2.4.1.3 IL-6 ELISA

As with TNF-α and IL-1β a Quantikine® ELISA kit was used for the quantification of IL-6 in amniotic fluid. The standard curve allowed for detection of 500 to 7.8pg/ml of IL-6. Samples were diluted 1 in 10 before being assayed.

2.4.1.4 IL-10 ELISA

A Quantikine® ELISA kit was used for the quantification of IL-10 in both amniotic fluid and serum. The standard curve allowed for detection of 1000 to 15.6pg/ml IL-10. Serum samples were diluted 1 in 2 and amniotic fluid samples were assayed neat.

2.4.2 Competitive binding ELISA

A competitive binding ELISA is based on labelled and unlabelled ligand competing for a limited number of antibody binding sites. A defined amount of labelled ligand is incubated with the sample and both the labelled ligand and any unlabelled ligand present in the sample compete to bind to the antibody binding sites on the plate. Substrate is then added and the resultant colour change can be measured by a spectrophotometer. If there is a high concentration of ligand in the sample then less labelled ligand will be bound and the colour change will be low, therefore in this ELISA the concentration of the protein of interest in the sample is inversely proportional to the optical density measured. As with the sandwich ELISA described above, plates were read using a plate reader and Softmax Pro software which automatically draws the standard curve and extrapolates protein concentrations.

2.4.2.1 Progesterone ELISA

A progesterone rat/mouse ELISA kit was used to quantify serum progesterone concentrations. The standard curve allowed for the detection of 100 to 0.4ng/ml progesterone. 25µl of standard and sample were added to the appropriate wells on the plate. 50µl of incubation buffer and 100µl of enzyme conjugate (progesterone conjugated to HRP) were also added to each well and the plate incubated at room temperature for 1 hour. The wells were then washed with 300µl of wash buffer and 200µl substrate solution (TMB and hydrogen peroxide solution) was added to each well and the plate incubated for 30 minutes at room temperature. 50µl of stop solution

was then added to quench the colour change reaction and the absorbance of each well was measured at 450nm.

2.5 IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was used to localise immune cells within the uterus. Details of solutions used are given in Table 2.6.

2.5.1 Paraffin embedding and slide cutting

Uterus samples were collected as detailed in section 2.1.3.1 and fixed overnight in 4% NBF before being transferred to 70% ethanol and embedded in paraffin blocks. Using a microtome, 5μm sections were cut and mounted onto slides. When cut, slides were incubated at 37°C for 24 hours to ensure sections were adhered to the slides before being used in immunhistochemistry experiments.

2.5.2 Staining protocol

Some staining was carried out using the automated Bond immunostaining robot service, where this was not used, the same conditions were used to achieve similar staining quality. Neutrophils were localised using either an anti-Gr-1 antibody, or the more specific anti-Ly-6G antibody; macrophages were localised using an anti-F4/80 antibody. Details of the source, dilution and antigen retrieval method for the different antibodies are given in Table 2.5.

Antibody name	Source	Dilution	Antigen retrieval method
Mouse Gr-1/Ly-6G, clone RB6-8C5	R&D Systems (MAB1037)	1:1000	None
Anti-mouse Ly-6G, clone 1A8	BioLegend, (127601)	1:500	None
Anti-mouse F4/80	eBioscience (14-4801)	1:250	0.5 mg/ml Trypsin for 10 minutes at 37°C

Table 2.5 - Antibody information for specific antibodies used in immunohistochemistry experiments.

Briefly, slides were dewaxed in xylene for 5 minutes, rehydrated in ethanol (absolute, 90%, 80% and then 70%), and washed in H_20. If required antigen retrieval was then performed. Slides were washed twice in PBS for 5 minutes. Following this, endogenous peroxidase activity was blocked by incubating the slides for 15 minutes in 3% H_20_2 in PBS. The two 5 minutes PBS washes were repeated prior to incubating the sections for 30 minutes in normal goat serum to block any non-specific binding. The primary antibody specific to the immune cell of interest was diluted in normal goat serum, added to the sections and the slides were incubated in a humidified chamber at 4^oC overnight. A negative control, where the primary antibody was replaced with normal goat serum, was included in each run.

The following day the slides underwent two 5 minutes PBS washes and were then incubated with the secondary antibody (ImmPRESS anti-Rat IgG reagent) for 30 minutes at room temperature. This reagent is a polymerized reporter enzyme staining system that conjugates a peroxidase micropolymer to the secondary antibody, allowing one-step detection. Following two further PBS washes, slides were incubated with 3, 3'-diaminobenzidine (DAB) substrate for peroxidase for a maximum of 5 minutes resulting in positive brown staining. Slides were then counterstained in haematoxylin, dehydrated in increasing concentrations of ethanol, incubated in xylene for 5 minutes and coverslips were mounted using pertex mounting medium.

Images of stained sections were obtained using the PROVIS microscope and AxioVision Rel 4.8 software.

Solution	Recipe
NBF fixative	100 ml 40% formaldehyde 4 g sodium dihydrogen phosphate monohydrate 6.5 g disodium hydrogen phosphate anhydrous 900 ml distilled H_20
Trypsin antigen retrieval solution	0.5 g trypsin 0.37g EDTA 1 L distilled H_20 pH 8
Normal goat serum	2ml non-immune goat serum 8 ml PBS 0.5 g BSA

Table 2.6 - Solutions used for tissue fixation and immunohistochemistry experiments.

2.5.3 Quantification of positively stained cells

Immune cell influx into the uterus six hours post-surgery was quantified stereologically. The myometrium and decidua of each uterus section were analysed separately. Each uterus section was tiled using a x5 objective and using Image-Pro Plus software, the area of interest (i.e., myometrium or decidua) was selected by drawing round the appropriate structure in the uterus. Using a x40 objective lens, a total of 10 randomised fields of view were visualised and the positive cells counted per section of myometrium or decidua, with 9 sections counted in total per mouse. The average number of positive cells counted per area was then calculated for each mouse and averaged across treatments for both the decidua and myometrium.

2.6 WESTERN BLOTTING

Western blotting was used to detect TNF-α protein in uterus, fetal membranes and placental tissue collected from mice as described in section 2.1.3.

2.6.1 Protein Extraction

0.5ml lysis buffer (Table 2.7) and a stainless steel bead were added to 2ml Eppendorfs tubes containing frozen tissue samples (collected as detailed in section 2.1.3.). Samples were homogenised at 25Hz for 5 minutes using a tissue lyser. The homogenates were then centrifuged at 8000g for 10 minutes at 4°C. Following this the clear aqueous phase was transferred to a fresh 2ml Eppendorf tube and stored at -80°C. Extracted protein from three pieces of the same tissue were pooled for each mouse.

2.6.1.1 Protein Quantification

The Bio-Rad *DC* Protein Assay was used to quantify the protein concentration in each sample. This protein assay is a colorimetric assay similar to the Lowry protein assay. The colour change results from the reaction of protein present in the sample with an alkaline copper tartrate solution and Folin reagent. The reaction between the protein and copper in the alkaline solution reduces the Folin reagent, resulting in a characteristic blue colour. The intensity of the blue colour is proportional to the amount of protein present in the sample and can be quantified by measuring the absorbance at 650 nm.

A standard curve of bovine serum albumin (BSA) was made up in lysis buffer (0, 2.5, 5, 10, 20 and 40 mg/ml of BSA). 2µl of standards or sample were added to wells in a 96-well plate, followed by 25µl Reagent A (alkaline copper tartrate solution) and 200µl Reagent B (dilute Folin reagent). The plate was then incubated at room temperature for 15 minutes. The plate was then read at 650 nm and the protein concentrations were extrapolated from the standard curve.

2.6.2 Method

Details of the solutions used for western blotting are given in Table 2.7.

A total of 100µg of protein was run in each lane. Loading buffer, in a volume of 5µl, was added to each sample and samples were denatured by heating at 100°C for 10 minutes, cooled on ice and quickly centrifuged at full speed for 10 seconds before being loaded onto the gel. Aliquots (15 or 20µl) of sample and 10µl of molecular weight marker (See Blue® Plus 2 Pre-Stained Standard) were loaded on to NuPAGE 4%-12% Bis-Tris Mini Gels (1mm, 15 well). The gel was run using MES running buffer at 200V for 30 – 45 minutes to separate out the proteins. The proteins were then transferred from the gel to a PVDF membrane in transfer buffer at 200mA for 2 hours. Following transfer, to

reduce the chance of non-specific binding, the membranes were blocked in 5% dry skimmed milk in TBST for 1 hour at room temperature. Membranes were then washed for 5 minutes in TBST, this step was repeated for a total of three washes. The membranes were then incubated overnight at 4°C with rabbit polyclonal anti-TNF-α antibody (1:800; Catalogue no. 3707, Cell Signaling) in 5% BSA in TBST.

The following day membranes were washed for 5 minutes in TBST for a total of three washes and incubated at room temperature for 1 hour with a goat-anti-rabbit IgG secondary antibody, which is conjugated to HRP, in 5% milk in TBST (1:1000). Following three final 5 minute washes in TBST, the membranes were incubated with 1ml chemiluminescent developing solution (ECL Western Blot Analysis System) for 30 seconds, placed in cassette and exposed to X-Ray film in the dark room for 5 seconds to 3 minutes, depending on the signal strength. The film was then exposed using an X-Ray developer. Following development of the TNF-α signal, membranes were then washed for 20 minutes in TBST for a total of 3 washes, and incubated overnight at 4°C with a rabbit polyclonal anti-β-Tubulin antibody (1:1000) in 5% BSA in TBST, as a protein loading control. The β-Tubulin signal was developed as described above for TNF-α.

2.6.3 Analysis

Image J software was used to quantify the intensity of the bands. The intensity of the TNF-α bands were then made relative to the intensity of the β-Tubulin bands, as a loading control for the amount of protein present in each lane. The seven PBS sample and LPS samples were analysed individually and then an average for PBS and LPS was calculated for each tissue.

Solution	Recipe
Lysis Buffer	50 mmol/l Tris (pH 7.4) 0.27 mol/l sucrose 1 mmol/l sodium orthovanadate (pH 10) 1 mmol/l EDTA 1 mmol/l EGTA 10 mmol/l sodium β-glycerophosphate 50 mmol/l NaF 5 mmol/l sodium pyrophosphate 1% [w/v] Triton X-100 0.1% [v/v] 2-mercaptoethanol protease inhibitors EDTA free tablets (Roche)
Loading Buffer (5x)	1.8 ml 2M Tris (pH 6.8) 3 ml Glycerol 0.3 ml 0.5% Bromophenol Blue 1.8 g SDS 925 mg DTT Made up to 10 ml with dH_20
Transfer Buffer (10x)	24.2 g Tris 112.3 g glycine Made up to 1 L with dH_20
Transfer Buffer (1x)	100 ml transfer buffer (10x) 800 ml dH_20 100 ml methanol
TBS (10x)	12.11 g Tris HCL 87.66g NaCl Made up to 1 L with dH_20 pH 7.6
TBST	1 L TBS 1 ml Tween-20

Table 2.7 - Solutions used for protein extraction and western blotting experiments.

2.7 FLOW CYTOMETRY

Flow cytometry is a technique used to analyse and count cell populations based on their size, granularity and, if labelled with a specific fluorophore-labelled antibody, their fluorescent emissions.

Flow cytometry was used to quantify the number of neutrophils present in mouse serum following neutrophil depletion experiments.

2.7.1 Sample preparation

Blood was collected 6 hours after surgery from the vena cava, as detailed in section 2.1.3.6. Following collection 20μl of whole blood was immediately mixed with an equal volume of the anticoagulant, sodium citrate and placed on ice. Aliquots of 20μl of the blood samples were then incubated with specific fluorophore-labelled antibodies diluted 1:100 in PBS for 30 minutes on ice, protected from the light (PE-labelled rat anti-mouse CD45 antibody; Pacific Blue-labelled rat anti-mouse Ly-6G antibody). Blood samples from each animal were incubated with both antibodies together (double stain). Single stains and an unstained control samples were incubated with pooled blood samples from multiple mice in order to check the specificity of the antibodies and control for basal fluorescence of the samples. After 30 minutes, 1ml of BD FACSLyse solution was added to each sample, to lyse red blood cells and fix the cells, and the samples were stored at 4°C until they were analysed on the flow cytometer. Prior to flow analysis, samples were centrifuged for 5 minutes at 350 *g*, the supernatant removed and the samples re-suspended in 200μl of PBS.

2.7.2 Flow cytometric analysis

Flow cytometry analysis was carried out using the BD LSR Fortessa. Immediately before analysis, 50μl of Flow-check bead suspension (approximately 50000 beads) was added to each sample to allow subsequent calculation of the absolute number of cells counted. The samples were then run on the flow cytometer and 5000-10000 bead events were counted. Cells were initially analysed based on their forward and side scatter characteristics, and the neutrophil population was gated based on expression of both CD45 and Ly-6G for comparison between blood collected from control mice and neutrophil depleted mice. Representative flow plots from a mouse treated with an IgG control antibody (Figure 2.3A) and a mouse treated with anti-Gr-1 (Figure 2.3B) are

shown below. Data were collected using BD FACSDiva software and analysed using FlowJo software (Treestar, Ashland, OR, USA).

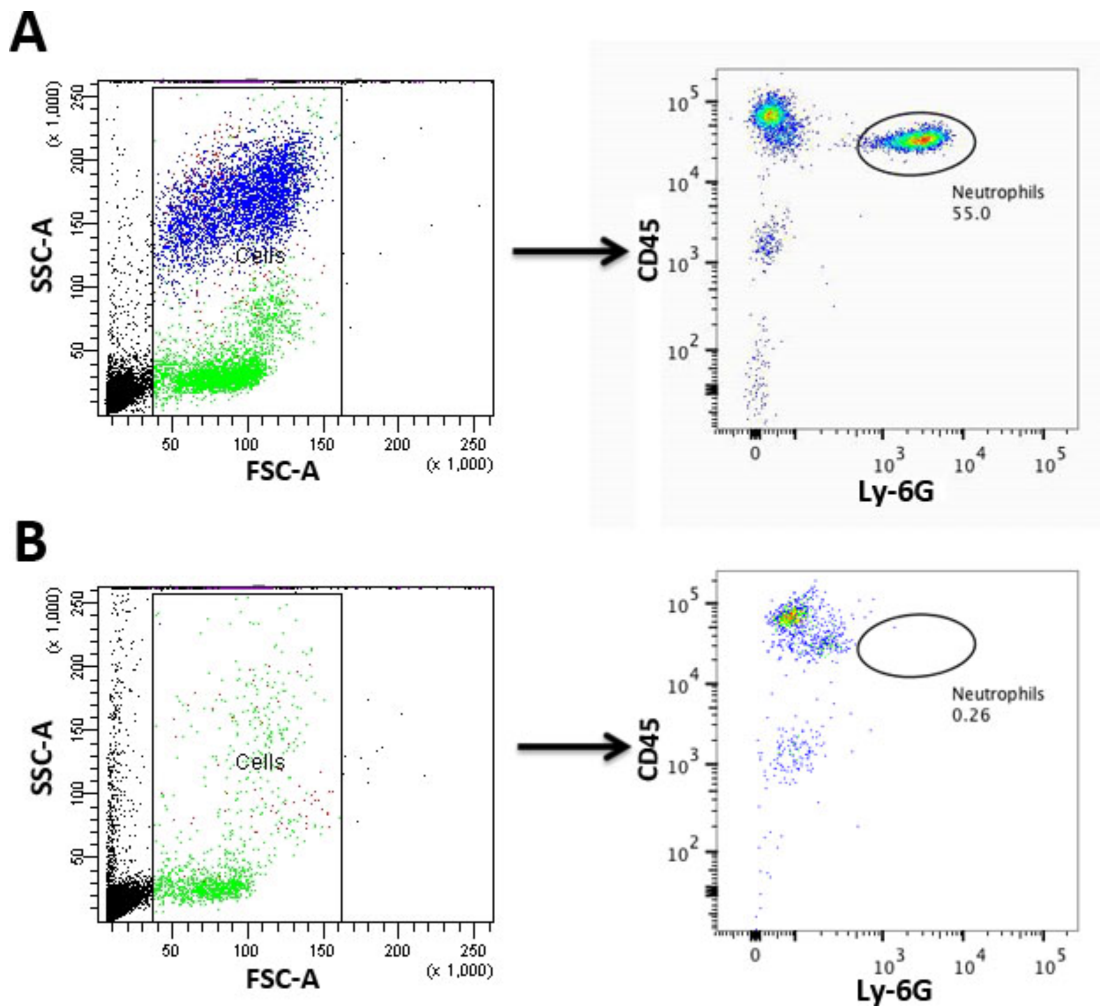

Figure 2.3 - Representative flow cytometry plots to measure circulating neutrophils in blood. Left panel of each shows FSC vs. SSC plots and gating on live cells. Right panel of each shows Ly-6G vs. CD45 staining of neutrophils in peripheral blood. **(A)** Representative flow plot from control mouse with high circulating neutrophil numbers. **(B)** Representative flow plot from a neutrophil depleted mouse (following anti-Gr-1 treatment), with a very low number of circulating neutrophils.

2.8 STATISTICAL ANALYSIS

Unless otherwise stated data are presented as mean ± standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Prism version 6.0 (GraphPad Software, San Diego, California, USA). The specific statistical tests used are described in each results chapter. $P<0.05$ was considered statistically significant.

Chapter 3 - Characterisation of the inflammatory and immune response in a mouse model of infection-induced PTL

3.1 INTRODUCTION

There is now strong evidence that labour is an inflammatory event, with the onset of labour at term being associated with increased production of inflammatory mediators, such as TNF-α, IL-1β and IL-6 at the maternal-fetal interface, as well as increased immune cells, particularly neutrophils and macrophages in the cervix, myometrium and fetal membranes (Denison *et al.* 1998, Sennstrom *et al.* 2000, Young *et al.* 2002, Osman *et al.* 2003). It is now widely accepted that intrauterine infection results in early activation of these inflammatory pathways normally initiated at term, resulting in preterm labour (Goldenberg *et al.* 2000). Indeed, intrauterine infection and inflammation are known to be an important cause of preterm labour, with studies estimating that up to 40% of preterm births occur in the presence of an intrauterine infection (Goldenberg *et al.* 2008). Furthermore, intrauterine infection and inflammation are strongly associated with adverse neonatal outcomes, such as brain injury (Shatrov *et al.* 2010).

Animal models have confirmed the role of infection and inflammation in preterm labour, where administration of bacterial products such as LPS have effectively induced preterm labour in mice (Elovitz *et al.* 2003, Pirianov *et al.* 2009), rabbits (McDuffie *et al.* 1992) and rhesus monkeys (Gravett *et al.* 1994). The use of animal models, which can be manipulated and studied at various time points during pregnancy, is invaluable to improve our understanding of the sequence of events that lead to preterm labour.

Using a mouse model of LPS-induced preterm labour, to mimic the presence of an intrauterine bacterial infection, this chapter aimed to characterise the local inflammatory and immune responses that result in preterm labour.

3.2 METHODS

3.2.1 Mouse model of PTL

Mice underwent surgery as detailed in section 2.1. Mice received an intrauterine injection of either 1μg (n=5), 5μg, (n=6) 10μg (n=11), 15μg (n=8) or 20μg (n=42) of LPS or PBS (n=35), as a control. Time to delivery and the proportion of live born pups was then monitored as detailed in sections 2.1.1 and 2.1.2. Time to delivery was also recorded in a second cohort of mice that did not undergo surgery (n=8), to determine if the surgery itself affected time to delivery. These mice were monitored from midday on D17 of gestation until delivery of the first pup to determine an average time to delivery for untreated mice.

3.2.2 Tissue collection

In a separate cohort of mice receiving intrauterine injection of 1μg (n=6), 5μg (n=5), 10μg (n=5), 15μg (n=5) or 20μg of LPS (n=11) or PBS (n=8), utero-placental tissues, maternal serum, amniotic fluid and fetal brains were collected 6 hours post-surgery as detailed in section 2.1.3, for analysis of the inflammatory and immune response.

3.2.3 qRT-PCR

The mRNA expression of several inflammatory genes and immune cell markers was examined using qRT-PCR as described in section 2.3. The expression of the following genes were measured in the uterus, fetal membranes and placenta of mice 6 hours post-surgery: *Cxcl1, Cxcl2, Cxcl5, Ccl2, Il-1β, Tnf-α, Il-6, Il-10, Cox-2, 15-Hpgd, Ngp* and *Emr1*. Expression of two genes associated with brain damage were also measured in the fetal brains 6 hours post-surgery, glial fibrillary associated protein (*Gfap*), and microtubule-associated protein (*Mtap2*).

As described in section 2.3.3, expression of the gene of interest was normalised to *β-actin* as the endogenous control gene in each sample and all samples were then compared relative to either a calibrator tissue or control sample. Analysis of all the genes examined in the uterus, fetal membranes and placenta was carried out relative to untreated D18 uterus as a calibrator. Analysis of the expression of genes within the brain was made relative to the PBS control group.

3.2.4 ELISA

ELISAs were used to quantify the concentration of inflammatory markers in both serum and amniotic fluid collected 6 hours post-surgery, as described in section 2.4. Briefly, the specific ELISAs performed were TNF-α, IL-10 and progesterone in maternal serum; and TNF-α, IL-1β, IL-6 and IL-10 in amniotic fluid. Details of each assay are given in section 2.4.

3.2.5 Western Blotting

Western blotting was used to detect TNF-α protein in the uterus, fetal membranes and placenta of mice harvested 6 hours post-surgery. Protein was extracted from mice who received an intrauterine injection of PBS (n=7) or 20μg LPS (n=7), as detailed in section 2.6.1. The protocol used for the western blotting experiments is described in section 2.6.2. The intensity of each band was normalised to β-tubulin as a loading control and then quantified using Image J software to measure the density of the bands, as detailed in section 2.6.3.

3.2.6 Immunohistochemistry

Immunohistochemistry was used to localise neutrophils and macrophages within the uterus of mice, as detailed in section 2.5. Neutrophils were localised using an anti-Gr-1 antibody. Macrophages were localised using an anti-F4/80 antibody. Staining protocols are detailed in section 2.5.2.

The three pieces of uterus collected from each mouse were fixed together in one block and a total of 3 slides were stained from each mouse. The number of positive cells was quantified as detailed in section 2.5.3 and compared between PBS (n=4) and 20μg LPS (n=5) treatment groups, with comparisons also made between the location of the cells within the uterus, whether they resided in the myometrium or decidua.

3.2.7 Statistical analysis

Data are presented as mean ± SEM. Where data were not normally distributed, it was transformed prior to analysis. Time to delivery data, and serum ELISA data were log

transformed before analysis; and the proportion of live pups born was arc-sin transformed prior to analysis. Normally distributed data were then analysed by one-way ANOVA to compare treatment groups, followed by Dunnett's multiple comparison tests to identify significant differences compared to the PBS control group. Preterm delivery rates were analysed by Fisher's exact test. Amniotic fluid cytokine levels were analysed non-parametrically using a Kruskal-Wallis test followed by Dunn's multiple comparison test to identify significant differences between treatment groups compared with the PBS control group. Comparisons between just PBS and 20μg LPS treated mice in western blot analysis were compared using unpaired t-tests. Immune cell counts were analysed by 2-way ANOVA. $P<0.05$ was considered statistically significant.

3.3 RESULTS

3.3.1 Intrauterine administration of LPS induced PTL in a dose-dependent manner

To investigate the ability of intrauterine LPS administration to induce preterm labour, a dose response experiment was carried out to identify the dose of LPS that reliably induced preterm labour with minimal variation.

3.3.1.1 Time to delivery

The time to delivery of mice that did not undergo surgery or treatment was not significantly different to the PBS control group, in which PBS was injected into the uterine horn (mean time to delivery of no surgery control 51.34 hours ± SEM 1.13 vs. PBS mean time to delivery 49.58 hours ± SEM 3.71; Figure 3.1). Mice receiving intrauterine LPS delivered earlier than those receiving PBS, in a dose-responsive manner. Administration of both 15μg and 20μg of LPS induced delivery significantly earlier than the PBS control group (15μg mean time to delivery 26.44 hours ± SEM 13.09, $p<0.001$ compared to PBS control; 20μg mean time to delivery 27.23 hours ± SE 3.37; $p<0.001$ compared to PBS control; Figure 3.1).

To analyse further the effect of LPS treatment on preterm delivery, the preterm delivery rate was calculated. This was defined as the proportion of dams in which there was delivery of the first pup within 36 hours of intrauterine injection. Preterm delivery rates for each treatment group are given in Table 3.1. The rate of preterm delivery in the PBS control group was 40%; higher preterm delivery rates were present in groups receiving doses of LPS at 5μg and higher. Mice receiving 20μg LPS were twice as likely to deliver preterm compared with the PBS control group, with a preterm delivery rate of 83.3% (relative risk 2.08 [95% CI 1.36 – 3.2]).

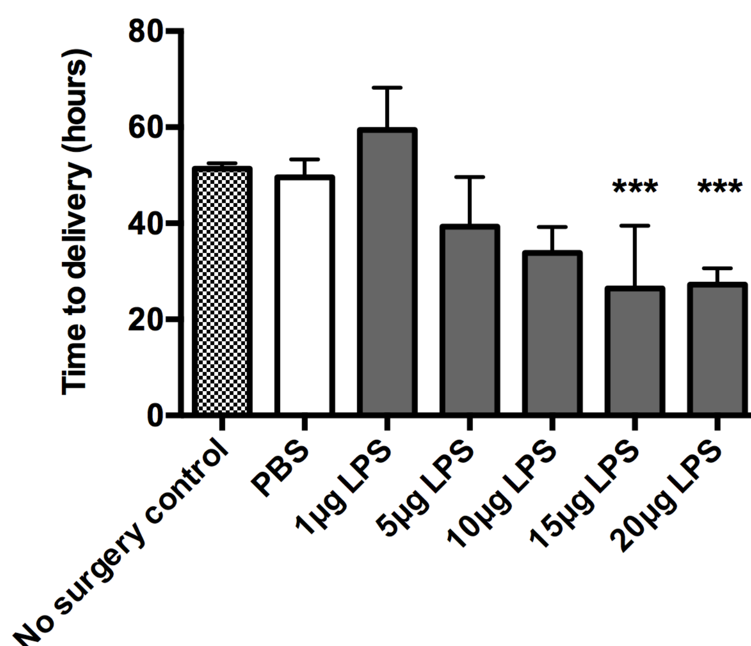

Figure 3.1 - Effect of intrauterine LPS administration on time to delivery. Time to delivery was monitored in mice receiving an intrauterine injection of PBS (n=35), 1µg LPS (n=5), 5µg LPS (n=6), 10µg LPS (n=11), 15µg (n=8) or 20µg LPS (n=42). Time to delivery was also monitored in a group of mice receiving no surgery as a control (n=8). Data are presented as mean ± SEM (error bars); ***p<0.001, compared to PBS

Treatment	Preterm delivery rate	Relative risk of preterm delivery (compared to PBS)
PBS (n=35)	40%	-
1µg LPS (n=5)	0%	-
5µg LPS (n=5)	60%	1.5 (95% CI 0.66 – 3.42)
10µg LPS (n=11)	54.5%	1.36 (95% CI 0.69 – 2.68)
15µg LPS (n=8)	75%	1.88 (95% CI 1.06 – 3.32)
20µg LPS (n=42)	83.3%	2.08 *** (95% CI 1.36 – 3.2)

Table 3.1 - Effect of intrauterine LPS administration on preterm delivery rate. Time to delivery was monitored in mice receiving an intrauterine injection of PBS, 1µg, 5µg, 10µg, 15µg and 20µg LPS. Preterm delivery was defined as delivery within 36 hours of intrauterine injection to calculate the preterm delivery rate in each group. Relative risk ratios calculated using Fisher's exact test. ***p<0.001, compared to PBS.

3.3.1.2 Proportion of live born pups

The proportion of live born pups reduced in response to increasing doses of LPS, with a significantly lower proportion of live pups born to mice receiving 15μg and 20μg of LPS, compared to the PBS control group (15μg mean proportion of live born pups 0.13 ± SEM 0.08, $p<0.01$; 20μg mean proportion of live born pups 0.25 ± SEM 0.05, $p<0.001$; compared with PBS mean proportion of live born pups 0.56 ± SEM 0.06; Figure 3.2A).

To investigate further whether the observed reduction in the proportion of live born pups in the 15μg and 20μg LPS treatment groups was simply due to a higher proportion of mice in these groups being born prematurely, rather than an effect of the LPS treatment, the proportion of live born pups was also examined in mice delivering preterm. Mice delivering preterm in the PBS group had a mean proportion of live born pups of 0.42 ± SEM 0.12, which was still significantly higher when compared to the proportion of live born pups in mice that delivered preterm in the 15μg LPS treatment group (mean proportion of live born pups 0; $p<0.05$); and the 20μg LPS treatment group (mean proportion of live born pups 0.14 ± 0.04; $p<0.05$; Figure 3.2B).

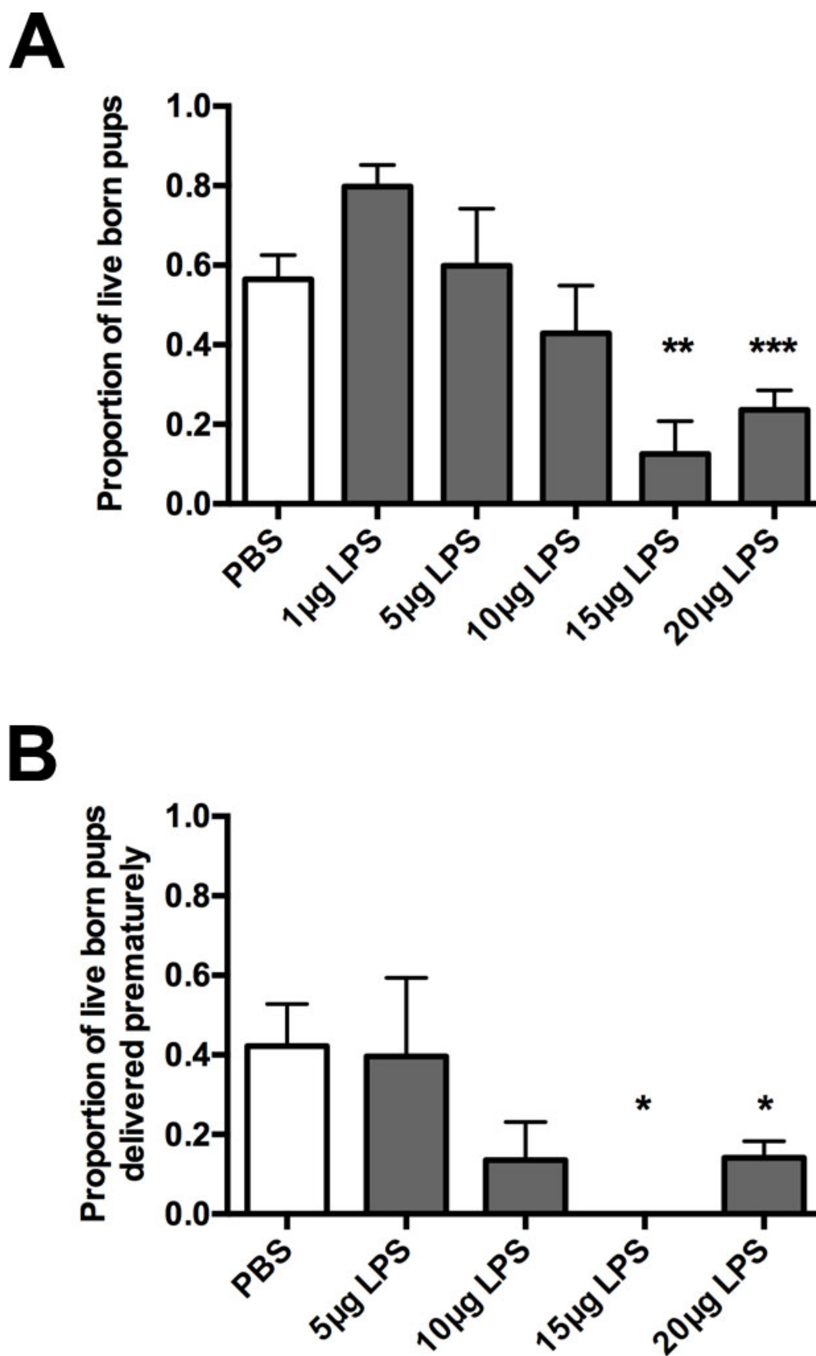

Figure 3.2 - Effect of intrauterine LPS administration on the proportion of live born pups. The proportion of live born pups was determined in mice receiving an intrauterine injection of PBS (n=35), 1µg LPS (n=5), 5µg LPS (n=6), 10µg LPS (n=11), 15µg (n=8) and 20µg LPS (n=42). **(A)** Proportion of live born pups in all mice. **(B)** The proportion of pups delivered prematurely (within 36 hours of surgery) who were born live; [PBS (n=14), 5µg LPS (n=3), 10µg LPS (n=6), 15µg LPS (n=6), 20µg LPS (n=35)]. Data presented as mean ± SEM (error bars); *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to PBS.

3.3.2 Expression of inflammatory mediators in the utero-placental tissues in response to intrauterine LPS

Utero-placental tissues were analysed for the expression of several inflammatory mediators 6 hours post-intrauterine injection using qRT-PCR. The genes measured were: the pro-inflammatory cytokines *Tnf-α, Il-1β* and *Il-6;* the major enzyme responsible for initiating prostaglandin synthesis, *Cox-2*; the enzyme responsible for the breakdown of prostaglandins, *15-Hpgd*; the anti-inflammatory cytokine, *Il-10*; the neutrophil chemokines *Cxcl1, Cxcl2* and *Cxcl5*; and the macrophage chemokine, *Ccl2.*

3.3.2.1 Inflammatory gene expression in the uterus

Uterine *Tnf-α* expression was significantly elevated in response to all doses of LPS administered compared to the PBS control ($p<0.001$; Figure 3.3A). In response to 20μg LPS, there was a 5.1-fold increase in *Tnf-α* expression compared to the PBS control group ($p<0.001$).

Il-1β expression was significantly elevated in response to all doses of LPS administered compared to the PBS control group ($p<0.001$; Figure 3.3B), with a 2.7-fold increase in uterine *Il-1β* expression in mice receiving 20μg of LPS compared to the control ($p<0.001$).

Il-6 expression was significantly increased in response to LPS treatment at all doses administered, with a mean 6.3-fold increase in *Il-6* expression in response to 20μg LPS compared to the PBS group ($p<0.001$; Figure 3.3C).

Expression of the anti-inflammatory cytokine, *Il-10* was also significantly elevated in response to intrauterine LPS administration in a dose-responsive manner, with a 2-fold increase in *Il-10* expression in the uterus of mice given 20μg of LPS, compared with mice receiving PBS ($p<0.001$; Figure 3.3D).

Given the important role of prostaglandins in the initiation of labour, the levels of *Cox-2* and *15-Hpgd* were also measured. Expression of *Cox-2* increased in response to increasing doses of LPS, with significantly elevated expression at 10μg ($p<0.05$), 15μg ($p<0.001$) and 20μg ($p<0.001$) of LPS, compared to the PBS control (Figure 3.3E). In contrast there was no significant change in the mRNA expression level of *15-Hpgd* in response to LPS treatment (PBS vs. 20μg LPS $p=0.38$; Figure 3.3F).

The expression of *Cxcl1*, *Cxcl2* and *Cxcl5*, all of which are potent murine neutrophil chemoattractants and activators was significantly elevated in the uterus in response to all doses of LPS administered compared to the PBS control group ($p<0.01$). In comparison to the PBS control group, administration of 20μg of LPS induced a 17.5-fold increase in *Cxcl1* expression ($p<0.001$; Figure 3.4A); a 14.7-fold increase in uterine *Cxcl2* expression ($p<0.001$; Figure 3.4B); and a 9.8-fold increase in *Cxcl5* expression ($p<0.001$; Figure 3.4C).

Expression of the macrophage chemokine, *Ccl2*, was also elevated in response to increasing doses of LPS, with significantly higher levels at 15μg and 20μg of LPS, compared with the PBS control ($p<0.01$; Figure 3.4D).

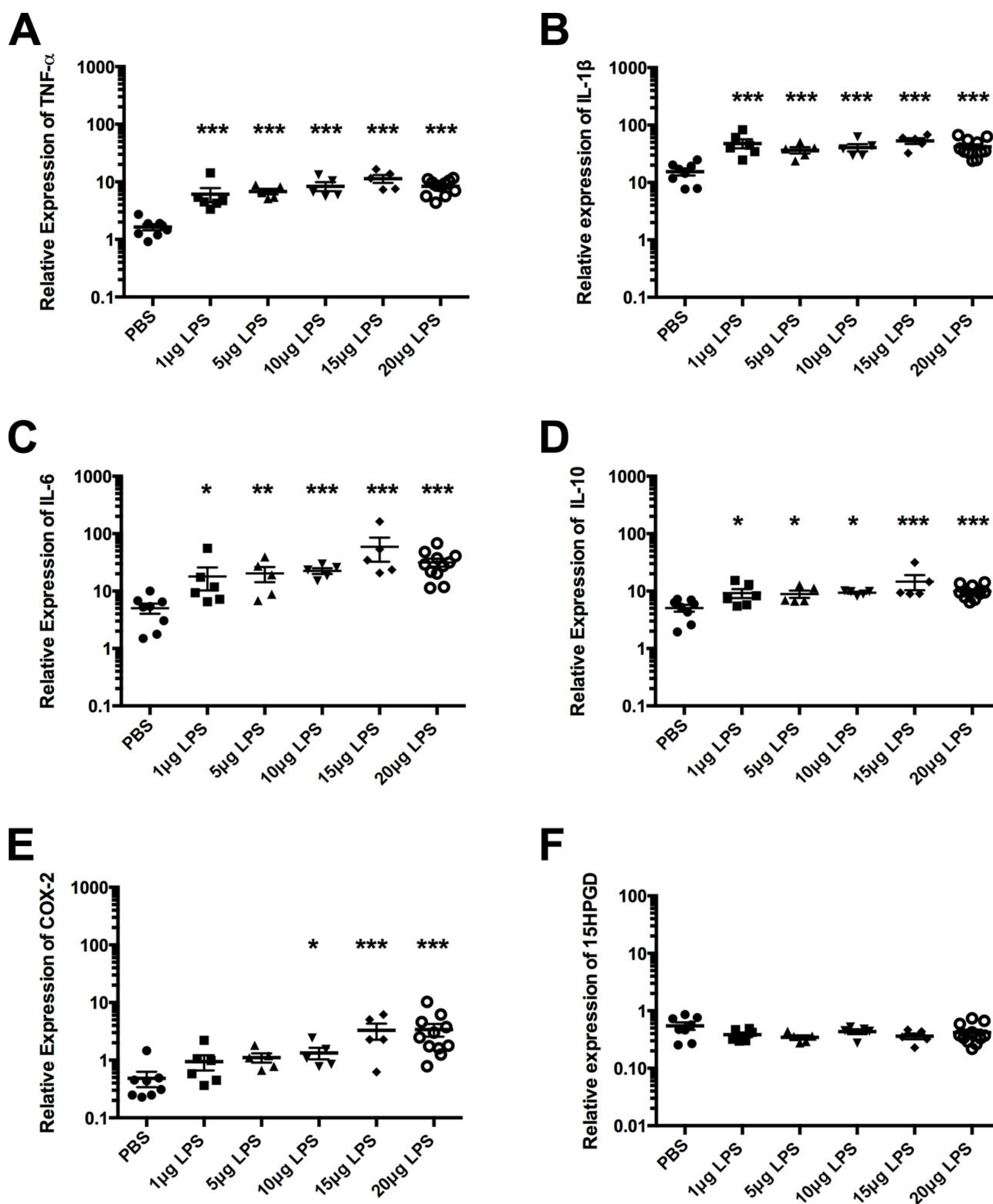

Figure 3.3 - Effect of intrauterine LPS administration on inflammatory gene expression in the uterus. Uterine tissue was collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of several inflammatory genes assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to PBS.

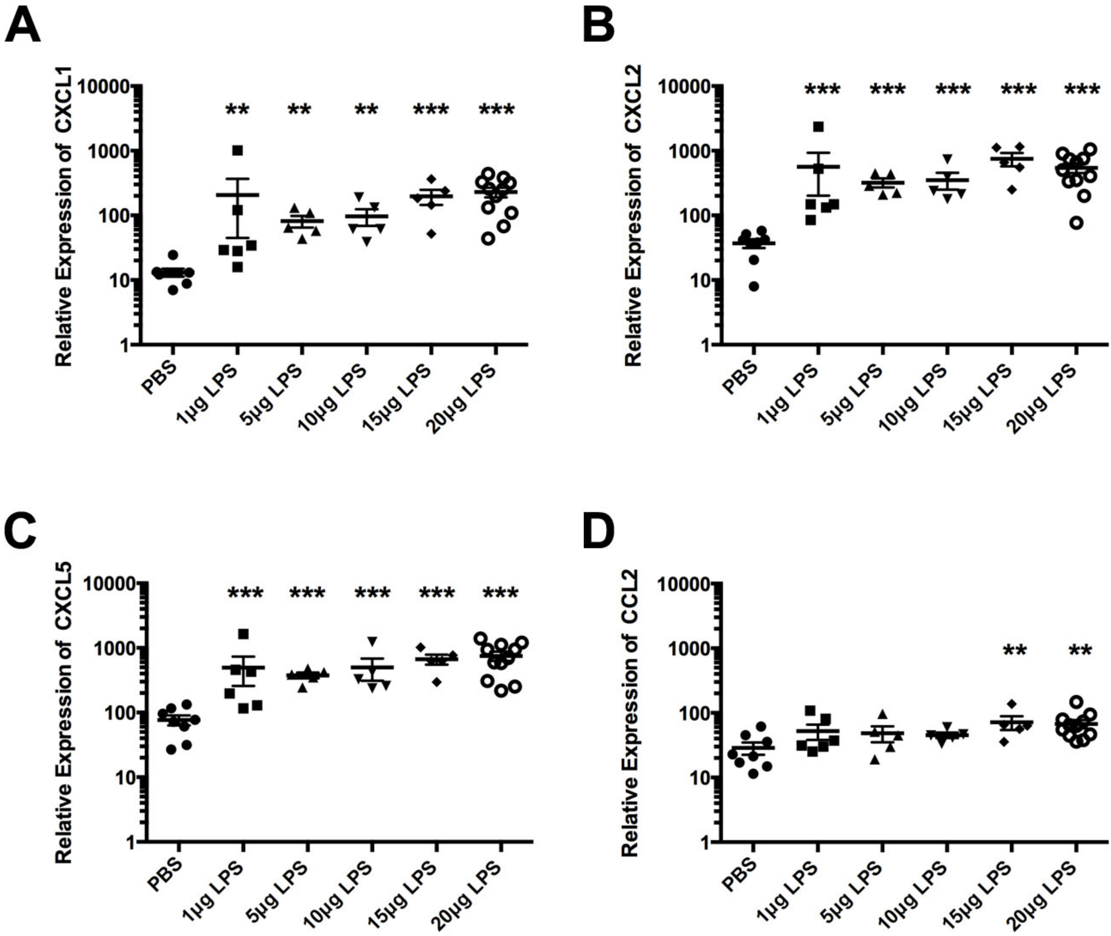

Figure 3.4 - Effect of intrauterine LPS administration on inflammatory chemokine expression in the uterus. Uterine tissue was collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of several inflammatory genes assessed by qRT-PCR**. (A**) *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold fold-change ± SEM (error bars); **p<0.01, ***p<0.001, compared to PBS.

3.3.2.2 Inflammatory gene expression in the fetal membranes

Tnf-α expression in the fetal membranes was significantly elevated in response to intrauterine LPS administration, at all doses (p<0.001; Figure 3.5A), with a 33-fold increase in expression in response to 20μg of LPS, compared to the PBS control.

Similarly *Il-1β* expression was also significantly elevated in the fetal membranes in response to all doses of LPS administered (p<0.001; Figure 3.5B), with 17.4-fold higher expression induced when 20μg of LPS was administered, compared to PBS.

Expression of *Il-6* was increased in a dose-responsive manner in the fetal membranes in response to increasing doses of LPS, with a 3.6-fold increase in expression in response to 20μg of LPS compared with the PBS control, however this was not statistically significant (PBS vs. 20μg LPS p=0.96; Figure 3.5C).

In response to increasing doses of LPS administered, *Il-10* expression in the fetal membranes was significantly increased (p<0.01 at 1μg, p<0.001 at all other doses of LPS; Figure 3.5D). Expression in response to 20μg of LPS was 8.2-fold higher compared with expression of *Il-10* in the fetal membranes of mice receiving PBS.

There was no significant change in *Cox-2* expression in the fetal membranes of mice receiving LPS, at any of the doses administered (PBS vs. 20μg LPS p=0.63; Figure 3.5E). In contrast, expression of *15-Hpgd* was significantly decreased in mice receiving LPS at each dose, with 2.5-fold lower expression of *15-Hpgd* in the fetal membranes of mice receiving 20μg LPS, compared to the PBS control (p<0.001; Figure 3.5F).

Expression of *Cxcl1* in the fetal membranes was significantly elevated in response to 5μg (p<0.01), 10μg (p<0.05), 15μg (p<0.001) and 20μg (p<0.01) of LPS, with 5.7-fold higher expression in response to 20μg of LPS compared to the PBS group (Figure 3.6A). *Cxcl2* expression was significantly higher in response to all doses of LPS administered compared to PBS (p<0.01 at 1μg, p<0.001 at all other doses of LPS; Figure 3.6B), with 8.8-fold increased expression in mice receiving 20μg of LPS, compared with PBS. Similarly fetal membrane *Cxcl5* expression was significantly elevated in response to all doses of LPS administered (p<0.001; Figure 3.6C), with 56.8-fold increased expression in response to 20μg of LPS, compared with PBS. In response to increasing doses of LPS administered, expression of *Ccl2* increased in the fetal membranes, with significantly elevated expression in response to 5μg (p<0.001), 10μg (p<0.01), 15μg (p<0.001) and

20μg ($p<0.001$) of LPS (Figure 3.6D). Expression levels of *Ccl2* in the fetal membranes were 7.4-fold higher in mice receiving 20μg of LPS, compared with those receiving PBS.

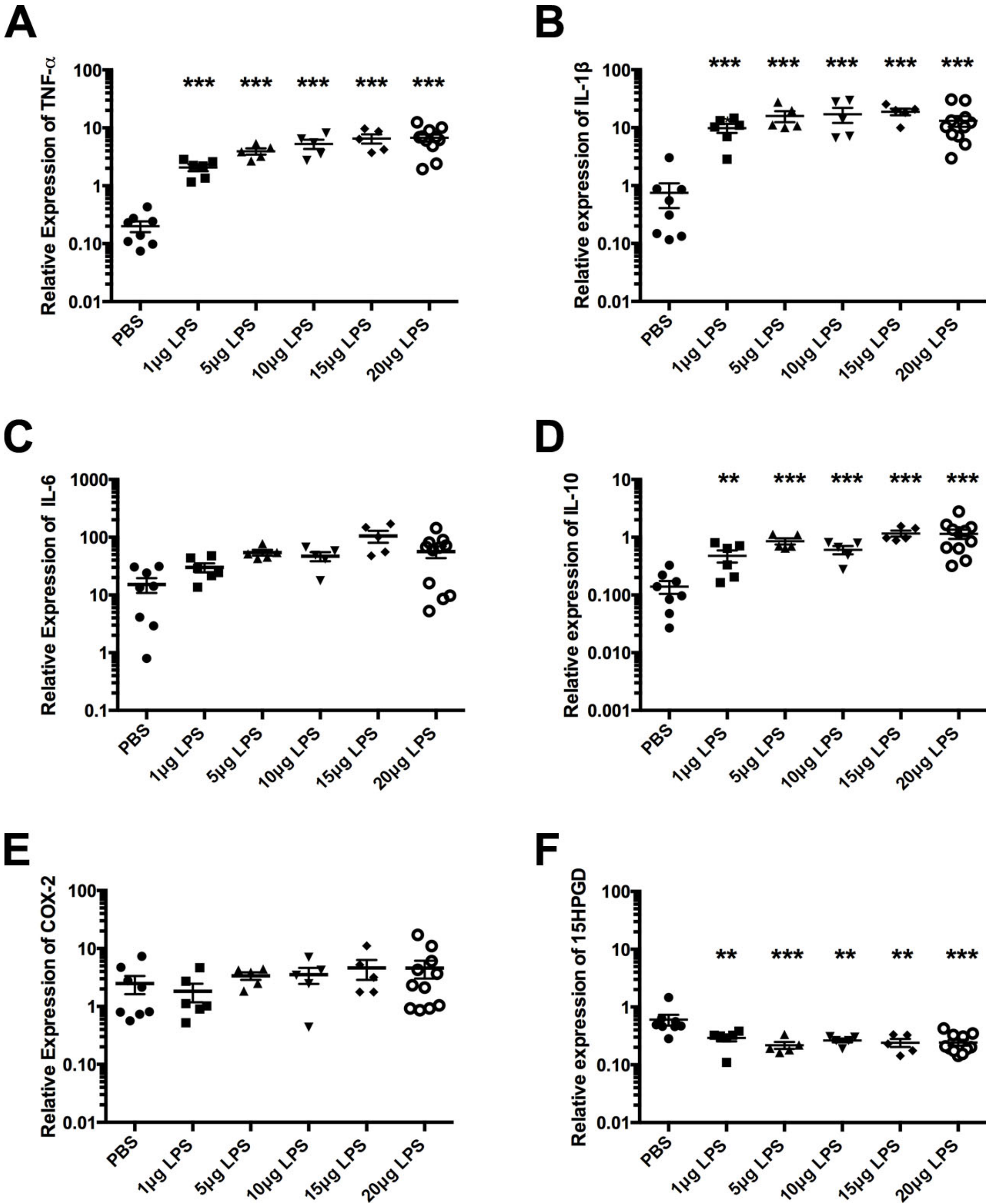

Figure 3.5 - Effect of intrauterine LPS administration on inflammatory gene expression in the fetal membranes. Fetal membranes were collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of several inflammatory genes assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold change ± SEM (error bars); **p<0.01, ***p<0.001, compared to PBS.

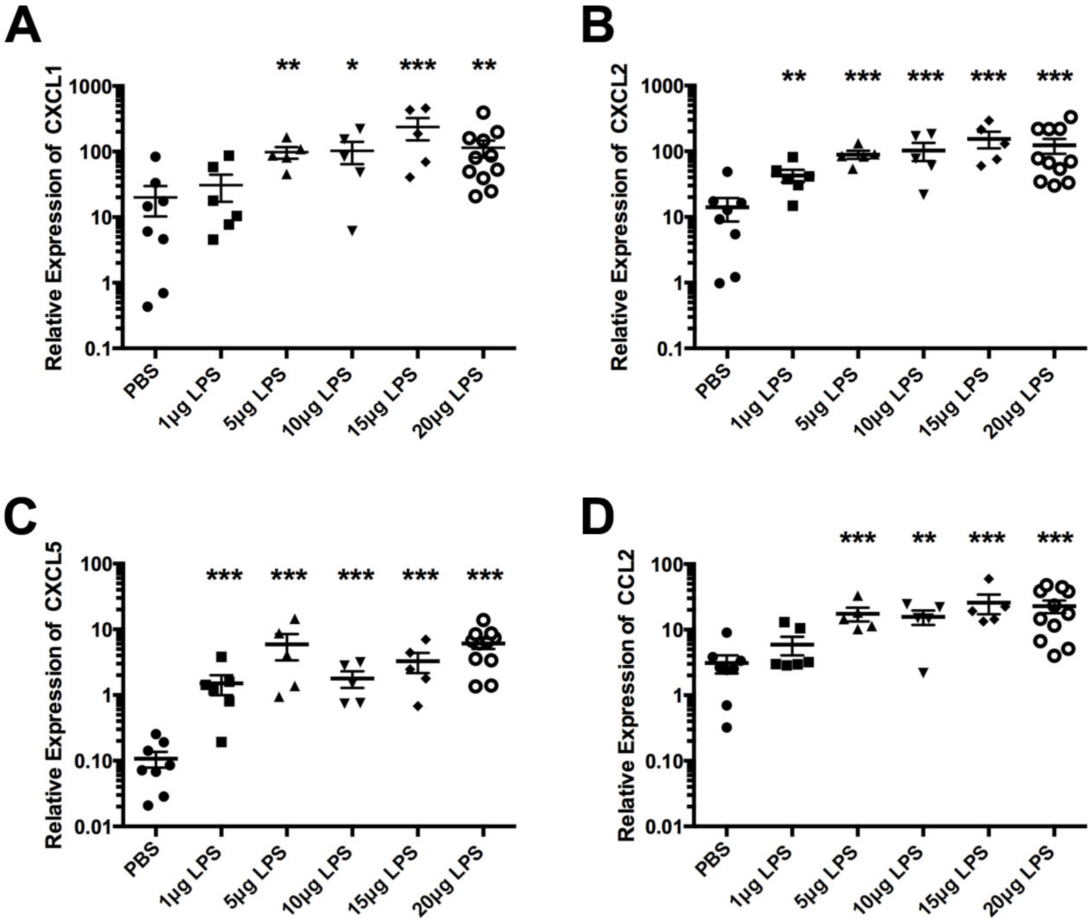

Figure 3.6 - Effect of intrauterine LPS administration on inflammatory chemokine expression in the fetal membranes. Fetal membranes were collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of several inflammatory genes assessed by qRT-PCR**. (A)** *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold fold-change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to PBS.

3.3.2.3 Inflammatory gene expression in the placenta

Tnf-α in the placenta was significantly elevated in response to all doses of LPS administered (p<0.001; Figure 3.7A), with 7-fold higher expression in the placenta of mice receiving 20μg of LPS, compared to those receiving PBS.

Similarly placental *Il-1β* expression was significantly increased at all doses of LPS administered, compared to the PBS control (p<0.001; Figure 3.7B), with 10-fold elevated expression in response to 20μg of LPS compared to the control.

Il-6 expression was also significantly elevated in the placenta in response to all doses of LPS, with 6.3-fold higher expression in response to 20μg LPS, compared to the PBS control (p<0.001; Figure 3.7C)

Expression of *Il-10* was significantly higher in the placentas of mice receiving LPS at all doses administered (p<0.01 at 10μg, p<0.001 at all other doses of LPS; Figure 3.7D), with a 3.4-fold increase in expression of IL-10 comparing the 20μg LPS dose with the PBS control.

No change in placental *Cox-2* expression was observed in response to intrauterine LPS administration (PBS vs. 20μg LPS p=0.99; Figure 3.7E). Similarly, *15-Hpgd* expression in the placenta did not change across treatment groups (PBS vs. 20μg LPS p=0.24; Figure 3.7F).

In response to increasing doses of LPS administered, expression of *Cxcl1* in the placenta was elevated. The level of *Cxcl1* expression was significantly higher in mice receiving 5μg, 10μg, 15μg and 20μg of LPS (p<0.001; Figure 3.8A), with 4.3-fold elevated expression at 20μg of LPS compared to PBS. Placental *Cxcl2* and *Cxcl5* expression was significantly elevated in response to each dose of LPS administered compared to the PBS control (p<0.001). At 20μg of LPS, *Cxcl2* expression was 12.7-fold higher than PBS (Figure 3.8B); and *Cxcl5* expression was 21.8-fold higher than the PBS control (Figure 3.8C).

Placental *Ccl2* expression was significantly higher in mice receiving all doses of LPS administered (p<0.001; Figure 3.8D), with 6.8-fold higher expression in mice receiving 20μg of LPS, compared with those receiving PBS.

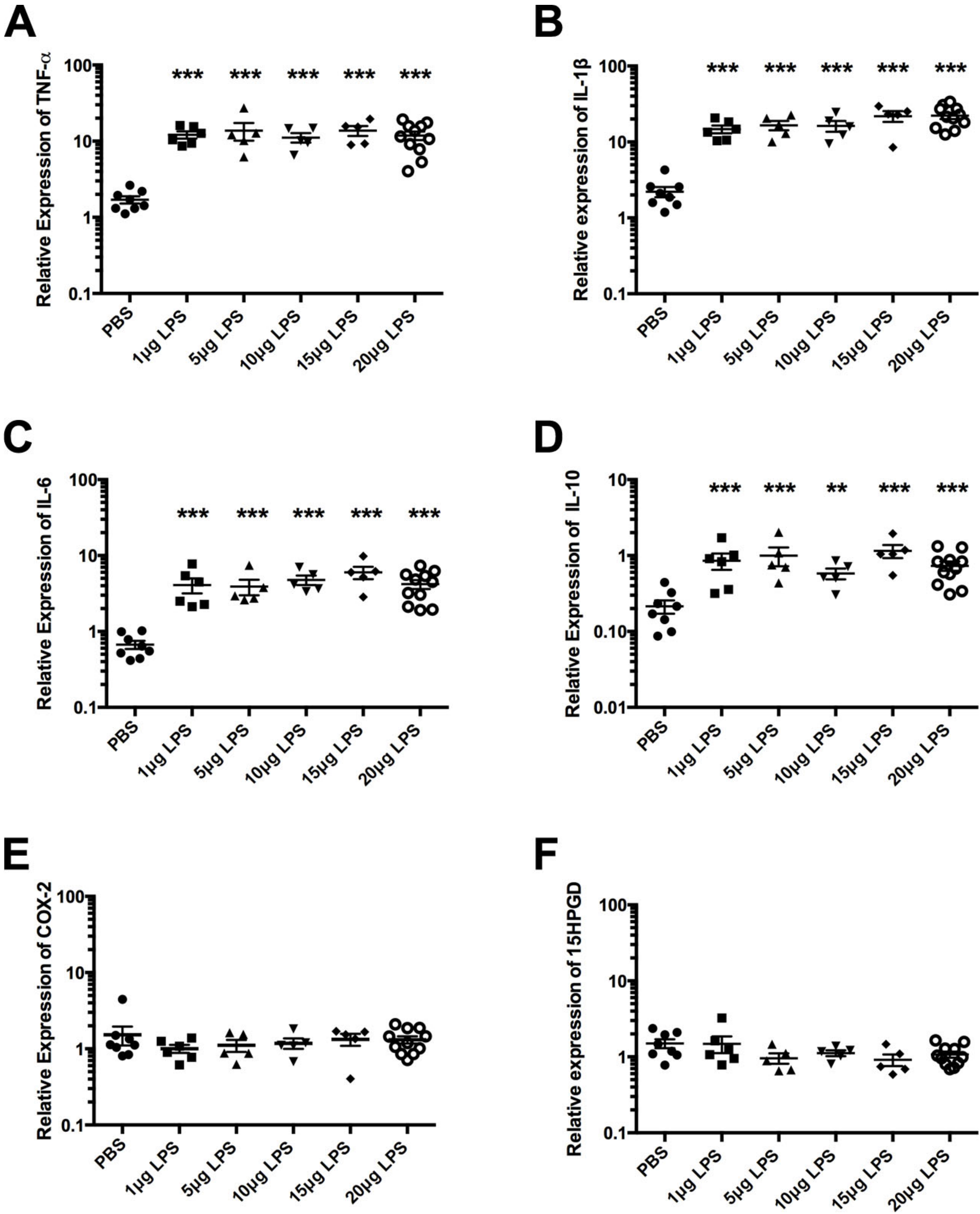

Figure 3.7 - Effect of intrauterine LPS administration on inflammatory gene expression in the placenta. Placental tissue was collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of several inflammatory genes assessed by qRT-PCR. **(A)** *Tnf-α* expression**. (B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression**. (E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold change ± SEM (error bars); **p<0.01, ***p<0.001, compared to PBS.

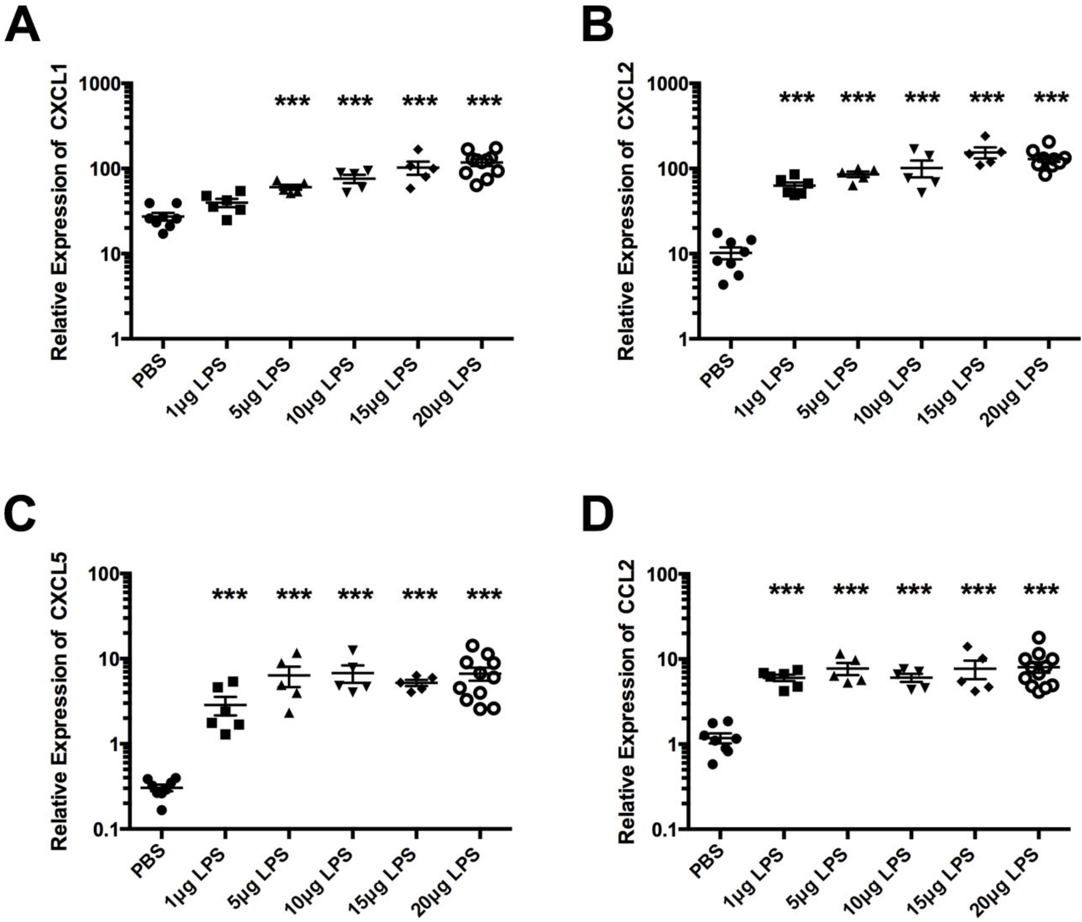

Figure 3.8 - Effect of intrauterine LPS administration on inflammatory chemokine expression in the placenta. Placental tissue was collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of several inflammatory genes assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold fold-change ± SEM (error bars); ***p<0.001, compared to PBS.

3.3.3 TNF-α protein levels in utero-placental tissues following LPS treatment.

To further investigate the inflammatory response induced by LPS treatment in the utero-placental tissues, western blotting was performed to determine whether the increased inflammatory gene expression measured by qRT-PCR was translated into the increased production of inflammatory proteins. TNF-α was chosen as the candidate inflammatory protein to examine because LPS treatment induced large fold-changes in *Tnf-α* mRNA expression in all three utero-placental tissues studied. TNF-α is initially produced as a 26kDa transmembrane protein that is then cleaved by tumour necrosis factor-alpha converting enzyme (TACE) into the mature TNF-α cytokine that weighs 17kDa (Black *et al.* 1997, Moss *et al.* 1997). Production of TNF-α protein was determined in the uterus, fetal membranes and placenta collected 6 hours post-surgery from mice receiving either intrauterine injection of either PBS or 20μg LPS, the antibody used should identify both precursor and mature TNF-α.

3.3.3.1 Uterine TNF-α protein production

Western blotting detected a protein weighing around 25kDa in the uterine tissue of mice receiving both PBS and LPS, mature TNF-α was not detectable in uterus from either treatment group (Figure 3.9A). β-tubulin was used as a loading control (Figure 3.9B). Densitometric analysis of the band intensity of TNF-α precursor relative to the amount of protein present in each sample showed there was no significant difference in the amount of TNF-α present in the uterine tissue of mice receiving PBS or LPS (Figure 3.9C; p=0.86).

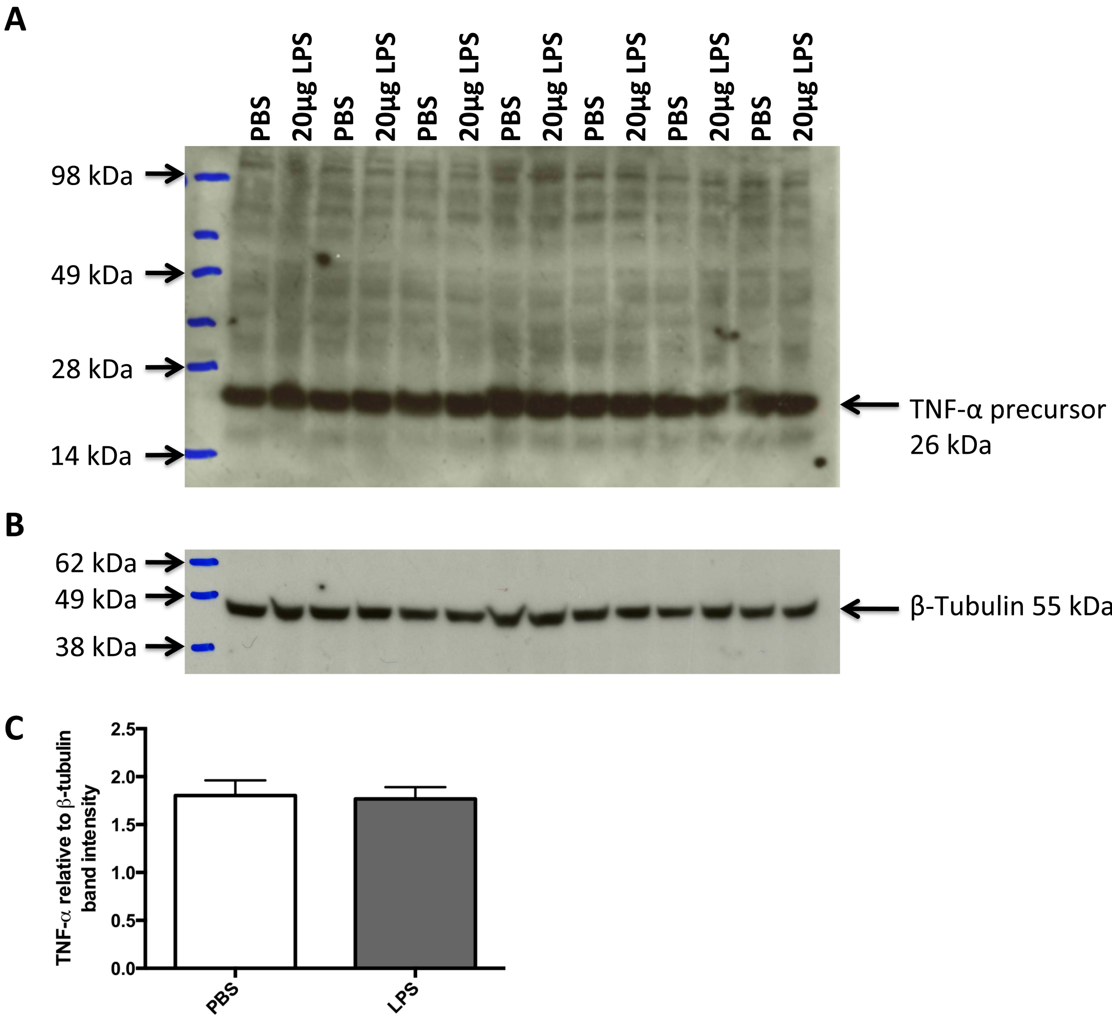

Figure 3.9 - Uterine TNF-α production. Uterine tissue was collected 6 hours post-surgery from mice receiving PBS (n=7) or 20μg LPS (n=7) and the production of TNF-α was examined by western blotting. **(A)** The 25kDa TNF-α precursor protein was detected in uterine tissue from mice receiving PBS and LPS. **(B)** β-tubulin was used as a protein loading control. **(C)** Densitometric analysis of TNF-α band intensity relative to β-tubulin showed there was no significant difference between PBS and LPS treatment in uterine TNF-α production. Data presented as mean ± SEM (error bars).

3.3.3.2 Fetal membrane TNF-α protein production

Western blotting detected a protein weighing around 25kDa in the fetal membranes of mice receiving both PBS and LPS, the 17kDa mature TNF-α protein was not detected in the fetal membranes from either treatment group (Figure 3.10A). β-tubulin was used as a loading control (Figure 3.10B). Densitometric analysis of the band intensity of TNF-α precursor relative to the amount of protein present in each sample showed there was a significant reduction in the amount of TNF-α precursor protein present in the fetal membranes of mice receiving LPS compared to the PBS control group (Figure 3.10C; $p<0.05$).

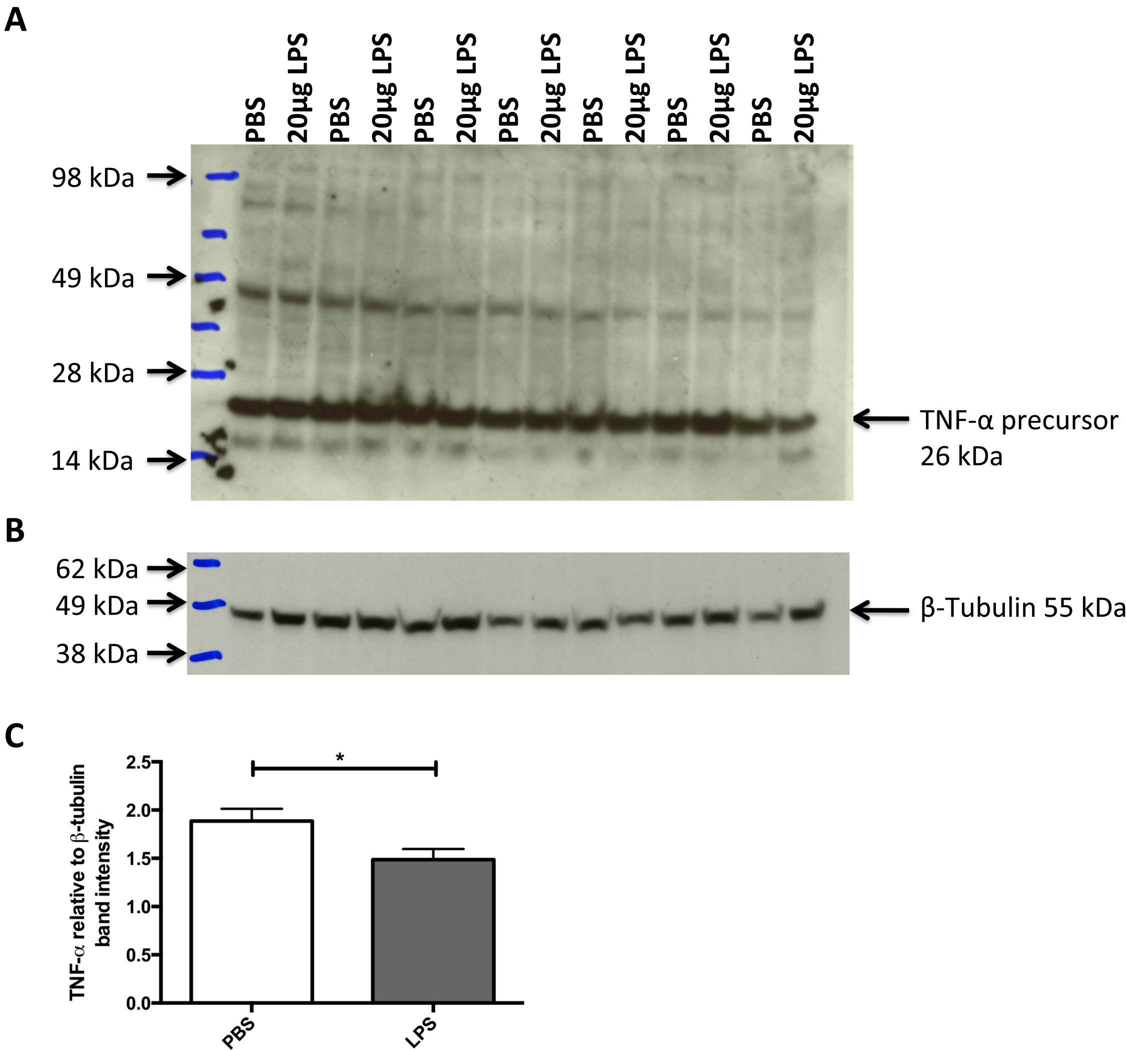

Figure 3.10 - Fetal membrane TNF-α production. Fetal membranes were collected 6 hours post-surgery from mice receiving PBS (n=7) or 20µg LPS (n=7) and the production of TNF-α was examined by western blotting. **(A)** The 25kDa TNF-α precursor protein was detected in fetal membranes from mice receiving PBS and LPS. **(B)** β-tubulin was used as a protein loading control. **(C)** Densitometric analysis of TNF-α band intensity relative to β-tubulin showed there was a significant reduction in the amount of TNF-α precursor in the fetal membranes of mice treated with LPS, compared to those receiving PBS. Data presented as mean ± SEM (error bars); *p<0.05.

3.3.3.3 Placental TNF-α protein production

Western blotting detected a protein weighing around 25kDa in the placental tissue of mice receiving both PBS and LPS. As in the uterus and fetal membranes, the 17kDa mature TNF-α protein was not detectable in the placental tissue from either treatment group (Figure 3.11A). β-tubulin was used as a loading control (Figure 3.11B). Densitometric analysis of the band intensity of TNF-α precursor relative to the amount of protein present in each sample showed there was no significant difference in the amount of TNF-α precursor protein present in the placental tissue of mice in either treatment group (Figure 3.11C; $p=0.42$).

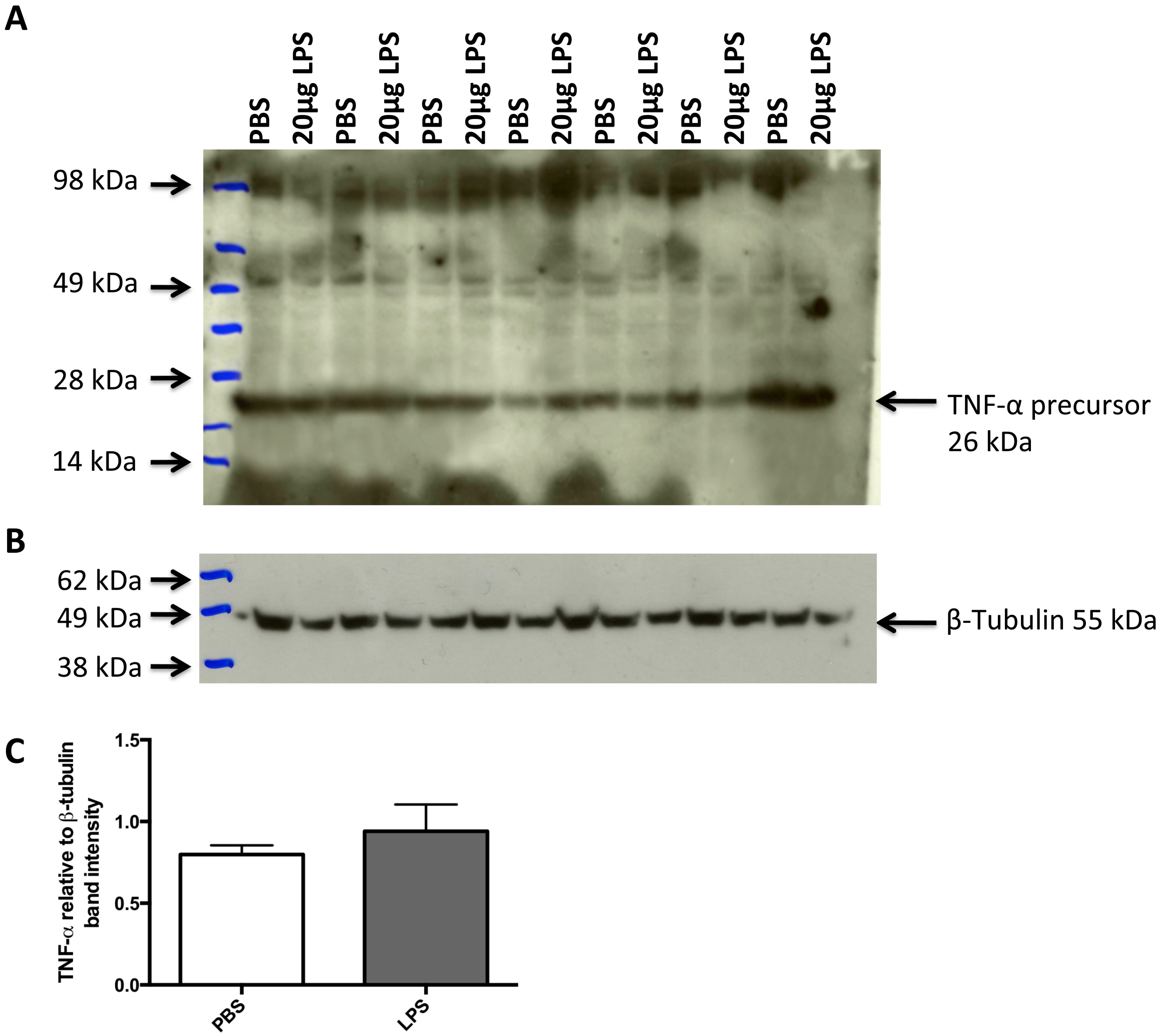

Figure 3.11 - Placental TNF-α production. Placental tissue was collected 6 hours post-surgery from mice receiving PBS (n=7) or 20µg LPS (n=7) and the production of TNF-α was examined by western blotting. **(A)** The 25kDa TNF-α precursor protein was detected in the placental tissue from mice receiving PBS and LPS. **(B)** β-tubulin was used as a protein loading control. **(C)** Densitometric analysis of TNF-α band intensity relative to β-tubulin showed there was no significant difference between PBS and LPS treatment in placental TNF-α production. Data presented as mean ± SEM (error bars).

3.3.4 Immune cell influx in response to intrauterine LPS administration

A number of studies have highlighted that both labour and preterm labour are associated with an immune cell influx in the utero-placental tissues (Thomson *et al.* 1999, Osman *et al.* 2003, Hamilton *et al.* 2012). Therefore, to determine whether immune cells played a role in our model of LPS-induced preterm labour, utero-placental tissues were collected 6 hours post-intrauterine injection and the expression of specific immune cell markers was measured by qRT-PCR and immune cells were localised in the uterus using immunohistochemistry.

3.3.4.1 Macrophage marker expression in the utero-placental tissues

To assess macrophage influx, expression of the *Emr1* gene was measured; this is the gene which codes for a specific antigen expressed on macrophages, F4/80. Uterine expression of *Emr1* was unaffected by intrauterine administration of LPS at any dose (Figure 3.12A). Similarly placental *Emr1* expression was unaffected by LPS treatment (Figure 3.12B). Expression of *Emr1* in the fetal membranes was significantly elevated in response to 20μg LPS, with 1.6-fold higher expression compared to the PBS control group ($p<0.05$; Figure 3.12C).

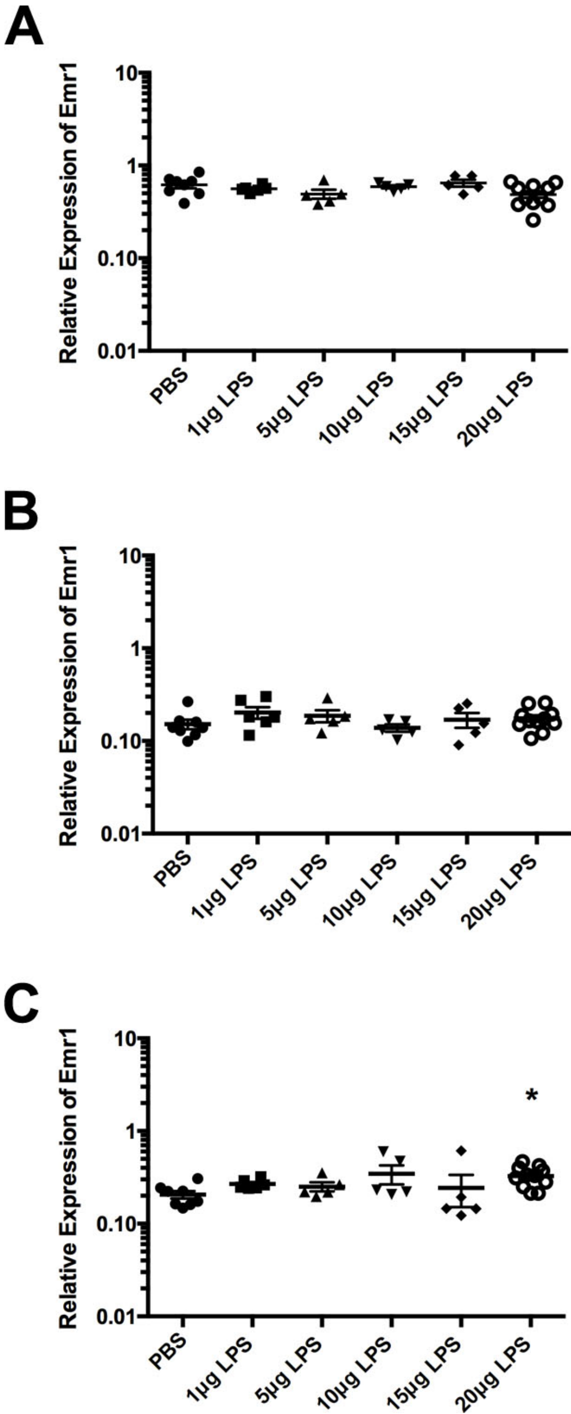

Figure 3.12 - Expression of *Emr1* mRNA in the uterus, placenta and fetal membranes. Uterus, placenta and fetal membranes were collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of *Emr1* was investigated using qRT-PCR. **(A)** *Emr1* mRNA expression in the uterus. (**B)** Placental *Emr1* mRNA expression. **(C)** *Emr1* mRNA expression in the fetal membranes. Data presented as mean fold change ± SEM (error bars); *p<0.05, compared to the PBS control.

3.3.4.2 Macrophage localisation in the uterus

To determine where in the uterus macrophages reside during pregnancy, and investigate whether surgery and/or LPS treatment affect their localisation, immunohistochemistry using an anti-F4/80 antibody was performed on uterine tissue collected from mice receiving PBS or 20μg LPS, 6 hours post-surgery and also from pregnant mice on D17 of gestation who had no surgery or treatment, as a comparison.

On D17 of gestation, in mice receiving no surgery or treatment, macrophages were localised to the myometrium (Figure 3.13A) and the decidua of these mice (Figure 3.13B). In mice receiving intrauterine PBS, macrophages were similarly found to be present in both the myometrium (Figure 3.13C) and decidua (Figure 3.13D), with more positively stained cells present in the endometrium compared to the myometrium. In mice treated with 20μg LPS, F4/80 positive macrophages were still present within the myometrium (Figure 3.13E) and decidua (Figure 3.13F). No positive staining was observed in negative control sections of myometrium (Figure 3.13G) and decidua (Figure 3.13H).

There were no clear differences in either the localisation or the number of macrophages present within the uterus when comparing PBS and LPS treatment; therefore stereological analysis was performed to further investigate macrophage localisation within the uterus, to determine if LPS treatment affected the distribution of F4/80 positive cells within the uterus. Ten randomised fields of view of decidua and myometrium were counted in each uterine section in order to give an estimate of the number of F4/80 positive cells in the uterus of mice receiving PBS or 20μg LPS. In response to intrauterine LPS, there was a mean 1.6-fold reduction in the number of F4/80 positive cells counted in the decidua, compared to the PBS control, however this did not reach statistical significance ($p=0.05$). Similarly, in the myometrium, LPS treatment resulted in a mean 2.1-fold decrease in the number of F4/80 positive cells counted, but again this reduction was not found to be statistically significant when compared with mice receiving PBS ($p=0.06$;Figure 3.14).

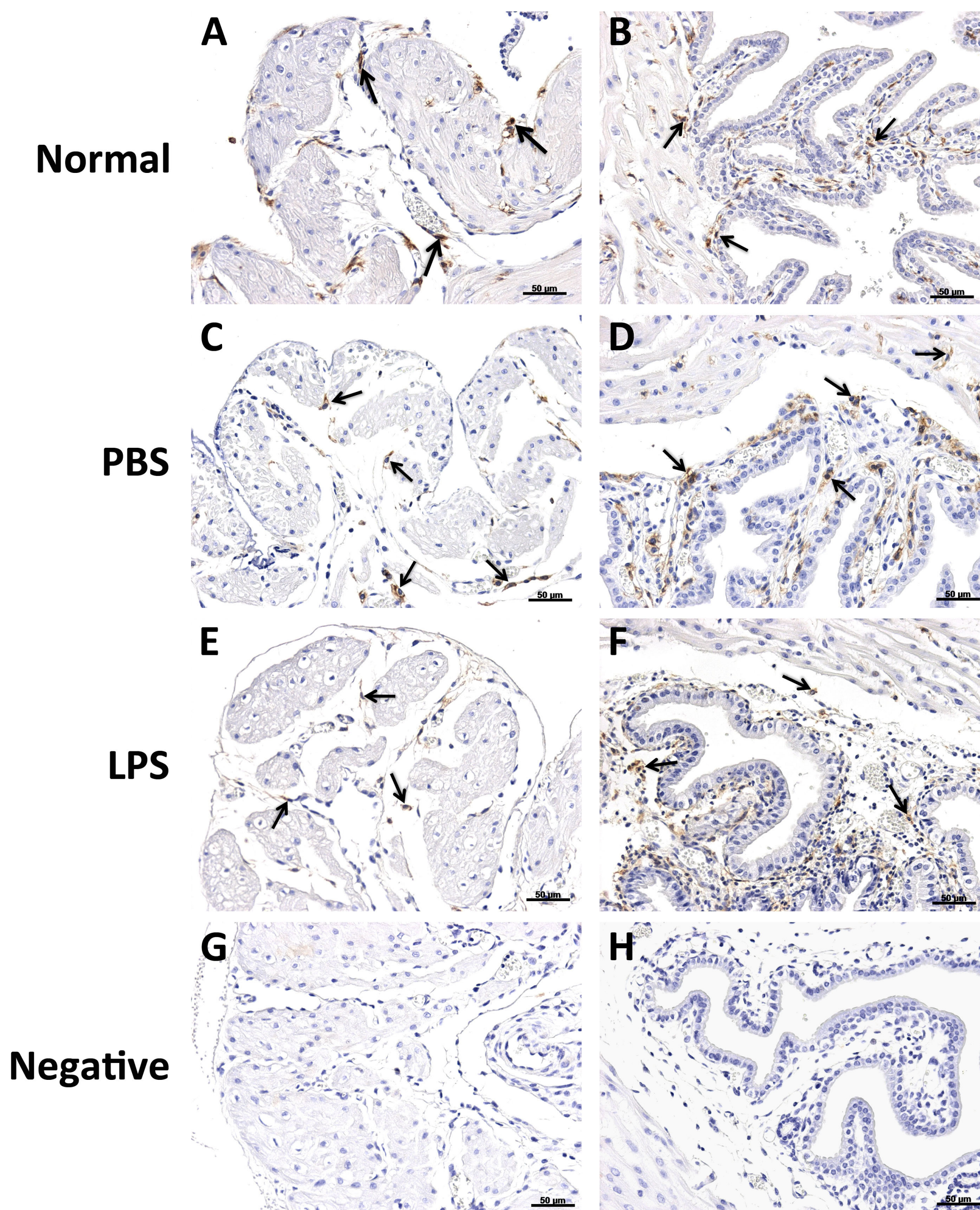

Figure 3.13 - Macrophage localisation in the uterus. Uterine tissue was collected 6 hours post-surgery from mice receiving PBS (n=4) or 20μg LPS (n=5). Representative images of macrophage localisation by immunohistochemical staining for F4/80. F4/80 positive cells were present in the myometrium **(A)** and decidua **(B)** of normal untreated D17 mice. Intrauterine injection of PBS did not appear to alter the localisation of F4/80 positive cells within the uterus, with macrophages still found within the myometrial tissue **(C)** and decidua tissue **(D).** Positive F4/80 macrophages were similarly located in the myometrium **(E)** and decidua **(F)** of mice receiving intrauterine LPS. No positive staining was observed in the negative control sections of myometrium **(G)** and decidua **(H).** Scale bars show 50μm, arrows indicate example of positively stained cells. All images taken with a x20 objective lens

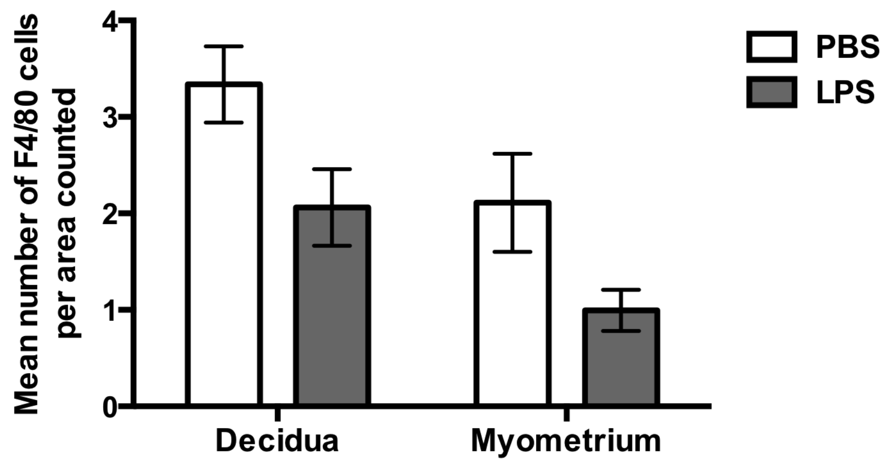

Figure 3.14 - Quantification of F4/80 positive cells in the uterus following surgery. Uterine tissue was collected 6 hours post-surgery from mice receiving PBS (n=4) or 20μg LPS (n=5). Decidua and myometrium were analysed separately. A total of ten randomised fields of view were counted per section, with nine sections of uterus counted in total per mouse. Data presented as mean ± SEM (error bars).

3.3.4.3 Neutrophil marker expression in the utero-placental tissues

To assess neutrophil influx into the utero-placental tissues, expression of the gene coding for neutrophilic granule protein, (NGP) was measured by qRT-PCR. Administration of LPS resulted in significantly elevated expression of *Ngp* in the uterus at all doses of LPS, compared to the PBS control (1μg and 5μg vs. PBS, p<0.01; 10μg, 15μg and 20μg vs. PBS, p<0.001; Figure 3.15A), with a 4.7-fold increase in expression in response to 20μg LPS compared to PBS. Similarly expression of *Ngp* in the fetal membranes was also significantly elevated in response to all doses of LPS administered (p<0.001; Figure 3.15B), with 5.7-fold higher expression of *Ngp* in response to 20μg LPS compared to PBS. Placental expression of *Ngp* was also significantly elevated in response to increasing doses of LPS, with significantly higher expression in the placenta in response to 15μg and 20μg LPS (p<0.001; Figure 3.15C), with a 2-fold increase in expression in response to 20μg LPS, compared to the PBS control.

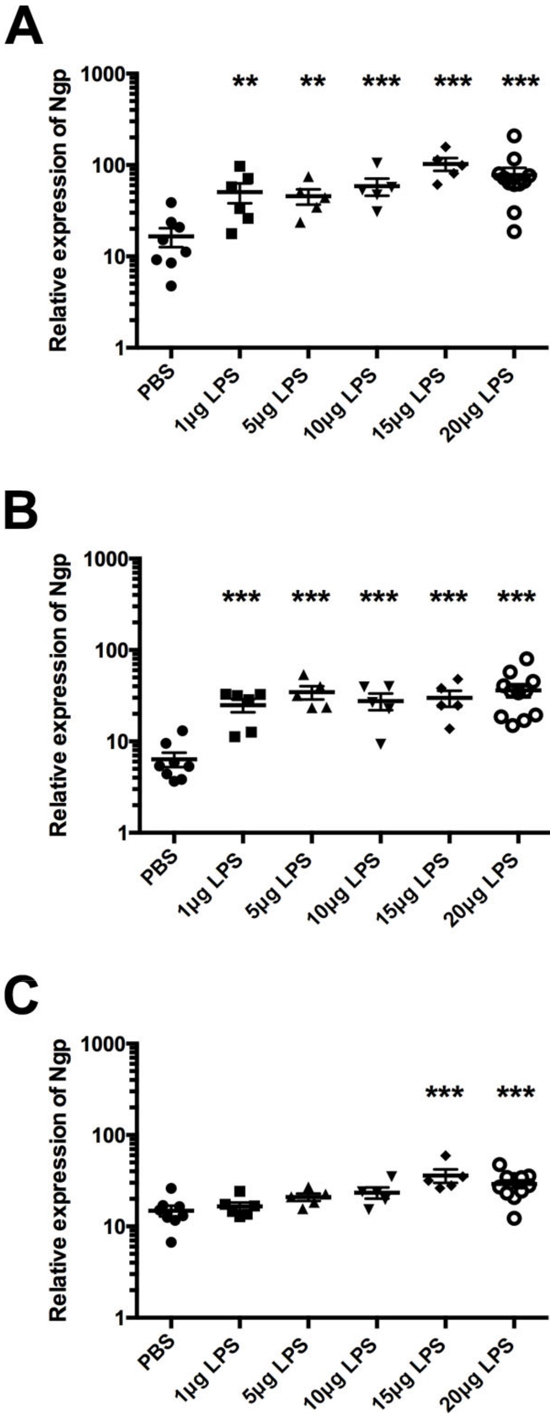

Figure 3.15 - Expression of *Ngp* mRNA in the uterus, fetal membranes and placenta. Uterus, fetal membranes and placenta were collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of *Ngp* was investigated using qRT-PCR. **(A)** *Ngp* mRNA expression in the uterus **(B)** *Ngp* mRNA expression in the fetal membranes**.** **(C)** Placental *Ngp* mRNA expression. Data presented as mean fold change ± SEM (error bars); **p<0.01, ***p<0.001, compared to the PBS control.

3.3.4.4 Neutrophil localisation in the uterus

To confirm that increased *Ngp* expression was due to neutrophil influx into the uterus and to determine where in the uterus neutrophils were being recruited to, immunohistochemistry using an anti-Gr-1 antibody was performed on uterine tissue collected from mice receiving PBS or 20μg LPS, 6 hours post-surgery and also from pregnant mice on D17 of gestation who had no surgery or treatment, as a comparison.

On D17 of gestation, in mice who received no treatment and who did not undergo any surgery, there were no neutrophils present in the myometrium (Figure 3.16A) and very few, if any, present in the decidua of these mice (Figure 3.16B). PBS treated mice had positive Gr-1 staining in the connective tissue layer surrounding the longitudinal myometrial muscle bundles, and within blood vessels of the myometrium (Figure 3.16C), but similarly to the untreated controls, there were very few, if any, positive cells located within the decidua (Figure 3.16D). Mice treated with LPS, still had Gr-1 positive cells within the myometrium, as with the PBS treated mice (Figure 3.16E), however there was a striking difference when comparing the decidua of the two treatment groups, where the LPS treated mice had a large influx of neutrophils into the decidua (Figure 3.16F). No positive staining was observed in negative control sections of myometrium (Figure 3.16G) and decidua (Figure 3.16H).

To investigate further the different localisation of neutrophils within the uterus of mice receiving PBS and LPS, stereological analysis was performed. Ten randomised fields of view of decidua and myometrium were counted in each uterine section in order to give an estimate of the number of Gr-1 positive cells and their distribution in the uterus of mice receiving PBS or 20μg LPS. In response to 20μg LPS, there was a significant increase in the number of Gr-1 positive cells present in the sampled areas of decidua, with approximately 7-fold more neutrophils present in the LPS treated group, compared to the PBS control group ($p=0.006$). There were no significant differences in the number of Gr-1 cells present in the sampled areas of myometrium when comparing between the PBS and LPS treated groups ($p=0.57$;Figure 3.17).

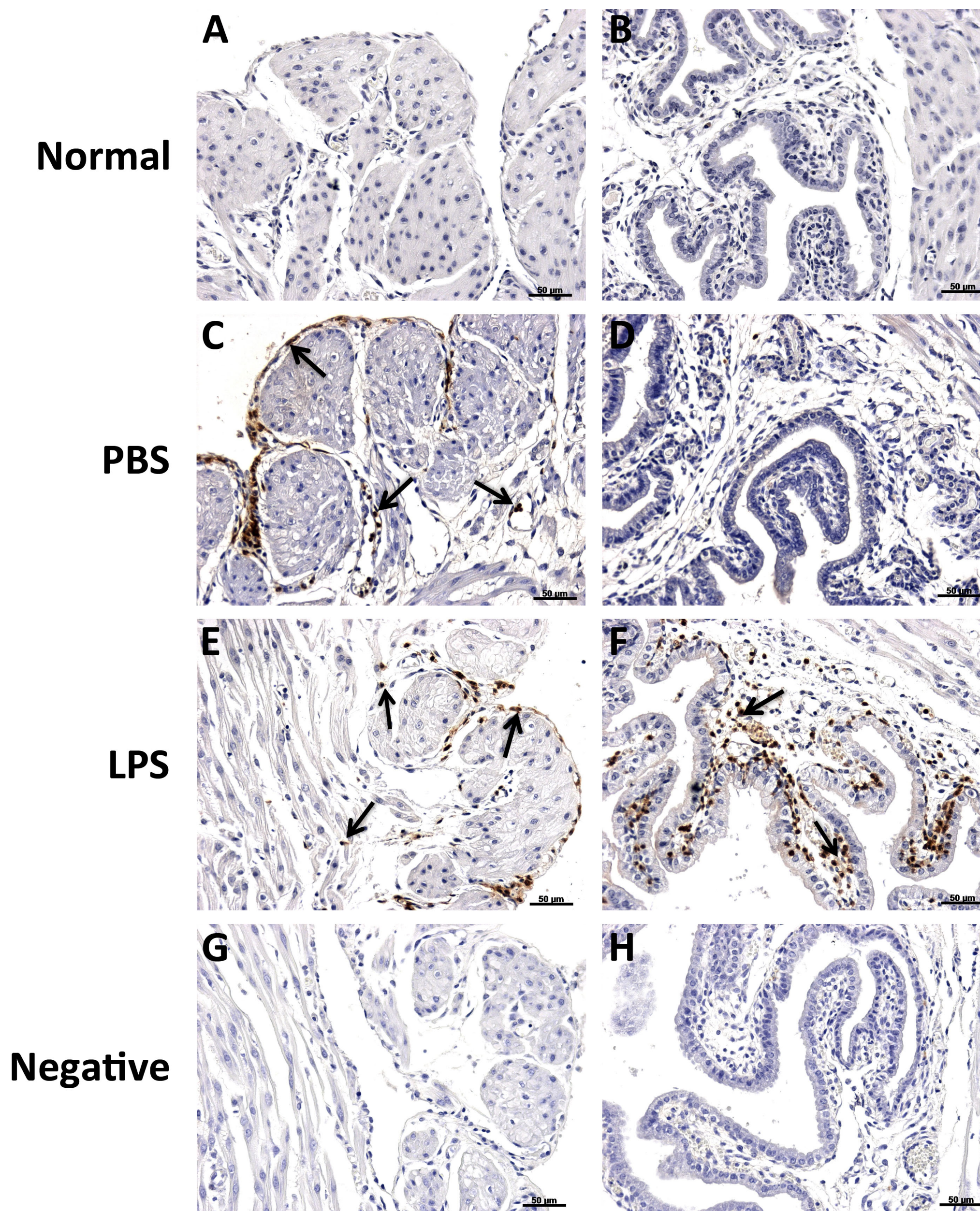

Figure 3.16 - Neutrophil localisation in the uterus. Uterine tissue was collected 6 hours post-surgery from mice receiving PBS (n=4) or 20μg LPS (n=5). Representative images of neutrophil localisation by immunohistochemical staining for Gr-1. No Gr-1 positive cells were present in the myometrium **(A)** and decidua **(B)** of normal untreated D17 mice. In response to intrauterine injection of PBS, Gr-1 positive cells were localised to the myometrium, surrounding the longitudinal muscle bundles, and within blood vessels **(C).** As in the untreated controls, there were no neutrophils present in the decidua of mice receiving PBS **(D).** Positive Gr-1 neutrophils were similarly localised to the myometrium in mice receiving LPS **(E).** An influx of neutrophils into the decidua of mice was observed in response to intrauterine injection of 20μg of LPS **(F).** No positive staining was observed in the negative control sections of myometrium **(G)** and decidua **(H).** Scale bars show 50μm, arrows indicate examples of positively stained cells. All images taken with a x20 objective lens.

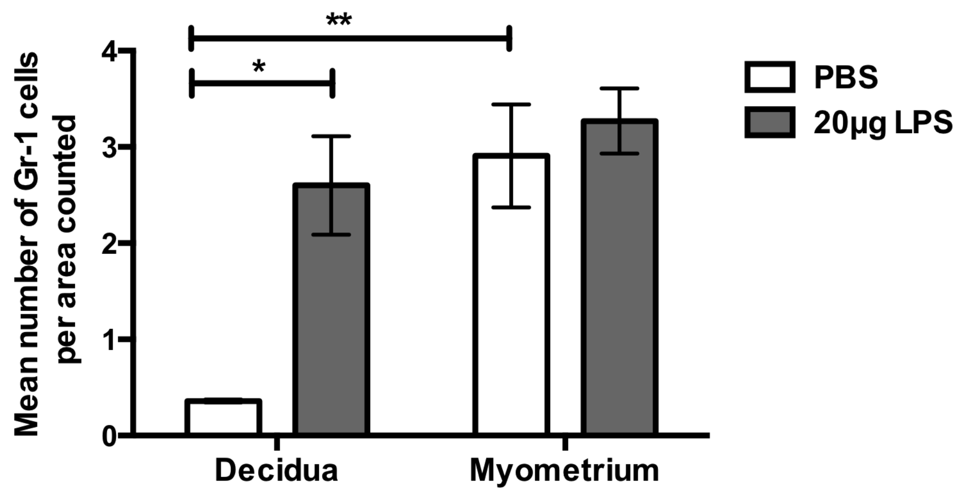

Figure 3.17 - Quantification of Gr-1 positive cells in the uterus following surgery. Uterine tissue was collected 6 hours post-surgery from mice receiving PBS (n=4) or 20μg LPS (n=5). Decidua and myometrium were analysed separately. A total of ten randomised fields of view were counted per section, with nine sections of uterus counted in total per mouse. Data presented as mean ± SEM (error bars). $*p<0.05$, $**p<0.01$.

3.3.5 Inflammatory markers in the amniotic fluid

Amniotic fluid was collected 6 hours post-surgery to examine the level of inflammatory cytokine production in the amniotic fluid in response to intrauterine LPS administration. ELISAs were used to measure the concentration of the cytokines TNF-α, IL-1β, IL-6 and IL-10. Where the concentration of cytokines was too low to be detected by the ELISAs, the value of the limit of detection of the specific ELISA was entered to give a value for analysis and the data were analysed by non-parametric ANOVA (Kruskal-Wallis).

TNF-α was undetectable in six PBS control samples, one 10μg LPS sample and one 20μg LPS sample. Administration of LPS resulted in increased amniotic fluid TNF-α levels, with significantly higher TNF-α production in response to 5μg LPS (mean 106.5 pg/ml ± SEM 38.23; $p<0.01$), 15μg LPS (mean 75.18 pg/ml ± SEM 30.71; $p<0.05$) and 20μg of LPS (mean 91.74 pg/ml ± SEM 28.75; $p<0.01$), compared with the PBS control group (mean 11.96 pg/ml ± SEM 1.06; Figure 3.18A)

IL-1β was undetectable in one 10μg LPS sample. Amniotic fluid levels of IL-1β were unaffected by intrauterine LPS administration, although slightly elevated IL-1β was measured in the amniotic fluid from mice receiving 20μg LPS (mean 54.59 pg/ml ± SEM 10.43) compared to PBS (mean 33.08 pg/ml ± SEM 5.08), this was not statistically significant ($p= 0.99$; Figure 3.18B).

Amniotic fluid levels of IL-6 were elevated in response to LPS treatment, with significantly higher levels of IL-6 measured in the amniotic fluid of mice receiving 20μg LPS (mean 2216 pg/ml ± SEM 484.7) compared with those receiving PBS (mean 473.9 pg/ml ± SEM 228.2; $p<0.01$; Figure 3.18C).

IL-10 was undetectable in five PBS samples, two 1μg LPS samples, three 10μg LPS samples, one 15μg LPS sample and four 20μg LPS samples. Intrauterine administration of LPS resulted in significantly elevated IL-10 production in response to 5μg LPS (mean 20.94 pg/ml ± SEM 15.5; $p<0.05$) and 15μg LPS (mean 24.38 pg/ml ± 4.29; $p<0.05$), compared with mice receiving PBS (mean 15.5 pg/ml ± SEM 0.37; Figure 3.18D).

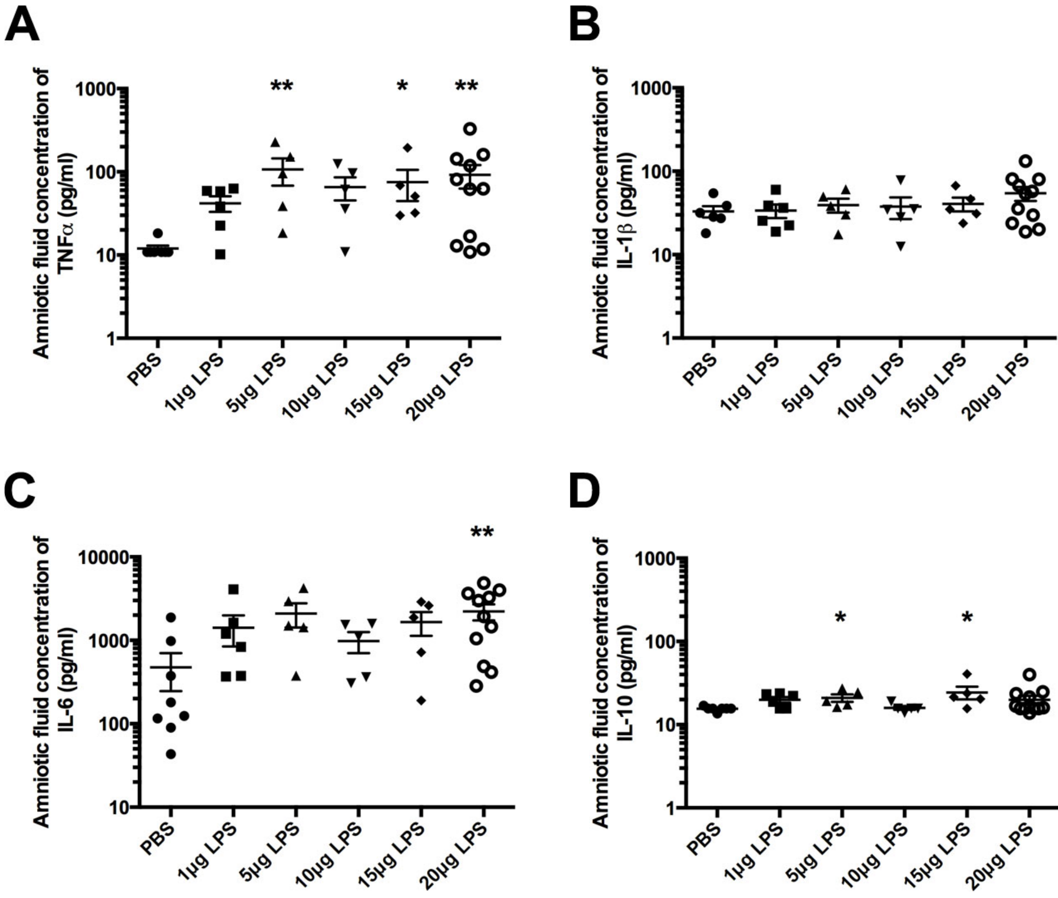

Figure 3.18 - Inflammatory markers in the amniotic fluid. Amniotic fluid was collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and ELISAs were used to quantify the concentration of inflammatory cytokines present in the amniotic fluid. **(A)** Amniotic fluid TNF-α concentration **(B)** Amniotic fluid IL-1β concentration**. (C)** Amniotic fluid IL-6 concentration. **(D)** Amniotic fluid IL-10 concentration. Data presented as mean ± SEM (error bars); *p<0.05, **p<0.01, compared to the PBS control.

3.3.6 Serum markers in response to intrauterine LPS administration

To investigate whether intrauterine LPS administration resulted in a systemic inflammatory response, the serum concentrations of two candidate cytokines measured in 3.3.5. were determined. The pro-inflammatory cytokine, TNF-α, and the anti-inflammatory cytokine, IL-10, were measured by ELISA in maternal serum collected 6 hours post-surgery. Also because a drop in circulating progesterone levels normally precedes labour in mice, serum progesterone concentration was also measured by ELISA.

Serum levels of TNF-α were elevated in response to increasing doses of LPS, with significantly higher levels of TNF-α measured when mice received doses of 10µg (mean 13.63 pg/ml ± SEM 2.72; $p<0.01$), 15µg (mean 25.54 pg/ml ± SEM 5.95; $p<0.001$) and 20µg of LPS (mean 19.07 pg/ml ± SEM 3.51; $p<0.01$), compared to the PBS control group (mean 4.06 pg/ml ± SEM 0.74; Figure 3.19A).

Intrauterine administration of LPS resulted in increased circulating levels of IL-10. Serum IL-10 was significantly elevated in response to both 15µg (mean 276.5 pg/ml ± SEM 59.0; $p<0.001$) and 20µg of LPS (mean 182.4 pg/ml ± SEM 23.89; $p<0.01$), compared to the PBS control (mean 52.04 pg/ml ± SEM 6.53; Figure 3.19B).

Serum progesterone levels were unaffected by intrauterine LPS administration. There was no significant difference in the mean progesterone concentration in the PBS group (mean 19.74 ng/ml ± SEM 1.58) compared with any of the doses of LPS administered (mean 20µg concentration 16.7 ng/ml ± SEM 1.48; $p=0.49$; Figure 3.19C).

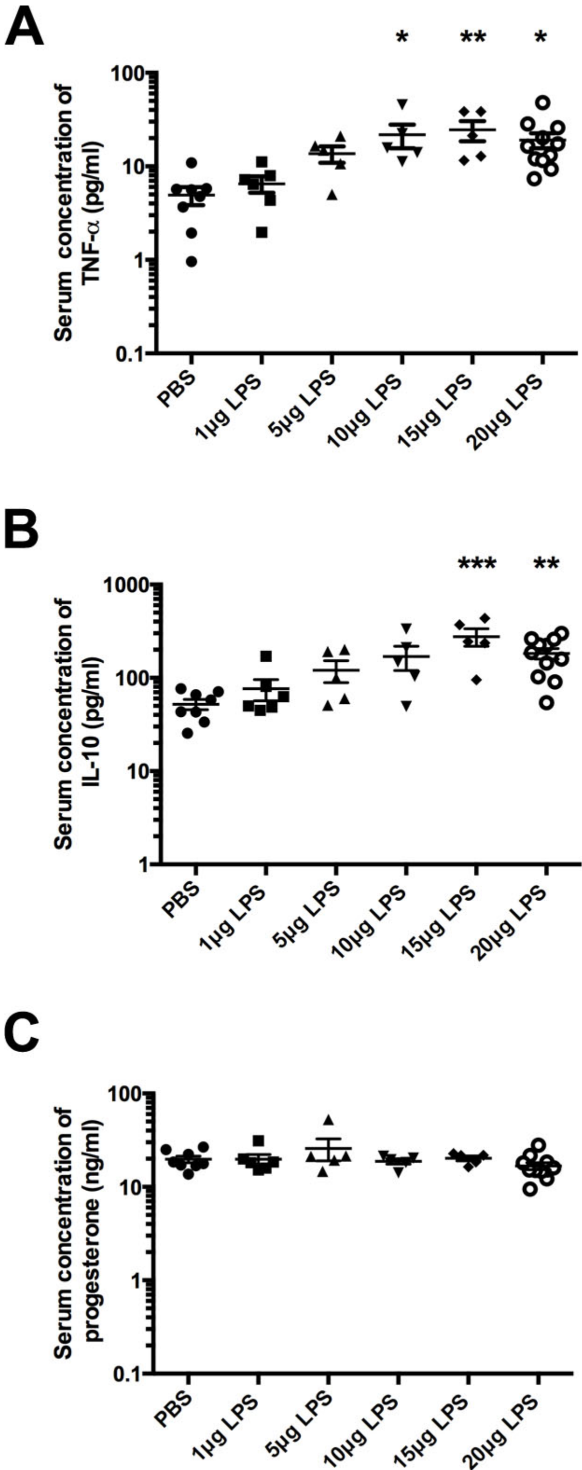

Figure 3.19 - Serum markers in response to intrauterine LPS administration. Maternal blood was collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and serum concentrations of TNF-α, IL-10 and progesterone were measured by ELISA. **(A)** Serum TNF-α concentration. **(B)** Serum IL-10 concentration. **(C)** Serum progesterone concentration. Data presented as mean ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to the PBS control.

3.3.7 Gene expression in the fetal brain following intrauterine LPS administration

The presence of an intrauterine infection is often associated with poor neonatal outcome and brain injury (Shatrov *et al.* 2010). Previous animal studies of preterm labour have demonstrated that administration of LPS can result in increased expression of inflammatory cytokines in the fetal brain, and alter the expression of neuronal markers, suggesting possible brain injury (Elovitz *et al.* 2006, Burd *et al.* 2011).

Fetal brains were collected 6 hours post-intrauterine injection and analysed for the expression of: the pro-inflammatory cytokines *Tnf-*α*, Il-1*β and *Il-6*; the anti-inflammatory cytokine, *Il-10*; and brain-specific markers glial fibrillary associated protein (*Gfap*), and microtubule-associated protein (*Mtap2*), to assess whether intrauterine LPS administration resulted in an increased inflammatory response within the fetal brain or any early signs of brain injury.

Expression of *Tnf-*α in the fetal brain was unaffected by intrauterine LPS treatment (Figure 3.20A). Similarly, LPS treatment did not induce a significant increase in *Il-1*β expression, although a small increase was observed in mice receiving 20μg LPS compared to the PBS control group, this was not statistically significant ($p=0.69$; Figure 3.20B). Unexpectedly *Il-6* expression in the fetal brain was significantly decreased in mice treated with 1μg LPS ($p<0.05$; Figure 3.20C), with 2-fold lower expression compared to the PBS control. *ll-10* expression in the fetal brain was too low to be detected in all treatment groups.

Expression of the marker of astrogliosis, *Gfap,* was not significantly altered in fetal brains of mice receiving LPS, compared to the PBS control (Figure 3.20D). Similarly, expression of the neuronal marker, *Mtap2* in fetal brains was also unaffected by intrauterine administration of LPS (Figure 3.20E).

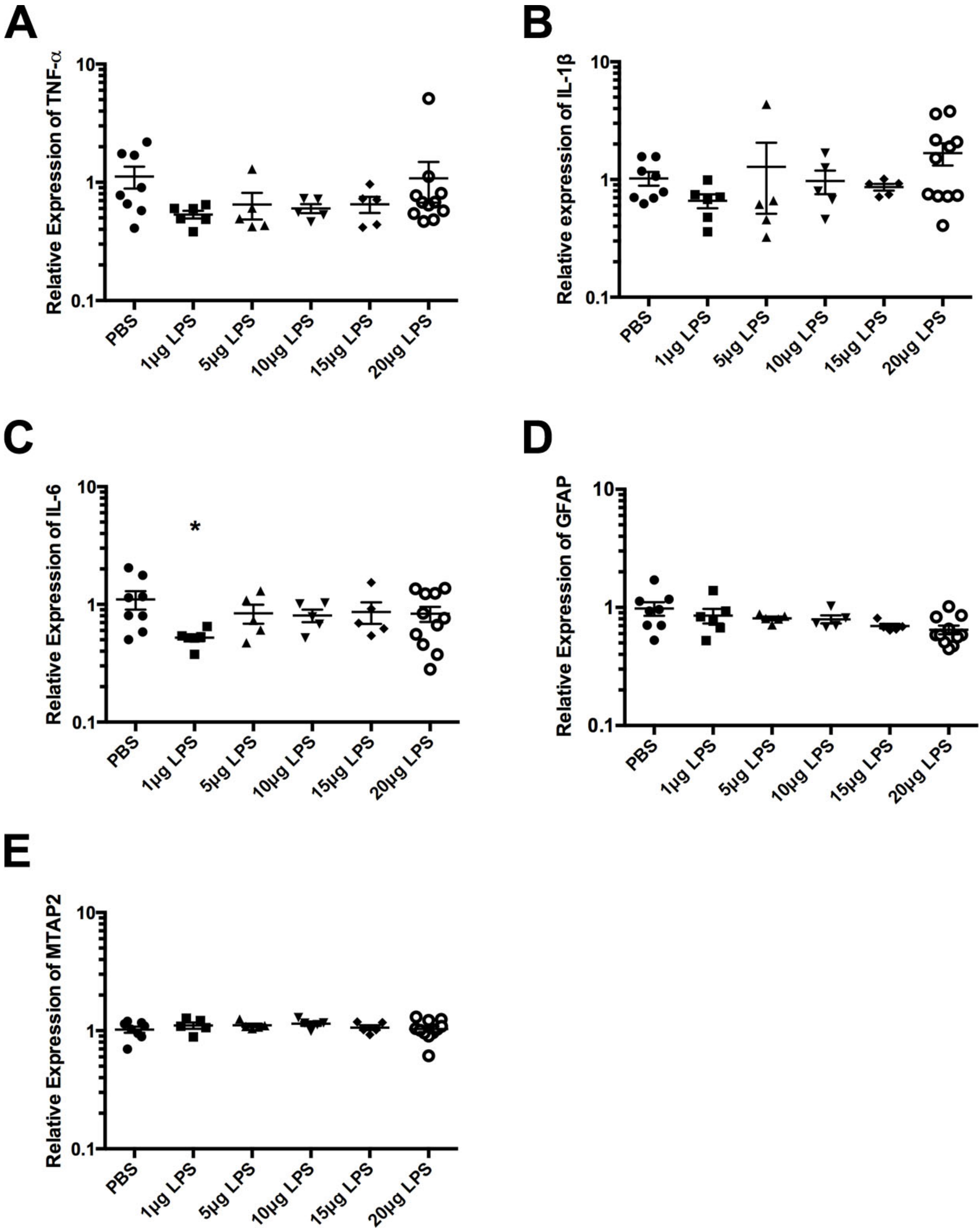

Figure 3.20 - Gene expression in the fetal brains following intrauterine LPS administration. Fetal brains was collected 6 hours post-surgery from mice receiving PBS (n=8), 1μg LPS (n=5), 5μg LPS (n=5), 10μg LPS (n=5), 15μg LPS (n=5) or 20μg LPS (n=11) and the mRNA expression of several inflammatory genes assessed by qRT-PCR. **(A)** *Tnf-α* mRNA expression. **(B)** *Il-1β* mRNA expression, **(C)** *Il-6* mRNA expression. **(D)** *Gfap* mRNA expression. **(E)** *Mtap-2* mRNA expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, compared to the PBS control.

3.4 DISCUSSION

Mouse model of infection induced preterm labour

Animal models of parturition have been used for many years to elucidate the mechanisms underlying the onset of spontaneous labour at term and to understand the causes of preterm labour. The pros and cons of various animal models for parturition research have been extensively discussed (Elovitz and Mrinalini 2004, Mitchell and Taggart 2009, Ratajczak *et al.* 2010). Clearly there are always limitations in the use of any animal model, as they can rarely replicate the exact clinical scenario in humans, but given the fact that collection of gestational tissues from women at various time points during pregnancy is clearly not possible, animal models are required to build a picture of the sequence of events involved in parturition (Mitchell and Taggart 2009). Aside from the clear physiological differences in the reproductive tissues, the main issue raised regarding the use of mice in parturition models, is that progesterone withdrawal is required for normal term labour in this species, which is not a feature of human parturition (Elovitz and Mrinalini 2004). However, recent studies suggest that the induction of preterm labour, by injection of bacterial products in mice, does not appear to rely on progesterone withdrawal (Hirsch and Muhle 2002, Murphy *et al.* 2009, Gonzalez *et al.* 2011), which may improve the suitability of mice in this context. The ability of mice to reproduce efficiently and rapidly with a short gestation, allows for studies to be carried out on a large number of animals over a short space of time, compared with studies using the more closely related non-human primate models (Elovitz and Mrinalini 2004, Mitchell and Taggart 2009, Ratajczak *et al.* 2010). Furthermore, as will be discussed in detail in this chapter, there are many similarities in the response to intrauterine infection between mice and humans, making them a reliable and cost-effective model to study infection-induced preterm labour.

This chapter aimed to use a mouse model of intrauterine LPS administration to induce preterm labour and investigate the associated inflammatory and immune responses. The use of a direct intrauterine injection of LPS to induce preterm labour was first described by Elovitz *et al.* (Elovitz *et al.* 2003), and is now routinely used as a model of preterm labour to investigate the role of inflammation and infection in preterm labour and to test out potential therapeutic drugs to delay delivery and improve neonatal outcome. Administration of bacterial products directly into the uterus, as opposed to via intra-peritoneal injection (Fidel *et al.* 1994, Kaga *et al.* 1996, Robertson *et al.* 2006),

provides a model which more closely reflects the clinical scenario in women, where the presence of an intrauterine infection is usually subclinical and results in a local, rather than a systemic, inflammatory and immune response (Goldenberg *et al.* 2000).

Clearly the surgical nature of the model itself does have drawbacks. Although generally the mice recovered well from the surgery, it is to be expected that performing relatively major surgery, late in gestation, may have an impact on time to delivery. Indeed, although no significant difference was observed in the mean time to delivery when comparing the no surgery control group with the PBS treated group, 40% of the PBS treated mice did go into preterm labour (i.e. they delivered within 36 hours of intrauterine injection), whilst no mice in the no surgery group delivered preterm. Although great care was taken during surgery, this rate of preterm birth in the PBS control group is likely due to the surgical procedures of pulling out and returning the uterine horns to the abdominal cavity.

Despite its limitations, the evidence described in this chapter supports that the model we used is an effective model of infection-induced preterm labour; which reliably induces preterm delivery and which can be used to analyse the inflammatory and immune responses occurring in response to a local intrauterine infection.

Intrauterine LPS administration dose-dependently induced preterm labour and reduced the proportion of live born pups

The LPS dose response experiments showed that intrauterine administration of LPS at doses of 15μg and 20μg induced delivery significantly earlier than the PBS control group. This result is similar to recently published data by Sykes *et al.* (Sykes *et al.* 2013), where they use the same mode of administration, same serotype of LPS and similar doses in a LPS dose response experiment measuring time to delivery. However, they show a significant effect of low doses of LPS (2.5μg, 5μg and 10μg) on reducing time to delivery, which were not seen in this study. These differences are possibly due to the potential variations in potency that can be associated with different batches of LPS. Whilst both 15μg and 20μg LPS both significantly reduced time to delivery, it was decided the 20μg LPS was the most appropriate dose to use in future experiments as this dose most consistently induced preterm labour and significantly increased the preterm delivery rate. For the purpose of describing the model in this chapter, time to

delivery and live pup data from all mice that received 20μg LPS and from all control mice that received PBS in all of our studies were pooled for analysis, hence the large *n* numbers in these groups.

The reduction in the proportion of live born pups associated with intrauterine LPS treatment has been demonstrated in previous studies in similar models (Elovitz *et al.* 2003, Pirianov *et al.* 2009, Sykes *et al.* 2013). This significant reduction in the number of live pups being born is likely to be due to both an effect of the LPS treatment, and the premature delivery of the pups. If the mice are delivered on D17 or D18, it is likely that they will not be developmentally competent to survive. However, evidence from this study suggests that receiving LPS treatment further compounds the negative effects of being born prematurely, as even when PBS control mice delivered preterm, they had a significantly greater proportion of live born pups compared to mice receiving 15μg and 20μg of LPS.

Intrauterine administration of LPS results in a local and systemic inflammatory response

Both spontaneous term labour and infection-associated preterm labour in humans have been associated with increased levels of pro-inflammatory mediators in the amniotic fluid (Yoon *et al.* 2001, Thomakos *et al.* 2010, Marconi *et al.* 2011); elevated expression of inflammatory cytokines within the utero-placental tissues (Denison *et al.* 1998, Sennstrom *et al.* 2000, Young *et al.* 2002, Osman *et al.* 2003); and increased activation of peripheral blood leukocytes (Yuan *et al.* 2009). In parallel, work in animal models has also demonstrated a similar inflammatory response within the intrauterine tissues both at term and in models of preterm labour (Robertson *et al.* 2010, Shynlova *et al.* 2013, Shynlova *et al.* 2013).

To investigate whether intrauterine LPS administration induced a local and systemic inflammatory response in our model, the expression of inflammatory cytokines was measured in the uterus, fetal membranes, placenta, amniotic fluid and maternal serum 6 hours post-surgery.

Expression of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in the myometrium, cervix, fetal membranes and placenta are elevated in women during term labour, compared to non-labouring women (Sennstrom *et al.* 2000, Young *et al.* 2002,

Osman *et al.* 2003, Osman *et al.* 2006). These cytokines are thought to be involved in stimulating the local production of other inflammatory mediators, such as MMPs (Riley *et al.* 1999, Xu *et al.* 2002) and prostaglandins (Pollard and Mitchell 1996, Keelan *et al.* 2003, Olson 2003); which can then go on to stimulate the processes required for labour progression, including myometrial contractions, fetal membrane rupture and cervical ripening and dilation. The role of these pro-inflammatory cytokines in preterm labour have also been confirmed in animal studies which demonstrated that intra-amniotic or intrauterine injection of TNF-α and IL-1β, induced preterm birth in animal models (Romero *et al.* 1992, Silver *et al.* 1994, Sadowsky *et al.* 2006); and that the rate of *E.coli*-induced preterm birth in IL-1 and TNF receptor double knockout mice was reduced when compared to wild-type mice (Hirsch *et al.* 2006). Additionally, the incidence of LPS-induced preterm labour was also significantly reduced in IL-6 knockout mice, compared with wild-types (Robertson *et al.* 2010).

Results from our model demonstrate that LPS treatment induced a robust increase in the expression of pro-inflammatory cytokines within 6 hours in the uterus, fetal membranes and placenta, with elevated expression of *Il-1β* and *Tnf-α* at all doses of LPS administered, when compared with the PBS control group. Similarly *Il-6* expression was significantly elevated at all doses of LPS administered in the uterus and placenta, but expression of *Il-6* was unchanged in the fetal membranes. In line with these data, similar models of LPS-induced preterm labour have reported increased expression of *Il-1β*, *Tnf-α* and *Il-6* in the uterus and placenta 6 hours after intrauterine LPS (Chang *et al.* 2011); and in the myometrium and decidua of mice during LPS-induced preterm labour (Shynlova *et al.* 2013, Shynlova *et al.* 2013).

Surprisingly, increased *Tnf-α* gene expression was not found to correlate with increased protein production in the utero-placental tissues, as measured by western blotting. TNF-α mRNA is translated into a 26kDa membrane-bound precursor protein, which is converted into the 17kDa secreted mature TNF-α protein by tumour necrosis factor-alpha converting enzyme (TACE) (Black *et al.* 1997, Moss *et al.* 1997). The western blot only detected the 26kDa TNF-α precursor protein, rather than the smaller mature protein. This may suggest a lack of mature TNF-α protein within these tissues, or it may be that the conditions were not optimised to identify the smaller mature protein.

In the uterus and placenta, there was no difference in TNF-α precursor protein levels when comparing tissue from mice receiving PBS with that from mice receiving 20μg LPS, and there was a reduction in the amount of TNF-α precursor protein in the fetal membranes. Given the significant increase in *Tnf-α* mRNA expression in uterus and placenta observed in response to 20μg LPS, compared to the PBS controls (5-fold increase in uterus, 7-fold increase in placenta), it was surprising that this did not correlate with increased TNF-α protein. However, other studies have reported LPS-induced increased *Tnf-α* expression in myometrium at the mRNA level without associated increased protein expression at the same time point (Shynlova *et al.* 2013). It could be that increased protein concentrations would be observed at a later time point, due to a delay in translation of the increased mRNA.

The observed reduction in TNF-α precursor protein levels detected in the fetal membranes, where *Tnf-α* mRNA was increased 33-fold in response to 20μg LPS, could be indicative of a higher rate of TACE activity in the fetal membranes converting the precursor protein into mature TNF-α in mice receiving LPS. Support for this hypothesis comes from a previous study by Hung *et al.* (Hung *et al.* 2006) which found increased TACE activity in placenta and fetal membranes collected from women who had evidence of chorioamnionitis, compared with tissues obtained from normal pregnancies. They also demonstrated that treatment of placental and fetal membrane samples with LPS *in vitro* increased TACE mRNA and protein levels (Hung *et al.* 2006). Additionally, a recent study highlights the importance of not only measuring inflammatory cytokines, but also their receptors when investigating the role of cytokines in labour (Alexander *et al.* 2012). They demonstrate that whilst there is inconsistent data regarding whether TNF-α levels increase in human myometrium with preterm or term labour, they found increased expression of the two TNF-α receptors, TNFR1A and TNFR1B with term and preterm labour, respectively, suggesting that the myometrium becomes more sensitive to TNF-α at the onset of labour, regardless of whether the levels of TNF-α change. Investigating cytokine receptor levels and quantification of TACE protein may have given a clearer picture of whether increased mRNA expression is translated into increased activity of the cytokines.

To investigate further the local inflammatory response to intrauterine LPS administration, the levels of TNF-α, IL-1β and IL-6 secreted into the amniotic fluid 6 hours after surgery were measured. Women who deliver preterm, with evidence of

intra-amniotic infection, have elevated levels of IL-1β, TNF-α and IL-6 in their amniotic fluid (Thomakos *et al.* 2010, Marconi *et al.* 2011). In line with these studies, intrauterine LPS administration in our model resulted in significantly elevated levels of both TNF-α and IL-6 in the amniotic fluid when compared with the PBS control group. Although the concentration of IL-1β was elevated in mice receiving 20μg LPS, this was not significantly greater than the amniotic fluid concentrations in PBS mice. These data suggest that, as in human pregnancy associated with an intra-amniotic infection, intrauterine administration of LPS results in a local inflammatory response within the amniotic fluid, and agrees with other reported mouse studies (Xu *et al.* 2005, Yang *et al.* 2009).

TNF-α levels were also measured in the maternal serum 6 hours post-surgery. Although in women, the presence of an intrauterine infection is normally associated with minimal clinical signs of systemic infection, elevated serum cytokine levels have been reported in women who deliver preterm (Murtha *et al.* 1998). Similarly, previous animal studies have reported up-regulation of serum cytokine levels in models of infection-induced preterm labour (Xu *et al.* 2005, Elovitz and Mrinalini 2006, Yang *et al.* 2009). In line with these studies, the serum concentration of TNF-α increased in response to increasing doses of intrauterine LPS, with significantly increased TNF-α in the serum of mice receiving doses of 10μg LPS and above.

Whilst there has been much focus on the role of pro-inflammatory signalling in initiating labour at term and preterm, more recent studies have begun to investigate the role of the classical anti-inflammatory cytokine, IL-10. IL-10 is expressed in gestational tissues (Roth and Fisher 1999, Hanna *et al.* 2000, Lidstrom *et al.* 2003); and whilst there are contrasting data regarding amniotic fluid concentrations across gestation (Greig *et al.* 1995, Dudley *et al.* 1997, Gotsch *et al.* 2008), it has been reported that IL-10 concentrations in the amniotic fluid are increased with spontaneous term and preterm labour and further increased in the presence of intra-amniotic infection (Greig *et al.* 1995, Gotsch *et al.* 2008). It is likely that IL-10 has an important role in controlling the inflammatory response during labour, and in the presence of an intra-amniotic infection, increased IL-10 in the amniotic fluid is likely a protective response to reduce fetal exposure to excess inflammation. Work using an IL-10 knockout mouse has confirmed that IL-10 plays an important role in protecting mice against LPS-induced fetal loss (Robertson *et al.* 2006). In response to intrauterine LPS

administration in our model, IL-10 expression was significantly elevated in response to all doses of LPS administered, even the lowest dose of 1μg, in the uterus, fetal membranes and placenta, compared to the PBS control tissues. Similarly, the concentration of IL-10 was significantly elevated in both the amniotic fluid and maternal serum in response to increasing LPS doses. These data are in agreement with other animal models of infection-induced preterm labour, which also report increased IL-10 expression in the utero-placental tissues and amniotic fluid (Elovitz and Wang 2004, Yang *et al.* 2009, Chang *et al.* 2011).

Given the important roles prostaglandins play in the induction of labour, including stimulating: myometrial contractions (Dyal and Crankshaw 1988, Senior *et al.* 1993); fetal membrane rupture (McLaren *et al.* 2000, Keelan *et al.* 2001) and cervical ripening and dilation (Fletcher *et al.* 1993, Keirse 1993); the expression of two important enzymes involved in regulating prostaglandin production and metabolism, COX-2 and 15-HPGD, was measured in intrauterine tissues collected from mice 6 hours post-surgery.

Intrauterine administration of LPS resulted in a dose-dependent increase in *Cox-2* expression in the uterus, with no change in the fetal membranes or placenta. Other studies in similar animal models have also demonstrated up-regulation of *Cox-2* in the myometrium following LPS treatment (Swaisgood *et al.* 1997, Pirianov *et al.* 2009, Shynlova *et al.* 2013, Sykes *et al.* 2013); and similarly following intrauterine injection with heat-killed *E.Coli* (Wang and Hirsch 2003). There have also been reports of increased *Cox-2* expression in the fetal membranes and placenta of mice 6-8 hours after i.p. LPS to induce preterm labour, which was not observed in our study (Swaisgood *et al.* 1997). However, in line with our results, other studies have also reported no change in *Cox-2* mRNA expression in the placenta and fetal membranes following intrauterine injection of bacteria (Wang and Hirsch 2003). Studies in humans have also demonstrated different expression profiles of COX-2 in the intrauterine tissues with labour onset, where COX-2 mRNA expression has been reported to: increase in the fetal membranes with the onset of labour (Bennett *et al.* 1993, Slater *et al.* 1995); not change in the placenta (Macchia *et al.* 1997); and there are conflicting reports as to whether COX-2 mRNA is affected by labour in the myometrium (Erkinheimo *et al.* 2000, Giannoulias *et al.* 2002).

Conversely, *15-Hpgd* mRNA expression was unchanged in uterus and placenta, but significantly decreased in the fetal membranes collected from mice 6 hours post-surgery. Decreased 15-HPGD expression has previously been reported in mouse uterus and fetal membranes following intrauterine injection of heat-killed *E.Coli* (Wang and Hirsch 2003); as well as in chorion and myometrium from women in preterm and term labour (VanMeir *et al.* 1996, VanMeir *et al.* 1997, Giannoulias *et al.* 2002).

The dose-dependent increase in uterine *Cox-2* expression and decrease in *15-Hpgd* in the fetal membranes, suggests that in response to intrauterine LPS administration, there is a shift towards increased prostaglandin synthesis and decreased metabolism within the intrauterine tissues, which could stimulate labour. Although the expression patterns of these enzymes are not exactly the same as in human utero-placental tissues in association with labour, where the amnion is proposed to be a major producer of prostaglandins at labour (Bennett *et al.* 1993), our results suggest there is an overall increase in prostaglandin activity in response to LPS, which is likely to play an important role in inducing preterm labour in our model. To further confirm this, it would be important to measure local prostaglandin levels within the intrauterine tissues.

In addition to increased cytokine production, labour has also been associated with increased chemokine signalling within the intrauterine tissues. Recent microarray analysis has identified that some of the most highly up-regulated genes in human myometrium and cervix during labour are chemokines, such as CXCL1, CXCL2, CXCL5, CXCL8 and CCL2 (Bollapragada *et al.* 2009). To investigate the role of chemokines in our mouse model of infection-induced preterm labour, the expression of three potent mouse neutrophil chemoattractants, *Cxcl1* (also known as keratinocyte chemokine; KC), *Cxcl2* (also known as macrophage inflammatory protein 2; MIP-2) and *Cxcl5* (also known as LPS-induced CXC chemokine; LIX) was measured, along with the expression of the mouse macrophage chemokine, *Ccl2* (also known as monocyte chemotactic protein 1; MCP-1). CXCL1, CXCL2 and CXCL5 have been proposed to be the murine homologs of human IL-8 (CXCL8) (Hol *et al.* 2010). Mouse CCL2 is thought to be a murine homolog of human MCP-1 (Fridlender *et al.* 2011).

In line with human data showing elevated neutrophil chemokine expression in association with labour (Keelan *et al.* 2004, Bollapragada *et al.* 2009); and previous mouse studies of LPS-induced preterm labour demonstrating up-regulation of *Cxcl1,*

Cxcl2 and *Cxcl5* in uterine tissue (Diamond *et al.* 2007, Pirianov *et al.* 2009, Shynlova *et al.* 2013), expression of all three neutrophil chemokines in the utero-placental tissues collected from our mice was significantly elevated by intrauterine administration of LPS at doses of 5μg and above, suggesting that neutrophil infiltration into the uterus, fetal membranes and placenta of mice receiving LPS is likely. Conversely, no change in the expression of either *Cxcl1* or *Cxcl5* was observed in uterus collected from mice labouring at term, compared with levels on D18 of gestation (Menzies *et al.* 2012), suggesting that these chemokines may be differentially expressed depending on the presence of an intrauterine infection.

Similarly, expression of the macrophage chemokine, *Ccl2* was elevated in response to increasing doses of intrauterine LPS in the uterus, fetal membranes and placenta tissue 6 hours post-surgery, suggesting potential macrophage recruitment to the intrauterine tissues. Previous studies have also demonstrated up-regulation of *Ccl2* in myometrium and decidua in association with LPS-induced preterm labour in mice (Diamond *et al.* 2007, Pirianov *et al.* 2009, Shynlova *et al.* 2013, Shynlova *et al.* 2013); as well as term labour in: mouse uterus and decidua (Diamond *et al.* 2007, Menzies *et al.* 2012, Shynlova *et al.* 2013); rat myometrium (Shynlova *et al.* 2008); and human labouring myometrium and fetal membranes (Esplin *et al.* 2005). The potential impact of increased expression of both potent neutrophil and macrophage chemokines in inducing immune cell recruitment into the intrauterine tissues will be discussed in greater detail below.

The mechanism by which intrauterine LPS induces increased production of these inflammatory mediators has not been discussed in this chapter. Studies suggest that the presence of an intrauterine infection initiates a maternal immune response via TLR recognition of bacterial products (Patni *et al.* 2007). Support for this hypothesis comes from evidence that increased TLR-2 and TLR-4 expression has been reported in fetal membranes associated with infection (Kim *et al.* 2004); and TLR-4 knockout mice studies demonstrated the essential role for TLR-4 in inducing *E.Coli*- and LPS-induced preterm labour (Elovitz *et al.* 2003, Wang and Hirsch 2003). Given that NF-κB is a key transcription factor activated in response to TLR signalling (Patni *et al.* 2007), and is well documented to play an important role in inflammatory signalling in labour (Lindstrom and Bennett 2005, Lappas and Rice 2007); it is likely that increased NF-κB signalling plays a role in the increased pro-inflammatory cytokine production

described in this model. Indeed, Pirianov *et al.* (Pirianov *et al.* 2009) reported intrauterine LPS administration activated both NF-κB and MAPK JNK signalling pathways in mouse myometrium after 6 hours. To confirm whether the same or other inflammatory transcription factors are involved in the LPS-induced up-regulation of pro-inflammatory signalling in our model, similar experiments would need to be performed.

Overall, in our model, intrauterine LPS administration induced a robust local inflammatory response within the utero-placental tissues, resulting in increased expression of both pro- and anti-inflammatory mediators compared to mice receiving PBS. It is not clear whether the increased inflammatory cytokine production is a result of increased expression by the utero-placental tissues themselves, or primarily from both resident and infiltrating immune cell populations. Whilst it is well accepted that immune cells in the utero-placental tissues are major producers of cytokines and chemokines, there is also evidence from women that extravillous trophoblasts, myometrial cells and stromal cells of the intrauterine tissues also produce inflammatory cytokines (Young *et al.* 2002, Gomez-Lopez *et al.* 2010, Mosher *et al.* 2012); which suggests that the increased pro-inflammatory signalling is likely due to both increased numbers of infiltrating immune cells and stimulation of non-immune cells.

Interestingly, the expression of many of the cytokines and chemokines measured here was found to be significantly elevated at the lowest dose of LPS administered, 1μg, where preterm delivery was not induced. In agreement with these data, Elovitz *et al*. also recently reported that administration of LPS at a dose insufficient to induce preterm labour, still elicited an inflammatory cytokine response in the fetal brain, amniotic fluid and placenta (Elovitz *et al.* 2011); and in a study investigating the role of TLR-4 in infection-induced preterm labour, Hirsch *et al*. reported that in response to intrauterine *E.coli* injection both TLR-4 mutant mice and wild-type mice had increased expression of inflammatory mediators in the myometrium and decidua, even though none of the TLR-4 mutant mice went into preterm labour (Hirsch and Wang 2005). Collectively, these data suggest that the increased expression of these inflammatory mediators is a response to, even low levels of, intrauterine infection, and they are not capable of inducing preterm labour alone. It may be that other factors not measured here, such as prostaglandins, may only be elevated in response to higher doses of LPS,

causing mice receiving 20μg LPS to reliably go into preterm labour. Another possibility is that the intrauterine environment is able to withstand a certain level of inflammation, but when the inflammation reaches a particular threshold level, the cascade of events resulting in labour is initiated; in our model this threshold may only be reached in response to the higher doses of LPS administered.

Intrauterine LPS administration induces changes in uterine immune cell populations

As described previously, parturition both at term and preterm are associated with a massive immune cell influx into the intrauterine tissues, particularly neutrophils and macrophages (Gomez-Lopez *et al.* 2010). There remains much debate as to whether these infiltrating immune cells are a causative factor in the initiation of labour or if the primary role of these cells is in the repair and remodelling of the uterus and cervix following parturition (Timmons *et al.* 2009, Shynlova *et al.* 2013, Shynlova *et al.* 2013). As discussed earlier, the increased expression of neutrophil and macrophage chemokines, suggests that infiltration of immune cells is likely in response to intrauterine LPS. In order to investigate this in our model, the expression of neutrophil and macrophage markers was assessed in the uterus, fetal membranes and placenta and the cells were localised in the uterus.

Previous studies examining expression of the macrophage marker, *Emr1* in the uterus of mice demonstrated significantly increased expression in association with labour, when compared to non-labouring D18 tissue (Menzies *et al.* 2012, Menzies *et al.* 2012). In contrast, no such increase in *Emr1* expression was observed in either the uterine tissue or the placental tissue of mice receiving intrauterine LPS when compared to those receiving PBS; however there was a dose-dependent increase in *Emr1* gene expression in the fetal membranes in response to LPS treatment. These differences suggest that perhaps in the context of intrauterine infection, in our model, macrophage recruitment is not induced in the uterus and placenta, unlike at term labour, which may support the hypothesis that different mechanisms could be responsible for inducing spontaneous labour at term and infection-induced preterm labour (Gonzalez *et al.* 2009, Holt *et al.* 2011, Shynlova *et al.* 2013); or it may be that if tissue had been collected at a later time point, or during labour we may have observed increased *Emr1* expression.

To further investigate whether the increased expression of *Ccl2* observed, resulted in macrophage influx into the uterus, macrophages were localised in the uterine tissue using F4/80. F4/80 is a glycoprotein that is highly expressed on the cell surface of mature macrophages and is commonly used to identify tissue-resident macrophages in mice (Austyn and Gordon 1981). Previous studies have reported that macrophages make up the largest immune cell population in the pregnant mouse uterus (Shynlova *et al.* 2013, Shynlova *et al.* 2013), where they have been estimated to account for around 22% of uterine cells during pregnancy (Hunt *et al.* 1985). There are conflicting reports on the dynamics of uterine macrophages during normal term labour in different species. In women it has been reported that there is an influx of macrophages into the myometrium, fetal membranes, decidua, placenta and cervix during spontaneous term labour (Thomson *et al.* 1999, Osman *et al.* 2003, Gomez-Lopez *et al.* 2009, Gomez-Lopez *et al.* 2010) and in preterm labour (Hamilton *et al.* 2012). In rats, macrophage numbers were found to increase: at term (Shynlova *et al.* 2008); during term labour in the myometrium; and in the 12 hours prior to labour and during labour in the decidua, relative to 2 days before term, with a significantly higher number of macrophages present within the decidua at term, compared to the myometrium (Hamilton *et al.* 2012). In mice it has previously been reported that uterine macrophage numbers peak around D15 and decrease on D18, before labour (Mackler *et al.* 1999); whilst more recent studies found macrophage numbers were increased on D18 of gestation compared to D15 and remained elevated during term labour in the myometrium and decidua (Shynlova *et al.* 2013, Shynlova *et al.* 2013). These differences may be explained by different mouse strains studied. In our model, we found that macrophages were present in normal D17 uterus, in both the myometrium and decidua; however since tissue was not collected at other time points, it was not possible to compare the number of macrophages present on D17 with different time points in pregnancy.

In a rat model of mifepristone-induced preterm labour, macrophage numbers have been reported to increase in the decidua during active labour (Hamilton *et al.* 2012). Conversely, mouse models of mifepristone- and LPS-induced preterm labour found no macrophage infiltration in the decidua or myometrium of mifepristone-treated mice, but did report increased macrophage numbers in the decidua of mice receiving LPS during labour (Shynlova *et al.* 2013, Shynlova *et al.* 2013). In contrast to these results, but in line with the lack of change in *Emr1* expression within the uterus, there was no

obvious increase in macrophage number associated with intrauterine LPS administration. In fact, when the number of F4/80 positive cells was quantified stereologically, there was a surprising decrease in the number of F4/80 macrophages within the myometrium and decidua of mice receiving LPS, although this decrease did not reach statistical significance. The results from other animal models refer to uterine tissues collected during active labour, as opposed to 6 hours post-surgery, as is the case in our model, therefore it is possible that an influx of macrophages may have been seen only at labour, as has been reported to occur at term in rats (Hamilton *et al.* 2012). Also, 6 hours may have been too short a time to allow for monocyte recruitment and differentiation into tissue-resident macrophages expressing F4/80. Additionally Shynlova *et al.* (Shynlova *et al.* 2013) describe macrophage recruitment into the decidua basalis, (which is the decidual tissue at the site of placental attachment), as opposed to the decidual parietalis (which describes the decidualised endometrial tissue lining the rest of the uterus). In this study, and in the work by Hamilton *et al.* (Hamilton *et al.* 2012), immunohistochemical studies localised macrophages in the decidua parietalis (referred to here as the decidua); therefore the differences observed may be due to macrophage infiltration to different regions of the uterus in response to LPS administration. Leukocyte infiltration into the intrauterine tissues during mouse pregnancy has been demonstrated to be a highly organised process (Kruse *et al.* 2002); and in support of this hypothesis, Shynlova *et al.* (Shynlova *et al.* 2013), report that the decidua basalis provides a much stronger chemotactic signal than the myometrium during term labour in mice. Detailed time-course analysis in all the areas of the uterus would be required to test this hypothesis. Even without infiltration, it is likely that macrophages already resident are playing an important role in the inflammatory environment given their abundance in the pregnant uterus, and the fact that they can be potent producers of inflammatory cytokines (Hunt and Pollard 1992).

The reason for the slight reduction in F4/80 cells in the LPS-treated decidua and myometrium observed in our model is unknown. As previously discussed, Mackler *et al.* described a reduction in the number of uterine macrophages just prior to labour (Mackler *et al.* 1999), which they suggested may be a mechanism to induce myometrial contractions, given that macrophages can produce an inducible form of nitric oxide synthase (iNOS), which has been shown to inhibit contractions and was found to decrease at term in the rat uterus (Buhimschi *et al.* 1996). It has also been previously reported that the F4/80 antigen is down-regulated in activated macrophages in the

presence of bacillus calmette-guerin infection (Ezekowitz and Gordon 1982); raising the possibility that LPS infection may also affect F4/80 expression. Whether there is indeed a reduction in the number of F4/80 cells in response to LPS in our model, or simply a reduction in expression of F4/80 needs further investigation. Immunohistochemical studies using a different antigen, such as CD68, may be useful in confirming macrophage expression in the intrauterine tissues.

Given the significant up-regulation of several potent neutrophil chemoattractants within the intrauterine tissues, neutrophil infiltration was examined by measuring expression of the gene *Ngp,* which encodes a neutrophilic granule protein expressed in mouse neutrophils. *Ngp* expression has previously been reported to remain unchanged in labouring mouse uterus, compared to non-labouring D18 tissue (Menzies *et al.* 2012). In contrast, in our model in response to intrauterine LPS administration a dose-dependent increase in *Ngp* expression was observed in the uterus, fetal membranes and placenta. Again these data provide further support for the theory that infection-induced preterm labour and term labour may occur via different mechanisms (Gonzalez *et al.* 2009, Holt *et al.* 2011, Shynlova *et al.* 2013).

To confirm that neutrophil influx was indeed responsible for the observed increase in *Ngp* expression in the uterus, neutrophils were localised using the marker, Gr-1. The RB6-8C5 clone of the Gr-1 antibody used in this chapter detects two antigens expressed on mouse neutrophils, Ly-6C and Ly-6G (Fleming *et al.* 1993). In women, neutrophils are proposed to play a role in cervical ripening (Bokström *et al.* 1997) and have been shown to infiltrate into the myometrium and cervix in association with labour (Thomson *et al.* 1999, Osman *et al.* 2003). Furthermore, increased neutrophil numbers have been reported in women in preterm labour with an infection, compared with women in idiopathic preterm or normal term labour (Hamilton *et al.* 2012). In mice, neutrophils make up a much smaller proportion of the immune cell population in the pregnant uterus compared with macrophages, where they have been reported to account for less than 2% of the total leukocyte population on D18 of gestation in the decidua and myometrium (Shynlova *et al.* 2013, Shynlova *et al.* 2013). In line with this, very few if any Gr-1 positive neutrophils were observed in D17 uterus from mice that did not undergo surgery or treatment. Interestingly, when this normal D17 uterus was compared with uterus collected from PBS treated mice there was a distinct pattern of neutrophils localised to the connective tissue layer surrounding the longitudinal myometrial muscle bundles. This finding suggests that the surgery itself induced a

local inflammatory response and immune cell influx to the uterus, which may in part explain why some mice in the PBS control group went into preterm labour. In a model of artificial stretch, Shynlova *et al*. (Shynlova *et al.* 2013), also reported neutrophil infiltration into the uterus of mice receiving sham surgery, supporting the theory that uterine surgery during pregnancy can induce a rapid neutrophil response.

Normal term labour in mice has been associated with a significant neutrophil infiltration into the decidua (Shynlova *et al.* 2013); but not into the myometrium, where neutrophil numbers were not found to be significantly elevated until the post-partum period (Shynlova *et al.* 2013). These results are in agreement with studies in the mouse cervix that also show neutrophil numbers in the cervix are significantly greater 2-4 hours after labour, compared to cervical tissue obtained from mice in late gestation before labour, and during labour (Timmons *et al.* 2009). In contrast, LPS-induced preterm labour has been shown to induce a massive neutrophil influx into both the decidua and myometrium of mice; which was not present in mifepristone-induced preterm labour (Shynlova *et al.* 2013, Shynlova *et al.* 2013). In our model, intrauterine LPS administration was found to alter both the number and the localisation of neutrophils present in the uterus of mice, compared to the PBS control group. Where previously neutrophils were absent from the decidua, LPS treatment induced a massive influx of neutrophils into the decidua, which was quantified as a 7-fold increase. In contrast, neutrophil numbers in the myometrium remained relatively stable between LPS and PBS treated mice. Although no neutrophil infiltration was observed in the myometrium at 6 hours, the influx may have been more apparent if tissues had been collected during active labour, as in other studies. Interestingly, this appears to be the first report of LPS-induced neutrophil influx into the decidua, as previous studies have focussed on localising neutrophils in the decidua basalis (Shynlova *et al.* 2013).

A limitation of the immunohistochemistry studies performed here is the use of Gr-1 as a neutrophil marker. Although commonly used as a marker of neutrophils in mice, as this antibody recognises both Ly-6G and Ly-6C antigens, it can also detect other Ly-6C expressing cells, which include some subsets of monocytes, dendritic cells and T-cells (Jutila *et al.* 1988, Kung *et al.* 1991, Shortman and Naik 2007). Therefore, to confirm neutrophil localisation in the uterine tissues, the more specific Ly-6G antibody (clone 1A8) should be the preferred marker in future studies.

A possible limitation of the quantification of F4/80 and Gr-1 positive cells within the uterus is that whilst the same number of fields of view were counted for each section, resulting in the same total area being counted, the total number of cells present were not accounted for. Hypertrophy of the uterus has been previously reported in late gestation (Shynlova *et al.* 2010); and furthermore, studies in cardiac myocytes demonstrate that LPS treatment induces hypertrophy of muscle cells (Liu *et al.* 2008). Therefore, LPS treatment may have altered the total number of cells present within the randomised fields. Although given the massive influx of neutrophils observed in the decidua, it is unlikely to have significantly altered this result, but may have masked more subtle changes. To confirm the changes observed in this study, counts relative to the total number of cells would need to be performed.

Intrauterine LPS administration induces the inflammatory and immune response without apparent progesterone withdrawal

In contrast to humans, the onset of labour in mice, as in many other species is preceded with a sharp drop in circulating serum progesterone levels; this decrease is thought to allow increased expression of pro-inflammatory factors and contraction-associated genes which can then go on to stimulate labour (Mitchell and Taggart 2009). However there have been conflicting reports as to whether progesterone withdrawal is required for infection-induced preterm labour in mice. Fidel *et al.* (Fidel *et al.* 1998) reported that intrauterine LPS administration in mice resulted in significantly reduced serum progesterone levels, compared to mice receiving PBS, within 1 hour of LPS administration prior to the onset of labour. Whereas, a number of more recent studies have demonstrated infection-induced preterm labour is not associated with progesterone withdrawal (Hirsch and Muhle 2002, Murphy *et al.* 2009, Gonzalez *et al.* 2011). To determine whether preterm labour in our model involved progesterone withdrawal, serum progesterone levels were measured 6 hours post-intrauterine injection. In line with these more recent studies, no drop in circulating progesterone levels were observed in response to intrauterine LPS administration, compared to the PBS control group. Although this measurement was taken after just 6 hours, and not during labour, the fact that at this time we already have a strong inflammatory response within the intrauterine tissues, suggests that the mechanisms involved in inducing preterm labour in our model are largely independent of the actions of progesterone.

Intrauterine LPS administration did not cause an inflammatory response in fetal brains

Premature birth is linked to adverse neonatal outcomes, in particular increased risk of brain injury. A recent meta-analysis highlighted the association between intrauterine infection and cerebral palsy, where they found the presence of clinical or histological chorioamnionitis was associated with a 140% or 80% increased risk for neonates to develop cerebral palsy, respectively (Shatrov *et al.* 2010). The exact mechanisms by which intrauterine infection results in neonatal brain injury are not clear, but a fetal systemic inflammatory response (FIRS) has been described in the presence of an intrauterine infection; which can result in elevated cytokine production and damage to neonatal organs (Gomez *et al.* 1998). Evidence from animal models suggests that intrauterine LPS administration results in rapid production of pro-inflammatory cytokines within the fetal brain itself (Elovitz *et al.* 2006). It has been proposed that these pro-inflammatory cytokines may cause brain damage both directly, by damaging oligodendrocytes and neurons; or indirectly, by activating microglia in the brain which can in turn secrete more cytokines (Burd *et al.* 2012).

To determine whether our model of infection-induced preterm labour caused adverse effects on the fetal brain, expression of pro-inflammatory cytokines and early markers of brain damage were examined 6 hours post intrauterine injection. Whilst previous studies have demonstrated up-regulated expression of inflammatory cytokines within 6 hours of intrauterine LPS administration (Elovitz *et al.* 2006, Chang *et al.* 2011, Elovitz *et al.* 2011); and NF-κB activation (Pirianov *et al.* 2009); in our model *Il-1*β and *Tnf-*α expression was not significantly altered, and surprisingly there was a small but significant reduction in *Il-6* expression in the brains of mice receiving the lowest dose of LPS. Furthermore, there was no change in the expression of the neuronal markers, *Gfap* or *Mtap2,* both of which have previously demonstrated altered expression in response to intrauterine LPS (Elovitz *et al.* 2006, Burd *et al.* 2011). The differences observed between these results and previous studies may relate to the different doses and serotypes of LPS used, which may elicit more potent responses. Some studies have also reported no significant effect of LPS administration on pro-inflammatory gene and neuronal marker expression after 6 hours (Burd *et al.* 2010), and in fact use of the same dose and serotype of LPS was recently not found to activate NF-κB in the fetal brain (Sykes *et al.* 2013); highlighting a variable response at this early time point.

The results presented here suggest that, in our model, there are no apparent early inflammatory effects on the fetal brain associated with intrauterine LPS administration. This was a surprising result and in contrast to some previously published studies in similar models. Given the robust inflammatory response induced in the intrauterine tissues, and in particular in the amniotic fluid surrounding the fetus, it seems unlikely that intrauterine LPS administration in our model will not induce an inflammatory response in the fetal brain. To further investigate whether this model would be suitable to examine the effects of intrauterine infection on the fetal brain, it would be interesting to examine both inflammatory signalling and markers of oligodendrocyte and neuronal damage in specific areas of the fetal brain using immunohistochemistry. These changes may be subtle and may be more apparent at time points later than 6 hours, for example at term, thus sampling fetal brains at a later stage may provide further information on the effects of intrauterine LPS on the fetal brain in our model.

Summary

This chapter provides further evidence that the immune and inflammatory responses of mice to intrauterine LPS administration, which ultimately results in preterm labour, share many similarities with the clinical scenario of bacterial infection-associated preterm labour in women. In agreement with previous studies, intrauterine administration of LPS resulted in the increased production of inflammatory cytokines and chemokines within the utero-placental tissues, amniotic fluid and maternal serum; however, this is the first study in which detailed analysis of the inflammatory response to a range of LPS doses has been carried out in the uterus, fetal membranes, placenta, amniotic fluid, maternal serum and fetal brains. Interestingly, these inflammatory responses appear to be largely independent of progesterone actions. The role of immune cells in inducing preterm labour in this model will be addressed in greater detail in a subsequent chapter, but these data presented here demonstrate that LPS administration induced a large neutrophil influx into the decidua of mice, which has not previously been reported. Using models, such as the one described here, are vital to improving our understanding of the events regulating the induction of preterm labour and will ultimately aid the search for novel therapeutic options to delay delivery and improve neonatal outcome.

Chapter 4 - Investigating the use of anti-inflammatory agents to delay preterm labour and improve pup survival in a mouse model

4.1 INTRODUCTION

Despite considerable medical advances and research, preterm birth remains the single biggest cause of neonatal mortality worldwide. A recent World Health Organisation report has estimated more than one million babies die each year due to the complications of their premature birth (March of Dimes 2012). Current treatment options for preterm labour are limited, and focus on the use of tocolytic agents such as calcium-channel blockers, oxytocin receptor antagonists and β-mimetics, to block myometrial contractions (Smith *et al.* 2009). Whilst these treatments have proved effective in delaying preterm birth for up to 7 days, it is not clear if they actually improve perinatal or neonatal morbidity (RCOG 2011). The limited effectiveness of tocolytic drugs in treating preterm labour is likely because they target myometrial contractions, which are likely to be an end-point of the inflammatory cascade leading to delivery. Attempting to delay preterm delivery is further complicated in the presence of an intrauterine infection, where leaving the fetus in a potentially hostile inflammatory environment could result in further adverse outcomes for the baby (Gotsch *et al.* 2007). Therefore, if novel treatments for preterm labour are to be more effective than the current options, they must attempt to block the inflammatory cascade earlier and have the potential to resolve the inflammatory intrauterine environment.

Given that many cases of preterm labour are thought to occur because of the premature initiation of the inflammatory cascade normally initiated during term labour, there is now growing interest in investigating the potential of anti-inflammatory agents to delay preterm delivery and improve neonatal outcome. The use of non-steroidal anti-inflammatory drugs (NSAIDS), such as indomethacin, and the steroid hormone progesterone in clinical practice for the treatment of preterm labour in women supports the hypothesis that targeting inflammatory pathways can reduce the incidence of preterm labour. Animal models of preterm labour have proved invaluable in investigating the potential of anti-inflammatory treatments to delay infection-induced preterm labour and improve pup survival. The therapeutic options examined to date include IL-10 (Terrone *et al.* 2001, Rodts-Palenik *et al.* 2004, Robertson *et al.* 2006), blocking TLR-4 signalling (Adams Waldorf *et al.* 2008, Li *et al.* 2010), NF-κB inhibitors (Buhimschi *et al.* 2003, Beloosesky *et al.* 2006, Nath *et al.* 2010, Chang *et al.* 2011), 15d-PGJ_2 (Pirianov *et al.* 2009), anti-TNF-α therapy (Fidel *et al.* 1997, Holmgren

et al. 2008) and blocking IL-1β signalling (Romero and Tartakovsky 1992, Yoshimura and Hirsch 2005).

The identification that the resolution of inflammation is an active process involving the production of mediators with specific anti-inflammatory and pro-resolution actions has provided new pathways to target in the search for novel treatments for inflammation-associated pathologies (Gilroy *et al.* 2004, Serhan *et al.* 2008). The arachidonic acid-derived lipid mediators, lipoxins, were the first family of mediators recognised to have dual acting anti-inflammatory and pro-resolution actions (Serhan *et al.* 1984, Serhan *et al.* 1984). Since their discovery in the 1980s, the therapeutic potential of lipoxins to treat inflammation-associated pathologies has been widely demonstrated in animal models of a range of pathologies including, asthma (Levy *et al.* 2002, El Kebir *et al.* 2009), arthritis (Zhang *et al.* 2008, Conte *et al.* 2010), and inflammatory bowel diseases (Gewirtz *et al.* 2002). The potential of lipoxins to regulate inflammation in the reproductive tissues has been less well explored (Hutchinson *et al.* 2011). However, a recent study from our laboratory reported that expression of the lipoxin receptor, FPR2/ALX, was increased in myometrial tissue obtained from women during term labour, compared to non-labouring women; and demonstrated that lipoxin treatment down-regulated LPS-induced inflammatory gene expression in myometrial explant culture *in vitro* (Maldonado-Perez *et al.* 2010). Therefore, given this evidence that lipoxins could be involved in regulating the inflammation associated with labour, and the evidence from a range of animal models of other inflammation-associated pathologies, we hypothesised that lipoxins could be useful novel therapeutic agents for the treatment of preterm labour.

Using our mouse model of LPS-induced preterm labour, the experiments described in this chapter aimed to investigate the potential of lipoxins, BML-111 (a stable lipoxin analogue) and the anti-inflammatory cytokine, IL-10, to delay LPS-induced preterm delivery and/or improve pup survival; and to examine whether lipoxin could regulate LPS-induced inflammatory signalling in the utero-placental tissues.

4.2 METHODS

4.2.1 Mouse model of PTL

Mice underwent surgery as detailed in section 2.1. The potential of three anti-inflammatory agents (epi-lipoxin, BML-111 and IL-10) to delay preterm delivery and improve pup survival were investigated. Based on the LPS dose response experiments described in chapter 3, all further experiments were carried out using 20μg LPS.

4.2.1.1 Epi-lipoxin experiments

To determine whether lipoxin administration could affect LPS-induced preterm labour, mice were pre-treated with 15-epi-lipoxin A_4. Briefly, 1-2 hours prior to intrauterine injection of LPS or PBS, mice received an intra-peritoneal (i.p.) injection of vehicle (PBS+1% ethanol) or epi-lipoxin (doses of 12.5ng or 125ng), both in a volume of 100μl. Therefore, there were five treatment groups: vehicle (i.p. injection of vehicle followed by intrauterine PBS, n=12); epi-lipoxin (i.p. injection of 125ng epi-lipoxin followed by intrauterine PBS, n=9); LPS (i.p. injection of vehicle followed by intrauterine LPS, n=12); 12.5ng epi-lipoxin+LPS (i.p. injection of 12.5ng epi-lipoxin followed by intrauterine LPS, n=11); and 125ng epi-lipoxin+LPS (i.p. injection of 125ng epi-lipoxin followed by intrauterine LPS, n=11).

4.2.1.2 BML-111 experiments

In BML-111 experiments, to determine whether BML-111 could affect LPS-induced preterm labour. Mice received an i.p. injection of vehicle (PBS+7% methanol) or BML at doses of 1mg/kg or 10mg/kg BML-111 in a volume of 500μl, 1-2 hours prior to intrauterine injection of LPS or PBS. Again this resulted in five treatment groups: vehicle (i.p. injection of vehicle followed by intrauterine PBS, n=10); BML-111 (i.p. injection of 1mg/kg or 10mg/kg BML-111 followed by intrauterine PBS, n=11; mice receiving both doses were pooled into one group for analysis as there was no significant difference between them); LPS (i.p. injection of vehicle followed by intrauterine LPS, n=22); 1mg/kg BML+LPS (i.p. injection of 1mg/kg BML-111 followed by intrauterine LPS, n=20); and 10mg/kg BML+LPS (i.p. injection of 10mg/kg BML-111 followed by intrauterine LPS, n=9).

4.2.1.3 IL-10 experiments

In IL-10 experiments, to determine whether IL-10 could affect LPS-induced preterm labour, mice received an i.p. injection of vehicle (PBS+sterile H_20) or IL-10 at doses of 2.5μg or 10μg, in a volume of 200μl, 1-2 hours prior to intrauterine injection of LPS or PBS. Again, there were five treatment groups: vehicle (i.p. injection of vehicle followed by intrauterine PBS, n=11); 10μg IL-10 (i.p. injection of 10μg IL-10 followed by intrauterine PBS, n=10); LPS (i.p. injection vehicle followed by intrauterine LPS, n=6); 2.5μg IL-10+LPS (i.p. injection of 2.5μg IL-10 followed by intrauterine LPS, n=5); and 10μg IL-10+LPS (i.p. injection of 10μg IL-10 followed by intrauterine LPS, n=6).

In each experiment, time to delivery and the proportion of live born pups were recorded following surgery, as detailed in sections 2.1.1. and 2.1.2.

4.2.2 Tissue collection

As detailed in section 2.1.3, utero-placental tissues and amniotic fluid were collected 6 hours post-surgery for analysis of lipoxin receptor expression in the utero-placental tissues; and analysis of the inflammatory response following treatment with LPS ± epi-lipoxin.

For analysis of the expression of the lipoxin receptor, Fpr2, uterus, fetal membranes and placental tissues were harvested 6 hours post-surgery from mice receiving intrauterine PBS (n=7) or 20μg LPS (n=10).

For analysis of the effect of epi-lipoxin on the LPS-induced inflammatory response, utero-placental tissues were collected 6 hours post-surgery from two separate cohorts of mice. In the first cohort, mice received i.p. pre-treatment with epi-lipoxin 1-2 hours prior to intrauterine LPS or PBS administration. Therefore, tissues were collected from the following groups: vehicle (n=3), 2.5μg epi-lipoxin (n=4), LPS (n=5), 0.25μg epi-lipoxin+LPS (n=5) and 2.5μg epi-lipoxin+LPS (n=5).

In a separate cohort of mice, the effect of intrauterine administration of epi-lipoxin was investigated. These mice received two 25μl intrauterine injections within 1 minute, either vehicle or epi-lipoxin, followed by PBS or 20μg LPS. Therefore, tissues were collected from mice receiving: vehicle (n=4), 250ng epi-lipoxin (n=4), LPS (n=5), 25ng epi-lipoxin+LPS (n=6) and 250ng epi-lipoxin+LPS (n=6).

4.2.3 qRT-PCR

The mRNA expression of the lipoxin receptor and several inflammatory genes was examined using qRT-PCR as described in section 2.3. The expression of the following genes were measured in the uterus, fetal membranes and placenta of mice 6 hours post-surgery: *Cxcl1*, *Cxcl2*, *Cxcl5*, *Ccl2*, *Il-1β, Tnf-α, Il-6*, *Il-10*, *Cox-2*, *15-Hpgd* and *Fpr2*.

As described in section 2.3.3, expression of the gene of interest was normalised to *β-actin* as the endogenous control gene in each sample and all samples were then compared relative to a calibrator tissue. Analysis of all the inflammatory genes examined in the uterus, fetal membranes and placenta was carried out relative to untreated D18 uterus as a calibrator. Analysis of the expression of *Fpr2* was carried out relative to untreated virgin spleen as a calibrator.

4.2.4 ELISA

ELISAs were used to quantify the concentration of TNF-α and IL-10 in amniotic fluid collected 6 hours post-surgery. Details of each assay are given in section 2.4.

4.2.5 Statistical analysis

Data are presented as mean ± SEM. Where data were not normally distributed they was transformed prior to analysis. Time to delivery data were log transformed before analysis; and the proportion of live born pups was arc-sin transformed prior to analysis. Normally distributed data were then analysed by one-way ANOVA to compare treatment groups, followed by Tukey's multiple comparison tests between treatment groups to identify significant differences. Preterm delivery rates were analysed by Fisher's exact test. Amniotic fluid cytokine levels were analysed non-parametrically using a Kruskal-Wallis test followed by Dunn's multiple comparison tests to identify significant differences between treatment groups. As *Fpr2* expression was only measured in two treatment groups, PBS and 20μg LPS, the expression was compared using unpaired t-tests. $P<0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Expression of the lipoxin receptor, Fpr2, in utero-placental tissues following intrauterine LPS administration

Uterus, fetal membranes and placenta were harvested from mice 6 hours post-intrauterine injection of PBS or 20μg LPS and qRT-PCR was performed to examine whether mRNA expression of the lipoxin receptor, *Fpr2*, was altered in response to intrauterine LPS.

Intrauterine LPS administration induced elevated expression of *Fpr2* in all three tissues (Figure 4.1). Uterine *Fpr2* expression was significantly elevated in mice receiving LPS, compared to those receiving PBS (2.4-fold increase; $p<0.001$; Figure 4.1A). In the fetal membranes, *Fpr2*, was also significantly elevated in response to intrauterine LPS compared to the PBS control group (29-fold increase; $p<0.001$; Figure 4.1B). Placental expression of *Fpr2* was also significantly higher in mice treated with LPS, compared to those receiving PBS (3.6-fold increase; $p<0.001$; Figure 4.1C).

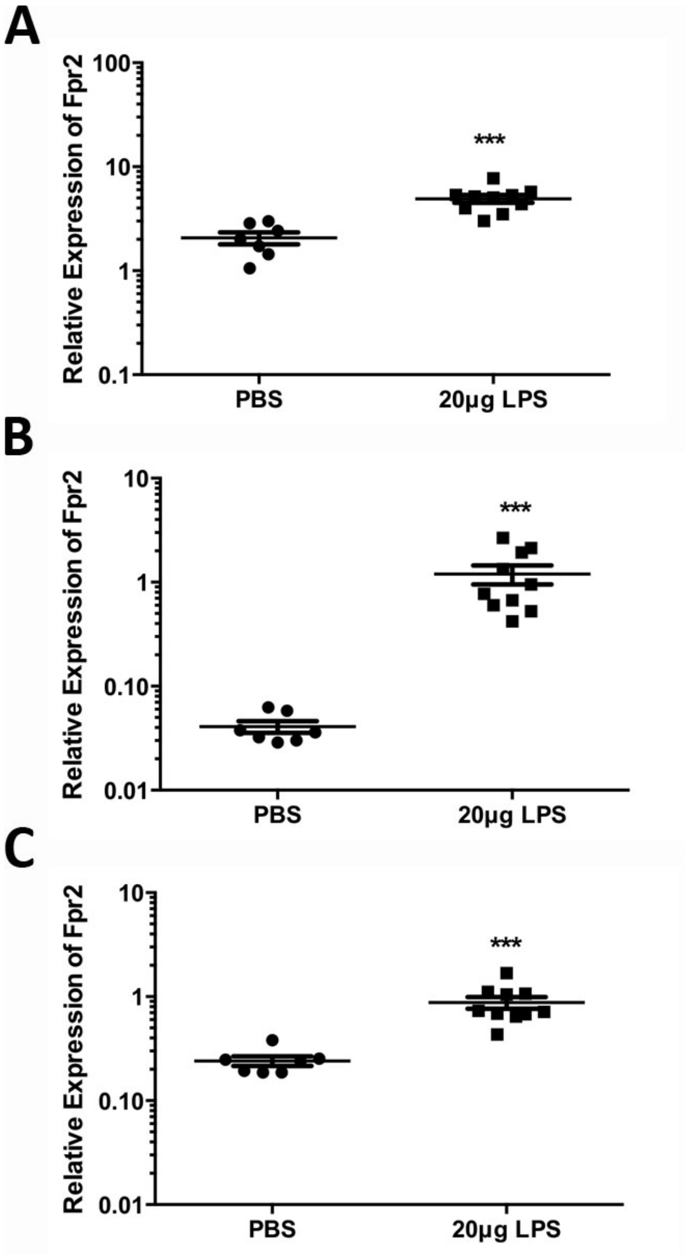

Figure 4.1 - Expression of *Fpr2* mRNA in uterus, fetal membranes and placenta. Uterus, fetal membranes and placenta were collected 6 hours post-surgery from mice receiving intrauterine PBS (n=7) or 20μg LPS (n=10) and the mRNA expression of *Fpr2* was investigated using qRT-PCR. **(A)** *Fpr2* expression in the uterus **(B)** *Fpr2* expression in the fetal membranes. **(C)** Placental *Fpr2* expression. Data presented as mean fold change ± SEM (error bars). ***p<0.001.

4.3.2 Effect of pre-treatment with epi-lipoxin on time to delivery and proportion of live born pups

Given that many studies have demonstrated that epi-lipoxins have anti-inflammatory and pro-resolution actions in other animal models of inflammation-associated pathologies (Levy *et al.* 2002, Conte *et al.* 2010, Borgeson *et al.* 2011); and that lipoxin receptor expression in the utero-placental tissues was increased in response to intrauterine LPS, we wanted to investigate the potential of epi-lipoxin to delay delivery and improve pup survival in our model. Mice were pre-treated with epi-lipoxin 1-2 hours prior to intrauterine LPS or PBS administration. As controls, mice were pre-treated with vehicle prior to intrauterine LPS or PBS injection.

4.3.2.1 Time to delivery

Pre-treatment with 125ng epi-lipoxin had no effect on time to delivery in mice receiving PBS, compared with the vehicle control group (Mean time to delivery of 125ng epi-lipoxin group 50.2 hours ± SEM 5.0 vs. vehicle mean 55.4 hours ± SEM 6.4; Figure 4.2). Mice receiving intrauterine LPS delivered significantly earlier than the vehicle control group (LPS mean time to delivery 27.54 hours ± SEM 6.3; $p<0.001$ vs. vehicle). Mice pre-treated with either 12.5ng or 125ng epi-lipoxin prior to intrauterine LPS still delivered significantly earlier compared to the vehicle control group (mean time to delivery in 12.5ng epi-lipoxin+LPS group 27.02 ± SEM 4.57; mean time to delivery in 125ng epi-lipoxin+LPS group 26.82 ± SEM 2.62; $p<0.01$ vs. vehicle). Mice receiving intrauterine LPS administration, either alone or with epi-lipoxin pre-treatment, also delivered significantly earlier than the 125ng epi-lipoxin control group (LPS group $p<0.01$, 12.5ng epi-lipoxin+LPS group $p<0.05$, 125ng epi-lipoxin+LPS group $p<0.05$).

To investigate further whether epi-lipoxin had any effect on preterm delivery, the preterm delivery rates in each group were calculated (Table 4.1). The preterm delivery rates were defined as the proportion of dams in which there was delivery of the first pup within 36 hours of intrauterine injection. All groups receiving intrauterine LPS had higher rates of preterm delivery compared with the vehicle and 125ng epi-lipoxin groups. Compared to the LPS group, the risk of preterm delivery was significantly lower in the vehicle ($p<0.05$) and 125ng epi-lipoxin ($p<0.01$) groups. Pre-treatment

with epi-lipoxin prior to intrauterine LPS administration did not have a significant effect on the rate of preterm delivery, compared to mice treated with LPS alone.

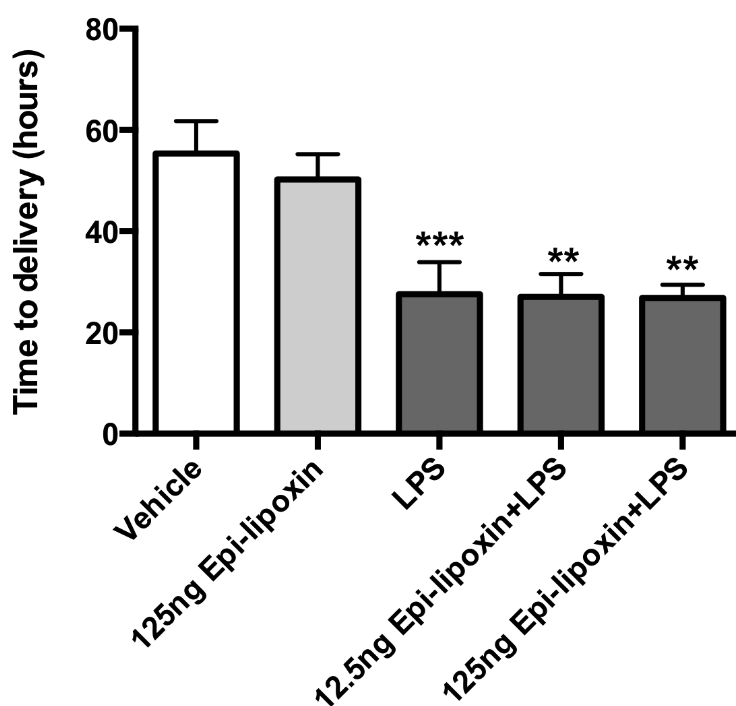

Figure 4.2 - Effect of epi-lipoxin pre-treatment on time to delivery. Time to delivery was monitored in mice pre-treated with vehicle (n=12) or 125ng epi-lipoxin (n=9), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=12), 12.5ng epi-lipoxin (n=11) or 125ng epi-lipoxin (n=11), prior to intrauterine LPS administration. Data are presented as mean ± SEM (error bars). $^{**}p<0.01$, $^{***}p<0.001$, compared to vehicle.

Treatment	Preterm delivery rate	Relative risk of preterm delivery (compared to LPS)
Vehicle (n=12)	0.25	0.3* [95% CI 0.11 – 0.83]
125ng Epi-lipoxin (n=9)	0.22	0.27** [95% CI 0.08– 0.93]
LPS (n=12)	0.83	-
12.5ng Epi-lipoxin+LPS (n=11)	0.64	0.76 [95% CI 0.46 – 1.28]
125ng Epi-lipoxin+LPS (n=11)	0.91	1.09 [95% CI 0.80 – 1.49]

Table 4.1 - Effect of epi-lipoxin pre-treatment on LPS-induced preterm delivery. Time to delivery was monitored in mice pre-treated with vehicle or 125ng epi-lipoxin prior to intrauterine PBS and in mice pre-treated with vehicle, 12.5ng epi-lipoxin or 125ng epi-lipoxin prior to intrauterine LPS administration. Preterm delivery was defined as delivery within 36 hours of intrauterine injection. Relative risk ratios calculated using Fisher's exact test. $^{*}p<0.05$, $^{**}p<0.01$, compared to LPS.

4.3.2.2 Proportion of live born pups

Mice pre-treated with 125ng epi-lipoxin prior to intrauterine PBS administration had a greater proportion of live born pups compared with the vehicle control group (125ng epi-lipoxin mean proportion of live born pups 0.87 ± SEM 0.05 vs. vehicle mean 0.58 ± SEM 0.1; Figure 4.3A), however this difference was not statistically significant ($p=0.2$). Mice in the LPS group had a significantly reduced proportion of live born pups compared with both the groups treated with intrauterine PBS, either those which had pre-treatment with vehicle ($p<0.01$) or those pre-treated with epi-lipoxin ($p<0.001$; mean proportion of live born pups in LPS group 0.16 ± SEM 0.09). Pre-treatment with 125ng epi-lipoxin prior to intrauterine LPS appeared to increase the proportion of live born pups (mean 0.45 ± SEM 0.11) compared to LPS treatment alone, but this did not reach statistical significance ($p=0.18$; Figure 4.3A). Compared to the 125ng epi-lipoxin control group, mice pre-treated with epi-lipoxin prior to intrauterine LPS still had a significantly reduced proportion of live born pups in both the 12.5ng epi-lipoxin+LPS group (mean proportion of live born pups 0.23 ± SEM 0.09; $p<0.001$) and the 125ng epi-lipoxin+LPS group ($p<0.05$).

To further investigate whether epi-lipoxin was having an effect on pup survival, particularly in mice delivering early, the proportion of live born pups was also investigated in mice delivering preterm (which we defined as delivery within 36 hours of intrauterine injection). Mice delivering prematurely in the vehicle group had a greater proportion of live born pups compared to LPS alone (mean proportion of live born pups in vehicle group 0.52 ± SEM 0.29 vs. LPS mean 0.03 ± SEM 0.02; Figure 4.3B), however this difference was not statistically significant ($p=0.08$). Pre-treatment with 125ng epi-lipoxin prior to LPS treatment significantly increased the proportion of live born pups, compared to LPS alone (mean proportion of live born pups in 125ng epi-lipoxin+LPS group 0.45 ± SEM 0.12; $p<0.05$, Figure 4.3B). Although a greater proportion of live born pups were born to mice receiving 12.5ng epi-lipoxin prior to intrauterine LPS (mean 0.12 ± SEM 0.09), this was not significantly greater compared to LPS alone. Only two mice in the 125ng epi-lipoxin control group delivered preterm, therefore, they were excluded from analysis; however both mice delivered litters with a high proportion of live born pups (0.78 and 1.0).

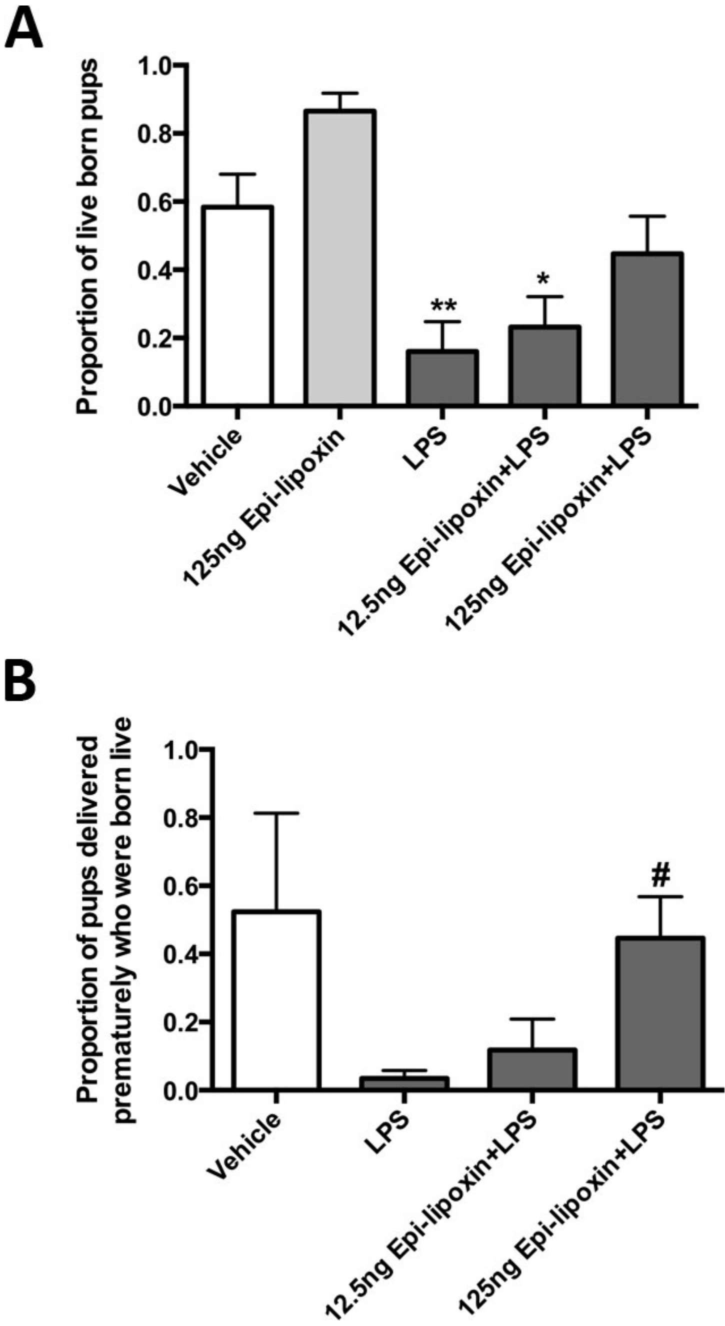

Figure 4.3 - Effect of pre-treatment with epi-lipoxin on the proportion of live born pups. The proportion of live born pups was determined in mice pre-treated with vehicle (n=12) or 125ng epi-lipoxin (n=9), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=12), 12.5ng epi-lipoxin (n=11) or 125ng epi-lipoxin (n=11), prior to intrauterine LPS administration. **(A)** Proportion of live born pups in all mice **(B)** The proportion of pups delivered prematurely (within 36 hours of surgery) who were born live; [vehicle (n=3), LPS (n=10), 12.5ng epi-lipoxin+LPS (n=7), 125ng Epi-lipoxin+LPS (n=10)]. Data presented as mean ± SEM (error bars). *p<0.05, **p<0.01 compared to vehicle; #p<0.05 compared to LPS.

4.3.3 Expression of inflammatory mediators in the utero-placental tissues following intra-peritoneal pre-treatment with epi-lipoxin

Given the results described in chapter 3, which demonstrated intrauterine LPS treatment induced a robust inflammatory response in the intrauterine tissues, resulting in the increased expression of a number of inflammatory genes, in a second cohort of mice, vehicle, epi-lipoxin, PBS and LPS were given as before, but mice were sacrificed 6 hours after intrauterine injection and utero-placental tissues were then analysed for the expression of the same inflammatory mediators using qRT-PCR to examine whether pre-treatment with epi-lipoxin affected LPS-induced inflammatory gene expression. As before, the genes examined were *Tnf-α, Il-1β, Il-6, Il-10, Cox-2, 15-Hpgd, Cxcl1, Cxcl2, Cxcl5* and *Ccl2*. To investigate the anti-inflammatory actions of epi-lipoxin, we administered higher doses of epi-lipoxin to try to maximise the anti-inflammatory effects, with 0.25μg and 2.5μg being used as the low and high doses in these experiments.

4.3.3.1 Inflammatory gene expression in the uterus

Compared to the vehicle control group, uterine *Tnf-α* expression was significantly elevated in mice treated with LPS (6.4-fold greater expression; $p<0.001$; Figure 4.4A). Pre-treatment with 0.25μg and 2.5μg epi-lipoxin, prior to intrauterine LPS, did not significantly alter LPS-induced *Tnf-α* expression, with mice in the 0.25μg epi-lipoxin+LPS and 2.5μg epi-lipoxin+LPS groups, still having significantly elevated *Tnf-α* expression compared to vehicle (4.9-fold and 6.8-fold greater expression respectively; $p<0.001$). Pre-treatment with 2.5μg epi-lipoxin prior to intrauterine PBS did not significantly affect *Tnf-α* expression, compared to the vehicle control group.

Expression of *Il-1β* in the uterus was significantly elevated in mice treated with LPS, compared to the vehicle control group ($p<0.05$; Figure 4.4B), with LPS inducing a 2.6-fold increase in expression. Again, mice pre-treated with 0.25μg and 2.5μg epi-lipoxin, prior to intrauterine LPS, still had significantly elevated *Il-1β* expression, compared to the vehicle control group (3.1-fold and 3.4-fold greater expression respectively; $p<0.01$). No significant difference in *Il-1β* expression was observed when comparing mice pre-treated with 2.5μg epi-lipoxin prior to intrauterine PBS to the vehicle control group.

Compared to the vehicle group, *Il-6* expression was significantly elevated in mice receiving LPS (7.7-fold greater expression; p<0.001). Pre-treatment with epi-lipoxin prior to intrauterine LPS did not significantly alter *Il-6* expression compared to LPS alone, and mice in these groups still had significantly greater expression of *Il-6* compared to vehicle (0.25μg epi-lipoxin+LPS: 7.9-fold greater expression; p<0.001; 2.5μg epi-lipoxin+LPS group 12.3-fold greater expression; p<0.001; Figure 4.4C). Treatment with epi-lipoxin alone did not significantly affect *Il*-6 expression, compared to the vehicle group.

Expression of *Il-10* was significantly elevated in the LPS treatment group compared to the vehicle group (2.2-fold greater expression; p<0.05; Figure 4.4D). Whilst pre-treatment with epi-lipoxin did not result in any significant effect on LPS-induced *Il-10* expression in the uterus, compared to either LPS alone or vehicle.

Compared to the vehicle group, *Cox-2* expression was significantly elevated in response to intrauterine LPS, in mice treated with LPS alone (9.2-fold greater expression, p<0.01) and in mice pre-treated with 0.25μg and 2.5μg epi-lipoxin prior to intrauterine LPS (16.1-fold and 20.8-fold increased expression respectively, p<0.001; Figure 4.4E). Interestingly, pre-treatment with 2.5μg epi-lipoxin prior to intrauterine PBS also significantly increased uterine *Cox-2* expression, compared to the vehicle group (6-fold greater expression, p<0.05).

Uterine expression of *15-Hpgd* was significantly reduced in mice pre-treated with 2.5μg epi-lipoxin prior to intrauterine PBS administration, compared to vehicle (2.8-fold reduced expression, p<0.05; Figure 4.4F). LPS treatment alone did not significantly alter *15-Hpgd* expression, compared to vehicle, however mice pre-treated 0.25μg and 2.5μg epi-lipoxin prior to intrauterine LPS also had significantly reduced *15*-Hpgd expression compared to the vehicle group (3.6-fold decrease (p<0.001) and 3.4-fold decrease (p<0.01) respectively). Mice in the 0.25μg epi-lipoxin+LPS and 2.5μg epi-lipoxin+LPS group also had significantly decreased expression of *15-Hpgd* compared to mice receiving LPS alone, with a mean 3.2-fold (p<0.001) and 3-fold (p<0.01) decrease, respectively.

Expression of the neutrophil chemokines *Cxcl1, Cxcl2* and *Cxcl5* in the uterus, were all significantly elevated in the LPS treatment group, compared with the vehicle control group. *Cxcl1* expression was 18.2-fold higher in the LPS treatment group compared to

mice in the vehicle group ($p<0.001$; Figure 4.5A). Intrauterine LPS administration induced a mean 16.8-fold upregulation in *Cxcl2* compared with the vehicle ($p<0.001$; Figure 4.5B). Expression of *Cxcl5* was 8.8-fold greater in the LPS treatment group compared to mice in the vehicle group ($p<0.001$; Figure 4.5C). Pre-treatment with epi-lipoxin, at either dose, prior to intrauterine LPS administration did not significantly alter LPS-induced expression of *Cxcl1*, *Cxcl2* or *Cxcl5*, with mice in these treatment groups still having significantly elevated expression of *Cxcl1, Cxcl2* and *Cxcl5* when compared to the vehicle group ($p<0.001$).

Although expression of the macrophage chemokine, *Ccl2*, was elevated 2.3-fold in response to intrauterine LPS, compared to the vehicle group, this difference was not found to be statistically significant ($p=0.07$; Figure 4.5D). Similarly, epi-lipoxin pre-treatment had no significant effect on *Ccl2* expression in the uterus.

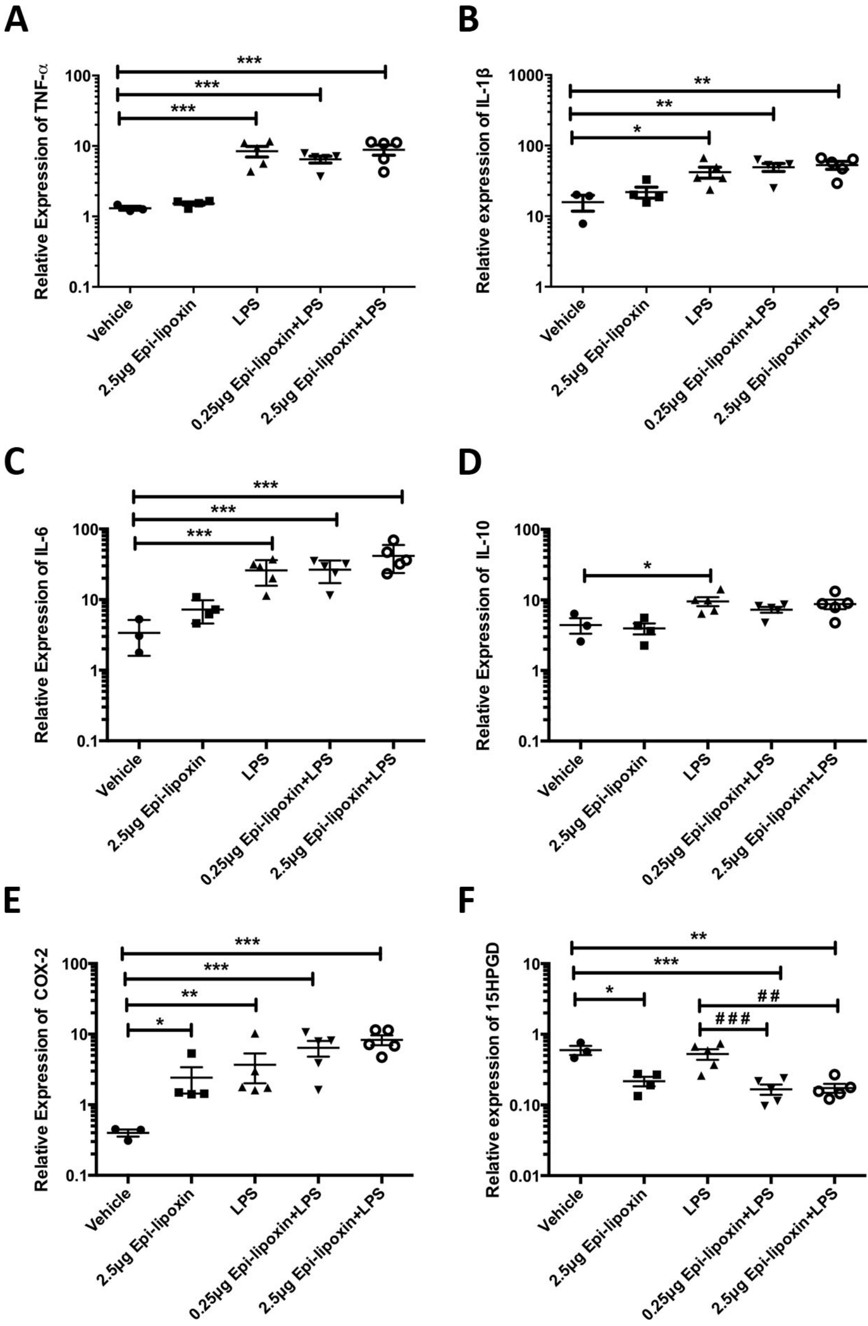

Figure 4.4 - Effect of i.p. epi-lipoxin pre-treatment on inflammatory gene expression in the uterus. Uterine tissue was harvested 6 hours post surgery from mice pre-treated with vehicle (n=3) or 2.5μg epi-lipoxin (n=4), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=5), 0.25μg epi-lipoxin (n=5) or 2.5μg epi-lipoxin (n=5), prior to intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to vehicle; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, compared to LPS.

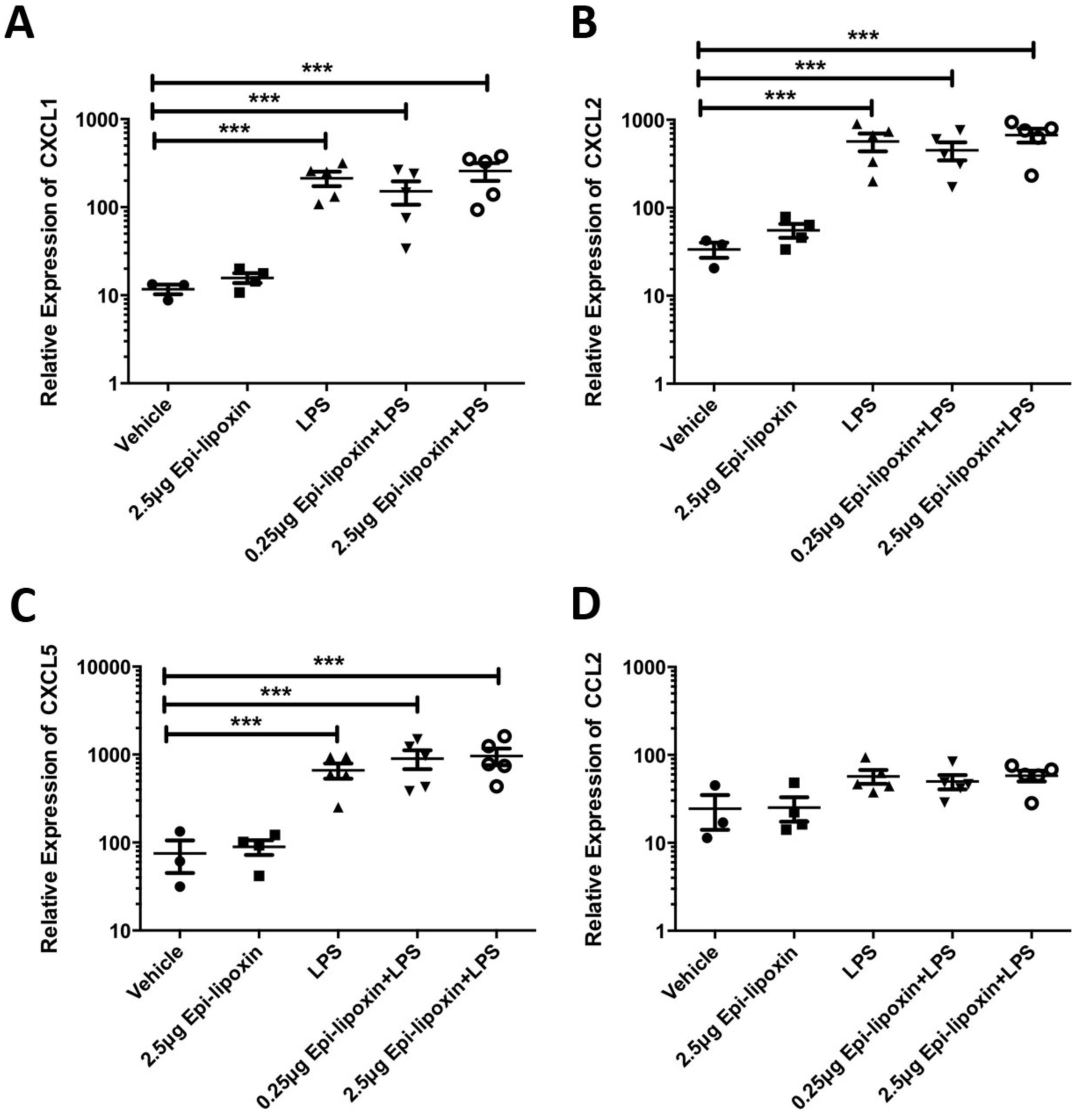

Figure 4.5 - Effect of i.p. epi-lipoxin treatment on inflammatory chemokine expression in the uterus. Uterine tissue was harvested 6 hours post surgery from mice pre-treated with vehicle (n=3) or 2.5μg epi-lipoxin (n=4), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=5), 0.25μg epi-lipoxin (n=5) or 2.5μg epi-lipoxin (n=5), prior to intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Cxcl1* expression**. (B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold fold-change ± SEM (error bars); ***p<0.001, compared to vehicle.

4.3.3.2 Inflammatory gene expression in the fetal membranes

Expression of *Tnf-α* in the fetal membranes was significantly elevated in response to intrauterine LPS administration, with 48-fold increased expression in the fetal membranes of mice receiving intrauterine LPS compared with the vehicle ($p<0.001$; Figure 4.6A). Pre-treatment with 0.25μg and 2.5μg epi-lipoxin, prior to intrauterine LPS did not significantly affect LPS-induced *Tnf-α* expression, compared to LPS alone, with *Tnf-α* expression in these groups still significantly greater compared to the vehicle control (58.9-fold and 60.4-fold increase, respectively, $p<0.001$). Pre-treatment with epi-lipoxin prior to intrauterine PBS administration did not significantly affect *Tnf-α* expression in the fetal membranes, compared to the vehicle control group.

Intrauterine administration of LPS resulted in significantly elevated *Il-1β* expression compared with the vehicle (29.5-fold increase; $p<0.001$; Figure 4.6B). Again pre-treatment with epi-lipoxin prior to intrauterine LPS administration did not significantly alter expression of *Il-1β*, compared to LPS alone, with mice in the epi-lipoxin+LPS groups still having significantly greater *Il-1β* expression, compared to the vehicle control group (0.25μg epi-lipoxin+LPS 29.5-fold greater expression; 50.9-fold greater expression; $p<0.001$).

Compared to vehicle, LPS alone did not significantly increase *Il*-6 expression (9.9-fold increase, $p=0.08$; Figure 4.6C). Pre-treatment with epi-lipoxin at both 0.25μg and 2.5μg epi-lipoxin prior to intrauterine LPS significantly increased expression of *Il*-6 in the fetal membranes compared to the vehicle control group (16.9-fold and 33.2-fold greater expression respectively, $p<0.01$); however these increases were not significantly greater when compared to LPS alone.

Il-10 expression was significantly elevated in response to intrauterine LPS administration compared with vehicle (15.8-fold increase; $p<0.001$; Figure 4.6D). Mice pre-treated with epi-lipoxin at both 0.25μg and 2.5μg epi-lipoxin prior to intrauterine LPS also had significantly elevated *Il-10* expression compared to the vehicle control group (9-fold ($p<0.01$) and 24.2-fold ($p<0.001$) greater expression, respectively); these differences were not statistically significant compared to LPS alone. Pre-treatment with epi-lipoxin prior to intrauterine PBS administration did not significantly alter *Il-10* expression, compared to vehicle.

Intrauterine LPS treatment alone, did not significantly alter *Cox-2* expression in the fetal membranes; however mice pre-treated with epi-lipoxin prior to intrauterine LPS administration had increased *Cox-2* expression compared to both vehicle and LPS alone. In response to 2.5μg epi-lipoxin+LPS, *Cox-2* expression in the fetal membranes was significantly greater, compared to the vehicle control group (14.3-fold increase, $p<0.05$; Figure 4.6E), and 4.4-fold higher expression was observed, compared to LPS alone, but this increase did not reach statistical significance ($p=0.07$).

Intrauterine administration of LPS reduced the expression of *15-Hpgd*, compared to vehicle (2.2-fold decrease; $p<0.01$; Figure 4.6F). Similarly, mice pre-treated with epi-lipoxin prior to intrauterine LPS administration had significantly reduced expression of *15-Hpgd* compared to vehicle control (0.25μg epi-lipoxin+LPS: 2.5-fold decrease, $p<0.01$; 2.5μg epi-lipoxin+LPS: 3-fold decrease, $p<0.001$). Pre-treatment with epi-lipoxin prior to intrauterine PBS did not alter *15-Hpgd* expression in the fetal membranes.

Compared to the vehicle control group, LPS treatment alone induced a 9.2-fold increase in *Cxcl1* expression, however this was not statistically significant ($p=0.08$; Figure 4.7A). Pre-treatment with 0.25μg and 2.5μg epi-lipoxin, prior to intrauterine LPS, resulted in significantly greater *Cxcl1* expression, compared to the vehicle control group (15.9-fold, $p<0.01$ and 18-fold, $p<0.001$, greater expression, respectively); however there were no significant differences when comparing pre-treatment with epi-lipoxin to LPS alone. *Cxcl2* expression in the fetal membranes was significantly elevated in all groups receiving intrauterine LPS, with and without pre-treatment with epi-lipoxin, compared to the vehicle group (LPS: 13.7-fold increase, $p<0.05$; 0.25μg epi-lipoxin+LPS: 20.7-fold increase, $p<0.01$; and 2.5μg epi-lipoxin+LPS: 23.7-fold increase, $p<0.01$; Figure 4.7B). Again pre-treatment with epi-lipoxin prior to intrauterine LPS did not significantly alter *Cxcl2* expression, compared to LPS alone. Similarly, all groups receiving intrauterine LPS had significantly greater *Cxcl5* expression, compared to vehicle (LPS: 87.5-fold increase; 0.25μg epi-lipoxin+LPS: 285.9-fold increase; 2.5μg epi-lipoxin+LPS: 154.8-fold increase; $p<0.001$; Figure 4.7C). Again, pre-treatment with epi-lipoxin prior to intrauterine LPS did not significantly alter *Cxcl5* expression, compared to LPS alone.

As with the other chemokines, *Ccl2* expression was significantly elevated in the fetal membranes of all groups of mice receiving intrauterine LPS, compared the vehicle group (LPS: 11-fold increase, $p<0.05$; 0.25μg epi-lipoxin+LPS: 15.2-fold increase,

$p<0.01$; 2.5μg epi-lipoxin+LPS: 15-fold increase; $p<0.01$; Figure 4.7D). Pre-treatment with epi-lipoxin, at either dose, did not significantly alter *Ccl2* expression compared to LPS alone.

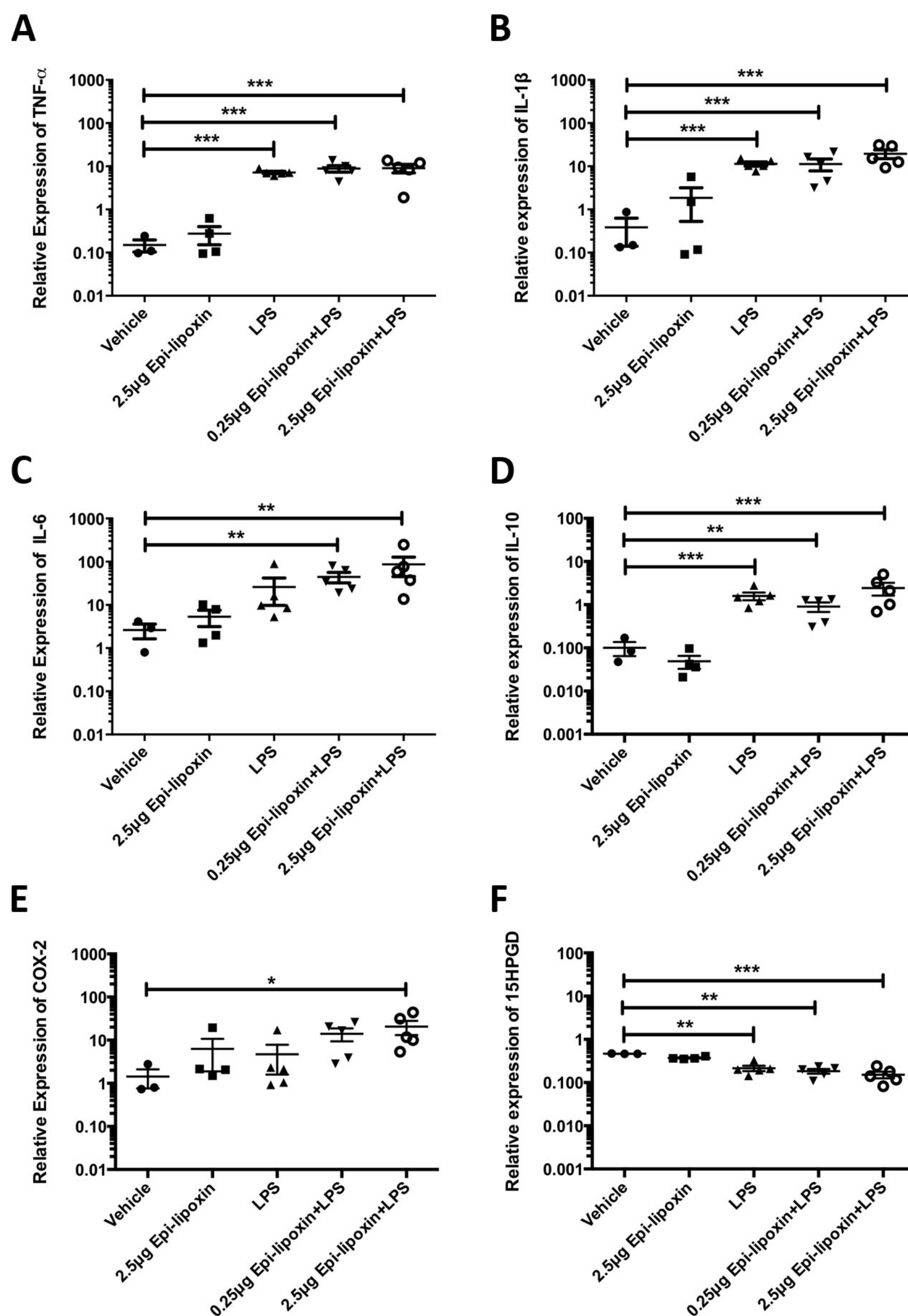

Figure 4.6 - Effect of i.p. epi-lipoxin pre-treatment on inflammatory gene expression in the fetal membranes. Fetal membranes were harvested 6 hours post surgery from mice pre-treated with vehicle (n=3) or 2.5μg epi-lipoxin (n=4), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=5), 0.25μg epi-lipoxin (n=5) or 2.5μg epi-lipoxin (n=5), prior to intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR**.** **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to vehicle.

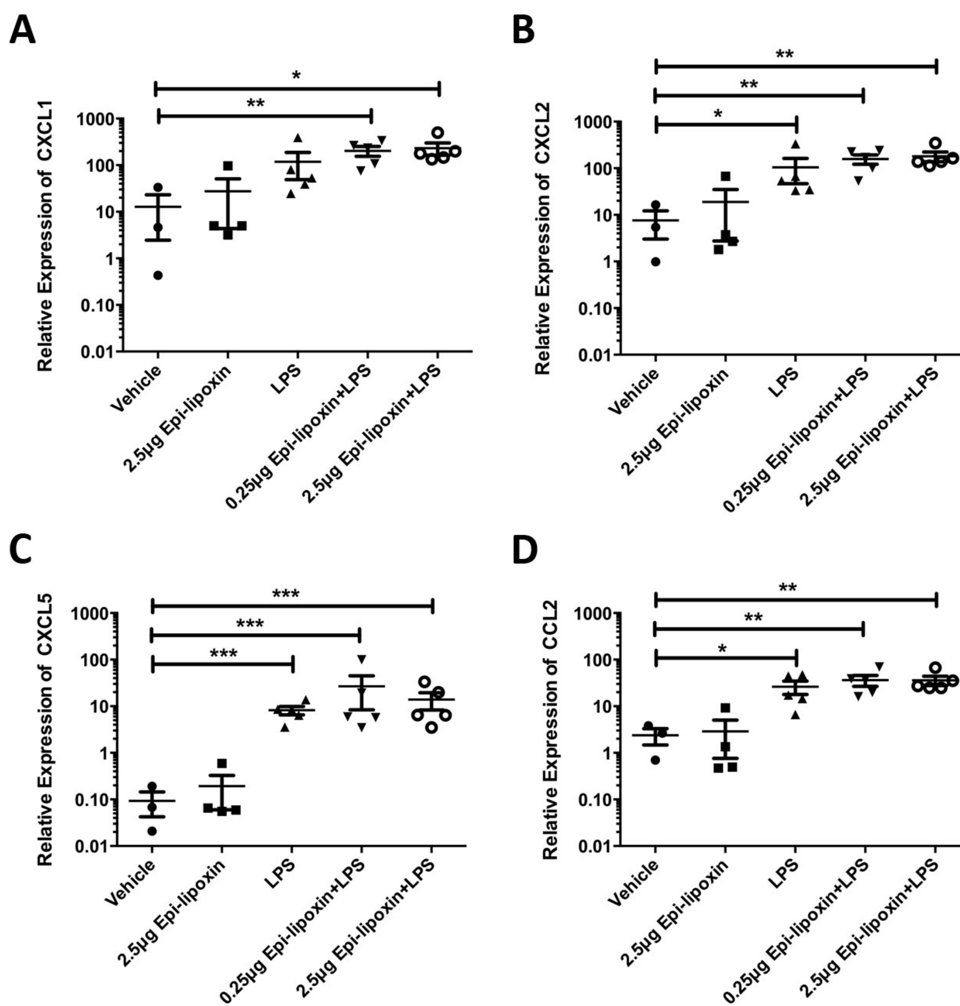

Figure 4.7 - Effect of i.p. epi-lipoxin treatment on inflammatory chemokine expression in the fetal membranes. Fetal membranes were harvested 6 hours post surgery from mice pre-treated with vehicle (n=3) or 2.5μg epi-lipoxin (n=4), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=5), 0.25μg epi-lipoxin (n=5) or 2.5μg epi-lipoxin (n=5), prior to intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold fold-change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to vehicle.

4.3.3.3 Inflammatory gene expression in the placenta

As was observed in the uterus and fetal membranes, placental *Tnf-α* expression was significantly up-regulated in mice receiving intrauterine LPS compared with the vehicle group (7.4-fold increase; p<0.001; Figure 4.8A). Similarly, mice pre-treated with epi-lipoxin, at both 0.25μg and 2.5μg, prior to intrauterine LPS, had significantly greater *Tnf-α* expression, compared to the vehicle control group (7.4-fold and 8.9-fold greater expression, respectively, p<0.001).

Expression of *Il-1β* in the placenta was also significantly elevated in the LPS group compared to the vehicle control group, with an 8.4-fold increase compared to vehicle (p<0.001; Figure 4.8B). Again, pre-treatment with epi-lipoxin prior to intrauterine LPS administration did not significantly attenuate LPS-induced *Il-1β* expression, and mice in these groups still had significantly elevated expression, compared to vehicle (0.25μg epi-lipoxin+LPS: 8.6-fold increase; 2.5μg epi-lipoxin+LPS: 15.5-fold increase; p<0.001).

Il-6 expression was significantly increased in response to intrauterine LPS administration compared to vehicle (7.7-fold increase, p<0.001; Figure 4.8C). Mice pre-treated with 0.25μg and 2.5μg epi-lipoxin prior to intrauterine LPS, also had significantly greater expression of *Il-6* compared to the vehicle control group (8.9-fold and 20.2-fold increase, respectively; p<0.001).

Similarly, placental *Il-10* expression was elevated in the LPS group compared with the vehicle group (4.5-fold increase; p<0.05; Figure 4.8D); and expression of *Il-10* was also significantly greater in mice pre-treated with epi-lipoxin, prior to intrauterine LPS, compared to vehicle (0.25μg epi-lipoxin+LPS: 4.2-fold increase, p<0.05; 2.5μg epi-lipoxin+LPS: 5.9-fold increase; p<0.01).

The expression of *Cox-2* was increased in the placenta of mice pre-treated with 2.5μg epi-lipoxin prior to intrauterine PBS, compared to vehicle, however this was not statistically significant (2.3-fold greater, p=0.17; Figure 4.8E). LPS treatment alone did not significantly affect *Cox-2* expression, but pre-treatment with epi-lipoxin at both 0.25μg and 2.5μg prior to intrauterine LPS administration significantly increased *Cox-2* expression, compared to the vehicle control group (5.4-fold increase, p<0.01; and 6.9-fold increase, p<0.001, respectively). Additionally, pre-treatment with epi-lipoxin, at both doses, prior to intrauterine LPS also significantly increased LPS-induced *Cox*-2

expression, compared to LPS alone (0.25μg epi-lipoxin+LPS: 5.2-fold increase; and 2.5μg epi-lipoxin+LPS: 6.7-fold increase; $p<0.001$).

Conversely, pre-treatment with 2.5μg epi-lipoxin prior to intrauterine PBS administration resulted in significantly reduced expression of *15-Hpgd* in the placenta, compared to vehicle (3.2-fold decrease; $p<0.001$; Figure 4.8F). LPS treatment alone did not significantly affect placental *15-Hpgd* expression, however mice pre-treated with 0.25μg epi-lipoxin prior to intrauterine LPS had significantly reduced *15-Hpgd* expression, compared to the vehicle control group (2.3-fold decrease, $p<0.01$).

Placental expression of the neutrophil chemokines *Cxcl1*, *Cxcl2* and *Cxcl5* was significantly elevated in response to intrauterine LPS administration. *Cxcl1* expression was significantly higher in the LPS treatment group compared to vehicle (4.2-fold increase, $p<0.001$; Figure 4.9A); and pre-treatment with epi-lipoxin prior to intrauterine LPS injection did not attenuate LPS-induced *Cxcl1* expression, with mice in these groups still having significantly elevated *Cxcl1*expression compared to the vehicle control group ($p<0.001$). Placental expression of *Cxcl2* was similarly elevated in response to intrauterine LPS administration compared with the vehicle (14-fold, $p<0.001$; Figure 4.9B); and again mice receiving pre-treatment with epi-lipoxin prior to intrauterine LPS still had significantly elevated *Cxcl2* expression, compared to vehicle ($p<0.001$). *Cxcl5* expression was also significantly elevated in response to intrauterine LPS administration compared to the vehicle group (24-fold, $p<0.001$; Figure 4.9C); however pre-treatment with epi-lipoxin prior to intrauterine LPS administration did not affect LPS-induced *Cxcl5* expression in the placenta, compared to treatment with LPS alone, with mice in these groups still having significantly elevated *Cxcl5* expression, compared to the vehicle control group ($p<0.001$).

Similarly, the macrophage chemokine, *Ccl2*, was found to have significantly elevated expression in response to intrauterine LPS administration, compared to vehicle (6.7-fold, $p<0.001$; Figure 4.9D). As observed with the other chemokines, pre-treatment with epi-lipoxin did not significantly attenuate LPS-induced *Ccl2* expression, and these mice were found to still have significantly greater *Ccl2* expression compared to the vehicle control group ($p<0.001$).

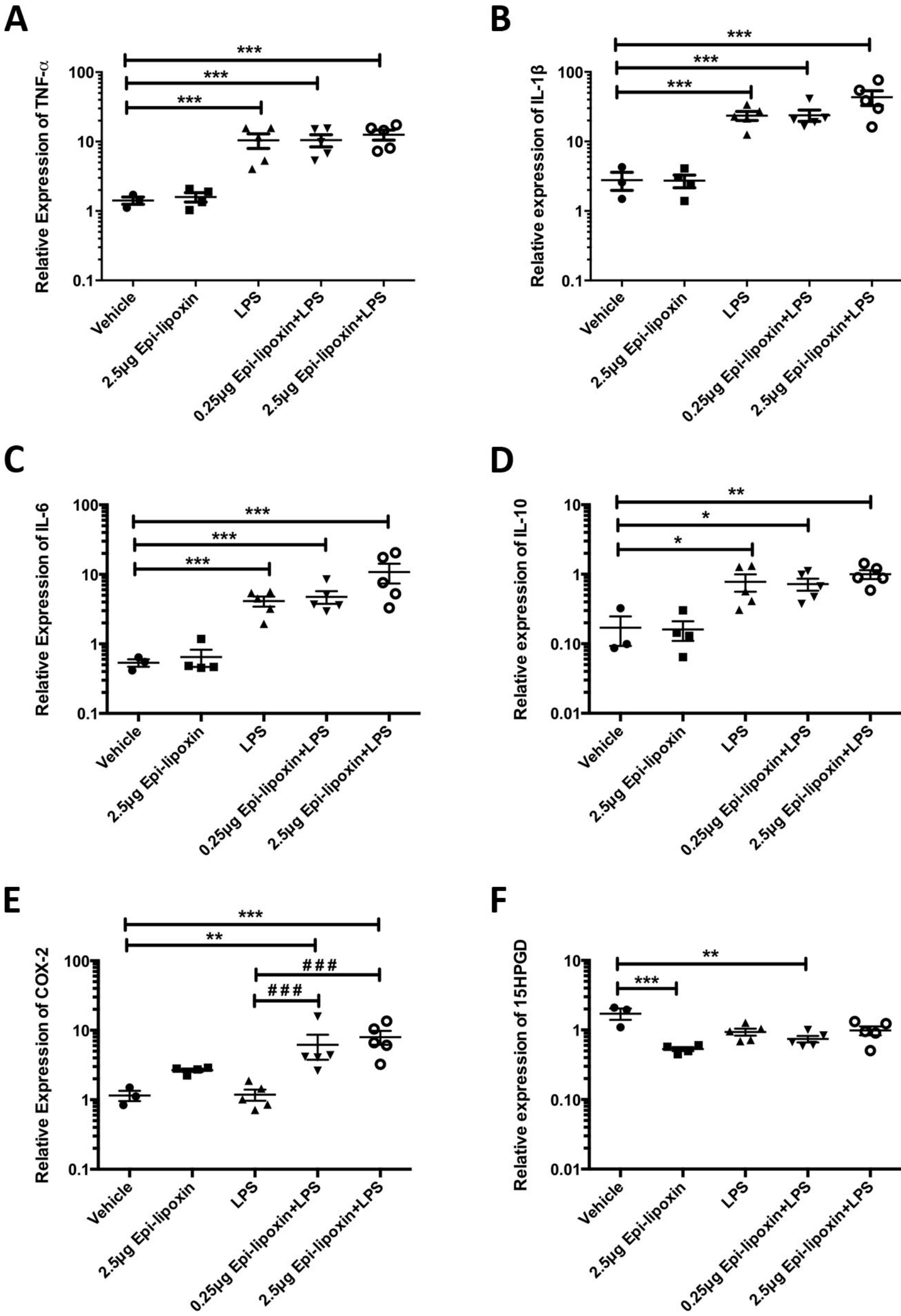

Figure 4.8 - Effect of i.p. epi-lipoxin pre-treatment on inflammatory gene expression in the placenta. Placental tissue was harvested 6 hours post surgery from mice pre-treated with vehicle (n=3) or 2.5μg epi-lipoxin (n=4), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=5), 0.25μg epi-lipoxin (n=5) or 2.5μg epi-lipoxin (n=5), prior to intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to vehicle; ###p<0.001, compared to LPS.

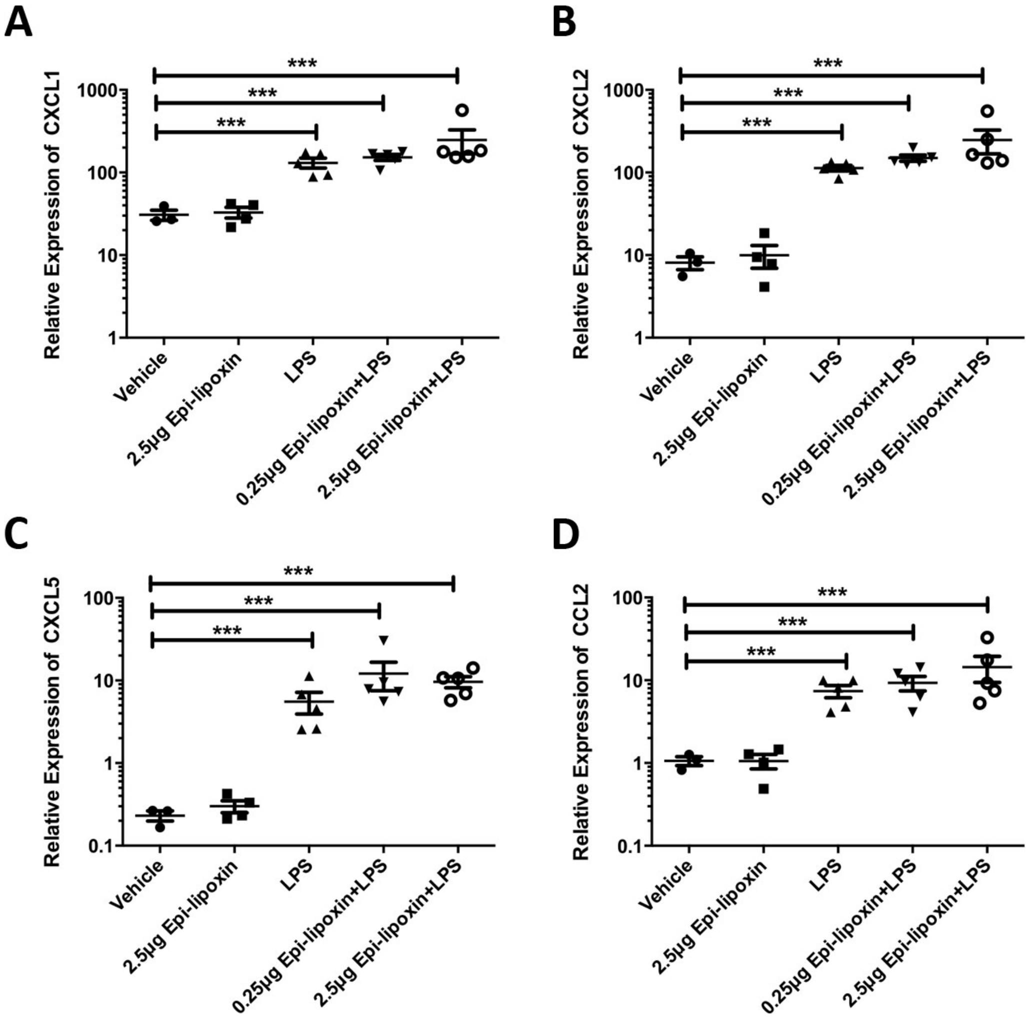

Figure 4.9 - Effect of i.p. epi-lipoxin treatment on inflammatory chemokine expression in the placenta. Placental tissue was harvested 6 hours post surgery from mice pre-treated with vehicle (n=3) or 2.5μg epi-lipoxin (n=4), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=5), 0.25μg epi-lipoxin (n=5) or 2.5μg epi-lipoxin (n=5), prior to intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold fold-change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001.

4.3.4 Effect of pre-treatment with epi-lipoxin on amniotic fluid cytokine levels

As described in chapter 3, intrauterine LPS administration also induced inflammatory cytokine production in the amniotic fluid, therefore, to investigate whether pre-treatment with epi-lipoxin was able to alter amniotic fluid cytokine levels, amniotic fluid was collected 6 hours post-surgery and the concentration of TNF-α and IL-10 was measured by ELISA. Where the concentration of cytokines was too low to be detected by the ELISAs, the value of the limit of detection of the specific ELISA was entered to give a value for analysis and the data was analysed by non-parametric ANOVA (Kruskal-Wallis).

TNF-α was undetectable in the vehicle and 2.5μg epi-lipoxin groups, which received intrauterine PBS; and also in one LPS sample. The concentration of TNF-α was elevated in mice receiving intrauterine LPS (97.63 pg/ml ± SEM 61.26), however this difference was not significantly greater compared to mice receiving intrauterine PBS. Pre-treatment with epi-lipoxin prior to intrauterine LPS administration did not significantly affect LPS-induced TNF-α production, although levels were higher in mice pre-treated with epi-lipoxin, compared to LPS alone (0.25μg epi-lipoxin+LPS mean 194.8 pg/ml ± SEM 32.76; 2.5μg epi-lipoxin mean 299.3 pg/ml ± SEM 100.8; Figure 4.10A).

IL-10 was also undetectable in the vehicle and 2.5μg epi-lipoxin groups, which received intrauterine PBS; and also in two LPS samples. LPS treatment did not significantly increase amniotic fluid IL-10 levels (mean 20.18 pg/ml ± SEM 4.97), compared to the vehicle and 2.5μg epi-lipoxin groups. Pre-treatment with epi-lipoxin again did not significantly affect LPS-induced IL-10 production compared to LPS alone, although the mean concentrations were slightly higher (mean 0.25μg epi-lipoxin+LPS 36.21 pg/ml ± 8.62; mean 2.5μg epi-lipoxin+LPS 34.63 pg/ml ± SEM 5.81; Figure 4.10B).

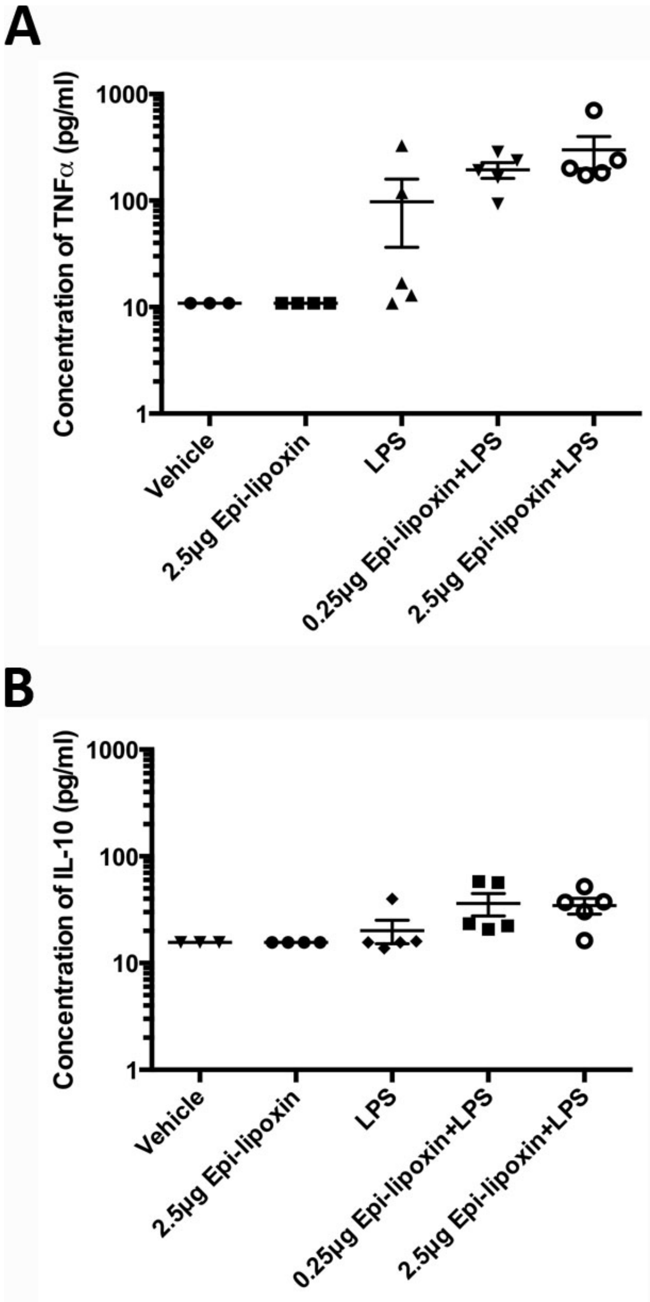

Figure 4.10 - Effect of pre-treatment with epi-lipoxin on inflammatory cytokine production in the amniotic fluid. Amniotic fluid was collected 6 hours post-surgery from mice pre-treated with vehicle (n=3) or 2.5μg epi-lipoxin (n=4), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=5), 0.25μg epi-lipoxin (n=5) or 2.5μg epi-lipoxin (n=5), prior to intrauterine LPS administration, and ELISAs were used to quantify the concentration of TNF-α and IL-10 in the amniotic fluid. **(A)** Amniotic fluid TNF-α concentration. **(B)** Amniotic fluid IL-10 concentration. Data presented as mean ± SEM (error bars).

4.3.5 Expression of inflammatory mediators in the utero-placental tissues following intrauterine administration of epi-lipoxin

Several studies have demonstrated that lipoxin administration locally, at the site of inflammation, can also have strong anti-inflammatory actions (Menezes-de-Lima *et al.* 2006, Conte *et al.* 2010). Therefore, given that intra-peritoneal pre-treatment with epi-lipoxin largely did not affect inflammatory gene expression in the utero-placental tissues, we administered epi-lipoxin directly into the uterus at the same time as either intrauterine LPS or PBS administration. As described before, utero-placental tissues were collected 6 hours post-surgery and examined for the expression of the same inflammatory genes using qRT-PCR. In this series of experiments epi-lipoxin was administered at lower doses compared to the i.p. epi-lipoxin 6 hour experiments, with doses of 25ng and 250ng as the low and high doses. The doses of epi-lipoxin were reduced because previous studies using local administration gave lower doses, compared to those given systemically.

4.3.5.1 Inflammatory gene expression in the uterus

As before, expression of *Tnf-*α was significantly elevated in response to intrauterine LPS administration, with 4.7-fold higher expression compared to the vehicle group ($p<0.001$; Figure 4.11A). Co-treatment of epi-lipoxin and LPS did not significantly alter LPS-induced *Tnf-*α expression in the uterus, compared to LPS alone, with expression of *Tnf-*α still significantly increased compared to the vehicle control group (25ng epi-lipoxin+LPS: 7.3-fold increase; 250ng epi-lipoxin+LPS 4.9-fold increase; $p<0.001$).

Similarly, *Il-1*β expression was also significantly greater in mice treated with intrauterine LPS, compared with the vehicle group (2.9-fold increase; $p<0.05$; Figure 4.11B). Compared to LPS alone, co-treatment with epi-lipoxin did not significantly affect LPS-induced *Il-1*β expression in the uterus; mice in the epi-lipoxin+LPS co-treatment groups still had significantly greater *Il-1*β expression, compared to the vehicle control group (25ng epi-lipoxin+LPS: 4.7-fold increase, $p<0.01$; 250ng epi-lipoxin+LPS: 3.3-fold increase, $p<0.005$).

Uterine *Il-6* expression was also significantly elevated in the presence of intrauterine LPS, compared to vehicle ($p<0.05$; 6.5-fold increase; Figure 4.11C). Again, co-administration of epi-lipoxin and LPS did not affect *Il-6* expression compared to LPS

alone; and mice treated with 25ng or 250ng epi-lipoxin prior to intrauterine LPS still had significantly elevated *Il-6* expression, compared to the vehicle control (10-fold increase, $p<0.05$; and 5.5-fold greater expression, $p<0.01$, respectively).

In this cohort of mice, expression of *Il-10* in the uterus was not significantly increased in response to intrauterine LPS administration, compared to the vehicle control group (Figure 4.11D). Similarly, compared to LPS treatment alone, co-treatment with epi-lipoxin had no significant effect on uterine *Il-10* expression.

Expression of *Cox-2* was significantly elevated in response to intrauterine LPS administration, compared with the vehicle control group, with 5.9-fold higher expression in LPS-treated mice ($p<0.05$; Figure 4.11E). Mice that received co-treatment of epi-lipoxin and LPS also had significantly greater expression of *Cox-2,* compared to the vehicle control group (25ng epi-lipoxin+LPS: 18.2-fold increase; 250ng epi-lipoxin+LPS: 14.5-fold increase; $p<0.001$). Additionally, co-treatment with either 25ng or 250ng epi-lipoxin at the same time as intrauterine LPS administration appeared to increase uterine *Cox-2* expression, compared to LPS alone (3-fold and 2.5-fold increase, respectively); however these differences were not statistically significant.

Intrauterine administration of LPS alone did not significantly affect uterine *15-Hpgd* expression. However, co-treatment with epi-lipoxin at either 25ng or 250ng, at the same time as LPS significantly reduced *15-Hpgd* expression, inducing a 2.3-fold and 2.5-fold decrease, compared to the vehicle control group ($p<0.05$; Figure 4.11F).

As observed previously, intrauterine administration of LPS significantly increased the expression of the three neutrophil chemokines, *Cxcl1*, *Cxcl2*, and *Cxcl5*. *Cxcl1* expression was 17.7-fold higher in LPS-treated mice, compared with the vehicle control group ($p<0.001$; Figure 4.12A). The LPS-induced expression of *Cxcl1* was unaffected by co-treatment with epi-lipoxin, with mice co-treated with 25ng and 250ng epi-lipoxin still found to have significantly elevated *Cxcl1* expression, compared to the vehicle control group ($p<0.001$). Similarly expression of *Cxcl2* was significantly elevated in the LPS group compared with the vehicle group (15.5-fold increase, $p<0.01$; Figure 4.12B); and co-treatment of 25ng or 250ng plus LPS was not found to significantly attenuate LPS-induced *Cxcl2* expression, with *Cxcl2* expression still found to be significantly greater in the 25ng epi-lipoxin+LPS ($p<0.01$) and 250ng epi-lipoxin+LPS ($p<0.001$) groups, compared to the vehicle control. *Cxcl5* expression was also significantly

increased in the LPS group compared with vehicle (12.2-fold increase; p<0.01; Figure 4.12C); and was still significantly elevated in the groups co-treated with 25ng epi-lipoxin+LPS (p<0.05), and 250ng epi-lipoxin+LPS (p<0.01), compared to the vehicle group. .

In this cohort of mice, although intrauterine LPS induced a 2.4-fold increase in *Ccl2* expression, this was not statistically significant (p=0.17; Figure 4.12D). Co-treatment with epi-lipoxin, either before intrauterine PBS or LPS, was not found to significantly alter uterine *Ccl2* expression.

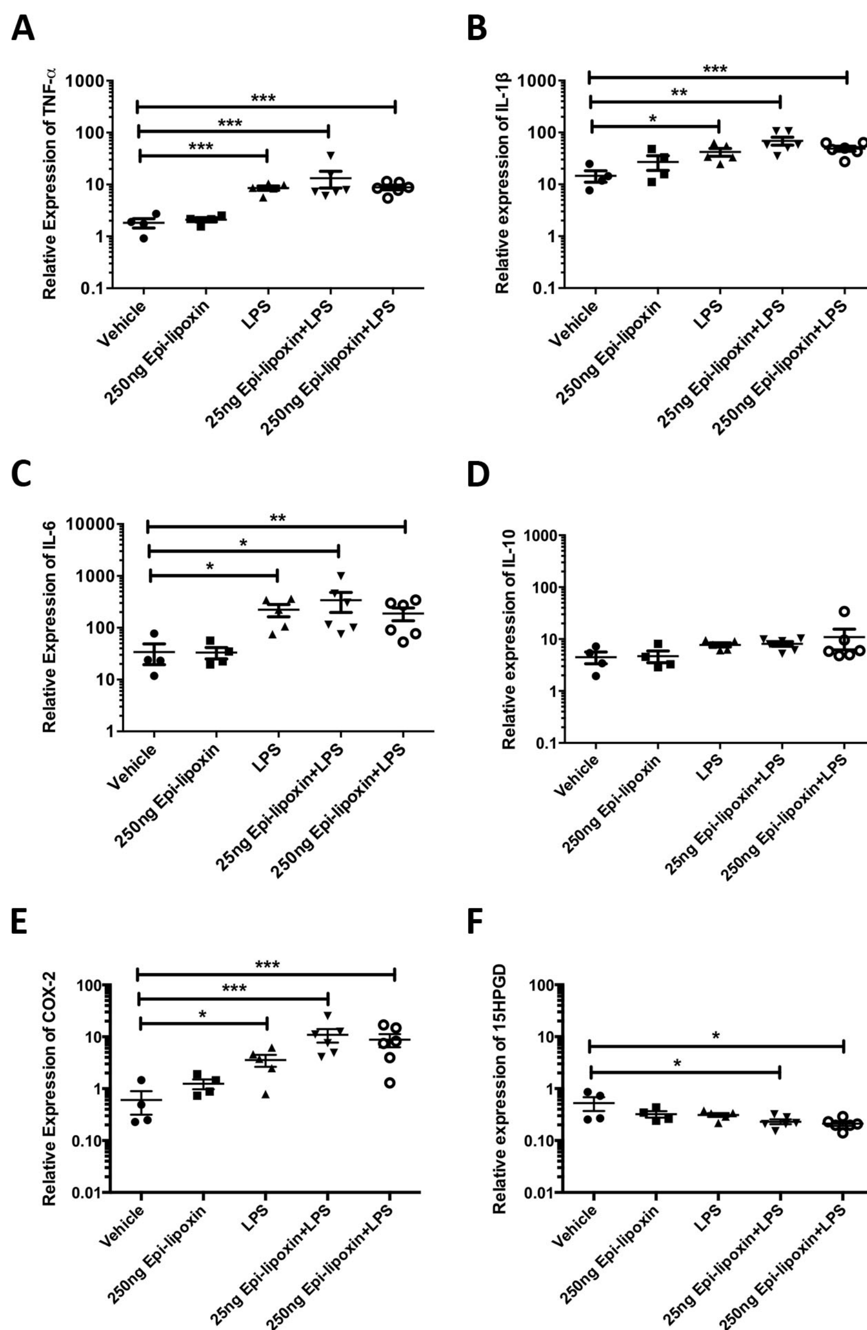

Figure 4.11 - Effect of co-treatment with epi-lipoxin on inflammatory gene expression in the uterus. Uterine tissue was harvested 6 hours post surgery from mice co-treated with vehicle (n=4) or 250ng epi-lipoxin (n=4), at the same time as intrauterine PBS; and in mice co-treated with vehicle (n=5), 25ng epi-lipoxin (n=6) or 250ng epi-lipoxin (n=6), at the same time as intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to vehicle.

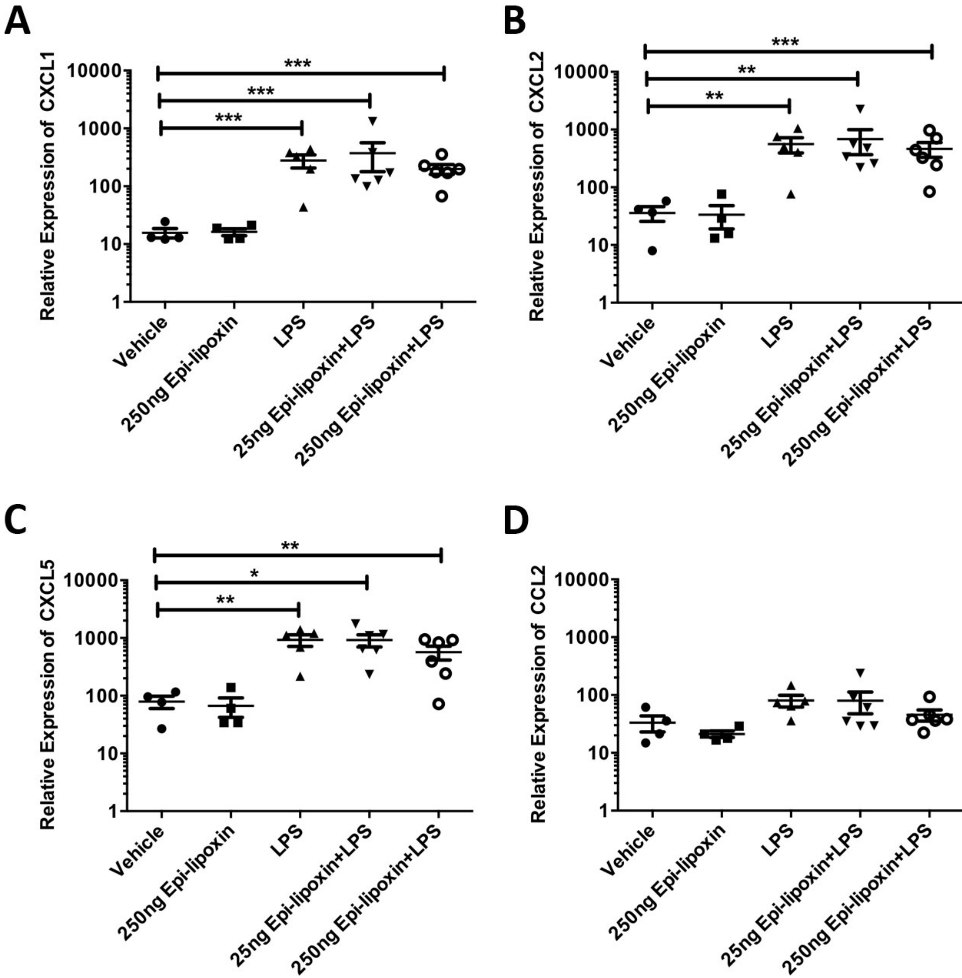

Figure 4.12 - Effect of co-treatment with epi-lipoxin on chemokine expression in the uterus. Uterine tissue was harvested 6 hours post surgery from mice co-treated with vehicle (n=4) or 250ng epi-lipoxin (n=4), at the same time as intrauterine PBS; and in mice pre-treated with vehicle (n=5), 25ng epi-lipoxin (n=6) or 250ng epi-lipoxin (n=6), at the same time as intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, ***p<0.001, compared to vehicle.

4.3.5.2 Inflammatory gene expression in the fetal membranes

Expression of *Tnf-α* in the fetal membranes was significantly increased in mice treated with LPS, compared to the vehicle (28.4-fold increase; $p<0.001$; Figure 4.13A). Intrauterine administration of epi-lipoxin at the same time as LPS did not significantly effect LPS-induced *Tnf-α* expression, compared with LPS alone; and mice treated with 25ng epi-lipoxin+LPS and 250ng epi-lipoxin+LPS still had significantly elevated *Tnf-α,* compared to the vehicle control group ($p<0.001$).

Similarly, expression of *Il-1β* was significantly elevated in mice receiving intrauterine LPS compared to the vehicle group (24.9-fold increase, $p<0.001$; Figure 4.13B). Co-treatment with epi-lipoxin did not affect LPS-induced *Il-1β* expression in the fetal membranes, compared to LPS alone; *Il-1β* expression in these groups was still significantly greater than the vehicle control ($p<0.001$).

In this cohort of mice, *Il-6* expression in the fetal membranes was not significantly altered in response to any treatments given (Figure 4.13C), although intrauterine LPS induced a 3.9-fold increase in *Il-6* expression compared with the vehicle group, these differences were not statistically significant.

As before, intrauterine LPS administration resulted in significantly increased expression of *Il-10* compared to the vehicle group (5.6-fold increase $p<0.05$; Figure 4.13D). Mice co-treated with epi-lipoxin and LPS also had significantly elevated *Il-10* expression compared with the vehicle control group (25ng epi-lipoxin+LPS: 14.1-fold increase, $p<0.001$; 250ng epi-lipoxin+LPS: 9-fold increase, $p<0.01$).

Intrauterine LPS administration alone did not significantly alter expression of *Cox-2* in the fetal membranes, compared to vehicle (Figure 4.13E). Co-treatment of 25ng or 250ng epi-lipoxin at the same time as intrauterine LPS, significantly increased expression of *Cox-2,* compared to the vehicle control group (7-fold and 6.7-fold greater expression, respectively; $p<0.05$). In mice treated with both epi-lipoxin and LPS, *Cox-2* expression appeared to also be increased compared to LPS alone (2.8-fold increase in the 25ng epi-lipoxin+LPS group; 2.6-fold increase in the 250ng epi-lipoxin+LPS group), however these differences were not statistically significant.

In this cohort of mice, expression of *15-Hpgd* in the fetal membranes was not significantly altered by either intrauterine LPS or epi-lipoxin treatment (Figure 4.13E).

As previously described, expression of *Cxcl1* was significantly increased in response to intrauterine LPS administration, compared to the vehicle group (12.6-fold increase, $p<0.05$; Figure 4.14A); and co-treatment with epi-lipoxin+LPS also resulted in increased *Cxcl1* expression, compared to the vehicle control group ($p<0.01$). Intrauterine LPS administration also significantly increased expression of *Cxcl2* compared to vehicle (15-fold increase, $p<0.01$; Figure 4.14B); and again LPS-induced *Cxcl2* expression was not significantly affected by co-treatment with epi-lipoxin, compared to LPS alone, with mice co-treated with epi-lipoxin+LPS still being found to have significantly elevated *Cxcl2* expression compared to the vehicle control group ($p<0.001$). Similarly, *Cxcl5* expression was significantly elevated in response to intrauterine LPS compared to the vehicle group (60.9-fold increase, $p<0.001$; Figure 4.14C). Co-treatment with epi-lipoxin did not significantly alter LPS-induced *Cxcl5* expression in the fetal membranes, compared to LPS alone, with *Cxcl2* expression in these groups still significantly elevated compared to the vehicle control group ($p<0.001$).

Expression of the macrophage chemokine, *Ccl2* was also increased in response to intrauterine LPS administration, compared with the vehicle (10.1-fold increase, $p<0.05$; Figure 4.14D). Again, compared to LPS alone, co-treatment of epi-lipoxin with LPS did not significantly affect LPS-induced *Ccl2* expression, and co-treatment of LPS and epi-lipoxin (at 25ng or 250ng) still resulted in significantly greater *Ccl2* expression, compared to the vehicle control group (25ng epi-lipoxin+LPS: 15.5-fold increase, $p<0.01$; 250ng epi-lipoxin+LPS: 11.6-fold increase, $p<0.05$).

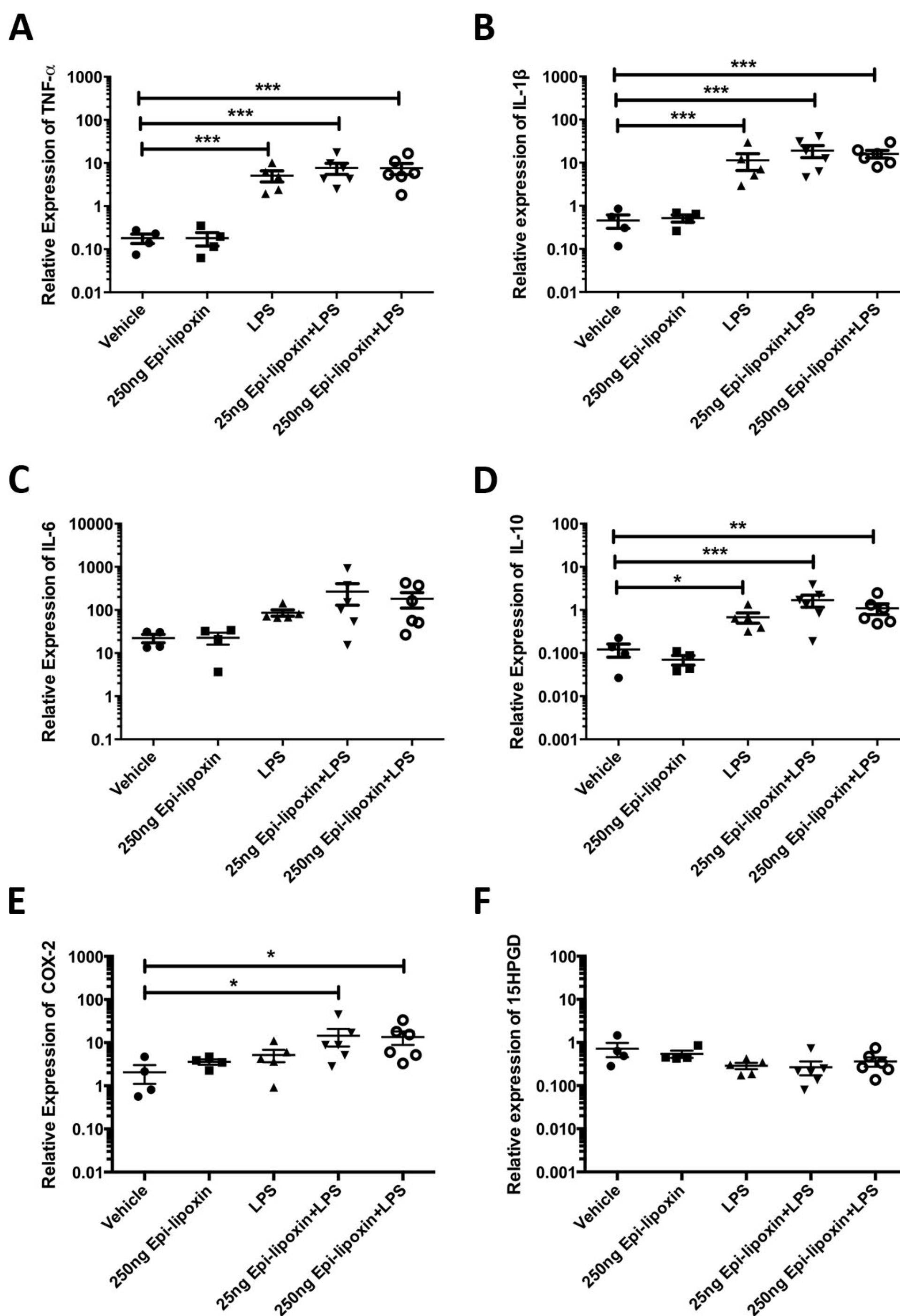

Figure 4.13 - Effect of co-treatment with epi-lipoxin on inflammatory gene expression in the fetal membranes. Fetal membranes were harvested 6 hours post surgery from mice co-treated with vehicle (n=4) or 250ng epi-lipoxin (n=4), at the same time as intrauterine PBS; and in mice co-treated with vehicle (n=5), 25ng epi-lipoxin (n=6) or 250ng epi-lipoxin (n=6), at the same time as intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold-change ± SEM (error bars); **p<0.01; ***p<0.001, compared to vehicle.

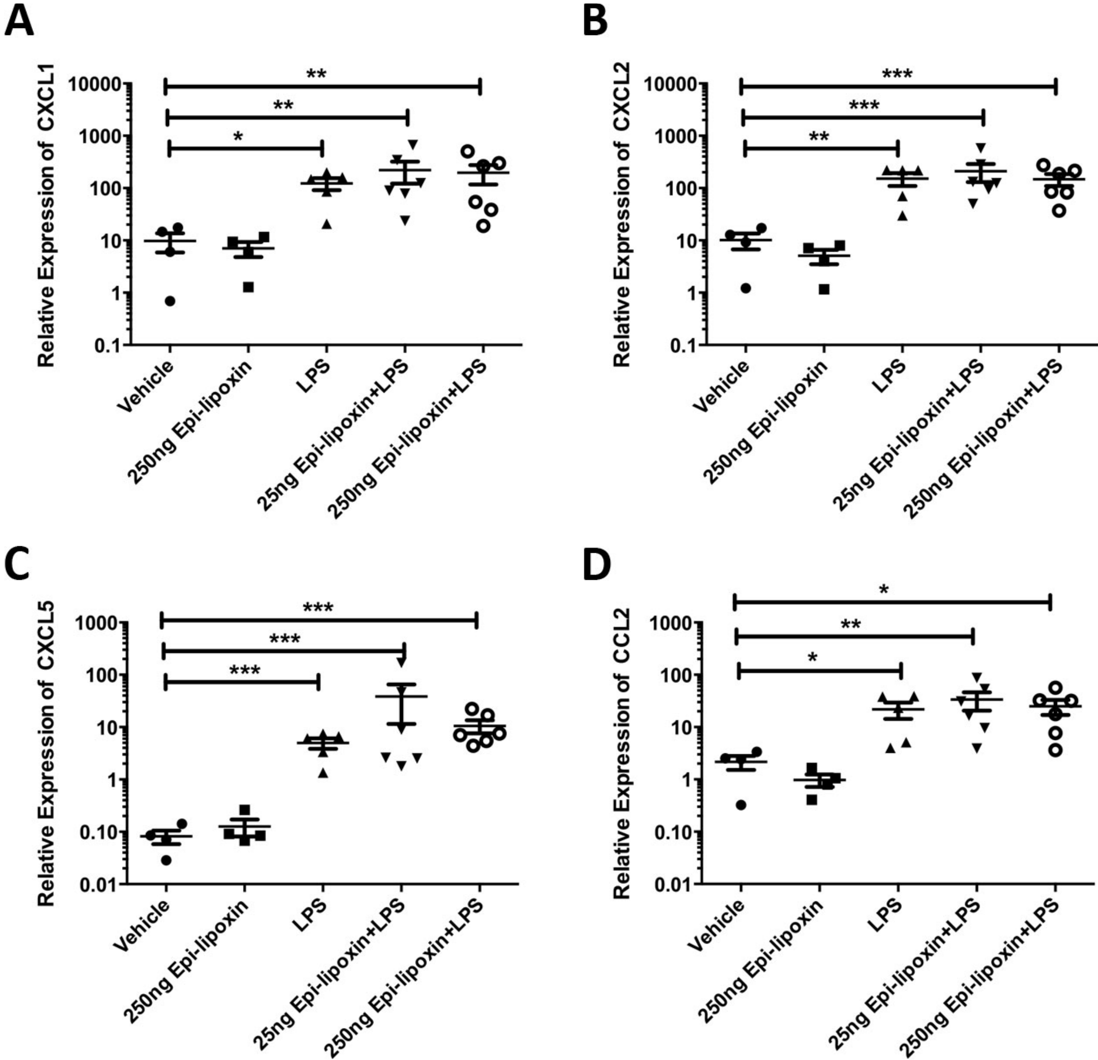

Figure 4.14 - Effect of co-treatment with epi-lipoxin on chemokine expression in the fetal membranes. Fetal membranes were harvested 6 hours post surgery from mice co-treated with vehicle (n=4) or 250ng epi-lipoxin (n=4), at the same time as intrauterine PBS; and in mice co-treated with vehicle (n=5), 25ng epi-lipoxin (n=6) or 250ng epi-lipoxin (n=6), at the same time as intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to vehicle.

4.3.5.3 Inflammatory gene expression in the placenta

As in previous cohorts, intrauterine LPS administration induced a significant increase in placental *Tnf-α* expression, compared with the vehicle control group (6.8-fold increase; p<0.001; Figure 4.15A). Intrauterine administration of epi-lipoxin did not significantly affect LPS-induced *Tnf-α* expression compared to LPS alone, with *Tnf-α* expression still significantly elevated in mice receiving epi-lipoxin+LPS, compared to the vehicle control (p<0.001).

Similarly, *Il-1β* expression was also significantly elevated in response to intrauterine LPS compared to the vehicle group (10.9-fold increase; p<0.001; Figure 4.15B). Again, co-treatment with epi-lipoxin did not alter LPS-induced *Il-1β* expression, compared to LPS alone, and mice in these groups still had significantly greater *Il-1β* expression compared to the vehicle control group (p<0.001).

Placental expression of *Il-6* was significantly increased in response to intrauterine LPS compared to the vehicle control group (5.9-fold increase; p<0.001; Figure 4.15C). Again, mice co-treated with 25ng and 250ng epi-lipoxin+LPS, still had significantly elevated *Il-6* expression, compared to the vehicle group (12-fold and 8.5-fold increase, respectively, p<0.001).

Il-10 expression was also significantly greater in response to intrauterine LPS administration compared with to the vehicle group (2.7-fold increase; p<0.01; Figure 4.15D). Compared to LPS treatment alone, intrauterine administration of epi-lipoxin at the same time as LPS did not affect LPS-induced *Il-10* expression; and mice in these groups still had significantly elevated *Il-10* expression compared to the vehicle control group (p<0.001).

LPS alone did not significantly alter placental *Cox-2* expression, compared to the vehicle control group. However co-treatment of epi-lipoxin, at both 25ng and 250ng, and LPS significantly increased *Cox-2* expression, compared to the vehicle group (25ng epi-lipoxin+LPS: 3-fold increase, p<0.05; 250ng epi-lipoxin+LPS: 3.9-fold increase, p<0.01; Figure 4.15E). Additionally, co-treatment with epi-lipoxin+LPS also significantly increased *Cox-2* expression compared to LPS alone, with 25ng epi-lipoxin+LPS inducing 3.8-fold higher expression (p<0.01) and 250ng epi-lipoxin+LPS inducing 4.9-fold greater expression (p<0.001).

Administration of 250ng epi-lipoxin prior to intrauterine PBS administration reduced placental *15-Hpgd* expression by 2.2-fold compared to the vehicle control group, however this difference did not reach statistical significance (p=0.07; Figure 4.15F). LPS treatment, either alone or with epi-lipoxin, did not significantly affect placental *15-Hpgd* expression.

As described earlier, intrauterine LPS injection significantly elevated expression of the three neutrophil chemokines *Cxcl1*, *Cxcl2* and *Cxcl5*. Expression of *Cxcl1* was significantly greater in mice treated with LPS compared to the vehicle group (4.5-fold increase; p<0.001; Figure 4.16A). Intrauterine administration of epi-lipoxin did not significantly affect LPS-induced *Cxcl1* expression compared to LPS alone, and these mice still had significantly elevated *Cxcl1* expression compared to the vehicle control group (p<0.001). *Cxcl2* was also significantly elevated in mice receiving LPS compared with the vehicle (13.5-fold increase; p<0.001; Figure 4.16B). Again, co-treatment of epi-lipoxin+LPS did not significantly alter *Cxcl2* expression compared to LPS alone, and *Cxcl2* expression was still significantly greater compared to the vehicle control group (p<0.001). Similarly, LPS induced a significant increase in placental *Cxcl5* expression compared to the vehicle group (22.8-fold increase; p<0.001; Figure 4.16C); however LPS-induced *Cxcl5* expression was unaffected by co-treatment with epi-lipoxin, compared to LPS alone, and mice receiving both epi-lipoxin and LPS still had significantly increased *Cxcl5* expression compared to the vehicle control group.

Ccl2 expression was also significantly increased in response to intrauterine LPS administration compared to the vehicle (5.2-fold increase; p<0.001; Figure 4.16D). Again, intrauterine administration of epi-lipoxin at the same time as LPS did not affect LPS-induced *Ccl2* expression in the placenta compared to LPS alone, and these mice still had significantly greater expression of *Ccl2* when compared to the vehicle control group (p<0.001).

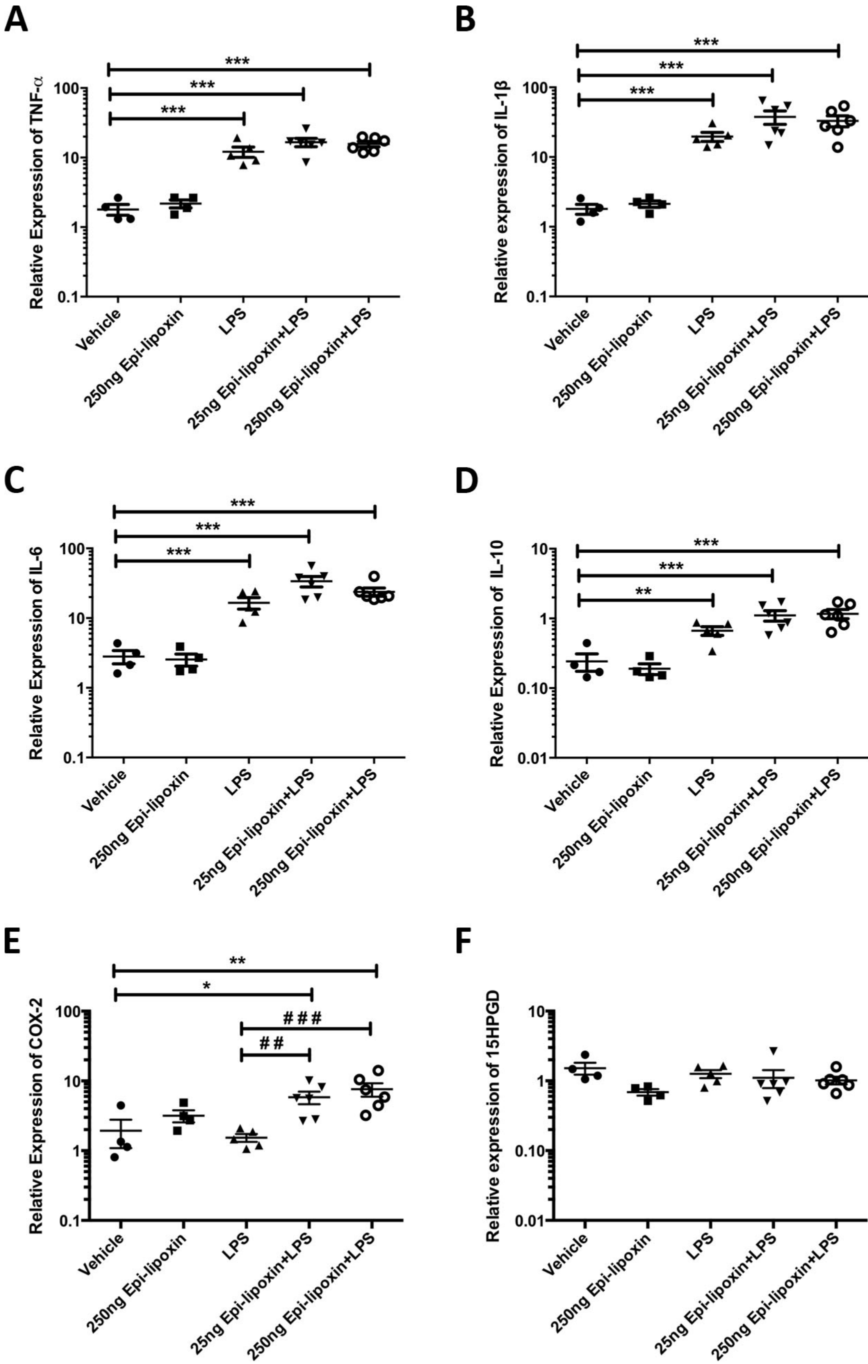

Figure 4.15 - Effect of co-treatment with epi-lipoxin on inflammatory gene expression in the placenta. Placental tissue was harvested 6 hours post surgery from mice co-treated with vehicle (n=4) or 250ng epi-lipoxin (n=4), at the same time as intrauterine PBS; and in mice co-treated with vehicle (n=5), 25ng epi-lipoxin (n=6) or 250ng epi-lipoxin (n=6), at the same time as intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression **(F)** *15-Hpgd* expression. Data presented as mean fold-change ± SEM (error bars); *p<0.05; **p<0.01; ***p<0.001, compared to vehicle; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, compared to LPS.

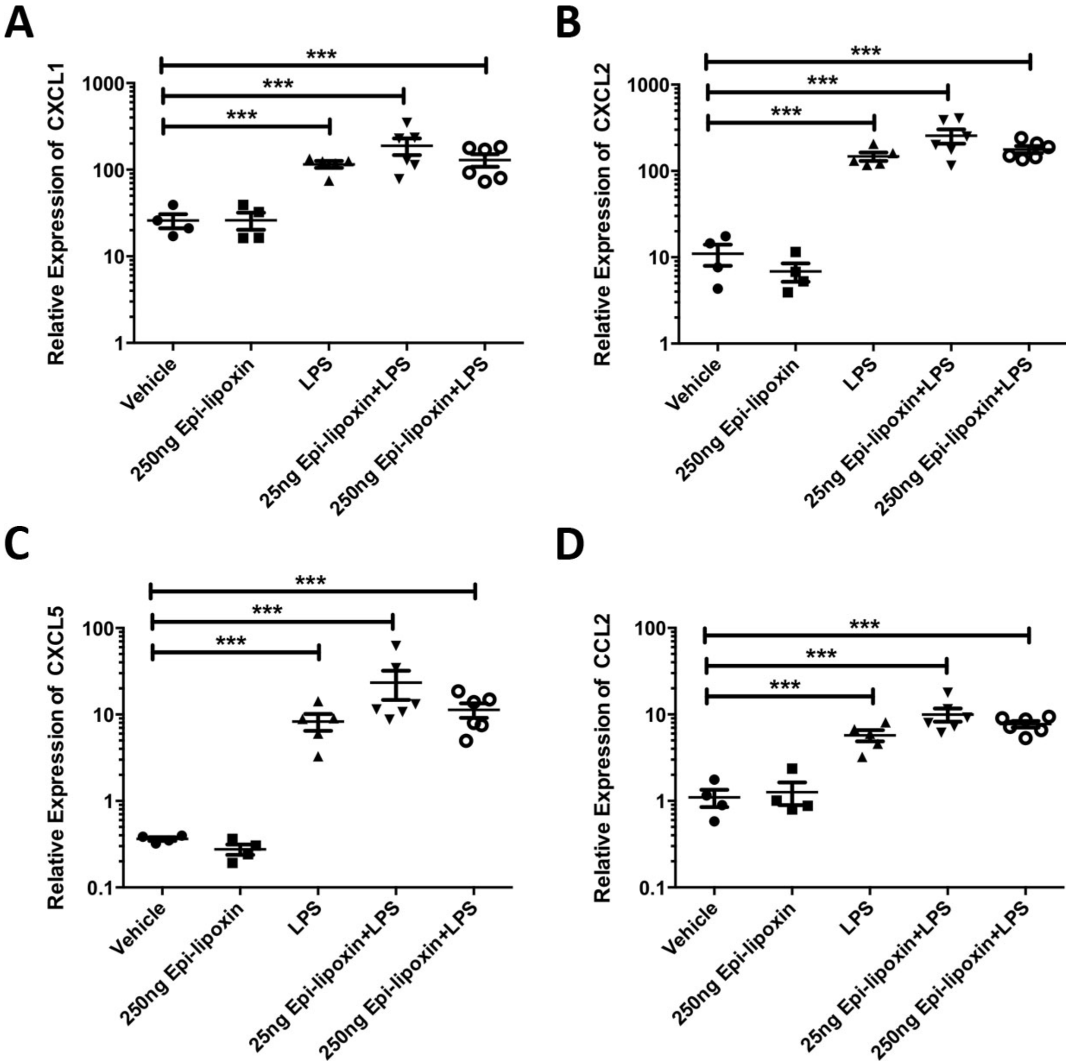

Figure 4.16 - Effect of co-treatment with epi-lipoxin on chemokine expression in the placenta. Placental tissue was harvested 6 hours post surgery from mice co-treated with vehicle (n=4) or 250ng epi-lipoxin (n=4), at the same time as intrauterine PBS; and in mice co-treated with vehicle (n=5), 25ng epi-lipoxin (n=6) or 250ng epi-lipoxin (n=6), at the same time as intrauterine LPS administration, and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, **p<0.01, ***p<0.001, compared to vehicle.

4.3.6 Effect of intrauterine epi-lipoxin administration on amniotic fluid cytokine levels

To determine whether co-treatment with epi-lipoxin and LPS directly into the uterus could affect inflammatory cytokine levels in the amniotic fluid, ELISAs were used to quantify the concentration of TNF-α and IL-10 in amniotic fluid collected 6 hours post surgery. As before, where the concentration of cytokines was too low to be detected by the ELISAs, the value of the lower limit of detection of the specific ELISA was entered to give a value for analysis and the data was analysed by non-parametric ANOVA (Kruskal-Wallis).

TNF-α was undetectable in three vehicle samples and all 250ng epi-lipoxin samples. Intrauterine LPS administration resulted in greater TNF-α concentration compared to the vehicle control group, however this difference was not statistically significant (mean vehicle concentration 12.75 pg/ml ± SEM 1.85; mean LPS concentration 91.73 pg/ml ± SEM 27.22). Co-treatment with epi-lipoxin at the same time as intrauterine LPS appeared to further increase amniotic fluid TNF-α levels, although again these differences were not statistically significant (25ng epi-lipoxin+LPS mean 203.5 pg/ml ± SEM 60.23; 250ng epi-lipoxin+LPS mean 231.5 pg/ml ± SEM 44.01; Figure 4.17A).

IL-10 was also undetectable in three vehicle samples, all 250ng epi-lipoxin samples, one LPS sample and one 25ng epi-lipoxin+LPS sample. Intrauterine LPS administration did not significantly increase the concentration of IL-10 compared to the vehicle control group (vehicle mean 15.93 pg/ml ± SEM 0.33; LPS mean 19.25 pg/ml ± SEM 2.03). Similarly, co-treatment with epi-lipoxin had no significant effect on the amniotic fluid IL-10 concentration compared to LPS alone (25ng epi-lipoxin+LPS mean 24.3 pg/ml ± SEM 3.5; 250ng epi-lipoxin+LPS mean 18.45 pg/ml ± SEM 1.56; Figure 4.17B).

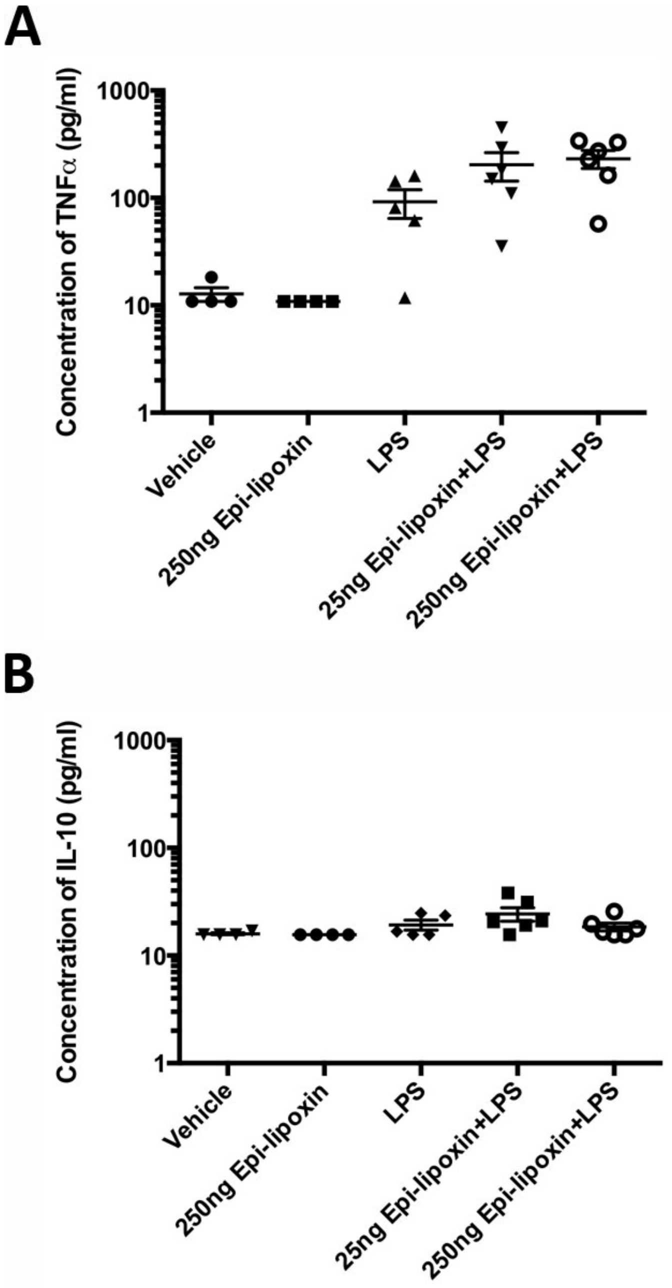

Figure 4.17 - Effect of co-treatment with epi-lipoxin on inflammatory cytokine production in the amniotic fluid. Amniotic fluid was collected 6 hours post surgery from mice co-treated with vehicle (n=4) or 250ng epi-lipoxin (n=4), at the same time as intrauterine PBS; and in mice co-treated with vehicle (n=5), 25ng epi-lipoxin (n=6) or 250ng epi-lipoxin (n=6), at the same time as intrauterine LPS administration, and ELISAs were used to quantify the concentration of TNF-α and IL-10 in the amniotic fluid**.** **(A)** Amniotic fluid TNF-α concentration. **(B)** Amniotic fluid IL-10 concentration. Data presented as mean ± SEM (error bars).

4.3.7 Effect of pre-treatment with BML-111 on time to delivery and the proportion of live born pups

Lipoxins are rapidly inactivated and broken down by the actions of 15-HPGD, therefore, a number of stable synthetic analogues have been developed which have a longer biological half-life (Serhan *et al.* 1995, Maddox *et al.* 1997). BML-111 is a stable synthetic analogue of lipoxin, which has been found to improve outcome in a number of animal models of inflammation-associated pathologies (Zhang *et al.* 2007, Chen *et al.* 2010, Gong *et al.* 2012), therefore, the potential of BML-111 to delay delivery and improve the proportion of live born pups was also investigated in our model. As with epi-lipoxin, mice received pre-treatment with BML-111 1-2 hours prior to intrauterine LPS or PBS administration. Mice were pre-treated with vehicle prior to intrauterine PBS or LPS administration as controls.

4.3.7.1 Time to delivery

Pre-treatment with BML-111 prior to intrauterine PBS administration did not affect time to delivery compared to the vehicle control group (mean time to delivery in BML group 47.5 hours ± SEM 7.3 vs. vehicle mean 48.48 ± SEM 7.6; Figure 4.18). Mice in the LPS treatment group delivered significantly earlier compared with the vehicle control group (mean time to delivery in LPS group 27.6 hours ± SEM 4.8, $p<0.01$). Pre-treatment with 1mg/kg or 10mg/kg BML-111 prior to intrauterine LPS administration did not significantly affect time to delivery compared to LPS alone, although time to delivery appeared to be increased in mice pre-treated with 1mg/kg BML-111 (mean time to delivery 42.0 hours ± SEM 6.5); however this was not significantly different to the LPS group ($p=0.24$; Figure 4.18). Mice pre-treated with 10mg/kg BML-111 prior to intrauterine LPS, still delivered significantly earlier compared to the vehicle control group (mean time to delivery 26.2 hours ± SEM 4.6, $p<0.05$). There were no significant differences when comparing time to delivery in mice pre-treated with BML-111 prior to LPS administration with the BML-111 control group.

To investigate further the effect of pre-treatment with BML-111 on LPS-induced preterm delivery, the preterm delivery rates in each group were calculated (Table 4.2). This was defined as the proportion of dams in which there was delivery of the first pup within 36 hours of intrauterine injection. All treatment groups receiving intrauterine LPS had higher preterm delivery rates compared to those receiving intrauterine PBS injection (LPS preterm delivery rate 0.86; vehicle preterm delivery rate 0.3; BML-111

preterm delivery rate 0.36). Compared to the LPS treatment group, the relative risk of preterm delivery was significantly reduced in both the vehicle and BML-111 groups ($p<0.01$). Additionally pre-treatment with 1mg/kg BML-111 prior to intrauterine LPS administration significantly reduced the risk of preterm delivery (0.55; RR 0.64, [95% CI 0.41 – 0.98]; $p<0.05$), compared to LPS alone. Perhaps surprisingly, higher doses of BML-111 failed to attenuate the adverse effects of LPS on the preterm delivery rate.

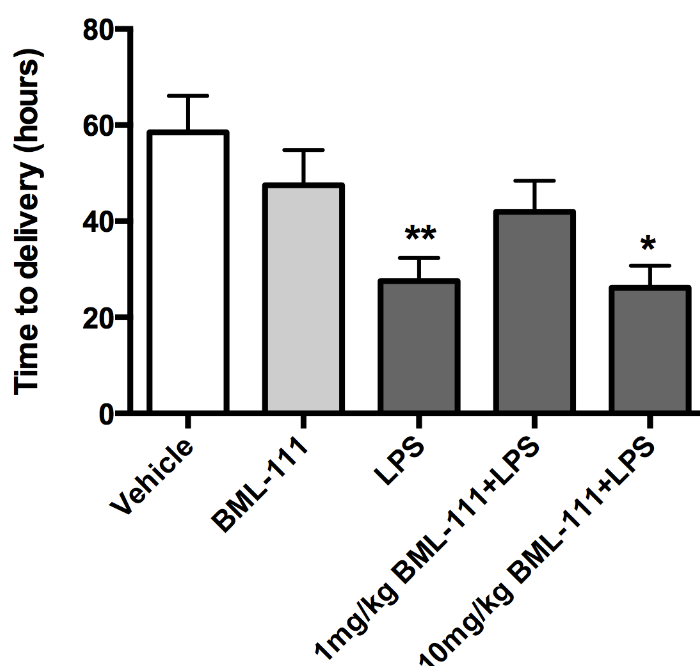

Figure 4.18 - Effect of pre-treatment with BML-111 on LPS-induced time to delivery. Time to delivery was monitored in mice pre-treated with vehicle (n=10) or BML-111 (n=11), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=22) 1mg/kg BML-111 (n=20) or 10mg/kg BML-111 (n=9), prior to intrauterine LPS administration. Data are presented as mean ± SEM (error bars); *p<0.05, **p<0.01, compared to vehicle.

Treatment	Preterm delivery rate	Relative risk of preterm delivery (compared to LPS)
Vehicle (n=10)	0.3	0.34** [95% CI 0.13 – 0.91]
BML-111 (n=11)	0.36	0.42** [95% CI 0.19 – 0.94]
LPS (n=22)	0.86	-
1mg/kg BML+LPS (n=20)	0.55	0.64* [95% CI 0.41 – 0.98]
10mg/kg BML+LPS (n=9)	0.77	0.90 [95% CI 0.61– 1.33]

Table 4.2 - Effect of pre-treatment with BML-111 on preterm delivery rate. Time to delivery was monitored in mice pre-treated with vehicle or BML-111 prior to intrauterine PBS or LPS administration. Preterm delivery was defined as delivery within 36 hours of intrauterine injection. Relative risk ratios calculated using Fisher's exact text. *p<0.05; **p<0.01.

4.3.7.2 Proportion of live born pups

The proportion of live born pups was reduced in the LPS group, compared to the vehicle control group (LPS mean proportion of live born pups 0.21 ± SEM 0.06; vehicle mean proportion of live born pups 0.55 ± SEM 0.11; Figure 4.19A); however this difference was not statistically significant ($p=0.1$). Mice in the BML-111 control group did have a significantly greater proportion of live born pups when compared to LPS alone (mean proportion of live born pups in BML-111 group 0.66 ± SEM 0.12; $p<0.05$). Pre-treatment with BML-111 at 1mg/kg and 10mg/kg prior to intrauterine LPS administration appeared to induce a dose-dependent increase in the proportion of live born pups (1mg/kg BML-111+LPS mean 0.31 ± SEM 0.08; 10mg/kg BML-111+LPS mean 0.48 ± SEM 0.15), however these differences were not statistically significant when compared to the LPS alone group (Figure 4.19A).

In mice delivering preterm a similar trend was observed, where LPS treatment resulted in a significantly reduced proportion of live born pups compared to mice in the BML-111 group (LPS mean proportion of live born pups 0.13 ± SEM 0.05, vs. BML-111 mean 0.64 ± SEM 0.22; $p<0.05$, Figure 4.19B). Mice pre-treated with 1mg/kg BML-111 prior to intrauterine LPS administration also had a significantly reduced proportion of live born pups compared to the BML control group (1mg/kg BML-111+LPS mean proportion of live born pups 0.03 ± SEM 0.03; $p<0.05$). Pre-treatment with 10mg/kg BML-111 prior to intrauterine LPS administration again appeared to increase the proportion of live born pups compared to mice receiving LPS alone (mean proportion of live born pups in 10mg/kg BML-111+LPS group 0.36 ± SEM 0.17), however again this was not statistically significant when compared to mice receiving LPS alone ($p=0.57$; Figure 4.19B).

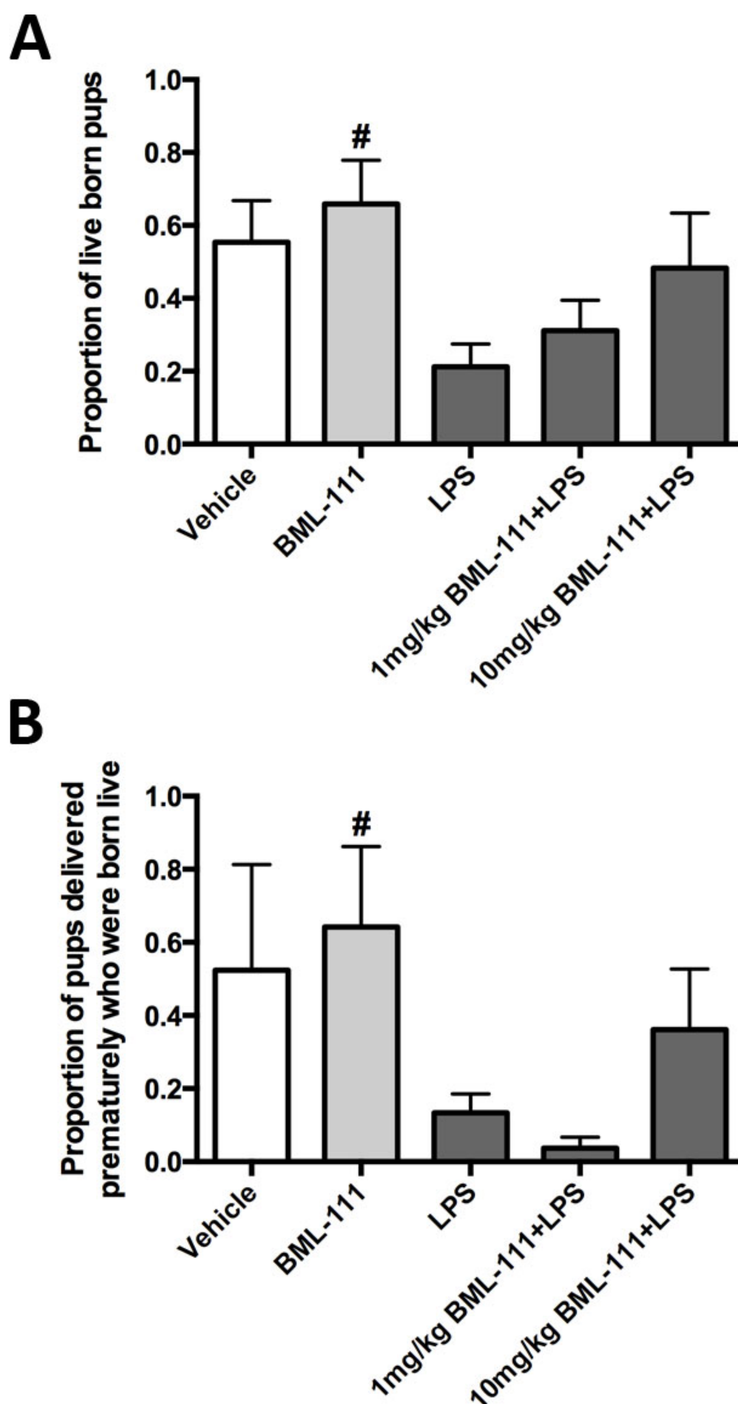

Figure 4.19 - Effect of pre-treatment with BML-111 on the proportion of live born pups. The proportion of live born pups was determined in mice pre-treated with vehicle (n=10) or BML-111 (n=11), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=22) 1mg/kg BML-111 (n=20) or 10mg/kg BML-111 (n=9), prior to intrauterine LPS administration. **(A)** Proportion of live born pups in all mice **(B)** The proportion of pups delivered prematurely (within 36 hours of surgery) who were born live; [vehicle (n=3), BML-111 (n=4) LPS (n=19), 1mg/kg BML-111+LPS (n=11), 10mg/kg BML-111+LPS (n=7)]. Data are presented as mean ± SEM (error bars); #p<0.05, compared to LPS.

4.3.8 Effect of pre-treatment with IL-10 on time to delivery and the proportion of live pups born

Administration of the classical anti-inflammatory cytokine, IL-10, has previously been reported to delay infection-induced preterm labour and improve pup survival in rodent models (Terrone *et al.* 2001, Rodts-Palenik *et al.* 2004, Robertson *et al.* 2006). Therefore, we wanted to investigate whether pre-treatment with IL-10 was capable of reversing the negative effects of LPS in our model. Mice were pre-treated with IL-10 1-2 hours prior to intrauterine LPS or PBS administration; control mice were pre-treated with vehicle prior to intrauterine LPS or PBS administration.

4.3.8.1 Time to delivery

Pre-treatment with IL-10 prior to intrauterine PBS administration did not significantly alter time to delivery, compared to the vehicle control group (mean time to delivery in 10μg IL-10 group 43.73 hours ± SEM 3.2 vs. vehicle mean 42.73 ± SEM 3.6; Figure 4.20). Mice receiving intrauterine LPS delivered significantly earlier than mice receiving intrauterine PBS in the 10μg IL-10 group (mean time to delivery in LPS group 28.46 hours ± SEM 9.7; $p<0.05$); although time to delivery in the LPS group was also decreased compared to the vehicle control group, this was not statistically significant ($p=0.1$). Pre-treatment with either 2.5μg or 10μg IL-10 prior to intrauterine LPS administration did not significantly alter time to delivery, compared with mice receiving LPS alone; and mice receiving 10μg IL-10 prior to intrauterine LPS still delivered significantly earlier compared to the 10μg IL-10 control group (mean time to delivery in 10μg IL-10+LPS group 23.63 hours ± SEM 3.04; $p<0.05$).

The rate of preterm delivery was also determined in each treatment group (Table 4.3). Preterm delivery rates were higher in all groups receiving intrauterine LPS, with all mice in the 2.5μg IL-10+LPS and 10μg IL-10+LPS groups delivering preterm; however in this cohort of mice, no statistically significant differences in preterm delivery rates were observed between treatment groups.

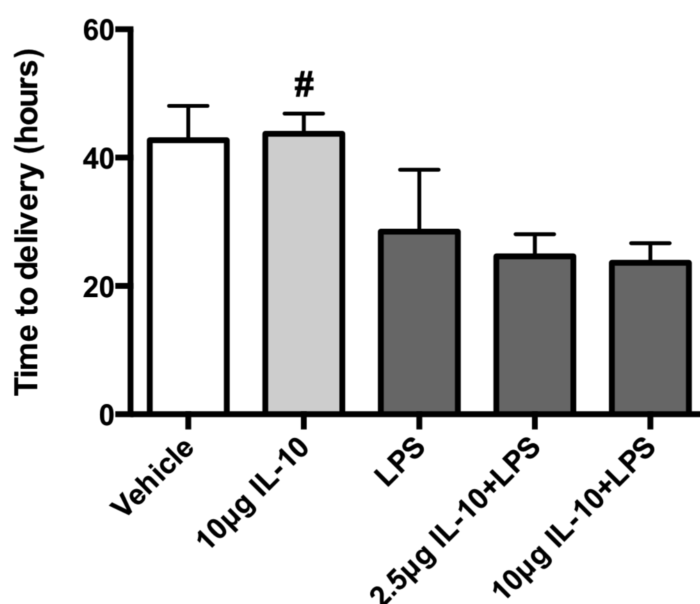

Figure 4.20 - Effect of pre-treatment with IL-10 on LPS-induced time to delivery. Time to delivery was monitored in mice pre-treated with vehicle (n=11) or 10µg IL-10 (n=10), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=6) 2.5µg IL-10 (n=5) or 10µg IL-10 (n=6), prior to intrauterine LPS administration. Data are presented as mean ± SEM (error bars); #p<0.05, compared to LPS.

Treatment	Preterm delivery rate	Relative risk of preterm delivery (compared to LPS)
Vehicle (n=11)	0.45	0.55 [95% CI 0.26 – 1.14]
10µg IL-10 (n=10)	0.3	0.36 [95% CI 0.13 – 0.99]
LPS (n=6)	0.83	-
2.5µg IL-10+LPS (n=5)	1	1.2 [95% CI 0.84 – 1.72]
10µg IL-10+LPS (n=6)	1	1.2 [95% CI 0.84 – 1.72]

Table 4.3 - Effect of pre-treatment with IL-10 on preterm delivery rate. Time to delivery was monitored in mice pre-treated with vehicle or *IL-10* prior to intrauterine PBS or LPS administration. Preterm delivery was defined as delivery within 36 hours of intrauterine injection. Relative risk ratios calculated using Fisher's exact text.

4.3.8.2 Proportion of live born pups

As previously observed in other experiments, LPS treatment resulted in a significantly reduced proportion of live born pups, compared to the 10μg IL-10 group (LPS mean proportion of live born pups 0.26 ± SEM 0.08 vs. 10μg IL-10 mean 0.77 ± SEM 0.09; $p<0.05$, Figure 4.21A). The proportion of live born pups in LPS treated mice was also reduced compared to the vehicle control group, however this was not statistically significant (mean proportion of live born pups in vehicle group 0.64 ± SEM 0.12; $p=0.18$ vs. LPS). Pre-treatment with 10μg IL-10 prior to intrauterine LPS administration appeared to increase the proportion of live born pups compared to LPS alone (mean proportion of live born pups in 10μg IL-10+LPS group 0.59 ± SEM 0.17), however this was not statistically significant (Figure 4.21A).

In mice delivering preterm, a similar trend was observed, with a greater proportion of live pups born to mice pre-treated with 10μg IL-10 prior to both intrauterine PBS or LPS administration, compared to LPS alone (10μg IL-10 mean proportion of live born pups 0.48 ± SEM 0.1; 10μg IL-10+LPS mean proportion of live born pups 0.59 ± SEM 0.17 vs. LPS mean 0.23 ± 0.09; Figure 4.21B); however these differences were again not found to be statistically significant.

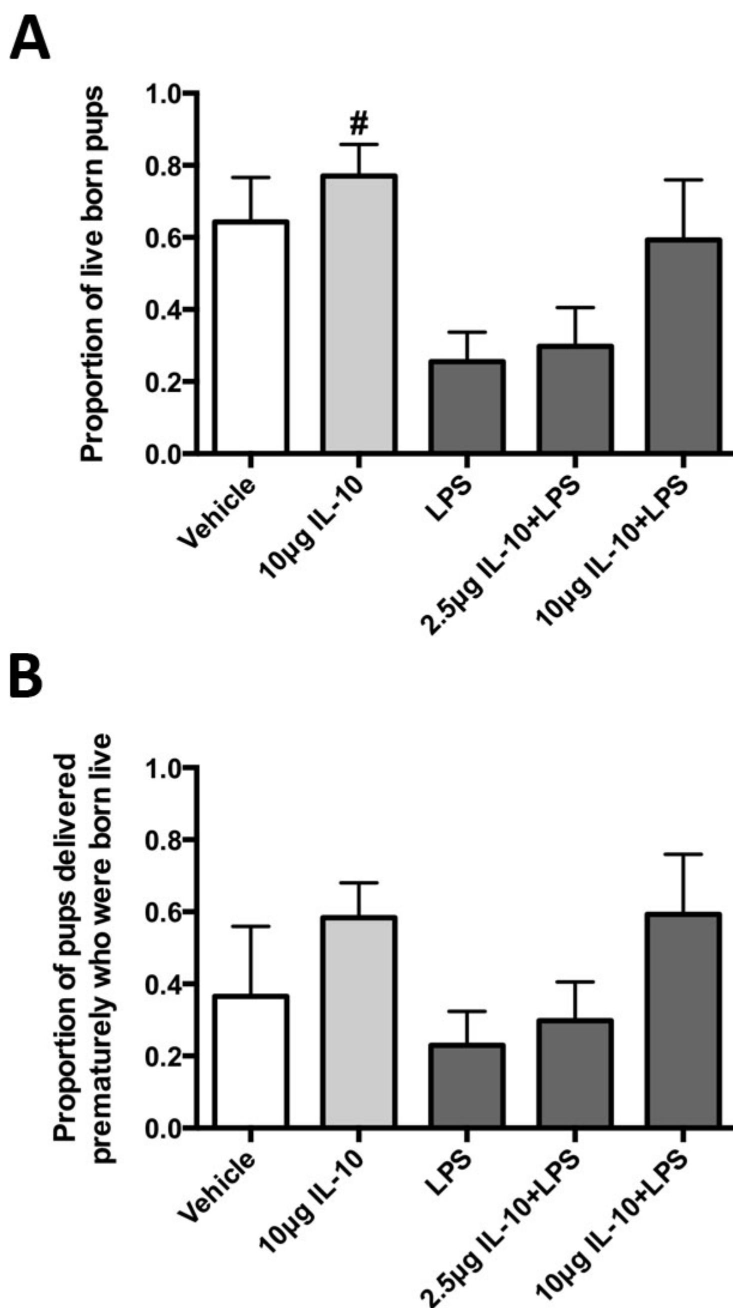

Figure 4.21 - Effect of pre-treatment with IL-10 on the proportion of live born pups. The proportion of live born pups was determined in mice pre-treated with vehicle (n=11) or 10µg IL-10 (n=10), prior to intrauterine PBS; and in mice pre-treated with vehicle (n=6) 2.5µg IL-10 (n=5) or 10µg IL-10 (n=6), prior to intrauterine LPS administration. **(A)** Proportion of live born pups in all mice. **(B)** The proportion of pups delivered prematurely (within 36 hours of surgery) who were born live; [vehicle (n=5), 10µg IL-10 (n=3) LPS (n=5), 2.5µg IL-10+LPS (n=5), 10µg IL-10+LPS (n=6)]. Data are presented as mean ± SEM (error bars); $^{\#}p<0.05$, compared to LPS.

4.4 DISCUSSION

The presence of an intrauterine infection is thought to be an important cause of preterm labour in many cases, which prematurely activates the inflammatory pathways normally initiated at term, resulting in the premature induction of labour (Goldenberg *et al.* 2000). Given this hypothesis, there is growing interest in examining whether anti-inflammatory treatments may be useful novel therapeutic agents to delay infection-induced preterm delivery and improve neonatal outcome. Therefore, the aims of the work in this chapter were to investigate whether the anti-inflammatory agents, epi-lipoxin, BML-111 and IL-10, could delay LPS-induced preterm labour and improve survival rates in our model of infection-induced preterm labour; and to examine whether epi-lipoxin could regulate LPS-induced inflammatory signalling in the utero-placental tissues.

Intrauterine LPS administration increased lipoxin receptor expression in the utero-placental tissues

In humans, lipoxins have been shown to act via a specific G-protein coupled receptor, FPR2/ALX, which belongs to the formyl peptide receptor family (Ye *et al.* 2009). Expression of FPR2/ALX has been described in a variety of cells and tissues including monocytes, macrophages, neutrophils, lymphocytes, microglial cells, fibroblasts, spleen, brain, lung and placenta (Migeotte *et al.* 2006). Whilst only three human formyl peptide receptors have been identified, the situation is more complex in the mouse, where to date, eight genes have been identified belonging to the mouse Fpr family (Gao *et al.* 1998, Ye *et al.* 2009). Two highly related mouse receptors, mFpr2 (encoded by the *Fpr2* gene) and mFpr3 (encoded by the *Fpr-rs2* gene), which have been reported to share high sequence homology with FPR2/ALX, have both been shown to be capable of binding lipoxin A_4 (Takano *et al.* 1997, Vaughn *et al.* 2002). Therefore, it is not clear whether both mFpr2 and mFpr3 are murine orthologs of the human lipoxin receptor, FPR2/ALX. The complexity of differentiating between mFpr2 and mFpr3 was highlighted in a recent study using Fpr2 knockout mice, where Dufton *et al*. originally believed they had generated an Fpr2 specific knockout mouse, only to later identify that both mFpr2 and mFpr3 were deleted (Dufton *et al.* 2010). Interestingly, this knockout study demonstrated that the anti-inflammatory and pro-resolution actions of lipoxin A_4 were severely attenuated providing further support that these receptors are

key lipoxin receptors in the mouse (Dufton *et al.* 2010). Similar to FPR2/ALX, expression of mFpr2 and mFpr3 have been reported in mouse leukocytes, spleen and lung tissue (Gao *et al.* 1998). The expression of both the human FPR2/ALX and mouse mFpr2 have been shown to be up-regulated by inflammatory cytokines and LPS (Gronert *et al.* 1998, Sodin-Semrl *et al.* 2000, Iribarren *et al.* 2003, Chen *et al.* 2007, Zheng *et al.* 2011, Mou *et al.* 2012).

In reproductive tissues, FPR2/ALX expression has been identified during the menstrual cycle in the endometrium and in first trimester decidua (Macdonald *et al.* 2011); and has also been reported in myometrial biopsies obtained from pregnant women at term, where FPR2/ALX expression was increased in myometrial samples obtained from women in labour, compared to non-labouring women (Maldonado-Perez *et al.* 2010); suggesting lipoxins may play an important role in regulating the inflammatory events surrounding the reproductive processes of menstruation and labour.

Therefore, given these data, we wanted to investigate whether lipoxin receptor expression was elevated in response to intrauterine LPS administration, to determine whether exogenous lipoxin administration may be a useful therapeutic option in the treatment of infection-induced preterm labour. In agreement with these previous studies demonstrating LPS can regulate *Fpr2* expression (Iribarren *et al.* 2003, Mou *et al.* 2012), qRT-PCR analysis on tissues harvested 6 hours post-surgery confirmed that intrauterine LPS administration increased the expression of *Fpr2* in the uterus, fetal membranes and placenta, suggesting that either endogenous or exogenous lipoxins may play an important role in regulating the LPS-induced inflammatory response within the utero-placental tissues.

Unfortunately, due to the lack of availability of a suitable mFpr2 antibody, it was not possible to localise mFpr2 expression in the utero-placental tissues. Therefore, it is not clear whether this increased *Fpr2* expression is largely due to the LPS-induced infiltration of immune cells expressing Fpr2, or whether the utero-placental tissues themselves express mFpr2. Furthermore, only the expression of *Fpr2* was examined, as the majority of studies examining the role of lipoxin in animal models of inflammation-associated pathologies focus on *Fpr2*; however as both *Fpr2* and *Fpr3* encode for receptors which can respond to lipoxin in the mouse, further studies to examine *Fpr3* expression should be carried out. There is evidence that lipoxins can also interact with other receptors including the cysteinyl leukotriene receptors (CysLT) (Badr *et al.* 1989,

McMahon *et al.* 2000, Gronert *et al.* 2001) and nuclear aryl carbon receptor (AhR) (Schaldach *et al.* 1999, Machado *et al.* 2006, McBerry *et al.* 2012), which may also mediate their anti-inflammatory effects. Whether these receptors play a role in our model has not been examined.

Effect of epi-lipoxin pre-treatment on LPS-induced PTL and neonatal outcome

In recent years, the therapeutic potential of lipoxins to improve disease outcome has been demonstrated in animal models of a range of inflammation-associated pathologies, such as, asthma (Levy *et al.* 2002), ulcerative colitis (Gewirtz *et al.* 2002), ischemia-reperfusion injury (Ye *et al.* 2010) and arthritis (Conte *et al.* 2010). This is the first study to investigate the potential of lipoxin as therapeutic agents in a model of infection-induced preterm labour. The role of lipoxins in regulating inflammatory events in the reproductive tissues, and in particular during labour, have been largely unexplored, however as discussed earlier, recent work from our laboratory demonstrated that pre-treatment with lipoxin A_4 was capable of down-regulating LPS-induced inflammatory gene expression in myometrial explants *in vitro (Maldonado-Perez et al. 2010)*, showing lipoxins can regulate LPS-induced inflammatory signalling in the myometrium. Therefore, using our mouse model of infection-induced preterm labour, we wanted to investigate whether pre-treatment with epi-lipoxin could delay LPS-induced preterm labour and/or improve neonatal outcome. As lipoxins are relatively unstable and rapidly broken down by the actions of 15-HPGD (Serhan *et al.* 1995), we chose to use the more stable 15-epi-lipoxin A_4 in this study. Previous reports have found that epi-lipoxins are inactivated by 15-HPGD at a slower rate than native lipoxins (Serhan *et al.* 1995), therefore, they display a longer half-life *in vivo*. Furthermore, several studies have shown that epi-lipoxin A_4 is more potent than native lipoxin A_4 in preventing neutrophil adhesion (Serhan 1997) and regulating production of the chemokine, IL-8, in intestinal epithelial cells (Gewirtz *et al.* 1998), thus making 15-epi-lipoxin A_4 more useful in *in vivo* models examining the anti-inflammatory and pro-resolution potential of lipoxin administration.

In our model, pre-treatment with epi-lipoxin, at the doses used in this study, did not delay LPS-induced preterm labour, or have any significant impact on the rate of LPS-induced preterm delivery. However, interestingly pre-treatment with epi-lipoxin at a

dose of 125ng, prior to either intrauterine PBS or LPS administration did increase the proportion of live born pups. Although not a significant increase, the finding that epi-lipoxin pre-treatment increased the proportion of live born pups even in mice just receiving intrauterine PBS, compared to the vehicle control group was unexpected and suggests the surgery itself may be having a negative effect on pup survival, which can be reversed by epi-lipoxin. Unfortunately, it was not possible to compare the proportion of live born pups with that of a no surgery control group, as the uterus of mice in the latter group was not opened up. Hence the number of pups in each dam prior to delivery in the "no surgery control" group was unknown, therefore, it was not possible to calculate the proportion of live born pups in these mice.

Pre-treatment with epi-lipoxin prior to intrauterine LPS administration significantly increased the proportion of live born pups in mice delivering preterm following LPS treatment. The fact that the same effect was not observed when examining the proportion of live born pups in the cohort as a whole could either be due to a selective effect of lipoxins on mortality in preterm pups, or is likely due to the fact that 2 mice in the LPS treatment group went to term and delivered a high proportion of live born pups (time to delivery in these mice was 68.75 and 75.75 hours with 82% and 77% of their pups born live, respectively), therefore, increasing the mean proportion of live born pups in the mice receiving intrauterine LPS in this cohort. Therefore, analysis was carried out on mice delivering preterm to determine whether pre-treatment with epi-lipoxin had an effect on the proportion of live born pups delivered prematurely. These data suggest that administration of epi-lipoxin may be protecting the fetus in some way from the negative effects of LPS, because even when mice pre-treated with epi-lipoxin delivered early, they had a greater proportion of live born pups.

Previous studies have also demonstrated the ability of anti-inflammatory treatments, such as an anti-TLR-4 antibody (Li *et al.* 2010), an anti-TNF antibody (Holmgren *et al.* 2008), IL-10 (Robertson *et al.* 2006) and 15d-PGJ_2 (Pirianov *et al.* 2009), to improve pup survival rates in mouse models of infection-induced preterm labour; however, in each case, the incidence of LPS-induced preterm labour was also reduced, which was not observed with epi-lipoxin treatment. This difference may be related to the fact that unlike other anti-inflammatory treatments used previously, epi-lipoxin is also known to have important pro-resolution actions (Serhan *et al.* 2008), therefore, epi-lipoxin could be promoting the resolution of the inflammatory intrauterine environment to an extent which protects the fetus from the negative effects of being born prematurely

and/or the LPS treatment, but cannot halt the inflammatory cascade which results in preterm labour. How epi-lipoxin is acting to increase the proportion of live born pups in this model is not clear at present, but possible mechanisms will be discussed in the next section examining the effect of epi-lipoxin on the expression of inflammatory mediators in the utero-placental tissues.

In this study, all assessments of live born pups were made within the first 24 hours of delivery. Therefore, to examine further whether epi-lipoxin treatment ultimately improves long-term survival, pups could be examined at later time-points to confirm whether survival is improved, or whether epi-lipoxin merely delays LPS-induced fetal death.

Effect of epi-lipoxin on the LPS-induced inflammatory response in utero-placental tissues

Previous studies have reported that treatment with lipoxin, epi-lipoxin or their stable analogues can reduce the expression of inflammatory mediators, such as TNF-α, IL-6, IL-1β, IL-8 and MMPs, whilst increasing expression of the anti-inflammatory cytokine IL-10, both in models of inflammation *in vitro* and in animal models of inflammation-associated pathologies *in vivo* (Hachicha *et al.* 1999, Sodin-Semrl *et al.* 2000, Wu *et al.* 2008, Baker *et al.* 2009, Kure *et al.* 2009, Maldonado-Perez *et al.* 2010, Borgeson *et al.* 2011, Macdonald *et al.* 2011, Chen *et al.* 2013). The mechanisms underlying these anti-inflammatory actions of lipoxins are thought to involve inhibition of NFκB signalling (Gewirtz *et al.* 2002, Wu *et al.* 2008, Kure *et al.* 2009, Wang *et al.* 2011); activation of PPAR-γ (Sobrado *et al.* 2009) and increased SOCS3 expression via STAT3 activation (Li *et al.* 2011).

Therefore, given the increased proportion of live born pups observed in mice pre-treated with epi-lipoxin, we hypothesised that administration of epi-lipoxin in our model would alter LPS-induced inflammatory signalling within the utero-placental tissues and amniotic fluid, thus providing a mechanism by which epi-lipoxin may be acting.

However, in contrast to these previous studies, we found that treatment with epi-lipoxin, either prior to or at the same time as intrauterine LPS administration, did not significantly reduce the LPS-induced expression of *Tnf-α, Il-1β, Il-6, IL-10, Cxcl1, Cxcl2,*

Cxcl5 or *Ccl2,* with the expression of these genes still found to be significantly elevated in mice treated with epi-lipoxin+LPS, when compared to the vehicle control group. Indeed, compared to vehicle, *Il-6* and *Cxcl1* in the fetal membranes were only up-regulated in response to epi-lipoxin+LPS, and were not significantly increased by LPS treatment alone. Rather than suggesting epi-lipoxin is having a pro-inflammatory effect, these results more likely reflect the variable responses observed between animals, which are more apparent with smaller sample sizes. Whilst the results described in chapter 3 demonstrated a robust inflammatory response to intrauterine LPS, these data in this chapter highlight that when examining small numbers of animals in each group, the response can be variable, such that although LPS increased the expression of the majority of inflammatory genes examined, due to the variability between animals this was not always statistically significant. Furthermore, whilst the expression of these two genes was significantly greater than the vehicle control group, the expression was not found to be significantly different to LPS alone, again suggesting this observed difference is likely due to a variable response to LPS between animals.

The reasons why epi-lipoxin administration in our model did not have similar anti-inflammatory effects to those reported in other models are not clear. Comparisons with other inflammatory models is complicated by the fact that the nature of an inflammatory response, in terms of its timing, magnitude and the specific inflammatory mediators involved, can be altered depending on the site of inflammation (Bannenberg 2010); and this is the first study to investigate the potential of lipoxins to regulate LPS-induced inflammatory signalling in the utero-placental tissues in a mouse model of preterm labour. It is possible that differences in the doses and timing of epi-lipoxin administration may in part explain why we did not observe inhibitory effects on pro-inflammatory signals. However, the doses used here were within the wide range of doses shown to be effective in other animal models administering lipoxin (Levy *et al.* 2002, El Kebir *et al.* 2009, Kure *et al.* 2009, Conte *et al.* 2010, Borgeson *et al.* 2011, Zhou *et al.* 2011).

Lipoxins have been shown to have potent anti-inflammatory actions when administered both locally at the site of inflammation (Clish *et al.* 1999, Menezes-de-Lima *et al.* 2006, Conte *et al.* 2010) or systemically (Clish *et al.* 1999, El Kebir *et al.* 2009, Kure *et al.* 2009, Conte *et al.* 2010, Borgeson *et al.* 2011). In this study the route of administration of epi-lipoxin, either systemically via i.p. injection or directly into the uterus, did not appear to alter its anti-inflammatory actions. However, it is possible

that alternative timings of lipoxin administration may have had a different effect. Pre-treatment with lipoxins prior to the inflammatory insult is a common protocol in animal studies using lipoxins (Levy *et al.* 2002, Kure *et al.* 2009, Borgeson *et al.* 2011), but several studies also give repeated doses of lipoxin after the inflammatory insult as well (Conte *et al.* 2010, Levy *et al.* 2011). This may be particularly important to consider given that although more stable than the native lipoxin, 15-epi-lipoxin is still rapidly metabolised *in vivo* (Serhan *et al.* 1995), and many of the studies mentioned above actually use synthetic lipoxin or epi-lipoxin analogues, which are more resistant to metabolism. Additionally, given that increased *Fpr2* expression in the utero-placental tissues is observed in mice 6 hours after LPS treatment, giving additional exogenous lipoxins at this later time point may have a greater impact on inflammatory signalling.

Having observed that epi-lipoxin treatment did not appear to alter inflammatory gene expression in the utero-placental tissues, we hypothesised that another potential mechanism which may explain the increased proportion of live born pups was that epi-lipoxin administration could be protecting the fetuses by either reducing the levels of inflammatory cytokines, such as TNF-α, or increasing the level of the anti-inflammatory cytokine, IL-10, in the amniotic fluid. Pre-treatment with epi-lipoxin prior to intrauterine LPS administration did not reduce LPS-induced amniotic fluid TNF-α levels; however, although in chapter 3, TNF-α levels were significantly elevated in the amniotic fluid of mice receiving 20μg LPS, compared to mice receiving intrauterine PBS, the same result was not observed in the cohort of mice used for the epi-lipoxin studies, although TNF-α levels were still greater in mice receiving intrauterine LPS, the difference was not statistically significant when compared with mice receiving intrauterine PBS. In terms of amniotic fluid IL-10 levels, again there was no significant difference observed between any treatment groups, and no trend that treatment with epi-lipoxin was altering LPS-induced IL-10 production.

Interestingly, pre-treatment with epi-lipoxin, prior to either intrauterine PBS or LPS administration, induced increased expression of *Cox-2* in the uterus, fetal membranes and placenta; and decreased expression of *15-Hpgd* in the uterus and placenta. As discussed in chapter 3, a change in the expression of these genes suggests increased prostaglandin synthesis and activity within the intrauterine tissues, which is commonly thought to be a pro-inflammatory event. However, there is now a large body of

evidence that suggests increased expression of COX-2, and subsequent prostaglandin production, as an important initial step in the resolution of inflammation. Studies in animal models have demonstrated anti-inflammatory roles for COX-2 and prostaglandins in the resolution of inflammation in the lung (Gilroy *et al.* 1999, Hodges *et al.* 2004, Fukunaga *et al.* 2005, Bonnans *et al.* 2006, Zheng *et al.* 2011) and colon (Reuter *et al.* 1996), where increased COX-2 expression and subsequently increased PGE_2, PGD_2 and 15d-PGJ_2 production was associated with the resolution phase, and inhibition of COX-2 led to exacerbated inflammation. Therefore, COX-2 is proposed to play opposing roles in the different phases of an inflammatory response. During the early phase of acute inflammation, elevated COX-2 expression is thought to be pro-inflammatory, whilst during the latter phase of the inflammatory response COX-2 has important anti-inflammatory and pro-resolution actions (Gilroy *et al.* 1999). The concept that prostaglandins can have dual roles in an inflammatory response is not novel, previous studies have demonstrated that depending on their dose, site of action and route of administration, prostaglandins can have either pro- or anti-inflammatory effects (Rampart and Williams 1986, Pons *et al.* 1994).

Lipoxins are proposed to be involved in mediating the anti-inflammatory actions of COX-2 and prostaglandins during the resolution phase. Studies in human neutrophils demonstrated that PGE_2 and PGD_2 are involved in a process termed 'lipid mediator class switching', whereby they can stimulate lipoxin production by activating transcription of 15-lipoxygenase resulting in neutrophils switching from the production of pro-inflammatory leukotrienes, to synthesis of lipoxins (Levy *et al.* 2001). Furthermore, in models of acute lung injury, increased COX-2 expression was associated with increased LXA_4 production, which was blocked when animals were treated with COX-2 inhibitors, resulting in exacerbated inflammation and longer recovery times (Fukunaga *et al.* 2005, Scully *et al.* 2012). PGE_2 has been shown to directly increase expression of the lipoxin receptor in bronchial epithelial cells *in vitro*, thus promoting lipoxin signalling, and this was again shown to be COX-2 dependent (Bonnans *et al.* 2006). Interestingly a recent study by Zheng *et al*, described a biphasic role of lipoxin A_4 in regulating LPS-induced COX-2 expression in rat pulmonary fibroblasts (Zheng *et al.* 2011). Their work demonstrated that LPS treatment induced increased COX-2 expression at 6 hours, with a second increase observed after 24 hours, where maximal COX-2 levels were reached. Treatment with lipoxin A_4 inhibited the initial LPS-induced increase in COX-2 production at 6 hours, but dose-dependently

increased COX-2 expression and PGD_2 after 24 hours (Zheng *et al.* 2011); again highlighting the diverse role COX-2 plays in the progression of an inflammatory response and how it can be regulated by lipoxin. Similar to the mechanisms proposed to mediate the anti-inflammatory actions of lipoxins, both PGD_2 and its metabolite, 15d-PGJ_2 have been found to have anti-inflammatory actions via inhibition of NFκB signalling and increased activation of PPAR-γ in immune cells (Ricote *et al.* 1998, Rossi *et al.* 2000, Ward *et al.* 2002); and in reproductive tissues both *in vitro* (Ackerman *et al.* 2005, Lindstrom and Bennett 2005, Lappas *et al.* 2006) and *in vivo* (Pirianov *et al.* 2009).

Therefore, it is possible that the epi-lipoxin-induced increase in both basal, and LPS-induced *Cox-2* expression observed in our model is a sign of the initiation of the resolution of the inflammatory response. The decreased *15-Hpgd* expression may be a mechanism to prolong lipoxin and prostaglandin action, since 15-HPGD is the main enzyme involved in the inactivation and breakdown of both lipoxins and prostaglandins. As observed in the studies discussed above, this may result in increased production of anti-inflammatory prostaglandins, such as PGD_2 and 15d-PGJ_2, which may act to resolve the inflammatory environment surrounding the fetus, thus leading to the observed increase in the proportion of live born pups. Support for this hypothesis comes from a recent study that demonstrated that administration of 15d-PGJ_2 was associated with increased pup survival in a mouse model of LPS-induced preterm labour (Pirianov *et al.* 2009). However, to confirm whether this mechanism is involved in our model, further work would need to be carried out to investigate prostaglandin production within the utero-placental tissues and amniotic fluid. Given that evidence suggests biphasic roles of COX-2 and prostaglandins during an inflammatory response, analysis at multiple time points, particularly later than 6 hours, will be important in future work to fully understand the role of COX-2 and the prostaglandins in the intrauterine inflammatory response to LPS and how epi-lipoxin administration may affect their expression.

Interestingly (as discussed in chapter 3), at 6 hours, LPS treatment is associated with a neutrophil influx into the uterus, with no significant effect on macrophage numbers. Again this finding supports the idea that perhaps by looking at 6 hours, we are looking early in the inflammatory response, because typically in the initial acute stage, neutrophils are the predominant immune cell present, whereas during the resolution

phase, monocytes and macrophages are the predominant cell type (Serhan *et al.* 2007). These data again suggest that looking at later time points may be more useful in understanding lipoxin actions. To further investigate whether COX-2 is involved in mediating the effect of epi-lipoxin in improving the proportion of live born pups, a selective COX-2 inhibitor, such as meloxicam (Cella *et al.* 2010) or nimesulide (Westover *et al.* 2012), could be given at different time points following epi-lipoxin and LPS administration to determine if inhibiting the actions of COX-2 reversed the beneficial effects of epi-lipoxin pre-treatment. However, the outcome of such an experiment may be difficult to interpret, given that COX inhibitors have been independently associated with adverse effects on the fetus. COX-2 deficient mice have a high rate of neonatal death, which has been linked to a patent ductus arteriosis (Loftin *et al.* 2001). Furthermore, evidence from a sheep model of intra-amniotic infection suggests that prostaglandins may play an important role in promoting fetal lung development, as inhibition of COX-2 was associated with decreased surfactant protein expression in the fetal lung (Westover *et al.* 2012). Thus it would be difficult to separate out an effect of COX-2 inhibitors on epi-lipoxin action, from a direct effect of COX-2 inhibitors themselves.

Aside from only looking at one time point, another limitation of our study here is that we focussed on examining the anti-inflammatory effects of epi-lipoxin and did not investigate its pro-resolution effects. In terms of their pro-resolution actions, lipoxins have been reported to have contrasting effects on different immune cell populations, including: inhibition of neutrophil activation, adhesion and chemotaxis (Lee *et al.* 1989, Colgan *et al.* 1993, Papayianni *et al.* 1996, Filep *et al.* 2005); stimulation of neutrophil apoptosis (El Kebir *et al.* 2007); stimulation of monocyte adhesion and migration (Maddox and Serhan 1996, Maddox *et al.* 1997); inhibition of monocyte apoptosis (Simoes *et al.* 2010); and promoting the non-phlogistic phagocytosis of apoptotic neutrophils by macrophages (Godson *et al.* 2000). Therefore, to further investigate the mechanisms by which epi-lipoxin may be acting, it would be important to examine whether epi-lipoxin administration in our model is associated with any of these inhibitory actions on neutrophils, or stimulation of monocytes and macrophages. Identification of the predominant immune cell population in mice treated with epi-lipoxin would help us to determine whether epi-lipoxin administration was indeed promoting resolution.

Additionally, to investigate whether the actions of epi-lipoxin in the utero-placental tissues are mediated by the lipoxin receptor, Fpr2, the experiments could be repeated using Fpr2 knockout mice (Dufton *et al.* 2010) or mice could be pre-treated with the specific lipoxin receptor antagonist butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc-2) (Menezes-de-Lima *et al.* 2006, Wang *et al.* 2011), to examine whether inhibiting Fpr2 signalling alters the effects of epi-lipoxin treatment in our model.

It should also be noted, that the doses of epi-lipoxin administered in the 6 hour analysis studies, were different to the original doses of lipoxin administered in the initial time to delivery experiments. The rationale behind increasing our doses of lipoxin was to maximise the potential anti-inflammatory actions of lipoxin. Similarly, when lipoxin was administered directly into the uterus, the doses were reduced as lower doses of lipoxin have been administered locally in other animal models, compared to the doses used for systemic administration (Clish *et al.* 1999, Conte *et al.* 2010). As stated earlier, all doses of epi-lipoxin administered in the course of this study were well within the range of doses which have been proved to be effective in other models (Levy *et al.* 2002, El Kebir *et al.* 2009, Kure *et al.* 2009, Conte *et al.* 2010, Borgeson *et al.* 2011, Zhou *et al.* 2011). Therefore, although the doses of lipoxin are not directly comparable between the different experiments, it is unlikely that this had a major impact on the results; however, it may be easier to elucidate the effects of lipoxin action if the same doses are used throughout future experiments.

Effect of BML-111 pre-treatment on LPS-induced PTL and neonatal outcome

As previously discussed, lipoxins are rapidly metabolised and have a short biological half-life *in vivo*, therefore, research has focused on developing stable synthetic lipoxin and epi-lipoxin analogues which are more resistant to metabolism (Serhan *et al.* 1995, Maddox *et al.* 1997). BML-111 (5(S),6(R),7-trihydroxymethyl heptanoate) is a commercially available truncated analogue of lipoxin A_4 which is an FPR2 agonist and has been shown to be as potent as lipoxin A_4 in inhibiting leukotriene-induced neutrophil migration *in vitro* (Lee *et al.* 1991) and has been found to have potent anti-inflammatory actions when administered *in vivo* in a number of animal models of inflammation-associated pathologies such as acute liver injury (Zhang *et al.* 2007), arthritis (Zhang *et al.* 2008, Conte *et al.* 2010), hepatocellular carcinoma (Chen *et al.*

2010, Hao *et al.* 2011), acute lung injury (Gong *et al.* 2012) and in a model of low-dose endotoxin induced pre-eclampsia (Lin *et al.* 2012). Therefore, we wanted to investigate whether administration of this stable lipoxin analogue in our model would delay LPS-induced preterm labour and improve the proportion of live born pups.

We found that pre-treatment with BML-111 prior to intrauterine PBS administration had no effect on the time to delivery compared to the vehicle control group. Pre-treatment with BML-111 prior to intrauterine LPS administration also did not significantly affect LPS-induced time to delivery, although the mean time to delivery in mice receiving 1mg/kg BML-111 prior to LPS was 14 hours later, compared to those mice receiving LPS alone, however this was not statistically significant. Interestingly, when examining the preterm delivery rates in the different treatment groups, we found that pre-treatment with 1mg/kg BML-111 significantly reduced the rate of preterm delivery from 86% to 55% (RR 0.64, 95% CI 0.41-0.98); thus although BMl-111 pre-treatment was not associated with an overall reduction in the time to delivery, fewer mice in this group delivered preterm.

Similar to the effect of epi-lipoxin pre-treatment, we observed that mice pre-treated with the higher dose of BML-111, 10mg/kg, had an increased proportion of live born pups, compared with LPS treatment alone, but this was not found to be a significant increase. However, the reduction in the proportion of live born pups associated with LPS treatment, was no longer significantly different compared to the BML-111 control group in mice pre-treated with BML-111 prior to intrauterine LPS, suggesting that BML-111 may be reducing the effects of LPS on pup death, however it is difficult to draw a firm conclusion from the current data. Similarly when examining the proportion of live born pups in mice delivery preterm, we found that pre-treatment with 10mg/kg BML-111 prior to intrauterine LPS administration appeared to increase the proportion of live born pups to a level which was no longer significantly different to the BML-111 control group. However, again this was not significantly greater than the proportion of live born pups in the LPS alone group, so whilst this may support the hypothesis that BML-111 is attenuating the LPS-induced effects on pup mortality, the data is currently not conclusive.

Given that BML-111 has been found to have similar actions to lipoxins *in vivo* and *in vitro*, as discussed above, it is possible that any beneficial effect of BML-111 on the proportion of live born pups may be occurring via the same mechanisms hypothesised

above for epi-lipoxin. These results require confirmation in other studies, but they do suggest that, similar to epi-lipoxin, pre-treatment with BML-111 may be protecting the pups from the negative effects of being born prematurely; and additionally appears to reduce the rate of LPS-induced preterm delivery. To better understand how BML-111 may be acting, tissue collection and analysis at a fixed time-point would need to be performed. Support for the therapeutic potential of BML-111 in treating infection-induced preterm labour, comes from a rat model of low-dose LPS-induced pre-eclampsia (Lin *et al.* 2012), which demonstrated that BML-111 administration can attenuate another inflammation-associated pregnancy pathology, that of pre-eclampsia. Interestingly Lin *et al.* also used repeated BML-111 administration, therefore, again highlighting repeat administration of lipoxins as a possible alteration of the study design used here.

Effect of IL-10 pre-treatment on LPS-induced PTL and neonatal outcome

IL-10 is a classical anti-inflammatory cytokine that has been previously shown to play an important role in protecting mice from LPS-induced preterm labour (Robertson *et al.* 2006). Robertson *et al* demonstrated that IL-10 knockout mice required a ten-fold lower dose of LPS to induce 50% preterm fetal loss compared to wild-type animals; and LPS treatment induced significantly greater production of inflammatory cytokines in the serum, uterus, placenta and fetus in IL-10 knockout animals, compared to wild-type animals receiving LPS. Exogenous administration of recombinant IL-10 to both knockout and wild-type animals reduced the negative effects of LPS on fetal loss and reduced LPS-induced inflammatory signalling. Also previous work in a rat models of infection-induced preterm birth demonstrated that administration of exogenous IL-10 significantly delayed LPS-induced preterm delivery and improved pup survival rates (Terrone *et al.* 2001); and inhibited *E.Coli*-induced white matter damage in pup brains (Rodts-Palenik *et al.* 2004, Pang *et al.* 2005). Furthermore, evidence from a non-human primate model showed that IL-10 treatment attenuated IL-1β-induced uterine contractions and reduced leukocyte influx and inflammatory mediator production in the amniotic fluid (Sadowsky *et al.* 2003). Collectively, these studies suggest that IL-10 treatment could be a useful therapeutic option in the treatment of infection-induced preterm labour. Therefore, we wanted to investigate whether IL-10 administration in our model had similar effects in reducing time to delivery and improving pup survival.

In contrast to these previous studies, we did not observe any beneficial effect of pre-treatment with IL-10 on LPS-induced preterm delivery or the proportion of live born pups. Similar to the results described above, administration of the higher dose of IL-10, 10μg, prior to intrauterine LPS treatment, did increase the proportion of live born pups to a level more similar to the 10μg IL-10 control group, compared to LPS treatment alone; however, the difference was not statistically significant. The reasons why IL-10 has not worked in our model, in comparison with previously published data, are unclear. However, we had problems with our vehicle control mice in the cohort of mice used in these experiments, where a higher than normal number of mice in this group delivered prematurely (45%), and we also observed increased variability in terms of the effectiveness of LPS to induce preterm labour as well, thus resulting in no treatment significantly affecting the preterm delivery rate in this cohort. The doses of recombinant IL-10 used were based on the dose used in the work by Robertson *et al*, where they demonstrated an effect of 2.5μg in the effects of LPS-induced preterm labour. However, our model differs from theirs, in that they administer LPS systemically, via i.p. injection; and also use a different type of LPS, which could explain some of the differences we have observed. Another possibility is that perhaps higher doses of IL-10 are required to attenuate LPS-induced preterm delivery in our model. Although there appears to be no effect on time to delivery, there is a trend towards an increased proportion of live born pups in mice pre-treated with 10μg IL-10 prior to intrauterine LPS, so it would be interesting to test whether increasing the does of IL-10 may further increase the proportion of live born pups. The 2.5μg dose was chosen based on the work by Robertson *et al* (Robertson *et al.* 2006), and the 10μg dose was chosen based a model demonstrating it as a suitable dose to protect mice from endotoxin-induced death (Howard *et al.* 1993), where they used the same strain of LPS as we have used in our study.

Although no significant effects of IL-10 treatment were observed in our study, IL-10 warrants further investigation as a potential therapeutic agent in the treatment of preterm labour as it appears capable of reducing the negative affects of LPS on pup mortality. The experiments would need to be repeated in another cohort of mice, perhaps using a new batch of LPS and examining a higher dose of IL-10 pre-treatment.

Summary

This chapter has investigated and discussed the potential of three anti-inflammatory agents to delay LPS-induced preterm delivery and improve pup survival in a mouse model of preterm labour. This is the first study to investigate the potential of the dual acting anti-inflammatory and pro-resolution mediators, lipoxins, and their stable analogue, BML-111 in a mouse model of infection-induced preterm labour. Interestingly although pre-treatment with epi-lipoxin prior to intrauterine LPS administration did not delay LPS-induced preterm labour, the mice that delivered prematurely had a significantly increased proportion of live born pups, compared with mice receiving LPS alone; suggesting that epi-lipoxin is in some way protecting the fetuses from the negative effects of preterm birth and/or LPS treatment. The mechanisms by which epi-lipoxin may be acting to improve pup survival in our model are not currently clear, as we only investigated one (early) time point after lipoxin administration, but since epi-lipoxin increases expression of *Cox-2,* which is known to be important in initiation the resolution phase of an inflammatory response, we propose that it may involve increased production of anti-inflammatory prostaglandins, such as PGD_2 and 15d-PGJ_2, which may attenuate LPS-induced inflammatory signalling. Similarly, the data presented here suggest that BML-111 may be another potentially useful anti-inflammatory therapeutic option, given that we observed a reduction in the preterm delivery rate of mice pre-treated with BML-111 compared to mice receiving LPS alone, and again we saw a trend for a greater proportion of live pups being born to mice pre-treated with BML-111 prior to intrauterine LPS, compared to mice receiving LPS alone. Finally, in our model, pre-treatment with IL-10 was not found to significantly attenuate LPS-induced preterm delivery or significantly improve the proportion of live born pups, however there was a similar trend as observed with both epi-lipoxin and BML-111, whereby a greater proportion of live pups were born to mice pre-treated with IL-10 prior to intrauterine LPS. Therefore, taken together, these data suggest that anti-inflammatory agents, in particular lipoxins, may be useful novel therapeutic options in the treatment of preterm labour, which may protect the fetus from the adverse effects of infection-induced preterm birth, thus improving neonatal outcome.

Chapter 5 - Investigating the role of immune cells in a mouse model of infection-induced preterm labour

5.1 INTRODUCTION

Parturition, both at term and preterm, is associated with an immune cell influx, particularly of neutrophils and macrophages, into the intrauterine tissues (Thomson *et al.* 1999, Osman *et al.* 2003, Gomez-Lopez *et al.* 2010). In women, macrophages have been reported to influx into the myometrium, fetal membranes, decidua, placenta and cervix during spontaneous term labour (Thomson *et al.* 1999, Osman *et al.* 2003, Gomez-Lopez *et al.* 2009, Gomez-Lopez *et al.* 2010), and also into the decidua during preterm labour (Hamilton *et al.* 2012). Neutrophils are proposed to play an important role in stimulating cervical ripening in women (Bokström *et al.* 1997) and have been shown to infiltrate into both the myometrium and cervix in association with normal term labour (Thomson *et al.* 1999, Osman *et al.* 2003). Interestingly, Hamilton *et al.* recently reported that neutrophil numbers were increased in the decidua of women in infection-associated preterm labour, when compared with women in either idiopathic preterm or normal term labour (Hamilton *et al.* 2012).

What role these immune cells play in the induction of labour, either in the presence of an intrauterine infection or normally at term is unclear; however, previous work has shown that infiltrating leukocytes are a major source of inflammatory mediator production, including IL-1β, TNF-α, IL-8, IL-6 and MMP-9 in the utero-placental tissues during labour (Roh *et al.* 2000, Helmig *et al.* 2002, Young *et al.* 2002), suggesting that immune cells are likely to contribute to the inflammatory response surrounding parturition.

Therefore, using antibody-mediated immune cell depletion in our mouse model of LPS-induced preterm labour, the work described in this chapter aimed to investigate the role of neutrophils and macrophages in infection-induced preterm labour. Specifically, this work aimed to investigate whether neutrophils or macrophages were required for the induction of preterm labour in response to intrauterine LPS; and to examine whether these immune cell populations contributed to the LPS-induced inflammatory response observed in the utero-placental tissues.

5.2 METHODS

5.2.1 Mouse model of PTL

To investigate the role of specific immune cell populations in LPS-induced preterm labour, antibody-based depletion strategies were used to deplete either neutrophils or macrophages and to examine the effect of such depletion on LPS-induced preterm labour and the inflammatory response within the utero-placental tissues. Neutrophils were depleted using two different antibodies, either anti-Gr-1 or anti-Ly-6G, and macrophages were depleted using anti-F4/80.

5.2.1.1 Anti-Gr-1 experiments

On D16 of gestation, mice received an intra-peritoneal injection of either a rat anti-mouse anti-Gr-1 antibody (clone RB6-8C5; n=5) or a rat IgG2b isotype control antibody n=5), at a dose of 250μg in a volume of 500μl. The following day, mice underwent surgery as detailed in section 2.1 and received an intrauterine injection of 20μg LPS.

5.2.1.2 Anti-Ly-6G experiments

On D16 of gestation, mice received an intra-peritoneal injection of either a rat anti-mouse anti-Ly-6G antibody (clone 1A8; n=8) or a rat IgG2a isotype control antibody (n=8), at a dose of 500μg in a volume of 500μl. The following day, mice underwent surgery as detailed in section 2.1 and received an intrauterine injection of 20μg LPS.

5.2.1.3 Anti-F4/80 experiments

On D17 of gestation, mice received an intra-peritoneal injection of either rat anti-mouse anti-F4/80 antibody (n=6) or a rat IgG2a isotype control antibody (n=5), at a dose of 300μg in a volume of 600μl. After 4 hours mice underwent surgery as detailed in section 2.1 and received an intrauterine injection of 20μg LPS.

In each experiment, time to delivery and the proportion of live born pups were recorded following surgery, as detailed in sections 2.1.1. and 2.1.2

5.2.2 Tissue collection

In separate cohorts of mice, utero-placental tissues and maternal blood were collected 6 hours post-surgery, as detailed in section 2.1.3, for confirmation of immune cell depletion and analysis of the inflammatory response following neutrophil or macrophage depletion. Some mice delivered before the 6 hour collection time point, therefore it was not possible to collect utero-placental tissues from these mice, but maternal blood, spleen, liver, lung and cervix were still collected to confirm depletion in these mice.

5.2.2.1 Anti-Gr-1 experiments

Utero-placental tissues were collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or anti-Gr-1+LPS (n=5) for qRT-PCR analysis and immunohistochemistry. Maternal blood was also collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=6) or anti-Gr-1+LPS (n=7) for flow cytometric and ELISA analysis.

5.2.2.2 Anti-Ly-6G experiments

Maternal blood and utero-placental tissues were collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or anti-Ly-6G+LPS (n=5) for analysis.

5.2.2.3 Anti-F4/80 experiments

Utero-placental tissues were collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=4) or anti-F4/80+LPS (n=6). Maternal blood, spleen, liver, lung and cervix were also collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or anti-F4/80+LPS (n=7) for analysis.

5.2.3 Flow cytometry

Flow cytometry was used to confirm neutrophil depletion following anti-Gr-1 and anti-Ly-6G treatment, as detailed in section 2.7. Maternal blood was collected 6 hours post-surgery and stained with anti-CD45 and anti-Ly-6G to identify circulating neutrophils.

5.2.4 Immunohistochemistry

Immunohistochemistry was used to localise neutrophils in the uterus, and macrophages in the uterus, cervix, spleen, liver and lung to examine whether administration of the immune-cell specific antibodies resulted in depletion of tissue-resident immune cells. To confirm depletion following anti-Gr-1 treatment, neutrophils were localised with anti-Gr-1 in the uterus of mice treated with either IgG control antibody+LPS (n=5) or anti-Gr-1+LPS (n=5). To confirm depletion following anti-Ly-6G treatment, neutrophils were localised with anti-Ly-6G in the uterus of mice treated with either IgG control antibody+LPS (n=7) or anti-Ly-6G+LPS (n=5). Macrophages were localised using anti-F4/80 in uterine tissue, spleen, liver, lung and cervix harvested from mice treated with IgG+LPS (n=4) or anti-F4/80+LPS (n=6). Staining protocols are detailed in section 2.5.2.

5.2.5 qRT-PCR

The mRNA expression of several inflammatory genes was examined using qRT-PCR as described in section 2.3. Expression of the following genes was quantified in the uterus, fetal membranes and placenta of mice 6 hours post-surgery: *Tnf-α, Il-1β, Il-6*, *Il-10*, *Cox-2*, *Cxcl1*, *Cxcl2*, *Cxcl5* and *Ccl2*.

As described in section 2.3.3, expression of the gene of interest was normalised to *β-actin* as the endogenous control gene in each sample and all samples were then compared relative to a calibrator tissue, which was untreated D18 uterus.

5.2.6 ELISA

The concentration of TNF-α in maternal serum following treatment with either the IgG control antibodies+LPS or the immune cell specific antibodies+LPS was determined by ELISA. Details of the TNF-α ELISA kit are given in section 2.4.

5.2.7 Statistical analysis

Data are presented as mean ± SEM. Where data were not normally distributed, they were transformed prior to analysis. Time to delivery data were log transformed before

analysis, and the proportion of live pups born was arc-sin transformed prior to analysis. Normally distributed data were then analysed by unpaired t-tests between the IgG control group and the group treated with the immune cell-specific antibody. $P<0.05$ was considered statistically significant.

5.3 RESULTS

5.3.1 Administration of anti-Gr-1 to deplete neutrophils on day 16 of gestation

To investigate whether neutrophils were involved in LPS-induced preterm labour and/or the LPS-induced inflammatory response in the utero-placental tissues in our model, neutrophils were depleted prior to intrauterine LPS administration using an antibody-based depletion strategy. The anti-Gr-1 antibody (RB6-8C5 clone) is commonly used to deplete neutrophils in *in vivo* mouse models (Kaitu'u-Lino *et al.* 2007, Tate *et al.* 2008, Gong and Koh 2010). In our model, mice received an intra-peritoneal injection with either anti-Gr-1 or an IgG control antibody on D16 of gestation, followed on D17 of gestation with intrauterine LPS administration to both groups.

To confirm that administration of the anti-Gr-1 antibody successfully depleted neutrophils in our model, circulating neutrophil numbers were assessed using flow cytometry and LPS-induced neutrophil recruitment to the uterus was examined using immunohistochemistry.

5.3.1.1 Depletion of circulating neutrophils

Blood samples were collected 6 hours post-intrauterine LPS administration and stained with anti-CD45 and anti-Ly-6G to assess the number of circulating neutrophils. Representative flow cytometry plots from two control mice that received the IgG control antibody and two mice that received anti-Gr-1 are shown in Figure 5.1. The flow cytometry plots confirmed that blood harvested from mice receiving the IgG control antibody contained a high percentage of circulating neutrophils (Figure 5.1A and Figure 5.1B), while blood harvested from mice treated with anti-Gr-1 had a very low percentage of circulating neutrophils (Figure 5.1C and Figure 5.1D). Additionally, quantification of the neutrophils confirmed that administration of anti-Gr-1 antibody significantly reduced the number of circulating neutrophils compared to the IgG control group (mean number of neutrophils/ml of blood in IgG control group: 3.67×10^6 ± SEM 0.21 vs. mean number of neutrophils/ml of blood in anti-Gr-1 group: 0.0063×10^6 ± SEM 0.0018, $p<0.001$; Figure 5.1E).

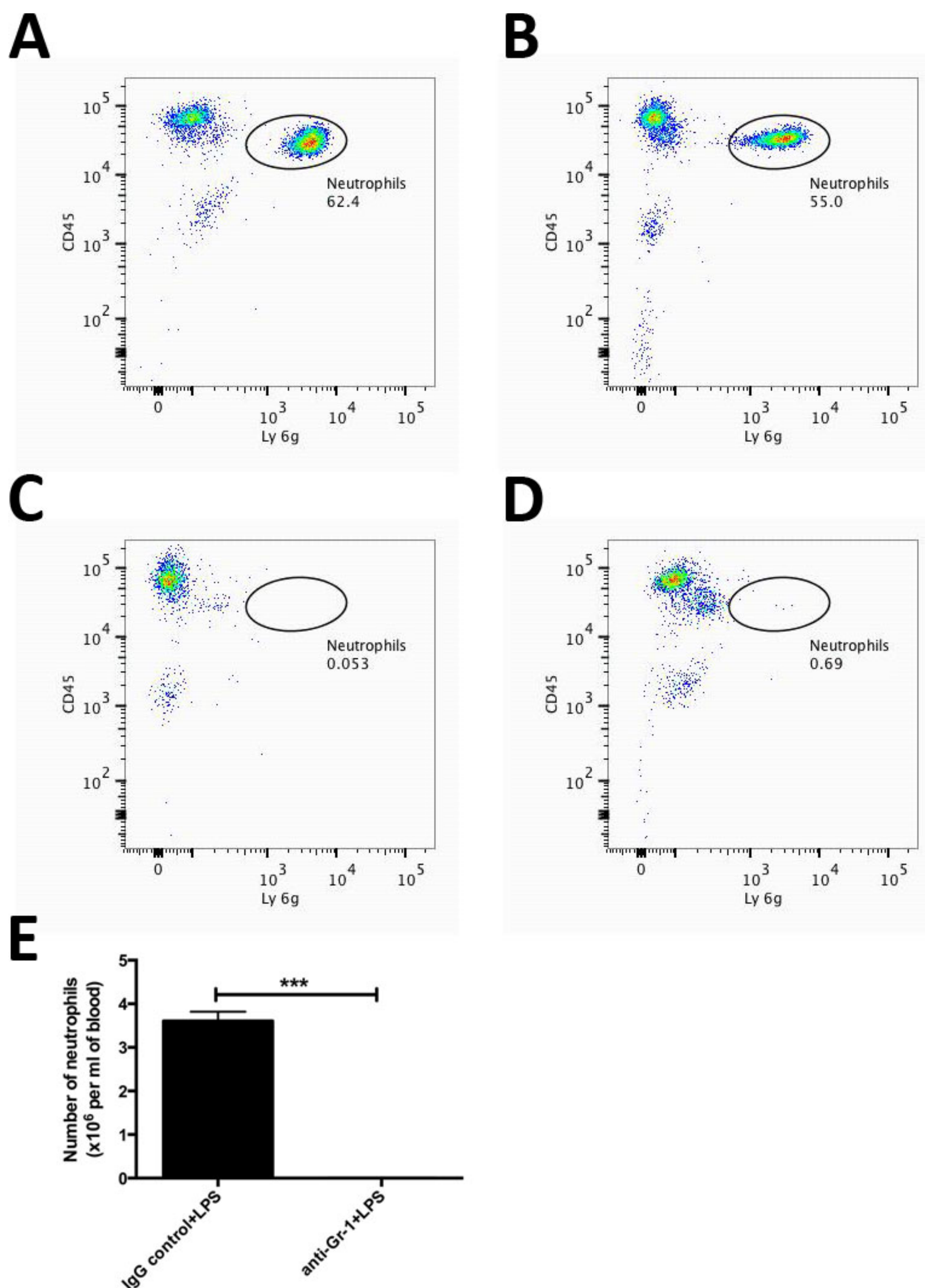

Figure 5.1- Effect of anti-Gr-1 administration on circulating blood neutrophils. Flow cytometric analysis of maternal blood collected 6 hours post-surgery from mice treated with either anti-Gr-1 antibody (n=7) or an IgG control antibody (n=6) prior to intrauterine LPS administration. Cells were gated based on expression of CD45 and Ly-6G. Representative flow plots from two mice treated with the IgG control antibody **(A)** and **(B)**; and from 2 mice treated with the anti-Gr-1 antibody **(C)** and **(D).** The number given beside each gated region indicates the percentage of neutrophils counted out of the total number of live cells. **(E)** The number of neutrophils per ml of blood. Data presented as mean ± SEM; ***$p<0.001$.

5.3.1.2 Effect of neutrophil depletion using anti-Gr-1 on LPS-induced neutrophil recruitment to the uterus

To confirm that depletion of neutrophils using anti-Gr-1 also blocked the LPS-induced recruitment of neutrophils into the uterus, uterine tissues was harvested on D17 of gestation 6 hours post-intrauterine LPS administration from mice that received an intra-peritoneal injection of either an IgG control antibody, or anti-Gr-1 on D16 of gestation. Immunohistochemistry using the anti-Gr-1 antibody was then performed on the fixed uterine tissue to localise Gr-1 positive cells within the uterus.

In mice treated with the IgG control antibody, intrauterine LPS treatment induced an influx of Gr-1 positive cells into the myometrial tissue (Figure 5.2A), and the decidua of these mice (Figure 5.2B). In contrast, no Gr-1 positive neutrophils were localised in uterine tissue harvested from mice treated with anti-Gr-1 prior to intrauterine LPS administration, with both the myometrium (Figure 5.2C) and the decidua (Figure 5.2D) clear of positive staining cells. Also, no positive staining was observed in negative control sections of myometrium, which were not treated with the anti-Gr-1 antibody, thus confirming specificity of the staining observed (Figure 5.2E) and decidua (Figure 5.2F).

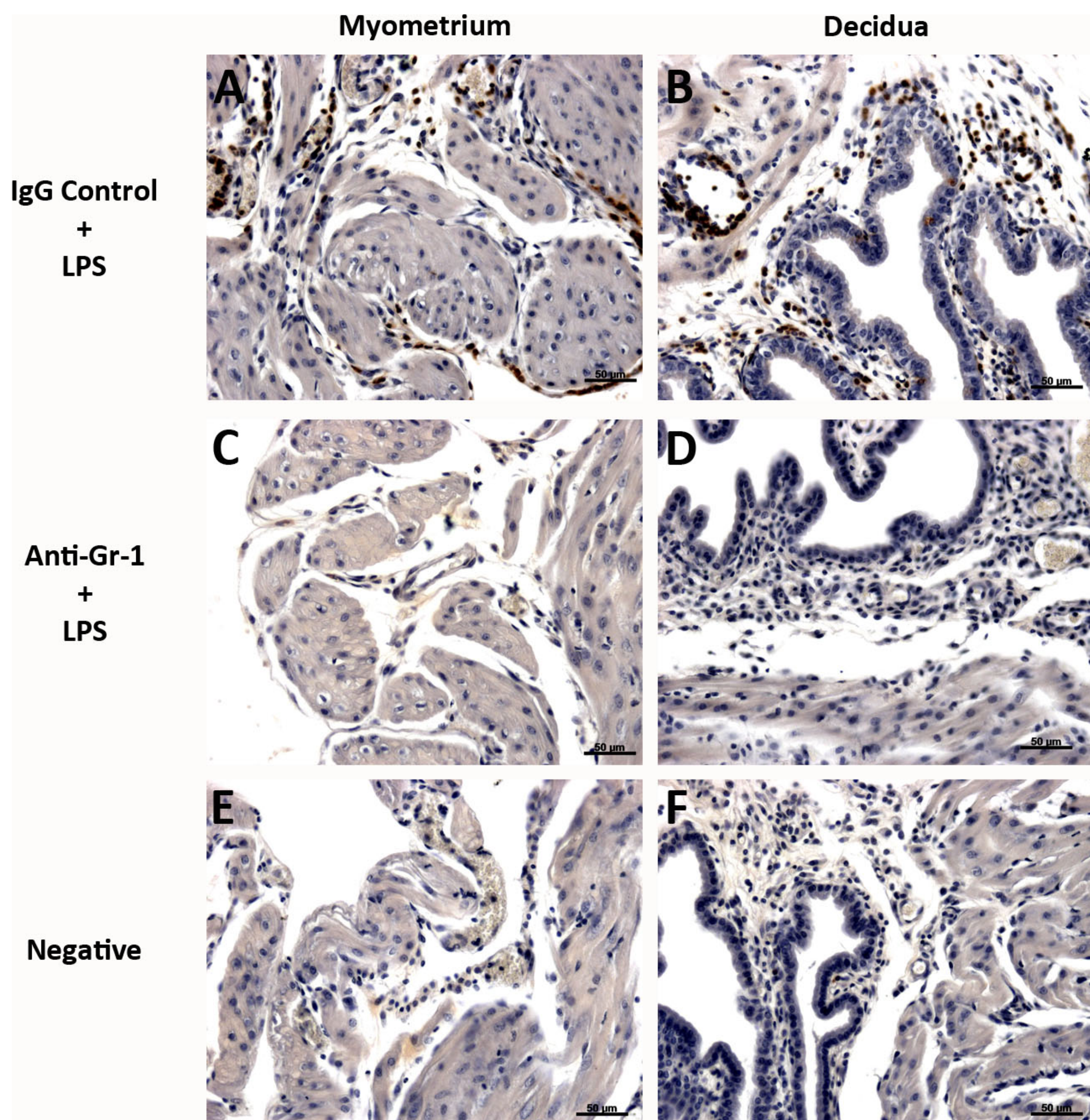

Figure 5.2 – Effect of anti-Gr-1 treatment on neutrophil localisation in the uterus. Uterine tissue was collected 6 hours post-LPS treatment from mice treated with either anti-Gr-1 (n=5) or the IgG control antibody (n=5). Representative images of neutrophil localisation by immunohistochemical staining for Gr-1. Gr-1 positive cells were localised to the myometrium **(A)** and decidua **(B)** of mice treated with the IgG control antibody+LPS. No Gr-1 positive cells were present in mice treated with the anti-Gr-1 antibody+LPS **(C)** and (**D).** No positive staining was observed in the negative control sections **(E)** and **(F).** Scale bars show 50μm. All images taken with a x20 objective lens.

5.3.2 Effect of neutrophil depletion using anti-Gr-1 on LPS-induced preterm labour

To investigate whether the observed LPS-induced neutrophil recruitment into the uterus of mice was required for the induction of LPS-induced preterm labour, on D16 of gestation mice received an intra-peritoneal injection of either an IgG control antibody or anti-Gr-1, followed on D17 with an intrauterine injection of 20μg LPS. The effect of neutrophil depletion on LPS-induced preterm labour was monitored.

5.3.2.1 Time to delivery

There was no statistically significant difference in time to delivery between the two treatment groups. If anything, a trend towards earlier delivery in the anti-Gr-1+LPS group (mean time to delivery in anti-Gr-1 group 8.95 hours ± SEM 1.91; mean time to delivery in IgG control group: 33.3 hours ± SEM 7.99; $p=0.12$; Figure 5.3A) was observed.

5.3.2.2 Proportion of live born pups

Similarly, mice treated with anti-Gr-1+LPS had a reduced proportion of live born pups compared to the IgG+LPS control group (no live pups were born to mice treated with anti-Gr-1+LPS, vs. mean proportion of live born pups in IgG+LPS group: 0.3 ± SEM 0.13; Figure 5.3B); however this difference did not quite reach statistical significance ($p=0.051$).

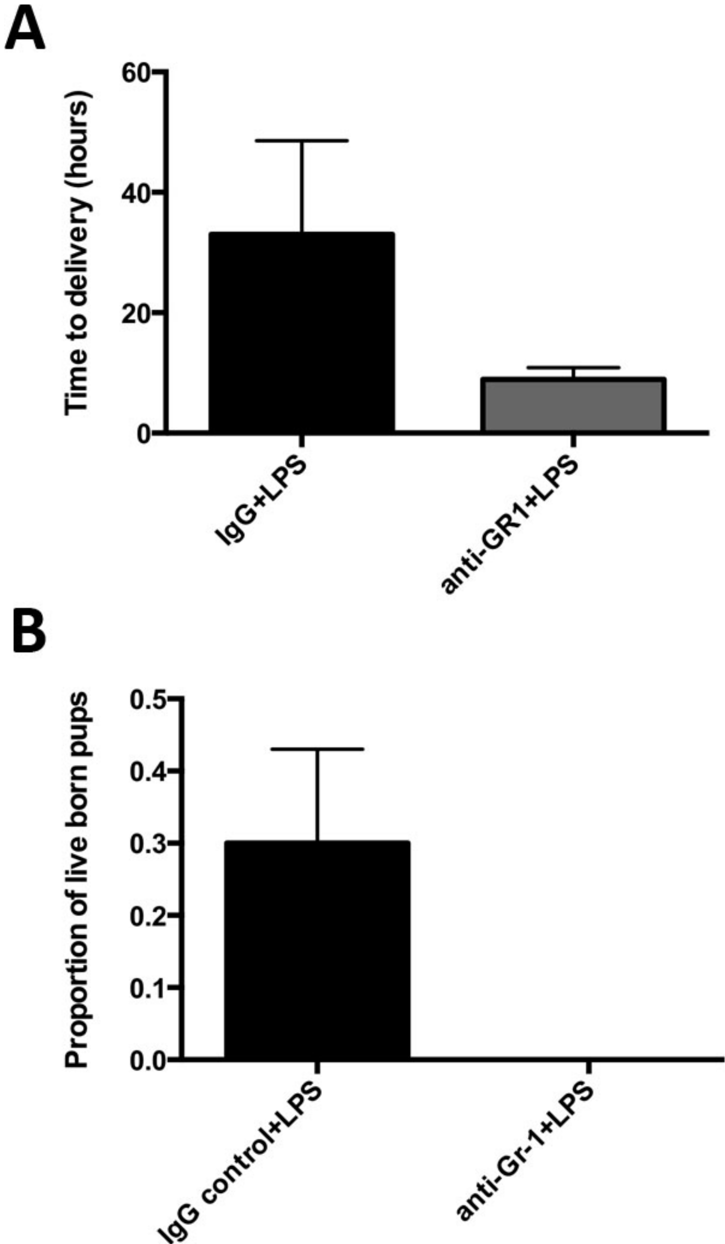

Figure 5.3 – Effect of neutrophil depletion with anti-Gr-1 on LPS-induced preterm delivery and the proportion of live born pups. Time to delivery and the proportion of live born pups was monitored in mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=5). **(A)** Time to delivery. **(B)** Proportion of live born pups. Data are presented as mean ± SEM (error bars).

5.3.3 LPS-induced expression of inflammatory mediators in the utero-placental tissues following neutrophil depletion with anti-Gr-1

To investigate whether neutrophil depletion using the anti-Gr-1 antibody affected LPS-induced inflammatory gene expression in the utero-placental tissues, treated mice were sacrificed 6 hours after intrauterine LPS injection. Utero-placental tissues were harvested and analysed by qRT-PCR to measure the expression of *Tnf-α, Il-1β, Il-6, Il-10, Cox-2*, *Cxcl1*, *Cxcl2*, *Cxcl5* and *Ccl2*.

5.3.3.1 Inflammatory gene expression in the uterus

There was no difference in *Tnf-α* expression in the uterine tissue harvested from anti-Gr-1+LPS mice, when compared to the IgG control+LPS group (Figure 5.4A).

Expression of *Il-1β* was significantly reduced in mice treated with anti-Gr-1+LPS, compared to the IgG+LPS group (2.9-fold lower expression, $p<0.01$; Figure 5.4B).

Uterine expression of *Il-6* (Figure 5.4C), *Il-10* (Figure 5.4D), *Cox-2* (Figure 5.4E) *Cxcl1* (Figure 5.5A), *Cxcl2* (Figure 5.5B), *Cxcl5* (Figure 5.5C) and *Ccl2* (Figure 5.5D) were also not significantly different when comparing the anti-Gr-1 treated mice with the IgG control mice.

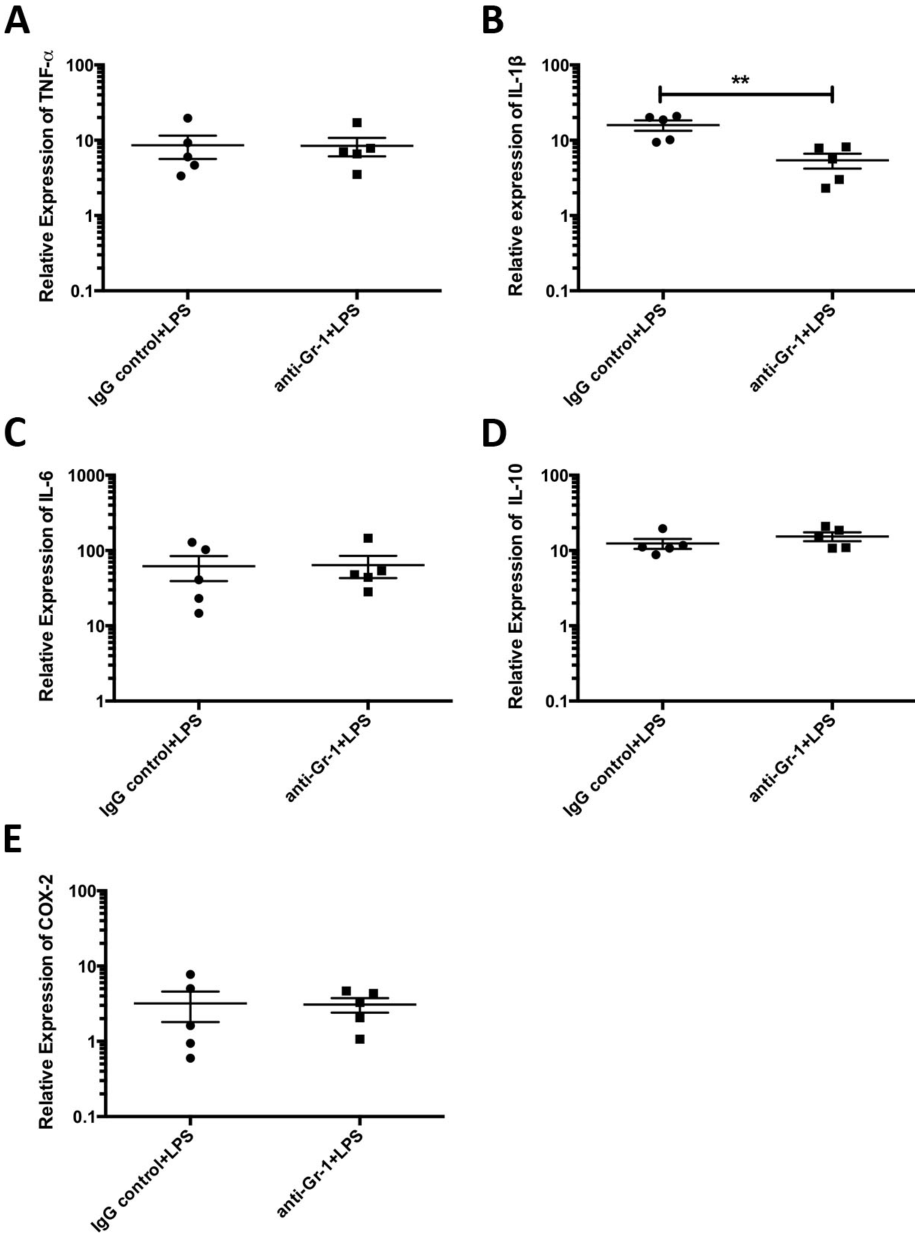

Figure 5.4 – Effect of anti-Gr-1 treatment on LPS-induced inflammatory gene expression in the uterus. Uterine tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=5) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars); **p<0.01.

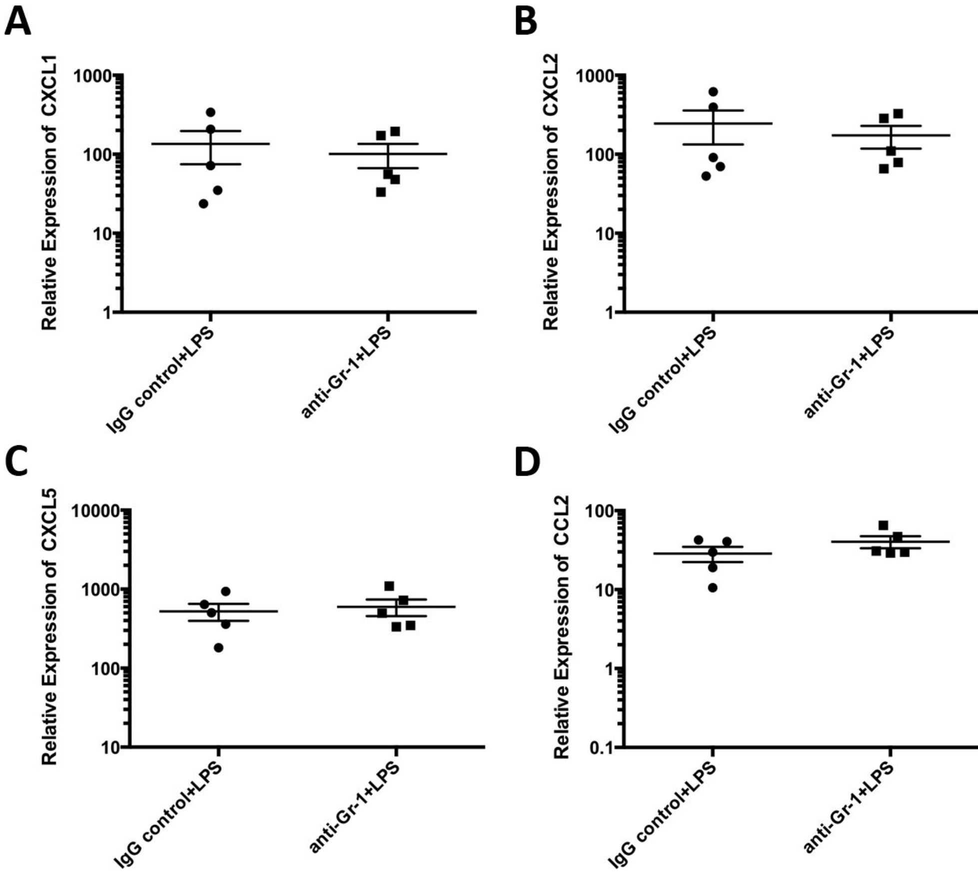

Figure 5.5 – Effect of anti-Gr-1 treatment on LPS-induced inflammatory chemokine expression in the uterus. Uterine tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=5) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars).

5.3.3.2 Inflammatory gene expression in the fetal membranes

In the fetal membranes, there was no difference in the expression of *Tnf-α* (Figure 5.6A) or *Il-1β* (Figure 5.6B) when comparing mice treated with anti-Gr-1+LPS and those treated with IgG+LPS.

Il-6 expression was significantly greater in the anti-Gr-1+LPS group compared to the IgG control+LPS (3.0-fold greater expression; $p<0.05$; Figure 5.6C).

Expression of *Il-10* was not altered by anti-Gr-1 treatment, compared to the IgG+LPS control group (Figure 5.6D).

Cox-2 expression was significantly up-regulated in mice treated with anti-Gr-1+LPS compared to the IgG control+LPS (2.1-fold greater expression; Figure 5.6E).

The expression of *Cxcl1, Cxcl2, Cxcl5* and *Ccl2* in the fetal membranes was significantly elevated in response to anti-Gr-1+LPS compared to the IgG control+LPS group. Expression of *Cxcl1* was 3.7-fold greater ($p<0.01$; Figure 5.7A). *Cxcl2* expression was 3.2-fold higher in the anti-Gr-1 group compared to the IgG group ($p<0.01$; Figure 5.7B). Expression of *Cxcl5* was 3.6-fold greater in the anti-Gr-1 group ($p<0.05$; Figure 5.7C). *Ccl2* expression was 3.3-fold greater in the anti-Gr-1 treatment group compared to the IgG control group ($p<0.05$; Figure 5.7D).

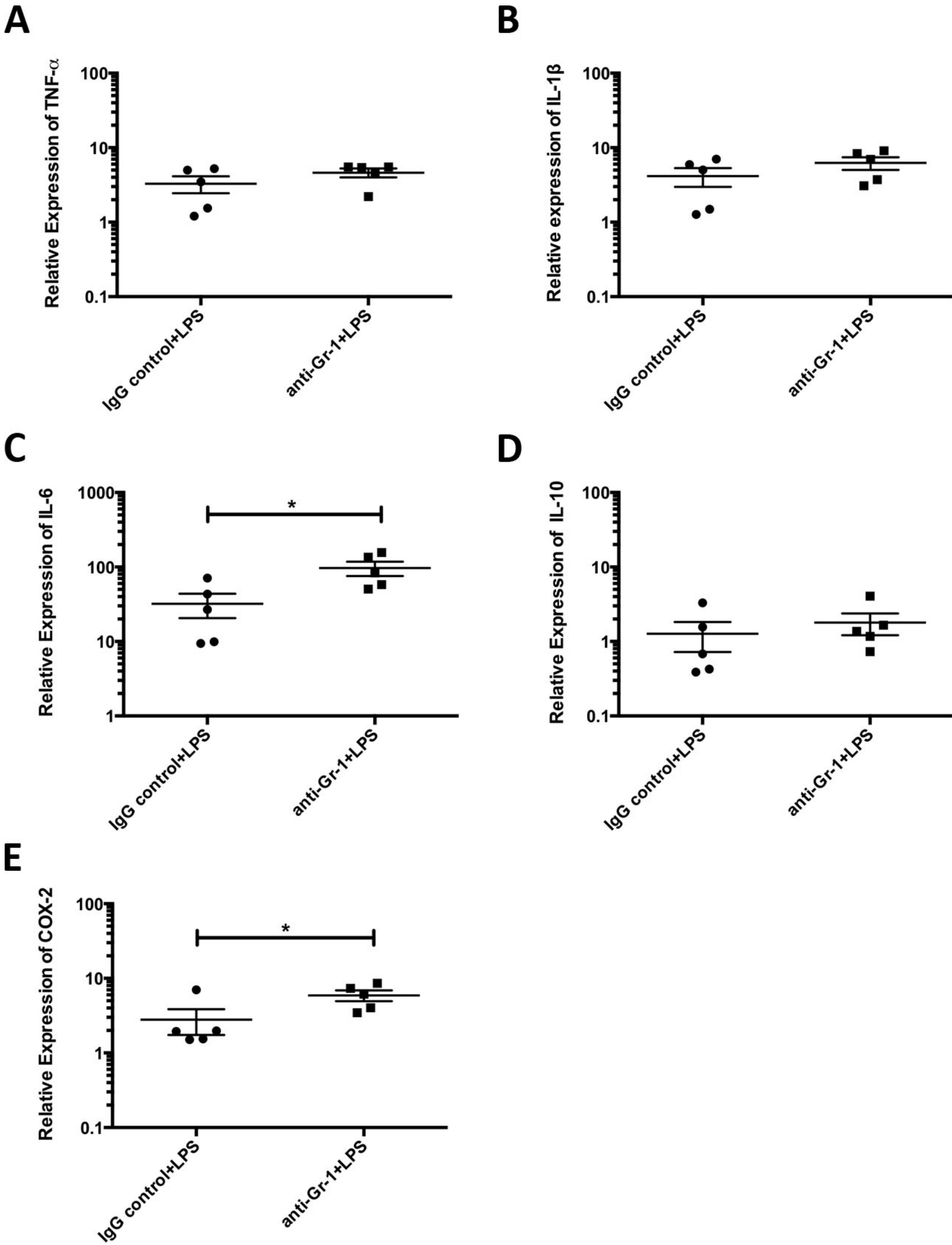

Figure 5.6 – Effect of anti-Gr-1 treatment on LPS-induced inflammatory gene expression in the fetal membranes. Fetal membranes were harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=5) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05.

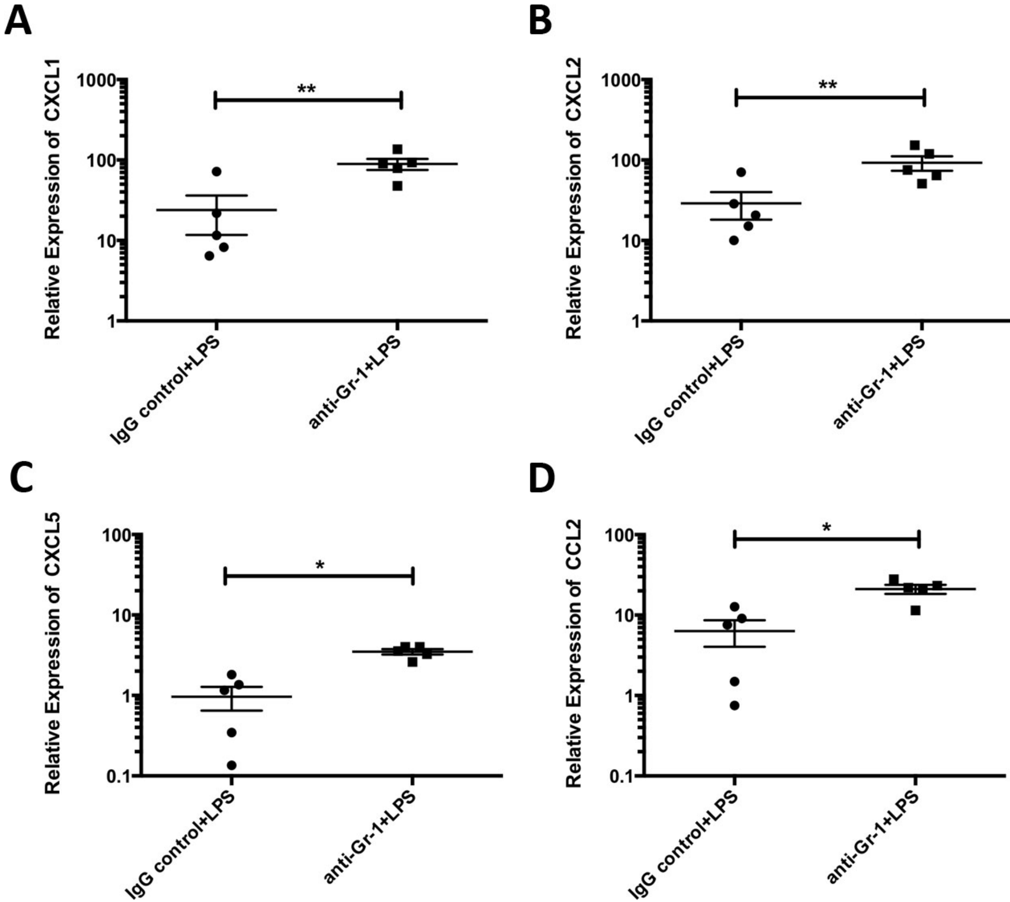

Figure 5.7 - Effect of anti-Gr-1 treatment on LPS-induced inflammatory chemokine expression in the fetal membranes. Fetal membranes were harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=5) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars); *p<0.05, **p<0.01.

5.3.3.3 Inflammatory gene expression in the placenta

There was no significant difference in *Tnf-α* expression in the placental tissue harvested from anti-Gr-1+LPS mice, when compared to the IgG control+LPS group (Figure Figure 5.8A).

Expression of *Il-1β* was significantly reduced in mice treated with anti-Gr-1+LPS, compared to the IgG+LPS group (1.7-fold lower expression, $p<0.01$; Figure 5.8B).

As was observed in the uterus, the expression of the remaining genes examined, *Il-6* (Figure 5.8C), *Il-10* (Figure 5.8D), *Cox-2* (Figure 5.8E), *Cxcl1* (Figure 5.9A), *Cxcl2* (Figure 5.9B), *Cxcl5* (Figure 5.9C) and *Ccl2* (Figure 5.9D) was unaffected by anti-Gr-1 treatment prior to intrauterine LPS, compared to those mice receiving IgG.

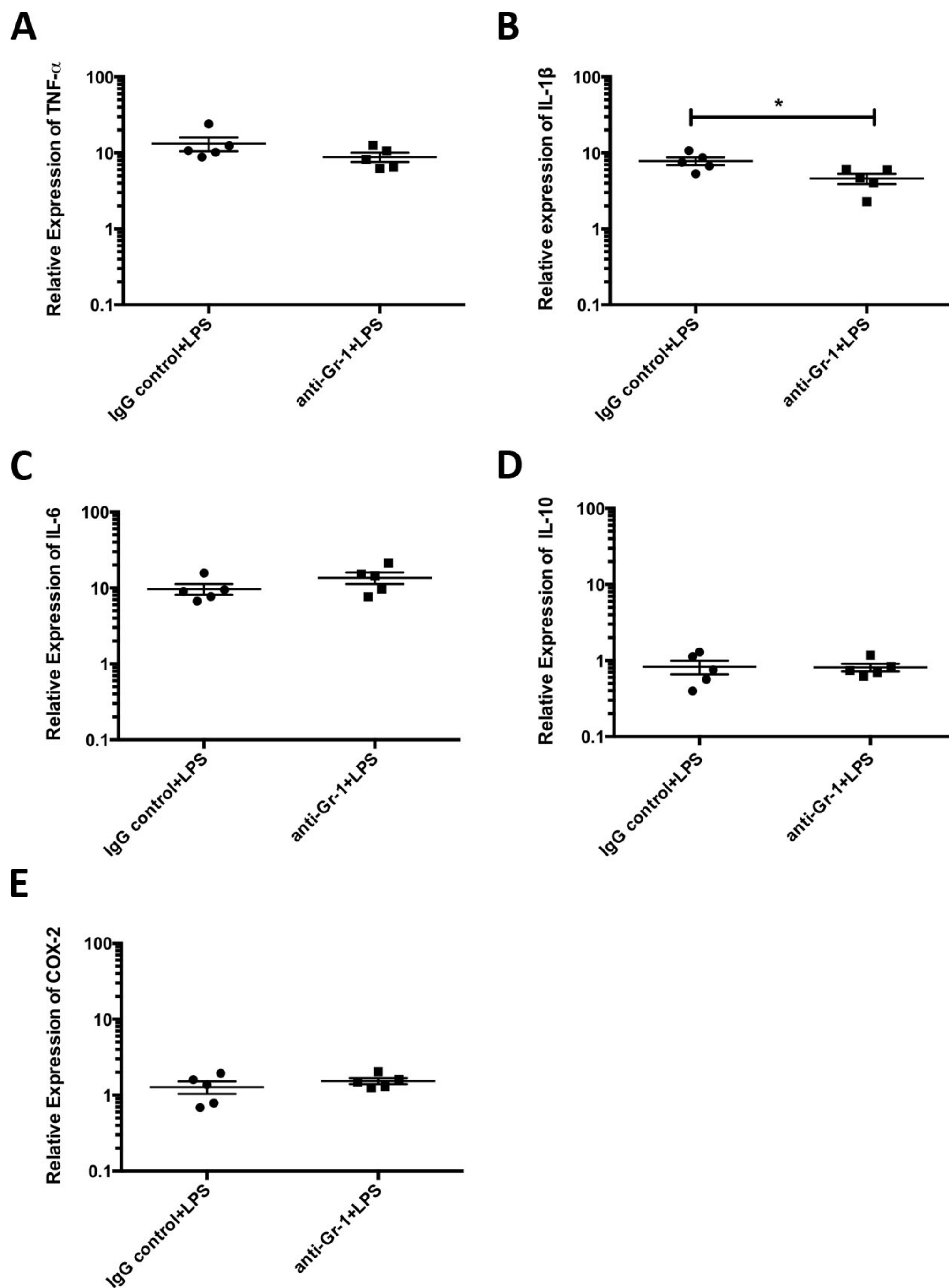

Figure 5.8 – Effect of anti-Gr-1 treatment on LPS-induced inflammatory gene expression in the placenta. Placental tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=5) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars); *$p<0.05$.

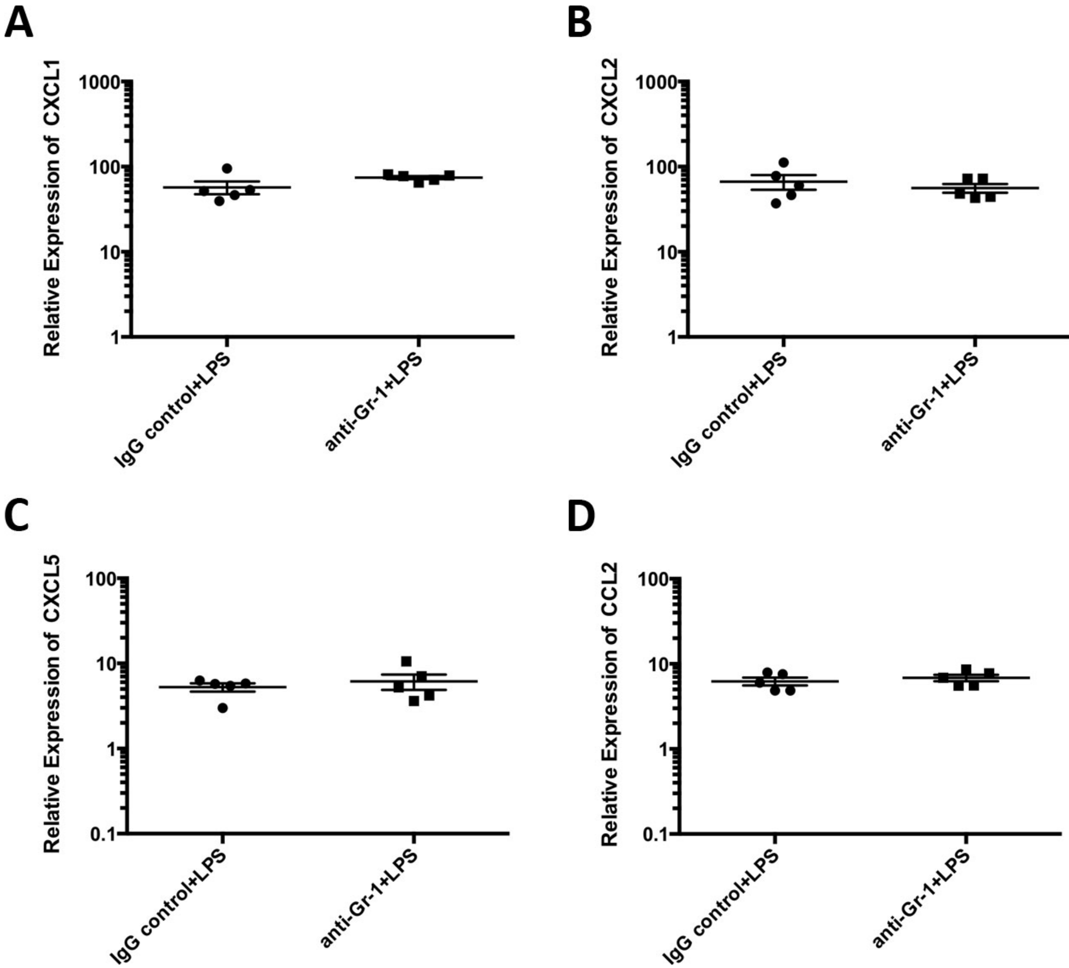

Figure 5.9 - Effect of anti-Gr-1 treatment on LPS-induced inflammatory chemokine expression in the placenta. Placental tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=5) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars).

5.3.4 Effect of anti-Gr-1 administration on LPS-induced serum TNF-α levels

As it has previously been reported that neutrophil depletion using the anti-Gr-1 antibody followed by LPS treatment resulted in elevated circulating TNF-α levels in a mouse model of endotoxaemia (Daley *et al.* 2008), we investigated whether a similar effect operated in our model. Blood was collected from mice 6 hours post-intrauterine LPS injection from mice treated with either the IgG control antibody or anti-Gr-1, and the serum concentration of TNF-α was measured by ELISA.

There was no statistically significant difference in the mean serum concentration of TNF-α in mice treated with anti-Gr-1+LPS compared to the IgG+LPS group (mean concentration anti-Gr-1 group: 47.6 pg/ml ± SEM 18.5 vs. IgG+LPS group: mean 28.5 pg/ml ± SEM 7.4; $p=0.36$; Figure 5.10).

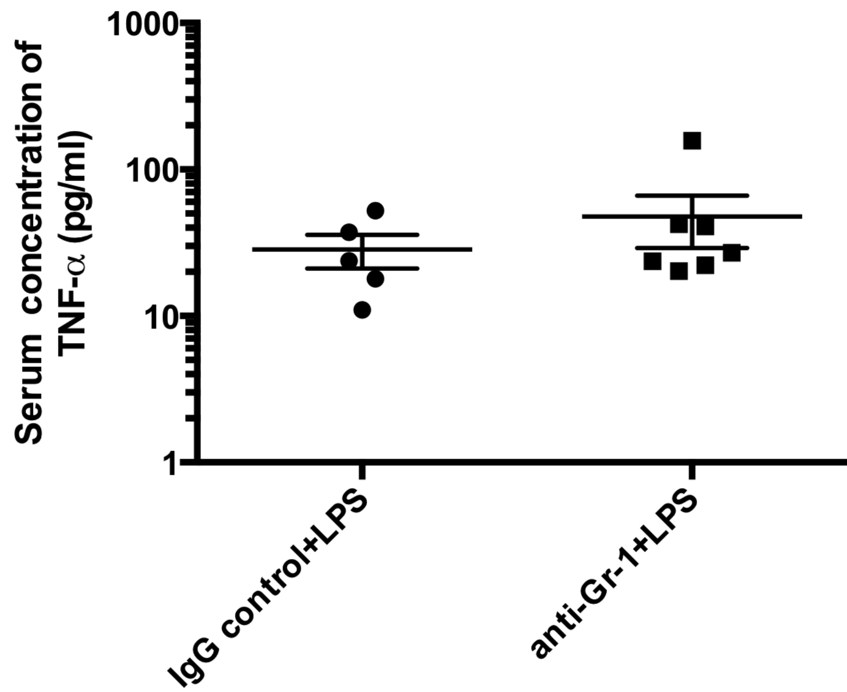

Figure 5.10 – Effect of anti-Gr-1 treatment on serum TNF-α levels. Maternal blood was collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=5) or the anti-Gr-1 antibody+LPS (n=7) and ELISA was used to quantify the serum concentration of TNF-α. Data presented as mean ± SEM (error bars).

5.3.5 Administration of anti-Ly-6G to deplete neutrophils on day 16 of gestation

Whilst anti-Gr-1 is commonly used to deplete neutrophils in *in vivo* models, it is now understood that as this antibody recognises both Ly-6G and Ly-6C antigens (Fleming *et al.* 1993). Anti-Gr-1 therefore not only depletes neutrophils, but may also deplete other cells expressing Ly-6C, such as some subsets of monocytes, T cells and dendritic cells (Jutila *et al.* 1988, Hestdal *et al.* 1991, Kung *et al.* 1991, Shortman and Naik 2007, Daley *et al.* 2008). In order to further investigate the specific role of neutrophils in our model of infection-induced preterm labour, we repeated the depletion experiments using a specific anti-Ly-6G antibody (1A8 clone), which has been shown to successfully remove neutrophils without altering other immune cell populations *in vivo* (Daley *et al.* 2008, Tate *et al.* 2009, Wojtasiak *et al.* 2010, Shi *et al.* 2011). Mice received an intra-peritoneal injection with either anti-Ly-6G or an IgG control antibody on D16 of gestation, followed on D17 of gestation with intrauterine LPS administration to both groups.

To confirm that administration of the anti-Ly-6G antibody successfully depleted neutrophils in our model, circulating neutrophil numbers were quantified using flow cytometry and LPS-induced neutrophil recruitment to the uterus was examined using immunohistochemistry.

5.3.5.1 Depletion of circulating neutrophils

Blood samples were collected 6 hours post-intrauterine LPS administration and stained with anti-CD45 and anti-Ly-6G to assess the number of circulating neutrophils. Representative flow cytometry plots from two control mice that received the IgG control antibody and two mice that received anti-Ly-6G are shown in Figure 5.11. Flow cytometry analysis confirmed that blood harvested from mice receiving IgG contained a high percentage of circulating neutrophils (Figure 5.11A and Figure 5.11B), while blood harvested from mice treated with anti-Ly-6G had a very low percentage of circulating neutrophils (Figure 5.11C and Figure 5.11D). Additionally, quantification of the neutrophils present in the blood sample confirmed that administration of anti-Ly-6G significantly reduced the number of circulating neutrophils compared to the IgG control group (mean number of neutrophils/ml of blood in IgG control group: $2.95 \times 10^6 \pm$ SEM 0.35 vs. mean number of neutrophils/ml of blood in anti-Ly-6G group: $0.04 \times 10^6 \pm$ SEM 0.02, $p<0.001$; Figure 5.11E).

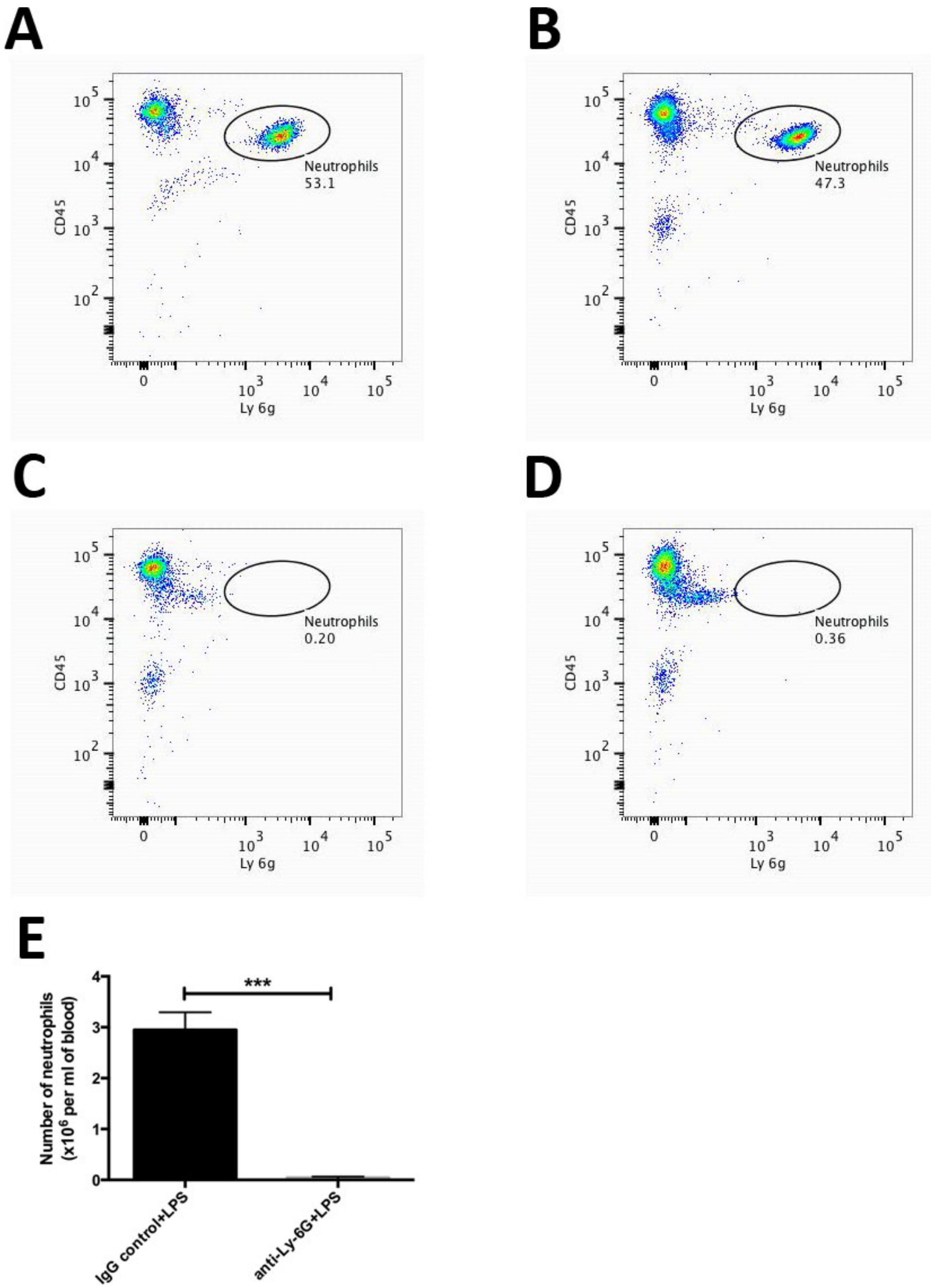

Figure 5.11 - Effect of anti-Ly-6G administration on circulating blood neutrophils. Flow cytometric analysis of maternal blood collected 6 hours post-surgery from mice treated with either anti-Ly-6G antibody (n=7) or an IgG control antibody (n=7) prior to intrauterine LPS administration. Cells were gated based on expression of CD45 and Ly-6G. Representative flow plots from two mice treated with the IgG control antibody **(A)** and **(B)**; and from 2 mice treated with the anti-Ly-6G antibody **(C)** and **(D).** The number given beside each gated region indicates the percentage of neutrophils counted out of the total number of live cells. **(E)** The number of neutrophils per ml of blood. Data presented as mean ± SEM; ***p<0.001.

5.3.5.2 Effect of neutrophil depletion with anti-Ly-6G on LPS-induced neutrophil recruitment to the uterus

As in the anti-Gr-1 experiments, immunohistochemistry was performed on uterine tissue harvested 6 hours post-intrauterine LPS administration, from mice treated with either the IgG control antibody or anti-Ly-6G, to confirm that depletion of neutrophils using anti-Ly-6G also blocked the LPS-induced recruitment of neutrophils into the uterus. Neutrophils were localised using the anti-Ly-6G antibody.

In mice treated with the IgG control antibody intrauterine LPS treatment induced an influx of Ly-6G positive neutrophils into the myometrium (Figure 5.12A), and decidua of these mice (Figure 5.12B). In contrast, no Ly-6G positive neutrophils were localised in uterine tissue harvested from mice treated with anti-Ly-6G prior to intrauterine LPS administration, with both the myometrium (Figure 5.12C) and the decidua (Figure 5.12D) clear of positive staining cells. Also no positive staining was observed in negative control sections of myometrium (Figure 5.12E) and decidua (Figure 5.12F).

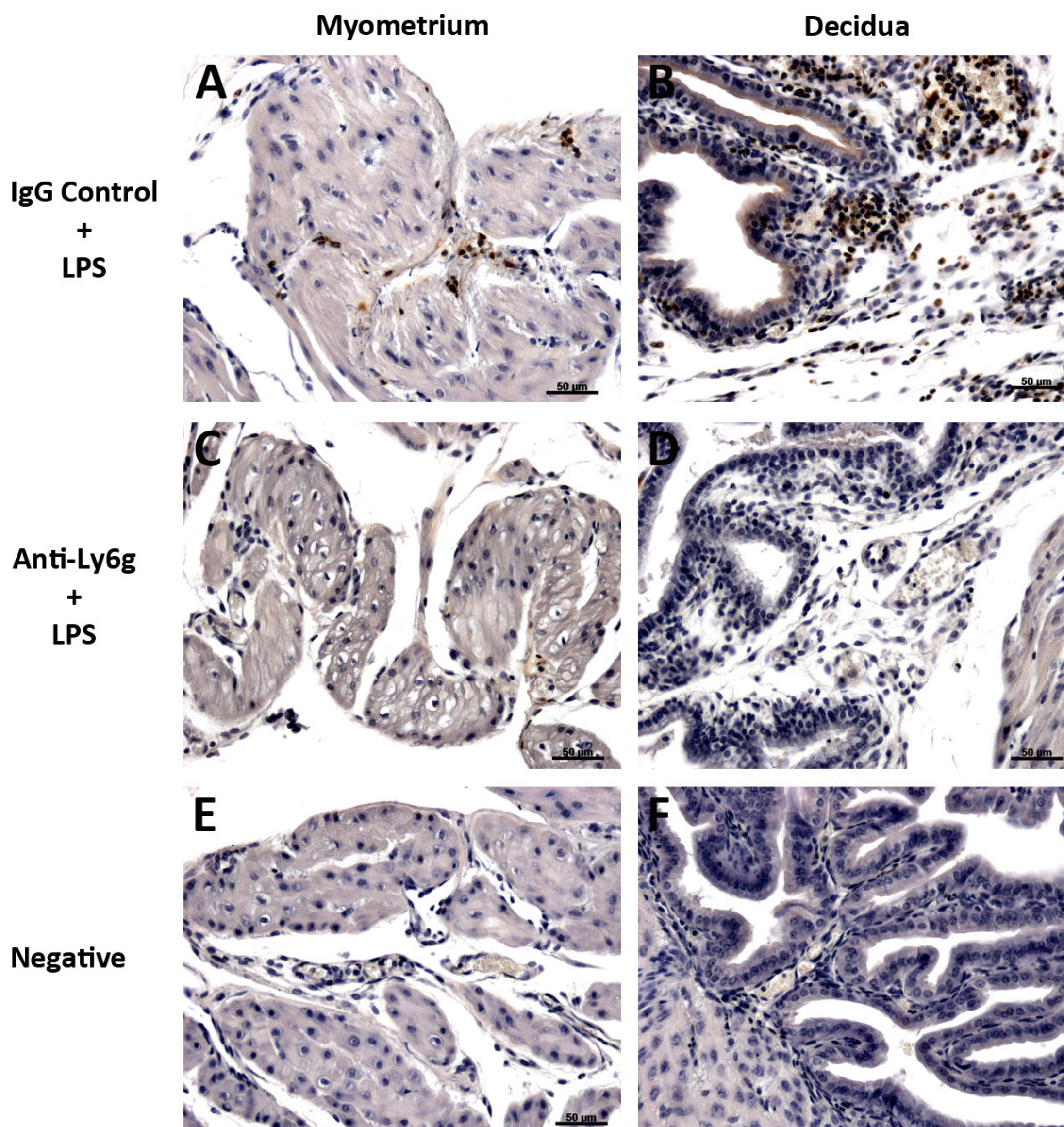

Figure 5.12 - Effect of anti-Ly-6G treatment on neutrophil localisation in the uterus. Uterine tissue was collected 6 hours post-LPS treatment from mice treated with either anti-Ly-6G (n=6) or the IgG control antibody (n=7). Representative images of neutrophil localisation by immunohistochemical staining for Ly-6G. Ly-6G positive cells were localised to the myometrium **(A)** and decidua **(B)** of mice treated with the IgG control antibody+LPS. No Ly-6G positive cells were present in mice treated with the anti-Ly-6G antibody+LPS **(C)** and (**D).** No positive staining was observed in the negative control sections **(E)** and **(F).** Scale bars show 50μm. All images taken with a x20 objective lens.

5.3.6 Effect of neutrophil depletion using anti-Ly-6G on LPS-induced preterm labour

To further investigate the specific role neutrophils may play in infection-induced preterm labour we determined the effect of anti-Ly-6G on LPS-induced preterm delivery. On D16 of gestation mice received an intra-peritoneal injection of either an IgG control antibody or anti-Ly-6G, followed on D17 with an intrauterine injection of 20μg LPS, and the effect of neutrophil depletion on LPS-induced preterm labour was monitored.

5.3.6.1 Time to delivery

There was no statistically significant difference when comparing the mean time to delivery between mice treated with anti-Ly-6G+LPS and those treated with IgG+LPS, if anything there was a trend towards a reduced time to delivery in those mice treated with anti-Ly-6G (mean time to delivery in anti-Ly-6G group: 19.25 hours ± SEM 9.35 vs. IgG group mean time to delivery: 33.47 hours ± 9.58; p=0.14 Figure 5.13A).

5.3.6.2 Proportion of live born pups

As was observed with anti-Gr-1 treatment, mice treated with anti-Ly-6G+LPS had a reduced proportion of live born pups compared to the IgG+LPS control group, (mean proportion of live born pups anti-Ly-6G group: 0.06 ± SEM 0.05 vs. mean proportion of live born pups IgG group: 0.29 ± 0.11; Figure 5.13B); however this difference was not found to be statistically significant.

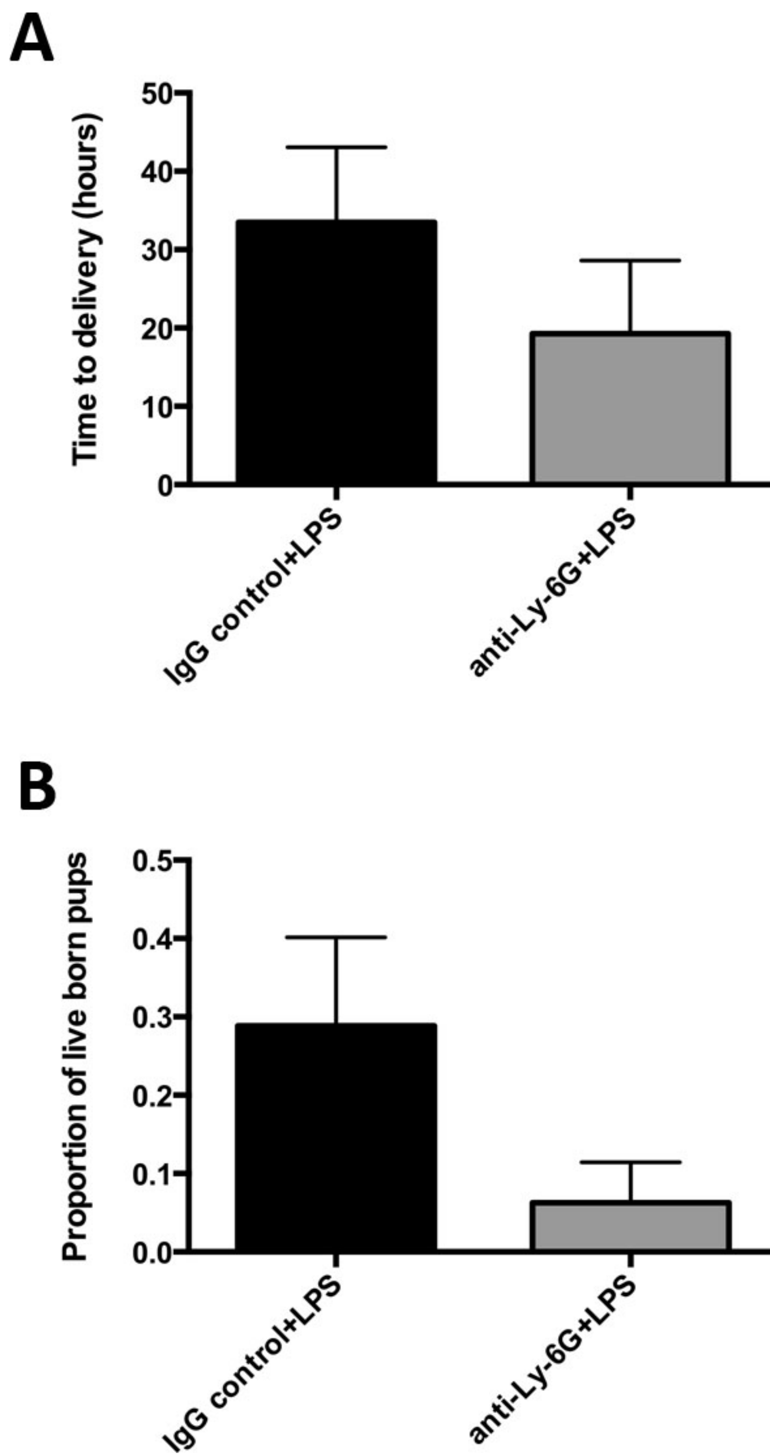

Figure 5.13 - Effect of neutrophil depletion with anti-Ly-6G on LPS-induced preterm delivery and the proportion of live born pups. Time to delivery and the proportion of live born pups was monitored in mice treated with either the IgG control antibody+LPS (n=8) or the anti-Ly-6G antibody+LPS (n=8). **(A)** Time to delivery. **(B)** Proportion of live born pups. Data are presented as mean ± SEM (error bars).

5.3.7 LPS-induced expression of inflammatory mediators in the utero-placental tissues following neutrophil depletion with anti-Ly-6G

To investigate whether neutrophil depletion using the anti-Ly-6G antibody also affected LPS-induced inflammatory gene expression in the utero-placental tissues, as observed with anti-Gr-1 treatment, mice were sacrificed 6 hours after intrauterine LPS injection and utero-placental tissues were harvested and analysed by qRT-PCR to measure the expression of *Tnf-*α*, Il-1*β*, Il-6, Il-10, Cox-2, Cxcl1, Cxcl2, Cxcl5* and *Ccl2*.

5.3.7.1 Inflammatory gene expression in the uterus

Expression of *Tnf-*α was significantly reduced in mice receiving treatment with anti-Ly-6G+LPS compared to the IgG+LPS control group (1.8-fold decrease, p<0.05; Figure 5.14A).

Similarly, uterine *Il-1*β expression was also significantly reduced in mice treated with anti-Ly-6G+LPS compared to those treated with IgG+LPS, with a 5.5-fold decrease in *Il-1*β expression (p<0.001; Figure 5.14B).

There was no significant differences in the expression of *Il-6* (Figure 5.14C), *Il-10* (Figure 5.14D), *Cox-2* (Figure 5.14E), *Cxcl1* (Figure 5.15A), *Cxcl2* (Figure 5.15B), *Cxcl5* (Figure 5.15C) and *Ccl2* (Figure 5.15D) in the uterus of mice treated with anti-Ly-6G+LPS, compared to those treated with IgG+LPS.

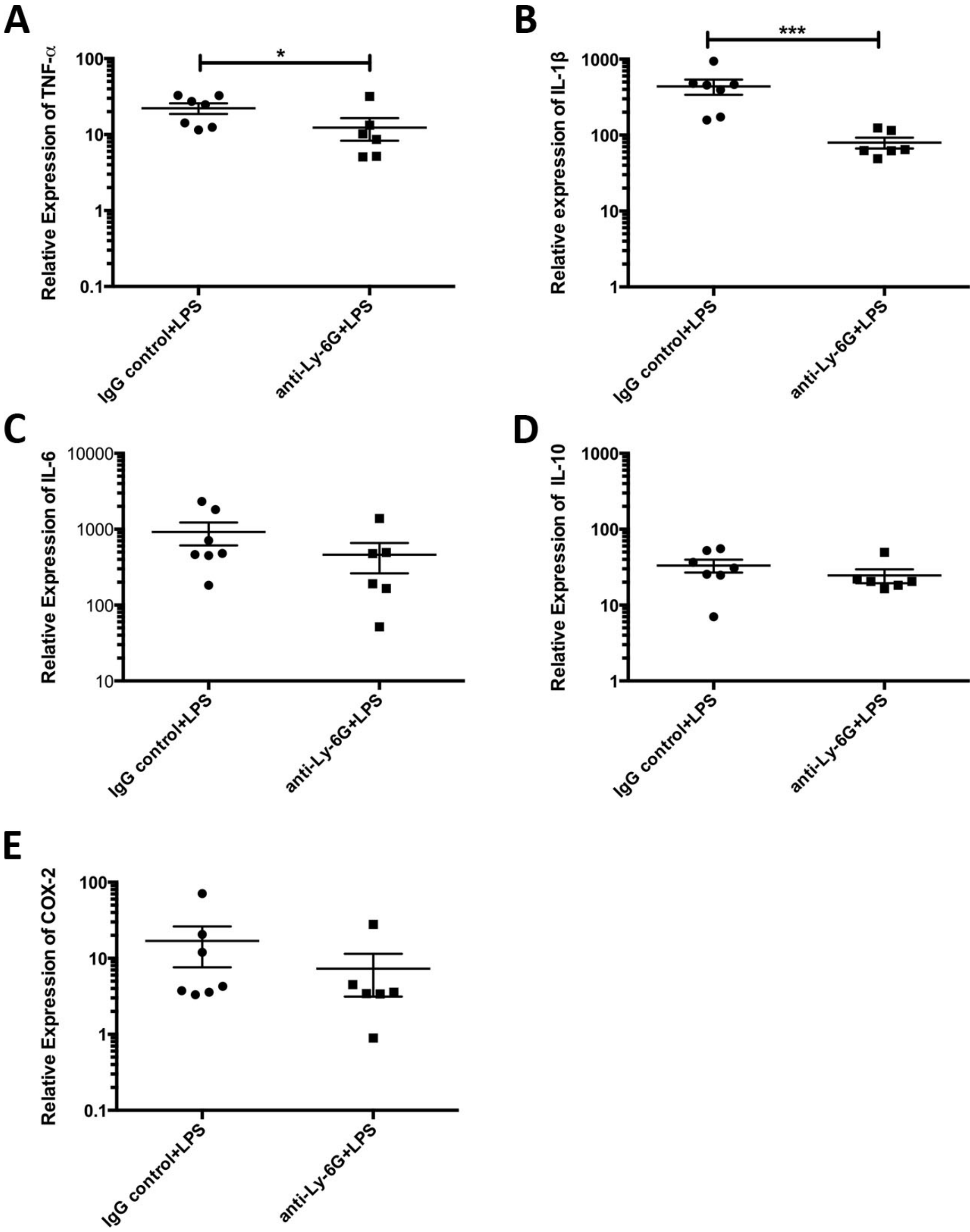

Figure 5.14 - Effect of anti-Ly-6G treatment on LPS-induced inflammatory gene expression in the uterus. Uterine tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-Ly-6G antibody+LPS (n=6) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars); *p<0.05, ***p<0.001.

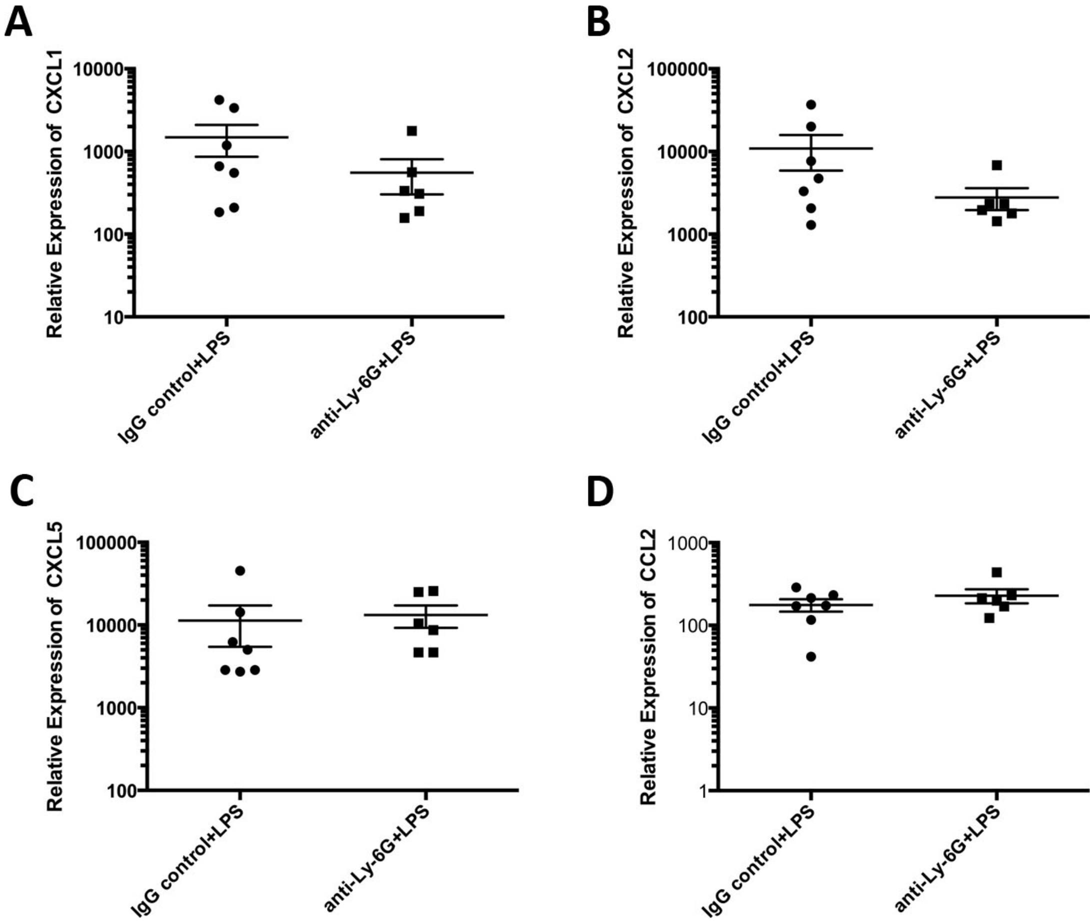

Figure 5.15 - Effect of anti-Ly-6G treatment on LPS-induced inflammatory chemokine expression in the uterus. Uterine tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-Ly-6G antibody+LPS (n=7) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars).

5.3.7.2 Inflammatory gene expression in the fetal membranes

Treatment with anti-Ly-6G+LPS did not significantly alter the expression of any of the inflammatory genes examined, with similar expression levels observed for *Tnf-*α (Figure 5.16A), *Il-1*β (Figure 5.16B), *Il-6* (Figure 5.16C), *Il-10* (Figure 5.16D) and *Cox-2* (Figure 5.16E), compared to the IgG+LPS control group.

Additionally, there were no significant differences in the expression of the chemokines *Cxcl1* (Figure 5.17A), *Cxcl2* (Figure 5.17B), *Cxcl5* (Figure 5.17C) and *Ccl2* (Figure 5.17D) when compared between anti-Ly-6G+LPS treatment and IgG control+LPS treatment.

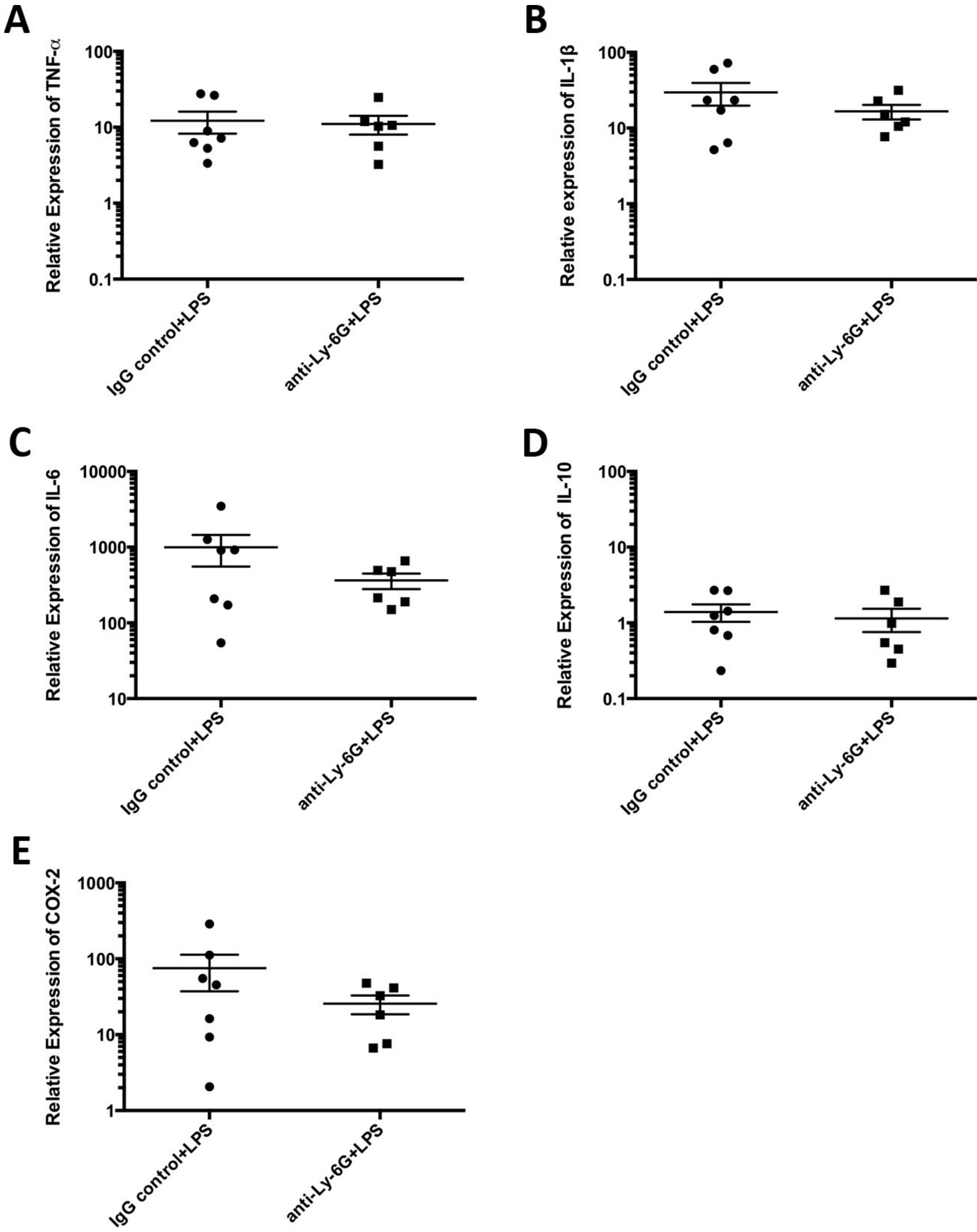

Figure 5.16 - Effect of anti-Ly-6G treatment on LPS-induced inflammatory gene expression in the fetal membranes. Fetal membranes were harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-Ly-6G antibody+LPS (n=6) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars).

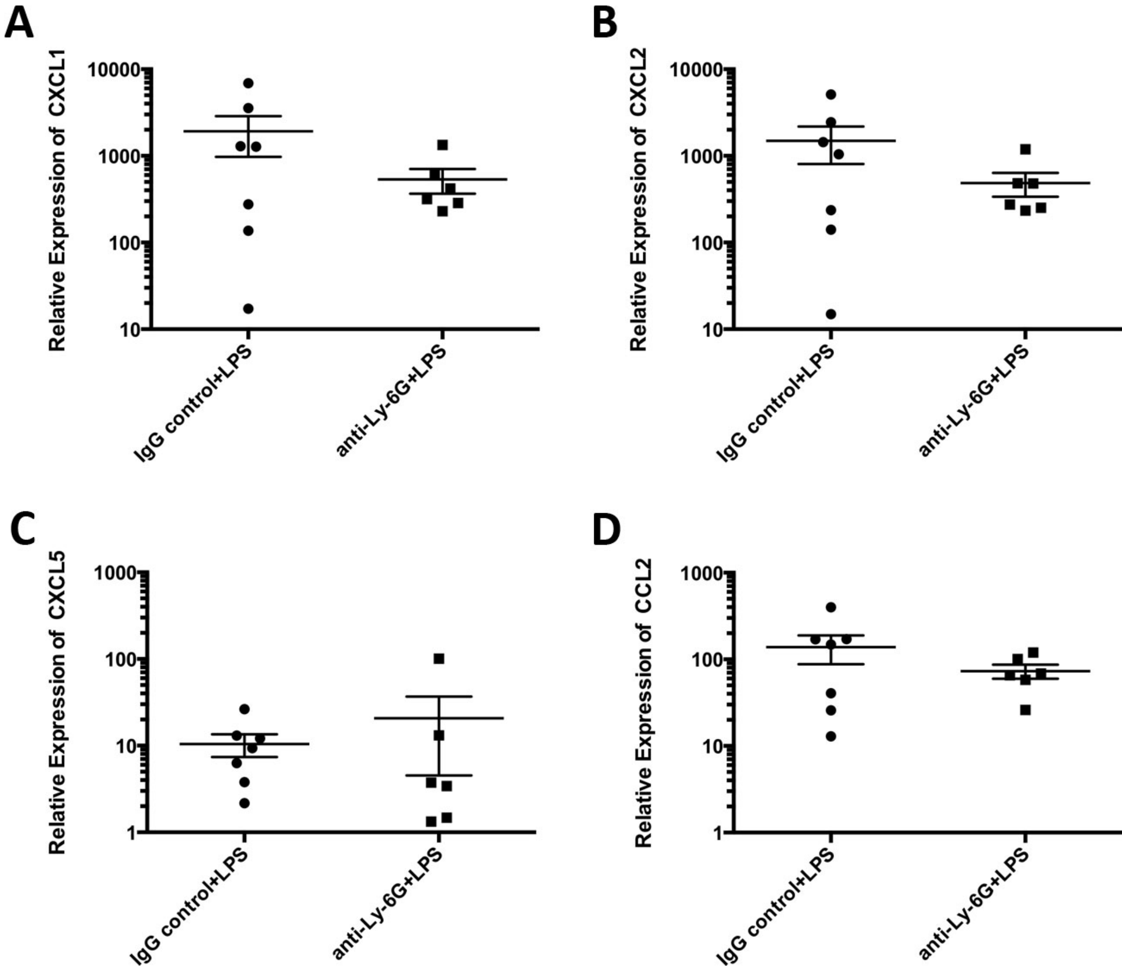

Figure 5.17 - Effect of anti-Ly-6G treatment on LPS-induced inflammatory chemokine expression in the fetal membranes. Fetal membranes were harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-Ly-6G antibody+LPS (n=7) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars).

5.3.7.3 Inflammatory gene expression in the placenta

Similar to the gene expression levels observed in the fetal membranes, there were no significant differences in the expression of *Tnf-*α (Figure 5.18A), *Il-1*β (Figure 5.18B), *Il-6* (Figure 5.18C), *Il-10* (Figure 5.18D) or *Cox-2* (Figure 5.18E) in the placental tissue of mice treated with anti-Ly-6G+LPS, compared with IgG control+LPS.

The expression of *Cxcl1* (Figure 5.19A) and *Cxcl2* (Figure 5.19B) was also unaffected by anti-Ly-6G treatment. However, placental *Cxcl5* expression was significantly elevated in mice treated with anti-Ly-6G+LPS, compared to the IgG+LPS control group (2.3-fold greater expression, $p<0.05$; Figure 5.19C). Expression of *Ccl2* was not significantly altered by anti-Ly-6G+LPS treatment, compared with the IgG+LPS group (Figure 5.19D).

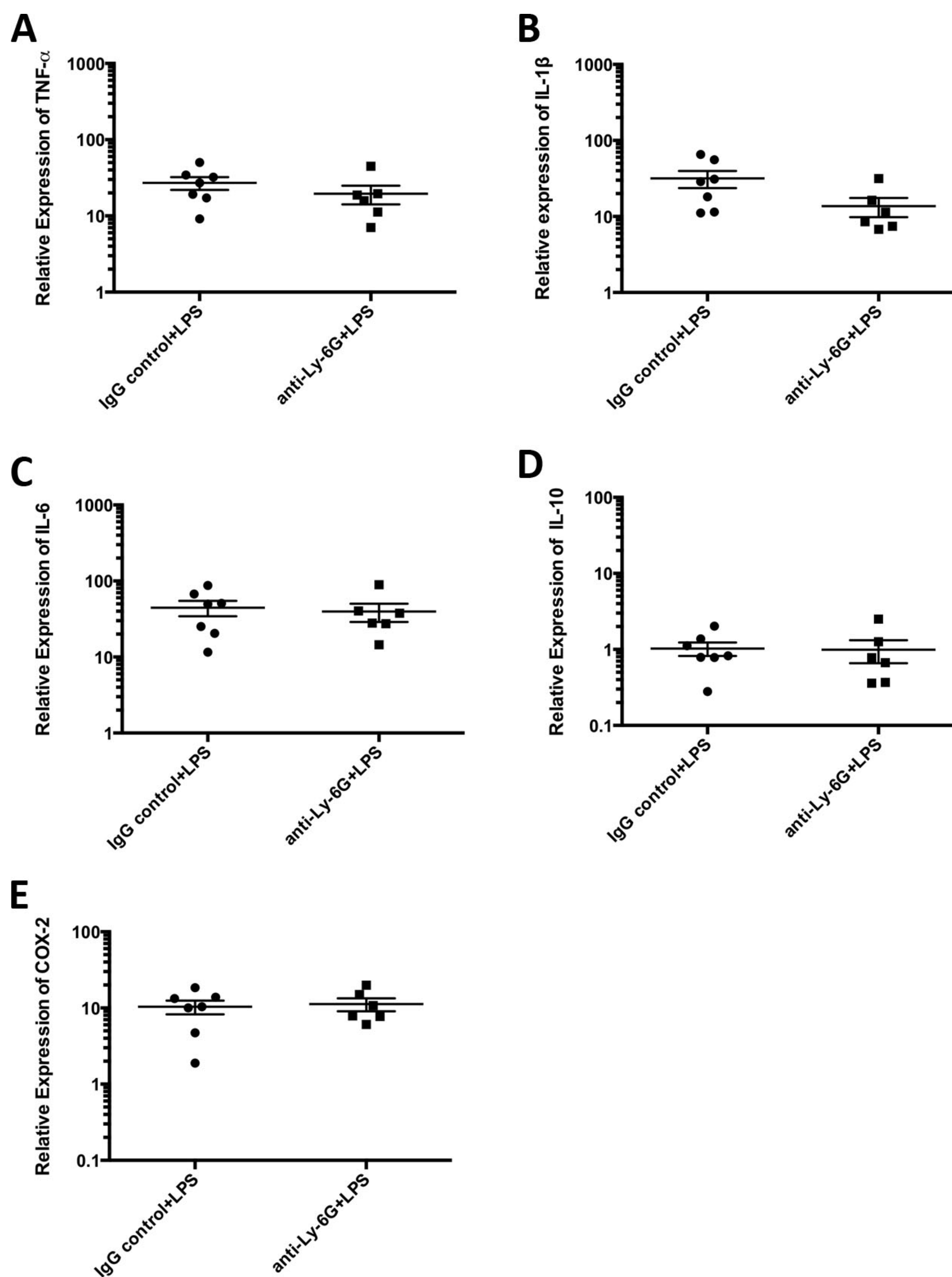

Figure 5.18 - Effect of anti-Ly-6G treatment on LPS-induced inflammatory gene expression in the placenta. Placental tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-Ly-6G antibody+LPS (n=6) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars).

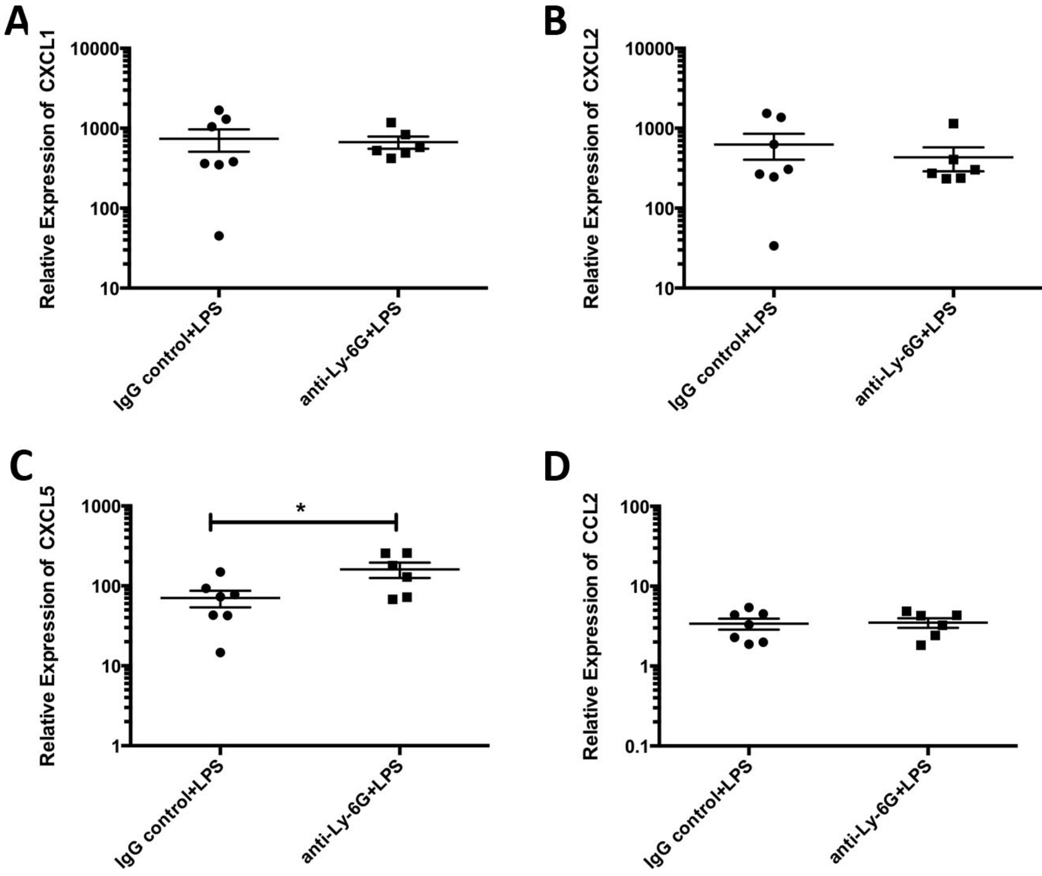

Figure 5.19 - Effect of anti-Ly-6G treatment on LPS-induced inflammatory chemokine expression in the placenta. Placental tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-Ly-6G antibody+LPS (n=7) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars); *p<0.05.

5.3.8 Effect of anti-Ly-6G treatment on LPS-induced serum TNF-α levels

As previously mentioned, neutrophil depletion using anti-Gr-1 followed by LPS treatment has been shown to result in elevated circulating TNF-α levels in a mouse model of endotoxaemia, however, depletion using anti-Ly-6G was not found to cause this increase in TNF-α levels (Daley *et al.* 2008). Therefore, we wanted to investigate whether anti-Ly-6G treatment altered circulating TNF-α levels in our model. As before, blood was collected from mice 6 hours post-intrauterine LPS injection from mice treated with either the IgG control antibody or anti-Ly-6G, and the serum concentration of TNF-α was measured by ELISA.

There was no difference in the serum concentration of TNF-α when samples obtained from mice treated with anti-Ly-6G+LPS were compared with mice receiving IgG+LPS (mean concentration anti-Ly-6G group: 19.4 pg/ml ± SEM 2.9 vs. mean concentration IgG group: 15.3 ± 1.6, $p=0.23$; Figure 5.20).

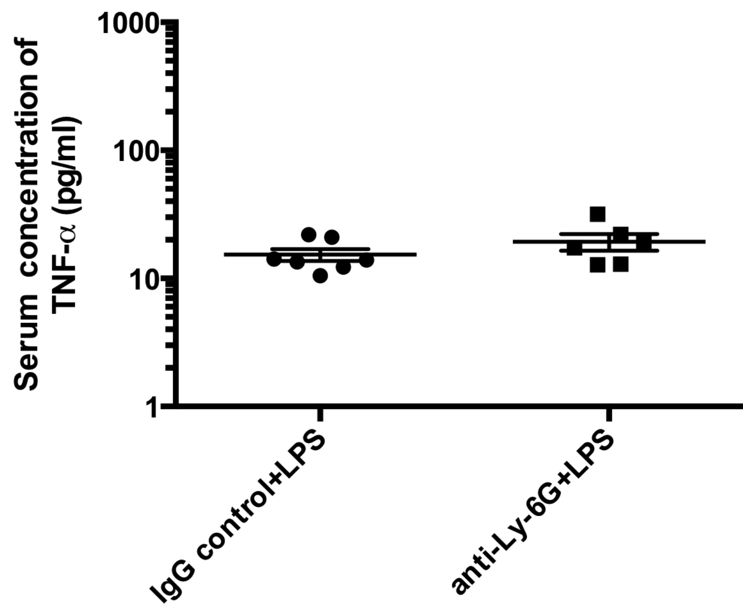

Figure 5.20 - Effect of anti-Ly-6G treatment on serum TNF-α levels. Maternal blood was collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-Ly-6G antibody+LPS (n=6) and ELISA was used to quantify the serum concentration of TNF-α. Data presented as mean ± SEM (error bars).

5.3.9 Administration of anti-F4/80 to deplete macrophages on day 17 of gestation

To investigate whether macrophages were involved in LPS-induced preterm labour and/or the LPS-induced inflammatory response in the utero-placental tissues in our model, mice were treated with anti-F4/80 prior to intrauterine LPS administration to deplete macrophages. Different methods of macrophage depletion have been found to selectively deplete different tissue-resident macrophage populations in the spleen, liver and lung (Bedoret *et al.* 2009, Dhaliwal *et al.* 2012, Ferenbach *et al.* 2012). Therefore spleen, liver and lung tissues were collected to examine whether anti-F4/80 administration in our model affected the macrophage populations in these tissues. Cervix was also collected, as a previous study has reported that anti-F4/80 administration depleted cervical macrophages in a mouse model of LPS-induced preterm labour (Gonzalez *et al.* 2011); and finally uterine tissue was also examined to determine if the resident uterine macrophage population was depleted following anti-F4/80 treatment. Mice received an intra-peritoneal injection of either anti-F4/80 or an IgG control antibody on D17 of gestation, followed 4 hours later with an intrauterine LPS injection. All tissues were harvested 6 hours post-LPS injection and fixed for immunohistochemical analysis. Macrophages were localised using the anti-F4/80 antibody.

Immunohistochemical analysis showed that anti-F4/80 treatment appeared to reduce the number of F4/80 positive cells in the spleen (Figure 5.21B), lung (Figure 5.21F) and cervix (Figure 5.21H), compared to the same tissues harvested from mice receiving the IgG control antibody (Figure 5.21A, Figure 5.21E and Figure 5.21G, respectively). The number of F4/80 positive cells appeared similar in liver tissue from mice treated with the IgG antibody, compared with mice treated with anti-F4/80 (Figure 5.21C and Figure 5.21D respectively).

Similarly, anti-F4/80 treatment did not result in an obvious reduction in the number of F4/80 positive cells with the myometrium (Figure 5.22C) or decidua (Figure 5.22D), when compared to F4/80 staining in uterine tissue harvested from mice treated with the IgG control antibody (Figure 5.22A and Figure 5.22B).

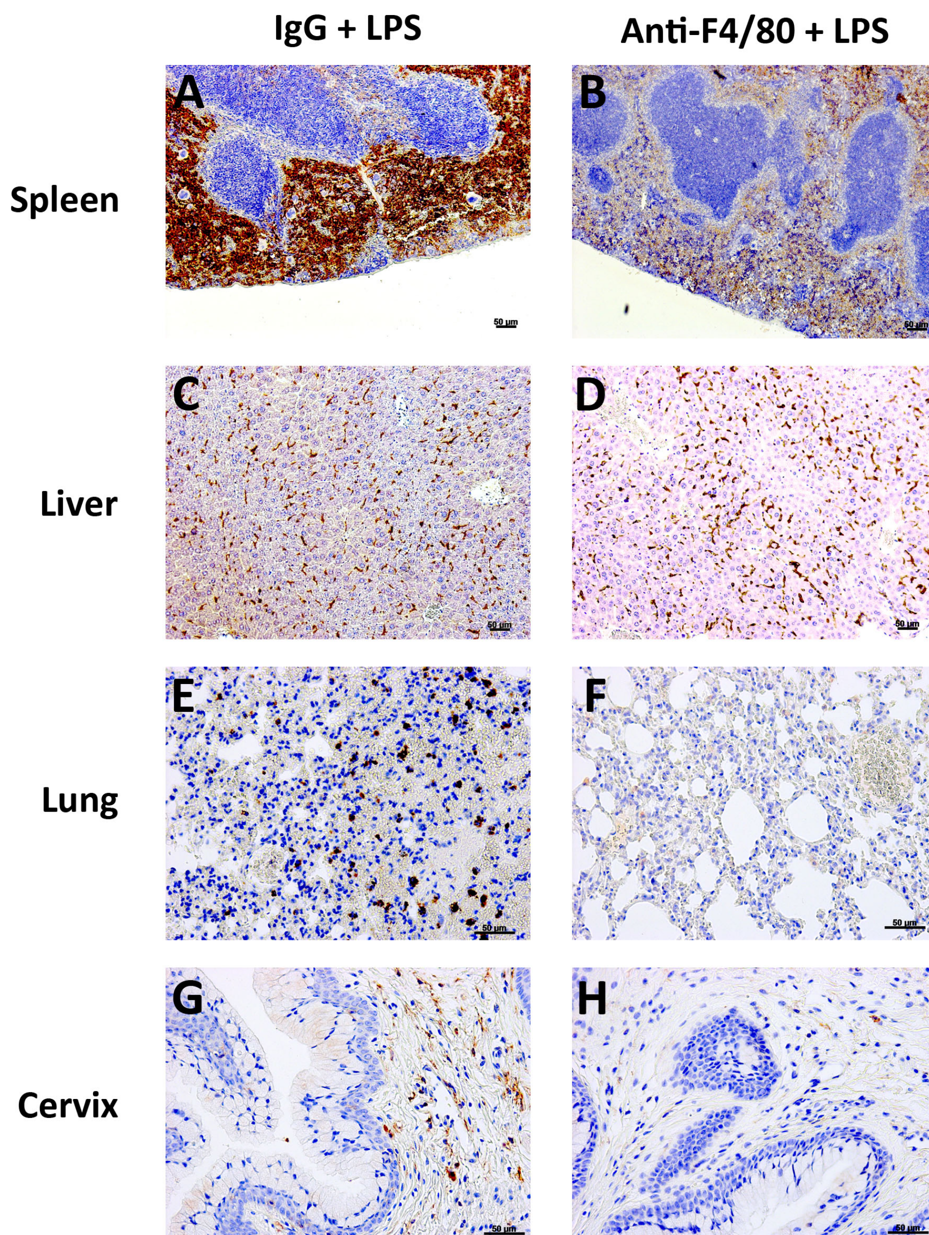

Figure 5.21 - Effect of anti-F4/80 treatment on macrophage populations in the spleen, liver, lung and cervix. Spleen, liver, lung and cervical tissue were collected 6 hours post-LPS treatment from mice treated with either the IgG control antibody (n=4) or the anti-F4/80 antibody (n=6). Representative images of macrophage localisation by immunohistochemical staining for F4/80. Macrophages were localised to the spleen in mice treated with IgG+LPS **(A)** and reduced macrophage staining was seen in spleen from mice treated with anti-F4/80+LPS **(B).** F4/80 positive cells present in liver tissue from both IgG+LPS treated **(C)** and anti-F4/80+LPS treated mice **(D).** Macrophages were present in the lung of mice in the IgG+LPS group **(E),** but fewer positively stained cells were present in the lung tissue from anti-F4/80+LPS treated mice **(F).** Similarly, macrophages were found in the cervical stroma of mice in the IgG+LPS group **(G),** with reduced staining present in the cervix from mice treated with anti-F4/80 **(H)**. Scale bars show 50μm. Images A-D were taken with a x10 objective lens, and images E-H were taken with a x20 objective lens.

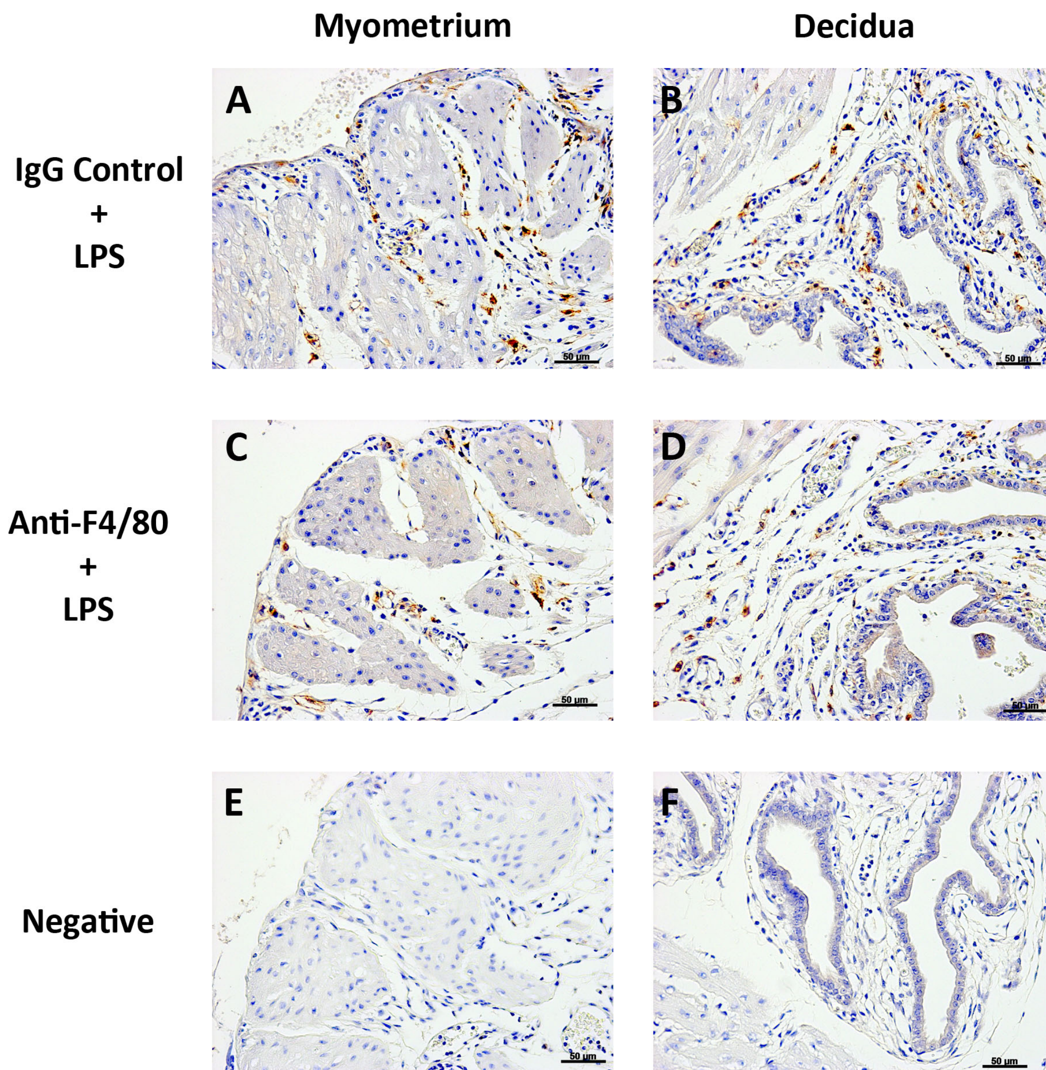

Figure 5.22 - Effect of anti-F4/80 treatment on macrophage localisation in the uterus. Uterine tissue was collected 6 hours post-LPS treatment from mice treated with either anti-F4/80 (n=6) or the IgG control antibody (n=4). Representative images of neutrophil localisation by immunohistochemical staining for F4/80. F4/80 positive cells were localised to the myometrium **(A)** and decidua **(B)** of mice treated with the IgG control antibody+LPS. Similarly, F4/80 postiive macrophage were still present in the myometrium **(C)** and decidua **(D)** of mice treated with anti-F4/80+LPS. No positive staining was observed in the negative control sections **(E)** and **(F).** Scale bars show 50μm. All images taken with a x20 objective lens.

5.3.10 Effect of anti-F4/80 treatment on LPS-induced preterm labour

Administration of anti-F4/80 antibody prior to intravaginal LPS administration has previously been shown to prevent preterm delivery in a mouse model (Gonzalez *et al.* 2011). Therefore, to investigate whether anti-F4/80 affected LPS-induced preterm delivery in our model, on D17 of gestation mice received either an intra-peritoneal injection of the appropriate IgG control antibody or anti-F4/80, then 4 hours later underwent surgery and received an intrauterine injection of 20μg LPS and the effect of anti-F4/80 depletion on LPS-induced time to delivery and the proportion of live born pups was determined.

5.3.10.1 Time to delivery

Treatment with anti-F4/80 prior to intrauterine LPS administration had no effect on LPS-induced time to delivery, compared with mice treated with the IgG control antibody (mean time to delivery in anti-F4/80 group: 20.08 hours ± SEM 2.23 vs. mean time to delivery in IgG group: 23.7 hours ± SEM 6.25; Figure 5.23A).

5.3.10.2 Proportion of live born pups

Similarly, there was no significant difference in the proportion of live born pups when compared between the anti-F4/80 group and the IgG control group (mean proportion of live born pups in anti-F4/80 group: 0.18 ± SEM 0.13 vs. mean proportion of live born pups in IgG control group: 0.23 ± 0.25; Figure 5.23B).

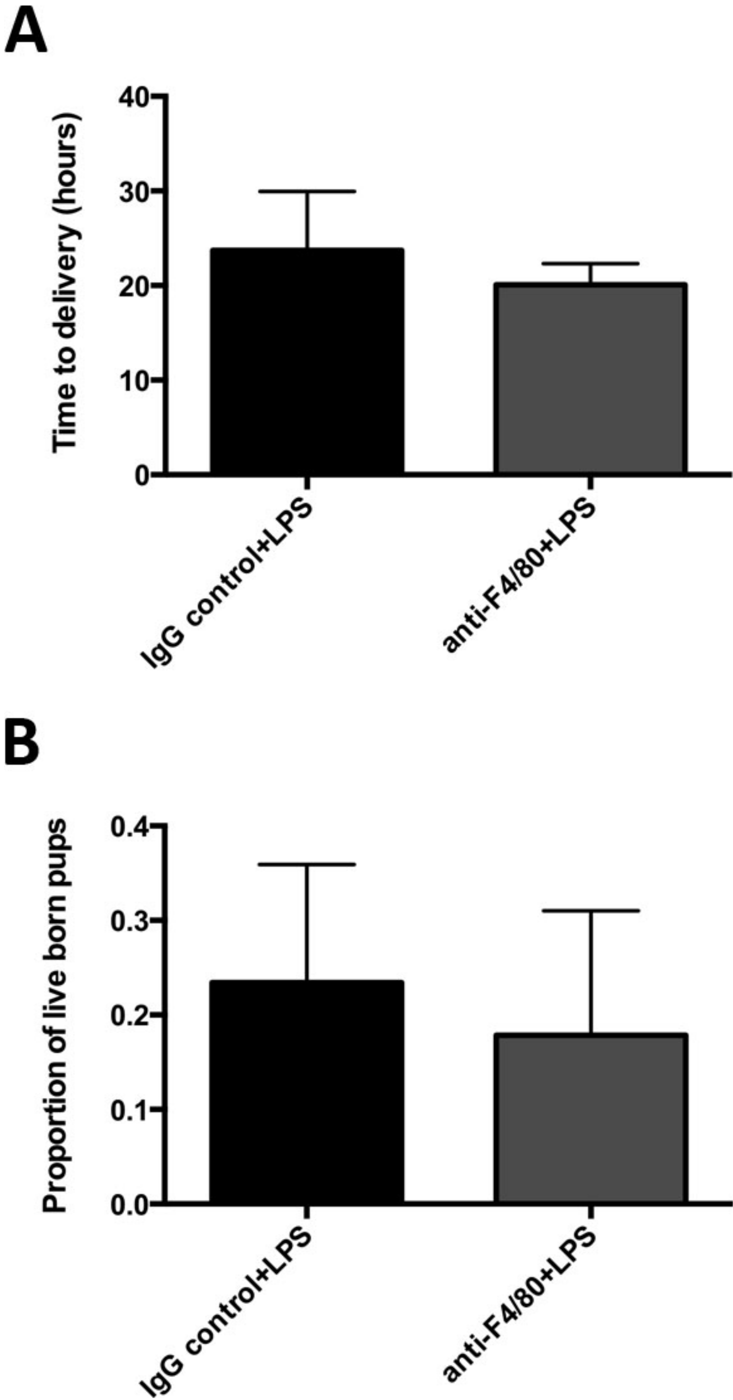

Figure 5.23 - Effect of anti-F4/80 treatment on LPS-induced preterm delivery and the proportion of live born pups. Time to delivery and the proportion of live born pups was monitored in mice treated with either the IgG control antibody+LPS (n=5) or the anti-F4/80+LPS (n=6). **(A)** Time to delivery. **(B)** Proportion of live born pups. Data are presented as mean ± SEM (error bars).

5.3.11 LPS-induced expression of inflammatory mediators in the utero-placental tissues following treatment with anti-F4/80

To examine wheher anti-F4/80 treatment altered LPS-induced inflammatory gene expression in the utero-placental tissues, mice were sacrificed 6 hours after intrauterine LPS injection and utero-placental tissues were harvested and analysed by qRT-PCR to measure the expression of: *Tnf-α, Il-1β, Il-6, Il-10, Cox-2, Cxcl1, Cxcl2, Cxcl5* and *Ccl2*.

5.3.11.1 Inflammatory gene expression in the uterus

Uterine *Tnf-α* expression was significantly reduced in mice treated with anti-F4/80+LPS, compared with the IgG+LPS control group, with 1.9-fold lower expression ($p<0.01$; Figure 5.24A).

Expression of *Il-1β* (Figure 5.24B), *Il-6* (Figure 5.24C), *Il-10* (Figure 5.24D), *Cox-2* (Figure 5.24E), *Cxcl1* (Figure 5.25A), *Cxcl2* (Figure 5.25B), *Cxcl5* (Figure 5.25C) and *Ccl2* (Figure 5.25D) was not significantly different when comparing uterine tissue collected from mice treated with anti-F4/80+LPS to the IgG+LPS group.

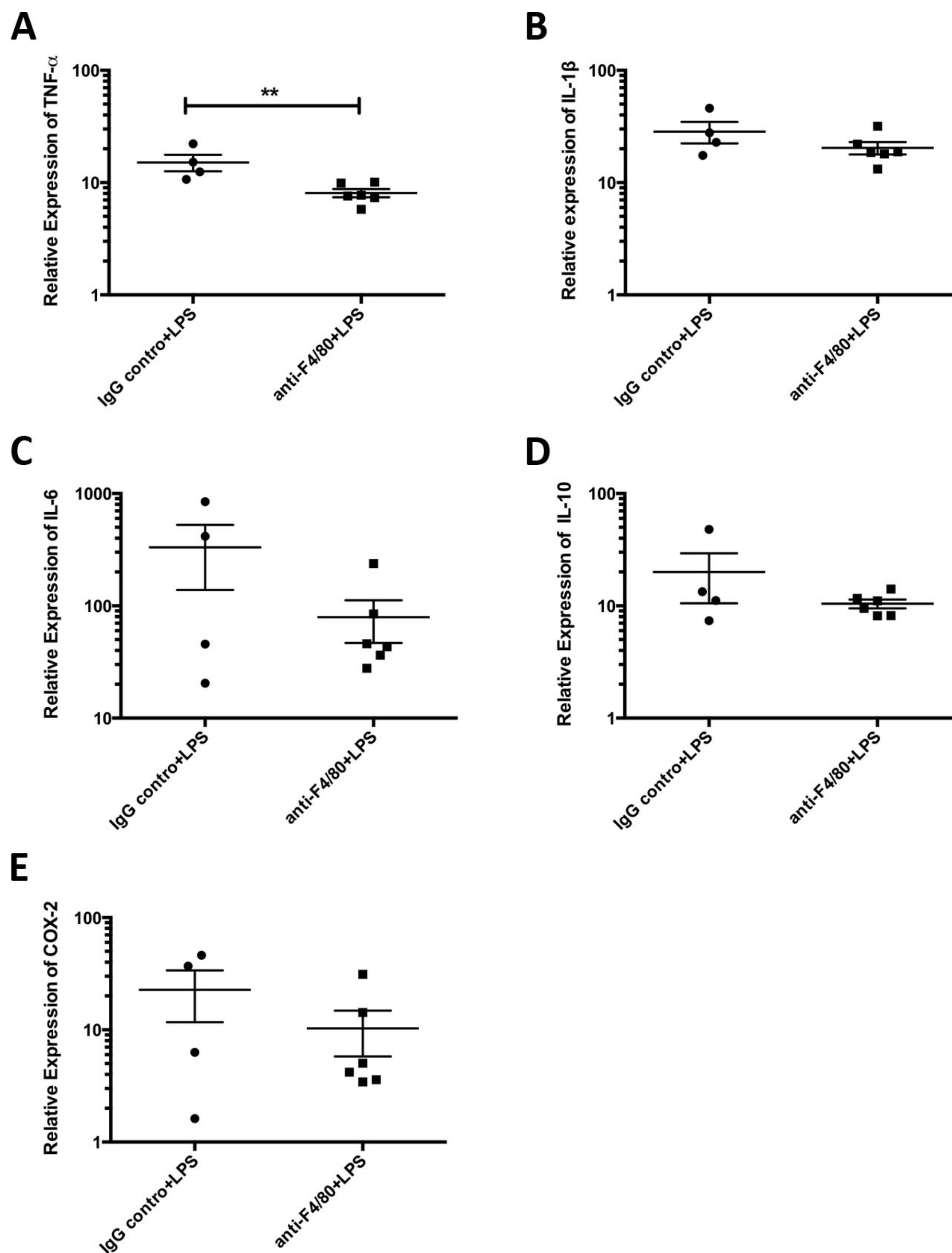

Figure 5.24 - Effect of anti-F4/80 treatment on LPS-induced inflammatory gene expression in the uterus. Uterine tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=4) or the anti-F4/80 antibody+LPS (n=6) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars); **p<0.01.

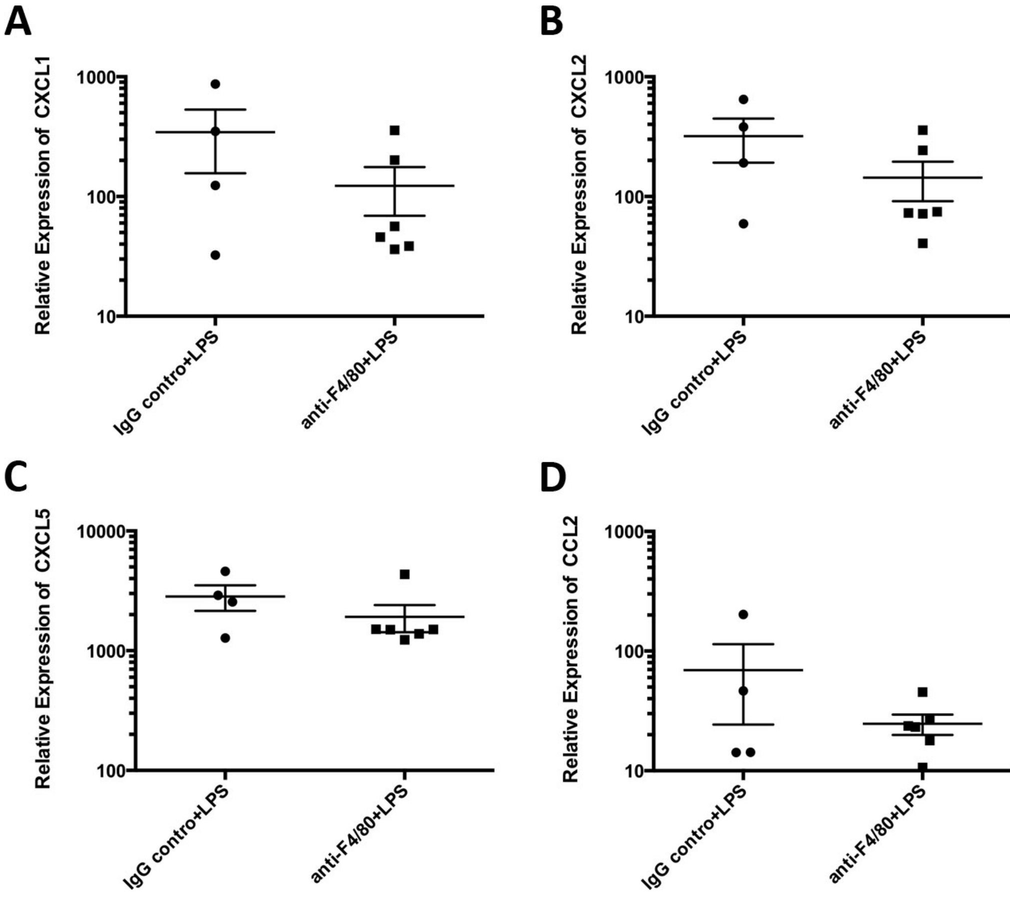

Figure 5.25 - Effect of anti-F4/80 treatment on LPS-induced inflammatory chemokine expression in the uterus. Uterine tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=4) or the anti-F4/80 antibody+LPS (n=6) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars).

5.3.11.2 Inflammatory gene expression in the fetal membranes

Anti-F4/80 treatment had no significant effect on the LPS-induced mRNA expression levels of any of the inflammatory mediators examined in the fetal membranes, when compared with the IgG+LPS control group: *Tnf-α* (Figure 5.26A), *Il-1β* (Figure 5.26), *Il-6* (Figure 5.26C), *Il-10* (Figure 5.26D), *Cox-2* (Figure 5.26E), *Cxcl1* (Figure 5.27A), *Cxcl2* (Figure 5.27B), *Cxcl5* (Figure 5.27C) and *Ccl2* (Figure 5.27D).

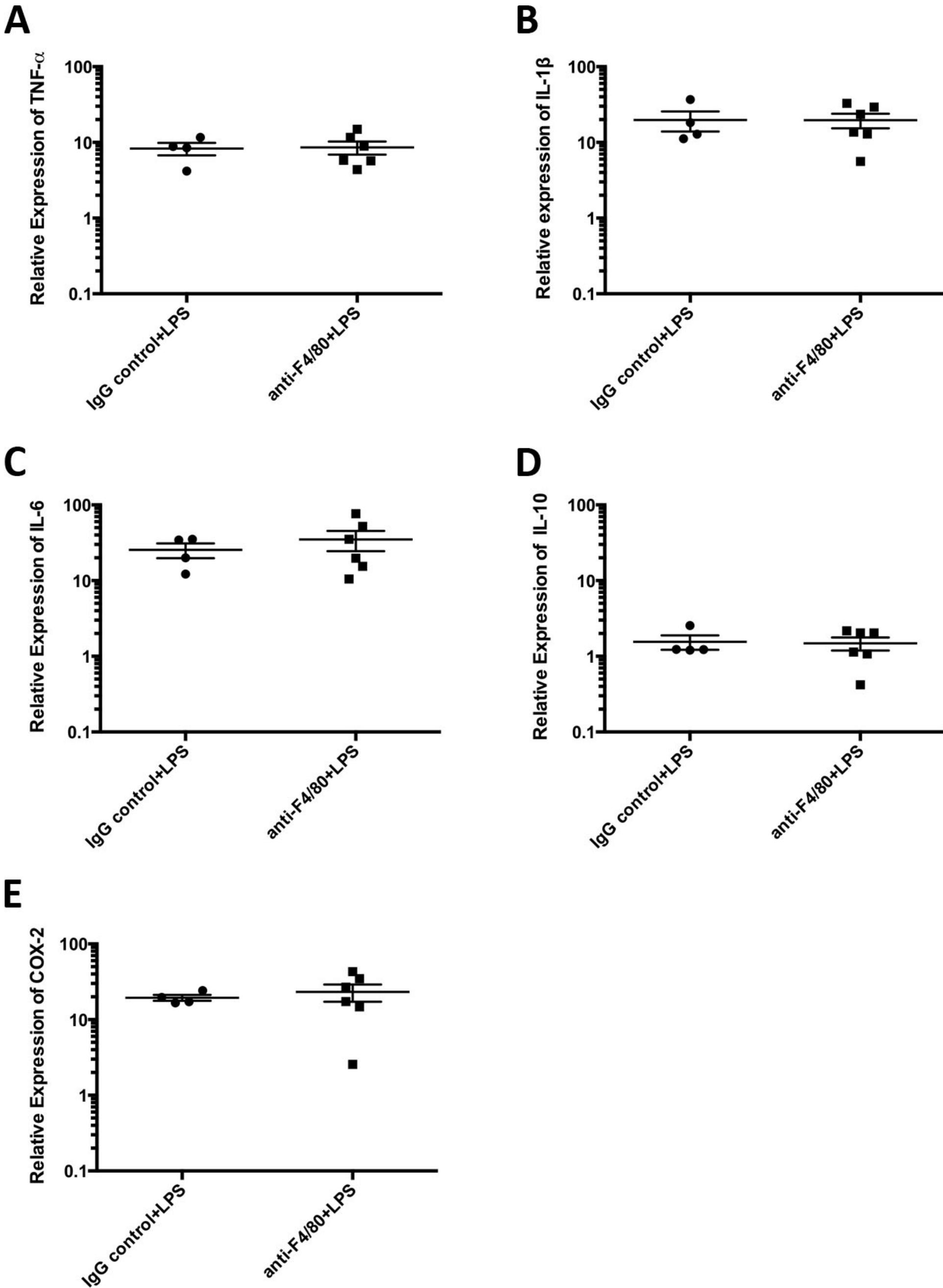

Figure 5.26 - Effect of anti-F4/80 treatment on LPS-induced inflammatory gene expression in the fetal membranes. Fetal membranes were harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=4) or the anti-F4/80 antibody+LPS (n=6) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars).

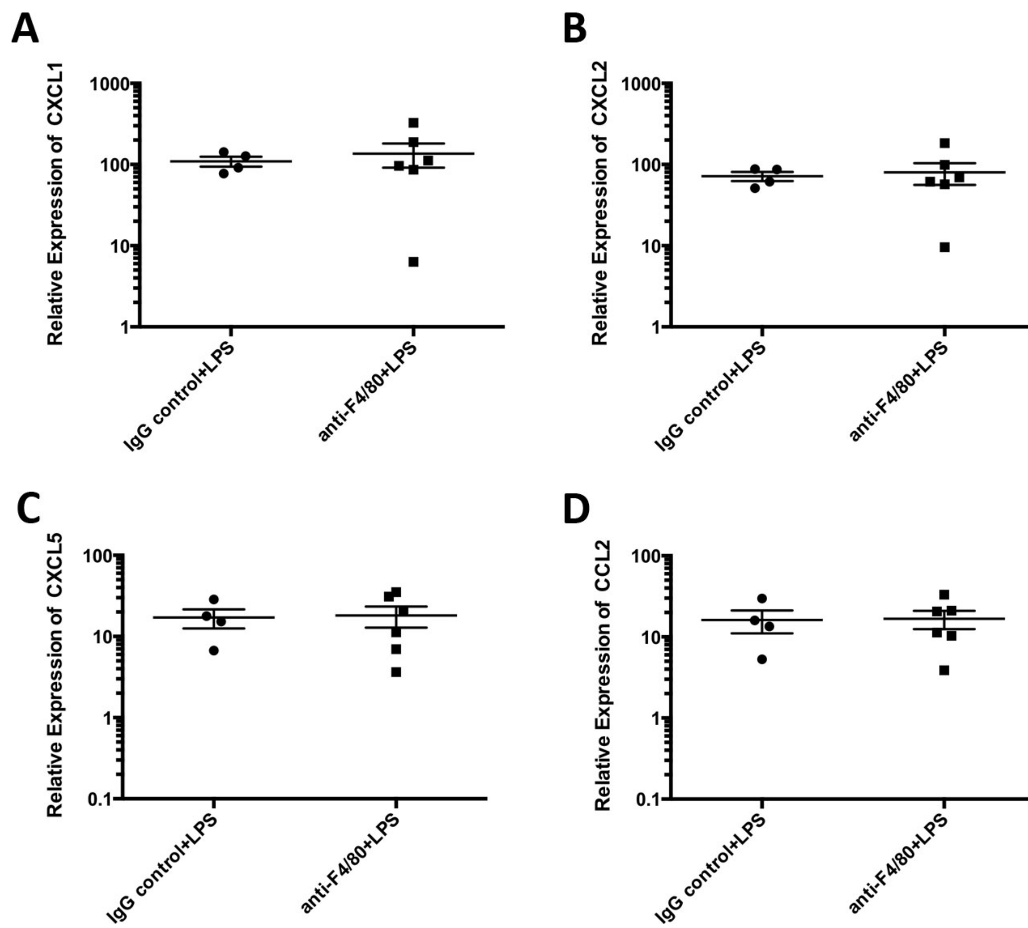

Figure 5.27 - Effect of anti-F4/80 treatment on LPS-induced inflammatory chemokine expression in the fetal membranes. Fetal membranes were harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=4) or the anti-F4/80 antibody+LPS (n=6) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars).

5.3.11.3 Inflammatory gene expression in the placenta

As observed in the fetal membranes, treatment with anti-F4/80 had no significant effect on LPS-induced mRNA expression levels of any of the inflammatory mediators examined, when compared with the IgG+LPS control group: *Tnf-*α (Figure 5.28A), *Il-1*β (Figure 5.28B), *Il-6* (Figure 5.28C), *Il-10* (Figure 5.28D), *Cox-2* (Figure 5.28E), *Cxcl1* (Figure 5.29A), *Cxcl2* (Figure 5.29B), *Cxcl5* (Figure 5.29C) and *Ccl2* (Figure 5.29D).

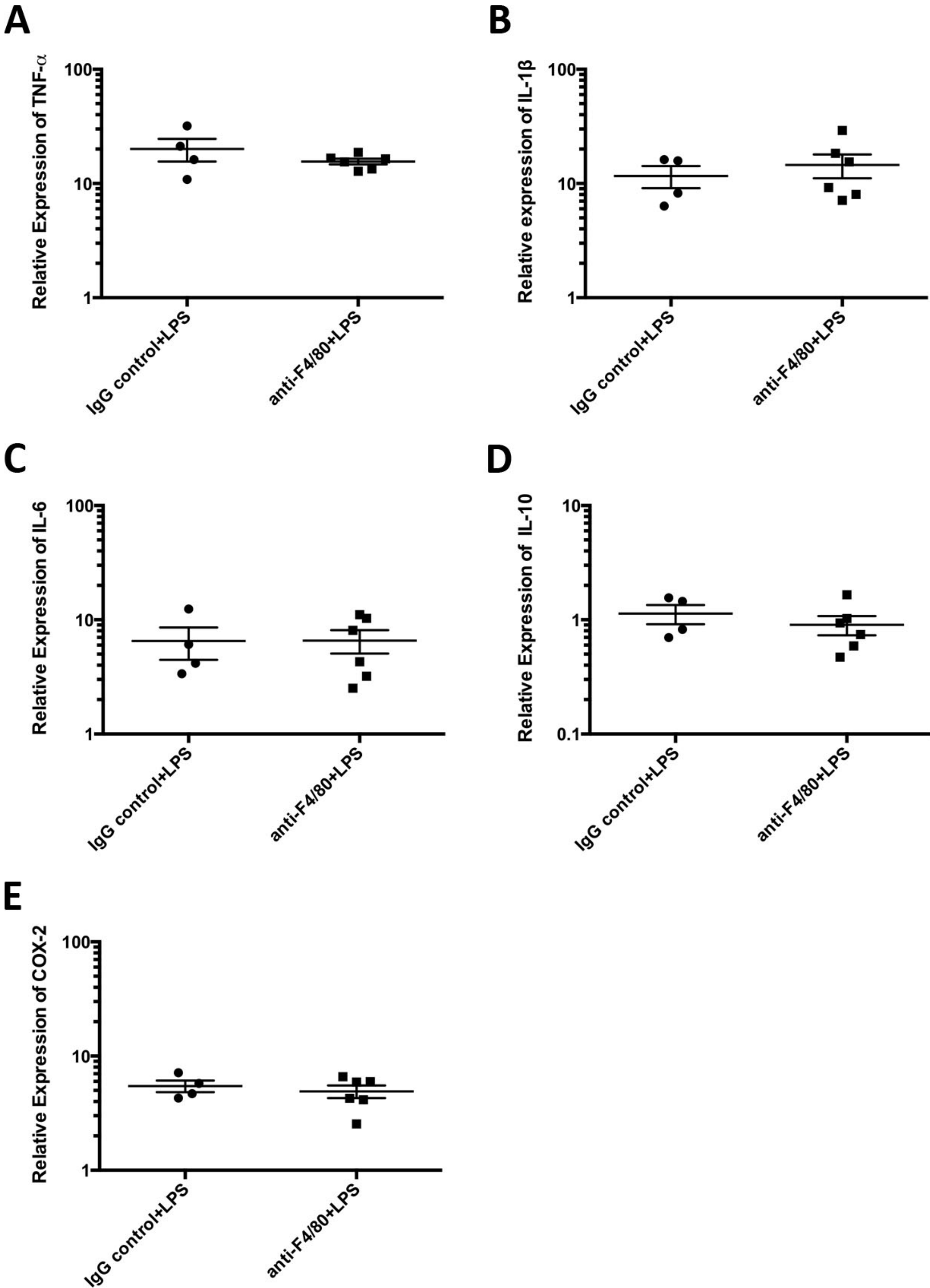

Figure 5.28 - Effect of anti-F4/80 treatment on LPS-induced inflammatory gene expression in the placenta. Placental tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=4) or the anti-F4/80 antibody+LPS (n=6) and the expression of inflammatory genes was assessed by qRT-PCR. **(A)** *Tnf-α* expression. **(B)** *Il-1β* expression. **(C)** *Il-6* expression **(D)** *Il-10* expression. **(E)** *Cox-2* expression. Data presented as mean fold change ± SEM (error bars).

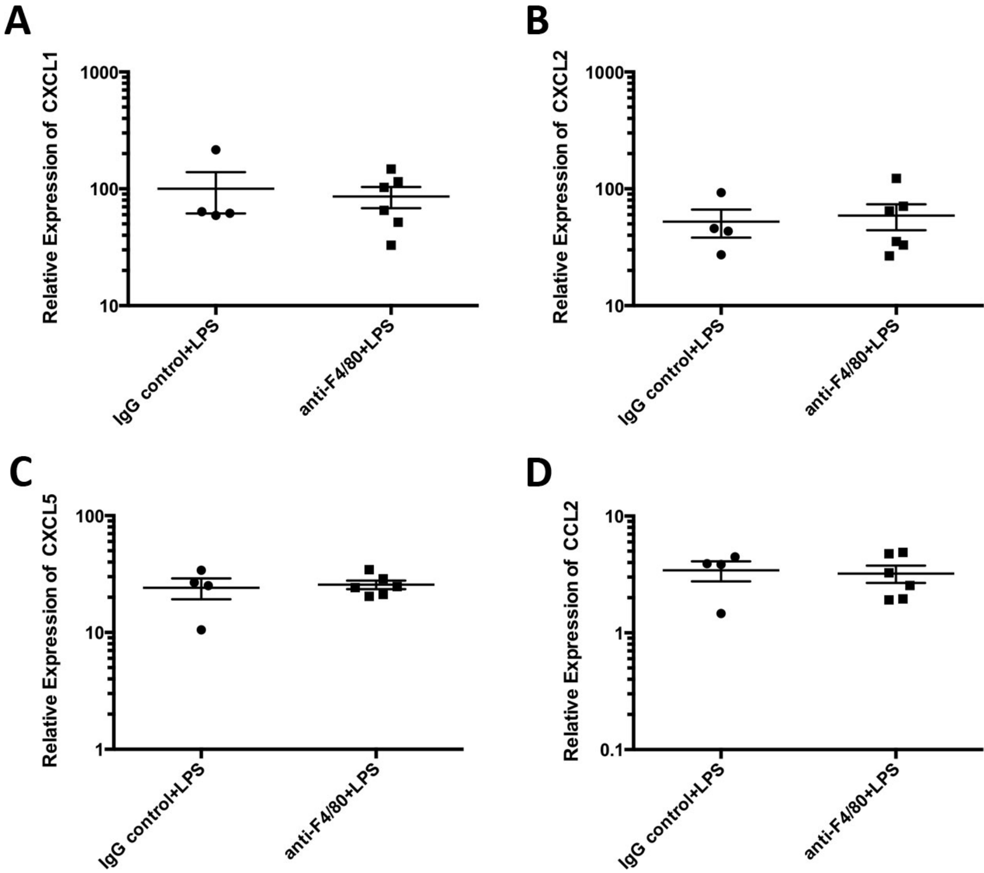

Figure 5.29 - Effect of anti-F4/80 treatment on LPS-induced inflammatory chemokine expression in the placenta. Placental tissue was harvested 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=4) or the anti-F4/80 antibody+LPS (n=6) and the expression of inflammatory chemokines was assessed by qRT-PCR. **(A)** *Cxcl1* expression. **(B)** *Cxcl2* expression. **(C)** *Cxcl5* expression. **(D)** *Ccl2* expression. Data presented as mean fold-change ± SEM (error bars).

5.3.12 Effect of anti-F4/80 treatment on LPS-induced serum TNF-α levels

To examine whether anti-F4/80 treatment altered the LPS-induced systemic inflammatory response, serum TNF-α levels were measured 6 hours post-surgery. As before blood was collected from mice 6 hours post-intrauterine LPS injection from mice treated with either the IgG control antibody or anti-F4/80, and the serum concentration of TNF-α was measured by ELISA.

There was no statistically significant difference in the serum concentration of TNF-α in mice treated with anti-F4/80+LPS, compared to the IgG control+LPS (mean TNF-α concentration in anti-F4/80 group: 28.14 pg/ml ± SEM 6.64 vs. mean TNF-α concentration in IgG control group: 69.66 pg/ml ± SEM 27.24; p=0.13 Figure 5.30).

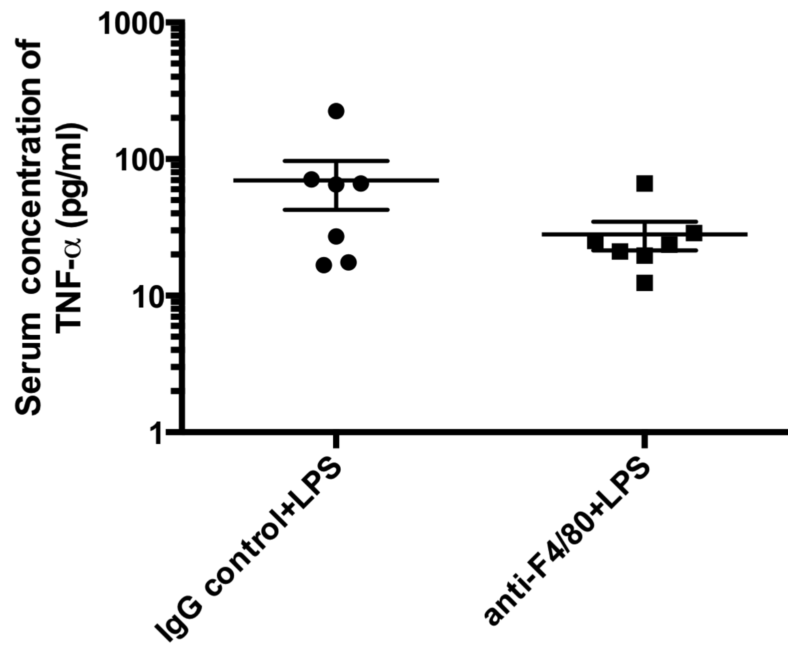

Figure 5.30 - Effect of anti-F4/80 treatment on serum TNF-α levels. Maternal blood was collected 6 hours post-surgery from mice treated with either the IgG control antibody+LPS (n=7) or the anti-F4/80 antibody+LPS (n=7) and ELISA was used to quantify the serum concentration of TNF-α. Data presented as mean ± SEM (error bars).

5.4 DISCUSSION

Studies in women have demonstrated that labour, both at term and preterm, is associated with an immune cell influx into the utero-placental tissues, particularly of neutrophils and macrophages (Young *et al.* 2002, Osman *et al.* 2003, Gomez-Lopez *et al.* 2010). Additionally, as described in chapter 3, we observed a massive neutrophil influx into the decidua of mice treated with intrauterine LPS within 6 hours of surgery, and we demonstrated that there is population of tissue-resident macrophages within the uterus, which we hypothesised might contribute to the LPS-induced inflammatory response. Therefore, the work in this chapter aimed to investigate the role of neutrophils and macrophages in both the induction of preterm labour in response to intrauterine LPS and the LPS-induced inflammatory response, using our mouse model of infection-induced preterm labour.

Antibody-mediated depletion strategies to study the role of specific immune cells in the inflammatory response

The administration of monoclonal antibodies *in vivo* to deplete specific cell types has been used for decades to investigate the role of immune cells in various disease models, including, acute lung injury (Tate *et al.* 2009, Dhaliwal *et al.* 2012), systemic infection (Daley *et al.* 2008, Shi *et al.* 2011) and carcinogenesis (Tazawa *et al.* 2003). Furthermore, therapeutic antibodies targeting specific immune cells are used clinically in the treatment of some types of cancer (Grillo-Lopez 2003) and autoimmune diseases, such as rheumatoid arthritis (Edwards and Cambridge 2006); and have also been trialled as a therapeutic option for the treatment of allergic asthma (Leckie *et al.* 2000, Nair *et al.* 2009). The mechanisms underlying antibody-based depletion of immune cells have been most well studied in the field of cancer research, investigating the mechanism of action of B-cell depleting antibodies, and are thought to involve antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, Fcγ receptors on effector immune cells, such as NK cells, monocytes or macrophages, recognise and bind to the Fc region on the monoclonal antibody, which is bound to the target immune cell. The binding of the Fcγ receptors to the Fc region of the antibody results in activation of the effector cell, which then releases cytotoxic granules resulting in apoptosis of the target immune cell (Boross and Leusen 2012). CDC involves the C1q protein complex binding to the Fc region of the

antibody, which triggers the classical complement activation pathway resulting in cell destruction via formation of the membrane attack complex (Boross and Leusen 2012). The exact depletion mechanisms of the antibodies used in this chapter are not clear, but are thought to involve the mechanisms already discussed, with other potential mechanisms still being investigated (Abbitt *et al.* 2009).

The anti-Gr-1 antibody (clone RB6-8C5) has been widely used to deplete neutrophils in mouse models to investigate the role they play in the development of inflammatory pathologies (Kaitu'u-Lino *et al.* 2007, Tate *et al.* 2008, Gong and Koh 2010). This antibody recognises two antigens expressed on the surface of some immune cells, Ly-6G and Ly-6C (Fleming *et al.* 1993). Whilst Ly-6G expression is restricted to murine neutrophils, Ly-6C is also expressed on subpopulations of monocytes, macrophages, dendritic cells and T-cells (Jutila *et al.* 1988, Hestdal *et al.* 1991, Kung *et al.* 1991, Shortman and Naik 2007). Therefore administration of anti-Gr-1 can also result in the depletion of other immune cell populations (Han and Cutler 1997, Tvinnereim *et al.* 2004, Daley *et al.* 2008), making the specific role of neutrophils in the inflammatory response difficult to interpret. For this reason, many studies now focus on using an anti-Ly-6G specific monoclonal antibody (clone 1A8) to investigate the role of neutrophils, as this antibody has been shown to selectively deplete neutrophils whilst preserving other immune cell populations (Daley *et al.* 2008, Tate *et al.* 2009, Wojtasiak *et al.* 2010, Shi *et al.* 2011).

In our model, flow cytometric analysis confirmed that administration of either anti-Gr-1 or anti-Ly-6G on D16 of gestation successfully depleted circulating neutrophils. Additionally immunohistochemical studies demonstrated that both anti-Gr-1 and anti-Ly-6G treatment prior to intrauterine LPS administration blocked the LPS-induced neutrophil recruitment into the uterus, which was still observed in mice receiving the IgG control antibody, thus again confirming successful depletion of neutrophils. A limitation of our analysis is that we did not examine the effects of depletion with either antibody on other immune cell populations. Given the evidence discussed above regarding the ability of anti-Gr-1 treatment to deplete other immune cell populations, it would be important to fully characterise the effects on other immune cells in order to understand any response to anti-Gr-1 treatment in our model.

Macrophage depletion using the anti-F4/80 antibody has been utilised in a number of animal models (Tidball and Wehling-Henricks 2007, Bedoret *et al.* 2009, Thaxton *et al.*

2009, Gonzalez *et al.* 2011, Sun *et al.* 2013). F4/80 is a glycoprotein that is highly expressed on the cell surface of mature macrophages and is a commonly used marker to identify tissue-resident macrophages in the mouse (Austyn and Gordon 1981). Another method of macrophage depletion is systemic liposomal clodronate (sLC) administration, which when ingested by macrophages induces apoptosis (Van Rooijen and Sanders 1994, Bedoret *et al.* 2009, Dhaliwal *et al.* 2012, Ferenbach *et al.* 2012). Lastly, some have used diphtheria toxin administration to CD11b diphtheria toxin receptor transgenic mice (CD11b-DTR), which are genetically modified to express the human diphtheria toxin receptor from the CD11b promoter sequence. In this scenario, diphtheria toxin administration results in conditional ablation of monocytes and macrophages (Duffield *et al.* 2005, Stoneman *et al.* 2007, Dhaliwal *et al.* 2012, Ferenbach *et al.* 2012). In contrast to circulating neutrophils, which can be rapidly depleted from the blood in response to antibody treatment, the depletion of macrophages is complicated by the fact that macrophages are tissue-resident cells and these different methods of macrophage depletion have been reported to deplete some tissue-resident macrophage populations and not others. For example, a recent study by Dhaliwal *et al*. (Dhaliwal *et al.* 2012), investigating the role of monocytes in LPS-induced lung injury, reported that sLC administration depleted peripheral blood monocytes and macrophages resident in both the liver and spleen, whilst the lung alveolar macrophage population remained unchanged. The same study also investigated the CD11b-DTR mouse as a method of depletion and found that there was 90% depletion of peripheral blood monocytes, with reduced depletion in the spleen, and with this method macrophage populations in the liver and lung were unaffected (Dhaliwal *et al.* 2012). Similar findings have also been reported by others (Bedoret *et al.* 2009, Ferenbach *et al.* 2012), again highlighting the complexity of macrophage depletion models.

Immunohistochemical analysis of F4/80 positive cells in our model demonstrated that anti-F4/80 treatment appeared to reduce the number of F4/80 positive cells in the spleen, lung and cervix, although some macrophages were still present; while the macrophage population of the liver appeared unaffected. In the uterus, macrophages were still present in both the myometrium and decidua of mice treated with the anti-F4/80 antibody. As formal quantification of F4/80 positive cells was not carried out in these preliminary studies we cannot definitively exclude the possibility that anti-F4/80 treatment did result in a small reduction in macrophage numbers in the uterus.

Macrophage depletion using the anti-F4/80 antibody has been previously investigated during pregnancy, where anti-F4/80 treatment has been reported to deplete macrophages in the cervix (Gonzalez *et al.* 2011) and in the utero-placental tissues (Thaxton *et al.* 2009, Sun *et al.* 2013). The reason why similar depletion of macrophages in the uterus was not observed in our study is not clear, but differences in the timing and duration of anti-F4/80 treatment may be important. In some of the previous studies, the anti-F4/80 antibody was administered twice during pregnancy (on D5 and D7; or D13 and D15), and depletion was examined two days later by flow cytometry (Thaxton *et al.* 2009, Sun *et al.* 2013). In contrast, in our study anti-F4/80 was administered once on D17, and tissues were harvested the same day. Perhaps if repeated doses of anti-F4/80 had been given, or if the tissues had been examined at a later time-point, we may have observed significant depletion of macrophages.

Effect of immune cell depletion on LPS-induced preterm labour

As it has become increasingly clear that labour, both at term and preterm, is an inflammatory event that is associated with immune cell influx into the utero-placental tissues (Thomson *et al.* 1999), there has been growing interest in understanding the role these immune cells play in the induction of labour. For that reason, *in vivo* depletion studies have become a useful tool in this field of research. In recent years, studies have used antibody-based depletion methods to deplete various immune cell populations including: macrophages (Thaxton *et al.* 2009, Gonzalez *et al.* 2011, Sun *et al.* 2013), neutrophils (Timmons and Mahendroo 2006, Thaxton *et al.* 2009, Sun *et al.* 2013) and uterine NK cells (Murphy *et al.* 2005, Murphy *et al.* 2009, Thaxton *et al.* 2009, Sun *et al.* 2013) to investigate their role in normal and adverse pregnancy outcomes.

These previous studies found that neutrophil depletion in mice using the anti-Gr-1 antibody (RB6-8C5) had no effect on normal term labour (Timmons and Mahendroo 2006) or in models of CpG oligodeoxynucleotide (CpG ODN)-induced preterm labour, in IL-10 knockout (Thaxton *et al.* 2009) and NK cell-deficient non-obese diabetic (NOD) mice (Sun *et al.* 2013). CpG ODN are short DNA molecules that are abundant in the microbial genome which induce an inflammatory and immune response via TLR-9 activation (Thaxton *et al.* 2009). As we had observed a massive recruitment of neutrophils in to the decidua of mice treated with LPS, but not in mice treated with PBS,

we hypothesised that this neutrophil influx may play an important role in the initiation of LPS-induced preterm labour. Therefore, we sought to investigate this using two different antibodies. In line with these previous studies discussed, we chose to deplete neutrophils using the anti-Gr-1 antibody, however we also used the more specific anti-Ly-6G antibody to ensure any effects we observed were due to neutrophil depletion and not the depletion of other subpopulations of immune cells. This is the first study to examine the effect of neutrophil depletion using anti-Gr-1 in a model of LPS-induced preterm labour, and also the first study to use the specific anti-Ly-6G antibody to investigate the role of neutrophils in a model of preterm labour. In line with these previous studies which investigated the effect of depletion with anti-Gr-1 in other models of preterm labour, we found that neutrophil depletion with anti-Gr-1 did not delay LPS-induced preterm delivery, compared to the IgG+LPS control group. In fact, the anti-Gr-1 treated mice seemed to deliver somewhat earlier than the control group, although this was not statistically significant. Depletion of neutrophils using anti-Ly-6G also did not delay LPS-induced preterm delivery, and again the mean time to delivery was slightly lower in the depleted mice compared to the control group, however not to the same extent as observed with anti-Gr-1 treatment. These data suggest that neutrophils are not required for the induction of LPS-induced preterm labour in our model, and in fact may have a role in controlling the LPS-induced inflammatory response, given that the depleted mice delivered earlier. Further studies would be needed to confirm this hypothesis.

As discussed previously (in chapter 3), neutrophils are proposed to play an important role in the repair and re-modelling of the uterus during the post-partum period (Timmons *et al.* 2009, Shynlova *et al.* 2013, Shynlova *et al.* 2013), therefore it would be interesting to examine what effect the neutrophil depletion has during the post-partum period, and to investigate whether repeated administration of the antibodies to maintain neutrophil depletion may impact uterine remodelling and therefore potentially hinder subsequent pregnancies. Support for this hypothesis comes from evidence from a mouse model of menstruation that demonstrated that neutrophil depletion (using the anti-Gr-1 antibody) significantly delayed endometrial repair following menstruation (Kaitu'u-Lino *et al.* 2007).

The proportion of live born pups was also lower in neutrophil depleted mice (with either antibody), compared to the respective control groups, however again this was not statistically significant. This could suggest that neutrophils play a role in protecting

the fetus from the adverse effects of LPS treatment, but may also just reflect the shorter time to delivery observed in the neutrophil depleted groups (particularly in the anti-Gr-1+LPS group), resulting in preterm delivery early on D17, when the pups are unlikely to be developmentally competent to survive.

Previous studies investigating macrophage depletion in adverse pregnancy outcomes have proved more promising. Depletion of macrophages with anti-F4/80 has been shown to rescue CpG ODN-induced early pregnancy loss and preterm birth in IL-10 knockout (Thaxton *et al.* 2009) and NK cell-deficient NOD mice (Sun *et al.* 2013). Additionally, Gonzalez *et al*. demonstrated that macrophage depletion prevented LPS-induced preterm delivery in a mouse model (Gonzalez *et al.* 2011). However, in contrast we found that anti-F4/80 treatment prior to intrauterine LPS administration had no effect on LPS-induced preterm delivery in our model. This difference was unexpected, as we used the same dose and timing of anti-F4/80 administration as that used by Gonzalez *et al,* but the different routes of LPS administration used may explain it. Gonzalez *et al.* administered LPS intravaginally on D15 of gestation (Gonzalez *et al.* 2011), while our model is of intrauterine LPS administration on D17. It is therefore possible, that intravaginal LPS administration, which results in a more local cervical inflammatory response, is more macrophage-dependent, than the inflammatory response induced by intrauterine LPS administration. It should also be noted, as discussed above, that anti-F4/80 treatment did not appear to cause depletion of macrophages from the body of the uterus, although there did appear to be depletion from the cervix, as reported by Gonzalez *et al.* (Gonzalez *et al.* 2011). Even if there were a small number of macrophages depleted from the uterine tissue, due to the large tissue-resident population of macrophages, there was still a large number of cells that could still respond to LPS. Therefore, although these data presented here suggest macrophages are also not necessary for the induction of LPS-induced preterm delivery in our model, it is difficult to draw a firm conclusion at present, as anti-F4/80 treatment did not appear to deplete the local macrophage population within the uterine tissue. To investigate further the role of macrophages in our model of infection-induced preterm labour, the macrophage depletion protocol would need to be optimised, either by administering additional doses of anti-F4/80 or by trying an alternative method of macrophage depletion such as sLC administration or using the CD11b-DTR mouse, as described above.

Effect of immune cell depletion on the LPS-induced inflammatory response

Having observed that neither neutrophil nor macrophage depletion had a significant effect on LPS-induced preterm delivery in our model, we investigated whether either of these immune cell populations contributed to the LPS-induced inflammatory response observed in the utero-placental tissues and serum. The previous studies investigating immune cell depletion in preterm labour models have focussed more on the outcome of preterm labour, rather than examining the effect of immune cell depletion on the inflammatory signalling of the utero-placental tissues. Of the studies mentioned above, only two have examined inflammatory signalling in the utero-placental tissues following depletion, and these studies only examined TNF-α production; both studies showed that depletion with anti-Gr-1 had no effect on TNF-α in utero-placental tissue, whereas anti-F4/80 treatment reduced TNF-α production (Thaxton *et al.* 2009, Sun *et al.* 2013). Therefore, we sought to examine the effect of immune cell depletion on a wider range of inflammatory markers in our model. Interestingly, depletion with the three different antibodies resulted in different inflammatory gene expression changes within the utero-placental tissues examined.

Depletion with the anti-Gr-1 antibody resulted in decreased *Il-1β* expression in the uterus and placenta, whilst the expression of several inflammatory mediators, including *Il-6*, *Cxcl1* and *Cxcl2* in the fetal membranes was significantly elevated in response to anti-Gr-1+LPS treatment, compared to the IgG+LPS control group. These results suggest that the Gr-1 positive depleted cells contribute to the observed LPS-induced increase in *Il-1β* expression in the uterus and placenta. In contrast, Gr-1 positive cells may play a role in inhibiting inflammatory signalling in the fetal membranes, as lack of Gr-1 positive cells induced increased expression of a number of inflammatory genes. Specific depletion of neutrophils using the anti-Ly-6G antibody resulted in fewer gene expression changes, with significantly reduced expression of *Tnf-α* and *Il-1β* in the uterus and increased *Cxcl5* expression in the placenta; no change was found in inflammatory gene expression in the fetal membranes following Ly-6G depletion.

The different responses observed to depletion using anti-Gr-1, compared to anti-Ly-6G again highlights that these two antibodies do not target the same immune cell population. It is possible that the large number of changes in inflammatory gene expression observed with anti-Gr-1, which are not apparent following anti-Ly-6G

treatment, are a result of depletion of the other subsets of immune cells expressing Ly-6C which are also depleted upon Gr-1 administration. Previous studies have also reported a more pro-inflammatory and more severe phenotype following Gr-1 administration, compared with Ly-6G administration, and it is proposed that the non-neutrophil Gr-1 positive cells may have an important inhibitory role in regulating inflammatory signalling (Daley *et al.* 2008, Wojtasiak *et al.* 2010). In line with these data suggesting anti-Gr-1 depletion results in a more pro-inflammatory response, in our model, anti-Gr-1 treatment resulted in a much shorter time to delivery compared to anti-Ly-6G treatment (mean time to delivery in anti-Gr-1+LPS group: 8.95 hours compared with mean time to delivery in anti-Ly-6G+LPS group: 19.25 hours) and induced a 2.5-fold (non-significant) increase in serum TNF-α levels, which was not observed following anti-Ly-6G treatment. Future work should focus on identifying the non-neutrophil Gr-1 positive cell populations that may play an important role in limiting the inflammatory response, as understanding more about how the infection-induced inflammatory response is regulated in the utero-placental tissues may help in the identification of novel targets for the treatment of preterm labour.

As has been previously reported (Thaxton *et al.* 2009, Sun *et al.* 2013), treatment with anti-F4/80 resulted in reduced expression of *Tnf-α* in the uterus and a 2-fold (non-significant) reduction in serum TNF-α levels. As macrophages are the major source of TNF-α (Riches *et al.* 1996), these results suggest that although not obvious by immunohistochemical analysis, anti-F4/80 treatment may have resulted in some macrophage depletion in the uterus.

Therefore, whilst immune cell depletion in our model suggests that neutrophils and macrophages are not required for the induction of preterm delivery in response to intrauterine LPS, they do have a role in contributing to the LPS-induced inflammatory response within the utero-placental tissues.

Summary

This chapter has described the use of antibody-mediated depletion of neutrophils and macrophages to investigate their role in LPS-induced preterm labour. This is the first study to investigate the effect of neutrophil depletion in a mouse model of LPS-induced preterm labour, and furthermore, the first study to use the specific anti-Ly-6G antibody

to investigate the role of neutrophils. Flow cytometry and immunohistochemical analysis confirmed successful depletion of neutrophils in response to both anti-Gr-1 and anti-Ly-6G treatment, however neutrophil depletion did not have a significant effect on LPS-induced preterm delivery. Similarly, macrophage depletion using anti-F4/80 treatment also did not affect LPS-induced preterm delivery in our model, although how effective the anti-F4/80 treatment was in depleting macrophages in our model remains unclear. Although no effect on LPS-induced preterm delivery was observed, depletion of both neutrophils and macrophages altered the LPS-induced inflammatory response in the utero-placental tissues, suggesting that whilst these immune cells appear not to be required for LPS-induced preterm delivery, they do contribute to the inflammatory response of the utero-placental tissues in response to intrauterine LPS administration. Immune cell depletion is a useful tool to investigate the role different immune cell populations play in infection-induced preterm labour, however, these data highlight the importance of choosing the most appropriate depletion strategy to ensure efficient depletion in the target tissues, and specific depletion of the immune cell population of interest. Using alternative depletion strategies may prove more useful to investigate the role of macrophages in preterm labour, and this work highlights that future work investigating the role of neutrophils should be carried out using the specific anti-Ly-6G antibody, as different responses were observed when comparing depletion with anti-Gr-1 and anti-Ly-6G.

Chapter 6 - General discussion

6.1 SUMMARY OF FINDINGS

Preterm labour remains a major clinical problem worldwide, with limited effective treatment options available. Given that many cases of preterm labour occur in the presence of an intrauterine infection, extensive research is being carried out to understand how the presence of an intrauterine infection triggers preterm labour and to investigate potential novel therapeutic options to stop preterm delivery that will ultimately improve neonatal outcome. This thesis has investigated the role of infection and inflammation in a mouse model of infection-induced preterm labour, with specific aims to: characterise the inflammatory and immune response to a bacterial intrauterine infection; investigate the potential of anti-inflammatory agents to delay preterm delivery and improve neonatal outcome; and to examine the role of immune cells in the induction of infection-induced preterm labour and the local inflammatory response. The main findings of this work are summarised below:

- Intrauterine administration of LPS at a dose of 20μg on D17 of gestation successfully induced preterm labour in a mouse model.
- Intrauterine administration of LPS induced increased expression of several key pro-inflammatory mediators within the utero-placental tissues, amniotic fluid and serum; highlighting the presence of an intrauterine infection induces a robust inflammatory response, even in response to doses of LPS that did not induce preterm delivery.
- The increased inflammatory response induced by LPS treatment appears to be largely independent of the actions of progesterone, as systemic progesterone withdrawal was not observed.
- In response to intrauterine LPS administration there was an influx of neutrophils into the decidua within 6 hours of LPS treatment, whilst macrophage numbers remained stable. Surgery itself appeared to induce neutrophil recruitment to the myometrium, even in mice receiving only intrauterine PBS.
- Pre-treatment with epi-lipoxin did not delay LPS-induced preterm delivery, but mice that delivered prematurely who were treated with epi-lipoxin (and LPS)

had a significantly increased proportion of live born pups, compared with mice receiving LPS alone.

- Epi-lipoxin increased the expression of *Cox-2* within the utero-placental tissues, and down-regulated *15-Hpgd* expression, suggesting that lipoxin may be promoting the production of anti-inflammatory prostaglandins.

- Pre-treatment with BML-111 prior to intrauterine LPS administration significantly reduced the preterm delivery rate, but did not have a significant impact on the mean time to delivery or proportion of live born pups.

- Pre-treatment with IL-10 prior to intrauterine LPS administration had no significant effect on LPS-induced preterm delivery or the proportion of live born pups.

- Neutrophil depletion using either the anti-Gr-1 or anti-Ly-6G antibody did not attenuate LPS-induced preterm delivery. Similarly, treatment with the anti-F4/80 antibody to deplete macrophages also did not significantly affect LPS-induced preterm delivery

- Although immune cell depletion did not attenuate LPS-induced preterm delivery, the inflammatory response induced by intrauterine LPS administration was altered, suggesting that although not required for LPS-induced preterm delivery neutrophils and macrophages are important in contributing to the LPS-induced inflammatory response within the utero-placental tissues.

6.2 MOUSE MODEL OF PRETERM LABOUR

Animal models have been used extensively in the field of parturition research to understand the basic mechanisms underlying the onset of spontaneous labour at term and to investigate potential novel therapeutic options for the treatment of preterm labour. As previously discussed, the pros and cons of using various animal models for parturition research have been extensively reviewed (Elovitz and Mrinalini 2004, Mitchell and Taggart 2009, Ratajczak *et al.* 2010). Whilst there are clear differences in

the reproductive physiology and the mechanisms required to initiate parturition between animal models and humans, the limited number of samples that can be feasibly and ethically collected from a woman throughout pregnancy and the high degree of inter-patient variability, make animal models invaluable to this field of research. A variety of animal models have been used in preterm birth research, including sheep, rats, non-human primates and guinea pigs, however by far the most commonly used animal model is the mouse. Indeed using the mouse as a model for parturition research has many advantages, including: a short gestational period; ease of breeding; a relatively well characterised immune system; and the availability of genetically modified mice in which a single factor can be either knocked out or over-expressed to investigate the role it may play in parturition (Hirsch and Wang 2005). In particular, the work presented in this thesis, and in other papers has highlighted the similarities between the inflammatory responses to a bacterial intrauterine infection. Limitations of using a mouse model to study parturition include physiological differences between mouse and human pregnancy, such as their bicornuate uterus, multiple pregnancies and short gestational period (Hirsch and Wang 2005); and the main issue often raised is the dependency of systemic progesterone withdrawal for the initiation of parturition in this model. However, several recent studies have now demonstrated that in the context of infection-induced preterm labour, systemic progesterone withdrawal does not appear to be integral to the induction of labour (Hirsch and Muhle 2002, Murphy *et al.* 2009, Gonzalez *et al.* 2011); similar to the results we obtained in our study. Therefore, whilst there are clear limitations to the use of any animal model, the mouse continues to provide a useful *in vivo* model with which to investigate further the molecular mechanisms of parturition and to test novel treatment options for preterm labour.

The model used in this thesis, based on the model originally described by Elovitz *et al.* (Elovitz *et al.* 2003), is a useful model to study the role of a local intrauterine infection in the induction of preterm labour. However, there are clear limitations to this model that became apparent during our work. The invasive nature of the surgery, during which the entire uterus is externalised to receive an intrauterine injection, prior to being returned to the abdominal cavity, resulted in a high preterm delivery rate even in our PBS control group. Therefore, the variable response to surgery became a confounding factor in the model. Whilst a certain degree of variability between animals is to be expected, particularly when performing surgery on pregnant animals close to

delivery, we feel that reducing the invasiveness of the surgery involved is key to improving the reliability of the model for future studies. Additionally, such an approach is consistent with the "3Rs" (refinement, reduction and replacement) philosophy which should underpin all studies involving animals. To this end, we have carried out a preliminary experiment investigating the potential of using ultrasound-guided intrauterine injection of bacterial agents, thus avoiding the requirement for invasive surgery. Currently we have only trialled injecting methylene blue dye and an ultrasound contrast agent as a 'proof-of-principle' experiment to test whether it was still possible to give a direct intrauterine injection between two gestational sacs, without externalisation of the uterus. We found that using ultrasound as a guide, it was possible to still inject specifically into the uterus, without entering either of the amniotic sacs. A representative image of this pilot data is given in Figure 6.1; this highlights that the UV contrast agent injected is localised to the space between two gestational sacs. When the uterus was opened up it was clear that the methylene blue dye was localised to the intrauterine space and had not spread elsewhere, including in the intra-amniotic space. Whilst preliminary, the results are promising and suggest that using ultrasound-guided injection in this model could provide a novel method with which to introduce LPS into the uterus in future experiments, which would remove the confounding effect of invasive surgery and improve the reliability, reduce the variability and ultimately decrease the number of animals required for experiments. The next step will be to perform some further experiments with PBS and LPS, to examine whether we still get reliable induction of preterm labour in response to ultrasound-facilitated LPS treatment, and with a reduced incidence of preterm labour in ultrasound-facilitated PBS-treated mice.

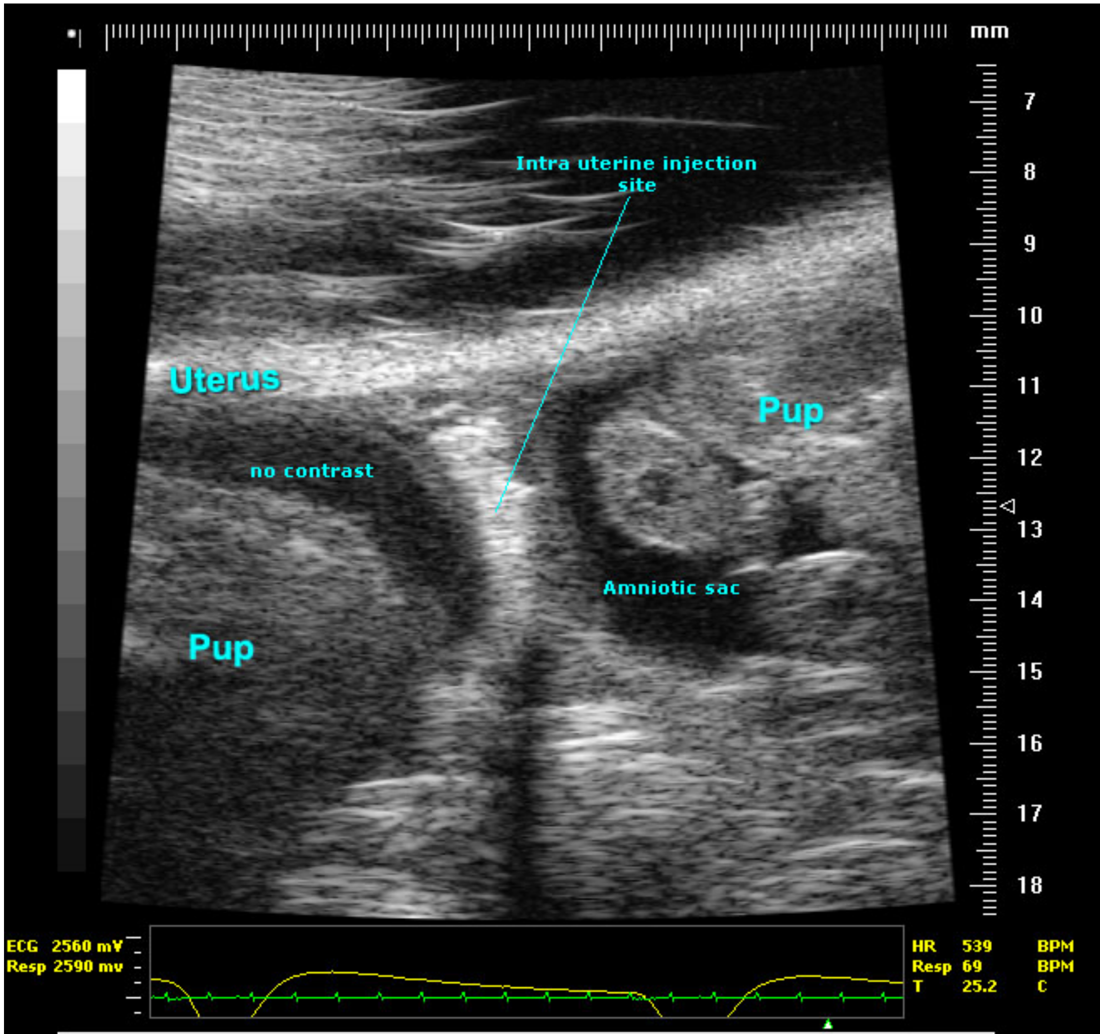

Figure 6.1 - Image of ultrasound-guided intrauterine injection. Ultrasound contrast agent (white) can be seen localised to the uterine space between two gestational sacs, with none present within the gestational sacs.

6.3 ROLE OF IMMUNE CELLS IN PRETERM LABOUR

Whilst there is now considerable evidence that labour is an inflammatory event associated with increased production of inflammatory cytokines and chemokines, and an influx of immune cells into the utero-placental tissues (Thomson *et al.* 1999, Sennstrom *et al.* 2000, Young *et al.* 2002, Osman *et al.* 2003), their precise role remains unclear. Work by Young *et al.* demonstrated that these infiltrating leukocytes produce inflammatory cytokines, thus contributing to the inflammatory environment (Young *et al.* 2002), however whether the infiltration of leukocytes is a key event in triggering the onset of parturition, or merely a consequence of the heightened inflammatory signalling remains to be elucidated.

To investigate the role that specific immune cell populations play in the induction of preterm labour, genetically modified mice that lack particular immune cell subsets, or antibody-based depletion of immune cells have been used. The results of these studies to date have been mixed. Although a number of studies have reported that depletion of some immune cell populations, including macrophages, NK cells and invariant NKT (iNKT) cells, attenuates infection-induced preterm delivery in mouse models (Murphy *et al.* 2009, Gonzalez *et al.* 2011, Li *et al.* 2012); the same result has not been observed in other studies examining the role of T and B lymphocytes and neutrophils (Bizargity *et al.* 2009, Thaxton *et al.* 2009). Similarly, the results presented in our study showed no benefit of depleting either neutrophils or macrophages prior to intrauterine LPS administration. Therefore, at present, it is unclear what role immune cells play in the onset of infection-induced preterm labour, as it appears that in some cases they are not required for the induction of parturition. The mixed success of these studies highlights the complex interactions of different immune cells and it is likely that different inflammatory stimuli will induce different immune cells to influx, thus making depletion of one single immune cell population difficult to interpret in each case.

Given that the primary role of immune cells is to defend the body against infectious diseases, depletion of immune cells could result in increased susceptibility to infectious stimuli, which may explain why the neutrophil-depleted mice in our study showed a trend for earlier delivery in response to intrauterine LPS administration, compared with the non-depleted mice. Interestingly, several other studies have reported that depletion of neutrophils actually results in increased susceptibility to infection and poorer outcomes in other models of infection-induced pathologies (Jarchum *et al.* 2012,

Ribes *et al.* 2013). Therefore, while the results from our study, alongside data from previously published work, suggest that the infiltrating immune cells contribute to the inflammatory response of the utero-placental tissues by producing inflammatory chemokines and cytokines, this response may be secondary to the protective role these cells may initially play in attempting to fight the infection.

Currently the precise role immune cells play in the onset of parturition, in particular in the context of an intrauterine infection remains poorly understood. Several studies suggest targeting some specific immune cell populations may be a novel therapeutic option, however these data come from mouse models, therefore further investigation into the relevance of these findings to women is required. Better characterisation of the immune cell populations that infiltrate the utero-placental tissues, both at term labour and in response to a pathological infection, and the factors they produce, will help us to understand the role these cells play in the process of parturition. There will clearly be differences in the role immune cells play in the presence of an intrauterine infection, compared to their role during spontaneous term labour, but improving our understanding of these roles will allow us to determine whether targeting immune cells and/or their products is likely to be a beneficial novel treatment option, and if so which specific immune cell populations should be targeted. The potential for immune cell depletion to heighten the inflammatory response to an intrauterine bacterial infection must also be carefully considered. Nevertheless, it is clear that continuing research into the role different immune cell populations play in the onset of term and preterm labour is required to elucidate the underlying mechanisms involved in the initiation of partition and to identify novel targets for the treatment of preterm labour.

6.4 NOVEL TREATMENT OPTIONS FOR PRETERM LABOUR: WILL ANTI-INFLAMMATORY DRUGS BE THE ANSWER?

Despite considerable research, preterm birth remains the single biggest cause of neonatal mortality worldwide. The currently available treatment options are limited, and although some can be effective at delaying labour for a short period, there is little evidence that these treatments result in an improved outcome for the baby. Therefore, the search continues for novel, more effective treatment options. A poor understanding

of the underlying causes of preterm labour has hindered the development of novel treatments, as without fully understanding why labour is initiated prematurely it is impossible to prevent. However, the identification of intrauterine infection and/or inflammation as important causative factors in many cases has led researchers to investigate whether targeting inflammatory signalling pathways may be a useful new option.

Most current treatments focus on blocking the clinical signs of labour, i.e. myometrial contractions, when they are already underway. Given that it now seems clear that myometrial contractions, along with fetal membrane rupture and cervical ripening, are strongly driven by an inflammatory signalling cascade, there is growing interest in using anti-inflammatory treatments. This interest in anti-inflammatory agents is driven by the hypothesis that targeting this inflammatory cascade earlier, prior to the onset of contractions, may prove to be more effective, as it could halt the inflammatory cascade before it has progressed too far.

An important consideration in the search for novel treatment options for preterm labour is that the primary goal must be to improve neonatal outcome and not merely to stop preterm delivery. For example, in the context of intrauterine infection, premature delivery may remove the baby from a potentially harmful *in utero* environment, and delaying this delivery could in fact worsen the outcome for the baby. However, another potential advantage of using anti-inflammatory treatments is the hope that some anti-inflammatory mediators that play important roles in driving the resolution of inflammation, such as lipoxins, may be able to resolve the inflammatory *in utero* environment, thus protecting the fetus from the adverse consequences associated with intrauterine infection and/or inflammation. Indeed, although the work presented here is still at an early stage, it is encouraging to note that treatment with epi-lipoxin did improve the survival of prematurely born pups in our mouse model.

Theoretically, the use of anti-inflammatory treatments during pregnancy could result in suppression of both maternal and fetal immune responses, which could result in increased susceptibility to infection. Whether this is a real concern would need further investigation, and could perhaps be limited by keeping the period of administration of the anti-inflammatory agent to a minimum. Another potential concern surrounding the use of anti-inflammatory drugs is that many of these pro-inflammatory factors have important roles outwith their role in the inflammatory cascade associated with labour,

for example NF-κB is a transcription factor for a large number of genes, not just those involved in parturition, therefore blocking this signalling pathway may have deleterious effects which would need to be investigated. Additionally, as with any drugs given during pregnancy, extensive research would be required to investigate any adverse effects on the fetus.

As research leads to a greater understanding of the underlying mechanisms responsible for initiating the events surrounding the onset of parturition, more potential targets will be identified; however, given the considerable evidence that inflammation plays an integral role in the onset of parturition, it seems clear that anti-inflammatory agents have the potential to be an exciting novel therapeutic option in the treatment of preterm birth. Although still at an early stage, there is now promising *in vivo* and *in vitro* data that demonstrate the potential of anti-inflammatory treatments. The possibility of a combination therapy approach may hold promise in the future, where agents such as lipoxin may prove beneficial as an adjuvant to classic tocolytic therapy or progesterone treatment. The heterogeneous nature of preterm labour means that it is both unlikely and unrealistic to believe that one treatment option will be effective in all women, and whether anti-inflammatory agents will ultimately improve neonatal outcome remains to be elucidated at present. However, while preterm birth remains the leading cause of neonatal mortality and morbidity worldwide, claiming the lives of more than one million children each year, continued research into all potential novel treatment options is urgently required.

6.5 CONCLUSIONS

The work presented in this thesis has demonstrated that in response to intrauterine LPS, as a surrogate model of infection, a robust inflammatory and immune response is mounted in the utero-placental tissues, involving the increased production of a number of inflammatory mediators and neutrophil influx into the decidua, which ultimately results in preterm delivery. The role immune cells play in the induction of infection-induced preterm labour remains unclear, as depletion of neutrophils and macrophages was not found to attenuate LPS-induced preterm delivery. Although the anti-inflammatory agents investigated here did not delay LPS-induced preterm delivery,

epi-lipoxin treatment did significantly improve pup survival in pups born preterm, suggesting it could be useful in protecting the fetus from the adverse effects of infection-induced preterm labour. Using animal models, such as the one described here, are vital to improving our understanding of the underlying mechanisms involved in the induction of preterm labour and are useful models with which to investigate the potential of novel therapeutic interventions. Collectively, these data confirm that the presence of an intrauterine infection is associated with a robust inflammatory response in the intrauterine tissues and suggest that anti-inflammatory agents, such as lipoxins, warrant further research as potentially promising new treatment options for preterm labour.

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APPENDICES

Appendix 1 - Materials

MOUSE SURGERY AND TISSUE COLLECTION	SOURCE
Isoflurane-Vet	Merial Animal Health Ltd, Harlow, Essex, UK
Hamilton syringe	Sigma-Aldrich, Poole, Dorset, UK
LPS (*E. Coli* 0111:B4)	Sigma-Aldrich, Poole, Dorset, UK
PBS	Gibco, Life Technologies Ltd, Paisley, UK
Mersilk Black 5/0 12mm 3/8 circle reverse cutting needle sutures	MidMeds Ltd, Waltham Abbey, UK
Coated Vicryl Violet 4/0 16mm 3/8 circle cutting needle sutures	MidMeds Ltd, Waltham Abbey, UK
Vetergesic Multidose	Alstoe Ltd, York, UK
Digital video recorder	AVerMedia Technologies Inc, UK
Mini Color IR Camera (SKC160IR)	Sunkwang Electronics Co., Ltd
RNA*later*®	Sigma-Aldrich, Poole, Dorset, UK

MOUSE SURGERY TREATMENTS	SOURCE
15-epi-lipoxin A_4 [5(S),6(R),15(R)-Lipoxin A_{4}]	Cayman Chemical, Ann Arbor Michigan, US
BML-111 [5(S),6(R)-7-trihydroxymethyl Heptanoate]	Cayman Chemical, Ann Arbor Michigan, US
Recombinant Murine IL-10	Peprotech EC Ltd, London, UK
Rat anti-mouse F4/80 antigen purified antibody (Clone BM8)	eBioscience Ltd, Hatfield, UK
Rat IgG2a K Isotype Control Purified antibody	eBioscience Ltd, Hatfield, UK
Mouse Gr-1/Ly-6G Antibody (Clone RB6-8C5)	R&D Systems, Abingdon, UK
Rat IgG2B Isotype Control antibody	R&D Systems, Abingdon, UK
Ultra-LEAF™ Purified anti-mouse Ly-6G (Clone 1A8)	BioLegend, Cambridge, UK
Ultra-LEAF™ Purified Rat IgG2a K Isotype Control	BioLegend, Cambridge, UK

RNA EXTRACTION AND qRT-PCR	**SOURCE**
TRI Reagent	Sigma-Aldrich, Poole, Dorset, UK
QIAzol Lysis Reagent	Qiagen, Crawley, West Sussex, UK
1-Bromo-3-chloropropane (BCP)	Sigma-Aldrich, Poole, Dorset, UK
RNeasy mini spin columns	Qiagen, Crawley, West Sussex, UK
DNase 1	Qiagen, Crawley, West Sussex, UK
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Life Technologies Ltd, Paisley, UK
Taqman gene expression assays	Applied Biosystems, Life Technologies Ltd, Paisley, UK
β-actin, Cox-2 and *Il-6* probes	Integrated DNA Technologies, Iowa, USA
2X Taqman mastermix	Applied Biosystems, Life Technologies Ltd, Paisley, UK

ELISA	SOURCE
Quantikine mouse TNF-α ELISA kit	R&D Systems, Abingdon, UK
Quantikine mouse IL-1β ELISA kit	R&D Systems, Abingdon, UK
Quantikine mouse IL-6 ELISA kit	R&D Systems, Abingdon, UK
Quantikine mouse IL-10 ELISA kit	R&D Systems, Abingdon, UK
Progesterone rat/mouse ELISA kit	Demeditec Diagnostics GmbH, Kiel, Germany

IMMUNOHISTOCHEMISTRY	SOURCE
Trypsin (from bovine pancreas)	Sigma-Aldrich, Poole, Dorset, UK
Mouse Gr-1/Ly-6G Antibody (Clone RB6-8C5)	R&D Systems, Abingdon, UK
Rat anti-mouse F4/80 antigen purified antibody (Clone BM8)	eBioscience Ltd, Hatfield, UK
Purified anti-mouse Ly-6G (Clone 1A8)	BioLegend, Cambridge, UK
ImmPRESS anti-Rat IgG reagent	Vector Laboratories Ltd, Peterborough, UK

WESTERN BLOTTING	**SOURCE**
Bio-Rad *DC* Protein Assay	Bio-Rad Laboratories Ltd, Hertfordshire, UK
See Blue® Plus 2 Pre-Stained Standard	Invitrogen, Life Technologies Ltd, Paisley, UK
NuPAGE Novex 4%-12% Bis-Tris Mini Gels	Invitrogen, Life Technologies Ltd, Paisley, UK
MES running buffer	Invitrogen, Life Technologies Ltd, Paisley, UK
Dried skimmed milk powder	Marvel, UK
Bovine Serum Albumin	Sigma-Aldrich, Poole, Dorset, UK
ECL Western Blot Analysis System	GE Healthcare, Buckinghamshire, UK
Rabbit polyclonal anti-TNF-α antibody	Cell Signaling Technology Inc., Danvers, MA, USA
Rabbit polyclonal anti-β-Tubulin antibody	Cell Signaling Technology Inc., Danvers, MA, USA
Goat anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology Inc., Danvers, MA, USA

FLOW CYTOMETRY	SOURCE
PE-labelled rat anti-mouse CD45 antibody	BioLegend, Cambridge, UK
Pacific Blue-labelled rat anti-mouse Ly-6G antibody	BioLegend, Cambridge, UK
BD FACSLyse solution	BD Biosciences, Oxford, UK
Flow-check Fluorospheres	Beckman Coulter, California, UK

Appendix 2 – Publications

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EXPERT REVIEWS

Anti-inflammatory mediators as physiological and pharmacological regulators of parturition

Expert Rev. Clin. Immunol. 7(5), 675–696 (2011)

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Increasing evidence highlights parturition as an inflammatory event characterized by leukocyte influx and proinflammatory mediator production in the intrauterine environment. While the mechanisms responsible for the initiation of this inflammatory cascade are not well understood, it is clear that these inflammatory events must be tightly regulated as the premature activation of these inflammatory signals is associated with adverse pregnancy outcomes, such as preterm labor, which is the leading cause of neonatal mortality and morbidity. In this article we highlight the importance of anti-inflammatory factors in regulating the inflammatory events surrounding parturition and discuss the use of anti-inflammatory mediators as potential novel therapeutic agents in the treatment of inflammation-induced preterm labor.

KEYWORDS: anti-inflammatory • inflammation • parturition • preterm labor • resolution

Despite decades of research, the molecular mechanisms responsible for regulating one of the most fundamental reproductive events, the process of parturition, remain relatively poorly understood. A successful outcome to parturition depends upon the appropriately timed initiation of the three main physiological processes associated with labor: cervical ripening and dilatation, rupture of the fetal membranes and development of myometrial contractions. The premature initiation of these processes results in preterm labor (<37 weeks gestation), leading to preterm birth, the most common cause of neonatal mortality and morbidity worldwide [1]. Improving our understanding of the underlying mechanisms of term labor is essential for the development of effective treatments to reduce the incidence of preterm labor and ultimately improve neonatal outcome.

Labor: an inflammatory event

There is now strong evidence indicating that the initiation and progression of labor in humans is an inflammatory event. The onset of labor requires a switch from the anti-inflammatory intrauterine state associated with the majority of gestation to an active, proinflammatory, pro-labor environment. The factors responsible for this dramatic switch include increased production of proinflammatory mediators by the gestational tissues, infiltration of immune cells and decreased responsiveness to the anti-inflammatory actions of progesterone.

Labor is associated with the increased expression of several proinflammatory mediators in the intrauterine tissues including: the proinflammatory cytokines, TNF-α, IL-1β, MCP-1, IL-6 and IL-8 in the cervix, decidua, fetal membranes, myometrium and placenta [2–5]; the prostaglandins, $PGF_{2\alpha}$ and PGE_2 in the myometrium, cervix and fetal membranes [6]; and the matrix metalloproteinases (MMP)-1 and MMP-9 in the cervix, fetal membranes and placenta [7,8]. The proinflammatory cytokines act to stimulate MMP expression, thereby promoting tissue remodeling in the cervix and fetal membranes [8]. IL-1β and TNF-α have also been shown to stimulate myometrial contractility by increasing calcium entry and concentration in myometrial smooth muscle cells [9,10] and increasing prostaglandin production in the myometrium [11]. Recent microarray analysis comparing gene expression in myometrial and cervical tissues obtained from women before and after

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the onset of labor at term confirmed that labor is associated with a core inflammatory response in these tissues [12]. Co-incident with the increased proinflammatory mediator expression, there is also an infiltration of immune cells, particularly macrophages and neutrophils, into the cervix, fetal membranes and myometrium at the time of labor [3,4,13–15]. Interestingly, there is now evidence that suggests that the nature of the inflammatory trigger may play an important role in determining which type of immune cells influx into the intrauterine tissues [16,17]. In addition to the activation and infiltration of the immune cells locally into the intrauterine environment, Yuan *et al.* also reported increased activation of peripheral blood leukocytes in association with labor [18].

Central to this dramatic increase in proinflammatory signaling is the transcription factor, nuclear factor-κB (NF-κB). NF-κB is involved in regulating the transcription of a wide number of genes, but is classically linked to inflammation and immune responses [19]. The role of NF-κB in labor has recently been comprehensively reviewed [20,21]. *In vitro* studies have demonstrated a role for NF-κB in the regulation of several key proinflammatory genes associated with labor. Blocking NF-κB activity inhibited expression of IL-8, IL-6 and TNF-α in isolated amnion epithelial cells [22], amnion and choriodecidua tissue [23] and cervical epithelial cells [22]; inhibited COX-2 expression and prostaglandin production in amnion and myometrial cells [24]; and decreased MMP activity in amnion and choriodecidual tissues [23]. Importantly, NF-κB can also be activated by several proinflammatory cytokines, such as TNF-α and IL-1β, thereby perpetuating the intrauterine inflammatory response [25]. However, there is conflicting evidence on changes in NF-κB activity in the intrauterine tissues in association with labor. While a number of studies have reported increased NF-κB activity in labor in the amnion [26–28], decidua [29] and myometrium [29,30], other studies have not observed any significant increases in NF-κB activity when comparing amnion and myometrial tissues obtained from women pre- and post-labor [29,31].

Another important contributing factor to the inflammation that occurs with labor is reduced responsiveness to the anti-inflammatory effects of progesterone. Progesterone is the hormone responsible for maintaining uterine quiescence throughout gestation and in many species labor is preceded by a sharp drop in serum progesterone levels. This decline in circulating progesterone levels is not seen in humans, but given the role of progesterone in maintaining uterine quiescence, it is now widely accepted that there must be mechanisms in place that result in a local decrease in progesterone action within the uterus to allow for the switch to proinflammatory signaling [32]. This has led to the proposal of a 'functional progesterone withdrawal', which is thought to be achieved through: changes in progesterone receptor isoform ratios [33], local progesterone metabolism [34] and negative interactions between NF-κB and the progesterone receptor [26].

To date, research has focused on elucidating the proinflammatory factors responsible for initiating parturition, with the role of anti-inflammatory mediators less well explored. Recent data have demonstrated the presence of several anti-inflammatory mediators during gestation and in association with labor. The classic anti-inflammatory cytokine, IL-10, is expressed in placental tissues, isolated cytotrophoblast cells [35,36], uterine natural killer (uNK) cells and decidual macrophages [37]; and amniotic fluid levels of IL-10 are increased in association with labor [38]. A recent study by our own group has reported both increased circulating serum levels of lipoxins (novel anti-inflammatory and proresolution lipid mediators) in women throughout gestation, compared with nonpregnant women and increased lipoxin receptor expression in the myometrium during labor [39]. Adverse pregnancy outcomes, including miscarriage, preterm labor and neonatal injury, have been linked with an exaggerated or inappropriately timed inflammatory response [40]. It is likely therefore that anti-inflammatory factors will have important roles in controlling the inflammatory state of the intrauterine environment and in protecting the fetus from the inflammatory events of labor.

Preterm labor

Preterm labor, defined as the initiation of labor prior to 37 weeks of gestation, is a major clinical problem estimated to affect between 5 and 12% of pregnancies worldwide each year [1]. Recent studies have demonstrated that in many countries, such as the USA, Scotland and Denmark, the incidence of spontaneous preterm labor is actually increasing [1,41,42]. Despite vast improvements in the care of preterm infants in recent decades, resulting in reduced mortality rates, premature birth remains the leading cause of neonatal mortality and morbidity. It is estimated that preterm birth accounts for up to 75% of neonatal deaths and more than half of long-term morbidities [1]. Premature infants who survive birth are at risk of developing a number of long-term problems including: respiratory disorders such as bronchopulmonary dysplasia; neurodevelopmental problems such as cerebral palsy; and behavioral and learning problems [43]. A recent study estimated the public sector cost of preterm birth over the first 18 years of life to be approximately GB£2.946 billion (US$4.567 billion) in England and Wales (at 2006 prices) [44]; highlighting the huge economic burden placed on the public sector by preterm-birth-associated morbidities over the long term, in addition to the major clinical problems of neonatal mortality and morbidity.

Causes of preterm labor

The causes of preterm birth are poorly understood and the majority of cases are unexplained. Preterm birth is a heterogeneous condition with a number of risk factors associated including: uteroplacental ischemia, uterine overdistention, cervical disorders and maternal stress [45]. However, the only factor for which a firm causal link has been established is intrauterine infection and inflammation [45,46]. Therefore, there is growing interest in the hypothesis that in many cases preterm birth may occur as a result of the premature activation of the inflammatory pathways normally initiated with labor at term, either idiopathically, or in response to a pathological intrauterine infection.

Studies estimate that intrauterine infection is present in between 25 and 40% of preterm births [1] and is more common in early preterm deliveries (prior to 34 weeks gestation) [46]. Supporting evidence for the role of intrauterine infection and inflammation in preterm labor includes the findings that women who deliver

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preterm have higher levels of proinflammatory mediators, such as IL-6, MMP-8 and TNF-α in amniotic fluid [47,48] along with a higher incidence of chorioamnionitis [1] compared with women who deliver at term. Evidence that infection is sufficient to cause inflammation has been provided by animal models where injection with bacterial products, such as lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls, effectively induced preterm labor in mice [49,50], rabbits [51] and rhesus monkeys [52]. IL-1 has also been shown to be capable of inducing preterm labor in a mouse and nonhuman primate model following intra-amniotic injection [53,54]. These studies demonstrate the potential for inflammatory activation to induce preterm labor, even in the absence of intrauterine infection. Not only has intrauterine infection and inflammation been implicated in triggering preterm birth, but it is also associated with a number of adverse neonatal outcomes such as neonatal brain injury. A recent meta-analysis study reported a significant correlation between chorioamnionitis and the development of cerebral palsy [55]. Increased levels of proinflammatory cytokines in the amniotic fluid and evidence of funisitis (indicative of a systemic fetal inflammatory response) have also been associated with increased risk of developing cerebral palsy [56].

The mechanism by which intrauterine infection is proposed to initiate preterm birth is through Toll-like receptor (TLR) recognition of bacterial products. TLRs are a family of pattern-recognition receptors involved in coordinating the innate immune reaction in response to pathogens. Expression of the TLRs has been reported in the myometrium [57], fetal membranes [58], decidua [59], placenta [60] and cervix [61] at term, with increased expression of TLR-2 and TLR-4 in response to infection reported in the fetal membranes [58]. Evidence from a mouse model highlights the critical role of TLRs, in particular TLR-4, in the initiation of preterm labor in response to intrauterine infection, given that *Escherichia coli* and LPS failed to induce preterm labor in TLR-4 mutant mice [62,63]. NF-κB is recognized as a key transcription factor that is activated in response to TLR activation [64] leading to the upregulation of proinflammatory cytokines, chemokines and prostaglandins. Therefore, TLRs are thought to be key initiators of the inflammatory cascade during intrauterine infection, where response to bacterial products such as LPS, or peptidoglycan, a component of Gram-positive bacterial cell walls, results in the subsequent activation of NF-κB leading to the inflammatory cascade already proposed to occur during term labor [64]. The inflammatory events involved in labor, both at term and in infection-induced preterm labor, are summarized in Figure 1.

Current treatments for preterm labor

In spite of considerable medical advances and research into the area, preterm birth remains the single biggest cause of neonatal mortality worldwide. Currently, treatments for preterm labor focus on blocking myometrial contractions using tocolytic drugs, including calcium-channel blockers, β-mimetics, oxytocin receptor antagonists and nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin [65]. The latest guideline published by the Royal College of Obstetricians and Gynaecologists on the topic of tocolysis for women in preterm labor summarizes the evidence regarding the effectiveness of tocolytic drugs to treat preterm labor stating that "use of a tocolytic drug is associated with a prolongation of pregnancy for up to 7 days but with no significant effect on preterm birth and no clear effect on perinatal or neonatal morbidity" [301]. Therefore, while these drugs have proved effective in delaying delivery in some cases, they do not significantly reduce the number of premature births and there is no clear evidence that they improve neonatal outcome. The limited effectiveness of tocolytic drugs in decreasing rates of preterm deliveries is perhaps due to the fact that the drugs are given when a woman is already in preterm labor, which may be too late for any therapy to take effect. Furthermore, tocolytics focus on inhibiting myometrial contractions rather than targeting the underlying cause of preterm labor, meaning that they are unlikely to be effective in each case. For example, in cases where the preterm labor has been triggered by inflammation and the proinflammatory events leading to delivery have already begun to progress rapidly, targeting the myometrial contractions may be too late in the proinflammatory cascade and the process of labor may, at this point, be irreversible.

A further complication in attempting to treat premature birth is that in many cases, such as in the presence of intrauterine infection, delaying delivery could result in prolonged exposure of the fetus to a hostile inflammatory environment. As discussed earlier, the presence of intrauterine infection and/or inflammation has been associated with adverse neonatal outcomes [55,56]. If the primary aim of delaying preterm labor is to ultimately improve neonatal outcome, treatments must aim not only to stop myometrial contractions but also to resolve the proinflammatory status of the uterus. Taking these factors into consideration, and given the hypothesis that in many cases preterm labor occurs as a result of the premature initiation of the inflammatory cascade normally initiated during labor at term, there is now growing interest examining whether targeting the inflammatory cascade at an earlier stage, either by blocking proinflammatory mediator production, upregulation of endogenous anti-inflammatory mediators, or exogenous administration of anti-inflammatory and/or pro-resolution mediators, could be a useful novel therapeutic approach in the treatment of preterm labor.

Anti-inflammatory interventions for the treatment of preterm labor

A vast number of pathologies are known to be associated with inflammation, ranging from rheumatoid arthritis, inflammatory bowel diseases, cardiovascular diseases, cancer, and the focus of this article, parturition [66,67]. Therefore, there is a large body of research investigating the potential of treatments to block inflammation. Indeed, understanding the potential of anti-inflammatory treatments to delay preterm labor has become a major focus of premature birth research in recent years. Evidence supporting the use of anti-inflammatory drugs to delay preterm labor comes from the use of NSAIDs, which inhibit prostaglandin synthesis, as tocolytic agents. Indomethacin is the most common NSAID that has been investigated as a tocolytic drug in a number of trials. While initial studies reported a promising reduction in preterm

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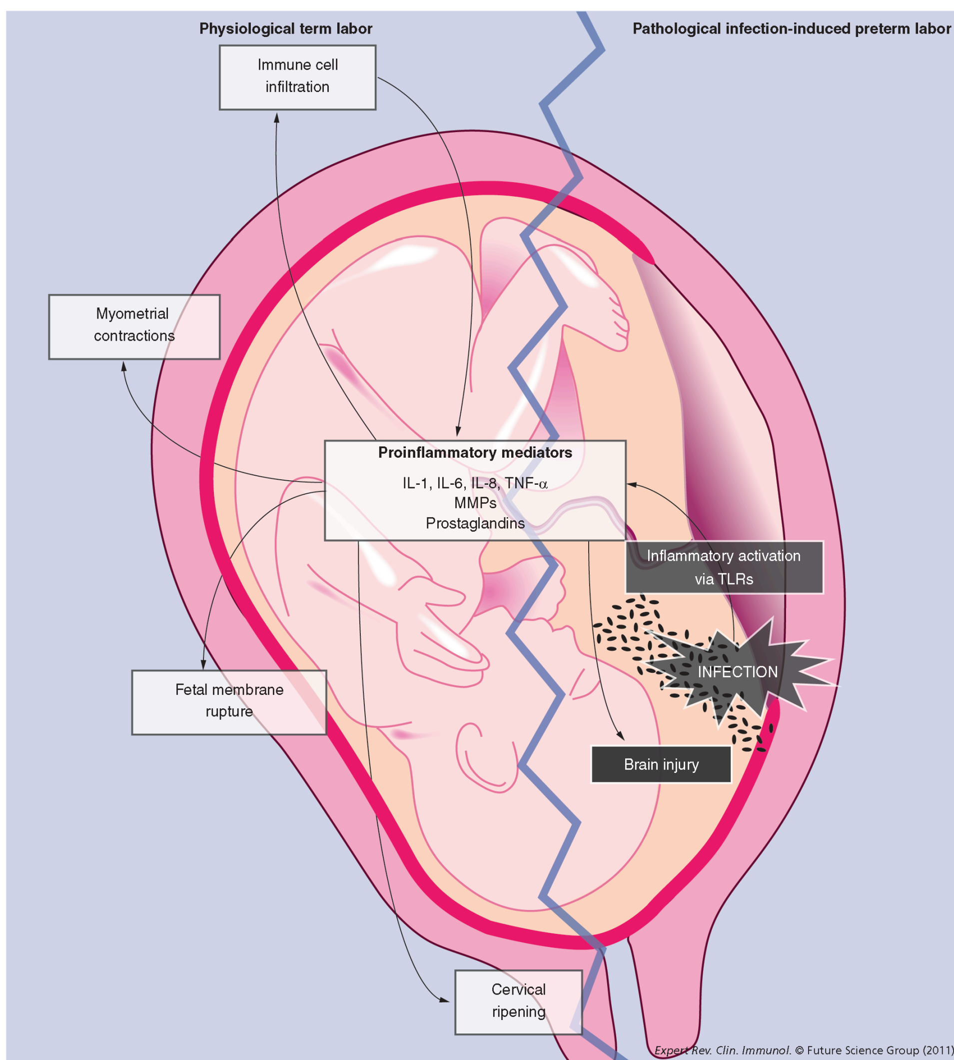

Figure 1. Inflammatory events associated with term labor and infection-induced preterm labor. The inflammatory cascade associated with labor at term is shown on the left and the events of infection-induced preterm labor on the right. Physiological term labor is associated with a leukocyte influx into the myometrium, cervix and fetal membranes and increased production of proinflammatory mediators, such as IL-1, IL-6, IL-8, MMPs and prostaglandins. These inflammatory mediators stimulate the three main processes of labor: fetal membrane rupture, cervical ripening and myometrial contractions. Preterm labor is thought to occur due to the premature activation of the same inflammatory cascade normally associated with physiological labor at term. In the presence of an intrauterine infection, TLRs activate an inflammatory response resulting in the pathological initiation of labor. In preterm labor, the presence of intrauterine infection can result in neonatal brain injury due to prolonged exposure to an inflammatory *in utero* environment. MMP: Matrix metalloproteinase; TLR: Toll-like receptor.

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birth following use of indomethacin [68–70], concerns over the safety of indomethacin have been raised given evidence associating treatment with indomethacin with a number of adverse neonatal outcomes, including necrotizing enterocolitis, premature closure of the ductus arteriousus and altered renal function [71,72]. Although a recent meta-analysis investigating the use of indomethacin as a tocolytic agent did not find increased risk of adverse neonatal outcomes, it emphasized that the small number of trials carried out to date investigating indomethacin as a tocolytic agent do not allow firm conclusions to be made on the safety of indomethacin [73]. Therefore, while some success of anti-inflammatory interventions to delay preterm delivery has been demonstrated by the use of indomethacin as an effective tocolytic agent, the risk of adverse neonatal outcomes associated with the use of this particular drug have led to continued research into alternative anti-inflammatory agents that could be useful in delaying preterm delivery and improving neonatal outcome.

This article will focus on discussing the evidence regarding the potential of anti-inflammatory treatments for preterm labor, first looking at treatments that aim to directly antagonize and inhibit proinflammatory signaling, including TLR antagonists, NF-κB inhibitors, IL-1 antagonists and anti-TNF-α agents; and second discussing the potential for exogenous administration of anti-inflammatory agents, including IL-10, progesterone, cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin-J_2 (15d-PGJ_2), lipoxins and resolvins.

Inhibition of proinflammatory signaling

TLR-4 antagonists

TLR-4 expression has been reported to increase in gestational tissues during both spontaneous term labor and infection-associated preterm labor [58,74]. Furthermore, there is evidence that impaired TLR-4 function, caused by a polymorphism in the *TLR-4* gene, is linked with preterm birth [75]. Therefore, given this evidence, along with reports from animal models indicating a critical role for TLR-4 in inducing infection-induced preterm labor [62,63], antagonism of TLR-4 has been investigated as a therapeutic option to delay premature labor. LPS interactions with TLR-4 are proposed to initiate the innate immune response and proinflammatory cascade associated with labor, leading to NF-κB activation and the subsequent increased production of proinflammatory mediators, therefore if TLR-4–LPS interactions were inhibited it could be possible to block the initiation of the inflammatory cascade altogether. The use of TLR antagonists to treat a variety of other inflammation- and infection-associated pathologies, such as sepsis, atherosclerosis and asthma, is being investigated [76].

In the context of preterm labor, use of a nonhuman primate model of LPS-induced preterm birth demonstrated the ability of TLR-4 antagonism to inhibit uterine contractility and significantly reduce TNF-α, IL-8, PGE_2 and $PGF_{2\alpha}$ concentrations in the amniotic fluid [77], with no apparent significant side effects of the TLR-4 antagonist. Recently, use of a monoclonal anti-TLR-4 antibody to block TLR-4–LPS interactions in a mouse model of preterm labor decreased the systemic and local activation of immune cells, resulting in decreased incidence of LPS-induced preterm labor from 50% in the LPS-treated mice, to 6.3% in mice receiving prior treatment with the anti-TLR-4 antibody and improved pup survival rates [78]. The results of these two *in vivo* studies provide encouragement for the concept of TLR-4 antagonism as a treatment for inflammation-induced preterm labor, although neither study investigated whether TLR-4 antagonism is capable of reducing fetal inflammation, which would be of particular importance in cases of inflammation-induced neonatal brain injury.

TLR-4 antagonists are at a relatively early stage of research, therefore further studies are required to investigate more thoroughly the potential adverse side effects of TLR-4 antagonism in pregnancy and any long-term effects on the fetus. Nevertheless, these preliminary studies in preterm labor, along with encouraging data in other pathologies, suggest that TLR-4 antagonists could be a useful therapeutic agent capable of inhibiting the innate immune response at the very start, thus inhibiting the resultant inflammatory cascade associated with preterm labor and fetal injury. While studies to date have focused on TLR-4 antagonism, this would only be an appropriate treatment in the context of Gram-negative bacteria-induced preterm labor, as TLR-4 specifically recognizes LPS. Agonists of other TLRs have also been shown to be capable of inducing preterm labor in animal models [79,80], and it would therefore be interesting to investigate whether the use of antagonists for other TLRs may also be a useful therapeutic approach in the treatment of preterm labor. This could be of particular importance in cases where there may be more than one pathogenic agent causing infection.

NF-κB inhibitors

As discussed earlier, the NF-κB signaling pathway appears to have a key role in regulating the inflammatory response associated with labor, making it an interesting therapeutic target for inflammation-associated preterm labor. Five different transcription factors are involved in the NF-κB pathway: p50, p52, p65, c-REL and RELB; these transcription factors interact with one another forming either homo- or hetero-dimers that bind to specific κB sequences to regulate target gene transcription. In unstimulated cells, NF-κB is held inactive in the cytoplasm by its endogenous inhibitor proteins, the IκB proteins. In response to inflammatory stimuli, such as IL-1 or LPS, the IκB kinase, IKKβ, phosphorylates the IκB protein, targeting it for degradation by the proteosome and allowing NF-κB dimers to translocate to the nucleus of the cell and regulate target gene transcription [19]. To date, more than 700 NF-κB inhibitors have been described that target the pathway at various points [81] and inhibition of NF-κB has been investigated as a therapeutic target in the treatment of a number of inflammation-associated pathologies including rheumatoid arthritis [82], inflammatory lung diseases [83,84] and inflammatory bowel disease [85]. The potential of a number of different NF-κB inhibitors to control inflammatory signaling in human gestational tissues and animal models of preterm labor has been investigated; this discussion will focus on evidence regarding sulfasalazine (SSZ), *N*-acetylcysteine (NAC) and a specific IKKβ inhibitor, [5-(p-fluorphenyl)-2-ureido] thiophene-3-carboxamide (TPCA-1).

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Sulfasalazine

Sulfasalazine is a 5-aminosalicylic acid drug that has been used for many years as an anti-inflammatory treatment for inflammatory bowel disease [86] and rheumatoid arthritis [87]. Although in use as an anti-inflammatory drug for many years it was only in 1998 that SSZ was identified as a potent NF-κB inhibitor [88]. SSZ is thought to block NF-κB translocation to the nucleus by directly inhibiting the IKK kinases, IKKα and IKKβ [89]. Lappas *et al.* first reported the ability of SSZ to act as an anti-inflammatory agent in human gestational tissues, demonstrating that SSZ treatment inhibited NF-κB binding activity in human placenta, amnion and choriodecidua explants *in vitro*, which in turn resulted in the inhibition of LPS-induced release of IL-6, IL-8 and TNF-α by these tissues [90]. These findings have been recently confirmed by Keelan *et al.*, where SSZ has been shown to block NF-κB p65 nuclear translocation and inhibit the release of IL-6, TNF-α, IL-8 and PGE_2 by fetal membranes in response to LPS *in vitro* [91]. In addition, SSZ treatment inhibited IL-8 production by endocervical epithelial cells in response to a variety of bacterial pathogens [92], illustrating the potential of SSZ to regulate infection-induced inflammation in the cervix. However, these studies also report that prolonged exposure (more than 20 h) of gestational tissues to SSZ is associated with increased levels of apoptosis [91] and reduced cell viability [92], which may raise concerns over the safety of administering SSZ during pregnancy.

The use of SSZ as a therapeutic agent to inhibit preterm labor has been recently investigated *in vivo* using a mouse model of preterm labor [93]. Mice were pre-treated with SSZ 1 h prior to intrauterine administration of *E. coli* on day 14.5 of gestation, followed with daily SSZ treatment until delivery. Treatment with SSZ was shown to significantly reduce the number of mice delivering preterm, where 81% of mice treated with *E. coli* alone delivered preterm compared with just 25% of mice receiving both SSZ and *E. coli*; and SSZ treatment significantly increased pup weight at delivery compared with dams receiving only *E. coli*. SSZ had a modest effect on pup survival, where the median number of pups per litter surviving more than 24 h was increased from six in the *E. coli*-alone treated dams, to 11 in dams receiving both *E. coli* and SSZ, although this increase was not statistically significant [93]. This study demonstrates for the first time the potential of SSZ to delay infection-induced preterm labor, although pup survival is not significantly improved.

Although the safety of SSZ as a treatment for preterm labor has not been assessed directly, SSZ is administered routinely to women during pregnancy to treat inflammation-associated pathologies including rheumatoid arthritis [94] and inflammatory bowel disease [95]. A recent meta-analysis investigating the effects of 5-aminosalicylic acid drugs used to treat inflammatory bowel disease on adverse pregnancy outcomes reported no significant impact of these drugs on congenital abnormalities [95], suggesting that the fetus should not be adversely affected by SSZ treatment. Yet, these studies do not take into consideration any longer term, more subtle effects on the fetus, therefore additional studies are required to elucidate the safety of SSZ treatment for the fetus. Further analysis of pup outcome in the mouse model of preterm labor used by Nath *et al.* could begin to answer these questions [93].

N-acetylcysteine

N-acetylcysteine is another NF-κB inhibitor that has been investigated as a potential therapeutic option to treat preterm labor. NAC is an antioxidant and an anti-inflammatory mediator involved in regulating oxidative stress by increasing glutathione levels [96]. NAC has been shown to inhibit nuclear translocation of NF-κB in a variety of cell systems [97,98]. Therapeutically, NAC has been used in the treatment of chronic obstructive pulmonary disease [83], influenza [99], pulmonary fibrosis [84] and paracetamol overdose [100]; and has also been investigated in the treatment of a number of reproductive pathologies including polycystic ovarian syndrome [101], recurrent pregnancy loss [102] and preeclampsia [103].

Lappas *et al.* reported the ability of NAC to regulate inflammatory signaling in human fetal membranes *in vitro* [23]. NAC treatment attenuated LPS-induced release of $PGF_{2\alpha}$, IL-8, IL-6, TNF-α and activity of MMP-9, by inhibiting NF-κB DNA-binding activity. *In vivo*, maternal administration of NAC to pregnant dams significantly delayed LPS-induced preterm labor and also improved pup survival rates: in dams receiving only LPS, 100% of pups were stillborn; however, in dams receiving LPS followed by NAC, 58% of pups were born alive [104]. NAC has also been shown to suppress fetal plasma levels of IL-6 and IL-1β in response to maternal LPS infection in rats [105]. More recently, Chang *et al.* demonstrated the ability of NAC to not only reduce LPS-induced preterm labor in a mouse model, but also to reduce the LPS-induced mRNA expression of IL-6, TNF-α and IL-1β in the myometrium and attenuate inflammation-induced fetal brain injury [106]. NAC was able to decrease the axonal injury and disturbances to myelination induced by LPS treatment in the mice [106], highlighting the potential of NAC to reduce infection-associated neonatal brain injury associated with preterm birth. In human pregnancy, NAC was administered orally in a randomized, double-blind, placebo-controlled clinical trial investigating the effect of NAC on recurrent preterm labor in women who had received treatment for bacterial vaginosis [107]. Women received NAC plus 17-hydroxyprogesterone caproate (17-OHPC) or placebo plus 17-OHPC from 16 to 18 weeks gestation until delivery. Allocation to sachet treatment number was by opening of a sealed envelope, with treatment codes known only to the trial pharmacist. Although this was a relatively small study (n = 140 in each arm), there was no loss to follow-up. NAC administration was associated with a significant reduction in the number of preterm births, with mean gestational age at delivery of 37.4 ± 0.4 in the NAC group compared with 34.1 ± 1.2 in the placebo group, increased fetal weight and a significant reduction in neonatal deaths [107]. No serious adverse maternal or fetal side effects were reported, although 11.4% of women did drop out of the trial due to nausea and vomiting associated with NAC treatment. This is the first clinical study to demonstrate an effect of NAC in delaying preterm labor and suggests that it could be a beneficial therapeutic option that, according to this preliminary study, appears to be relatively well tolerated by both mother and baby, although longer term follow-up of babies receiving NAC treatment would be required to fully investigate any potential side effects.

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TPCA-1

TPCA-1 is a specific IKKβ inhibitor that has been shown to inhibit IL-1β-induced MMP expression in corneal fibroblasts [108], LPS-induced proinflammatory gene expression in human monocytes and reduce disease severity in a mouse model of collagen-induced arthritis [109]. A recent study by De Silva *et al.* reported the ability of TPCA-1 to block NF-κB p65 nuclear translocation, inhibit LPS-induced IL-6 and TNF-α production and inhibit expression of a number of inflammatory genes including MMP-2, TNF-α and MCP-1 in primary choriodecidual cells treated with LPS *in vitro* [110]; suggesting it could be a useful drug in inhibition of choriodecidual inflammation in the presence of intrauterine infection.

Taken together these studies demonstrate the potential of targeting the NF-κB signaling pathway as a mechanism to inhibit inflammatory signaling in the intrauterine tissues and do suggest that this could be a useful therapeutic approach in the treatment of preterm labor. The fact that some NF-κB inhibitors have already been safely administered during pregnancy suggests that they should be well accepted by mother. However, given the wide range of genes controlled by NF-κB, it must be considered that inhibition of this pathway could lead to downstream effects on other processes such as cell differentiation, apoptosis and proliferation [25].

Targeting TNF-α & IL-1β

The well-documented role of proinflammatory cytokines during labor and preterm labor has led to research into whether directly blocking these cytokines might be a useful target in the prevention of preterm labor. IL-1β and TNF-α are two proinflammatory cytokines that have been strongly associated with the inflammatory cascade of labor both at term and preterm [4,40,47]. Both cytokines have been shown to have key roles in regulating prostaglandin production in myometrial cells [11] and both are potent activators of NF-κB signaling [21].

TNF-α

Anti-TNF-α therapy has been investigated as a treatment for inflammatory diseases including arthritis and Crohn's disease and has previously been administered during pregnancy to treat these pathologies [111,112]. The roles of TNF-α in term and preterm labor have been well documented: TNF-α upregulates expression of prostaglandins, MMPs and proinflammatory cytokines at the maternal–fetal interface [11,54]; increased TNF-α concentration in the amniotic fluid of women delivering preterm has been reported [47]; and intra-amniotic infusion of TNF-α induces preterm labor in animal models [54,113]; all of which make TNF-α an attractive candidate target for therapeutic intervention.

Blocking TNF-α, either using anti-TNF-α antibodies, soluble TNF receptors or pentoxifylline, an inhibitor of TNF-α synthesis, has been shown to improve infection-mediated adverse pregnancy outcomes in mice including fetal resorption, intrauterine growth restriction and fetal mortality [113–115]. The use of anti-TNF-α therapy to inhibit preterm labor has also been examined using mouse models with mixed success. Fidel *et al.* reported that blocking of TNF-α using a soluble TNF-α receptor Fc fusion protein did not prevent LPS-induced preterm delivery [116]. Holmgren *et al.* recently reported pretreatment with an anti-TNF-α antibody significantly decreased the number of LPS-induced preterm deliveries as well as improving pup survival and decreasing the expression of several proinflammatory mediators including IL-6 and IL-1β [117]. Although these studies provide conflicting evidence on the potential of anti-TNF-α therapy in preterm labor, the Holmgren *et al.* [117] study is encouraging as it used a commercially available antibody that has been previously used to treat arthritis and Crohn's disease in humans, demonstrating its anti-inflammatory potential in different clinical settings.

The use of anti-TNF-α agents to treat preterm labor or other reproductive disorders has not been investigated clinically but, as mentioned earlier, anti-TNF-α antibodies and soluble TNF receptors are routinely administered to pregnant women suffering from pathologies including rheumatoid arthritis and inflammatory bowel disease. A recent review examining the evidence on the safety of administering anti-TNF therapy to treat rheumatoid arthritis in pregnant women concluded that while some studies reported no association between anti-TNF-α therapy and adverse outcomes, there have been reports of congenital abnormalities in women taking anti-TNF-α drugs, thus the safety of these drugs during pregnancy is not yet clear [111]. Interestingly, a recent study by Zelinkova *et al.* reported evidence that high levels of infliximab, an anti-TNF-α antibody used in the treatment of inflammatory bowel conditions, were found in the cord blood of newborn babies born to mothers taking infliximab until week 30 of gestation, confirming that infliximab can cross the placenta and that maternal ingestion leads to direct fetal exposure to this drug [118]. Although no immediate adverse side effects were apparent after 6 months, the long-term implications are unknown [118].

IL-1β

Blocking IL-1 signaling has proved to be a successful therapeutic approach in the treatment of some pathologies, and the use of the endogenous IL-1 receptor antagonist (IL-1Ra) is clinically approved for the treatment of rheumatoid arthritis [119]. Studies into the role of IL-1Ra in regulating inflammatory signaling in gestational tissues began in 1992 when Romero *et al.* demonstrated the ability of IL-1Ra to decrease IL-1β-induced PGE_2 production in cultured amnion and chorion explants *in vitro* [120]. This finding was rapidly followed with evidence from an *in vivo* mouse model of preterm labor where pretreatment with IL-1Ra was reported to prevent IL-1-induced preterm birth [53]. However, since these two encouraging results, conflicting data have been reported on the usefulness of blocking IL-1β signaling as a strategy to prevent preterm labor: administration of IL-1Ra is not capable of preventing LPS-induced preterm labor in a mouse model [116]; overexpression of IL-1Ra in a transgenic mouse does not prevent IL-1β-induced preterm delivery [121]; and furthermore deletion of either *IL-1β* or the *IL-1 receptor* in transgenic knockout mice is not reported to reduce rates of preterm delivery when compared with wild-type mice [122,123]. Taken together these studies suggest that simply targeting IL-1β signaling is unlikely to be sufficient to inhibit

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preterm labor, although IL-1β does play an important role in the inflammatory cascade associated with parturition, and is capable of inducing preterm labor, it is not actually essential for the induction of preterm labor. Interestingly, while studies with IL-1 receptor knockout mice did not show any difference in susceptibility to infection-induced preterm labor [123], a double knockout mouse model lacking both the IL-1 and TNF-α receptor has been reported to show decreased rates of *E. coli*-induced preterm birth compared with wild-type mice [124], although preterm labor was not completely abolished. This study highlights the complexity of cytokine interactions associated with preterm labor and suggests that although some success has been seen using animal models, targeting individual cytokines may not be the most useful therapeutic strategy.

Prokineticins

Prokineticins are peptides with a wide range of functions, including stimulation of smooth muscle contractility, angiogenesis, amplification of inflammatory responses, and regulation of hemopoiesis [125]. We have previously shown that prokineticin 1 (PROK1) and its receptor (PROKR1) are expressed in the placenta and that PROK1 upregulates IL-8 and COX-2 expression [126]. More recently, we have shown upregulation of PROK1 in the human myometrium and placenta during labor, with no change in expression of the receptor PROKR1 [127]. Importantly, infection of myometrial explants with lentiviral miRNA targeting *PROK1* reduced LPS-induced expression of inflammatory genes, raising the possibility that PROKR1 antagonism might be an effective mechanism to prevent infection/inflammation-induced preterm birth.

Endogenous anti-inflammatory agents

Another potential strategy that is being investigated in the search for novel treatments to delay preterm labor involves upregulation of endogenous anti-inflammatory agents. The administration of endogenous anti-inflammatory mediators may be more beneficial than attempting to block specific points of the inflammatory cascade, as they have the potential to have broader, more widespread anti-inflammatory actions. Furthermore, in recent decades it has become clear that the resolution of inflammation is an active process involving the production of mediators with specific pro-resolution and anti-inflammatory actions [128,129]. The identification of mediators that not only block inflammation, but are also capable of promoting complete resolution and return to normal tissue homeostasis, has opened up a whole new field of research into treatments for inflammation-associated pathologies. Targeting these pro-resolution pathways could be an exciting novel therapeutic option in the treatment of preterm labor as they have the potential to not only inhibit the inflammatory cascade, but also to resolve the inflammatory, and often hostile, intrauterine state associated with so many cases of preterm birth, therefore potentially being more useful than an anti-inflammatory agent alone in improving neonatal outcome. The anti-inflammatory agents that will be discussed here are IL-10, progesterone, 15d-PGJ_2, lipoxins and resolvins.

IL-10

Originally termed 'cytokine synthesis inhibitory factor' after its discovery in 1989 as a novel product of mouse Th2 cell clones capable of inhibiting Th1-derived IL-12 and IFN-γ [130], IL-10 is now considered as the classic anti-inflammatory cytokine. The anti-inflammatory effects of IL-10 on a range of immune cells, including monocytes, macrophages, T cells and NK cells are well summarized in a recent review by Sabat *et al.*, which highlights monocytes and macrophages as the main target of IL-10 [131]. The key actions of IL-10 on monocytes and macrophages include: inhibiting their production of proinflammatory mediators, such as TNF-α, IL-6, IL-8 [132,133]; promoting their phagocytic ability [134]; and stimulating the production of other anti-inflammatory mediators, such as soluble TNF-α receptor and IL-1Ra [135,136]. The anti-inflammatory actions of IL-10 are largely mediated through activation of the signal transducer and activation of transcription (STAT) transcription factors, specifically STAT3, which upon activation by IL-10 promotes the expression of a number of genes including the suppressor of cytokine signaling 3 (SOCS3) and IL-10 itself [131]. IL-10 has also been shown to inhibit NF-κB activation in a number of different cell systems [137–139]. Research into IL-10 as a therapeutic option to treat inflammation-associated pathologies, including Crohn's disease [140], psoriasis [141] and chronic hepatitis C [142], has progressed to clinical trial stage with mixed success in terms of reducing disease severity, but in general administration of IL-10 was well tolerated in the individuals, with minimal side effects [143].

The important role of IL-10 in pregnancy has been discussed extensively in two recent reviews [144,145]. During gestation IL-10 is expressed in placental tissues, isolated cytotrophoblast cells [35,36], uterine NK cells and decidual macrophages [37]. Changes in IL-10 expression in pregnancy have been demonstrated in placental tissues, with higher IL-10 in the first and second trimesters followed by downregulation at term [36]. IL-10 has also been detected in the amniotic fluid of women during pregnancy with one study reporting increased amniotic fluid IL-10 at term compared with second trimester levels [146], while other studies have reported no change across gestation [38,147]. Gotsch *et al.* describe increased amniotic fluid IL-10 levels associated with spontaneous labor both at term and in cases of preterm labor, compared with gestation matched nonlaboring women, with a further increase in amniotic fluid IL-10 levels measured in the presence of intra-amniotic infection [38]. These studies suggest that IL-10 has an important role to play in regulating inflammatory signaling during labor at term and preterm, particularly in response to infection. Increasing levels of IL-10 in the amniotic fluid are likely to act as an important protective mechanism to prevent fetal exposure to excess inflammation. The importance of IL-10 in controlling the inflammatory response associated with intrauterine infection has been demonstrated by studies with the *IL-10* knockout mouse, where *IL-10* knockout mice required a tenfold lower dose of LPS to induce 50% fetal loss compared with wild-type animals and serum levels of TNF-α and IL-6, induced by LPS treatment, were much greater in the knockout animals compared with the wild-type mice. The differences between *IL-10* knockout mice and

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wild-type mice were attenuated by administration of recombinant IL-10 [50]. Additionally, IL-10 deficiency has been associated with a number of adverse pregnancy outcomes, such as preeclampsia, recurrent spontaneous abortions and preterm labor [148–150], highlighting the importance of IL-10 in regulating the intrauterine inflammatory environment throughout gestation.

The ability of IL-10 to attenuate proinflammatory signaling in human gestational tissues has been investigated *in vitro*. In the fetal membranes, IL-10 has been shown to be capable of downregulating the production of the proinflammatory mediators TNF-α [151], PGE_2, IL-1β [152] and MMP-2 and -9 [153]. In choriodecidual explants, IL-10 treatment significantly reduced LPS-induced IL-1β and TNF-α [154]. In cultured placental trophoblasts, IL-10 downregulated IL-1β-induced COX-2 and PGE_2 production [155] and inhibited MMP-9 production [156]. Similarly, using placental explants collected at term, IL-10 treatment reduced LPS and lipoteichoic-induced proinflammatory cytokine synthesis and PGE_2 production [157]. Whether these anti-inflammatory effects of IL-10 in gestational tissues are mediated via inhibition of NF-κB or through other mechanisms is unclear.

Animal models have been invaluable in investigating the effect of IL-10 administration on preterm labor. As discussed earlier, Robertson *et al.* demonstrated that administration of exogenous IL-10 to both pregnant *IL-10* knockout and wild-type mice resulted in a significant reduction in the number of LPS-induced fetal losses in both mice and also attenuated proinflammatory cytokine synthesis in gestational tissues in knockout mice [50]. In a rat model of preterm birth, IL-10 treatment, either at the same time as LPS or 24 h later, was found to significantly delay the time to delivery and importantly improve pup survival rates [158]. IL-10 treatment has also been shown capable of inhibiting *E. coli*-induced white matter damage in rat pup brains [159]. This protective effect of IL-10 on infection-induced neonatal brain injury is thought to involve suppression of microglial/macrophage activation [160]. Additionally, in a nonhuman primate model investigating inflammation-induced preterm birth, IL-10 administration attenuated IL-1β-induced uterine contractility, amniotic fluid TNF-α and prostaglandin levels and reduced leukocyte influx into the amniotic fluid. Furthermore, intravenous and intra-amniotic infusion of IL-10 was not associated with any changes in baseline fetal heart rate [161], suggesting that IL-10 administration was not having any immediate adverse effects on the fetus.

The *IL-10* knockout mouse has been utilized as a model to investigate the role of particular immune cell subtypes in preterm labor in response to different inflammatory triggers. Treatment with low-dose LPS has demonstrated a role for TNF-α in the infiltration of cytotoxic uNK cells associated with adverse pregnancy outcome in these animals [17]. However, administration of CpG oligodeoxynucleotide, a TLR-9 agonist, to *IL-10* knockout mice leads to macrophage-dependent, uNK cell-independent preterm delivery [16]. These studies suggest that particular immune cell subtypes respond differentially to different inflammatory triggers, but an important caveat is that much of this data has yet to be replicated in wild-type animals. Notably the uNK cell infiltration seen in response to LPS-treatment in knockout animals is not observed in wild-type mice [17]. This diminished role of uNK cells in wild-type animals is consistent with studies in the human where decidual uNK cell numbers decline from 60–70% in the first trimester to around 3% at term [162].

Taken together these studies highlight the potential of IL-10 as a novel anti-inflammatory treatment for preterm labor that can target early events in the inflammatory cascade leading to labor. Although these are promising studies, and IL-10 administration appears to be relatively well tolerated in other inflammation-associated pathologies, any long-term effects on the fetus are unknown and would need to be investigated further. Furthermore, given the widespread role of IL-10 during pregnancy, it is possible that exogenous administration of IL-10 would not only be beneficial in cases of preterm labor, but could also be a useful treatment for other inflammation-associated pathologies that lead to adverse pregnancy outcomes, such as preeclampsia [145].

Progesterone

As discussed earlier, progesterone plays a key role during pregnancy in maintaining uterine quiescence and functional withdrawal of progesterone in the uterus is thought to precede the initiation of labor in humans [32]. This suggests that maintaining intrauterine progesterone concentrations might be a useful therapeutic strategy to delay preterm delivery.

Progesterone has been evaluated as a treatment for preterm labor since the 1960s [163], with clinical trials still ongoing evaluating the effectiveness of different progesterone derivatives in high-risk women. Many clinical studies have reported that progesterone supplementation to high-risk women is associated with reduced preterm delivery rates [164–166]; although progesterone prophylaxis appears to be less effective in women with multiple pregnancies [167–169] and a recent study also reported that administration of 17-α-hydroxyprogesterone did not inhibit preterm delivery in women with preterm premature rupture of the fetal membranes [170]. Importantly, although in use as a treatment for preterm labor for many years, the mechanisms by which progesterone delays preterm delivery are relatively poorly understood; improving understanding of how progesterone acts will aid in the definition of which groups of women in particular may benefit from progesterone treatment and may also explain why progesterone is more effective in some cases, such as in singleton pregnancies, but not others.

In vitro studies and animal models of preterm labor have been used to try to understand the mechanisms of progesterone action. Using a mouse model of LPS-induced inflammation where pretreatment with the progestational agent medroxyprogesterone acetate (MPA) delayed preterm delivery, Elovitz *et al.* reported that MPA treatment downregulated LPS-induced expression of COX-2, TNF-α, IL-1β and connexin-43, which is a protein associated with myometrial gap junctions, and also inhibited cervical ripening, indicating that in the context of intrauterine infection progesterone has anti-inflammatory actions and can also act to decrease myometrial contractility [171]. Using the same model, Elovitz *et al.* subsequently reported that MPA treatment inhibited LPS-induced TLR-2 upregulation in the cervix and placenta, which may explain another mechanism by which progesterone

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may act to delay preterm delivery [172]. This inhibitory effect of MPA on LPS-induced TLR expression has more recently also been reported in human myometrial samples *in vitro* [57]. Another potential anti-inflammatory mechanism of progesterone action in the intrauterine tissues could be explained by the negative interactions between the progesterone receptor and NF-κB, which have been previously reported in other cell systems [173] and in human amnion cells [26]. Furthermore, a study investigating the effect of progesterone treatment on fetal membranes *in vitro* suggested inhibition of apoptosis in the fetal membranes may be another mechanism of progesterone action [174]. Progesterone pretreatment has also been shown to be capable of modulating IL-6 production in feto–placental arteries [175] and in fetal mononuclear cells [176,177], which the authors suggest may indicate progesterone can also inhibit the fetal inflammatory response. A recent study by Norman *et al.* demonstrates that prolonged administration of progesterone *in vivo* results in decreased myometrial expression of connexin 43 and decreased CD11b expression on circulating monocytes, suggesting that progesterone may act to inhibit synchronous myometrial contractility and reduce leukocyte influx into the myometrium [178].

It must be noted that, although several studies have demonstrated the use of progesterone administration to prevent preterm delivery, there is still little convincing evidence that it actually improves neonatal outcome [179]. However, there are a number of randomized control trials ongoing examining the long-term outcomes in children whose mothers received progesterone treatment, one such UK-based study is the OPPTIMUM trial (ISRCTN14568373); thus in the near future the benefits of progesterone treatment on neonatal outcome should become clearer.

15d-PGJ$_2$

15d-PGJ$_2$ is a metabolite of PGD$_2$ that has been shown to have potent anti-inflammatory actions in a variety of cell systems [180]. 15d-PGJ$_2$ is produced in the later phases of an inflammatory response *in vivo* and is therefore postulated to have an important role in the resolution of the inflammatory response [181]. Studies have demonstrated that 15d-PGJ$_2$ stimulates leukocyte apoptosis [182] and promotes macrophage clearance [183], thus promoting the resolution of inflammation. A major anti-inflammatory mechanism of 15d-PGJ$_2$ action is through inhibition of the NF-κB signaling pathway either directly via inhibition of IKKβ [184] or indirectly via activation of PPAR-γ [185].

During pregnancy both the placenta and decidua have been shown to be sources of PGD$_2$ [186,187] and both PGD$_2$ and 15d-PGJ$_2$ have been detected in the amniotic fluid of women at term [188]. *In vitro* studies have demonstrated the ability of 15d-PGJ$_2$ to: downregulate TNF-α-induced COX-2 expression, PGE$_2$ release and NF-κB activity in human amnion and WISH cells [189]; inhibit IL-1β-induced COX-2 and NF-κB activation and activity in primary amnion and myometrial cells [24]; and inhibit the LPS-induced activation of NF-κB and the subsequent release of proinflammatory cytokines and prostaglandins from human placenta, amnion and choriodecidua explants [190]. Following on from these *in vitro* studies, Pirianov *et al.* recently reported that treatment with 15d-PGJ$_2$ significantly delayed preterm delivery and improved fetal survival rates in a mouse model of LPS-induced preterm labor [49]. Confirming data from the *in vitro* work, 15d-PGJ$_2$ treatment was associated with decreased IKKβ activity in the myometrium; inhibition of LPS-induced myometrial proinflammatory cytokine production; and inhibition of cPLA2 expression in the myometrium suggesting a negative feedback mechanism on prostaglandin synthesis [49]. Furthermore, intrauterine administration of 15d-PGJ$_2$ also resulted in decreased IKKβ activity in fetal brains [49], suggesting that aside from inhibiting inflammatory signaling in the myometrium, 15d-PGJ$_2$ may also be capable of protecting the fetus from inflammation-associated brain injury.

Research into the use of 15d-PGJ$_2$ as an anti-inflammatory therapeutic agent is still at a relatively early stage, but *in vivo* data from animal models demonstrating a beneficial effect of 15d-PGJ$_2$ treatment in a wide variety of inflammation-associated diseases, including chronic lung injury [191], arthritis [192] and ulcerative colitis [193], provide encouragement for the concept that exogenous administration of 15d-PGJ$_2$ could be a useful therapeutic strategy. The ability of 15d-PGJ$_2$ to inhibit NF-κB-regulated inflammatory gene expression in intrauterine tissues *in vitro*, inhibit infection-induced preterm labor and improve fetal outcome *in vivo* suggest that it could be an exciting novel therapeutic target in the treatment of inflammation-induced preterm labor. Further research is required to examine this potential and investigate any adverse fetal effects.

Lipoxins

Lipoxins were the first family of lipid mediators recognized to have dual-acting pro-resolution and anti-inflammatory properties. Lipoxins were first identified as novel products of 15-HPETE in human leukocytes [194,195], and have since been reported to have a range of anti-inflammatory and pro-resolution actions in a wide number of cell types. In neutrophils, lipoxins have been reported to inhibit neutrophil activation, adhesion and chemotaxis and promote neutrophil apoptosis [196–200]. In contrast to the inhibitory effects of lipoxins reported in neutrophils, lipoxins have been reported to stimulate monocyte adhesion and migration [201,202], inhibit monocyte apoptosis [203] and promote the clearance of apoptotic neutrophils by macrophages [204]. In addition to the effects of lipoxin on immune cells, lipoxin treatment has also been shown to: inhibit the leukotriene-stimulated activation and proliferation of endothelial cells [205]; block platelet-derived growth factor-induced migration of smooth muscle cells [206]; decrease MMP production and increase TIMP production by fibroblasts [207]; and decrease NF-κB activation in epithelial cells [208]. These varied actions of lipoxins in the resolution of inflammatory responses are summarized in Figure 2. Lipoxins are synthesized from arachidonic acid via the action of their specific lipoxygenase enzymes: 5-lipoxygenase (ALOX-5), 12-lipoxygenase (ALOX-12) and 15-lipoxygenase (ALOX-15). Lipoxin synthesis involves transcellular interactions between leukocytes and either epithelial cells or platelets. Aspirin can also be involved in lipoxin synthesis, whereby in the presence of aspirin COX-2 becomes acetylated and drives the synthesis of aspirin-triggered lipoxins via ALOX-5 [209]. It has been demonstrated that during the course of an acute inflammatory response,

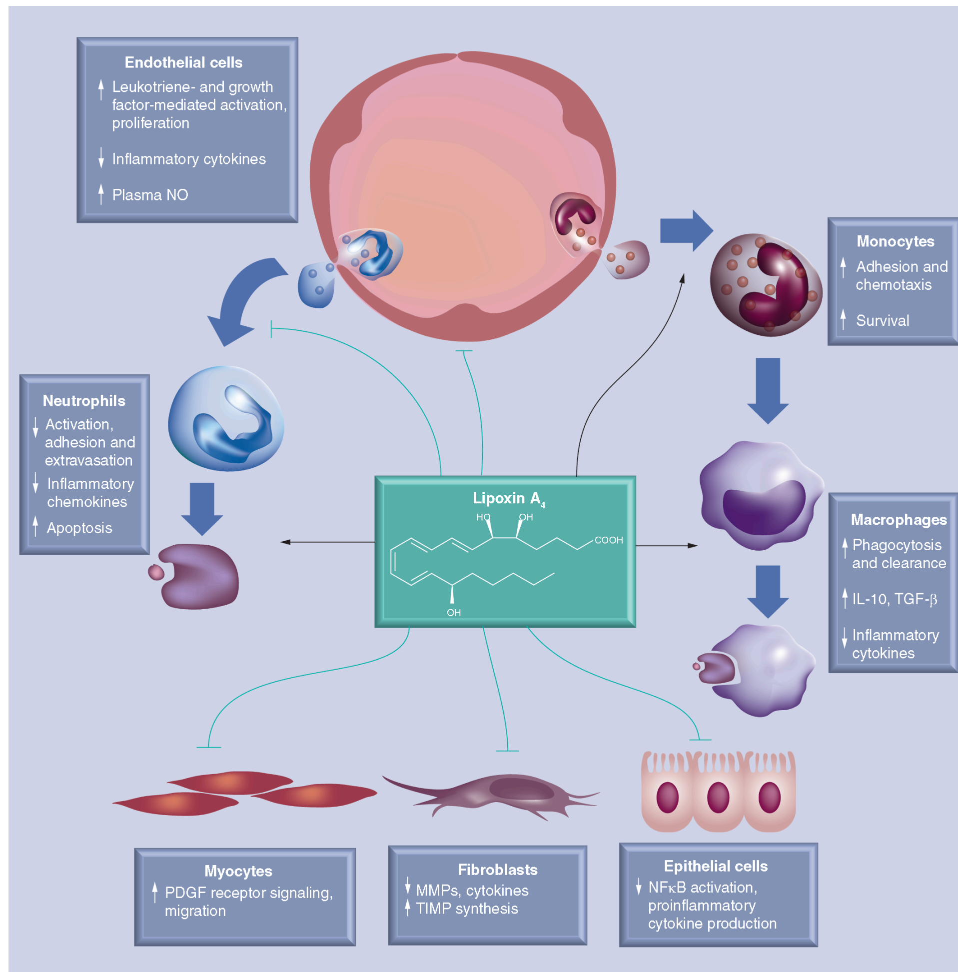

Figure 2. Anti-inflammatory and pro-resolution actions of lipoxin A_4. Summary of lipoxin A_4 actions on key cell types in the inflammatory response.
MMP: Matrix metalloproteinase; NO: Nitric oxide; TIMP: Tissue inhibitor of matrix metalloproteinase.

polymorphonuclear neutrophils undergo a process termed 'lipid-mediator class switching', where they switch from production of proinflammatory lipid mediators such as leukotrienes and prostaglandins, to production of lipoxin A_4 [210].

In a variety of cell systems lipoxins have also been shown to downregulate a range of proinflammatory mediators *in vitro*, including many of those involved in the inflammatory cascade of labor, such IL-8, IL-6, IL-1β, TNF-α and MMPs [207,211–213]. Lipoxins have been shown to inhibit the NF-κB pathway in a number of cell systems [211,213,214]; and activation of PPAR-γ is reported as another potential mechanism for the anti-inflammatory actions of lipoxins [215]. Additionally, a recent study demonstrated the activation of STAT3 and increased SOCS3 expression in response to lipoxin treatment in a macrophage cell line *in vitro* [216], which could underlie the downregulation of proinflammatory gene expression observed with lipoxin treatment. The therapeutic potential of

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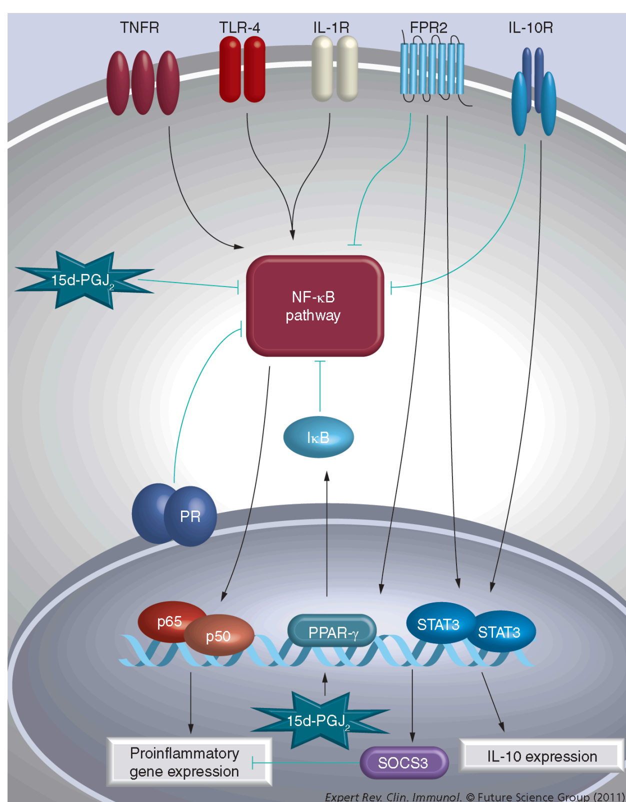

Figure 3. Overview of the inflammatory signaling pathways that could be potential targets for the treatment of preterm labor. Black arrows show activation of signaling pathways and green arrows show inhibition of proinflammatory processes. The NF-κB pathway is central to many of the inflammatory events surrounding parturition and is therefore a major target for inhibiting inflammation-induced preterm labor, with many of the anti-inflammatory treatments discussed in this article converging on the NF-κB pathway. TNF-α, LPS and IL-1 can activate NF-κB by binding to their receptors, TNFR, TLR-4 and IL-1R, respectively. Upon activation, NF-κB is released from its endogenous inhibitor protein, IκB, allowing translocation of the NF-κB heterodimer, p65/p50, to the nucleus, where it promotes the transcription of proinflammatory genes, such as IL-1 and TNF-α. The anti-inflammatory actions of lipoxins acting on FPR2 are thought to involve: direct inhibition of the NF-κB pathway; activation of the transcription factor PPAR-γ; and activation of the transcription factor STAT3 resulting in the expression of SOCS3, which acts to downregulate proinflammatory gene expression. The anti-inflammatory actions of IL-10, acting via its receptor IL-10R, also involves direct inhibition of the NF-κB pathway and STAT3 activation leading to increased expression of SOCS3 and further IL-10 expression. The anti-inflammatory prostaglandin, 15d-PGJ$_2$, does not have a specific cell membrane receptor and is thought to act intracellularly to inhibit NF-κB either directly or indirectly via PPAR-γ. The ability of progesterone to downregulate inflammation is thought to be mediated in part through negative interactions between its receptor, PR, and NF-κB, leading to inhibition of NF-κB signaling.
STAT3: Signal transducer and activator of transcription 3; SOCS3: Suppressor of cytokine signaling 3.

lipoxins and their stable analogues has been demonstrated in a number of animal models of inflammation-associated pathologies such as asthma [217], ulcerative colitis [208], ischemia/reperfusion injury [218] and arthritis [219] where lipoxin administration was associated with reduced severity of disease and improved outcome in each case.

The role of lipoxins in regulating inflammation in the intrauterine tissues, and in particular in parturition, has been largely unexplored. ALOX enzyme expression has been detected in gestational tissues: in amnion and choriodecidua tissues, ALOX-5 expression has been reported, with increased ALOX-5 expression in the choriodecidua in association with labor [220]; decreased ALOX-15 has been reported in myometrial smooth muscle cells at term and during labor [221]; and ALOX-5 and ALOX-12 have also been detected in baboon myometrium, cervix, chorion and decidua at term [222]. A recent study examining the role of lipoxins in pregnancy reported significantly higher levels of circulating serum lipoxin A$_4$ levels in pregnant women compared with non-pregnant women, and increased expression of FPR2 in myometrium in association with labor [39]. Furthermore, the same study also demonstrated the ability of lipoxin A$_4$ to downregulate LPS-induced IL-6 and IL-8 expression in myometrial explants *in vitro* [39]. A recent review also discusses the evidence regarding lipoxin action in other reproductive events, such as ovulation and menstruation, highlighting that these relatively novel mediators are likely to be involved in regulating the inflammatory events surrounding a number of physiological reproductive events [223].

Given the evidence from animal models that lipoxins are useful in treating inflammation-associated pathologies, along with the data of these preliminary studies examining the role of lipoxins in labor-associated inflammation, it seems clear that lipoxin treatment could be an exciting novel therapeutic strategy in the treatment of inflammation-induced preterm labor. The pro-resolution actions of lipoxins, in stimulating neutrophil apoptosis and promoting macrophage phagocytosis, suggest that they could be more useful than simple anti-inflammatory agents alone at resolving a hostile inflammatory intrauterine

environment, thereby limiting inflammation-induced neonatal injury. Interestingly, recent studies have also demonstrated the ability of lipoxins to regulate inflammatory signaling in the brain – in a human astrocytoma cell line *in vitro* [213] and also *in vivo* using a rat model of brain injury [218] – suggesting that lipoxins may also be beneficial in inhibiting inflammation-induced neonatal brain injury. Indeed, work is now being undertaken in our lab investigating the efficacy of lipoxins to delay preterm delivery and improve neonatal outcome using a mouse model of preterm labor.

Resolvins

Resolvins were first isolated and identified in murine inflammatory exudates formed in response to a dorsal air pouch model of inflammation [224]. They are synthesized from Ω-3 poly-unsaturated fatty acids, specifically from the precursor fatty acids, eicosapentaenoic acid and docosahexaenoic acid, which give rise to E-series and D-series resolvins, respectively. Resolvins share similar anti-inflammatory and proresolution actions as those already described for lipoxins. Animal models have been invaluable in demonstrating the therapeutic potential of resolvins in treating a variety of inflammation-associated pathologies including peritonitis [225,226], ischemia/reperfusion injury [227] and colitis [228].

Little is known about the role resolvins play in regulating inflammatory responses in gestational tissues; however, dietary supplementation with their precursor molecules, poly-unsaturated fatty acids, has been implicated in decreasing rates of preterm birth [229]. Ω-3 fatty acid supplementation has long been recognized as a beneficial treatment for a number of pathologies including cardiovascular disease [230,231], inflammatory bowel diseases [232,233] and arthritis [234,235]; but the underlying molecular mechanisms of how Ω-3 fatty acids were beneficial were not well understood until the identification of resolvins [129].

Clinical trials have also been carried out examining whether Ω-3 fatty acid supplementation during pregnancy is associated with prolonged gestation and, while some studies have reported a significant decrease in preterm deliveries in women taking Ω-3 supplements [236–238], two recent meta-analyses reviewing current evidence concluded that there is not yet sufficient evidence to recommend the use of Ω-3 supplementation to delay preterm delivery and treat other adverse pregnancy outcomes, with the relative risks of preterm birth in each study reported as 0.92 (95% CI: 0.79–1.07) and 0.99 (95% CI: 0.9–1.1) [239,240]. Interestingly, the mechanisms by which Ω-3 fatty acids are proposed to delay preterm delivery are unclear, but it seems probable that any benefits observed could be mediated by the anti-inflammatory, pro-resolution actions of the Ω-3 derivatives, resolvins. To date there have been no reports on the role of resolvins in regulating inflammation in intrauterine tissues, but given some reports of Ω-3-fatty acid supplementation delaying delivery, along with the preliminary data suggesting lipoxins may be a useful therapeutic agent in this context, it is interesting to speculate that resolvins may equally be promising new therapeutic treatments for inflammation-induced preterm labor.

The inflammatory pathways discussed here as potential targets for the treatment of preterm labor are summarized in Figure 3.

Expert commentary

Despite decades of research, preterm birth remains the leading cause of neonatal mortality worldwide and can lead to a number of long-term health consequences for those babies who do survive. Currently used tocolytic drugs have been largely ineffective in reducing the incidence of preterm birth and there is little evidence that these drugs improve neonatal outcome. Given the growing body of evidence that labor at term is an inflammatory event and the hypothesis that in many cases preterm birth is triggered by the premature activation of similar inflammatory pathways, either idiopathically or in response to intrauterine infection, we have discussed the role of anti-inflammatory mediators in parturition and their potential as therapeutic agents to delay preterm labor and improve neonatal outcome.

With preterm labor remaining a major obstetrical problem worldwide, there is increasing research into novel treatments that could have a significant impact on preterm birth rates and improve neonatal survival. In particular the anti-inflammatory agents discussed in this article have been the focus of much research, but there are many other promising therapeutic targets being investigated. Examples of other promising mediators with anti-inflammatory properties that are being studied include sirtuins (SIRT), antioxidants and vitamins. SIRT1, which possess histone deacetylase activity, has recently been shown to have anti-inflammatory actions in human gestational tissues *in vitro* [241]. In addition, the vitamin B_3 derivative, nicotinamide, has been reported to have anti-inflammatory and antioxidative actions in human placental explants *in vitro* [242]; and furthermore a recent clinical trial investigating the effects of L-arginine and antioxidant vitamin supplementation of women at high-risk of preeclampsia reported a reduction in the overall rate of preterm birth [243].

Anti-inflammatory mediators have the potential to be more effective than tocolytic drugs alone in the treatment of preterm labor, as rather than simply blocking myometrial contractions, anti-inflammatory agents target the underlying mechanisms thought to trigger spontaneous preterm labor in a vast number of cases and, more importantly, they target events occurring earlier in the inflammatory cascade before the onset of myometrial contractions. Furthermore, some anti-inflammatory agents, in particular those with proresolution actions, also have the potential to resolve the inflammatory *in utero* environment, therefore limiting the exposure of the fetus to the hostile inflammatory signals and preventing associated inflammation-induced pathology. Animal models have been invaluable to research in this field and have demonstrated the ability of anti-inflammatory agents to not only effectively delay infection- and inflammation-induced preterm labor, but also to reduce infection-induced neonatal brain injury, suggesting that these agents could be useful in decreasing the incidence of preterm labor, but more importantly could improve neonatal outcome. While extrapolation of evidence from animal models to human systems is complicated by differences in parturition mechanisms and physiology, they do provide encouragement for the concept of using anti-inflammatory therapies.

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Theoretically, the use of anti-inflammatory agents to treat preterm labor could result in suppression of the maternal and fetal immune responses, impairing the ability to mount a defense to infection. Whether this possible suppression of immune response is a real concern would need more investigation. Furthermore, targeting inflammatory factors, such as NF-κB, could lead to other downstream effects given the role of NF-κB in regulating the transcription of a wide range of genes, not just those related to inflammation. Any potential long-term side effects of the anti-inflammatory therapies discussed here are unknown and further research would be required to confirm the safety of administering these drugs during pregnancy to ensure that they do ultimately result in an improved fetal outcome.

The heterogeneous nature of preterm labor means it is unlikely that there will be one effective treatment for all cases, therefore it is important to continue research into a range of therapeutic strategies. Further investigations into markers that can be used to predict the onset of preterm labor will aid in the identification of which treatments will be appropriate to specific subgroups of women. We feel that given the key role of the inflammatory signaling in parturition, anti-inflammatory agents have the potential to be exciting novel therapeutic agents in the treatment of preterm birth.

Five-year view

It is likely that agents that inhibit intrauterine inflammation will become an important part of preterm labor prevention and treatment strategies within the next 5 years. Progesterone, as discussed previously, is known to have an anti-inflammatory effect. It is already in established use in the USA for prevention of preterm birth in women at high risk. The UK approach is more cautious, with a desire to demonstrate improved long-term outcomes for the baby, rather than just prevention of preterm birth, before introduction into routine clinical practice. Notwithstanding, identification of subgroups of women who will benefit from progesterone prophylaxis remains a challenge: to date a reduction in preterm birth has only been demonstrated in those either with a previous spontaneous preterm birth or with a short cervix on scan in the second trimester. A strategy to identify nulliparous women at high risk, together with studies to demonstrate that such women would benefit from progesterone, is urgently needed.

N-acetylcysteine has been shown to be beneficial in one study – again further evidence on risks and benefits for the baby is needed prior to adoption into clinical use.

Beyond progesterone, other anti-inflammatory agents in clinical trials for nonobstetric indications include 15d-PGJ_2 and IL-10. Although additional preclinical work is needed, it is hoped that these agents could be tested in clinical trials to reduce preterm birth and improve outcome within the next 5 years. The results of such trials may take some time to accrue: a difficulty in preterm birth research is that the end point of preterm birth prevention is merely a surrogate for the more important outcome of neonatal and childhood mortality and morbidity. The crucially important outcome of offspring neurological function may not be readily assessable until several years of age – thus outcome ascertainment can take many years after randomization. However, the importance of preterm birth, and the rising extent of the death and disability it causes should encourage clinicians, researchers and funders to meet this important challenge, and to identify effective therapies for preterm birth prevention and treatment as soon as possible.

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Key issues

- Increasing evidence highlights parturition as an inflammatory event involving leukocyte influx into the myometrium, cervix and fetal membranes and increased production of proinflammatory mediators, such as IL-6, IL-8 and TNF-α at the time of labor.
- Preterm labor is a major obstetric problem that accounts for up to 75% of neonatal deaths. The causes of preterm labor are poorly understood; however, the presence of intrauterine infection and/or inflammation are thought to be important factors. Therefore, there is growing interest in the hypothesis that in many cases preterm labor occurs due to the premature activation of the inflammatory cascade normally associated with labor at term, either idiopathically, or in response to intrauterine infection.
- Current treatments for preterm labor focus on the use of tocolytic agents to inhibit myometrial contractions. While these agents have had some success in delaying labor, they have not had a significant impact on the number of preterm births and there is little evidence that they improve neonatal outcome.
- The use of anti-inflammatory agents to delay preterm labor offers a novel therapeutic strategy in the treatment of preterm labor. These anti-inflammatory mediators have the potential to be more effective at delaying preterm labor and, more importantly, at improving neonatal outcome as they target the underlying mechanisms thought to trigger preterm labor.
- The pro-resolution actions of some of the anti-inflammatory mediators discussed in this article, such as the lipoxins and resolvins, are particularly interesting mediators to consider as they have the potential to not only reduce inflammatory signaling but also resolve the inflammatory intrauterine environment, therefore limiting fetal exposure to often harmful inflammatory signals.

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