Seminal plasma regulation of the post-coital inflammatory response in the human cervix

David James Sharkey

Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, Australia

A thesis submitted to the University of Adelaide in fulfilment of the requirements for admission to the degree Doctor of Philosophy

August 2005
Abstract

In mice and other mammalian species, deposition of semen into the female reproductive tract elicits a local inflammatory response. Whether a comparable response occurs within the human cervix has not previously been studied. The experiments described in this thesis demonstrate, using cervical tissue biopsies taken before and after intercourse, that exposure to semen elicits an infiltration of leukocytes into the cervical tissue of peri-ovulatory women. Immunohistochemical analysis identified macrophages and dendritic cells as the predominant leukocytes recruited into the cervical epithelium and stroma following intercourse. Cytotoxic / suppressor T lymphocytes and memory T cells were also increased. Comparable responses were not detected following condom-protected intercourse. Quantitative real-time PCR was performed on duplicate tissue biopsies to investigate the molecular regulation of this response. Expression of GM-CSF, a potent stimulator of myeloid cell recruitment, was found to increase by 2.5-fold following unprotected intercourse. Trends towards increased IL-6 and IL-8 mRNA were also observed. Condom-protected intercourse did not activate cytokine expression, further suggesting that exposure to semen, as opposed to mechanical trauma, provides the inflammatory stimulus.

In an in vitro model using the immortalised Ect-1 cell line, TGFβ was identified as a candidate active seminal factor. All three TGFβ isoforms were capable of mimicking the stimulatory ability of seminal plasma in Ect-1 cells and were comparable in their capacity to stimulate both GM-CSF and IL-6 expression in a dose-responsive manner. The addition of TGFβ isoform-specific neutralising antibodies inhibited seminal plasma-induced increases in these cytokines. However TGFβ was unable to stimulate IL-8 production. Addition of IFNγ was found to strongly inhibit TGFβ-stimulated GM-CSF production, and 19-OH PGE₁ was found to increase IL-6 and IL-8, but not GM-CSF production. Responses to seminal plasma constituents were almost exactly replicated in primary cultures of human ectocervical cells. These results identify TGFβ as the major active constituent in human seminal plasma and indicate that other seminal agents, 19-OH PGE₁ and IFNγ, interact with TGFβ to differentially regulate cervical cytokine expression.

Finally, whether human seminal plasma cytokine content was associated with fertility in men was examined. No relationship between seminal plasma TGFβ₁, TGFβ₂, TGFβ₃, IL-8 or bacterial endotoxin content and fertility status was observed. However, there was an increased likelihood of high IFNγ

Sharkey
content in the male partners of couples experiencing infertility, most notable in recurrent miscarriage. The discriminating value of IFNγ was increased when evaluated as a ratio of total TGFβ content.

Inflammatory changes after exposure of the female reproductive tract to seminal plasma are implicated in ‘conditioning' the maternal immune response, to facilitate successful embryo implantation and pregnancy. The studies described in this thesis provide a mechanistic basis for the observations linking exposure to semen with pregnancy success in humans and have expanded our knowledge of the cellular and molecular events that occur within the female reproductive tract following intercourse. Seminal plasma can therefore no longer be thought of as merely a transport medium for spermatozoa, rather as a means for communication between the male and female reproductive tissues, potentially required for optimal pregnancy success.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I further grant my consent to the University of Adelaide to make this thesis available for loan and photocopying once accepted for the degree.

David James Sharkey

August 2005
Acknowledgements

I wish to sincerely thank Assoc. Prof. Sarah Robertson for allowing me the privilege of being involved in the studies presented in this thesis. Her expert guidance and unwavering enthusiasm in the pursuit of scientific discovery was, and continues to be a source of inspiration. I can not express strongly enough just how grateful I am to have had such an amazing mentor and friend.

I would also like to thank Prof. Robert Norman for co-supervising me throughout the course of these studies and for allowing me the use of the facilities at both the Queen Elizabeth Hospital and Repromed.

I would also like to acknowledge the Department of Obstetrics and Gynaecology at the University of Adelaide for providing me with the support and resources required to perform this research. I would especially like to express my gratitude to Prof. Jeffrey Robinson for his support and encouragement during these studies. Thank you also goes to the Postgraduate Coordinator, Assoc Prof David Kennaway and the staff and students of the department of Obstetrics and Gynaecology (both past and present). I am especially grateful for the support and strong friendships I have developed with members of the SAR laboratory.

I would like to express my sincere thanks to Dr Kelton Tremellen for all of his assistance, help and guidance with these studies. Kelton played a very important role in the design of the studies described in chapters 3 and 4 and was instrumental in the recruitment of patients for the study described in chapter 6. His dedication and enthusiasm throughout the course of these studies is greatly appreciated.

I also wish to thank our clinical collaborator in Sweden, Dr Kristina Gemzell-Danielsson for her efforts in the recruitment of patients, and collection of samples, for the studies described in chapters 3 and 4. A big thank you also goes to George Miari and the staff at Repromed for their invaluable and sustained efforts in the collection and processing of clinical samples for use in chapter 6. Thanks also go to Fred Amato for the extended use of his facilities at the Queen Elizabeth Hospital as well as for his advice and assistance in the validation of the assays used in chapter 6. Thank you also to Dr Melinda Jasper for all of her help in teaching me how to perform real-time quantitative PCR and also for her assistance in the analysis of data presented in chapter 4.
The studies described in this thesis were financially supported by grants from the University of Adelaide and the National Health and Medical Research Council. I would also like to express my gratitude to the University of Adelaide for providing me with a University of Adelaide Postgraduate Scholarship as well Assoc. Prof. Sarah Robertson for providing me with additional financial support.

To my fiancée Emma, I would like to express my tremendous appreciation for all of her support, both emotional and financial, and encouragement over the course of these studies. Her patience, understanding and perseverance with me over the last few years has been nothing short of remarkable. Finally, I would like to thank my parents for their continued support throughout this journey.

Sharkey
Publications arising from these and related studies

1. SA Robertson and DJ Sharkey.
The role of semen in induction of maternal immune tolerance to pregnancy (review).

2. SA Robertson, WV Ingman, S O'Leary, DJ Sharkey, KP Tremellen.
Transforming growth factor β - a mediator of immune deviation in seminal plasma (review).

Insemination evokes changes in leukocyte recruitment and pro-inflammatory cytokine production in the human cervix.

4. DJ Sharkey and SA Robertson (in preparation)
Human seminal plasma constituents induce changes in pro-inflammatory cytokine production by cervical epithelial cells in vitro.

5. DJ Sharkey, KP Tremellen, GA Dekker, SA Roberston (in preparation).
The relationship between seminal plasma cytokine content and fertility status in men.

Patient

1. Treatment and diagnosis of a reproductive disorder by measuring or inhibiting Interferon gamma.
International publication number IP0240US. Published 20th September 2002.
Abstracts arising from these studies

*Presenting author underlined*

2000

- **DJ Sharkey, KP Tremellen, K Gemzell-Danielsson, SA Robertson.**

- **DJ Sharkey, KP Tremellen, K Gemzell-Danielsson, SA Robertson.**
  "Insemination evokes an inflammatory response within the human cervix". Australian Society for Medical Research National Conference, Melbourne, Australia.

- **DJ Sharkey, KP Tremellen, K Gemzell-Danielsson, SA Robertson.**
  "The inflammatory response to semen in the human cervix". Australian Society for Medical Research South Australian Conference, Australia.

2001

- **DJ Sharkey, KP Tremellen, SA Robertson, GA Dekker.**
  "The role of male-derived immunoregulatory molecules in early human pregnancy". Australian Society of Immunology (South Australian Division) Annual Meeting, Adelaide, South Australia.

2002

- **DJ Sharkey, KP Tremellen, GA Dekker, SA Robertson.**
  "Seminal cytokine concentration and human reproductive outcome". Society for Reproductive Biology. 33rd Annual Conference, Adelaide, South Australia.

- **DJ Sharkey, KP Tremellen, GA Dekker, SA Robertson.**
  "The effect of seminal plasma cytokine content on human reproductive outcome". Australian Society of Immunology (South Australian Division) Annual Meeting, Adelaide, South Australia.
• DJ Sharkey, MJ Jasper, KP Tremellen, K Gemzell-Danielsson, SA Robertson.
"Insemination induces pro-inflammatory cytokine mRNA expression within the human cervix". Society for Reproductive Biology, 34th Annual Conference, Melbourne, Australia.

• DJ Sharkey, KP Tremellen, GA Dekker, SA Robertson.
"Human seminal cytokine concentration and pregnancy outcome". Australian Society for Medical Research (South Australian Division) Scientific Conference, Adelaide, Australia.

2004

• DJ Sharkey, MJ Jasper, KP Tremellen, K Gemzell-Danielsson, SA Robertson.
"Pro-inflammatory cytokine mRNA expression is induced within the human cervix following insemination". Society for the Study of Reproduction, 37th Annual meeting, Vancouver, Canada.

• DJ Sharkey and SA Robertson.
"Seminal plasma TGFβ activates pro-inflammatory cytokine synthesis in human cervical epithelial cells". Society for Reproductive Biology, 35th Annual Conference, Sydney, Australia.
## Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Declaration</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Publications arising from these and related studies</td>
<td>vi</td>
</tr>
<tr>
<td>Patent</td>
<td>vi</td>
</tr>
<tr>
<td>Abstracts arising from these studies</td>
<td>vii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>ix</td>
</tr>
<tr>
<td>List of figures</td>
<td>xvii</td>
</tr>
<tr>
<td>List of tables</td>
<td>xx</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xxi</td>
</tr>
</tbody>
</table>
Chapter 1  Literature review ................................................................. 1

1.1  INTRODUCTION .................................................................................... 2

1.2  THE ROLE OF CYTOKINES IN THE FEMALE REPRODUCTIVE TRACT ...... 3

1.2.1  Introduction .................................................................................... 3

1.2.2  Transforming growth factor beta (TGFβ) ......................................... 5
   1.2.2.1  TGFβ isoforms ........................................................................ 5
   1.2.2.2  Regulation of TGFβ activation ................................................ 6
   1.2.2.3  TGFβ signalling .................................................................... 8
   1.2.2.4  Role of TGFβ and its receptors in early pregnancy .................... 10

1.2.3  Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) .......... 13
   1.2.3.1  Introduction ............................................................................ 13
   1.2.3.2  Role of GM-CSF and its receptors in early pregnancy ............... 13

1.2.4  Interleukin-1 (IL-1) ........................................................................ 16
   1.2.4.1  Role of IL-1 in early pregnancy ............................................. 16

1.2.5  Interleukin-6 (IL-6) ......................................................................... 17
   1.2.5.1  Role of IL-6 in early pregnancy ............................................. 17

1.2.6  Interleukin-8 (IL-8) ......................................................................... 19
   1.2.6.1  Role of IL-8 in early pregnancy ............................................. 19

1.2.7  Tumour Necrosis Factor alpha (TNFα) ............................................. 21
   1.2.7.1  Role of TNFα in early pregnancy .......................................... 21

1.2.8  Interferon gamma (IFNγ) ................................................................. 23
   1.2.8.1  Role of IFNγ in early pregnancy ............................................ 23

1.3  THE ROLE OF LEUKOCYTES IN THE FEMALE REPRODUCTIVE TRACT .... 25

1.3.1  The female reproductive tract as an immunocompetent site ............... 25

1.3.2  Leukocyte populations within the uterus and cervix ......................... 26
   1.3.2.1  Introduction .......................................................................... 26
   1.3.2.2  Neutrophils .......................................................................... 26
   1.3.2.3  Macrophages ....................................................................... 27
   1.3.2.4  Dendritic cells .................................................................... 30
THE MATERNAL IMMUNE RESPONSE TO PREGNANCY

1.4.1 The fetus as an allograft

1.4.2 Post-mating inflammatory response to semen in the murine uterus

1.4.3 Post-coital inflammatory response in the human cervix

1.4.4 The inflammatory response and implantation

ROLE OF SEMINAL PLASMA IN REPRODUCTION

1.5.1 Introduction

1.5.2 The role of seminal plasma in reproduction in animals

1.5.3 The role of seminal fluids in human pregnancy

1.5.4 The immunomodulatory role of seminal plasma

1.5.5 Antigens contained within semen

1.5.6 Role of immune-deviating cytokines in seminal plasma

Chapter 2 Materials and Methods

2.1 HUMAN STUDIES

2.1.1 Ethics approval

2.2 CERVICAL BIOPSIES

2.2.1 Subject population

2.2.2 Experimental design
2.2.3 Tissue histology

2.2.3.1 Monoclonal antibodies

2.2.3.2 Tissue sectioning

2.2.3.3 Immunohistochemistry

2.2.4 Quantitation of mRNA expression

2.2.4.1 General precautions for RNAse-free conditions

2.2.4.2 Isolation of total cellular RNA

2.2.4.3 Reverse transcription

2.2.4.4 Oligonucleotide primer design

2.2.4.5 Polymerase chain reaction (PCR)

2.2.4.6 Gel electrophoresis of PCR products

2.2.4.7 Sequencing of PCR products

2.3 IN VITRO CULTURE OF HUMAN CERVICAL CELLS

2.3.1 Patient participation and tissue collection

2.3.2 Cell culture

2.3.2.1 General

2.3.2.2 Cell lines

2.3.2.3 Culture medium

2.3.2.4 Cell quantification

2.3.2.5 Recombinant cytokines and cytokine neutralising antibodies

2.3.2.6 Primary ectocervical epithelial cell cultures

2.3.2.7 Immortalised epithelial cell cultures

2.3.3 Cervical epithelial cell culture supernatant ELISAs

2.3.3.1 GM-CSF

2.3.3.2 IL-6

2.3.3.3 IL-8

2.3.3.4 IL-10

2.4 SEMINAL PLASMA CYTOKINE ASSAY

2.4.1 Subject population

2.4.2 Seminal plasma collection and processing

2.4.2.1 Seminal plasma collection
2.4.2.2 Acid activation of latent TGFβ ........................................... 79
2.4.3 Seminal plasma cytokine ELISAs and endotoxin assay .................................. 80
  2.4.3.1 IL-8 ........................................................................ 80
  2.4.3.2 IFNγ .................................................................... 81
  2.4.3.3 TGFβ1 .................................................................... 81
  2.4.3.4 TGFβ2 .................................................................... 81
  2.4.3.5 TGFβ3 .................................................................... 82
  2.4.3.6 Bacterial endotoxin (LPS) ........................................... 82

Chapter 3  The effect of intercourse on leukocyte recruitment and activation within the human cervix ................................................. 83
  3.1 INTRODUCTION ................................................................. 84
  3.2 THE EFFECT OF INTERCOURSE ON LEUKOCYTE RECRUITMENT AND ACTIVATION IN THE EPITHELIUM AND STROMA OF THE HUMAN CERVIX ................. 86
  3.3 DISCUSSION ................................................................. 101

Chapter 4  Exposure to semen following intercourse induces pro-inflammatory cytokine mRNA expression within the human cervix ................. 107
  4.1 INTRODUCTION ................................................................. 108
  4.2 THE EFFECT OF INTERCOURSE ON GM-CSF, IL-1α, IL-6 AND IL-8 mRNA EXPRESSION WITHIN THE HUMAN CERVIX ................................................................. 110
  4.3 THE EFFECT OF INTERCOURSE ON TNFα AND IFNγ mRNA EXPRESSION WITHIN THE HUMAN CERVIX ................................................................. 114
  4.4 THE EFFECT OF INTERCOURSE ON CCR5 AND CXCR4 CHEMOKINE RECEPTOR mRNA EXPRESSION IN THE HUMAN CERVIX ................................................................. 116
  4.5 DISCUSSION ................................................................. 118
Chapter 5  Pro-inflammatory activity of seminal plasma constituents .............................................. 125

5.1 INTRODUCTION .................................................................................................................. 126
5.2 THE EFFECT OF SEMINAL PLASMA ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY HUMAN FEMALE REPRODUCTIVE TRACT CELL LINES ........................................ 129
5.3 THE DOSE RESPONSE TO SEMINAL PLASMA INDUCED GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO ................................. 132
5.4 THE EFFECT OF SEMINAL PLASMA ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY PRIMARY CERVICAL EPITHELIAL CELLS IN VITRO .............................................. 134
5.5 THE EFFECT OF RECOMBINANT TGFβ1, TGFβ2 AND TGFβ3 ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO .............................................. 137
5.6 THE EFFECT OF RECOMBINANT TGFβ1, TGFβ2 AND TGFβ3 ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY PRIMARY CERVICAL EPITHELIAL CELLS IN VITRO ......................... 140
5.7 THE EFFECT OF IFNγ ON GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO ........................................................................... 143
5.8 THE EFFECT OF RECOMBINANT TGFβ1 AND IFNγ ON GM-CSF, IL-6 AND IL-8 PRODUCTION BY PRIMARY CERVICAL EPITHELIAL CELLS IN VITRO ........................................ 146
5.9 THE EFFECT OF TGFβ ISOFORM-SPECIFIC NEUTRALISING ANTIBODIES ON SEMINAL PLASMA-INDUCED INCREASE IN GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO .............................................. 149
5.10 THE EFFECT OF 19-HYDROXY PROSTAGLANDIN E1 ON GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO .............................................. 152
5.11 DISCUSSION ....................................................................................................................... 155

Chapter 6  The relationship between seminal plasma cytokine content and fertility status in men ........ 162

6.1 INTRODUCTION .................................................................................................................. 163
6.2 STUDY DESIGN.................................................................................................................... 165
6.3 GENERAL SEMEN CHARACTERISTICS OF PARTICIPANTS IN THIS STUDY ........................... 167
6.4 THE RELATIONSHIP BETWEEN SEMINAL PLASMA CYTOKINE CONTENT AND FERTILITY STATUS IN MEN .................................................................................................................. 169

6.4.1 Validation and optimisation of ELISA assays for measuring total TGFβ1 content in human seminal plasma samples ................................................................................................................. 169
6.4.2 Validation and optimisation of ELISA assays for measuring biologically active TGFβ1, and biologically active and total TGFβ2 .............................................................................................. 172
6.4.3 The relationship between seminal plasma TGFβ1 content and fertility status .................................................................................................................. 173
6.4.4 The relationship between seminal plasma TGFβ2 content and fertility status .................................................................................................................. 175
6.4.5 The relationship between seminal plasma TGFβ3 content and fertility status .................................................................................................................. 177
6.4.6 The relationship between seminal plasma total TGFβ content and fertility status ........................................................................................................... 178

6.5 THE RELATIONSHIP BETWEEN IL-8 CONTENT IN SEMINAL PLASMA AND FERTILITY STATUS IN MEN.................................................................................. 180
6.6 THE RELATIONSHIP BETWEEN IFNγ CONTENT AND FERTILITY STATUS IN MEN............................................................................................................. 181
6.7 EFFECT OF SEMINAL PLASMA BACTERIAL ENDOTOXIN CONCENTRATION ON PREGNANCY OUTCOME.................................................................................. 183
6.8 ASSOCIATIONS BETWEEN CYTOKINE AND SPERM PARAMETERS IN SEMEN .... 185
6.8.1 Correlation between individual cytokine content ................................................................................................................................. 185
6.8.2 Correlation between cytokine content and sperm parameters ............................................................................................................ 186
6.8.3 Correlation between cytokine content and leukocytes ...................................................................................................................... 186

6.9 VARIATION IN SEMINAL PLASMA TGFβ CONTENT WITHIN INDIVIDUALS OVER TIME .......................................................................................... 187
6.10 VARIATION IN SEMINAL PLASMA IFNγ, IL-8 AND ENDOTOXIN CONTENT WITHIN INDIVIDUALS OVER TIME ................................................................. 189
6.11 DISCUSSION .......................................................................................................................... 190

Chapter 7 General discussion and conclusions ........................................... 197
7.1 DISCUSSION AND CONCLUSION ................................................................. 198

Appendix A ........................................................................................................... 210
A.1 CERVICAL IMMUNE RESPONSE TO TGFβ PATIENT INFORMATION SHEET .......... 211
A.2 CERVICAL IMMUNE RESPONSE TO TGFβ STUDY CONSENT FORM ............ 212
List of figures

FIGURE 1.1 The formation of latent TGFβ complexes ................................................................. 6
FIGURE 1.2 Schematic illustration of the main TGFβ receptors involved in TGFβ binding and signal transduction .................................................................................................................. 9
FIGURE 1.3 Schematic illustration of the molecular and cellular events which occur during the post-mating inflammatory response in mice .............................................................................. 45
FIGURE 2.1 A time line showing the relative times at which cervical biopsies were collected with respect to both intercourse (or abstinence) and day after the LH peak ................................................. 60
FIGURE 2.2 RT-PCR amplification products from human ectocervical cDNA using primers specific for TNFα, IFNγ, GM-CSF, CXCR4, CCR5, β-actin, IL-6, IL-8 and IL-1α .................. 70
FIGURE 2.3 Acid activation of pooled human seminal plasma samples ........................................ 80
FIGURE 3.1 The effect of exposure to semen following intercourse on leukocyte numbers in the epithelium of the cervix .................................................................................................................. 88
FIGURE 3.2 The effect of exposure to semen following intercourse on leukocyte numbers in the stroma of the cervix ..................................................................................................................... 90
FIGURE 3.3 The effect of intercourse on leukocyte recruitment in the human cervix .................... 99
FIGURE 4.1 Effect of intercourse on GM-CSF, IL-1α, IL-6 and IL-8 cytokine mRNA expression within the human cervix .................................................................................................................. 112
FIGURE 4.2 Effect of intercourse on TNFα and IFNγ cytokine mRNA expression within the human cervix ........................................................................................................................................... 115
FIGURE 4.3 Effect of intercourse on CCR5 and CXCR4 chemokine receptor mRNA expression within the human cervix .................................................................................................................. 117
FIGURE 5.1 Effect of seminal plasma on Ect1, End1 and Vk2 epithelial cell line GM-CSF, IL-6 and IL-8 production in vitro .................................................................................................................. 131
FIGURE 5.2 Effect of seminal plasma on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro ................................................................. 133

FIGURE 5.3 Effect of seminal plasma on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro ................................................................. 136

FIGURE 5.4 Effect of recombinant TGFβ on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro ................................................................. 139

FIGURE 5.5 Effect of recombinant TGFβ on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro ................................................................. 142

FIGURE 5.6 Effect of recombinant TGFβ1 and IFNγ on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro ................................................................. 145

FIGURE 5.7 Effect of recombinant TGFβ1 on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro ................................................................. 148

FIGURE 5.8 Effect of TGFβ isoform-specific neutralising antibodies on seminal plasma-induced increase in GM-CSF, IL-6 and IL-8 production by Ect1 cervical epithelial cells in vitro ................................................................. 150

FIGURE 5.9 Effect of 19-OH PGE on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro ................................................................. 153

FIGURE 6.1 Test of similarity between recombinant TGFβ1 standard and seminal plasma TGFβ1 content in a TGFβ1-specific ELISA assay ................................................................. 170

FIGURE 6.2 The effect of dilution on TGFβ1 inter-assay coefficient of variation ................................................................. 171

FIGURE 6.3 Concentration of total and biologically active TGFβ1 in human seminal plasma ................................................................. 174

FIGURE 6.4 Concentration of total and biologically active TGFβ2 in human seminal plasma ................................................................. 176

FIGURE 6.5 Concentration of total TGFβ3 in human seminal plasma ................................................................. 178

FIGURE 6.6 Concentration of total TGFβ in human seminal plasma ................................................................. 179

FIGURE 6.7 The effect of IL-8 concentration on pregnancy outcome ................................................................. 180

FIGURE 6.8 The effect of IFNγ concentration on fertility status ................................................................. 182

FIGURE 6.9 Concentration of bacterial endotoxin within human seminal plasma samples ................................................................. 184
FIGURE 6.10 Variation in seminal plasma TGFβ content within individuals over time ........................................... 188

FIGURE 6.11 Variation in individual cytokine content within individuals over time ........................................... 190

FIGURE 7.1 Schematic illustration of the cellular and molecular events that occur within the female reproductive tract following intercourse ................................................................. 201

FIGURE 7.2 Schematic illustration of the potential consequences of the post-coital inflammatory response in the human cervix ................................................................. 207
List of tables

TABLE 2.1 The antigenic specificities and cell lineage specificities of the mouse anti-human mAbs used for the immunohistochemical analysis of cervical tissue samples ............... 62

TABLE 2.2 PCR primers used to quantify cytokine and chemokine mRNA expression .................. 67

TABLE 2.3 Primer amplification efficiencies and linear regression equations for individual oligonucleotide primers used in this study ......................................................... 69

TABLE 2.4 The species, lineage, origin and culture media for cell lines used in this thesis ............... 72

TABLE 3.1 Proportion of positively labelled cells detected within the cervical epithelium before and after either abstinence, condom-protected or unprotected intercourse ................... 95

TABLE 3.2 Proportion of positively labelled cells detected within the cervical stroma before and after either abstinence, condom-protected or unprotected intercourse ................... 97

TABLE 5.1 The effect of seminal plasma, TGFβ, 19-OH PGE1 and IFNγ on pro-inflammatory cytokine expression from cervical epithelial cells in vitro .......................................... 155

TABLE 6.1 Characteristics of human semen samples included in this study .......................... 168

TABLE 6.2 Typical performance characteristics of human cytokine assay kits used in this study .... 172

TABLE 6.3 Comparison of seminal plasma IFNγ concentrations between proven fertile men and male partners of couples experiencing infertility .................................................... 183
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BPE</td>
<td>Bovine pituitary extract</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CIRT</td>
<td>Cervical immune response to TGFβ</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine tetrachloride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified minimal essential medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's PBS</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbency assay</td>
</tr>
<tr>
<td>EU</td>
<td>Equivalent units</td>
</tr>
<tr>
<td>FACs</td>
<td>Fluorescence-activated cell scanning</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotrophin</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth retardation</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilisation</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-dalton</td>
</tr>
<tr>
<td>KSFM</td>
<td>Keratinocyte serum-free medium</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate assay</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency associated protein</td>
</tr>
<tr>
<td>LCA</td>
<td>Leukocyte common antigen</td>
</tr>
<tr>
<td>LGL</td>
<td>Large granular lymphocytes</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent transforming growth factor β binding protein</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
</tbody>
</table>
| MMP          | Matrix metalloproteinase  
| MQ           | Milli-Q  
| mRNA         | Messenger RNA  
| NHS          | Normal human serum  
| 19-OH PGE    | 19-hydroxy prostaglandin E  
| NK           | Natural killer  
| NWAHS        | North Western Allied Health Service  
| °C           | Degrees celcius  
| PBMC         | Peripheral blood mononuclear cell  
| PBS          | Phosphate buffered saline  
| PCR          | Polymerase chain reaction  
| PGE          | Prostaglandin  
| PSA          | Prostate specific antigen  
| RNA          | Ribonucleic acid  
| RNase        | Ribonuclease  
| rpm          | Revolutions per minute  
| RT-PCR       | Reverse transcriptase polymerase chain reaction  
| SDS          | Sodium dodecyl sulphate  
| SEPO         | Semen exposure and pregnancy outcome  
| T            | Thymine  
| TGF          | Transforming growth factor  
| TIMP         | Tissue inhibitor of metalloproteinase  
| TLR          | Toll-like receptor  
| TLX          | Trophoblast / lymphocyte cross-reactive antigen  
| TNF          | Tumour necrosis factor  
| TSP-1        | Thrombospondin-1  
| U            | Uracil  

Sharkey
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>VIA</td>
<td>Video image analysis</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
Chapter 1

Literature review
1.1 INTRODUCTION

Studies in mice have shown that the deposition of semen into the female reproductive tract evokes changes in leukocyte behaviour and cytokine expression in the oestrogen-primed uterine endometrium, which closely resemble a 'classical' inflammatory response. The key active molecule in seminal plasma is identified as transforming growth factor beta (TGFβ) (Tremellen et al. 1998), which together with other factors present in seminal plasma is known to stimulate the production of pro-inflammatory cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 6 (IL-6) by uterine epithelial cells. GM-CSF as well as various chemokines, induced during this response are key factors in the recruitment of inflammatory leukocytes, predominantly neutrophils and macrophages, into the underlying uterine stroma and lumen. These cells are then activated to release interleukin 1 (IL-1) and tumour necrosis factor alpha (TNFα) in association with upregulated expression of cell surface molecules such as major histocompatibility complex (MHC) class II and B7 involved in antigen uptake and presentation (reviewed by Robertson et al. 2001a). To date, our knowledge of whether a similar response occurs in women is very poor. Two studies have shown that after donor insemination, there is an infiltration of neutrophils into the cervical lumen (Pandya et al. 1985, Thompson et al. 1992). However, these studies examined only the superficial surface of the cervical epithelium, and there have been no studies to formally examine whether exposure to semen following intercourse causes inflammatory changes in the underlying cervical tissue.

Seminal plasma has already been identified to have an immuno-modulatory role in reproductive processes, and these observations suggest a further role for semen in providing a 'priming' stimulus to the pre-implantation uterus. To date, our knowledge of the physiological significance of seminal plasma in pregnancy has come largely from mice and studies in other animal species. As in the mouse, TGFβ is contained within human seminal plasma at concentrations higher than those observed in any other biological fluid (Nocera et al. 1993, Srivastava et al. 1996, Loras et al. 1999). Studies comparing the levels of TGFβ within human seminal plasma obtained from fertile men and infertile men with various testicular pathologies (including secretory pathology and epididymal occlusion) found no significant differences in biologically active TGFβ, however a small increase in total TGFβ was detected in men with secretory testicular pathologies (Loras et al. 1999). To date, there has been no investigation into the relationship between TGFβ concentrations within seminal plasma of overtly fertile men and its effect on pregnancy outcome in their partners. Epidemiological studies have however linked lack of exposure to semen, through short-periods of sexual cohabitation or the use of barrier methods of

Sharkey Chapter 1
contraception with an increased risk of pre-eclampsia (Klonoff-Cohen et al. 1989, Robillard et al. 1995).

There are currently several proposed mechanisms by which the post-mating inflammatory response may facilitate embryo implantation and establishment of optimal pregnancy. TGFβ is a potent type 2 immune-deviating molecule and has been shown to be a key tolerance-inducing molecule in the ocular, intestinal and respiratory mucosae (Letterio et al. 1998). There is some evidence suggesting that this response may prime the maternal immune system and induce tolerance to paternal transplantation antigens, thereby promoting placental development through inhibiting the potentially detrimental effects of a cell-mediated immune response against the placenta. Secondly, this response may promote endometrial tissue remodelling, for example breakdown of extracellular matrix (ECM) or changes in epithelial integrin expression to accommodate embryo attachment and trophoblast invasion. Thirdly, seminal plasma may induce changes in either the production or composition of embryotrophic growth factors within the oviduct or uterine luminal fluid, thereby facilitating the attachment and growth of the embryo. A fourth, but still rather speculative role for this response may be in promoting ovulation and corpus luteum development.

This chapter will review the current status of our understanding of the immune response in early pregnancy, with a focus on the potential contribution of seminal plasma in regulating the changes necessary to allow establishment of pregnancy. The discussion will centre on both human and mouse studies.

1.2 THE ROLE OF CYTOKINES IN THE FEMALE REPRODUCTIVE TRACT

1.2.1 Introduction

Cytokines are known to play a central role in cell-cell communication in reproductive processes ranging from ovulation, blastocyst maturation, implantation, placental growth and parturition (Robertson et al. 1994). A better understanding of the roles of cytokines in reproductive tissues, and the regulation of their expression and function is required.

Cytokines act as soluble intercellular signalling molecules capable of effecting cell proliferation, differentiation, adhesion, migration and chemotaxis. The binding of a cytokine to its membrane bound receptor usually activates an associated protein kinase, the resulting phosphorylated proteins then
stimulate the production of nuclear transcription factors which in turn activate cellular proto-oncogenes, culminating in the generation of a cellular response (Taga et al. 1995). Cytokine receptors commonly consist of a heterodimeric combination \( \alpha \) and \( \beta \) subunits, with the \( \alpha \) subunit providing cytokine specificity while the \( \beta \) subunits increase the binding affinity of the cytokine to the \( \alpha \) subunit, and facilitate signal transduction through their long intra-cytoplasmic domains (Miyajima et al. 1992).

The ability of cytokines to act at very low concentrations (often in the range of pico-nano molar), along with their short half-life, localised site of action and tightly regulated production at both the transcriptional and translational level make them the ideal intercellular signalling system. Cytokines do not act in isolation, as most cells are exposed to several cytokines at any one time. The same cytokine may have either a synergistic or antagonist effect on a particular cell depending on the cell type, its location and the surrounding microenvironment (i.e. other cytokines, prostaglandins etc). The effect a cytokine exhibits may also be modified by the presence of soluble receptors and binding proteins, which may enhance or prevent binding of the cytokine to its receptor.

Cytokine redundancy is an important factor governing the function of the cytokine signalling system in nature (Kelso 1994). The concept of cytokine redundancy describes the phenomena by which many different cytokines can act on the same or similar cell type to exert the same or a similar effect. The removal of any one cytokine may not result in an observable effect on the cells phenotype, since it is functionally compensated for by other cytokines. In recent times, the molecular basis underlying this 'cytokine redundancy' has been investigated which has allowed potential mechanisms for this action to be proposed. An example of this is the observation that despite the cytokines IL-6, Leukaemia inhibitory factor (LIF) and IL-11 being recognised by distinct receptors, they all use a common gp130 subunit signalling pathway. Due to this 'shared' signalling pathway, it is possible that LIF or IL-11 may be capable of substituting for IL-6 and provide the same effect (Gearing et al. 1992).

Several cytokines are thought to be of particular relevance during early pregnancy, with increasing evidence suggesting that TGF\( \beta \), GM-CSF, IL-1, IL-6 and TNF\( \alpha \) have important roles in facilitating the correct permissive maternal immune environment to allow successful pregnancy to occur. This section of the literature review will look at the potential roles of each of these cytokines during early pregnancy.

Sharkey Chapter 1
1.2.2 Transforming growth factor beta (TGFβ)

1.2.2.1 TGFβ isoforms

TGFβ belongs to a large family of growth factors with roles in cell growth, differentiation and migration, as well as in the formation of the extracellular matrix and regulation of the expression of cell surface molecules. TGFβ₁ was the first of this family of growth factors to be isolated and was named as such due to its ability to induce differentiation of rat fibroblast cells to an anchorage-independent state, when administered in concert with transforming growth factor-alpha (TGFα) (Roberts et al. 1981). The best characterised members include the five isoforms of TGFβ, while activin, inhibin, mullerian inhibitory substance, the bone morphogenetic proteins and the growth and differentiation factors are part of the broader family group. TGFβ₁, TGFβ₂ and TGFβ₃ are expressed in mammals, while TGFβ₄ and TGFβ₅ are expressed in birds and amphibians such as Xenopus laevis respectively (Ohta et al. 1987, Derynck et al. 1988, Jakowlew et al. '1988, Roberts et al. 1990). At the molecular level, proteins belonging to the TGFβ family display between 30 and 80% homology in their amino acid sequences along with sharing an almost identical protein structure consisting of an approximately 25 kDA disulphide-linked homodimer with several conserved cysteine residues (Ruscetti et al. 1991). The emphasis of this section of the literature review will be on the different isoforms of TGFβ as this will be a major focus of the current study.

The mammalian isoforms of TGFβ₁, β₂ and β₃ are encoded by individual genes with distinct promoters and are located on chromosomes 19q13, 1q41 and 14q24 in the human, and on chromosomes 7,1 and 2 in the mouse. The three isoforms of TGFβ found in mammalian species share over 70% amino acid sequence homology at their N-terminal ends, and more than 97% identity in their physical structure (Daopin et al. 1992), suggesting their likely origin was through duplication of a common ancestral gene. Interestingly, there is considerable conservation between TGFβ₁ cDNAs from different mammalian species, with almost complete amino acid sequence identity within the entire carboxy-terminal domain, which gives rise to cross-species bioactivity.

In vitro studies once led to the belief that the different isoforms of TGFβ were functionally redundant and had interchangeable biological activities, a notion supported by the finding that each isoform signals through the same receptors. However, distinct differences contained within the promoter region along with differing expression patterns provided insight into their having isoform specific functions in vivo (reviewed by Lawrence 1996). The expression of TGFβ₁ appears to be under
the influence of oncogenes and immediate early genes as well as autocrine regulation, while TGFβ\(_2\) and TGFβ\(_3\) are predominantly hormonally and developmentally regulated.

### 1.2.2.2 Regulation of TGFβ activation

The TGFβ genes encode pre-proproteins of 390-412 amino acids that requires a number of processing steps intracellularly before it is secreted by the cell. A key step in this process is the proteolytic cleavage into two subunits that assemble into a heterodimer termed latent TGFβ. One subunit is a 65-75 kDa glycosylated protein termed the 'latency associated protein' (LAP), and the other is a 12.5 kDa C-terminal derived protein that corresponds to mature bioactive TGFβ (Derynck et al. 1985). This complex is then linked covalently to a 125-160 kDa latent TGFβ binding protein (LTBP) to produce a 225-260 kDa complex (see Figure 1.1) (Wakefield et al. 1988, Miyazono et al. 1993). The LTBP has been implicated in facilitating secretion from the cell, after which the 12.5 kDa C-terminal subunits dissociate from the latent precursor and the 25kDa biologically active form of TGFβ is produced when the two monomer subunits bind together via the formation of disulphide bonds, forming homodimer (although heterodimers are encountered on rare occasions). Interestingly, the LAPs of TGFβ\(_1\), β\(_2\) and β\(_3\) are significantly more disparate than their C-terminal fragments sharing only 30–40% homology, with in vitro studies indicating that TGFβ\(_1\) LAP is capable of conferring latency on TGFβ\(_2\) and β\(_3\), providing further evidence that complex interactions exist between isoforms of TGFβ that may help to further regulate activation (Bottinger et al. 1996). Therefore, the release of active TGFβ from its latent precursor form is likely an important factor in limiting the biological activity of TGFβ.

![Diagram of TGFβ complex formation](image)

**FIGURE 1.1 The formation of latent TGFβ complexes.** Details are given in text. LAP, latency associated protein; LTBP, latent TGFβ binding protein.
There are various ways in which the biologically active form of TGFβ can be released from its latent precursor form. In vitro, these include low pH through transient acidification, heating or treatment with urea (Lawrence et al. 1985, Lyons et al. 1988, Miyazono et al. 1989, Schultz et al. 1993). In vivo, activation is more likely to be achieved through the actions of biological enzymes. Physiological release of biologically active TGFβ within the female reproductive tract could partially be accounted for by the acidic environment of the vagina, commonly at pH 3.5 – 5, though clearly other mechanisms also exist to enhance this activation. Enzymatic activation by proteases including plasmin, thrombospondin, cathepsins B and D, calpain and glycosidases such as endoglycosidase F, sialidase and neuraminidase have more recently been described (Chu et al. 1998, Khalil 1999). Thrombospondin-1 (TSP-1) has a key role in activation of TGFβ in vivo through its interaction with the LAP of latent TGFβ, resulting in structural changes that expose TGFβ receptor binding sites (Schultz et al. 1993). The physiological importance of TSP-1 in activation of TGFβ was further confirmed by the observation that mice lacking a functional TSP-1 gene developed inflammatory lesions within the lungs and pancreas and that these lesions were significantly alleviated when mice were subsequently injected with KRFK, a region of TSP-1 responsible for TGFβ activation (Crawford et al. 1998). TSP-1 may also be important in the activation of TGFβ within the human female reproductive tract since cervical and endometrial epithelial cells express TSP-1 throughout the menstrual cycle and during pregnancy (Wu et al. 2004, Kawano et al. 2005). Therefore, it is possible that TSP-1 may also contribute to the activation of the large amounts of latent TGFβ deposited into the female reproductive tract following intercourse. Recently another protease independent mechanism for the activation of TGFβ has been identified using epithelial cells that express αvβ6 integrin (Munger et al. 1999). The RGD sequences in the LAP domain of L-TGFβ appear to complex with the αvβ6 integrin on epithelial cells resulting in conformational changes that permit type II TGFβ receptor binding (Munger et al. 1999). Furthermore, this binding was observed to occur without the release of LAP, or active TGFβ (Khalil 1999). Whether integrin αvβ6 contributes to TGFβ activation in the human female reproductive tract is yet to be investigated, however it is possible since it is constitutively expressed by endometrial epithelial cells (Bowen et al. 2000). The precise physiological significance of each of these molecular pathways in activating TGFβ in vivo is yet to be determined.
1.2.2.3 TGFβ signalling

TGFβ is able to exert its biological effects over a broad range of cell types so it is not surprising that receptors for TGFβ are expressed by almost all tissues throughout the body, including the human endometrium and cervix (Piestrzeniewicz-Ulanska et al. 2002). The type I and type II receptors (termed Tβ-RI and Tβ-RII respectively) are transmembrane glycoprotein signal transducers that contain cytosolic domains with serine/threonine kinase activity (see Figure 1.2) (Wrana et al. 1992) and are expressed at high levels on all cells. TGFβ1 and β3 are unable to bind to Tβ-RI in the absence of Tβ-RII, although they are capable of binding with high affinity directly to Tβ-RII even in the absence of Tβ-RII (Laiho et al. 1991, Massague 1998). Conversely, TGFβ2 has a low affinity for Tβ-RII and therefore is unable to bind directly to this receptor, instead relying on the co-expression of either Tβ-RI or additional transmembrane molecules such as betaglycan to facilitate its binding to Tβ-RII (Rodriguez et al. 1995, del Re et al. 2004).

Three additional membrane-bound receptors have been identified as having the ability to bind TGFβ, however, none of these receptors have the ability to induce signal transduction and are believed to play a role in sequestering TGFβ to the plasma membrane (Andres et al. 1989). These additional receptors include firstly, the type III receptor commonly referred to as Betaglycan which can bind all three mammalian isoforms of TGFβ. Betaglycan has only a very short cytoplasmic tail and has no apparent signalling motif (see Figure 1.2) (Wang et al. 1991), and along with localising TGFβ to the cell surface it may also play a pivotal role in stabilising the interaction between TGFβ2 and Tβ-RII (Lopez et al. 1994, Massague 1998) by enhancing TGFβ signalling through its interaction with the cytoplasmic region of Tβ-RII (Blobe et al. 2001). Some cells are also capable of secreting a soluble (non-membrane bound) form of the type III receptor that may serve as a reservoir for ligand retention (Miyazono et al. 1993). Another transmembrane receptor known as Endoglin (or CD105) is unable to bind TGFβ on its own, however it is capable of binding TGFβ1 and β3 with high affinity in the presence of Tβ-RI and Tβ-RII receptors (Yamashita et al. 1994). It is suggested that through interactions with its extracellular and cytoplasmic domains with the signalling receptors, endoglin may help to modulate TGFβ responses (Guerrero-Esteo et al. 2002) though just what effect this has on TGFβ signalling may depend on the type of target cell. For example, it has been shown that endoglin antagonises the inhibitory effect of TGFβ on human endothelial cells (Li et al. 2000) yet is able to enhance TGFβ-induced inhibition of human trophoblast differentiation (Caniggia et al. 1997). Finally, the transmembrane pseudoreceptor BAMBI which is related to the TGFβ Tβ-RI receptor but lacks an intracellular kinase domain has
recently been identified as a negative regulator of not only TGFβ signalling but also of activin and bone morphogenic protein (BMP) signalling (Onichtchouk et al. 1999). It is thought to exert this inhibitory effect by competing for ligand binding. More recently it has been discovered that the transcription of BAMBI is regulated by TGFβ signalling through direct binding of SMAD3 and SMAD4 to the BAMBI promoter (Sekiya et al. 2004). Clearly the large and diverse range of TGFβ receptors and binding proteins described above allow for strict and complex regulation of TGFβ signalling by controlling the binding of TGFβ ligand to its receptor complexes.

![Diagram of TGFβ receptors](image)

**FIGURE 1.2 Schematic illustration of the main TGFβ receptors involved in TGFβ binding and signal transduction.**

TGFβ responses are generally cell-type specific and are dependent on both the concentrations of TGFβ signalling components and the activity of other signal transduction pathways, which can either synergise with or antagonise the TGFβ pathway. TGFβ signal transduction is induced by the phosphorylation of SMAD proteins (Kretzschmar et al. 1998) by serine-threonine kinases such as MAD (Chen et al. 1996, Liu et al. 1997) and TAK1 (Wang et al. 1997). The phosphorylation of SMAD2 and SMAD3 results in the formation of a hetero-oligomeric complex with SMAD4 before being translocated to the nucleus, where they exert their effect by regulating transcriptional responses (Nakao et al. 1997, Zhang et al. 1997). Other SMAD proteins, namely SMAD6 and SMAD7, are also produced following TGFβ receptor activation and complicate the TGFβ signalling pathway by preventing the phosphorylation of SMAD2 and SMAD3, effectively blocking signal transduction (Imamura et al. 1997, Sharkey Chapter 1)
Nakao et al. 1997).

1.2.2.4 Role of TGFβ and its receptors in early pregnancy

Transforming growth factor beta has been shown to be involved in various reproductive processes including spermatogenesis in the male, ovulation in the female, along with embryogenesis (Shull et al. 1994).

All three mammalian isoforms of TGFβ are produced by the somatic and germinal compartments of the human testis (Watrin et al. 1991, Zhang et al. 2004). In vitro studies have shown that TGFβ produced by Sertoli cells inhibits the production of testosterone by Leydig cells in response to human chorionic gonadotropin (hCG) and cyclic adenosine monophosphate (cAMP) (Avallet et al. 1987, Morera et al. 1988). It appears unlikely that TGFβ has a major effect on mature sperm since the addition of recombinant TGFβ to human sperm cultures does not affect their motility, nor their ability to penetrate zona pellucida-free hamster oocytes (Naz et al. 1991). In addition, mice deficient in TGFβ1 that survive to reproductive age produce viable sperm able to fertilise oocytes, therefore suggesting that TGFβ1 is not an absolute requirement for normal testicular function. However these mice appear to have compromised testosterone synthesis and impaired ability to mount and intromit, leading to profound infertility (Ingman 2002).

The expression of TGFβ in the peri-implantation uterus, along with its established roles in the control of cell proliferation, differentiation, migration, tissue remodelling and angiogenesis, are suggestive of TGFβ playing a major role in the implantation process. The use of immunohistochemical and mRNA expression techniques has identified all three mammalian isoforms of TGFβ to be present within the human cervical epithelium (Soufla et al. 2005) and the luminal and glandular epithelium of the endometrium (Tang et al. 1994) as well as the uterine and vaginal epithelium in the mouse (Tamada et al. 1990, Takahashi et al. 1994). In the human endometrium, the expression of TGFβ1, β2 and β3 mRNA and protein within the human endometrium peaks during the late proliferative and early secretory phases of the menstrual cycle (Chegini et al. 1994), with corresponding uterine luminal fluid TGFβ levels rising after ovulation and remaining elevated until just prior to menstruation (Bulletti et al. 1994). This temporal pattern of TGFβ production suggests that TGFβ may be involved in the initiation of endometrial maturation during the transition from proliferative to secretory endometrium. Interestingly, the pre-implantation embryo has been identified as being a source of TGFβ production.
since analysis of culture media containing early human embryos has also been shown to contain bioactive TGFβ (Austgulen et al. 1995). In mice, TGFβ₁ mRNA expression has been detected in unfertilised oocytes, disappearing immediately following fertilisation then returning at the two cell stage where it remains present to the blastocyst stage (Chow et al. 2001).

The three main receptors for TGFβ (Tβ-RI, Tβ-RII and Tβ-RIII) are all present within the human cervical epithelium, with Tβ-RI and Tβ-RIII mRNA and protein being present at much higher concentrations than Tβ-RII (Soufla et al. 2005), though whether their expression is altered throughout the menstrual cycle is yet to be determined. Similarly, these three TGFβ receptors have been shown to be expressed by endometrial epithelial cells (Piestrzeniewicz-Ulanska et al. 2002). Receptors for TGFβ have been identified on murine pre-implantation embryos from as early as the one cell stage, with receptor density gradually increasing as the embryo develops (Paria et al. 1992, Chow et al. 2001). Several studies have investigated the effect of adding exogenous TGFβ to the culture medium of early pre-implantation murine and bovine embryos, with results suggesting that TGFβ exhibits a mitogenic effect on these embryos by promoting the development of these embryos into blastocysts (Paria et al. 1990, Larson et al. 1992, Lim et al. 1993). Arrested development at morula stage in TGFβ₁ heterozygote embryos generated after mating TGFβ null females with wild type males may be interpreted as evidence for a role for maternally derived oocyte TGFβ mRNA transcripts in embryo development (Ingman 2002). Additionally, TGFβ has also been shown to increase oncofetal fibronectin production by trophectoderm cells, which is suggested to promote the attachment of the pre-implantation embryos to the uterine epithelium (Feinberg et al. 1991, Feinberg et al. 1994). Experimental evidence supporting a role for TGFβ in vivo comes from studies in mice which have shown that administration of TGFβ neutralising antibody to day 3.5 murine pre-implantation blastocysts results in an approximately 50% decrease in implantation rate (Slager et al. 1993).

In humans, there is increasing evidence to suggest that the three isoforms of TGFβ may also contribute to controlling the depth of trophoblast invasion. TGFβ is thought to exert this effect through its anti-proliferative capacity on trophoblast, and its ability to initiate remodelling of the decidual ECM, while enhancing the differentiation of invasive trophoblast into non-invasive multi-nucleate giant cells (Dungy et al. 1991, Graham et al. 1991). TGFβ₁ and TGFβ₂ have been shown to be expressed locally within the decidua, the cytoplasm of villous syncytiotrophoblast and in extravillous trophoblast cells throughout pregnancy, with a shift in decidual TGFβ from the ECM to decidual cells as gestation proceeds (Lysiak et al. 1995, Simpson et al. 2002). TGFβ₃ mRNA and protein however is only very

Sharkey Chapter 1
weakly expressed within the placenta and is diffusely expressed within the decidua (Ando et al. 1998, Lash et al. 2005). In vitro studies using placental explants obtained at various stages of gestation have shown that neutralisation of each of the three isoforms of TGFβ increases the invasive capacity of extravillous trophoblast cells. Conversely, in the same study the addition of exogenous TGFβ1, β2 or β3 resulted in a dose-dependent inhibition of trophoblast invasion (Lash et al. 2005). It has been suggested that TGFβ controls trophoblast invasion by reducing the secretion of matrix metalloproteinase (MMP) 9 and urokinase plasminogen activator by trophoblast cells since all three isoforms of TGFβ are capable of inhibiting the production of these proteases by placental trophoblast cells in vitro (Lash et al. 2005). Other key factors involved in the TGFβ-mediated control of trophoblast invasion have been identified within human placental tissue including endoglin, which is a component of the TGFβ receptor system (Cheifetz et al. 1992), and decorin, a natural inhibitor of TGFβ activity (Yamaguchi et al. 1990). Endoglin levels are low in the areas in close proximity to cytotrophoblast, transiently increased on invading intermediate cytotrophoblast, and present at high concentrations on syncytiotrophoblast (St-Jacques et al. 1994). Decorin is present throughout pregnancy within the ECM of the chorionic villi but is completely absent from the decidual tissue (Lysiak et al. 1995).

The interplay between localisation of TGFβ, endoglin and decorin results in the facilitation of trophoblast invasion early in pregnancy, while inhibiting excessive invasion at later stages. Apart from controlling trophoblast invasion, TGFβ is reported to up-regulate hCG and placental lactogen production by human and murine trophoblast cells in vitro (Aoki et al. 1991, Yamaguchi et al. 1994).

Studies in humans and mice have also suggested that TGFβ may play an additional role during early pregnancy, by facilitating the induction of maternal immune tolerance toward the semi-allogeneic conceptus. A group of CD56+ lymphoid cells found in the human decidua (Clark 1994) and small granular lymphocytes in murine decidua (Clark et al. 1990, Lea et al. 1992) have been identified as producing a suppressor factor that is similar to TGFβ2 (ie. has the same biological activity, but a slightly smaller molecular weight). It is hypothesised that this TGFβ2-related molecule contributes to the cytokine milieu at the decidual-trophoblast interface, which may help to prevent destructive cytotoxic immune responses against the semi-allogeneic trophoblast. In the human significantly reduced numbers of TGFβ2-producing CD56+ decidual lymphocytes have been linked to recurrent pregnancy loss (Lea et al. 1995). A key role for TGFβ also seems likely in the induction and expansion of regulatory T (Treg) cells, which have been shown in mice to have an essential role in immune tolerance to the semi-allogeneic conceptus (Aluvihare et al. 2004).
1.2.3 Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)

1.2.3.1 Introduction

GM-CSF, a pro-inflammatory cytokine, is a member of the colony stimulating factor family of cytokines, a group discovered due to their capacity to control the proliferation, differentiation and function of granulocytes and macrophages / monocytes. GM-CSF is secreted as a glycoprotein monomer of 127 amino acids in the human and 124 amino acids in the mouse, giving rise to a 15kDa protein core, which in turn is variably glycosylated to produce a stable protein of 22kDa in the human and 23kDa in the mouse (Burgess et al. 1977, Gasson et al. 1984, Moonen et al. 1987). Both human and murine GM-CSF are encoded by genes that display a high level of homology within both the coding and non-coding regions (Miyatake et al. 1985) and while these proteins share 54% identity at the amino acid level, they exhibit no cross-species biological activity.

1.2.3.2 Role of GM-CSF and its receptors in early pregnancy

GM-CSF is a potentially important intercellular communication molecule in the female reproductive tract, with major influences over the development of the pre-implantation embryo, placental trophoblast, and the large population of myeloid leukocytes present within the cycling and pregnant uterus. The female reproductive tracts of humans and mice are potent sources of GM-CSF production. In humans, cervical epithelial cells have been shown to constitutively express GM-CSF (Woodworth et al. 1993, Hubert et al. 1999), with these cells also secreting active protein into the cervical mucous (Shobokshi et al. 2002). In the endometrium, luminal and glandular epithelial cells are the major sites of GM-CSF mRNA and protein production, with maximal secretion observed during the mid-secretory phase compared to the proliferative phase, with a greater reduction observed during the post-menopausal period (Giacomini et al. 1995, Zhao et al. 1999). This temporal pattern of GM-CSF production suggests that GM-CSF production within the endometrium is regulated by sex steroid hormones. In addition, receptors for GM-CSF are expressed within the endometrium, with the α-receptor being expressed predominantly by endometrial stromal cells and leukocytes, at much higher levels than the β-receptor, which is expressed predominantly by endometrial epithelial cells (Zhao et al. 1999). In the mouse, GM-CSF is produced by the uterine epithelium, with maximal levels being observed in the mouse during oestrus, under the positive influence of oestrogen and following mating (Robertson et al. 1990, Robertson et al. 1996b). In situ hybridisation and quantitative RT-PCR have shown that on day 1 of pregnancy, there is a twenty-fold increase in the transcription of GM-CSF
messenger RNA (mRNA) localised in the uterine epithelium (Robertson et al. 1990, Robertson et al. 1992a). Increased expression in GM-CSF mRNA extends to the oviductal epithelium (Robertson et al., unpublished data). This increase in uterine epithelial GM-CSF production plays a key role in initiating the post-mating influx of neutrophils, macrophages, dendritic cells, and eosinophils into the murine endometrium (termed ‘post-mating inflammatory response’), as it has been shown that the addition of recombinant GM-CSF to the uterine lumen of ovariectomised mice produces an inflammatory response comparable to that observed following mating (Robertson et al. 1994). The post-mating surge in GM-CSF production by uterine epithelial cells has been identified as being a direct consequence of exposure of the uterine epithelium to seminal vesicle secretions both in vivo and in vitro (Robertson et al. 1996a), with more recent studies identifying TGFβ1 as being the major eliciting factor within seminal plasma (Tremellen et al. 1998). Interestingly, preliminary in vitro studies have shown that seminal plasma and recombinant TGFβ1 can each stimulate increased production of GM-CSF by human cervical and endometrial epithelial cells (Tremellen 1998), however whether exposure to semen following intercourse induces similar changes in vivo remains to be examined.

GM-CSF has been identified as an embryotrophic factor, stimulating the development of pre-implantation embryo development. Both human and murine pre-implantation embryos express mRNA for the α-subunit of the GM-CSF receptor and are capable of binding GM-CSF, but the means by which GM-CSF mediates biological effects is unclear, since the mRNA for the β-subunit required for signal transduction is not expressed by embryos in either species (Robertson et al. 1994, Sjoblom et al. 2002). Murine pre-implantation embryos cultured in media supplemented with recombinant GM-CSF display accelerated in vitro development, particularly hatching and attachment (Robertson et al. 1992b). The beneficial effect of GM-CSF is thought to be mediated through inhibiting apoptosis and facilitating glucose uptake (Robertson et al. 2001b). Furthermore, in vivo-induced murine blastocysts from GM-CSF ‘knockout’ mice contain fewer cells than those blastocysts obtained from wild-type mice (Robertson et al. 1999). GM-CSF has also been shown to be important for human embryo development, with the proportion of human embryos developing to blastocysts more than doubling in the presence of rhGM-CSF, with blastocysts containing approximately 30% more cells compared to control embryos, primarily due to an increase in size of the inner cell mass (Sjoblom et al. 1999).

GM-CSF has also been shown to play an important role in fetal and placental development. The production of GM-CSF by the murine uterine epithelium decreases considerably by day 3 of pregnancy under the negative influence of rising progesterone levels (Robertson et al. 1996b). From this stage on, decidual cells such as stromal fibroblasts, endothelial cells, and leukocytes such as NK cells may be principally responsible for GM-CSF production at the maternal-placental interface in both mice and
humans (Crainie et al. 1990, Kanzaki et al. 1991, Jokhi et al. 1994b), with human trophoblast cells also acting as a source of GM-CSF production (Jokhi et al. 1994b). Experiments using GM-CSF deficient female mice have shown that a lack of GM-CSF at the fetal-maternal interface has no observable effect on implantation rates, yet leads to litter sizes that are 25% smaller compared to those of the wild type controls at the time of weaning. This finding is attributed to an increased rate of death in the late fetal and early post-natal period (Robertson et al. 1999). Another important observation is that pups born to GM-CSF deplete mothers are significantly growth restricted, presumably due to abnormalities in placental development (Robertson et al. 1999). More specifically, the labyrinth of the placenta in GM-CSF deplete placentae is decreased in area when compared to wild-type placentae. These observations are consistent with previous studies indicating that GM-CSF promotes proliferation, differentiation and enhances the secretory activity (hCG, placental lactogen) of rodent and human cytotrophoblasts, both in vitro (Athanassakis et al. 1987, Armstrong et al. 1989, Garcia-Lloret et al. 1994) and in vivo (Chaouat et al. 1990).

Another way in which GM-CSF may benefit pregnancy outcome is through modification of uterine leukocyte trafficking and behaviour. While GM-CSF has been implicated as the principal initiator of the post-mating influx of inflammatory leukocytes in mice (Robertson et al. 1994), an absence of GM-CSF in GM-/- females does not have any significant effects on the numbers or spatial distribution of leukocytes within the uterine endometrium following mating or in the mid-term decidua (Robertson et al. 1999). Factors other than GM-CSF, more specifically the chemokine family of molecules, have now been identified as being involved in this compensatory cytokine redundancy (Pollard et al. 1998, Robertson et al. 1998). Recent studies in GM-CSF null mutants have identified subtle defects in uterine macrophages and dendritic cells, including a reduction in membrane expression of MHC class II antigen and the co-stimulatory molecule B7-2, both of which are known to be critical for antigen presenting activity (Robertson et al. 2000). The diminished functional capacity of these cells has been postulated to impede the development of an immuno-permissive environment within the decidua towards the semi-allogeneic placenta (Robertson et al. 1997). Further evidence supporting the concept that GM-CSF plays an essential role in the initiation of immune responses beneficial to reproductive outcome comes from studies which found that a single dose of GM-CSF can reverse the high rate of immune-mediated fetal loss that occurs spontaneously in the matings between CBA/J and DBA/2 mice (Tartakovsky et al. 1991), while also protecting against LPS or IFNγ induced resorption (Chaouat et al. 1990). This beneficial effect of GM-CSF is believed to be mediated through changes to the maternal immune axis rather than by a direct enhancement of trophoblast growth, since depletion of CD8+ T lymphocytes prior to GM-CSF administration ameliorates the beneficial effect of this cytokine and is
without effect if CD8+ T cells are depleted prior to GM-CSF administration (Clark et al. 1994).

1.2.4 Interleukin-1 (IL-1)

1.2.4.1 Role of IL-1 in early pregnancy

IL-1 exists as two 17kDa ligands (IL-1α and IL-1β) that share approximately 22% sequence homology and act through a common receptor (IL-1Rt1). Both isoforms of IL-1 are believed to have similar biological functions (di Giovine et al. 1990) and are predominantly produced by cells of the monocyte lineage in response to pro-inflammatory signals (TNF-α, lipopolysaccharide or IL-1 itself), with its major activity being to promote T cell activation following antigen exposure.

In the lower human female reproductive tract, IL-1 is produced by both vaginal and cervical epithelial cells (Woodworth et al. 1993, Fichorova et al. 2004) and is present in detectable concentrations within cervical mucous (Kanai et al. 1997). The concentration of IL-1 and its naturally occurring receptor antagonist (IL-1ra) within cervical mucous has been shown to be hormonally regulated, with higher concentrations throughout the post-ovulatory period compared to the follicular phase of the menstrual cycle (Kanai et al. 1997). Endometrial epithelial cells are also a major site of IL-1α and IL-1β production, with low levels of IL-1 also expressed by endometrial lymphoid, endothelial and stromal cells, with epithelial IL-1 receptor density reaching maximal levels during the luteal phase (Simon et al. 1993a, Simon et al. 1993b). In the murine uterus, the production of both IL-1α and IL-1β is localised to endometrial macrophages and endothelial cells, with a transient increase in production peaking 24 hours after mating (Sanford et al. 1992). Whether exposure to seminal plasma following intercourse can induce a similar increase in IL-1 within the cervical and endometrial tissues of women in vivo is yet to be determined, however in vitro studies have recently shown that seminal plasma can stimulate IL-1β mRNA production by primary endometrial epithelial cells (Gutsche et al. 2003).

Both human and murine pre-implantation embryos have been shown to produce and secrete IL-1 (Austgulen et al. 1995), with embryo-derived IL-1 capable of triggering up-regulated IL-1 receptor expression by neighbouring endometrial cells in vitro (Simon et al. 1994). Furthermore, a crucial role for IL-1 in implantation is suggested by the observation that intra-peritoneal injection of an IL-1 antagonist between days 3-9 of pregnancy is found to almost completely abolish implantation in the mouse (Simon et al. 1994). The observation that IL-1 and its receptor are expressed at maximal levels during the luteal phase of the menstrual cycle, the time when embryonic attachment and implantation occurs may
suggest that IL-1 is also important human implantation. In addition, a positive correlation between high levels of IL-1α and IL-1β production by human pre-implantation embryos and their successful implantation following embryo transfer has been observed (Sheth et al. 1991). The precise manner by which IL-1 mediates an effect on implantation is unknown, but may be related to its ability to increase cytotrophoblast invasion of decidual tissue (Librach et al. 1994) given it is expressed by trophoblast cells at the maternal-fetal interface in humans (Hu et al. 1992).

1.2.5 Interleukin-6 (IL-6)

1.2.5.1 Role of IL-6 in early pregnancy

IL-6 is a 26kDa cytokine produced by monocytes, endothelial cells, fibroblasts and epithelial cells in response to different stimuli including IL-1, IL-2, TNF-α and products of viral infection (Lee 1992). There are several possible immunological roles for IL-6, including the initiation of antibody production by triggering maturation of B cells into plasma cells, initiating the differentiation of monocytes into macrophages, and increasing the ability of macrophages to activate T cells (Ruppert et al. 1991).

In humans, vaginal and cervical epithelial cells have been shown to constitutively express IL-6 (Woodworth et al. 1993, Donders et al. 2003), with IL-6 also being secreted into the cervical mucus (Naz et al. 1996, Sagawa et al. 1996, Franklin et al. 1999). The levels of IL-6 within the vagina and cervix do not appear to be regulated by steroid hormones since IL-6 concentrations do not vary significantly throughout the menstrual cycle (Kutteh et al. 1998). It has been suggested that the presence of IL-6 in cervical mucus may play a role in the capacitation of human sperm, since IL-6 has been shown to trigger sperm capacitation in vitro (Naz et al. 1994b, Naz et al. 1996, Laflamme et al. 2005) and the IL-6 receptor is expressed on the surface of ejaculated sperm (Laflamme et al. 2005).

Both murine and human uterine epithelial and stromal cells have been shown to express IL-6 (Jacobs et al. 1992, Tabibzadeh et al. 1992). In the human endometrium, IL-6 mRNA and protein expression is low during the proliferative phase of the menstrual cycle and increases progressively, reaching a maximum during the mid-late secretory phase (Tabibzadeh et al. 1992, von Wolff et al. 2002b). Interestingly, this menstrual cycle-dependent increase in IL-6 production does not appear to be regulated by steroid hormones, since no increase in IL-6 production is observed when endometrial epithelial or stromal cells are incubated with estrogen or progesterone in vitro (von Wolff et al. 2002a).
Instead, there is evidence to suggest that endometrial IL-6 production is regulated by pro-inflammatory cytokines such as IL-1 and TNFα, both of which are also elevated during the mid-late secretory phase of the menstrual cycle and have been shown to stimulate the production of IL-6 by endometrial epithelial and stromal cells in vitro (Tabibzadeh et al. 1989, von Wolff et al. 2002a, Kawano et al. 2004). The maximal expression of endometrial IL-6 during the mid-late secretory phase together with the observation that the endometrium expresses IL-6 receptors (Sharkey et al. 1995) and secretes IL-6 into the uterine cavity (von Wolff et al. 2002a) suggests that IL-6 plays a role in embryo implantation. Further evidence supporting a role for IL-6 in human implantation comes from the observation that human pre-implantation embryos express both IL-6 and IL-6 receptors (Sharkey et al. 1995). In addition, elevated expression of IL-6 within the endometrium has been observed in women experiencing unexplained infertility and endometriosis (Tseng et al. 1996), while decreased levels have been observed in women experiencing recurrent miscarriage (Lim et al. 2000).

The murine uterus also produces IL-6, with exposure to semen following mating triggering an increase in IL-6 production (Robertson et al. 1992a). In mice, the dramatic increase in uterine IL-6 mRNA production observed following mating declines on day 2 of pregnancy, before again rising on day 3, then remaining elevated for the rest of gestation (Sanford et al. 1992). The addition of IL-6 to cultured murine blastocysts has been shown to inhibit their attachment and outgrowth on laminin-coated culture dishes (Jacobs et al. 1992), suggesting IL-6 may play a role in the timing of blastocyst implantation. The surge in IL-6 production following mating may serve to enhance the maternal immune response to paternal antigens introduced at the time of mating, since intra-uterine administration of IL-6 to ovariectomised rats has been reported to increase antigen presentation and antibody production by the uterine mucosa (Prabhala et al. 1995). Seminal plasma may also stimulate similar responses in the human endometrium since seminal plasma and TGFβ1 (present in abundance in human seminal plasma) are capable of stimulating marked increases in IL-6 mRNA production by endometrial epithelial cells in vitro (Gutsche et al. 2003). interestingly, endometrial cells obtained during the mid-secretory phase of the menstrual cycle were more responsive to stimulation by seminal plasma (Gutsche et al. 2003).
1.2.6 Interleukin-8 (IL-8)

1.2.6.1 Role of IL-8 in early pregnancy

IL-8 is a 72 amino acid α-chemokine produced by a variety of cell types including monocytes, fibroblasts (Dudley et al. 1993), lymphocytes, epithelial and endothelial cells (Baggiolini et al. 1994). IL-8 is a potent chemoattractant and activator of inflammatory cells such as neutrophils (Mukaida et al. 1989) and T lymphocytes (Larsen et al. 1989) whose function is mediated by binding to the cell-surface receptors, CXCRI and CXCRII (Ulukus et al. 2005). Interestingly, the tissue concentration of IL-8 has been shown to be important in determining whether neutrophils, T lymphocytes or both are recruited to specific tissues. At low concentrations, T lymphocytes are 2-10 times more sensitive to the chemotactic effect of IL-8 than neutrophils (Larsen et al. 1989). To date, IL-8 has been implicated as having functional roles in reproductive processes including menstruation, parturition and cervical ripening as well as in pathological conditions such as pre-term delivery and endometriosis (Cocchi et al. 1995, Simon et al. 1997).

The human female reproductive tract has been shown to be a rich source of IL-8, with functional protein being detected within the vaginal (Fichorova et al. 1999, Yamada et al. 2002, Donders et al. 2003), ectocervical (Barclay et al. 1993, Fichorova et al. 1999) and endocervical epithelium (Fichorova et al. 1999) as well as cervical mucous (Sakai et al. 2004). Interestingly, seminal plasma has been shown to induce a marked increase in IL-8 production by cervical epithelial cells in vitro (Denison et al. 1999b). The precise role of this increase in IL-8 has not been determined, however it has been postulated that IL-8 may act to stimulate the recruitment of neutrophils into the upper vagina, cervical mucous and epithelium where they would be ideally positioned to phagocytose non-fertilising spermatozoa (Denison et al. 1999b). Further supporting this notion is the observation that exposure to semen following donor insemination stimulates the recruitment of neutrophils into the cervical mucous and superficial layers of the epithelium (Pandya et al. 1985, Thompson et al. 1992). It is likely that factors contained within human seminal plasma are responsible for stimulating this increase in IL-8 and the resultant influx of neutrophils since seminal plasma has been shown to contain IL-8 (Koumantakis et al. 1998) and extraordinarily high concentrations of prostaglandin E2 (PGE2) (Kelly et al. 1997b), which has been shown to increase IL-8 and IL-10 production by cervical epithelial cells in vitro (Denison et al. 1999a). In addition, it has been proposed that an increase in cervical IL-8 production could also trigger the local recruitment of T lymphocytes, where they could then be presented with seminal antigens in the presence of potent immunomodulatory molecules such as IL-10, PGE2 and TGFβ, which may be conducive to the development of antigen-specific anergy (Groux et al. 1996). Whether
exposure to semen following intercourse stimulates similar increases in IL-8 production within cervical tissue in humans in vivo has not yet been examined.

The human endometrium also produces IL-8, with mRNA and protein levels peaking during the late secretory and early to mid-proliferative phase of the menstrual cycle, with lower levels detected midcycle (Arici et al. 1993, Arici et al. 1998). This increase in IL-8 during the late secretory phase of the menstrual cycle may serve to facilitate recruitment of neutrophils before menstruation, where these cells are likely to be involved in the degradation of endometrial tissue (García-Velasco et al. 1999). Immunohistochemical analysis has localised immunoreactive IL-8 protein to endometrial epithelial cells and to a lesser extent within the glands, with very little IL-8 immunoreactivity observed within the stromal tissue (Araci et al. 1998). IL-8 production by epithelial cells appear to be regulated either directly or indirectly by steroid hormones, particularly progesterone (Araci et al. 1993), however high concentrations of progesterone have been shown to reduce IL-8 production in endometrial explants (Kelly et al. 1994). Additionally, cytokines such as IL-1 and TNFα are capable of stimulating IL-8 production by endometrial stromal cells (Arici et al. 1993) whereas TGFβ1 has the ability to inhibit IL-8 mRNA expression and protein synthesis (Arici et al. 1996). The precise function of IL-8 within the human endometrium is not well understood, however the observation that unstimulated female reproductive tract epithelial cells preferentially express IL-8 at their apical surface (Shen et al. 2004) may encourage the movement of neutrophils from the epithelium into the lumen where they are likely to be involved in the defence against pathogenic organism invasion (Arici et al. 1998). Further supporting this is the observation that endometrial (Eckmann et al. 1993) and cervical epithelial cells (Watari et al. 2003, Sakai et al. 2004) have been shown to increase their production of IL-8 in response to bacterial entry. It has also been speculated that IL-8 production by epithelial cells may also play a role beyond that of regulating maternal leukocyte recruitment and may exert an effect on sperm function or on the blastocyst (Arici et al. 1998).

IL-8 has is also produced in the human ovary (Runesson et al. 1996, Chang et al. 1998), fallopian tube (Palter et al. 2001, Mulayim et al. 2003), decidua and placenta (Saito et al. 1994a). The precise function of IL-8 in human pregnancy, particularly in the decidua and placenta remains to be determined, but has been implicated in the processes of cervical ripening and the onset of labour given that cervical IL-8 production increases approximately 6-fold immediately prior to the onset of labour, consistent with IL-8 playing a role in connective tissue remodelling that occurs during cervical ripening (Sennstrom et al. 1997). In addition, placental and fetal membrane expression of IL-8 receptors increases markedly following the onset of labour supporting a role for IL-8 in parturition.
1.2.7 Tumour Necrosis Factor alpha (TNFα)

1.2.7.1 Role of TNFα in early pregnancy

TNFα is a 51kDa homotrimer protein principally produced by activated macrophages; although antigen-stimulated T cells, activated NK cells and mast cells may also secrete this cytokine. It is a pleiotrophic cytokine that exerts a variety of effects including pro-inflammatory activity, growth promotion and inhibition, angiogenesis, cellular toxicity and immunomodulation (Beutler et al. 1989).

In the lower human female reproductive tract, TNFα is constitutively expressed by vaginal (Steele et al. 2002) and cervical epithelial cells (Mota et al. 1999) and is also present within cervical mucous (Naz et al. 1995). The precise function of TNFα within these tissues remains unknown but is believed to contribute to the prevention of infection by intracellular pathogens within these sites (Wira et al. 1998, Ahmed et al. 2001).

In the endometrium, epithelial cells have been found to be a major source of TNFα protein and mRNA production throughout the menstrual cycle with minimal protein production through the proliferative, early and mid-secretory phases while increased through the late-secretory and menstrual phase (Tabibzadeh et al. 1999). In contrast, TNFα mRNA expression is maximal during the proliferative phase, lower during the secretory phase and significantly decreases during the menses and the post-menopausal period (Hunt et al. 1992, Chegini et al. 1999). The temporal expression of TNFα throughout the menstrual cycle suggests its production is regulated by steroid hormones (Hunt et al. 1992, Tabibzadeh et al. 1999) with elevated estrogen and progesterone both inhibiting TNFα production. The role of TNFα within the normal cycling endometrium is not well understood, however its elevation during the secretory phase of the menstrual cycle, coinciding with estrogen withdrawal (Tabibzadeh et al. 1999) and the increased expression of the TNFα type I and II receptors (Hunt et al. 1992) within the endometrium at this time suggests it may play a role in menstruation.

The presence of TNFα within the human ovary (Roby et al. 1990), fallopian tube (Fahey et al. 2005), decidua (Chen et al. 1991), placenta (Chen et al. 1991, Hayashi et al. 2005) and pre-implantation embryo (Sharkey et al. 1995) suggests that TNFα may play a role in numerous...
reproductive processes. The precise function of TNFα within these sites and particularly its role in pregnancy is not well understood however studies in mice and humans have provided some insight.

It has been suggested that endometrial TNFα may play a role in implantation and also in pre-implantation embryo development. The addition of exogenous TNFα to cultured human luteal phase endometrial cells has been shown to induce prostaglandin release (Chen et al. 1995), which may impact on the vascular permeability of the endometrium (Peek et al. 1992) and therefore have an effect on the implantation process. Evidence supporting a role for TNFα in pre-implantation embryo development comes from studies which have shown that human embryos themselves express TNFα from the two cell to morula stage (Zolti et al. 1991, Lachapelle et al. 1993) and both the type I and II receptors (Sharkey et al. 1995) and the endometrium actively secretes high levels of TNFα during the late-proliferative and early-mid secretory phases of the menstrual cycle (von Wolff et al. 1999). The effect of TNFα on the pre-implantation embryos is not clear with studies in mice showing that culturing embryos in the presence of TNFα does not alter blastocyst formation (Wincek et al. 1991) or attachment rates (Haimovici et al. 1991). However, murine blastocysts exposed to TNFα display a reduction in the number of cells contained within the inner cell mass (Pampfer et al. 1994). Whether TNFα exerts similar effects on human embryos is not known and it is possible that species differences exist since human blastocysts do not express receptors for TNFα (Sharkey et al. 1995).

The murine uterus is also a rich source of TNFα, with uterine luminal and endometrial glandular epithelial cells, along with stromal macrophages being a source of production (De et al. 1991). TNFα synthesis is transiently increased following mating (Sanford et al. 1992), and similarly to the human, its regulation is under the control of sex hormones (Roby et al. 1994). In mice, a pregnancy-associated increase in TNFα production is observed, with uterine epithelial cell TNFα mRNA production increasing immediately after implantation and remaining high until day 14 of pregnancy (Hunt et al. 1993).

Investigators have postulated that TNFα may influence placental development by regulating trophoblast invasion. This idea is supported by the observation that in mice, decidual macrophages present at the fetal-maternal interface release TNFα, which could then activate cytolytic NK cells against trophoblast cells, hence preventing over invasion of the trophoblasts into the maternal tissue (Parr et al. 1995). Similar mechanisms may also operate in humans, since the decidua contains an abundance of macrophages, NK cells and T lymphocytes (all capable of producing TNFα).
(Vince et al. 1992), and has previously been shown to contain immunoreactive TNFα.

(Chen et al. 1991). Further supporting this, in vitro studies have demonstrated that TNFα is a negative regulator of human trophoblast invasion in normal placentae (Yui et al. 1994) and is expressed by trophoblast cells during the first trimester, then decreasing throughout gestation until it again increases at term (Chen et al. 1991). Interestingly, stress-induced abortion in mice (Arck et al. 1995) and endometriosis in humans (Syrop et al. 1987) have both been associated with increased levels of TNFα, suggesting that high levels of TNFα may have adverse effects on viability and/or function of placental trophoblast, leading to pregnancy failure.

1.2.8 Interferon gamma (IFNγ)

1.2.8.1 Role of IFNγ in early pregnancy

IFNγ is a 34kDa homodimeric glycoprotein predominantly secreted by activated NK cells and T lymphocytes that has important regulatory effects on many cell types (Boehm et al. 1997, Kurago et al. 1998). IFNγ has been shown to regulate processes such as smooth muscle cell proliferation, cell adhesion, apoptosis, and processing pathways for protein synthesis and packaging (Boehm et al. 1997, Boehm et al. 1998).

In humans, IFNγ mRNA has been detected within the vaginal and cervical epithelium and has been largely attributed to the large populations of T lymphocytes within these sites (El-Sherif et al. 2001). Furthermore, in vitro studies using immortalised vaginal and cervical epithelial cells failed to observe the spontaneous release of IFNγ protein from these cells, further supporting the role resident leukocytes play in regulating IFNγ production (Fichorova et al. 1999). IFNγ has also been detected within cervical mucous, with significantly higher levels detected in women with idiopathic infertility (Naz et al. 1995).

In the upper female reproductive tract, IFNγ mRNA and protein has been detected in the endometrium (Tabibzadeh 1994) and within the decidua (Saito et al. 1993, Jones et al. 1997) and placenta (Berkowitz et al. 1990, von Rango et al. 2003) during early pregnancy, while IFNγ mRNA has been shown to be present in healthy human implantation sites (Delassus et al. 1994, Jokhi et al. 1994c). In both humans and mice, natural killer (NK) cell subsets known as uterine natural killer (uNK) cells, are the most abundant maternal lymphocyte population within the uterus (King et al. 1991, Croy

Sharkey Chapter 1
and are among the cells expressing IFNγ (Saito et al. 1993, Platt et al. 1998). In the human decidua, IFNγ receptors have been detected on macrophages, extravillous trophoblast cells and vascular endothelial cells suggesting that IFNγ has a complex influence on the decidua and fetal trophoblast (Jokhi et al. 1997, Loke et al. 1997).

While the precise function of IFNγ in human pregnancy is unknown, it is suggested that IFNγ may contribute to placental development and maintenance of pregnancy. IFNγ has been shown to influence the antigenicity of tissue by regulating the expression of HLA class I and II antigens, with IFNγ up-regulating the expression of HLA-C and HLA-G antigens on human trophoblast cells (Grabowska et al. 1990, King et al. 2000b) presumably preventing them from maternal attack. In addition, IFNγ has also been associated with stimulating increased NK cell cytotoxic activity against trophoblast (Haimovici et al. 1991, Yui et al. 1994). Interestingly, HLA-G has been shown to reduce the release of IFNγ by NK cells through an interaction with the killer-cell inhibitory receptor (Loke et al. 1997), with decidual IFNγ content decreasing late in the first trimester when trophoblast invasion is complete (von Rango et al. 2003). These findings suggest that IFNγ may be involved in the control of trophoblast outgrowth by inhibiting excessive trophoblast invasion.

Further evidence supporting a role for IFNγ in pregnancy comes from studies in mice. In both normal and SCID (T and B cell deficient) mice, the concentration of IFNγ within the mesometrial triangle, where uNK cells aggregate, was found to increase in early pregnancy with peak levels of expression being detected at around the time of implantation on day 10 of pregnancy (Ashkar et al. 1999). Experiments using tge26 (uNK-NK-T-) transgenic female mice showed that the mesometrial triangle increase in IFNγ associated with pregnancy was ten-fold lower compared with wild-type or SCID mice (Ashkar et al. 1999), which suggests that uNK cells are the major source of IFNγ in the pregnant mouse uterus. Mice deficient for either IFNγ or the ligand-binding component of the IFNγ receptor (IFNGR1), originally referred to as the IFNγ receptor α chain, are found to have histologic anomalies within their implantation sites at mid-gestation. The anomalies included firstly, an excessive number of uNK cells within the implantation site and that were uncharacteristically small and had limited numbers of cytoplasmic granules (Ashkar et al. 1999). Secondly, the major decidual arteries, equivalent to the uterine spiral arteries of women, were not observed to have undergone normal gestation-induced remodelling and finally, there was evidence that decidualization had been initiated, but the cellularity of the decidua was not maintained. These observations suggest that uNK cell-derived IFNγ may contribute to the initiation of pregnancy-induced uterine vascular modification, the continual
maintenance of decidual integrity, and the regulation of maturation and senescence of the uNK cell population. IFNγ may also contribute to the integrity of the human decidua by facilitating the remodelling of spiral arteries since it has been shown to selectively influence vascular endothelial adhesion factors, inducing the migration of monocytes and lymphocytes and inhibiting the invasion of neutrophils (Melrose et al. 1998). This may then mediate the recruitment of leukocytes into the tissue or may modulate the interaction with endovascular trophoblast cells (von Rango et al. 2003).

1.3 THE ROLE OF LEUKOCYTES IN THE FEMALE REPRODUCTIVE TRACT

1.3.1 The female reproductive tract as an immunocompetent site

The uterus is part of the common mucosal immune system and is unique in its ability to support a semi-allogenic conceptus whilst retaining its capacity to provide a barrier to foreign antigens and pathogens. As such, it shares many structural and functional similarities with other mucosal tissues including the repertoire of lymphoemopoietic cells and cytokine networks present, lymphocyte trafficking pathways and lymphatic drainage systems (reviewed by Robertson 2000). Despite the uterus lacking specialised secondary lymphoid nodules, the uterine mucosa is capable of mounting antigen-specific immune responses towards foreign antigens resulting from exposure to sexually transmitted infections, seminal plasma constituents and paternal transplantation antigens (Quayle et al. 1989, Kelly et al. 1997a, Robertson 2000).
1.3.2 Leukocyte populations within the uterus and cervix

1.3.2.1 Introduction

The human and murine female reproductive tracts are densely populated by a diverse range of leukocytes. Different tissues within the female reproductive tract harbour different populations of cells, and there are changes in the relative abundance and phenotypes of different populations at different stages of the cycle and in pregnancy. These are regulated by fluctuations in expression of chemotactic and regulatory cytokines driven by ovarian steroid hormones and also factors in semen. Insemination causes alterations in the composition and phenotype of these leukocytes in a pattern with similarities to that seen during inflammatory reactions in other mammalian tissues (De et al. 1991, Thompson et al. 1992, Robertson et al. 1996a). Leukocytes within the human endometrium for example make up approximately 10% of all stromal cells during the proliferative phase, 20% in the secretory phase and 30% of endometrial cells during early pregnancy (Bulmer et al. 1991). The human cervix has also been shown to contain a large population of leukocytes, with increased numbers being reported within the outer-most layers of the epithelium and cervical mucous following exposure to semen (Pandya et al. 1985, Thompson et al. 1992). The female reproductive tract is therefore a competent site for the initiation of local and systemic immune responses against microbial pathogens and seminal antigens introduced at the time of copulation (Parr et al. 1985), as well as antigens expressed by the semi-allogeneic conceptus.

1.3.2.2 Neutrophils

Neutrophils constitute the major proportion of leukocytes recruited into sites of infection or injury under the influence of cytokines such as GM-CSF, TGFβ, IL-1 and TNFα, as well as other inflammatory mediators such as IL-8. Their primary function appears to cross the endothelial barrier and act as the first line of defence in response to tissue damage or infection and also have the capacity to recruit additional types of leukocytes (Nathan 2002).

Within the female reproductive tract of humans, neutrophils have been identified as being present in considerable numbers within the endometrium, accounting for almost 15% of all endometrial cells during the late secretory phase of the menstrual cycle (Salamonsen et al. 2000). Neutrophils are also present in abundance within the human cervical lumen, accounting for approximately 70% of leukocytes present within cervical mucous of both pregnant and non-pregnant women (Okamura et al. 1985).
Interestingly an influx of neutrophils has been observed within the cervical epithelium and mucous of humans following the administration of donor semen (Pandya et al. 1985, Thompson et al. 1992), as well as after mating in the cervical and uterine tissues of other mammalian species including the mouse, rat, rabbit and pigs (Lovell et al. 1968, Phillips et al. 1977, De et al. 1991, O'Leary et al. 2004). This post-coital associated neutrophilia is short-lived, lasting for only 12 - 16 hours before the number of neutrophils decline back to basal levels (Pandya et al. 1985). It has been suggested that the primary role of this influx of neutrophils appears to be in the phagocytosis of sperm and seminal debris, as well as the removal of potentially harmful bacteria introduced at the time of mating (Tomlinson et al. 1992). Interestingly, the phagocytic ability of neutrophils has been assessed in women during various stages of the menstrual cycle where it was found that neutrophils obtained from pregnant women had a higher phagocytic capacity than their non-pregnant controls. Additionally, this phagocytic capacity was also observed to be greater during the ovulatory stage of the menstrual cycle of non-pregnant women than at any other stage, including menstruation (Saikia et al. 2003). These findings may provide additional evidence for neutrophils playing a role in phagocytosis of sperm, cellular debris or other antigens contained within the ejaculate.

1.3.2.3 Macrophages

Macrophages are multi-functional cells, which differentiate from blood-borne monocytes. Generally macrophages serve four main functions: they phagocyte and destroy invading organisms, process and present antigens to T lymphocytes, produce inflammatory mediators such as cytokines, prostaglandins, and histamine and release proteases and other enzymes capable of extracellular matrix digestion and tissue remodelling.

In humans, macrophages are present in relatively small numbers within the vaginal epithelium and are convincingly outnumbered by other cells types such as CD4+ and CD8+ T lymphocytes, with very little variation in their relative abundance observed throughout the menstrual cycle (Patton et al. 2000). Macrophages are also present within the ecto- and endocervical tissues of women and represent a greater proportion of the total cells present compared to the vagina. Immunohistochemical analysis has revealed these cells are most commonly located within the stromal tissue in close proximity to the basement membrane (Prakash et al. 2001) and their relative densities and distribution do not fluctuate significantly throughout the menstrual cycle (Poppe et al. 1998). Exposure to semen following intercourse or donor insemination has been shown to result in a small and transient increase in macrophage numbers within the superficial layers of the cervical epithelium and lumen (Pandya et al.)
1985, Thompson et al. 1992, Prakash et al. 2003). Furthermore, mucous sampling from the endocervix has revealed that macrophage numbers continue to increase, reaching maximal levels at approximately 3 days post-coitus at which time their numbers exceed that of CD4+ T lymphocytes (Prakash et al. 2003). The precise role of this increase in the relative abundance of macrophages within the cervix is yet to be determined although it has been speculated to be of importance in the clearance of seminal debris and in the prevention of infection following intercourse (Parr et al. 1985).

In the human endometrium, macrophages represent approximately 20% of the total endometrial cell population, with little variation between the proliferative and early secretory phase, though macrophages were found to increase only marginally during the late secretory phase of the menstrual cycle (Rieger et al. 2004), suggesting that these cells are less responsive to changing steroid hormone levels than other leukocytes (Starkey et al. 1991). Endometrial macrophage numbers also increase significantly during early pregnancy in humans (Bulmer et al. 1985), and unlike cells such as natural killer cells, remain high throughout pregnancy (Khong 1987). These cells have also been shown to constitute approximately 30% of the total leukocyte population present at the implantation site (Kabawat et al. 1985) and are subsequently spatially associated with the first trimester extravillous cytotrophoblast and the fetal membranes throughout pregnancy (Hu et al. 1992).

There is evidence that successful pregnancy requires a maternal immune environment biased towards Th2-type immune responses. Supporting this, human decidual tissue has been shown to secrete high levels of IL-10, a molecule with roles in establishing Th2-type immune responses (Roth et al. 1996). In addition, macrophages are capable of promoting Th1 or Th2-type immune responses depending on their type of activation though recently decidual macrophages have been identified as having increased phagocytic ability and an 'alternatively activated' phenotype (Cupurdija et al. 2004). These cells have also been described as expressing high levels of HLA class II, along with receptors for GM-CSF (Jokhi et al. 1994a), M-CSF (Jokhi et al. 1993) and IFNγ (Loke et al. 1996), as well as the co-stimulatory molecules CD86 and low levels of CD80 (Petroff et al. 2003), and the inhibitory HLA class Ia and Ib receptors CD85d and CD85j (Petroff et al. 2002). The high levels of HLA class II expression compared to the low levels of the co-stimulatory molecules CD86 and CD80 suggests that decidual macrophages are more likely to induce tolerance of maternal T cells by failing to give a sufficient co-stimulatory signal (Heikkinen et al. 2003). Also, the blockade of the CD86-CD28 interaction has been shown to give rise to antigen-specific immunoregulatory T cells able to suppress alloreactive T cells (Koenen et al. 2000). Furthermore, decidual macrophages have also been shown to
spontaneously secrete high levels of IL-10 (Heikkinen et al. 2003), which further supports a possible role for these cells in the maintenance of maternal tolerance to the developing fetus.

Macrophages are also present in the murine endometrium comprising approximately 10% of endometrial cells. Within 8 hours after mating, macrophages increase in number to 25% of all endometrial cells and their location within the tissue alters, such that they accumulate in the stromal tissue in close proximity to the luminal and glandular epithelium (Robertson et al. 1994). This influx of macrophages into the endometrium is believed to be due to an increase in epithelial production of a number of cytokines (IL-1, IL-6, TNFα and GM-CSF) as well as chemokines (RANTES, MIP-1α and MIP-1β) (Robertson et al. 1990, Sanford et al. 1992, Brandon 1993, Robertson et al. 1998). By day 3 of pregnancy, the numbers of macrophages return to basal levels, being distributed evenly throughout the endometrium.

Studies in mice have demonstrated that the influx of leukocytes within the endometrial tissues and lumen can exert effects other than by activating immune responses. There is evidence that these cells also produce an array of soluble signalling molecules and potent enzymes likely to affect the turnover of the ECM along with altering the characteristics of endothelial cells in the endometrium, a process referred to as tissue remodelling. Macrophages have been described as the major leukocyte population responsible for the regulation of endometrial remodelling to facilitate implantation and placental development (Robertson 2005). Part of this endometrial restructuring involves the process of angiogenesis, a process which is tightly regulated and driven by macrophages. Activated macrophages have been observed to play crucial roles in all aspects of the angiogenic process, including alterations to the extracellular matrix, induction of endothelial cell migration and proliferation, and formation of capillaries (Sunderkotter et al. 1994). Macrophage-secreted cellular products have also been suggested to target the ECM of endometrial stroma, which is known to be remodelled prior to and during decidualisation (Aplin et al. 2002), with MMPs being key regulators of this process (Curry et al. 2003). MMPs exert this influence after coordinated increases in their transcription, secretion and proteolytic activation, along with their regulatory proteins, the tissue inhibitors of metalloproteinases (TIMPs).

In addition to roles in tissue remodelling, macrophages may play a role in facilitating implantation, by selectively targeting luminal epithelial cells involved in embryo attachment during the early stages of implantation. The fluctuating expression of adhesion and anti-adhesion molecules throughout the cycle provides a physical barrier, preventing embryo attachment until the window of
implantation when selective changes in the expression of integrins and mucins by epithelial cells (Aplin 1997) allow the close apposition and then attachment between the blastocyst and the luminal surface, just prior to invasion. In vitro studies using uterine epithelial cells have demonstrated that leukocytes are also capable of regulating epithelial adhesive properties in humans (Kosaka et al. 2003). Macrophages may further contribute to implantation success by virtue of their ability to alter transport properties and epithelial cell barrier integrity, thereby facilitating trophoblast invasion through the epithelial surface (Robertson 2005).

1.3.2.4 Dendritic cells

Dendritic cells play an important role in priming T cell responses. Despite these cells have only a limited phagocytic capacity, their ability to store antigen on their cell surface for extended periods together with their high MHC class II expression, allows them to present antigens to T cells with high efficiency (Hart 1997).

In humans, dendritic cells are also present in the vaginal epithelium and represent approximately 5% of the total cell population within these tissues (Bjercke et al. 1983), with little to no variation in their abundance observed throughout the menstrual, pre-ovulatory and post-ovulatory stages of the menstrual cycle (Patton et al. 2000). Dendritic cells are also present within the cervical tissue of women, where they are present in their highest numbers within the ectocervical epithelium and transformation zone and in lower numbers within the endocervical epithelium (Poppe et al. 1998). Immunohistochemical analysis has also revealed these cells are predominantly distributed throughout the epithelium and are detected in lesser numbers within the stromal tissue (Poppe et al. 1998) while their relative densities and distribution do not fluctuate significantly throughout the menstrual cycle (Poppe et al. 1998). The precise role of dendritic cells within the cervix and vagina is yet to be determined, however it is thought they may play a role in the defense against sexually transmitted pathogens (White et al. 1997) presumably by processing viral or bacterial antigens then migrating to the draining lymph nodes where they mature, and subsequently present these antigens on their cell surface in the context of MHC class II, culminating in the activation of T lymphocytes (Soilleux et al. 2002). It is interesting to speculate that the presence of dendritic cells within the lower reproductive tract would see them ideally positioned to sample seminal antigens introduced following intercourse and potentially play a role in the initiation of immune responses to paternal antigens.
Dendritic cells are also present within the human endometrium with their numbers remaining relatively constant throughout the menstrual cycle, though a slight increase in endometrial dendritic cells has been reported towards the end of the menstrual cycle (Rieger et al. 2004). Whether this increase is a result of altered steroid hormones or changes in the local cytokine environment is yet to be determined. Dendritic cells are also present in the decidua during pregnancy, with numbers typically increasing during the first trimester, reaching levels higher than those observed during any stage of the menstrual cycle (Rieger et al. 2004). The literature contains conflicting reports as to the phenotype of these cells during pregnancy, with discrepancies between investigators likely arising through a lack of specific cell surface markers expressed only by dendritic cells. For example, a unique population of DC-SIGN+ (CD209, an ICAM-3 receptor) dendritic cells have been shown to increase 4-fold in the pregnant decidua, compared to the cycling endometrium (Kammerer et al. 2003, Rieger et al. 2004), but confusion exists as to whether this marker is also expressed by a subset of macrophages (Soilleux et al. 2002, Gardner et al. 2003). It has been proposed that these cells may be of an 'alternative' activation phenotype since molecules known to influence dendritic cell function and induce tolerogenic responses in other tissues, such as TGFβ, prostaglandin E2 and IL-10 are all present in abundance in the decidua (Gardner et al. 2003). More recently, these DC-SIGN+ dendritic cells have been described as closely resembling immature dendritic cells, both in phenotype and function and are the most rapidly proliferating dendritic cell in the decidua in early pregnancy (Kammerer et al. 2003). Also, the number of CD83+ mature dendritic cells has been shown to decrease in early pregnancy compared to the non-pregnant endometrium (Rieger et al. 2004). From a functional viewpoint, the presentation of antigens to T cells by immature dendritic cells has been shown to induce antigen-specific T cell tolerance (Steinman et al. 2003). This finding is similar to the mouse where uterine dendritic cells have also been described as having an immature phenotype during pregnancy (Keenihan et al. 2004).

The precise functions of decidual dendritic cells and whether they play a role pregnancy remains to be determined. However, the proliferation of dendritic cells in the pregnant endometrium (Rieger et al. 2004), their powerful ability to sample external antigens, and their close proximity to the invading trophoblast cells of the developing placenta (Gardner et al. 2003) suggests that these cells may play a role in uterine immunity, with potential roles in maintaining the delicate balance between defence against pathogens and tolerance towards the fetus.

Dendritic cells are also present in the uterus of cycling mice, however they are of an immature phenotype resulting in only a limited antigen-presenting capacity (Keenihan et al. 2004). An increase in GM-CSF release from uterine epithelial cells following mating is believed to be responsible for the
recruitment of dendritic cells into the murine uterus (Robertson et al. 1994), as well as phenotype switching to a mature antigen-presenting state. GM-CSF up regulates dendritic cell expression of MHC class II antigen and the co-stimulatory molecule B7-2, thereby initiating antigen-presenting capacity in these cells.

1.3.2.5 Natural killer cells

Natural killer cells are a lymphocyte lineage distinct from B and T lymphocytes, as they do not express either the T cell receptor or CD3 molecules. The main function of NK cells is to act as the first line of defence against microbial pathogens, and release cytokines including GM-CSF and CSF-1 that modify immune functions (Trinchieri 1989). NK cells can distinguish "self" from "non-self", lysing any cells which have diminished MHC class I antigen expression due to viral infection, or those which express MHC that is different to that expressed by the host (Ljunggren et al. 1990).

In humans, NK cells have been detected within the vaginal epithelium (Edwards et al. 1985) and the cervix, however within the cervical tissue, only small numbers are present within the stromal tissue and are absent from the epithelial compartment (McKenzie et al. 1991). The human uterine environment is also a rich source of NK cells, the largest of which is the large granulated lymphocytes (LGLs) located within the endometrium (Bulmer et al. 1991) accounting for more than 70% of endometrial leukocytes in the first trimester decidua. A unique population of NK cells, the uterine NK cells (uNK) have been implicated as playing a role during human and murine pregnancy by virtue of their ability to secrete a diverse range of cytokines thought important for establishing a uterine microenvironment conducive to implantation and subsequent pregnancy. In the human endometrium, uNK cells have been shown to increase during the secretory phase, peaking during early pregnancy at which time they represent a massive 75% of uterine leukocytes (Bulmer et al. 1991). As gestation proceeds the number of uNK cells present within the endometrium declines to such an extent that they are absent from the endometrium at term (Moffett-King 2002).

The exact function of uNK cells remains to be determined however it has been suggested that they may be involved in endometrial decidualisation due to their increase in numbers during the mid-secretory phase of the menstrual cycle and close proximity to stromal cells, glands and blood vessels (King 2000). During pregnancy, uNK cells are thought to play a role in regulating the maternal immune response to the conceptus and also in controlling the trophoblast growth and invasion (Dosiou et al. 2004), a notion proposed following the observation that uNK cells are located in close proximity to the
implantation site during early human pregnancy, intimately associated with the invading trophoblast cells (Dosiou et al. 2004). Insight into the possible role of uNK cells come from experiments using mice lacking uNK cells. Pregnancies resulting from these mice were found to give rise to placentas displaying hypocellularity, oedema of the decidua basalis and abnormal thickening of the spiral arteries, however the pregnancy appears to continue normally without fetal loss (Croy et al. 2003). Therefore these finding suggest that at least in the mouse, while uNK cells appear to be important in establishing the appropriate vasculature of the placenta, successful pregnancies can still occur in their absence. The precise role of these cells during human pregnancy is not yet clear.

1.3.2.6 T lymphocytes

T cells can be divided into CD4+ helper cells (Th cells) which promote cell-mediated (Th1), antibody-mediated (Th2), or suppressive (Th3) responses through their differential release of certain cytokines, and CD8+ cytotoxic cells (Tc cells), which lyse antigen-bearing target cells.

Th1 immune responses, characterised by the production of cytokines including IL-2, IFNγ and TNFα result in the activation of macrophages, CD8+ cytotoxic T cells and cell-mediated responses important for resistance to infection by intracellular pathogens and delayed-type hypersensitivity (DTH) reactions as well as being potentially dangerous for the fetus (Tezabwala et al. 1989). Th2 responses are characterised by the production of cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 results in vigorous antibody production and are therefore commonly observed in the presence of antibodies directing at fighting infections caused by extracellular pathogens (Mosmann et al. 1989). The antibodies produced are typically non-complement fixing IgG1, with this cytokine milieu also resulting in the suppression of cytotoxic cell-mediated immune responses (Carter et al. 1996). These Th1 and Th2 cells have been identified as being mutually suppressive to generation of the other. The products of Th1 cells such as IFNγ can prevent the activation of Th2 cells whilst IL-10 produced by Th2 cells prevents the activation of antigen-presenting cells, thereby blocking the activation of Th1 cells (Wegmann et al. 1993). CD4+ Th3 cells, also referred to as regulatory T cells are characterised by their expression of TGFβ and their lack of expression of molecules such as IL-2, IFNγ, IL-4 and IL-10. These cells appear to be a unique subpopulation of T cells whose function is believed important in down-regulating Th1 cell immune responses (Raghupathy 2001).

In humans, T lymphocytes are present within the vaginal epithelium in numbers greater than any other leukocyte population, representing approximately 50% of all leukocytes present (Johansson et al.)
In peripheral blood, the normal ratio of CD4+:CD8+ T cells is approximately 1.7, however within the vagina this ratio is dramatically reduced to approximately 0.3 with CD8+ T cells clearly outnumbering CD4+ T cells (Johansson et al. 1999). Very little variation in the relative abundance of these cells is observed throughout the menstrual cycle (Patton et al. 2000). Both CD4+ and CD8+ T lymphocytes are also present within the epithelial and stromal compartments of the ecto- and endocervical tissues of women with CD4+ T cells outnumbering CD8+ T cells within the ectocervix and the opposite occurring within the endocervix (Poppe et al. 1998). Immunohistochemical analysis has revealed that both CD4+ and CD8+ T lymphocytes are present in higher concentrations within the cervical stroma than in the epithelium, in close proximity to the basement membrane (Johansson et al. 1999) and their relative densities and distribution remains consistent throughout the menstrual cycle (Poppe et al. 1998, Johansson et al. 1999). Exposure to semen following intercourse or donor insemination has been shown to result in transient increases in CD8+ and to a lesser extent CD4+ T lymphocytes within the superficial layers of the cervical epithelium and lumen (Thompson et al. 1992). It has been proposed that these CD8+ T cells are of a suppressive phenotype however further experiments are required to confirm this. Furthermore, mucous sampling from the endocervix has revealed that CD4+ T lymphocytes are present in higher concentrations in the superficial layers of the cervical epithelium and mucous of women who reported having sexual intercourse less than 3 days ago, compared to those who had their last sexual contact greater than 3 days ago (Prakash et al. 2003). The precise role of the large numbers of T lymphocytes within the vagina and cervix is yet to be determined though their presence and cytolytic activity (White et al. 1997) suggests that T cell mediated immunity is important within these tissues and may therefore play a role in the prevention of infection. It is also interesting to speculate that they may also play a role in the development of tolerogenic immune responses to seminal antigens contained within the ejaculate, however further phenotypic analysis would be required to confirm this function.

In the human endometrium, T lymphocytes represent approximately 10% of the total endometrial cell population (Starkey et al. 1991), with little variation in their density and distribution observed between the various stages of the menstrual cycle, suggesting that their recruitment is not under steroid hormone control (Starkey et al. 1991). During early pregnancy, endometrial T lymphocyte numbers remain relatively constant (Vassiliadou et al. 1996), though a number of phenotypic changes have been observed. These include the down-regulation of T cell receptor expression on decidual T cells (Morii et al. 1993, Saito et al. 1993), the expression of activation markers such as CD69, HLA-DR and CD45RO (Saito et al. 1992, Saito et al. 1994b) and the finding that decidual T cells do not proliferate in vitro when mixed with trophoblast or allogeneic peripheral blood lymphocytes as would be
seen in a mixed lymphocyte reaction (King et al. 1996). T cells clearly play an important role in pregnancy, highlighted by the observation that decidual T cells obtained from women experiencing recurrent miscarriage produce lower concentrations of Th2 cytokines such as IL-4 and IL-10 compared to decidual T cells recovered from women undergoing elective termination (Piccinni et al. 1998a), thereby suggesting a role for T cells in the maintenance of pregnancy.

In both human and murine pregnancies, a subset of T cells known as γδ T cells which are believed to be important in the generation of immunological tolerance are present in elevated numbers adjacent to trophoblast cells at the fetal-maternal interface (Heyborne et al. 1992, Mincheva Nilsson et al. 1997). It has been postulated that the main function of γδ T cells within the decidua is in the prevention of αβ T cell activation at the fetal-maternal interface, since γδ T cells have been shown to inhibit the proliferation of αβ T cells in a one-way mixed lymphocyte reaction (Suzuki et al. 1995). The potential importance of this inhibition is highlighted by studies in rodents which have demonstrated that αβ T cells have higher cytotoxic activity (Morita et al. 1991), an increased ability to recognise allogeneic MHC antigens than γδ T cells (Hunig et al. 1989) and are therefore more capable of eliciting detrimental immune responses towards fetal antigens. Conversely, γδ T cells recognise a distinct group of ligands and antigens in an MHC-unrestricted manner suggested to be important in the immunological recognition of pregnancy (Mincheva-Nilsson 2003). Importantly, trophoblast cells do not express polymorphic MHC and class II MHC expression can not be induced following stimulation with IFNγ, thereby avoiding direct stimulation of maternal αβ T cells (Mincheva-Nilsson 2003). The ability of γδ T cells to recognise antigens expressed by trophoblasts may also implicate these cells in having a role in controlling placental trophoblast invasion. In addition, human γδ T cells are believed to contribute to the overall Th2 cytokine bias within the decidua by virtue of their exclusive expression of the immunosuppressive cytokines TGFβ1 and IL-10 (Nagaeva et al. 2002). The interaction between decidual γδ T cells and trophoblast and the constitutive release of immunosuppressive molecules such as TGFβ1 and IL-10 by these cells may act to modulate the maternal immune system towards tolerance of the fetus by either directly inhibiting effector cell function at the fetal-maternal interface (Seo et al. 1999) or indirectly, by stimulating the generation of regulatory T cells (T_{reg}), which also produce TGFβ1 and IL-10 and suppress effector T cell function (Drobyski et al. 2000).

Recently, another specialised subset of T cells, known as CD4⁺CD25⁻ T_{reg} cells, have received significant attention from reproductive immunologists. CD4⁺CD25⁻ T cells have been shown to be crucial in the prevention of autoimmunity (Sakaguchi 2000, Takahashi et al. 2003, Sakaguchi 2005), the
development of transplantation tolerance (Graca et al. 2002, Bluestone et al. 2003) and have been shown to have potent regulatory properties in both the induction and maintenance phases of in vivo tolerance to alloantigens in humans (Baecher-Allan et al. 2001, Jonuleit et al. 2001) and mice (Sakaguchi et al. 1995). Despite CD4+CD25+ T_{reg} cells having similar regulatory properties in humans and in mice, species differences clearly exist since only CD4+CD25^{high} T_{reg} cells exhibit regulatory function in humans while CD4+CD25^{low} cells having no ability for immunosuppression (Baecher-Allan et al. 2001, Baecher-Allan et al. 2004).

CD4+CD25^{high} T_{reg} cells have also been implicated as playing a crucial role in the establishment and maintenance of tolerance towards the fetus in both humans and mice (Aluvihare et al. 2004, Somerset et al. 2004). In early human pregnancy, the proportion of decidual CD4+CD25^{high} T cells has been shown to increase to approximately 20% of the total decidual T cell population compared to approximately 8% in non-pregnant women (Heikkinen et al. 2004, Sasaki et al. 2004, Somerset et al. 2004), with similar changes also observed within peripheral blood samples (Sasaki et al. 2004). This increase is maximal during the first and second trimesters with CD4+CD25^{high} T cell numbers declining shortly before labour and postpartum (Somerset et al. 2004). Similar increases are also observed within the uterine tissues of mice, with CD4+CD25^{high} T_{reg} cell expansion occurring as early as 2 days following mating where they comprise up to 30% of the T cell population. Interestingly, it has recently been demonstrated in mice, that an absence of CD4+CD25^{high} T_{reg} cells leads to pregnancy failure only in allogeneically and not syngeneically mated mice, suggesting that CD4+CD25^{high} T_{reg} cells are only important for materno-fetal tolerance when the fetus expresses distinct paternal antigens (Aluvihare et al. 2004). Studies investigating the role of the cells in human pregnancy are limited, however it has recently been reported that there is a significant reduction in decidual CD4+CD25^{high} T cells in vivo in the women experiencing spontaneous abortion compared to women undergoing elective termination of pregnancies (Sasaki et al. 2004).

The factors regulating CD4+CD25^{high} T cells within the decidua are unknown, however studies in mice have shown that estrogen can increase CD4+CD25^{high} T cells in mice (Polanczyk et al. 2004). Also, the precise mechanisms by which CD4+CD25^{high} T cells exert their immunosuppressive effects in humans are still being investigated however it is believed that CD4+CD25^{high} T cells inhibit the immunostimulation of conventional T cells through either direct cell-to-cell contact (Sasaki et al. 2004) and/or the release of immunosuppressive cytokines such as IL-10 and TGFβ (Saito et al. 2005). In addition, the high intracellular and surface expression of CTLA-4 by CD4+CD25^{high} T_{reg} cells, which is increased during human pregnancy (Sasaki et al. 2004), may also be important since this molecule up-
regulates the expression of indoleamine 2,3-dioxygenase (IDO) and tryptophan catabolism in antigen-presenting cells (Grohmann et al. 2001) which has been implicated as having an important role in the maintenance of tolerance (Munn et al. 1998, Mellor et al. 2002, Fallarino et al. 2003). It is likely that decidual CD4+CD25+ Treg cells also operate via similar mechanisms during human pregnancy since Treg cells are increased within the decidua, the immunosuppressive cytokines IL-10 and TGFβ are present in abundance at the fetal-maternal interface and the levels of tryptophan have been observed to decline throughout normal human pregnancy (Fuchs et al. 1996, Schrocksnadel et al. 2003).

1.3.2.7 Non-professional antigen presenting cells

Cells such as keratinocytes are capable of acting as non-professional antigen presenting cells (Nickoloff et al. 1994). Interestingly, uterine epithelial cells have been identified as having the ability to present antigen on their cell surface. These cells have been shown to express the non-classical MHC class 1 protein, CD1d (Canchis et al. 1993). This protein has known functions in the presentation of antigen to T cells and therefore it is plausible that the uterine epithelium may also be involved in antigen presentation and induction of pregnancy-associated immune responses. Furthermore, human ecto- and endocervical epithelial cells have also been shown to constitutively express human leukocyte antigen (HLA) class I (Ljunggren et al. 1998, Mota et al. 1999) with in vitro studies also demonstrating that HLA class II expression can also be induced following treatment with IFNγ (Ljunggren et al. 1998). The ability to express class II molecules is of particular interest, as it is normally a feature of ‘professional’ APCs, whose function is to stimulate CD4+ T lymphocytes which in turn provide essential signals for other immune cells. Despite ectocervical and endocervical epithelial cells expressing differing amounts of class II molecules, these cells may possess the ability to directly stimulate CD4+ T lymphocytes, and therefore take part in immune regulation. Interestingly, this IFNγ-induced class II expression can be down-regulated by other cytokines present at sites of inflammation / immune responses, namely by TNFα and TGFβ1. It is therefore possible that these cells may play a role in human pregnancy given that the ejaculate is deposited at the keratinized epithelial surface of the the vagina and cervix.
1.4 THE MATERNAL IMMUNE RESPONSE TO PREGNANCY

1.4.1 The fetus as an allograft

1.4.1.1 Introduction

The mechanism by which the developing fetus evades rejection by the mother has perplexed reproductive immunologists for almost a century. In 1953, Peter Medwar was the first to articulate that mammalian pregnancy defies the rules of transplantation. Medwar proposed that this lack of fetal rejection might be explained by one or more of the following hypotheses. Firstly, the fetus may be antigenically immature, suppressing the expression of antigens that the maternal immune system might recognise as being foreign and target for destruction. Secondly, the mother and the fetus might remain anatomically separated, thereby preventing the exposure of the maternal immune system to the allogenic antigens expressed by the fetus. Thirdly, the maternal immune system might be immunologically inert during pregnancy and therefore not capable of mounting an immune response against fetal antigens.

Throughout the years, each of Medwar’s hypotheses have been systematically challenged as new insight into the immunological relationship between the mother and fetus emerges. This section of the literature review will focus on explaining our current understanding of the various mechanisms which exist to prevent the immunological rejection of the fetus by the mother.
1.4.1.2 Hypothesis 1: is the fetus antigenically immature?

A mechanism that may account for the limited immune responses to the fetus during pregnancy is decreased expression of classical MHC antigens by fetal cells. MHC is a group of extremely polymorphic genes that encode cell surface antigens that play a central role in distinguishing self from non-self, with these antigens being primarily responsible for mediating immune rejection of transplanted tissue between unrelated donor and recipients. The developing fetus expresses MHC molecules shared by both its mother and father and therefore half of the genes it expresses are foreign to the mother.

Classical MHC class I and II molecules are known to play a role in inhibiting NK cell-mediated cytotoxicity and since these molecules are not present on trophoblast cells that are in direct contact with maternal circulation then additional mechanisms must exist that protect the trophoblast against this NK-cell mediated attack. The lack of expression of classical MHC molecules on trophoblast cells could explain the failure of maternal effector cells to destroy fetal cells that are in contact with maternal blood. However, decreased expression of MHC can not fully explain the survival of the fetus as studies using mice that have increased expression of MHC on exposed trophoblast do not have increased fetal rejection (Koch et al. 2003). Other researchers have investigated this further by using transgenic mice that express allogenic MHC class I on various trophoblast populations, including labyrinthine trophoblast exposed to the maternal circulation also observing no increase in fetal loss (Rogers et al. 1998).

In the human, the classical MHC class Ia antigens (HLA-A, -B and -C) are not expressed by syncytiotrophoblast whereas the non-classical MHC class Ib molecules HLA-G and HLA-E, and to a lesser extent a form of HLA-C (thought to be non-immunogenic) are expressed by many trophoblast cell types including the extravillous cytotrophoblast (King et al. 2000c). Interestingly, these non-classical MHC antigens exhibit very little polymorphism throughout the population and are therefore unlikely to be responsible for initiating a destructive response within the mother (Geraghty 1993). The presence of this non-polymorphic HLA-G (either soluble or membrane-bound) on trophoblasts therefore may protect these cells from destruction by NK cells given that cells not expressing class I antigen are typically targeted for destruction by NK cells, and without initiating T cell responses (Chumley et al. 1994). HLA-G may protect the fetus from T cell mediated attack as demonstrated by its ability to inhibit the proliferation of allogenic T cells by up to 95% in mixed lymphocyte reactions using cells transfected with HLA-G (Riteau et al. 1999). Recently it has also been shown that cells that are normally susceptible to NK cell-mediated lysis become resistant to this when transfected with an isoform of the membrane-bound form of HLA-G1 (Pazmany et al. 1996), with evidence suggesting that this protection is...
The human blastocyst has also been shown to express HLA-G (Jurisicova et al. 1996). It was found that in vitro fertilized human embryos secreting soluble HLA-G gave rise to a 24% pregnancy rate following transfer, whereas no pregnancies were achieved following the transfer of embryos not expressing HLA-G (Fuzzi et al. 2002). These observations help to emphasise the important role that HLA antigens may play in establishing a successful pregnancy.

HLA class II antigens are not expressed by human trophoblast cells that are in direct contact with the maternal circulation (Raghupathy 1997). This lack of HLA class II may be important since upregulated trophoblast expression of MHC class II antigen by treatment with IFNγ results in immune-mediated fetal loss in mice (Vassiliadis et al. 1994). In the human, placental tissue obtained from women experiencing recurrent miscarriage was found to express HLA class II molecules, whereas placental tissue obtained from normal pregnancies were found not to express these molecules (Athanassakis et al. 1995).

Medwar's first hypothesis is therefore not sustainable, since modern research has demonstrated that the fetus is not antigenically immature, and in fact does express HLA antigens capable of stimulating a maternal immune response. In fact, it may be that reproductive outcome is actually enhanced by maternal awareness of the fetal-placental unit, since pregnancies between HLA disparate couples result in a heavier fetal-placental unit (Ober et al. 1983). Additionally, a weak maternal immune
response towards antigens expressed by the fetus due to a high degree of HLA antigen concordance between parents has been suggested associated with an increased risk of spontaneous miscarriage in humans (Gill 1983, Ober et al. 1998).

1.4.1.3 Hypothesis 2: is the fetus anatomically separated from the mother?

Medwar's second hypothesis suggesting that the fetus evades rejection by the maternal immune system by being anatomically separated from the mother by a physical barrier is void from an anatomical viewpoint, as paternal antigen expressing trophoblast cells do come in contact with the maternal tissues. However functional barriers do exist. Shortly after fertilisation, the pre-implantation embryo quickly becomes surrounded by the zona pellucida, an impenetrable glycoprotein coat completely surrounding the embryo and conferring protection against attack from cytotoxic lymphocytes (Ewoldsen et al. 1987). During the implantation period this protective coating surrounding the embryo is lost and replaced by extruded extra-villous cytotrophoblast cells which are known to express non-classical HLA class I antigens thereby producing yet another protective mechanism against immune attack. Compartmentalisation of leukocytes within the decidua may provide a further layer of protection, since leukocytes with potent antigen presenting capabilities or cytolytic activity are excluded from decidual tissues in close proximity to the fetal-maternal interface. However, there are studies in mice (Nagarkatti et al. 1983, Manjunath et al. 1985, Zhou et al. 1998) and humans (Ahrons 1971, Mogil et al. 1989) which show evidence of systemic maternal immune priming to paternal MHC antigens in pregnancy, clearly illustrating that any physical separation is incomplete.
1.4.1.4 Hypothesis 3: is the mother immunologically inert during pregnancy?

The cytokine environment within the female reproductive tract is of critical importance to the success of pregnancy. If the hypothesis that the maternal immune system is suppressed throughout pregnancy was correct, this would leave the pregnant female in the dangerous position of being more susceptible to infection and having a reduced ability to detect tumor cells. Clearly, pregnant women are able to mount an immune response following vaccination, and reject allogenic tissue transplants, which suggests their immune system remains responsive to challenge. In light of this, pregnancy is no longer thought to induce generalised suppression of the maternal immune system, instead it is now thought of as a state of immunological tolerance towards trophoblast antigens.

A state of immunological tolerance towards a specific antigen typically results in the down-regulation or elimination of T lymphocytes which recognise this antigen. The suggestion that pregnancy results in the development of tolerance against paternal antigens expressed by trophoblast cells come from studies in mice that demonstrated that T cells reactive with paternal MHC class I became unable to recognise this antigen due to the internalisation of T-cell receptors (Tafuri et al. 1995).

Tom Wegmann and others have proposed that a shift in the maternal immune environment from a Th1 type response to a Th2/Th3 type response is necessary for successful fetal outcome with evidence of this shift occurring in both human and mouse pregnancies (Wegmann et al. 1993). There is evidence to indicate that a failure to establish and/or maintain this Th1-Th2/3 balance is a major cause of fetal loss both in animals and humans (Raghupathy 2001). The importance of this delicate balance in the maternal immune system comes from studies using abortion-prone mice that are unable to generate their own Th2 cytokines and as a result have a 20-50% fetal loss rate. When pregnant female mice are administered either of the Th2 cytokines GM-CSF or IL-10, the high rate of fetal loss is overcome (Clark et al. 1994, Chaouat et al. 1995). Production of type-1 cytokines has adverse effects on pregnancy, with studies showing that the injection of IL-2, IFNγ or TNFα into pregnant mice results in an increase in fetal loss (Chaouat et al. 1995).

Human pregnancy is also characterised by a Th2 shift with elevated concentrations of Th2 type cytokines such as IL-4 and IL-10 being produced by trophoblast cells of the placenta during pregnancy (Cadet et al. 1995). Investigators measuring serum IL-10 levels have shown that pregnant women who have successful pregnancies have far higher concentrations of IL-10 than women who have pregnancies that end in miscarriage (Jenkins et al. 2000, Wilson et al. 2004). These investigators also reported that women with a history of recurrent miscarriage had serum IFNγ levels both before and
during pregnancy that were much higher than women who had a subsequent normal pregnancy (Jenkins et al. 2000, Wilson et al. 2004). Further evidence supporting this Th2 shift was obtained by comparing the cytokine profiles of peripheral blood mononuclear cells (PBMCs) obtained from normal pregnant women and women with recurrent miscarriage. It was found that PBMCs from normal pregnant women consistently express higher levels of IL-4, IL-5, IL-6 and IL-10 than PBMCs from recurrent miscarriage patients. Women experiencing miscarriage uniformly expressed higher levels of the Th1 type cytokines IL-2, IFNγ and TNFα than during normal pregnancies (Makhseed et al. 2000). Recently, CD4+ T cells expressing IL-4 and IL-10 have shown to be present in greater numbers within the decidua of normal pregnant women than those with recurrent miscarriage (Raghupathy 2001). A similar failure to establish the appropriate immune environment through up-regulated expression of Th1 cytokines has also been suggested as being a contributing factor to the development of pre-eclampsia in women. This Th1 to Th2 shift is further supported by the observation that women suffering from cell-mediated immune problems such as arthritis often report that their symptoms are dramatically reduced during pregnancy (Varner 1991), whereas humoral-mediated conditions such as lupus erythematosis are exacerbated (Da Silva et al. 1992).

These observations together help to confirm that the predominance of Th2 type cytokines within the female tissues is important in initiating the type 2 immune response shift observed during normal pregnancy and that a failure to establish this shift toward a Th2 immune response may also contribute the development of pathologies of pregnancy such as pre-eclampsia and recurrent miscarriage. For example, in normal pregnancies activated decidual macrophages secrete high levels of Th2 type cytokines, including IL-4, IL-6 and IL-10, while the secretion of Th1 type cytokines is suppressed. In pregnancies complicated by pre-eclampsia or intrauterine growth retardation (IUGR), activated macrophages have been observed to secrete cytokines such as IFNγ and TNFα, potentially leading to apoptosis of extravillous trophoblast (Mor et al. 2003). In addition, an increased incidence of clusters of TNFα secreting macrophages have been detected in the placental bed of women experiencing severe forms of pre-eclampsia (Pijnenborg et al. 1998).
1.4.2 Post-mating inflammatory response to semen in the murine uterus

Recognition that the female immune response begins to adapt to pregnancy from the time of implantation, or even earlier, has led researchers to focus on local changes occurring at that time. Interestingly, dramatic changes in cytokine and leukocyte parameters are seen to be induced from the time of conception, when semen is introduced into the female tract. The post-mating inflammatory response in the mouse has been shown to involve an infiltration of neutrophils, macrophages and eosinophils into the endometrial stroma and lumen within hours of mating (De et al. 1991, Kachkache et al. 1991, McMaster et al. 1992). The influx of neutrophils is relatively short-lived and is resolved within 24 hours, while increased macrophage numbers persist until day 2-3 of pregnancy, at which time their numbers begin to decline due to increasing progesterone concentrations (Kachkache et al. 1991). This infiltrate is still observed following mating with a vasectomized male (Choudhuri et al. 1992), yet is absent following matings with seminal vesicle-deficient males (Robertson et al. 1996a). This finding led to the discovery that seminal vesicle-derived components of the seminal plasma, most notably TGFβ, trigger the post-mating inflammatory response (Tremellen et al. 1998).

The levels of pro-inflammatory cytokines produced by the murine uterus are closely associated with leukocyte migration during the first three days of pregnancy. This observation is consistent with the notion that TGF-β and other seminal vesicle secretions mediate an influx of leukocytes into the uterus by initiating the release of pro-inflammatory cytokines, including GM-CSF and IL-6 (Tremellen et al. 1998), from the oestrogen primed uterine epithelium, rather than the seminal vesicle secretions being directly chemotactic (shown in Figure 1.3). Cytokine transcripts obtained from murine uteri during the first few days of pregnancy were analysed to reveal an increased expression of mRNA for the cytokines IL-1, IL-6, TNFα and GM-CSF (Robertson et al. 1990, Robertson et al. 1992a, Sanford et al. 1992), as well as the chemokines RANTES, eotaxin, monocyte chemotactic protein (MCP-1) and macrophage inflammatory proteins (MIP-1α and MIP-1β) (Pollard et al. 1998, Robertson et al. 1998). This increase in cytokine / chemokine expression lasts for only 1-2 days before subsiding and returning to baseline levels by day 4 of pregnancy, by this time the infiltration of inflammatory cells has also diminished.
1.4.3 Post-coital Inflammatory response in the human cervix

The local response to insemination is not as well characterised in humans as it is in mice. There have been three previous studies in humans, with all investigating the cellular changes in the lumen, or at best the superficial epithelial cell layers of the cervix. Sampling of the cervical cells achieved using an Ayre’s spatula, as is used when performing a Papanicolaou smear test, has revealed that within four hours following exposure to semen there is a large influx of neutrophils into the cervix, with increased numbers of macrophages and lymphocytes also detected (Pandya et al. 1985, Thompson et al. 1992, Prakash et al. 2003) (refer to section 1.3 for more details). This cervical leukocytosis lasts for approximately 24 hours before subsiding. However, whether inflammatory cells traffic into deeper layers of the cervical tissue, and whether altered cytokine and chemokine expression occurs, has not been investigated.

It is currently unknown whether the human post-coital inflammatory response extends to the endometrium in vivo. The distribution of seminal material within the female reproductive tract after...
intercourse would limit the tissues infiltrated by inflammatory cells and thus the range of downstream effects in a species-specific manner. In rodents and pigs the ejaculate fills the uterine lumen where it gains direct access to the implantation site, but in humans the ejaculate is deposited at the external os of the cervical canal. In humans, emerging evidence obtained from in vitro studies suggest that the effects of seminal plasma are likely to extend to the uterus since endometrial epithelial and stromal cells are responsive to stimulation by seminal plasma (Tremellen et al. 1997, Gutsche et al. 2003). In vivo studies have also demonstrated that active seminal constituents, including TGFβ, are present on the post-acrosomal region of the sperm head and are thereby transported into the uterine environment (Chu et al. 1996). This movement into the higher female tract is facilitated by rapid and sustained peristaltic uterine contractions capable of transporting macromolecular material as high as the fallopian tube (Kunz et al. 2002). The transport of progesterone and other mediators from the cervix to the endometrial tissues are also facilitated by unique vascular connections which may also allow the transportation of inflammatory mediators (Bulletti et al. 1997).

1.4.4 The inflammatory response and implantation

There are currently several proposed mechanisms by which the post-mating inflammatory response may facilitate implantation. This inflammatory response may play a role in establishing the cascade of cellular and biochemical events initiated by the ejaculate and may be critical for the production of a 'receptive' endometrial microenvironment. This influence of semen on the state of receptivity may be achieved as a direct consequence of the number or behaviour of local leukocyte populations, increased local angiogenic activity, or even by changes in the composition or growth factor content of the local luminal fluid; the latter being of great importance to the attachment and growth of the embryo. This concept is supported by the finding that reduced implantation rates are associated with failure of the post-mating surge in GM-CSF release, for example, following superovulation in mice (Beaumont et al. 1975, Robertson et al. 1990), or when pregnancies are initiated after mating with seminal vesicle deficient mice (Pang et al. 1979, Chow et al. 2003, Bromfield et al. 2004).

Perhaps the most significant and well-characterised role for the inflammatory response is in its interaction with the maternal immune system. The maternal immune response likely to be of key importance in facilitating defence from potentially harmful pathogens from the female reproductive tract introduced at the time of mating (Parr et al. 1985). But perhaps just as importantly, the immune response activated at insemination may 'prime' the maternal immune system against paternal antigens.
present in the ejaculate (Thaler 1989) with consequences for tolerance in pregnancy. Antigen-presenting cells such as macrophages and dendritic cells are present in high numbers in the murine endometrium shortly after mating, and therefore are ideally positioned to sample paternal antigens. Following mating, mice sterilised by tubal-ligation can be demonstrated to have functional tolerance as measured by their inability to reject syngeneic skin (Lengerova et al. 1966) expressing the same antigens as those expressed by the original mate, yet these females are capable of rejecting allografts from a third party. In addition, female mice exposed to seminal plasma even without the presence of sperm or a conceptus become transiently tolerant to male MHC antigens and fail to reject challenge with male tumour cells (Robertson et al. 1997). The protection of male antigens from rejection by the maternal immune system is afforded by MHC antigens and the minor histocompatibility antigen H-Y, but only when sperm is delivered in the presence of seminal plasma (Beer et al. 1974, Hancock et al. 1986). Furthermore, studies with seminal vesicle-deficient mice suggest that immunoregulatory components within seminal plasma may affect the phenotype of antigen-presenting cells so that they present paternal antigens contained within the ejaculate to maternal T cells in a way that induces antigen-specific immunological tolerance. This immune deviation may benefit the growth and survival of the semi-allogeneic conceptus by providing an immunological environment that favours embryo implantation and placental development. Whether the post-mating inflammatory response plays a role in priming the maternal immune response in humans is yet to be elucidated.
1.5 ROLE OF SEMINAL PLASMA IN REPRODUCTION

1.5.1 Introduction

For decades it was thought that the only function of seminal plasma was to transport sperm and promote their survival in the female reproductive tract. The studies described above suggest that seminal plasma may have a further role in interacting directly with epithelial cells and leukocytes in the cervix and endometrium, initiating a cascade of cellular and molecular changes that may promote embryo implantation and a successful pregnancy outcome. The composition of semen consists of spermatozoa, non-germ cells such as leukocytes and genitourinary tract epithelial cells, all suspended in a viscous fluid known as seminal plasma. Male accessory sex glands including the prostate, seminal vesicle, bulbourethral and coagulating glands all contribute to the production of seminal plasma. The vast majority (approximately 70%) of seminal plasma secretions produced by mice and humans is derived from the seminal vesicle, with prostatic secretions making up the majority of the remaining volume (Mann et al. 1981, Aumuller et al. 1992). In addition, seminal plasma contains secretions derived from the epithelium of the epididymus and vas deferens. The average volume of ejaculate produced by fertile men is typically between 1.5 and 4ml and contains approximately 50-200 million sperm (Rowe et al. 1993). In comparison, male mice typically deposit approximately 6 million sperm into the uterine lumen following mating (Tucker 1980).

Seminal plasma is a complex mixture of ions, proteins, peptides, sugars and hormones (Mann et al. 1981, Blank 1988, Aumuller et al. 1992). It has also been shown to contain all of the ions normally contained within blood plasma, however potassium and zinc are present at concentrations much higher than those seen in blood. The use of sodium dodecyl-sulphate (SDS)-page gel electrophoresis has allowed over twenty-five major protein species to be identified as being present within murine seminal plasma (Bradshaw et al. 1977), while approximately nineteen major proteins have been identified in human seminal plasma (Verma et al. 1993). Despite many of the proteins present within seminal plasma being derived from serum (ie. albumin, transferrin and immunoglobulin), a large proportion of these proteins are involved in the processes of semen coagulation and subsequent liquefaction. Fibronectin and semenogelin are the primary structural proteins are the principal structural proteins involved in semen coagulation, while prostatic enzymes such as Prostate Specific Antigen (PSA), a kallikrein-like serine protease, is involved in coagulum liquefaction (Lilja et al. 1987). The observation that seminal vesicle-derived secretions contain large amounts of fructose, along with lesser amounts of other sugars including glucose, fructose and ribose, has been associated with providing an energy
source for sperm (Phadke et al. 1973).

1.5.2 The role of seminal plasma in reproduction in animals

The primary mechanism by which seminal plasma may enhance reproductive potential in mice and humans is by increasing the numbers of ejaculated sperm that reach the oocyte. In rodent species this is achieved by increasing the sperm's forward motility (Peitz et al. 1986). Shortly after mating, a vaginal plug, consisting of seminal plasma proteins, is formed within the rodent vagina/cervix and is believed to facilitate the trans-cervical passage of sperm into the uterine lumen (Carballada et al. 1992, Carballada et al. 1993). In the human, seminal plasma is believed to enzymatically modify the cervical mucus resulting in an increased capacity of sperm to traverse the cervix and pass into the uterus (Overstreet et al. 1980).

Seminal plasma is not an absolute requirement for fertilisation, since successful pregnancy can be initiated using epididymal or washed ejaculated sperm in artificial insemination, or in vitro fertilisation/embryo transfer. However, studies in other species have shown that prospective mothers that have not been exposed to seminal plasma suffer compromised pregnancies (Peitz et al. 1986). For example, murine embryos transferred to recipients prepared without exposure to male mice usually have a survival rate to term of approximately 50%, which is less than that seen when recipients are prepared by mating with vasectomized males (Rafferty 1970). Studies in rats show that implantation is improved when females are inseminated prior to embryo transfer (Carp et al. 1984). Experiments in which the seminal vesicles, prostate or coagulating glands are surgically removed from stud mice, rats and hamsters prior to mating each show that the seminal vesicle secretions are the most important component of seminal plasma necessary for the establishment of pregnancy (Pang et al. 1979, Queen et al. 1981, Chen et al. 1988). Although it is generally thought that these effects are mediated through the maternal tract (Chen et al. 1988), the embryo may also be directly affected since female hamsters mated with accessory sex gland-deficient males produced embryos that displayed retarded pre-implantation development and decreased implantation rates (Chen et al. 1988). New studies in our laboratory show that initiating pregnancy in the absence of seminal plasma can also compromise placental development and fetal growth trajectory (Bromfield et al. 2004).

Further evidence supporting the concept that seminal plasma plays an important role in reproductive fecundity comes from experiments in pigs. When sows are exposed to additional volumes of seminal plasma during estrous cycles prior to or at the time of artificial insemination, either as a
result of infusion into the uterus of freeze-thaw killed semen, or mating with vasectomised boars, farrowing rate is improved and litter size as well as piglet viability and weight are increased (Murray et al. 1983, Stone et al. 1987, Flowers et al. 1993).

1.5.3 The role of seminal fluids in human pregnancy

There are limited studies investigating the regulatory effect of seminal plasma on reproductive outcome in humans. Epidemiological studies link semen exposure with the occurrence of various clinical manifestations of 'shallow placentation' including pre-eclampsia, IUGR and recurrent spontaneous abortion. Each of these conditions are thought to have an immunological basis and in some instances has been associated with an excessive type 1 (cell-mediated) immunity against either paternal or trophoblast antigens (Hill et al. 1995, Raghupathy 1997, Piccinni et al. 1998b). Currently it is speculated that either an insufficient or inappropriate immune response to paternal antigens introduced into the female reproductive tract at insemination may be a major determinant of this 'immune mal-adaptation' (reviewed by Dekker et al. 1998). This concept is supported by the observation that women with only limited sexual experience, a short duration of sexual cohabitation with their partner or used barrier methods of contraception prior to conception have been shown to have an increased risk of implantation failure, recurrent spontaneous abortion and pre-eclampsia (Marti et al. 1977, Klonoff-Cohen et al. 1989, Robillard et al. 1995), suggesting there may be cumulative benefits of semen exposure on pregnancy outcome. Interestingly, the cumulative benefits of semen exposure appear to be partner specific, and are lost in a subsequent pregnancy resulting from the semen of a new partner where the risk of pre-eclampsia is comparable with that of primiparity (Robillard et al. 1995, Trupin et al. 1996). Further evidence demonstrating the beneficial effect of semen exposure comes from studies in which women exposed to semen around the time of embryo transfer during assisted reproduction techniques such as IVF or GIFT treatment, have a greater pregnancy success rate with significantly improved implantation rates and increased live birth rates compared to women not exposed to semen (Maroni et al. 1973, Bellinge et al. 1986, Tremellen et al. 2000). Furthermore, treatment of women experiencing recurrent miscarriage with seminal plasma pessaries has been reported to moderately enhance implantation rates (Coulam et al. 1993).
1.5.4 The immunomodulatory role of seminal plasma

Semen may also play a role in modulating the maternal immune response in preparation for the acceptance of the resulting conceptus. The presence of cytokines and prostaglandins in seminal plasma may ensure that the female response to paternal antigens is hyporesponsive in the Th1 (cell-mediated) compartment, or ‘tolerogenic’ in nature (Robertson et al. 1997). This proposal is supported by the finding that when female mice are mated with seminal vesicle-deficient males the ‘tolerogenic’ effect of mating is lost, suggesting that Th1 hyporesponsiveness is dependant on a seminal vesicle derived factor, with recent studies identifying TGF-β as being the most likely candidate responsible for deviating the immune response to seminal antigens (Tremellen et al. 1999).

1.5.5 Antigens contained within semen

The controversy surrounding whether or not MHC class I and II antigens are present on the surface of sperm has been disputed for many years, with several investigators being unable to identify these antigens on human (Anderson et al. 1982a, Haas et al. 1986, Schaller et al. 1993) or murine sperm (Harding et al. 1992), while others reportedly have been able to demonstrate low levels of expression of MHC class I and II antigens on human sperm (Bishara et al. 1987, Kurpisz et al. 1987, Ohashi et al. 1990). Recent studies using reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry techniques on purified human ejaculated spermatozoa have demonstrated the expression of MHC class I transcripts within and on the membrane surface of mature spermatozoa of fertile and infertile men (Paradisi et al. 2001). Similar levels of expression were observed in fertile and infertile men suggesting that the fertilizing capacity of the spermatozoon is independent of MHC class I expression on their surface. The investigators also demonstrated the presence of MHC class II antigens on purified human ejaculated sperm (Paradisi et al. 2000). Interestingly, approximately 88% of the spermatozoa samples obtained from infertile men were positive for MHC class II expression, compared to 20% of the fertile group (Paradisi et al. 2000).

Semen also contains a vast number of leukocytes and genitourinary epithelial cells which are believed to provide additional paternal antigenic stimulus to the female reproductive tract (Collins et al. 1994, Wolff 1995). In mice, the cervical-vaginal mucosa has been demonstrated as being immunologically competent by its ability to phagocytose and uptake foreign lymphocytes introduced into the vagina (Ibata et al. 1997). These somatic cells express a high density of MHC antigens on their cell surface and therefore are likely to contribute a strong antigenic stimulus, despite being present in much
lower numbers compared to sperm. Non-germ cells within the human ejaculate have been reported to be more potent than sperm in initiating the activation of maternal lymphocytes (Rodriguez-Cordoba et al. 1982). Somatic cells may therefore be an important antigenic stimulus to the female, since between 10 and 20% of men have in excess of $10^6$ leukocytes per ml within their semen (Wolff 1995).

Various soluble antigens, meaning those not associated with either sperm or somatic cells are also found within semen. These include soluble MHC class I antigens (Schaller et al. 1993, Koelman et al. 2000), and a group of antigens referred to as Trophoblast Lymphocyte Cross- Reactive (TLX) antigens. TLX antigens were first discovered when polyclonal rabbit antisera was found to cross react with trophoblast cells and lymphocytes in an allotypic manner (McIntyre et al. 1983). This observation led to various investigators postulating that exposure of women to seminal plasma TLX antigens at the time of insemination may serve as a source of allogenic recognition, which may then lead to the immunological acceptance of the semi-allogeneic conceptus (Kajino et al. 1988, Thaler 1989). This theory was later invalidated when TLX antigens were found to be identical to CD46, a complement receptor that is believed to protect the placenta from complement-mediated attack (Purcell et al. 1990).

Although exposure to semen is associated with improved reproductive outcome, antigens contained within semen are also capable of triggering adverse immunological events in some individuals. A small proportion of women exhibit an allergy towards semen, with the severity of this allergy ranging from post-coital oedema and pruritus, through to an anaphylactic reaction (Jones 1991, Ebo et al. 1995, Drouet et al. 1997). Interestingly, allergies triggered by semen do not necessarily manifest following first intercourse, but may occur following prostatectomy or vasectomy in the male partner, or pregnancy and reproductive tract surgery in the female (Jones 1991). Provocation of this type of allergy is generally not partner-specific, however a high degree of HLA compatibility between sexual partners has been noted in a small series of couples reporting semen allergy (Bernstein et al. 1981).
1.5.6 Role of immune-deviating cytokines in seminal plasma

The immunosuppressive or more precisely, the ‘immune-deviating’ activity within seminal plasma, meaning the ability to induce tolerance or to skew the class of an immune response generated, has been extensively studied over the last 20 years (James et al. 1984, Alexander et al. 1987, Kelly 1995). The investigation into whether semen contained potentially immune-deviating molecules was initiated as an attempt to explain why it is uncommon for detrimental immune responses to be generated against foreign paternal antigens introduced into the female reproductive tract following sexual intercourse (McShane et al. 1985, Isojima 1988). One of the first studies to demonstrate the presence of immune-deviating activity within human seminal plasma was performed by Stites and Erickson in 1975. Since this initial observation, seminal plasma has been reported to suppress T cell responses to mitogen (Lord et al. 1977, Marcus et al. 1978, Majumdar et al. 1982, Vallely et al. 1986), inhibit primary and secondary antibody responses to vaginally administered antigens (Anderson et al. 1982b), decrease natural killer cell activity (James et al. 1985, Rees et al. 1986), inhibit the ability of macrophages to phagocytose and present antigens to T cells (Stankova et al. 1976, James et al. 1983), decrease neutrophil phagocytic and killing capacity (Petersen et al. 1980), and interfere with complement-mediated immunity (Petersen et al. 1980, Witkin et al. 1983).

To date, the major factors contained within seminal plasma likely to be responsible for initiating and propagating the establishment of immunological tolerance within the female reproductive tract, as a result of their observed immune-deviating activities, are members of the TGFβ and PGE families (Kelly 1999, Robertson et al. 2002). Various other immuno-active substances including complement regulatory proteins, cytokine inhibitory factors and cytokines including IL-1 (Srivastava et al. 1996), IL-6 (Naz et al. 1994a), IL-8 (Friebe et al. 2003), IL-10 (Camejo 2003), IL-12 (Huleihel et al. 1999), TNFα (Kocak et al. 2002) and IFNγ (Fujisawa et al. 1998) have also been shown to be present in human seminal plasma. To date, investigators measuring these molecules in human seminal plasma have mainly focused on comparing cytokine levels between fertile and infertile men, where altered sperm parameters have been identified as the major cause of infertility. For example, several studies have observed elevated IL-1 (Srivastava et al. 1996), IL-6 (Furuya et al. 2003) and IFNγ (Paradisi et al. 1996) concentrations in infertile men compared to fertile individuals. Of these, only IL-6 was found to have an association with sperm parameters, with an inverse relationship identified between IL-6 content and sperm concentration (Furuya et al. 2003). Conversely, IL-10 content was found to be reduced in infertile compared to fertile men, with no correlations with sperm parameters detected (Camejo 2003). In addition, no differences in IL-12 (Huleihel et al. 1999) or TNFα (Kocak et al. 2002) levels have been
observed between fertile and infertile men although a positive correlation between TNFα content and sperm motility and morphology has been observed (Kocak et al. 2002). Finally, no differences in IL-8 concentrations have been detected between fertile and infertile individuals (Friebe et al. 2003), however men with leukocytospermia have been reported as having significantly increased IL-8 content, positively correlated with both leukocyte count and the presence of infection (Koumantakis et al. 1998).

These molecules have established roles in immune-deviation within other biological fluids and mucosal surfaces, and their presence within seminal plasma suggests they may play a similar role in reproduction. TGFβ, contained within murine and human seminal plasma at concentrations higher than those observed in any other biological fluid (approximately 100-200 ng/ml, compared to approximately 5 ng/ml in serum) (Nocera et al. 1993, Tremellen et al. 1998), is a potent type 2 immune-deviating molecule and has been shown to be a key tolerance-inducing molecule mucosal tissues (Letterio et al. 1998). Studies comparing the levels of TGFβ within human seminal plasma obtained from fertile men and infertile men with various testicular pathologies (including secretory pathology and epididymal occlusion) found no significant differences in biologically active TGFβ, however a small increase in total TGFβ was detected in men with secretory testicular pathologies (Loras et al. 1999). A small sample size may have prevented any significant differences from being detected. To date, there has been no investigation into the relationship between TGFβ concentrations within seminal plasma and its effect on reproductive outcome. PGE, predominantly the 19-hydroxy forms that are present within seminal plasma at concentrations approximately 100 000 times higher than those seen in an inflammatory response (Kelly et al. 1997a), act to program a type 2-inducing phenotype in dendritic cells through its capacity to inhibit IL-12 synthesis (Kapsenberg et al. 1999), capable of inhibiting T cell responses and NK cell activity (Bankhurst 1982, Rappaport et al. 1982). Evidence is emerging to suggest that TGFβ and PGE are involved both directly and indirectly through the actions of IL-10, in the induction and differentiation of various suppressor lymphocyte populations such as Th3 and Treg cells, which are characterized by their lack of antigen specificity (Weiner 1997, Groux et al. 1999). The immune-deviating effects of seminal plasma derived cytokines within the female reproductive tract may be intensified by locally expressed TGFβ and PGE, along with the production of additional type 2 and Th3 deviating cytokines such as IL-4, IL-6 and IL-10, which have been found to be expressed by endocervical and endometrial cells in women (Lim et al. 1998, Fichorova et al. 1999), and in rodents (Tamada et al. 1990). The expression of IL-10 and IL-6 has been shown to be induced by factors contained within seminal plasma (Robertson et al. 1992a, Denison et al. 1999b). Studies have shown IL-6 levels within human seminal plasma are higher in infertile men than those observed samples collected from fertile individuals, however the significance of this finding to reproduction remains...
unclear (Naz et al. 1994a). However, the addition of recombinant IL-6 to human sperm has been shown to enhance their ability to fertilize zona-free hamster ova, possibly by increasing the capacitation of sperm thereby facilitating acrosomal reactions (Naz et al. 1994b).

The relative effectiveness of TGFβ and PGE in stimulating immune-deviation within the cervix and uterus remains unknown. Experimental evidence supporting the notion that these molecules can influence the maternal immune response in such a manner comes from the observation that the addition of exogenous TGFβ to immunizing sperm preparations results in diminished type 1 immunity to sperm antigens, which leads to larger fetal placental weights in subsequent pregnancies (Tremellen et al. 1999). The precise mechanism by which TGFβ and other immunoregulatory molecules found in seminal plasma elicit this response remains unclear, however evidence resulting from studies conducted in the oral and ocular mucosa strongly implicate antigen presenting cells as being involved (Kapsenberg et al. 1999).

1.6 SUMMARY

In summary, there is considerable published work suggesting that seminal plasma may have an active role in organising the female immune response in preparation for pregnancy. This appears to occur after interaction between specific factors present in semen and female reproductive tract epithelial cells, in a response reminiscent of a classical inflammatory cascade. To date, most of our knowledge comes from studies in rodents and other animals. The purpose of the studies outlined in this thesis was to examine whether exposure to semen at natural intercourse might elicit a similar response in women, and to investigate the molecular and cellular basis of this response.
1.7 HYPOTHESES

The experiments described in this thesis aim to address the following hypotheses:

- Human seminal plasma cytokines evoke immunological changes, both in leukocyte abundance and cytokine expression within the human cervix following intercourse.

- Seminal plasma derived cytokines interact with cervical epithelial cells to generate the post-coital inflammatory response in the human cervix.

- Fertility status in men is influenced by seminal plasma cytokine content.

1.8 AIMS

The aims of the experiments described in this thesis are to investigate:

- The effect of insemination on recruitment and activation of leukocyte populations within the human cervix.

- The effect of insemination on pro-inflammatory cytokine / chemokine mRNA in the cervix.

- The identity of seminal plasma constituents active in stimulating cytokine production in human cervical epithelial cells in vitro.

- The relationship between the cytokine content of human seminal plasma samples and reproductive outcome.
Chapter 2

Materials and Methods
2.1 HUMAN STUDIES

2.1.1 Ethics approval

The ethics committee of the Karolinska Hospital, Stockholm, Sweden approved the human study aimed at investigating the inflammatory response to semen in the human cervix. This study was undertaken in collaboration with Dr Kristina Gemzell-Daniellson, Department of Women and Children's Health, Karolinska Hospital, Stockholm, Sweden. Subjects were recruited and cervical tissue was recovered in Stockholm, then shipped to Adelaide for analysis.

Ethics approval for all other human studies described in this thesis was obtained from the combined human ethics committees of the University of Adelaide and North Western Allied Health Service (NWAHS). Two separate study protocols were approved:


In all studies, informed consent was obtained from each subject prior to inclusion in the study.

2.2 CERVICAL BIOPSIES

2.2.1 Subject population

Eight healthy women aged 18-40 years, with regular menstrual cycles (between 25 and 35 days) and current involvement in a sexual relationship with a proven fertile regular partner, volunteered for the study. None of the women had used steroidal contraceptives or an intrauterine device for a minimum of three months preceding the study. A complete gynaecological examination of each subject was performed upon admission. Couples were also screened for any sign of bacterial or viral infection including, but not limited to, chlamydia, hepatitis B/C, human immunodeficiency virus and human papilloma virus. A negative test result was a prerequisite for inclusion in the study. Subjects with male partners who were either vasectomised or known azoospermic were excluded from the study.

Sharkey Chapter 2
2.2.2 Experimental design

Upon acceptance into the study, subjects were randomly allocated into one of three groups. Group A, the 'abstinent' group (n=7), completely abstained from sexual intercourse, Group B, the 'intercourse' group (n=6), had unprotected intercourse and Group C, the 'condom' group (n=5), had condom-protected intercourse during the study period.

All couples totally abstained from penetrative intercourse for two days prior to the onset of the study, whilst condoms were used for five days prior to this period of time. This protocol was employed to ensure that no sperm would be present in the cervical region at the time of the first biopsy.

In order to synchronise the timing of the first biopsy, all subjects determined their LH peak in urine samples taken twice daily from approximately day 10 after the onset of menstruation to the time at which an LH increase was detected (LH0-LH+1) by using a rapid self-test (Clearplan, Searle Unipath Ltd., Bedford, UK).

Subjects presented for the first biopsy during the peri-ovulatory stage of the menstrual cycle (LH0 to LH+1). Before the biopsies were taken, the cervix was washed with 10ml of saline solution to clear mucous and debris. The cervical washings of all women were collected and analysed for the presence of sperm to confirm that abstinence had been adhered to. Duplicate needle biopsies (4 mm x 6 mm) were taken from the left hand side of the cervix, approximately 1 cm from the transformation zone (the 'initial' biopsy). One cervical biopsy, destined for immunohistochemical analysis was immediately embedded in O.C.T. Tissue Tek (Miles Scientific, Elkhart, IN), frozen by immersion in isopropanol (Ajax Chemicals, Auburn, AU) cooled by liquid nitrogen (N₂) and then stored at -70°C until use. The second biopsy, to be analysed for cytokine / chemokine expression using quantitative reverse transcription-polymerase chain reaction (RT-PCR), was immediately placed into a 2 ml eppendorf tube (Eppendorf, Hamburg, Germany) and snap frozen in liquid N₂ and stored at -70°C until use.

Following the first biopsy, couples were asked to abstain from intercourse for 36 hours to enable haemostasis and healing of the biopsy site. Intercourse couples were asked to have intercourse (either condom-protected or unprotected) on one occasion approximately 36 hours after the first biopsy and return for a second ('repeat') biopsy approximately 12 hours after intercourse (on day LH+2 to LH+3). Abstinent couples also presented 48 hours after the first biopsy for a repeat biopsy (refer to Figure 2.1).
Prior to the repeat biopsies being taken, the cervix was again washed with 10 ml of saline solution, which was collected and analysed for the presence / absence of sperm in order to confirm compliance. The procedure used for the repeat biopsy was essentially the same as was used for the first biopsy, except they were positioned 180° from the initial site, on the right hand side of the cervix. One of each of the cervical biopsies was then either embedded in O.C.T. Tissue Tek (Miles Scientific, Elkhart, IN) or snap frozen in liquid N₂, as described previously.

![Figure 2.1 A time line showing the relative times at which cervical biopsies were collected with respect to both intercourse (or abstinence) and day after the LH peak. Details are given in the text. LH = luteinizing hormone.]

Some couples involved in the study volunteered for multiple rounds, for example, those who were in the intercourse group the first time they participated were included in the abstinent group the second time, and vice versa. Couples who had already been included in the study were required to wait for a minimum of 3 months (from their last biopsy) before they were considered eligible for re-inclusion in the study.
2.2.3 Tissue histology

2.2.3.1 Monoclonal antibodies

Monoclonal antibodies (mAbs) specific for a variety of cell lineages (Table 2.1) were used to determine the distribution and activation status of reactive cells in intact tissue. Mouse anti-human mAbs including OKT3 (α-CD3), OKT4 (α-CD4), OKT8 (α-CD8), HB202 (α-CD11), FMC17 (α-CD14), FMC13 (α-CD15), FMC51 (α-CD45), FMC71 (α-CD45RA), UCHL1 (α-CD45RO), TIB200 (α-CD57) and FMC52 (α-MHC Class II) were kindly provided as hybridoma supernatants by P. McCardle, Flinders Medical Centre, Adelaide, Australia. Other mouse anti-human mAbs including L307.4 (α-CD80) and IT2.2 (α-CD86) were purchased from PharMingen (San Diego, US).

The specific reactivity of the monoclonal antibodies used in this study was validated using fluorescence-activated cell scanning (FACS) analysis (not shown), and immunohistochemical staining of fresh frozen human peripheral blood mononuclear cell smears (not shown). The optimal working concentration of each antibody was then determined by immunohistochemical staining of fresh frozen human endometrium and cervical tissue samples using the protocol described in 2.1.2.5.
<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Antigenic specificity</th>
<th>Reactive cell lineages</th>
<th>Dilution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMC51</td>
<td>CD45 (LCA)</td>
<td>pan leukocytes</td>
<td>1:10</td>
<td>(Macardle et al. 1996)</td>
</tr>
<tr>
<td>OKT3</td>
<td>CD3</td>
<td>pan T-lymphocytes</td>
<td>1:10</td>
<td>(Ashman 1984)</td>
</tr>
<tr>
<td>OKT4</td>
<td>CD4</td>
<td>T-lymphocytes (helper)</td>
<td>1:10</td>
<td>(Crockard et al. 1983)</td>
</tr>
<tr>
<td>OKT8</td>
<td>CD8</td>
<td>T-lymphocytes (cytotoxic / suppressor)</td>
<td>1:10</td>
<td>(Leskinen et al. 1986)</td>
</tr>
<tr>
<td>FMC71</td>
<td>CD45RA</td>
<td>T-lymphocytes (helper and cytotoxic / suppressor)</td>
<td>1:5</td>
<td>(Tworek et al. 1998)</td>
</tr>
<tr>
<td>UCHL1</td>
<td>CD45RO</td>
<td>activated / memory T-lymphocytes</td>
<td>1:5</td>
<td>(Vignola et al. 1998)</td>
</tr>
<tr>
<td>HB202</td>
<td>CD11 (LFA-1α)</td>
<td>macrophages, dendritic cells and some lymphocytes</td>
<td>1:10</td>
<td>(Egner et al. 1993)</td>
</tr>
<tr>
<td>FMC17</td>
<td>CD14</td>
<td>macrophages / dendritic cells</td>
<td>1:10</td>
<td>(Triglia et al. 1985)</td>
</tr>
<tr>
<td>FMC52</td>
<td>MHC Class II</td>
<td>activated macrophages / dendritic cells, some B-cells</td>
<td>1:20</td>
<td>(Zola et al. 1996)</td>
</tr>
<tr>
<td>L307.4</td>
<td>CD80</td>
<td>activated macrophages / dendritic cells</td>
<td>1:100</td>
<td>(Koulova et al. 1991)</td>
</tr>
<tr>
<td>IT2.2</td>
<td>CD86</td>
<td>activated dendritic cells</td>
<td>1:100</td>
<td>(Azuma et al. 1993)</td>
</tr>
<tr>
<td>TIB200</td>
<td>CD57</td>
<td>Natural killer cells</td>
<td>1:10</td>
<td>(Abo et al. 1982)</td>
</tr>
<tr>
<td>FMC13</td>
<td>CD15</td>
<td>Neutrophils</td>
<td>1:10</td>
<td>(Tetteroo et al. 1992)</td>
</tr>
</tbody>
</table>

**TABLE 2.1** The antigenic specificities and cell lineage specificities of the mouse anti-human mAbs used for the immunohistochemical analysis of cervical tissue samples. The source of each antibody is given in the text. LCA = leukocyte common antigen; LFA = leukocyte functional antigen.
2.2.3.2 Tissue sectioning

Six-micrometer (6 μm) tissue sections were cut from endometrial or cervical biopsies (described in section 2.1.2.2) and placed onto glass slides. Slides were allowed to air dry for 2 hours (room temperature), then packed into plastic slide boxes with dehydrated silica gel (Ajax Chemicals, Auburn, AU), which were sealed with electrical tape and stored at -70°C until staining was performed. Slide boxes were removed from the -70°C freezer and equilibrated to room temperature prior to staining. All slides to be stained for a given mAb were prepared and stained together.

2.2.3.3 Immunohistochemistry

Sections of cervical tissue were fixed in 96% ethanol (4°C, 10 min), then washed three times in phosphate buffered saline (PBS). Slides were then placed in a 1% (w/v) bovine serum albumin (BSA) solution, to block non-specific protein binding sites, for 2 min. Duplicate sections were incubated with 40 μl of mouse anti-human mAbs [hybridoma supernatants diluted in PBS with 1% BSA and 10% normal human serum (NHS)] in a humidified chamber (4°C, 2 hours) (refer to Table 2.1 for mAb dilutions). Negative control sections were incubated with an irrelevant, isotype matched mouse mAb. Following incubation, slides were washed three times in PBS and placed in 1% BSA for 2 min. A biotinylated goat anti-mouse secondary antibody (Dako Corporation, Carpinteria, US; 40 μl of 1:300 in PBS with 1% BSA and 10% NHS) was then added to each of the tissue sections and incubated in a humidified chamber (4°C, 45 min). Following incubation, slides were washed three times in PBS and placed in 1% BSA for 2 min. Horseradish peroxidase (HRP) conjugated avidin (Dako Corporation, Carpinteria, US; 40 μl of 1:400 in PBS with 1% BSA and 10% NHS) was then added to each tissue section. Following incubation (4°C, 45 min), slides were once again washed three times in PBS. To visualise HRP, slides were incubated in diaminobenzidine tetrachloride (DAB) (Sigma, Castle Hill, AU; 5 mg/ml in 0.05 M Tris-HCl [pH 7.2] plus 0.02% hydrogen peroxide) for 8 min at room temperature. Tissue sections were counterstained in haematoxylin (Sigma, Castle Hill, AU), dehydrated in two changes of absolute ethanol, cleared in two changes of Safsolvent (Ajax Chemicals, Auburn, AU), mounted in Depex (BDH Laboratory Supplies, Toronto, CA) and stored at room temperature until viewing and analysis.

Video image analysis (VIA) software (Video Pro 32) (Leading Edge Pty. Ltd, Blackwood, AU) was employed to quantify the density of positive (DAB) staining in the cervical tissue sections. Staining was analysed in both the cervical epithelium and cervical stroma. Cervical stroma was defined as being the region of tissue located directly beneath the basement membrane, and was analysed to a maximum
depth of 2 fields (700 μm). The mean area of positive staining (% positivity) in 10 low power fields (x10 objective), was calculated as:

\[
\text{% positivity} = \frac{\text{DAB stained area}}{\text{total (hematoxylin + DAB) stained area}} \times 100
\]

Prior to each session of data collection, the VIA system was calibrated using a standard field of tissue. The inter-assay coefficient of variation was determined to be 4.8%, calculated by analysing an individual field (10 times) on 5 separate sessions. The intra-assay coefficient of variation was 0.7%.

2.2.4 Quantitation of mRNA expression

Quantitative reverse transcription PCR was employed to analyse the quantity of cytokine and chemokine receptor messenger ribonucleic acid (mRNA) within the cervical tissue samples. The cytokines and chemokine receptors analysed were GM-CSF, IL-1α, IL-6, IL-8, TNFα, IFNγ, CCR5 and CXCR4.

2.2.4.1 General precautions for RNase-free conditions

Ribonuclease (RNase)-free conditions were maintained for all materials, chemicals and solutions used in the generation and handling of cells used for extraction of mRNA. Precautions taken in order to prevent RNase contamination included the use of sterile disposable plastic pipettes and tubes and the baking of all glassware and the tissue homogenisation probe for 3 hours at 180°C prior to use. Disposable latex gloves were also used, and were replaced at regular intervals throughout the experiment. All reagents and samples were prepared in a laminar flow hood in order to minimise the potential for contamination.

2.2.4.2 Isolation of total cellular RNA

Cervical tissue biopsies were collected as described previously (in section 2.1.2.2) and immediately frozen in liquid N₂ with samples being stored at -70°C until processing.

Upon removal from storage, 500 μl of RNAzol B (Tel-Test, Friendswood, US) was immediately added to the cervical tissue samples. The tissue was then disrupted by homogenisation using a pestle mixer (Scientific Specialties Inc, Randallstown, US). Once the tissue was dissolved, 50 μl of chloroform

Sharkey Chapter 2 64
(Sigma, Castle Hill, AU) was added and the samples were then gently vortexed and incubated on ice for 5-10 min. Following a 15 min spin at 13000 rpm (11000g) at 4°C, the aqueous ribonucleic acid (RNA) phase was removed using a pipette and placed into a fresh eppendorf tube. The RNA was then precipitated from the aqueous phase by the addition of an equal volume of isopropanol (Sigma, Castle Hill, AU) followed by incubation overnight at -20°C. The following day, samples were centrifuged at 14000 rpm (13000g) for 30 min at 4°C and the pellet was subsequently washed twice with 500 μl of ice cold 75% ethanol, with 15 min 13000 rpm (11000 g) spins at 4°C. The pellet was dried on the bench (within the laminar flow hood) for 30 - 45 min before being resuspended in 100 μl Milli-Q (MQ) water.

To remove any contaminating chromosomal deoxyribonucleic acid (DNA), RNA was incubated with Deoxyribonuclease (DNase) master mix comprising of 25 μl 5x DNA buffer, 1 μl RNAse inhibitor and 5 μl 10U/μl DNase I (both from Roche, Basel, CH) was added to each of the samples and incubated at 37°C for 1 hour. RNA was then recovered by the addition of 125 μl of phenol (H₂O saturated) (Sigma, Castle Hill, AU), 125 μl of chloroform (Sigma, Castle Hill, AU) and isoamyl alcohol (24:1) (Sigma, Castle Hill, AU) and centrifugation at 13000 rpm (11000 g) for 10 min at room temperature. The aqueous phase was then transferred to a fresh eppendorf tube together with 2.5x sample volume of 100% ethanol and 0.1x sample volume of 2M sodium acetate (Sigma, Castle Hill, AU). The tube was invered to mix and stored at -20°C until reverse transcription.

2.2.4.3 Reverse transcription

Samples of extracted RNA were centrifuged at 14000 rpm (13000 g) for 30 min at 4°C. The supernatant was carefully removed and washed twice with 500 μl cold 70% ethanol, with 10 min 13000 rpm (11000 g) spins in between at 4°C. The pellet was then dried on the bench for approximately 30 min before being resuspended in 50 μl MQ water.

The quantity and purity of RNA in the sample was determined by spectrophotometry (DU-50, Beckman). Samples were considered sufficiently pure if the ratio of OD₂₆₀ / OD₂₈₀ was >1.7. The quantity of RNA in each of the samples was determined by the following equation:

\[ \text{OD}_{260} \times 40 \text{RNA} \times \text{dilution factor (50)} = \text{RNA } \mu\text{g/ml} \]

RNA samples were diluted in MQ water to give a concentration of 1 μg/10 μl, of which 10 μl was then used for reverse transcription. The remaining sample was reprecipitated in 2.5x volume 100% ethanol and 0.1x volume 2M sodium acetate and stored at -20°C. 1 μl of random hexamers (Geneworks, Sharkey Chapter 2
Adelaide, AUS) (500 μg/ml) was added to the RNA and incubated at 70°C for 10 min, then chilled on ice for 5 min. A reaction mixture containing 4 μl RT buffer (Gibco BRL, Melbourne, AU; 250 nM Tris-HCl pH 8.3, 300 mM KCl, 15 mM MgCl₂), 2 μl DTT (Gibco BRL, Melbourne, AU) and 2 μl 2′-deoxynucleotide 5′-triphosphate (dNTPs) (AMRAD Pharmica Biotech, Melbourne, AU; 10 mM each dATP, dTTP, dCTG and dGTP) was added to the tube and incubated at 43°C for 2 min. 1 μl of Superscript II RNAse-H reverse transcriptase (Invitrogen, Melbourne, AU; 200 U/μl) was added and the sample incubated for a further 90 min at 43°C. Finally samples were incubated at 94°C for 5 min and then on ice for a further 5 min. The complimentary DNA (cDNA) sample was made up to 100 μl by the addition of MQ water and stored at -20°C.

2.2.4.4 Oligonucleotide primer design

Primers for real time PCR were designed with PrimerExpress 2.0 software (Applied Biosystems, Warrington, UK) using Genbank cDNA sequences. The sequence, Genbank accession number, nucleotide position and PCR product length of the primers are given in Table 2.2.
TABLE 2.2 PCR primers used to quantify cytokine and chemokine mRNA expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nt position</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>Genbank</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>91 (fwd) 167 (rev)</td>
<td>5' AGCCCTGGGAGCATGTGACAGCTCAGGATTC</td>
<td>77 bp</td>
<td>NM_000758</td>
</tr>
<tr>
<td>IL-1α</td>
<td>263 (fwd) 453 (rev)</td>
<td>5' CCAAGGAGAAGTCTGAGCAACAGGATTC</td>
<td>191 bp</td>
<td>NM_000575</td>
</tr>
<tr>
<td>IL-6</td>
<td>188 (fwd) 470 (rev)</td>
<td>5' ACTACCTCTGACGAGGATTC</td>
<td>283 bp</td>
<td>M54894</td>
</tr>
<tr>
<td>IL-8</td>
<td>131 (fwd) 285 (rev)</td>
<td>5' GCCAGCTTCTGAGTCTGGAAGTTC</td>
<td>155 bp</td>
<td>YOO787</td>
</tr>
<tr>
<td>TNFα</td>
<td>2531 (fwd) 2626 (rev)</td>
<td>5' GCCGACTATCTCGACGAGGATTC</td>
<td>96 bp</td>
<td>X02910</td>
</tr>
<tr>
<td>IFNγ</td>
<td>459 (fwd) 530 (rev)</td>
<td>5' GAAACGAGATGACTCTGAAAGCT</td>
<td>72 bp</td>
<td>M29383</td>
</tr>
<tr>
<td>CCR5</td>
<td>31 (fwd) 144 (rev)</td>
<td>5' AAAGCTTCCGAGGATTC</td>
<td>114 bp</td>
<td>U54994</td>
</tr>
<tr>
<td>CXCR4</td>
<td>245 (fwd) 406 (rev)</td>
<td>5' TGCCGACGTCTGCTGGATTC</td>
<td>162 bp</td>
<td>AF025375</td>
</tr>
<tr>
<td>β-actin</td>
<td>1350 (fwd) 1511 (rev)</td>
<td>5' TGATGTCGATGGATTC</td>
<td>162 bp</td>
<td>M10277</td>
</tr>
</tbody>
</table>

2.2.4.5 Polymerase chain reaction (PCR)

Real-time quantitative PCR was performed in a ABI Prism 5700 Sequence Detection System (Applied Biosystems, Warrington, UK) according to the manufacturers instructions to allow amplicon quantification. The PCR amplification employed reagents supplied in a SYBR green PCR master mix (Applied Biosystems, Warrington, UK), and each reaction volume (20 μl) consisted of 0.5 - 2.0 μM 5' (forward) and 3' (reverse) primer and 3 μl of cDNA. The negative control included in each reaction consisted of H2O substituted for cDNA.

The optimal concentration of primers was determined in a preliminary experiment using serial dilutions of primer pairs and control cervical and endometrial tissues. The most appropriate concentration of primers was determined by selecting the concentration at which the difference between the threshold
cycle (Ct) of the sample (signal) and the Ct of the non template control (noise) was greatest. The GM-CSF, IL-8, TNFα, IFNγ, CCR5 and CXCR4 primers were diluted to give a final concentration of 0.5 μM, whilst the IL-1α and IL-6 primers were diluted to give a final concentration of 1 μM and 2 μM respectively. The optimal PCR reaction conditions for all of the primers except for β-actin and IL-6 was 50°C for 2 min, 95°C for 5 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min. For β-actin the following conditions were used; 50°C for 2 min, 95°C for 5 min, 40 cycles of 95°C for 20 sec, 58°C for 20 sec, 72°C for 1 min and for IL-6; 50°C for 2 min, 95°C for 5 min, 40 cycles of 95°C for 20 sec, 60°C for 20 sec, 72°C for 1 min.

The absence of aberrant PCR products and primer dimers in the PCR product was demonstrated by a single dissociation peak and further confirmed by visualisation of the PCR product on an agarose gel. Data were normalised for β-actin mRNA expression and expressed as a percent of the mean of the control ('initial biopsy') sample for each individual using the arithmetic equation 2^ΔΔCt x 100 / K (Applied Biosystems User Bulletin No.2), where K is the normalising constant. The values were multiplied by this constant to give an arbitrary mRNA quantity so that the average of the control in each group was 100 arbitrary units. The PCR product was then purified and sequenced to verify the identity of the PCR product.

To determine the concentration range of cDNA at which Ct increase is linear, serial 1:8 dilutions of cervical and endometrial cDNA were amplified using each of the primers. It was determined that 3 μl of neat cervical and endometrial cDNA was required for quantitative analysis of all cytokines and chemokines.

The efficiency of each PCR reaction was determined by regression analysis of the fluorescence of the sample at each cycle number. All reactions occurred at a minimum of 80% efficiency (Individual primer efficiencies and linear regression equations are given in Table 2.3).
### TABLE 2.3 Primer amplification efficiencies and linear regression equations for individual oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer efficiency (%)</th>
<th>Equation describing linear regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>96</td>
<td>$Y = -3.39x + 22.31$, $r = 0.98$</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>96</td>
<td>$Y = -3.39x + 32.65$, $r = 0.92$</td>
</tr>
<tr>
<td>IL-1α</td>
<td>92</td>
<td>$Y = -2.91x + 30.94$, $r = 0.97$</td>
</tr>
<tr>
<td>IL-6</td>
<td>96</td>
<td>$Y = -4.3x + 29.39$, $r = 0.80$</td>
</tr>
<tr>
<td>IL-8</td>
<td>93</td>
<td>$Y = -2.85x + 28.50$, $r = 0.99$</td>
</tr>
<tr>
<td>IFNγ</td>
<td>89</td>
<td>$Y = -2.79x + 30.59$, $r = 0.97$</td>
</tr>
<tr>
<td>TNFα</td>
<td>88</td>
<td>$Y = -3.13x + 23.47$, $r = 0.90$</td>
</tr>
<tr>
<td>CCR5</td>
<td>89</td>
<td>$Y = -3.43x + 19.95$, $r = 0.99$</td>
</tr>
<tr>
<td>CXCR4</td>
<td>87</td>
<td>$Y = -4.24x + 22.96$, $r = 0.98$</td>
</tr>
</tbody>
</table>

#### 2.2.4.6 Gel electrophoresis of PCR products

For confirmation of product size the PCR products were separated by gel electrophoresis. PCR products (20 μl PCR product, 4 μl 6X loading buffer) were run on a 2% agarose gel (w/v) (Promega, Madison, US) containing 0.5 mg/ml ethidium bromide (Sigma, MO, USA) diluted in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2) for 40 min.

PCR products were visualised by transillumination with UV light. Gels were photographed using a Kodak DC120 digital camera (Eastman Kodak Company, Rochester, US) and the size of the PCR products were determined by comparison of their relative mobility to the DNA molecular weight marker, *Hpa II*-digested pUC19 (Bresatec, Adelaide, AU) (Figure 2.2).
2.2.4.7 Sequencing of PCR products

To confirm the identity of PCR products, the products of PCR reactions were sequenced.

All PCR products were sequenced by excising the corresponding bands from the agarose gel and recovering using a SpinBind PCR purification kit (FMC Biogenics, MA, USA) according to the manufacturers instructions. The purified PCR products were amplified with ABI Prism Dye Terminator reaction mixture (Applied Biosystems, Warrington, UK), using the forward primer (320 pmol) and 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. The sequencing reaction was then clarified by adding 20 μl of PCR reaction mixture to a tube containing 2 μl of 3 M sodium acetate and 50 μl 95% ethanol. The resultant mixture was mixed thoroughly for 10 sec and centrifuged for 20 min at 4°C at 11000 rpm (10000 g). The supernatant was carefully removed and the pellet washed with 1 ml cold 70% ethanol. The tubes were dried in a SpeedVac concentrator (RH200-13, Savant instruments).

The sequence of the PCR products were analysed at the IMVS, Adelaide, AU. Homology with all known sequences was determined by BLAST nucleotide sequence analysis ((Altschul et al. 1997) / www.ncbi.nlm.nih.gov/blast/blast.cgi).
2.3 IN VITRO CULTURE OF HUMAN CERVICAL CELLS

2.3.1 Patient participation and tissue collection

Human cervical tissue was obtained from women undergoing routine vaginal or total abdominal hysterectomy at the Lyell McEwin, Queen Elizabeth or Women's and Childrens Hospitals (Adelaide), for benign, non-cervical pathology (dysfunctional uterine bleeding, fibroids, endometriosis, pelvic pain, prolapse etc). All women gave informed consent. All women were premenopausal, however no distinction was made regarding the stage of menstrual cycle at the time of surgery.

2.3.2 Cell culture

2.3.2.1 General

All experiments carried out using primary tissue and cell lines were carried out using aseptic technique, under sterile conditions and in a laminar flow hood in order to minimise the potential for microbial contamination. Cell cultures were assessed daily to ensure that the cells were both viable and free from contamination using phase contrast microscopy (Nikon TMS, Japan).

2.3.2.2 Cell lines

The cell lines used to perform the experiments described within this thesis were obtained from the sources indicated and maintained in media as described in Table 2.4.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species</th>
<th>Lineage</th>
<th>Cellular products</th>
<th>Origin</th>
<th>Culture media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mv1Lu</td>
<td>Mink</td>
<td>Lung fibroblast</td>
<td>N/A</td>
<td>Phil Elliott, Groep, Adelaide</td>
<td>DMEM + 5% FCS</td>
</tr>
<tr>
<td>3T3</td>
<td>Mouse</td>
<td>Fibroblast</td>
<td>Laminin, fibronectin</td>
<td>A. Lopez, Hanson centre, Adelaide</td>
<td>DMEM + 10% FCS</td>
</tr>
<tr>
<td>Ect1</td>
<td>Human</td>
<td>Ectocervical epithelium (squamous)</td>
<td>Cytokeratin 8, 10, 13, 18 and 19</td>
<td>ATCC CRL-2615, Rockville, MD</td>
<td>Keratinocyte serum free media + human EGF (0.1 ng/ml) + BPE (0.05 mg/ml) + CaCl₂ (0.4 mM final)</td>
</tr>
<tr>
<td>End1</td>
<td>Human</td>
<td>Endocervical epithelium (columnar)</td>
<td>Cytokeratin 8, 18 and 19</td>
<td>ATCC CRL-2614, Rockville, MD</td>
<td>Keratinocyte serum free media + human EGF (0.1 ng/ml) + BPE (0.05 mg/ml) + CaCl₂ (0.4 mM final)</td>
</tr>
<tr>
<td>Vk2</td>
<td>Human</td>
<td>Vaginal epithelium (squamous)</td>
<td>Cytokeratin 8, 10, 13, 18 and 19</td>
<td>ATCC CRL-2616, Rockville, MD</td>
<td>Keratinocyte serum free media + human EGF (0.1 ng/ml) + BPE (0.05 mg/ml) + CaCl₂ (0.4 mM final)</td>
</tr>
</tbody>
</table>

**TABLE 2.4** The species, lineage, origin and culture media for cell lines used in this thesis. N/A = not applicable.

2.3.2.3 **Culture medium**

3T3 and Mv1Lu cells were propagated in media containing Dulbecco’s Modified Minimal Essential Medium (DMEM) (low glucose) (Gibco-BRL, New York, US) supplemented with 0.05 mM β-mecaptoethanol (BDH laboratory supplies, Poole, UK), 2 mM L-glutamine (BDH laboratory supplies, Poole, UK) and antibiotics (penicillin / streptomycin) (Sigma, Castle Hill, AU) and heat-inactivated FCS (CSL, Parkville, AU). Primary cervical epithelial cell cultures were grown in media containing DMEM, Hams – F12, Neutridoma-SP, FCS, 2 mM L-glutamine and 0.5 µg / ml hydrocortisone. The Ect1, End1 and Vk2 cells were cultured using keratinocyte serum-free media supplemented with 0.1 ng /ml epidermal growth factor (EGF), 0.05 mg/ml bovine pituitary extract (BPE) (Gibco-BRL, New York, US; Cat # 17005-042) and additional calcium chloride (CaCl₂; 0.4 mM).

2.3.2.4 **Cell quantification**

A Neubauer haemocytometer (Assistant, DE) and an Olympus BH-2 phase contrast microscope (Olympus, Melville, US) was used to determine cell concentrations for use in cell line and primary cell cultures.
2.3.2.5  
Recombinant cytokines and cytokine neutralising antibodies

Recombinant Chinese Hampster Ovary (CHO) cell – derived human TGFβ1 was purchased from R & D Systems (Minneapolis, US; Cat # 240 – B). Recombinant mouse myeloma cell line (NS0) expressed human TGFβ2 was purchased from R & D Systems (Minneapolis, US; Cat # 302 – B2). Recombinant Sf 21 insect cell expressed human TGFβ3 was purchased from R & D Systems (Minneapolis, US; Cat # 243 – B3). Recombinant E. coli expressed human IFNγ was also purchased from R & D Systems (Minneapolis, US; Cat # 285 – IF). Recombinant human 19 – hydroxy prostaglandin E1 (19-OH PGE1) was purchased from Cayman chemicals (Michigan, US; Cat # 13910). All cytokines were reconstituted in PBS / 0.1% BSA / 4 mM HCl, and stored in small 50 µl aliquots at – 70°C to prevent repeated freeze thaw cycles. 19-OH PGE1 was provided in a solution of methyl acetate with the stock solution (50 µg / ml) being aliquoted and stored at – 20°C until use. Each aliquot was only used once and the 19-OH PGE1 was used immediately after dilution in PBS.

A mouse monoclonal antibody to human TGFβ1 was used to neutralise human TGFβ1 bioactivity and was purchased from R & D Systems (Minneapolis, US; Cat # MAB240). Goat polyclonal antibodies to human TGFβ2 and TGFβ3 were also purchased from R & D Systems (Cat # AF-302-NA and AB-244-NA respectively). A pan-specific mouse anti-human TGFβ monoclonal antibody was used to neutralise all mammalian isoforms of TGFβ (R & D Systems, Minneapolis, US; Cat # MAB1835).

2.3.2.6  
Primary ectocervical epithelial cell cultures

Human ectocervical epithelial cells were cultured using a modification of the technique described by Rheinwald and Green (1975). Cervical tissue biopsies were washed twice in ice cold Hank’s Balanced Salt Solution (HBSS) (in order to remove excess red blood cells) then placed into 5 ml of DMEM culture media containing 5 U dispase (Boehringer Mannheim, Mannheim, DE) and incubated overnight at 4°C to dissociate the epithelial surface from the stromal tissue. The next morning, the cervical biopsy / dispase solution was incubated at room temperature for 1 hour (hr) after which, large sheets of epithelial cells were mechanically removed from the biopsy using sterile forceps and a scalpel. The sheets were then incubated in a total volume of 10 ml DMEM / 1 ml trypsin (2.5%) / 2.5 ml collagenase (0.05%) at 37°C for 30 min to disaggregate the sheets of epithelial cells into small clumps of cells. The disaggregation was aided by aspirating the epithelial cells into a 10 ml syringe, then extruding the cells through hypodermic needles of incrementally decreasing gauge until no large sheets of cells remained. The epithelial cells were then treated with 0.01% EDTA in 10ml DMEM before being removed from the enzyme mixture by
centrifugation at 1,400 rpm (350 g). Once the supernatant had been removed, the epithelial cells were resuspended in 'ectocervical culture media – high (7%) fetal calf serum' consisting of 68% DMEM / 22% Hams-F12 / 7% FCS / 1% Neutridoma-SP (Boehringer Mannheim, Mannheim, DE) and 0.5 ml of 2 mM glutamine and 0.5 ml of 0.5 μg/ml hydrocortisone (Upjohn, Rydalmere, AU).

Human cervical epithelial cells were suspended in ectocervical culture media at a density of approximately 2 x 10^5 cells/ml, and 500 μl of this suspension was added to 1.5 ml culture wells (Nunc, Weisbaden, DE) seeded 24 hrs prior with murine 3T3 fibroblast cells. The fibroblast cells act as a feeder layer by secreting extracellular matrix components such as laminin and fibronectin required for epithelial cell attachment. The 3T3 fibroblast cells were rendered mitogenically inactive by exposure to 4 μg/ml mitomycin C (Sigma, Castle Hill, AU) for 2 hrs prior to their addition to the culture wells. Immediately following mitomycin C treatment, the cells were washed three times in sterile PBS, then detached from the culture flask by incubating the cells in 0.25% trypsin in 10 ml PBS for 10 min at 37°C. Cells were then centrifuged for 5 min at 1,400 rpm (350 g), washed again and finally resuspended in DMEM at a final concentration of 8 x 10^4 cells/ml. 500 μl of this suspension was then added to each well to give a final concentration of 4 x 10^4 cells/well. The completed cervical epithelial cell cultures were then incubated for 5 – 7 days to enable the majority of epithelial cells to attach to the culture well floor and displace the 3T3 fibroblasts. Once good attachment had been observed, the culture supernatant containing desquamated epithelial cells and 3T3 cells was removed carefully and exchanged for 500 μl of fresh 'ectocervical culture medium – low (2%) fetal calf serum'. Twelve hours later, this 'pre-treatment' supernatant was collected and replaced with 500 μl of fresh media containing various concentrations of recombinant human TGFβ1, TGFβ2, TGFβ3, IFNγ, combinations of these molecules or culture media alone (control), which in turn was collected 12 hrs later as the '0 – 12 hr post-treatment' supernatant. Again, 500 μl of fresh media was added to the wells and collected an additional 12 hrs later as the '12 – 24 hr post-treatment' supernatant. The supernatants were then centrifuged to remove cellular debris and stored at –70°C until assayed using commercially available GM-CSF, IL-6 and IL-8 Enzyme-Linked Immunosorbancy Assay (ELISA) kits (R & D Systems, Minneapolis, US). Refer to section 2.3.3 for description of protocols used for these assays.
2.3.2.7 Immortalised epithelial cell cultures

Human Ect1, End1, and Vk2 cells were cultured according to protocols described by Fichorova et al. (Fichorova et al. 1997). All cell lines were propagated in culture flasks (Nunc, Weisbaden, DE) containing keratinocyte serum–free media (KSFM) supplemented with 0.1 ng/ml recombinant human EGF, 0.05 mg/ml BPE and additional CaCl$_2$ (0.4 mM) until reaching approximately 70 – 80% confluence. The cells were then washed in PBS and incubated with a solution of PBS containing 0.25% trypsin, 0.53 mM EDTA at 37°C / 5% CO$_2$ for 10 mins or until all cells had detached from the bottom of the culture flask. The trypsin was then neutralised by adding a 1:1 mixture of DMEM and Ham's-F12 medium containing 10% FCS. Cells were then centrifuged for 10 min at 1 000 rpm (180 g) and resuspended in fresh keratinocyte serum–free media to a concentration of $2 \times 10^5$ cells/ml. 500 µl of this cell suspension was then added to 1.5 ml culture wells (Nunc, Weisbaden, DE) and incubated at 37°C / 5% CO$_2$ for 2-3 days to generate a confluent monolayer, when the culture supernatant was carefully removed and exchanged for 500 µl of fresh KSFM. Twelve hours later, this 'pre-treatment' supernatant was collected and replaced with 500 µl of fresh media containing various concentrations of recombinant human TGFβ1, TGFβ2, TGFβ3, IFNγ, combinations of these molecules or culture media alone (control), which in turn was collected 12 hrs later as the '0 – 12 hr post-treatment' supernatant. Again, 500 µl of fresh media was added to the wells and collected an additional 12 hrs later as the '12 – 24 hr post-treatment' supernatant. The supernatants were then centrifuged to remove cellular debris and stored at −70°C until assay by commercial ELISA for GM-CSF, IL-6, IL-8 and IL-10 content (see section 2.3.3).

2.3.3 Cervical epithelial cell culture supernatant ELISAs

2.3.3.1 GM-CSF

A GM-CSF specific sandwich ELISA (R&D Systems, Minneapolis, US) was used to measure human GM-CSF in cervical epithelial cell culture supernatants, according to the manufacturer's instructions. Briefly, a mouse anti-human GM-CSF antibody was coated onto a 96 well microtitre plate to capture GM-CSF from either recombinant standard or assay samples. A biotinylated mouse anti-human detection antibody was then used and bound antibody was then quantified by the addition of streptavidin conjugated HRP, followed by the addition of a chromagen substrate. After incubation for 20 min at room temperature, the substrate product was acidified by the addition of 50 µl of 1M HCl, and absorbance at 450 nm (reference wavelength 570 nm) was measured using a Benchmark™ microplate reader (Bio-Rad Laboratories, Hercules, US). The concentration of GM-CSF within biological fluids was then calculated.
from a standard curve (4-parameter logistic curve) using known concentrations of recombinant GM-CSF. This assay was reported by the manufacturer to have a minimum detection limit of 15 pg/ml, with intra- and inter-assay precision of approximately 5%.

2.3.3.2 IL-6

The IL-6 content of cervical epithelial cell culture supernatants was determined using an IL-6 specific sandwich ELISA (R&D Systems, Minneapolis, US) according to the manufacturer’s instructions. This assay is similar to that described in 2.3.3.1, with the exception that a mouse anti-human IL-6 capture antibody was used, followed by a biotinylated goat anti-human IL-6 detection antibody. This assay was reported by the manufacturer to have a minimum detection limit of 15 pg/ml, with intra- and inter-assay precision of approximately 5%.

2.3.3.3 IL-8

The IL-8 content of cervical epithelial cell culture supernatants was determined using an IL-8 specific sandwich ELISA (R&D Systems, Minneapolis, US) according to the manufacturer’s instructions. This assay is similar to that described in 2.3.3.1, with the exception that a mouse anti-human IL-8 capture antibody was used, followed by a biotinylated goat anti-human IL-8 detection antibody. This assay was reported by the manufacturer to have a minimum detection limit of 15 pg/ml, with intra- and inter-assay precision of approximately 5%.

2.3.3.4 IL-10

The IL-10 content of cervical epithelial cell culture supernatants was determined using an IL-10 specific sandwich ELISA (R&D Systems, Minneapolis, US) according to the manufacturer’s instructions. This assay is similar to that described in 2.3.3.1, with the exception that a mouse anti-human IL-10 capture antibody was used, followed by a biotinylated goat anti-human IL-10 detection antibody. This assay was reported by the manufacturer to have a minimum detection limit of 15 pg/ml, with intra- and inter-assay precision of approximately 5%.
2.4 SEMINAL PLASMA CYTOKINE ASSAY

2.4.1 Subject population

Seminal plasma samples were collected from male partners of couples undergoing investigation for various fertility problems at the Reproductive Medicine Unit of The University of Adelaide (Repromed). All couples approached to be involved in this study were undergoing investigation for fertility problems at the University of Adelaide's reproductive medicine unit (Repromed Pty Ltd.). All potential participants were given an information sheet outlining the purpose of the study and given sufficient time (approximately 48 hours) to consider participation in the study. Written informed consent was then obtained from interested couples before their enrolment in the study. Patients were also made aware that they could decline to be involved in the study without affecting their current treatment.

In order to be successfully recruited into the study, male partners who would be providing semen samples had to satisfy a number of selection criteria including:

1. good general health
2. aged 18 - 50 years of age
3. not taking any medications with the potential to modify semen parameters
   (ie. non-steroidal anti-inflammatory drugs such as aspirin, immune modifying agents such as steroids etc)

A number of exclusion criteria also existed and couples were unable to participate in this study if any of the exclusion criteria were present before or developed during the course of the study. Participants were also informed that they could withdraw from the study at any time at the request of either partner without their current treatment being affected. Reasons for exclusion from this study included:

1. Either partner unable to give informed consent.
2. Male partner had symptoms suggestive of a urinary or genital tract infection (dysuria, penile discharge).
3. Male partner was taking medication that may affect semen parameters (immune modifying medication such as methotrexate, NSAIDs).
4. Female patients experiencing recurrent miscarriage in which a potential cause has been found (uterine abnormality, chromosomal abnormality, thrombophilia, hyperhomocysteinaemia, diabetic).
After having met the selection criteria described above and after obtaining informed consent, participants were allocated into one of seven fertility classifications depending on their reproductive history and according to the following descriptions:

1. Proven fertile - This group contained men who had either fathered a child naturally in their current relationship and / or had fathered a child through Repromed's donor insemination program. Only males who had fathered children from normal pregnancies, defined as the delivery (normal vaginal delivery, instrumental or caesarean section) of an infant at term (37 completed weeks) in which there was no evidence of preeclampsia or intrauterine growth restriction during the antenatal course of the pregnancy were included.

2. Male infertility - This group consisted of men who had any defect in their semen parameters that resulted in values below the reference ranges according to WHO standards and where there was no evidence of a female problem as the cause of their infertility.

3. Female infertility - This group contained men who had normal semen analyses but whose female partners had been identified as having a condition such as anovulation, tubal blockage or endometriosis as their potential cause of their infertility.

4. Combined male / female infertility - This group was comprised of male partners of couples where both male and female factors had been identified as their likely cause of infertility.

5. Multiple IVF Failure - This group contained male partners of couples who failed to have a successful pregnancy despite the transfer of 10 good quality embryos during in-vitro fertilization treatment, even if they had a male or female factor as well.

6. Recurrent miscarriage - This group contained male partners of couples who had experienced the successive loss of three or more fetuses in the first trimester of pregnancy, in the absence of an identifiable cause (normal maternal and paternal karyotype; normal uterine cavity according to hysteroscopy / ultrasound or HSG; negative lupus anticoagulant, anticardiolipin antibody and ANA; normal thyroid and glucose function tests; no evidence of hyperhomocysteinaemia; normal protein C, protein S and Activated Protein C activity). Couples experiencing recurrent miscarriages where a cause had been identified (ie. genetic, thrombophilia, severe maternal disease such as diabetes etc) were not included.
7. Infertility of unknown origin - This group was made up of male partners of couples who had been unable to conceive spontaneously, and where tests on both the male (semen defect ruled out) and female (anovulation and tubal blockage excluded) partners failed to identify a potential cause for their infertility.

2.4.2 Seminal plasma collection and processing

2.4.2.1 Seminal plasma collection.

Semen samples were produced by masturbation, without the use of lubricants or condoms, following a period of at least 48 hours abstinence. The samples were produced at Repromed or were delivered to the Andrology Laboratory at Repromed within 30 min of production. The volume of semen was measured and divided equally into two portions. Semen analysis was performed according to World Health Organisation (WHO) standards (WorldHealthOrganisation 1999) on one portion with parameters such as sperm concentration, motility, morphology, pH etc being recorded. The other portion was centrifuged for 15 min at 14 000 rpm (13 000g) to remove sperm and other cellular debris. The supernatant was then removed, transferred to a fresh tube and stored at -70°C. Once the seminal plasma samples were received at the medical school they were immediately thawed on ice, aliquoted into 100μl volumes (to prevent repeated freeze thaw cycles) and then refrozen and stored at -70°C until assayed by ELISA for cytokine content.

2.4.2.2 Acid activation of latent TGFβ

In order to measure the total amount of TGFβ1, TGFβ2 and TGFβ3 present within human seminal plasma, the samples first needed to be transiently acidified in order to release the biologically active protein from its latent form (Nocera et al. 1995). Acid activation was achieved by adding 100 μl of seminal plasma to 300 μl of Dulbecco's Phosphate-Buffered Saline (DPBS), followed by addition of 50 μl of 1M HCl, flick spinning to ensure thorough mixing and incubation for 20 min at room temperature. Immediately following addition of acid, the pH of the resultant solution was measured to ensure it had a pH of approximately 3.0. Following incubation, the samples were neutralised by the addition of 50 μl of 1M NaOH to result in a final dilution of 1:5 (100 μl of seminal plasma in a total volume of 500 μl).
To determine the optimal amount of acid to add to the samples in order to obtain maximal activation in preliminary experiments a varying amount of acid was added to pooled seminal plasma samples and total TGFβ₁ content was measured using a TGFβ₁ specific ELISA (R & D Systems, Minneapolis, US). The highest concentration of TGFβ₁ was observed when 50 μl of acid was added to 100 μl of seminal plasma samples (Figure 2.3).

![Figure 2.3](image)

**FIGURE 2.3** Acid activation of pooled human seminal plasma samples. Seminal plasma was activated using varying amounts of 1M HCl to determine the optimal volume of acid required for maximal activation. The concentration of total TGFβ₁ within the sample was then measured using a TGFβ₁ specific ELISA. The optimal volume of acid for addition to 100 μl of seminal plasma was determined to be 50 μl (indicated by an asterix).

### 2.4.3 Seminal plasma cytokine ELISAs and endotoxin assay

#### 2.4.3.1 IL-8

The amount of IL-8 present within human seminal plasma samples was measured using the protocol described previously in 2.3.3.3.
2.4.3.2 IFNγ

The IFNγ content of human seminal plasma was determined using a high-sensitivity IFNγ-specific ELISA (Amersham Biosciences, Uppsala, SE). All assays were performed according to the manufacturer's instructions. This assay is based on a solid phase ELISA, similar to that described in 2.3.3, with the exception that a mouse anti-human IFNγ capture antibody was used, followed by a biotinylated goat anti-human IFNγ detection antibody and subsequent signal amplification using the supplied Amdex™ reagent. This assay was stated by the manufacturer as having a minimal detectable limit of 0.1 pg/ml, with intra-assay precision of < 10% and an inter-assay precision of < 10%.

2.4.3.3 TGFβ1

A TGFβ1-specific ELISA (Promega, Madison, US) was used to measure biologically active and total TGFβ1 in human seminal plasma samples according to the manufacturers' instructions. This assay is similar to that described in 2.3.3.1, with the exception that the 96-well plate was coated with soluble type II TGFβ receptors able to bind all isoforms of TGFβ, with TGFβ1 specificity conferred by the detection reagent, a polyclonal TGFβ1 specific antibody (< 2% cross-reactivity with other TGFβ isoforms). The amount of specifically bound polyclonal antibody was then detected using a species-specific TGFβ HRP conjugate as a tertiary reactant. This assay was stated by the manufacturer as having a minimal detectable limit of 32 pg/ml, with intra-assay precision of < 5% and an inter-assay precision of < 12%.

2.4.3.4 TGFβ2

A TGFβ2-specific ELISA (Promega, Madison, US) was used to measure biologically active and total TGFβ2 in human seminal plasma samples according to the manufacturer's instructions. This assay is similar to that described in 2.4.3.3, in that a 96-well plate was coated with soluble type II TGFβ receptors that were able to bind all isoforms of TGFβ, with use of a polyclonal TGFβ2 specific antibody (< 0.7% cross-reactivity with other TGFβ isoforms), to confer TGFβ2 specificity. The amount of specifically bound TGFβ2 polyclonal antibody was then detected using a species-specific TGFβ HRP conjugate as a tertiary reactant. This assay was stated by the manufacturer as having a minimal detectable limit of 32 pg/ml, with intra-assay precision of < 5% and an inter-assay precision of < 12%.
2.4.3.5 TGFβ3

A TGFβ3-specific ELISA (R&D Systems, Minneapolis, US) was used to measure biologically active and total TGFβ3 in human seminal plasma samples according to the manufacturer's instructions. This assay is similar to that described in 2.3.3.1, with the exception that a mouse anti-human TGFβ3 specific coating antibody was used, followed a biotinylated goat anti-human TGFβ3 specific polyclonal antibody (exhibiting no cross-reactivity with other TGFβ isoforms). Again, bound antibody was then quantified by the addition of streptavidin conjugated HRP. This assay was stated by the manufacturer as having a minimal detectable limit of 15.6 pg/ml, with intra-assay precision of < 5% and an inter-assay precision of < 10%.

2.4.3.6 Bacterial endotoxin (LPS)

The gram-negative bacterial endotoxin content of human seminal plasma samples was determined using a chromogenic limulus amebocyte lysate (LAL) assay (Cambrex bioproducts, East Rutherford, US). This assay was performed according to the manufacturer's instructions which required that all reagents including the microtitre plate be maintained at 37°C for the duration of the assay and that pyrogen-free plastic and glassware was used throughout. Briefly, either LAL-free water (control), standard or seminal plasma was added to a 96-well plate. 50 μl of limulus amebocyte lysate solution was then added to each of the wells (T = 0 min) and then incubated for 10 min (T = 10 min) at 37°C. The amount of endotoxin was quantified by addition of a chromogenic substrate for 6 min (T = 16 min) at 37°C, followed by acidification of the substrate product by the addition of 100 μl of 25% acetic acid (v/v), and measurement of absorbance at 405 nm. The concentration of endotoxin within seminal plasma was then calculated from a standard curve (4 - parameter logistic curve) using known concentrations of E. coli derived endotoxin. This assay was stated by the manufacturer as having a minimal detectable limit of 0.1 EU / ml, with intra-assay precision of <4% and an inter-assay precision of <10%.
Chapter 3

The effect of intercourse on leukocyte recruitment and activation within the human cervix
3.1 INTRODUCTION

A post-mating leukocytosis is characterised by an influx of leukocytes into the female reproductive tract following local exposure to semen. This response has been observed in many species including mice (De et al. 1991, Robertson et al. 1996), rabbits (Phillips et al. 1977), pigs (Lovell et al. 1968, O’Leary et al. 2004) and humans (Pandya et al. 1985, Thompson et al. 1992, Prakash et al. 2003). In intrauterine ejaculators such as mice and pigs, the majority of the ejaculate is deposited within the uterine cavity, and the major site of the response is within the uterine endometrium. In intravaginal ejaculators such as rabbits and humans, the majority of the ejaculate is deposited within the vagina in close proximity to the cervix and cellular changes indicative of a cervical leukocytosis are observed.

The effects of insemination on local cellular parameters are best characterised in the mouse, where a process resembling a classical inflammatory response appears to be the underlying mechanism. In the mouse, the post-mating inflammatory response has been shown to involve an infiltration of neutrophils, macrophages and eosinophils into the endometrial stroma and lumen within hours of mating (De et al. 1991, Kachkache et al. 1991, McMaster et al. 1992). The influx of neutrophils is relatively short-lived and is usually resolved within 24 hours, while increased macrophage numbers persist until day 2-3 of pregnancy, at which time their numbers begin to decline due to increasing progesterone concentrations (Kachkache et al. 1991). The activation status of these cells also fluctuates, with increased expression of molecules involved in antigen presentation such as MHC class II and B7-2 occurring after exposure to semen (Keenihan et al. 2004). This infiltration of leukocytes is still observed following mating with vasectomized male mice (Choudhuri et al. 1992), yet is absent following matings with seminal vesicle-deficient males (Robertson et al. 1996). This observation led to the discovery that seminal vesicle-derived components, most notably TGFβ, are responsible for triggering the murine post-mating inflammatory response (Tremellen et al. 1998).

The local response to insemination is not as well characterised in humans as it is in mice. There have been three previous studies in humans, with all investigating the cellular changes in the lumen, or at best the superficial epithelial cell layers of the cervix. Sampling of the cervical cells achieved using an Ayre’s spatula, as is used when performing a Papanicolaou smear test, or an endocervical brush, has revealed that within four hours following exposure to semen through artificial insemination, there is a large influx of neutrophils into the cervix, with increased numbers of macrophages and lymphocytes also detected (Pandya et al. 1985, Thompson et al. 1992, Prakash et al. 2003). These studies are deficient however, in that only the outermost layers of the cervical mucosa were investigated.
addition, these studies gave no indication of the cellular changes occurring deeper within the tissue, in the deeper layers of the epithelium and stroma, where on the basis of studies in the mouse it might be expected that the major cellular changes are occurring. Therefore there is a need for an accurate identification of the leukocyte populations involved in the leukocytic response in humans.

The experiments described in this chapter were therefore undertaken to investigate whether exposure to semen following intercourse stimulates changes in leukocyte recruitment and activation within the cervical tissue of women, comparable to those observed in the reproductive tracts of other mammalian species, such as mice. To investigate this, cervical tissue biopsies were collected from peri-ovulatory women before and after abstinence, condom-protected intercourse or unprotected intercourse. Cervical tissue samples were processed as described in section 2.2.3, then immunohistochemical analysis was performed on fresh-frozen cervical tissue sections using an extensive panel of 13 monoclonal antibodies specific for a number of cell lineages (refer to Table 2.1 for more details).
3.2 THE EFFECT OF INTERCOURSE ON LEUKOCYTE RECRUITMENT AND ACTIVATION IN THE EPITHELIUM AND STROMA OF THE HUMAN CERVIX

To investigate whether exposure to semen following intercourse can alter leukocyte recruitment and activation status within the human cervix, cervical tissue biopsies were collected both before and after abstinence, condom-protected intercourse and unprotected intercourse.

Briefly, all couples totally abstained from penetrative intercourse for two days prior to the onset of the study, whilst silicon condoms were used for five days prior to this period of time. This protocol was employed to ensure that no sperm would be present in the cervical region at the time of the first biopsy. In order to synchronise the timing of the first biopsy, all subjects determined their LH peak in urine samples taken twice daily from approximately day 10 after the onset of menstruation to the time at which an LH increase was detected (LH0-LH+1) by using a rapid self-test (Clearplan, Searle Unipath Ltd., Bedford, UK). Subjects presented for the first biopsy during the peri-ovulatory stage of the menstrual cycle (LH0 to LH+1) and duplicate needle biopsies were taken from the left hand side of the cervix, approximately 1 cm from the transformation zone (the 'initial' biopsy). For this aspect of the study, the first biopsy was immediately embedded in O.C.T. Tissue Tek (Miles Scientific, Elkhart, IN), frozen by immersion in isopropanol cooled by liquid nitrogen and then stored at -70°C until use. Following the first biopsy, couples were asked to abstain from intercourse for 36 hours to enable haemostasis and healing of the biopsy site. Intercourse couples were asked to have intercourse (either unprotected or condom-protected) on one occasion, approximately 36 hours after the first biopsy and return for a second ('repeat') biopsy approximately 12 hours after intercourse (on day LH+2 to LH+3). Abstinent couples also presented 48 hours after the first biopsy for a repeat biopsy (refer to Chapter 2 for more details).

Cervical tissue samples were processed as described in section 2.2.3, then immunohistochemistry was performed on fresh-frozen tissue sections using a panel of 13 monoclonal antibodies specific for a variety of cell lineages (refer to Table 2.1 for more details). The amount of positive staining in cervical tissue sections was then quantified using video image analysis. Staining was analysed in both the cervical epithelium and cervical stroma. The cervical stroma was defined as being the region of tissue located directly beneath the basement membrane, and was analysed to a maximum depth of 2 fields (700 μm). The mean area of positive staining (% positivity) in 10 low power fields (x10 objective), was calculated as:

Sharkey Chapter 3
Data were analysed by paired T-tests to establish the significance of the effect of sampling time on differences in cell numbers within individuals, and by non-parametric Mann-Whitney U test to establish the effect of treatment group on cell numbers at each of the two sampling times.

Representative data showing the effects of abstinence, condom-protected intercourse and unprotected intercourse on leukocyte populations within individuals are shown in Figures 3.1 (epithelium) and 3.2 (stroma), while summaries of the total data are given in Tables 3.1 (epithelium) and 3.2 (stroma). Figure 3.3 shows representative photomicrographs of fresh frozen cervical tissue sections incubated with mAbs specific for different leukocyte populations both before and after unprotected intercourse.

CD45+ leukocytes were detected within the cervical epithelium and stroma of all samples tested, with relatively small levels of variation observed between individuals. For example, the proportion of CD45+ cells in the cervical epithelium and stroma ranged from 70% - 117% and 81% - 119% (in the epithelium and stroma respectively) of the mean value in the combined cohort of ‘before’ samples (n=18) (data not shown). In addition, there was no significant difference in ‘initial’ CD45+ leukocyte numbers between the abstinent, condom-protected intercourse and unprotected intercourse groups in their ‘before’ biopsies in both the epithelium and stromal tissues. Exposure to semen following intercourse was found to result in statistically significant increases in total leukocyte numbers, with mean increases of 42±13% and 79±14% observed in the epithelium and stroma respectively (Figure 3.1 A and 3.2 A respectively)(Refer to Figure 3.3 for representative photomicrograph). Increased CD45+ leukocyte numbers were detected in each of the individuals within this group following insemination. In contrast, condom-protected intercourse was found to elicit a much smaller, 13±11% increase in CD45+ cells within the epithelium (not significant) (Figure 3.1A), while a small yet statistically significant increase of 13±7% was observed in the stroma (Figure 3.1A). Repeat biopsies in the abstinent group also displayed slight increases of 4±13% and 14±17% in the epithelium and stroma respectively (not significant) (Figure 3.1 A).
FIGURE 3.1 The effect of exposure to semen following intercourse on leukocyte numbers in the epithelium of the cervix. Mean proportion of cells detected within the epithelium before and after either abstinence (n=7), condom-protected (n=5) or unprotected intercourse (n=6) for CD45 (A), CD11 (B), MHC class II (C) and CD45RO (D). Mean increases are shown in parentheses and are presented as Mean ± SD.
FIGURE 3.2 The effect of exposure to semen following intercourse on leukocyte numbers in the stroma of the cervix. Mean proportion of cells detected within the epithelium before and after either abstinence (n=7), condom-protected (n=5) or unprotected intercourse (n=6) for CD45 (A), CD11 (B), MHC class II (C) and CD45RO (D). Mean increases are shown in parentheses and are presented as Mean ± SD.
Macrophages and dendritic cells were identified as being the most abundant leukocytes present within the cervical tissue. The cell surface marker CD14, present on monocytes, macrophages and dendritic cells showed a very large and statistically significant increase in the proportion of labelled cells in both the epithelium and stroma following unprotected intercourse. The mean increases detected within the epithelium and stroma was 104±21% and 116±18% respectively (refer to Tables 3.1 and 3.2 respectively). An increase in CD14+ leukocytes was detected in each of the individuals within this group following insemination. Condom-protected intercourse was again found to elicit much smaller, yet statistically significant increases of 24±7% and 16±13% in the epithelium and stroma respectively. Repeat biopsies in the abstant group also displayed slight increase of 12±15% in the epithelium (not significant) and a statistically significant increase of 9±7% in the stroma. Relatively small levels of variation were again observed between individuals. For example, the proportion of CD14+ cells in the cervical epithelium and stroma ranged from 71% - 131% and 77% - 122% (in the epithelium and stroma respectively) of the mean value in the combined cohort of ‘before’ samples (n=18) (data not shown).

The observed increase in macrophages and dendritic cells was further supported by the results of CD11 staining, a second monoclonal antibody specific for macrophages and dendritic cells. Unprotected intercourse was found to result in very large and statistically significant increases in the proportion of CD11+ cells in both the epithelium and stroma, with mean increases of 130±89% and 133±62% respectively (Figure 3.1 B and 3.2 B respectively)(Refer to Figure 3.3 for representative photomicrograph).

The effect of insemination on the activation status of leukocyte populations within the cervix was also examined using a monoclonal antibody specific for MHC class II. This antibody was specific for activated macrophages, dendritic cells and some B cells. The amount of variation observed between individuals was again relatively small, with the proportion of MHC class II+ cells in the cervical epithelium and stroma ranging from 71% - 131% and 75% - 132% (in the epithelium and stroma respectively) of the mean value in the combined cohort of ‘before’ samples (n=18) (data not shown). Exposure to semen following intercourse was found to result in statistically significant increases in the proportion of cells expressing MHC class II in both the epithelium and stroma, with mean increases of 54±25% and 117±24% respectively (Figure 3.1 C and 3.2 C, epithelium and stroma respectively)(Refer to Figure 3.3 for representative photomicrograph). In contrast, small changes in the mean proportion of MHC class II+ labelled cells were observed in the epithelium and stroma following either condom-protected intercourse (mean increases of 13±30% and 17±22% respectively) or abstinence (mean increase of 15±23% and decrease of -1±14% respectively) and this was not statistically significant.
Monoclonal antibodies specific for other signs of macrophage and dendritic cell activation such as the co-stimulatory molecules B7-1 (anti-CD80) and B7-2 (anti-CD86) were also examined. Both CD80 and CD86 were only detectable on a very small proportion of cells. Exposure to semen following unprotected intercourse was found to stimulate very small but statistically significant increase in the proportion of cells expressing CD80 and CD86 in the epithelium (mean increases of 14±14% and 54±37% respectively), while no changes were observed in the stromal tissue (refer to Tables 3.1 and 3.2). Even smaller increases in the proportion of CD80 and CD86 positively labelled cells were observed in the epithelium and stroma following abstinence or condom-protected intercourse but these increases were not statistically significant when data was compared using paired T-tests.

T lymphocytes were also identified as comprising a major proportion of leukocytes present within the cervix using the pan-T lymphocyte marker, CD3. The amount of variation observed between individuals was again relatively small, with the proportion of CD3+ cells in the cervical epithelium and stroma ranging from 68% - 121% and 69% - 113% (in the epithelium and stroma respectively) of the mean value in the combined cohort of ‘before’ samples (n=18) (data not shown). Exposure to semen following intercourse was found to elicit statistically significant increases in the proportion of T cells detected, with mean increases of 51±13% and 38±9% observed in the epithelium and stroma respectively (refer to Tables 3.1 and 3.2). The proportion of CD3+ T cells detected within the cervical tissue was also increased following condom-protected intercourse, with mean increases of 16±6% and 14±4% detected within the epithelium and stroma respectively. While the magnitude of this increase was far less than that observed following unprotected intercourse, it was found to be statistically significant when data was compared by paired T-test. In contrast, no changes in the proportion of CD3+ T cells were observed in the epithelium or stroma following abstinence.

Cell markers specific for different T lymphocyte subsets were also used. The proportion of positively labelled CD8+ cytotoxic / suppressor T lymphocytes significantly increased following intercourse, with mean increases of 51±11% and 85±20% detected in the epithelium and stroma respectively (refer to Tables 3.1 and 3.2). Smaller, but statistically significant increases in CD8+ T cells were also observed in the stromal tissue of women following both condom-protected intercourse and abstinence (mean increases of 16±11% and 17±20% respectively). No change in the proportion of CD8+ T cells in the epithelium was observed in the 'repeat' biopsies of women following condom-protected intercourse or abstinence. The amount of variation observed between individuals in the proportion of CD8+ T cells detected in the cervical epithelium and stroma ranged from 72% - 142% and
65% - 133% (in the epithelium and stroma respectively) of the mean value in the combined cohort of 'before' samples (n=18) (data not shown).

A CD4-specific monoclonal antibody was used to detect helper T lymphocytes. Exposure to semen following intercourse was found to result in a statistically significant decrease in the proportion of CD4+ helper T cells within the cervical stroma. A similar decrease was also observed in the epithelium, however this was not statistically significant when data was compared by paired T-test. In contrast, small increases in CD4+ T cells were observed in the epithelium and stroma following both condom-protected intercourse and abstinence (refer to Tables 3.1 and 3.2) which were not statistically significant.

Activated and memory T lymphocytes were also examined using a monoclonal antibody specific for CD45RO. A statistically significant influx of CD45RO+ T lymphocytes into the cervical epithelium and stroma was detected following unprotected intercourse, with mean increases of 131±34% and 118±30% respectively. Much smaller increases in CD45RO+ T cells were observed in epithelium and stroma of the 'repeat' biopsies of women following condom-protected intercourse and abstinence. These increases were not found to be statistically significant when data was compared by paired T-test (Figure 3.1 D and 3.2 D, epithelium and stroma respectively) (Refer to Figure 3.3 for a representative photomicrograph). Naïve or virgin T lymphocytes (CD45RA+) represented only a small proportion of the total cells present, however a statistically significant increase (29±19%) in these cells was detected in the cervical epithelium following unprotected intercourse. Smaller increases were observed in the epithelium and stroma following condom-protected intercourse and abstinence, though these increases were not statistically significant (refer to Tables 3.1 and 3.2).

Neutrophils were detected using a CD15-specific monoclonal antibody and were found to represent only a very small proportion of the total leukocytes present in the cervix. Exposure to semen following intercourse was found to stimulate increases in the proportion of CD15+ neutrophils within the cervical epithelium (50±73%) and stroma (35±44%), however these were not statistically significant. Smaller increases in CD15+ cells were also observed following both condom-protected intercourse and abstinence, in both the epithelium and stroma, and similarly to the intercourse group these increases were not statistically significant (refer to Tables 3.1 and 3.2).

Natural killer cells were identified using a monoclonal antibody specific for CD57. These cells were found to represent only a minor proportion of the total leukocytes present in the cervix. Statistically significant increases in natural killer cell numbers were observed following unprotected intercourse.
intercourse in both the epithelium and stroma, with mean increases of $93\pm63\%$ and $101\pm30\%$ respectively. There was no significant increase in the proportion of CD57+ cells observed within the epithelium of the 'repeat' biopsies of women following condom-protected intercourse or abstinence, compared to their 'before' biopsies. A statistically significant increase was however observed in the stromal tissue of women following condom-protected intercourse (mean increase $40\pm16\%$), though its magnitude was far less than that observed following unprotected intercourse (refer to Tables 3.1 and 3.2).

TABLE 3.1 Proportion of positively labelled cells detected within the cervical epithelium before and after either abstinence, condom-protected or unprotected intercourse. Data are presented as mean ± SD.

- Before and after data within an experimental group were analysed statistically using paired T-tests. * Significant difference between before and after data, $p < 0.05$.
- To determine if there was an effect of group, on leukocyte numbers, before and after data between experimental groups were analysed statistically using Mann-Whitney U test. ** Significant difference between condom-protected intercourse or unprotected intercourse and abstinent group, $p < 0.05$. 

Sharkey Chapter 3 95
<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Group</th>
<th>Proportion of labelled cells in the epithelium:</th>
<th>Mean increase</th>
<th>P value a (paired T test)</th>
<th>P value b (Mann-Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before-After</td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>Abstain</td>
<td>$22 \pm 2%$</td>
<td>$23 \pm 2%$</td>
<td>$2 \pm 7%$</td>
<td>0.493</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$19 \pm 3%$</td>
<td>$22 \pm 3%$</td>
<td>$16 \pm 6%$</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$21 \pm 4%$</td>
<td>$31 \pm 6%$</td>
<td>$51 \pm 13%$</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD4</td>
<td>Abstain</td>
<td>$8 \pm 1%$</td>
<td>$8 \pm 1%$</td>
<td>$4 \pm 19%$</td>
<td>0.756</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$6 \pm 2%$</td>
<td>$6 \pm 2%$</td>
<td>$6 \pm 16%$</td>
<td>0.235</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$7 \pm 1%$</td>
<td>$4 \pm 3%$</td>
<td>$-41 \pm 45%$</td>
<td>0.097</td>
</tr>
<tr>
<td>CD8</td>
<td>Abstain</td>
<td>$18 \pm 4%$</td>
<td>$18 \pm 3%$</td>
<td>$4 \pm 26%$</td>
<td>0.884</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$17 \pm 2%$</td>
<td>$18 \pm 2%$</td>
<td>$6 \pm 13%$</td>
<td>0.394</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$15 \pm 4%$</td>
<td>$23 \pm 6%$</td>
<td>$51 \pm 11%$</td>
<td>0.001*</td>
</tr>
<tr>
<td>CD11</td>
<td>Abstain</td>
<td>$23 \pm 8%$</td>
<td>$24 \pm 8%$</td>
<td>$6 \pm 28%$</td>
<td>0.723</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$19 \pm 6%$</td>
<td>$24 \pm 5%$</td>
<td>$35 \pm 46%$</td>
<td>0.185</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$16 \pm 6%$</td>
<td>$34 \pm 9%$</td>
<td>$130 \pm 86%$</td>
<td>0.002*</td>
</tr>
<tr>
<td>CD14</td>
<td>Abstain</td>
<td>$17 \pm 3%$</td>
<td>$19 \pm 4%$</td>
<td>$12 \pm 15%$</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$16 \pm 3%$</td>
<td>$20 \pm 4%$</td>
<td>$24 \pm 7%$</td>
<td>0.004*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$18 \pm 3%$</td>
<td>$36 \pm 3%$</td>
<td>$104 \pm 21%$</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD15</td>
<td>Abstain</td>
<td>$5 \pm 1%$</td>
<td>$6 \pm 1%$</td>
<td>$11 \pm 21%$</td>
<td>0.165</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$5 \pm 2%$</td>
<td>$6 \pm 1%$</td>
<td>$33 \pm 73%$</td>
<td>0.597</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$5 \pm 2%$</td>
<td>$7 \pm 3%$</td>
<td>$50 \pm 73%$</td>
<td>0.132</td>
</tr>
<tr>
<td>CD45</td>
<td>Abstain</td>
<td>$50 \pm 4%$</td>
<td>$52 \pm 7%$</td>
<td>$4 \pm 13%$</td>
<td>0.479</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$42 \pm 7%$</td>
<td>$48 \pm 9%$</td>
<td>$13 \pm 11%$</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$47 \pm 5%$</td>
<td>$66 \pm 6%$</td>
<td>$42 \pm 13%$</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD45RA</td>
<td>Abstain</td>
<td>$3 \pm 0%$</td>
<td>$3 \pm 0%$</td>
<td>$4 \pm 11%$</td>
<td>0.488</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$2 \pm 1%$</td>
<td>$2 \pm 0%$</td>
<td>$8 \pm 24%$</td>
<td>0.758</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$2 \pm 1%$</td>
<td>$3 \pm 1%$</td>
<td>$29 \pm 19%$</td>
<td>0.013*</td>
</tr>
<tr>
<td>CD45RO</td>
<td>Abstain</td>
<td>$9 \pm 2%$</td>
<td>$9 \pm 2%$</td>
<td>$8 \pm 26%$</td>
<td>0.733</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$7 \pm 2%$</td>
<td>$8 \pm 2%$</td>
<td>$22 \pm 31%$</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$9 \pm 3%$</td>
<td>$20 \pm 4%$</td>
<td>$131 \pm 34%$</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD57</td>
<td>Abstain</td>
<td>$3 \pm 1%$</td>
<td>$3 \pm 1%$</td>
<td>$-14 \pm 38%$</td>
<td>0.134</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$2 \pm 1%$</td>
<td>$3 \pm 1%$</td>
<td>$23 \pm 47%$</td>
<td>0.681</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$2 \pm 0%$</td>
<td>$5 \pm 2%$</td>
<td>$93 \pm 63%$</td>
<td>0.025*</td>
</tr>
<tr>
<td>CD80</td>
<td>Abstain</td>
<td>$0.2 \pm 0.1%$</td>
<td>$0.2 \pm 0.1%$</td>
<td>$11 \pm 17%$</td>
<td>0.242</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$0.2 \pm 0.1%$</td>
<td>$0.2 \pm 0.1%$</td>
<td>$11 \pm 6%$</td>
<td>0.344</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$0.2 \pm 0.1%$</td>
<td>$0.3 \pm 0.1%$</td>
<td>$14 \pm 14%$</td>
<td>0.039*</td>
</tr>
<tr>
<td>CD96</td>
<td>Abstain</td>
<td>$0.2 \pm 0%$</td>
<td>$0.2 \pm 0%$</td>
<td>$11 \pm 12%$</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$0.2 \pm 0%$</td>
<td>$0.2 \pm 0%$</td>
<td>$10 \pm 7%$</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$0.1 \pm 0%$</td>
<td>$0.2 \pm 0%$</td>
<td>$54 \pm 37%$</td>
<td>0.012*</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Abstain</td>
<td>$23 \pm 3%$</td>
<td>$26 \pm 3%$</td>
<td>$15 \pm 23%$</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>$21 \pm 3%$</td>
<td>$24 \pm 5%$</td>
<td>$13 \pm 30%$</td>
<td>0.466</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>$27 \pm 4%$</td>
<td>$41 \pm 5%$</td>
<td>$54 \pm 25%$</td>
<td>0.001*</td>
</tr>
</tbody>
</table>
TABLE 3.2 Proportion of positively labelled cells detected within the cervical stroma before and after either abstinence, condom-protected or unprotected intercourse. Data are presented as mean ± SD.

a Before and after data within an experimental group were analysed statistically using paired T-tests.* Significant difference between before and after data, p < 0.05.

b To determine if there was an effect of group, on leukocyte numbers, before and after data between experimental groups were analysed statistically using Mann-Whitney U test. * Significant difference between condom-protected intercourse or unprotected intercourse and abstinence group, p < 0.05.
<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Group</th>
<th>Proportion of labelled cells in the stroma:</th>
<th>Mean Increase</th>
<th>P value a (paired T test)</th>
<th>P value b (Mann-Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before-After</td>
<td>Before</td>
</tr>
<tr>
<td>CD3</td>
<td>Abstain</td>
<td>26 ± 2%</td>
<td>27 ± 3%</td>
<td>3 ± 4%</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>24 ± 3%</td>
<td>27 ± 3%</td>
<td>14 ± 4%</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>25 ± 3%</td>
<td>34 ± 4%</td>
<td>38 ± 9%</td>
<td>0.180</td>
</tr>
<tr>
<td>CD4</td>
<td>Abstain</td>
<td>9 ± 2%</td>
<td>9 ± 2%</td>
<td>4 ± 6%</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>8 ± 3%</td>
<td>9 ± 3%</td>
<td>9 ± 18%</td>
<td>0.033*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>8 ± 3%</td>
<td>4 ± 1%</td>
<td>-39 ± 45%</td>
<td>0.043*</td>
</tr>
<tr>
<td>CD8</td>
<td>Abstain</td>
<td>15 ± 3%</td>
<td>17 ± 2%</td>
<td>17 ± 20%</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>18 ± 2%</td>
<td>21 ± 3%</td>
<td>16 ± 11%</td>
<td>0.049*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>16 ± 3%</td>
<td>30 ± 4%</td>
<td>85 ± 20%</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD11</td>
<td>Abstain</td>
<td>27 ± 5%</td>
<td>30 ± 5%</td>
<td>13 ± 29%</td>
<td>0.022*</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>25 ± 6%</td>
<td>30 ± 9%</td>
<td>24 ± 21%</td>
<td>0.046*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>23 ± 9%</td>
<td>51 ± 5%</td>
<td>133 ± 62%</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD14</td>
<td>Abstain</td>
<td>19 ± 3%</td>
<td>21 ± 2%</td>
<td>9 ± 7%</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>20 ± 3%</td>
<td>23 ± 2%</td>
<td>16 ± 13%</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>21 ± 3%</td>
<td>44 ± 3%</td>
<td>116 ± 18%</td>
<td>0.022*</td>
</tr>
<tr>
<td>CD15</td>
<td>Abstain</td>
<td>6 ± 1%</td>
<td>7 ± 1%</td>
<td>18 ± 24%</td>
<td>0.221</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>6 ± 2%</td>
<td>6 ± 1%</td>
<td>18 ± 20%</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>8 ± 2%</td>
<td>11 ± 3%</td>
<td>35 ± 44%</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD45</td>
<td>Abstain</td>
<td>42 ± 5%</td>
<td>47 ± 4%</td>
<td>14 ± 17%</td>
<td>0.078</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>44 ± 6%</td>
<td>49 ± 4%</td>
<td>13 ± 7%</td>
<td>0.003*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>44 ± 4%</td>
<td>79 ± 5%</td>
<td>79 ± 14%</td>
<td>0.000*</td>
</tr>
<tr>
<td>CD4SRA</td>
<td>Abstain</td>
<td>5 ± 1%</td>
<td>5 ± 1%</td>
<td>4 ± 9%</td>
<td>0.392</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>5 ± 1%</td>
<td>5 ± 1%</td>
<td>19 ± 42%</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>5 ± 1%</td>
<td>6 ± 2%</td>
<td>27 ± 27%</td>
<td>0.775</td>
</tr>
<tr>
<td>CD4SRO</td>
<td>Abstain</td>
<td>11 ± 3%</td>
<td>12 ± 3%</td>
<td>10 ± 35%</td>
<td>0.081</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>10 ± 3%</td>
<td>13 ± 4%</td>
<td>27 ± 19%</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>12 ± 3%</td>
<td>25 ± 4%</td>
<td>118 ± 30%</td>
<td>0.217</td>
</tr>
<tr>
<td>CD57</td>
<td>Abstain</td>
<td>4 ± 1%</td>
<td>4 ± 1%</td>
<td>10 ± 21%</td>
<td>0.023*</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>3 ± 1%</td>
<td>4 ± 1%</td>
<td>40 ± 16%</td>
<td>0.006*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>3 ± 1%</td>
<td>6 ± 3%</td>
<td>101 ± 30%</td>
<td>0.035*</td>
</tr>
<tr>
<td>CD80</td>
<td>Abstain</td>
<td>0.5 ± 0.3%</td>
<td>0.7 ± 0.6%</td>
<td>41 ± 78%</td>
<td>0.185</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>0.4 ± 0.2%</td>
<td>0.5 ± 0.2%</td>
<td>20 ± 6%</td>
<td>0.176</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>0.5 ± 0.3%</td>
<td>2 ± 2%</td>
<td>182 ± 242%</td>
<td>0.851</td>
</tr>
<tr>
<td>CD86</td>
<td>Abstain</td>
<td>0.2 ± 0%</td>
<td>0.2 ± 0%</td>
<td>4 ± 22%</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>0.2 ± 0.1%</td>
<td>0.2 ± 0.1%</td>
<td>15 ± 2%</td>
<td>0.082</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>0.2 ± 0%</td>
<td>2 ± 2%</td>
<td>828 ± 242%</td>
<td>0.082</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Abstain</td>
<td>25 ± 3%</td>
<td>25 ± 3%</td>
<td>-1 ± 14%</td>
<td>0.129</td>
</tr>
<tr>
<td></td>
<td>Condom</td>
<td>24 ± 4%</td>
<td>28 ± 7%</td>
<td>17 ± 22%</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>Intercourse</td>
<td>25 ± 3%</td>
<td>55 ± 3%</td>
<td>117 ± 24%</td>
<td>0.851</td>
</tr>
</tbody>
</table>

Sharkey Chapter 3
FIGURE 3.3 The effect of intercourse on leukocyte recruitment in the human cervix. Tissue sections obtained before (A, C, E, G) and after (B, D, F, H) intercourse were incubated with a selection of monoclonal antibodies including CD45 (A and B respectively), CD11 (C and D respectively), MHC class II (E and F respectively) and CD45RO (G and H respectively). Ep; Epithelium, St; Stroma. Scale bar = 50 µm.
The experiments described in this chapter aimed to investigate whether exposure to semen following intercourse can induce changes in leukocyte recruitment and activation within the cervix of women in vivo. The recruitment of leukocytes within the female reproductive tract has been thought to be largely regulated by ovarian steroid hormones or the presence of micro-organisms. These experiments have generated the novel finding that exposure to semen following intercourse is also capable of inducing changes in leukocyte recruitment and activation within the cervical stroma, and to a lesser extent into the cervical epithelium of peri-ovulatory women in vivo. Macrophages and dendritic cells were identified as being the predominant leukocytes recruited into the ectocervix following intercourse, while relatively high numbers of T lymphocytes (particularly CD8+ and CD45RO+ T cells) were also detected. This marked infiltration of leukocytes was observed in all of the seven women following unprotected intercourse and was evident across the full thickness of the cervical epithelium and subjacent stromal tissue. In contrast to semen exposure, condom-protected intercourse was found to elicit much smaller increases in leukocyte numbers within the cervix. The magnitude of this increase was only marginally higher that that observed in the abstinent group following the 'repeat' biopsy, and was considerably lower than that observed following exposure to semen. This suggests that mechanical trauma sustained by the cervix following intercourse is not responsible for generating the post-coital inflammatory response. Small increases in leukocyte numbers were also observed in some of the 'repeat' biopsies of women who abstained from intercourse, suggesting that either the 'initial' biopsy caused some slight inflammation, or that the slight changes were due to natural variation within individuals due to the stage of the menstrual cycle. These results therefore suggest that a direct interaction between seminal constituents and cervical epithelial cells is required to generate the post-coital inflammatory response in humans. Furthermore, they also demonstrate that the cellular components required to initiate a new immune response are present in the cervix after insemination, consistent with the idea that the cervix is an active and competent immune 'inductive' site.

The ectocervix was chosen as the preferred site to investigate this response since preliminary in vitro studies in our laboratory had identified that cells from the ectocervix were more responsive to seminal factors than cells from the endocervix (Tremellen 1998).

Macrophages and dendritic cells are antigen-presenting cells which are primarily responsible for generating new immune responses, and regulating existing responses, toward foreign antigens. Previous studies in other laboratories have identified both of these cell types as being present within the ectocervical epithelium and stroma (Poppe et al. 1998, Prakash et al. 2001). Their relative densities
and distribution has been shown not to fluctuate significantly throughout the menstrual cycle (Poppe et al. 1995). In the current study, macrophages and dendritic cells appeared to be the predominant leukocyte in the cervix before and after intercourse. Following unprotected intercourse, considerable increases in the relative abundance of macrophages and dendritic cells were observed both in the epithelium and stroma. Their physical location within the tissue also altered, such that insemination caused their accumulation in the stromal tissue in close proximity to the basement membrane. These findings are in contrast to previous studies which found much smaller increases in macrophage numbers within the superficial layers of the cervical epithelium and lumen following intercourse or donor insemination (Pandya et al. 1985, Thompson et al. 1992, Prakash et al. 2003). These differences are not entirely surprising, given that macrophages and dendritic cells would be expected to remain within the stromal tissue rather than traversing the epithelial surface. The increase in antigen-presenting cells observed in this study is consistent with studies in mice, where an influx of macrophages and dendritic cells into the uterine tissues has been observed following mating (Robertson et al. 1996). Exposure to semen following intercourse was also found to elicit an increase in the activation status of macrophages and dendritic cells within cervical tissue. In the mouse, the activation of these cells after mating has also been associated with increased membrane expression of MHC class II antigen and enhanced antigen-presenting capacity (Hunt et al. 1996). The precise role of the increases in macrophages and dendritic cells within the cervix following intercourse is yet to be determined. However it is speculated that their increase in activation status, together with their physical location, may facilitate the processing and presentation of antigens associated with sperm or micro-organisms, contained within the ejaculate (Parr et al. 1985).

Neutrophils were found to account for only a small proportion of the total cell population within the cervical epithelium and stroma. Exposure to semen following unprotected intercourse was found to result in only a small increase in the proportion of neutrophils detected within the epithelium and stroma, while even smaller increases were observed following condom-protected intercourse or abstinence (none of which were statistically significant). The number of neutrophils detected both before and after intercourse were considerably lower than those observed in previous studies in humans (Pandya et al. 1985, Thompson et al. 1992), however this is perhaps not surprising given that neutrophils have been shown to comprise approximately 70% of all leukocytes in the cervical mucous of non-pregnant women (Okamura et al. 1988). In contrast to our findings, an influx of neutrophils has been observed within the cervical epithelium and mucous of humans following the administration of donor semen (Pandya et al. 1985, Thompson et al. 1992), as well as after mating in the cervical and uterine tissues of other mammalian species including the mouse, rat, rabbit and pigs (Lovell et al. 1968, Sharkey Chapter 3 102
Phillips et al. 1977, De et al. 1991, O’Leary et al. 2004). In humans, previous studies have shown the post-coital associated neutrophilia within the cervical mucous and superficial epithelium to be rapid, yet short-lived, occurring as early as 4 hours after intercourse and lasting for approximately 24 hours before subsiding (Thompson et al. 1992). A possible explanation for the lack of neutrophils observed in this current study may be that the neutrophils had already migrated from the stroma to the epithelium and into the lumen, before the ‘repeat’ biopsy was taken (approximately 12 hours after intercourse). This possibility could be evaluated by collecting cervical biopsies at an earlier time point, for example 4-8 hours after intercourse. This may then reveal an influx of neutrophils within the tissues comparable to those seen in other studies. A second possibility is that the neutrophils were still present in the superficial layers of the epithelium and cervical mucous at the time of biopsy, but were not detected due to washing of the cervix prior to biopsy to confirm compliance. Previous investigators have postulated that the increase in neutrophils within the cervical mucous and superficial epithelium following insemination may play a role in the phagocytosis of sperm and seminal debris, as well as aid in the removal of potentially pathogenic bacteria introduced at intercourse (Tomlinson et al. 1992).

T cells can be divided into CD4+ helper T cells which promote cell-mediated (Th1), antibody-mediated (Th2), or suppressive (Th3) immune responses through their differential release of certain cytokines, and CD8+ suppressor / cytotoxic T cells which are now also known to exhibit both Th1 and Th2 cytokine secretion profiles.

T lymphocytes have been shown to be present in relatively high numbers within the epithelial and stromal compartments of the ectocervical and endocervical tissues in women (Poppe et al. 1998). This is consistent with the findings in this study which identified T lymphocytes (using a CD3-specific antibody) as comprising a major proportion of the leukocytes present in the cervix before and after intercourse. Exposure to semen following intercourse was found to elicit a significant increase in the proportion of T lymphocytes present within the epithelium and stroma. Significant increases were also observed in the epithelium and stroma following condom-protected intercourse; however the magnitude of this infiltration was far less than following unprotected intercourse. No change in T lymphocyte abundance was observed following abstinence in either the epithelium or stroma. Again, this contrasts to previous studies that reported only minor increases in the number of T cells present in the superficial samples taken following exposure to semen in humans (Thompson et al. 1992).

The effect of intercourse on specific subsets of T lymphocytes within the cervix was also examined. Interestingly, CD8+ T cells were found to represent the largest proportion of T cells within the cervix both before and after intercourse. This finding is in contrast to a previous study that identified
CD4+ T cells as outnumbering CD8+ T cells in the ectocervix (Poppe et al. 1998). Exposure to semen was found to trigger a significant increase in the number of CD8+ T cells within the epithelium and stroma. Statistically significant increases were also observed in the cervical stroma following condom-protected intercourse and abstinence, however the magnitude of this influx was again far less than that observed following unprotected intercourse. These findings are consistent with a previous study in humans where an influx of CD8+ T cells was observed in the superficial epithelium and cervical mucous (Thompson et al. 1992). It has been proposed that these CD8+ T cells are of a 'suppressive' phenotype, as elevated numbers of cytotoxic T cells reactive with sperm antigens in the reproductive tract might be detrimental to conception or an ensuing pregnancy. In addition, in the same study, cytotoxic T lymphocytes were found to be almost completely absent following insemination (Thompson et al. 1992). Therefore, it would be interesting to perform further phenotypic analysis on these cells to determine if the CD8+ T cells detected in our study were in fact of a suppressive phenotype, as opposed to being cytotoxic T cells. In order to investigate this, the cells would need to be extracted from the tissue and purified using, for example CD8 antigen specific beads. The resultant cells could then be assessed for cytokine production by either FACS analysis or ELISA, allowing a cytokine profile for the cell population to be determined.

CD4+ helper T cells are responsible for assisting the activation and proliferation of B cells or CD8+ T cells. In contrast to a previous study identifying CD4+ T cells as being the predominant lymphocyte present within the ectocervix (Poppe et al. 1998), these cells were detected in only very low numbers in this current study. Exposure to semen was found to result in a significant decrease in the proportion of CD4+ T cells within the cervical stroma, while a smaller decrease was detected in the epithelium. In contrast to semen exposure, no changes in CD4+ T cells were observed following either condom-protected intercourse or abstinence. This finding is consistent with previous studies both in humans and mice, which also showed low levels of CD4+ T cells being detected following insemination (Pandya et al. 1985, Pace et al. 1991, Thompson et al. 1992). Interestingly, a recent study using mucous sampling from the endocervix reported finding higher concentrations of CD4+ T lymphocytes in the cervical mucous of women who reported having sexual intercourse less than 3 days ago, compared to those who had their last sexual contact greater than 3 days ago (Prakash et al. 2003).

This study is the first to identify a relative abundance of CD45RO+ memory T cells within the cervix, with significant increases observed in both the epithelium and stroma after insemination. In contrast, no changes in CD45RO+ T cells were observed following condom-protected intercourse or abstinence. Memory T cells are derived from both naïve and effector T cells elicited during a prior
exposure to their cognate antigen. Therefore, the relative abundance of memory T cells within the cervical tissue would suggest that the seminal antigens contained within the ejaculate had been encountered previously, implying that the inflammatory response observed in the cervix following intercourse is a recurring phenomenon. Naïve or virgin T cells were found to represent only a small proportion of the total T cell population present in the cervix, with only a small, but statistically significant increase detected within the epithelium after insemination. Again, no differences were observed in the epithelium or stroma following condom-protected intercourse or abstinence.

The precise role of the large numbers of T lymphocytes within the cervix is yet to be determined though their presence and cytolytic activity (White et al. 1997) suggests that T cell mediated immunity is important within these tissues and may therefore play a role in the prevention of infection. It is interesting to speculate that they may also play a role in the development of tolerogenic immune responses to seminal antigens contained within the ejaculate, however further phenotypic analysis would be required to confirm this function.

Natural killer cells were also found to represent a minor proportion of the leukocytes present in the cervix, a finding consistent with studies in other laboratories (McKenzie et al. 1991). This study is the first to investigate whether natural killer cells are involved in the human post-coital inflammatory response. A small increase in natural killer cells was detected following intercourse, with comparable numbers seen in the epithelium and stroma. This finding is consistent with studies in mice, where no additional influx of natural killer cells is observed within the endometrium following mating (Parr et al. 1985). The precise function of the small number of natural killer cells detected within the cervical tissue is currently unknown, however it is believed they may play a role in the defence against microbial pathogens.

The results described in this chapter demonstrate for the first time that exposure to semen following intercourse elicits a clear and dramatic influx of leukocytes within the cervix of peri-ovulatory women in vivo, with changes in resident leukocyte populations comparable to those observed in the female reproductive tract of other mammalian species. These data also provide experimental evidence that exposure to semen, as opposed to mechanical trauma, is responsible for generating the post-coital inflammatory response in humans. The results also confirm that a number of similarities do exist between mice and humans in the cellular changes that occur within the female reproductive tract following exposure to semen. While the precise physiological function(s) of this response in humans has not yet been established, it is likely that the influx of leukocytes into the female tissues may assist in the removal of potentially harmful pathogens introduced into the female reproductive tract at
insemination (Parr et al. 1985). It has also been suggested that this response may promote the survival of viable, fertilising sperm by selectively phagocytosing dead, abnormal or non-fertilising sperm (Tomlinson et al. 1992). Finally, the post-coital inflammatory response occurring within the cervix, would be ideally positioned to sample sperm antigens contained within the ejaculate.

While this study has demonstrated that a post-coital inflammatory response occurs within the ectocervical tissue of peri-ovulatory women, it would be interesting to use the same experimental approach to investigate the effect of steroid hormone status on this response. For example, it would be interesting to examine whether a comparable response occurs in post-menopausal women, women using the contraceptive pill or in women at different stages of the menstrual cycle. Also, as this study investigated the inflammatory response in the ectocervix, it would be of interest to examine whether a comparable response also occurs in other sites such as the endocervix.

It is currently unknown whether the semen-induced recruitment of leukocytes observed within the cervical tissue is also be mimicked within the endometrial tissues following intercourse. It seems reasonable to speculate that this may occur since the endometrium is also densely populated by diverse array of leukocytes and two recent studies have demonstrated that seminal plasma can induce pro-inflammatory cytokine and chemokine production by endometrial epithelial cells in vitro (Tremellen 1998, Gutsche et al. 2003). This could be investigated using a variation of the experimental protocol used in this current study, where the only alteration required would be the collection of endometrial tissue biopsies before and after unprotected intercourse, condom-protected intercourse or abstinence. If it was found that the effects of semen do extend to the endometrium then it may be that the resultant leukocyte recruitment could then impact on the local environment of the endometrium, potentially influencing events such as tissue remodelling as well as facilitating implantation.
Chapter 4

Exposure to semen following intercourse induces pro-inflammatory cytokine mRNA expression within the human cervix
4.1 INTRODUCTION

Experiments described in the previous chapter identified a clear and dramatic influx of leukocytes within the cervix following exposure to semen in peri-ovulatory women, with changes in resident leukocyte populations comparable to those observed in the female reproductive tract of other mammalian species. The inflammatory cascade initiated following intercourse was evident across the full thickness of the cervical epithelium and subjacent stromal tissue with a striking infiltration of macrophages, dendritic cells and lymphocytes in both compartments. Furthermore, the recruitment of leukocytes into the cervical tissue required direct interaction between seminal constituents and cervical epithelial cells, since no inflammatory reaction was observed following condom-protected intercourse.

In the mouse, the semen-induced recruitment of leukocytes into the uterine endometrium following mating has been shown to be regulated by the induction of a surge in pro-inflammatory cytokine synthesis by these uterine epithelial cells (Robertson et al. 1996, Robertson et al. 1998). GM-CSF, a potent stimulator of myeloid cell recruitment, has been identified as being as one of the key mediators of this inflammatory response. Following mating, there is an approximate 20-fold increase in GM-CSF expression both at the mRNA and protein level. In addition, insemination also leads to an increase in the synthesis of IL-6 and an array of chemokines (Robertson et al. 1996, Robertson et al. 1998). Interestingly, similar changes in cytokine synthesis are also seen in pigs following insemination, where seminal plasma also induces uterine expression of GM-CSF and IL-6, along with the chemokine MCP-1 (O'Leary et al. 2004). As observed in the mouse, the changes in pro-inflammatory cytokine and chemokine production are accompanied by an influx of macrophages and dendritic cells into the endometrial stroma (O'Leary et al. 2004).

The experiments described in this chapter were undertaken to determine whether similarities exist between mice and humans in regard to the cytokine regulation of the inflammatory response following insemination. Such similarities are reasonable to postulate since the human female reproductive tract produces GM-CSF, and limited in vitro studies have shown that seminal plasma is capable of inducing cytokine production in cervical and endometrial epithelial cells (Kelly et al. 1997, Denison et al. 1999, Gutsche et al. 2003). However it has not previously been determined whether exposure to semen following intercourse can induce changes in pro-inflammatory cytokine production within the cervix of women in vivo. To investigate this, cervical tissue biopsies were obtained from peri-ovulatory women before and after abstinence, condom-protected intercourse or unprotected intercourse. The tissues were collected as duplicate biopsies from the same subjects at the same time.
as biopsies for immunohistochemical analysis as described in Chapter 3. Cervical tissue samples were then processed (as described in section 2.2.4), and quantitative real-time PCR was performed using oligonucleotide primers specific for the pro-inflammatory cytokines GM-CSF, IL-1α, IL-6 and IL-8. The effect of intercourse on additional pro-inflammatory and immune-deviating cytokines TNFα and IFNγ was also examined.

In addition, we evaluated the effect of semen exposure on expression of two chemokine receptors CXCR4 and CCR5. These chemokine receptors are expressed by activated macrophages, dendritic cells and CD4+ T lymphocytes (Sallusto et al. 1998, Juffermans et al. 2002, Prakash et al. 2004a, Prakash et al. 2004b) and are implicated in sexual transmission of the human immunodeficiency (HIV) virus by providing cell surface receptors for HIV binding and entry. It was of interest, as a related research question, to evaluate whether the inflammatory response to insemination may act to facilitate HIV transmission by increasing the availability of cell surface receptors available to the virus.
4.2 THE EFFECT OF INTERCOURSE ON GM-CSF, IL-1α, IL-6 AND IL-8 mRNA EXPRESSION WITHIN THE HUMAN CERVIX

To investigate whether exposure to semen following intercourse can alter GM-CSF, IL-1α, IL-6 and IL-8 mRNA expression within the human cervix, cervical tissue biopsies were collected both before and after abstinence, condom-protected intercourse and unprotected intercourse.

Briefly, all couples totally abstained from penetrative intercourse for two days prior to the onset of the study, whilst condoms were used for five days prior to this period of time. This protocol was employed to ensure that no sperm would be present in the cervical region at the time of the first biopsy. In order to synchronise the timing of the first biopsy, all subjects determined their LH peak in urine samples taken twice daily from approximately day 10 after the onset of menstruation to the time at which an LH increase was detected (LH0-LH+1) by using a rapid self-test (Clearplan, Searle Unipath Ltd., Bedford, UK). Subjects presented for the first biopsy during the peri-ovulatory stage of the menstrual cycle (LH0 to LH+1) and duplicate needle biopsies were taken from the left hand side of the cervix, approximately 1 cm from the transformation zone (the 'initial' biopsy). For this aspect of the study, the second biopsy was immediately snap frozen in liquid N₂ and stored at -70°C until use. Following the first biopsy, couples were asked to abstain from intercourse for 36 hours to enable haemostasis and healing of the biopsy site. Intercourse couples were asked to have intercourse (either condom-protected or unprotected) on one occasion approximately 36 hours after the first biopsy and return for a second ('repeat') biopsy approximately 12 hours after intercourse (on day LH+2 to LH+3). Abstinent couples also presented 48 hours after the first biopsy for a repeat biopsy (refer to Chapter 2 for more details).

Cervical tissue samples were processed as described in section 2.2.4, then quantitative real-time PCR was performed on cDNA generated from cervical tissue biopsies using oligonucleotide primers specific for GM-CSF, IL-1α, IL-6 and IL-8. Data were normalised for β-actin mRNA expression and expressed as a percent of the mean of the control ('initial biopsy') sample for each individual using the arithmetic equation $2^{\Delta \Delta C_T} \times 100 / K$ (Applied Biosystems User Bulletin No.2), where K is the normalising constant. The values were multiplied by this constant to give an arbitrary mRNA quantity so that the average of the control in each group was 100 arbitrary units. Data was also normalised to an alternative house-keeping gene, 18s, for comparison to the β-actin normalised data.

Sharkey Chapter 4

110
GM-CSF mRNA expression was detected in all of the samples tested, with relatively high levels of variation observed between individuals. For example, absolute GM-CSF content ranged from 11% to 554% of the mean value in the combined cohort of 'before' samples (n = 18) (data not shown). Unprotected intercourse was found to significantly increase GM-CSF mRNA expression (paired T-Test, p = 0.004), with a 2.5-fold mean increase in individuals detected in the 'after' biopsies when compared to the 'before' biopsies (Figure 4.1 A). Increased GM-CSF mRNA expression was observed in each of the individuals within this group following insemination. In contrast, condom-protected intercourse was found to elicit a smaller, 42% mean increase in relative GM-CSF mRNA expression, compared to before biopsies and this was not statistically significant. No increase in GM-CSF mRNA expression was observed in the after biopsies of women from the abstinent group, compared to before samples.

IL-1α mRNA expression was also detected in all of the samples tested with a variance of 10% to 391% of the mean value in the combined cohort of 'before' samples (n = 18) (data not shown). Unprotected intercourse was found to induce a mean 2.5-fold increase in IL-1α mRNA expression in individuals within 'after' biopsies, when compared to 'before' biopsies (Figure 4.1 B). Increased IL-1α mRNA expression was observed in each of the individuals within this group following insemination, however due to the relatively high degree of variation between individuals, this increase failed to reach significance when data was evaluated by paired T-test. Similarly to the results observed for GM-CSF, condom-protected intercourse was found to elicit a smaller mean increase (34%) in relative IL-1α mRNA expression than observed following unprotected intercourse, which was not statistically significant. A comparable change in IL-1α mRNA expression to those observed following condom-protected intercourse were detected following abstinence, with a mean increase of 45% observed in the after biopsies of women from the abstinent group compared to their before samples. However this change was not statistically significant when data was analysed by paired-T test.

IL-6 mRNA expression was also detected in all of the samples tested with a variance of 5% to 331% of the mean value in the combined cohort of 'before' samples (n = 18) (data not shown). Unprotected intercourse was found to increase IL-6 mRNA expression, with a mean 2.6-fold increase detected in the 'after' biopsies of individuals when compared to the 'before' biopsies (Figure 4.1 C). Increased IL-6 mRNA expression was observed in each of the individuals within this group following insemination, however due to the relatively high amount of variation between individuals, this result failed to reach significance when data was compared by paired T-test (p = 0.06). Condom-protected intercourse was associated with a 53% decrease in mean relative IL-6 mRNA expression (not
significant), when compared to before biopsies, and no difference in the after biopsies of women from the abstinent group.

IL-8 mRNA expression was also detected in all of the samples tested with a variance of 1% to 883% of the mean value in the combined cohort of ‘before’ samples (n = 18) (data not shown). Unprotected intercourse was found to elicit a marked increase in IL-8 mRNA expression, with a mean 17-fold increase detected in the ‘after’ biopsies when compared to the ‘before’ biopsies (Figure 4.1 D). Increased IL-8 mRNA expression was observed in each of the individuals within this group following insemination, however due to the high degree of variation between individuals in the magnitude of this induction, statistical significance was not achieved when data was compared by paired T-test. Condom-protected intercourse was also found to induce IL-8 mRNA expression within the cervix, though to a lesser extent than detected following unprotected intercourse. Condom-protected intercourse was associated with a 2.2-fold increase in mean relative IL-8 mRNA expression, when compared to before biopsies (not significant). No change in IL-8 mRNA expression was detected in cervical tissue obtained from women from the abstinent group.

**FIGURE 4.1** Effect of intercourse on GM-CSF, IL-1α, IL-6 and IL-8 cytokine mRNA expression within the human cervix. Cervical tissue biopsies were collected from women during the peri-ovulatory period and again 48 hours later following abstinence, condom-protected or unprotected intercourse. Messenger RNA was extracted and reverse transcribed, then quantitative PCR was performed. Relative mRNA expression was calculated by normalising data to β-actin mRNA expression. Cytokine mRNA expression in ‘after’ biopsies is given relative to mRNA expression in the ‘before’ biopsy for the same individual, where the mean mRNA expression in ‘before’ biopsies for the group is arbitrarily given a value of 100. Data are presented as mean ± SEM, with statistical analysis performed using paired T-test, followed by Mann-Whitney U test, with significance inferred when *p < 0.05. The number of individuals included in each group is shown in parentheses.
4.3 THE EFFECT OF INTERCOURSE ON TNFα AND IFNγ mRNA EXPRESSION WITHIN THE HUMAN CERVIX

To investigate whether exposure to semen following intercourse can alter the mRNA expression of additional pro-inflammatory and immune-deviating cytokines TNFα and IFNγ within the human cervix, quantitative real-time PCR was performed on cDNA generated from cervical tissue biopsies collected both before and after abstinence, condom-protected intercourse and unprotected intercourse.

TNFα mRNA expression was also detected in all of the samples tested with variance of 23% to 218% of the mean value in the combined cohort of the ‘before’ samples (n = 18) (data not shown). Unprotected intercourse was found to have little impact on TNFα mRNA expression, with only a small mean increase of 13% detected in the ‘after’ biopsies when compared to the ‘before’ biopsies (Figure 4.2 A). Small increases were detected in each of the individuals within this group following insemination, with levels of variation in TNFα mRNA expression observed between individuals similar to those observed for other cytokines measured. Similar small increases in expression were evident after condom-protected intercourse and in tissues from abstinent women, but none of these changes in TNFα mRNA expression were found to be significant when data was compared using paired T-test.

IFNγ mRNA expression was detected in all of the samples tested with a variance of 13% to 431% of the mean value in the combined cohort of the ‘before’ samples (n = 18) (data not shown). IFNγ mRNA expression was found to decrease following unprotected intercourse, with a mean decrease of 61% detected in the ‘after’ biopsies when compared to the ‘before’ biopsies (Figure 4.2 B). Decreased IFNγ mRNA expression was observed in each of the individuals within this group following insemination, though no significant difference was observed when data was compared by paired T-test. Similar small decreases in IFNγ mRNA expression was seen following condom-protected intercourse, and in cervical tissue obtained from women in the abstinent group, but statistical significance was not reached. Greater variation in relative IFNγ mRNA expression between individuals was observed in the before biopsies than in the after biopsies in each of the groups.
FIGURE 4.2 Effect of intercourse on TNFα and IFNγ cytokine mRNA expression within the human cervix. Cervical tissue biopsies were collected from women during the peri-ovulatory period and again 48 hours later after abstinence, condom-protected or unprotected intercourse. Messenger RNA was extracted and reverse transcribed, then quantitative PCR was performed. Relative mRNA expression was calculated by normalising data to β-actin mRNA expression. Cytokine mRNA expression in ‘after’ biopsies is given relative to mRNA expression in the ‘before’ biopsy for the same individual, where the mean mRNA expression in ‘before’ biopsies for the group is arbitrarily given a value of 100. Data are presented as mean ± SEM, with statistical analysis performed using paired T-test, followed by Mann-Whitney U test, with significance inferred when *p < 0.05. The number of individuals included in each group is shown in parentheses.
4.4 THE EFFECT OF INTERCOURSE ON CCR5 AND CXCR4 CHEMOKINE RECEPTOR mRNA EXPRESSION IN THE HUMAN CERVIX

To investigate whether exposure to semen following intercourse can alter CCR5 and CXCR4 chemokine receptor mRNA expression within the human cervix, quantitative real-time PCR was performed on cDNA generated from cervical tissue biopsies collected both before and after abstinence, condom-protected intercourse and unprotected intercourse.

CCR5 mRNA expression was detected in all of the samples tested with a variance of 12% to 292% of the mean value in the combined cohort of before' samples (n = 18) (data not shown). Unprotected intercourse was found to have little effect on CCR5 mRNA expression (Figure 4.3 A). Small but not statistically significant decreases in CCR5 mRNA expression were observed following condom-protected intercourse and with abstinence. The CCR5 mRNA content of tissues was greater in the after' biopsies in the intercourse group compared with the after' biopsies in the abstain' group (independent samples T-test, p = 0.04).

CXCR4 mRNA expression was also detected in all of the samples tested with variance of 6% to 392% in the combined cohort of before' samples (n = 18) (data not shown). Unprotected intercourse was found to elicit small increases in relative CXCR4 mRNA expression in each individual (Figure 4.3 B) but this was not found to be significant when data was compared by paired T-test (p = 0.25). In contrast, decreases in relative CXCR4 mRNA expression were observed in cervical tissues of women following condom-protected intercourse and with abstinence (not significant). There was a significant increase in the mean CXCR4 mRNA content of tissues in the after' biopsies in the intercourse group compared with the after' biopsies in the abstain' group (independent samples T-test, p = 0.05) or the after' biopsies in the condom group (independent samples T-test, p = 0.019).
FIGURE 4.3 Effect of intercourse on CCR5 and CXCR4 chemokine receptor mRNA expression within the human cervix. Cervical tissue biopsies were collected from women in the peri-ovulatory period and again 48 hours later after abstinence, condom-protected or unprotected intercourse. Messenger RNA was extracted and reverse transcribed, then quantitative PCR was performed. Relative mRNA expression was calculated by normalising data to β-actin mRNA expression. Cytokine mRNA expression in 'after' biopsies is given relative to mRNA expression in the 'before' biopsy for the same individual, where the mean mRNA expression in 'before' biopsies for the group is arbitrarily given a value of 100. Data are presented as mean ± SEM, with statistical analysis performed using paired T-test, followed by Mann-Whitney U test, with significance inferred when *p < 0.05. The number of individuals included in each group is shown in parentheses.
DISCUSSION

The experiments described in this chapter aimed to investigate whether exposure to semen following intercourse can induce changes in pro-inflammatory cytokine production within the cervix of women in vivo. The recruitment and distribution of leukocytes within the female reproductive tract has been thought to be largely regulated by ovarian steroid hormones or the presence of micro-organisms. These experiments have generated the novel finding that semen is also capable of inducing changes in leukocyte distribution and activation within the cervical tissue of women following unprotected intercourse in vivo. More specifically, the alterations in leukocyte density and activation status induced by semen appears to be mediated through the up-regulation of pro-inflammatory cytokine synthesis within the female tissues, with marked increases in cervical GM-CSF, IL-6 and IL-8 mRNA expression observed following unprotected intercourse. In contrast to semen exposure, condom-protected intercourse elicited little change in cervical cytokine synthesis, suggesting that mechanical trauma sustained by the cervix during intercourse is not responsible for generating the post-coital inflammatory response. Similarly, no significant change in pro-inflammatory cytokine mRNA synthesis was observed in women who abstained from intercourse, indicating that the influx of leukocytes observed following unprotected intercourse was not due to changes in ovarian steroid hormone levels through the menstrual cycle.

Previous studies in other laboratories have identified GM-CSF as being constitutively expressed by cervical epithelial cells throughout the menstrual cycle (Woodworth et al. 1993, Hubert et al. 1999). This study has generated the novel finding that exposure to semen following sexual intercourse results in a significant (2.5-fold) increase in GM-CSF mRNA expression within the cervical tissue of peri-ovulatory women in vivo. In contrast, condom-protected intercourse and abstinence were found to have no effect on GM-CSF mRNA expression. This finding is consistent with preliminary in vitro studies which have shown that seminal plasma can stimulate increased production of GM-CSF by human cervical epithelial cells (Tremellen 1998). Furthermore, these results are also consistent with studies in mice, where GM-CSF mRNA production by uterine epithelial cells is also up-regulated following exposure to semen (Robertson et al. 1990). In the murine uterus, the post-mating surge in GM-CSF mRNA and protein production has been associated with triggering the recruitment of macrophages and dendritic cells along with neutrophils into the uterine tissues (Robertson et al. 2000). Consistent with published studies showing the importance of GM-CSF in regulating the functional maturation and antigen-processing ability of dendritic cells, experiments in GM-CSF null mutant mice show the essential role of this cytokine for regulating uterine dendritic cell phenotype and for activating optimal
immune responses to seminal antigens (Robertson and Molden-Hauer, unpublished data). It is likely that the post-coital surge in GM-CSF plays a similar role in recruiting and activating leukocytes of the myeloid lineage into the human cervix, since macrophages and dendritic cells have been identified as being the predominant leukocytes recruited into the cervical tissue following intercourse (as shown in Chapter 3).

In mice, the post-mating increase in GM-CSF mRNA expression has been shown to extend to the oviductal epithelium (Robertson et al., unpublished data). Whether semen exposure evokes similar changes in cytokine expression within the upper reproductive tract of humans, within the endometrial tissues, has not yet been examined. Emerging evidence from in vitro studies suggest that the effects of semen exposure are likely to extend to the uterus in humans, since endometrial epithelial and stromal cells have been shown to up-regulate pro-inflammatory cytokine production in response to stimulation with seminal plasma (Tremellen et al. 1997, Gutsche et al. 2003). In addition, in vivo studies have demonstrated that active seminal constituents, including TGFβ, are present on the post-acrosomal region of the sperm head and are thereby transported into the uterine environment (Chu et al. 1996). This movement into the higher female tract is facilitated by rapid and sustained peristaltic uterine contractions capable of transporting macromolecular material as high as the fallopian tube (Kunz et al. 2002).

IL-1 is also produced by the cervical epithelium throughout the menstrual cycle (Woodworth et al. 1993, Fichorova et al. 2004). Similarly to the results obtained for GM-CSF, exposure to semen following unprotected intercourse was found to elicit a greater increase in cervical IL-1α mRNA expression compared to women who had condom-protected intercourse or those who abstained from intercourse. The observation of a 45% increase in IL-1α mRNA expression in the repeat biopsies of abstinent women may indicate that IL-1α expression within the cervix is at least partially regulated by sex steroid hormones. This suggestion is supported by the observation that cervical mucous IL-1α concentrations fluctuate throughout the menstrual cycle, reaching maximal levels during the post-ovulatory period (Kanai et al. 1997). The precise role of the post-coital increase in cervical IL-1α mRNA production within the cervix in humans remains unknown, however given its known biological functions in other tissues, including the activation of T cells, it may be postulated that the increase in IL-1α facilitates the activation of maternal T cells exposed to seminal antigens contained within the ejaculate. The human endometrium is also a source of IL-1α, with endometrial epithelial cells identified as being the major site of production (Kanai et al. 1997). Whether seminal plasma is capable of up-regulating IL-1α mRNA expression within these cells has not yet been examined, however in vitro studies have shown that...
another closely related isoform with similar biological functions, IL-1β, is increased following incubation with seminal plasma (Gutsche et al. 2003). In mice, an increase in uterine IL-1α and IL-1β mRNA expression has been observed following mating, reaching maximal levels after 24 hours, and its production has been primarily localised to endometrial macrophages and endothelial cells (Sanford et al. 1992). It is also likely that macrophages contribute to the increase in cervical IL-1α mRNA production in humans since these cells are recruited in large numbers into the cervical tissue following unprotected intercourse, but this would need to be confirmed by in situ localisation of mRNA or protein. In mice, the increase in uterine IL-1 production following mating has been implicated in facilitating embryo implantation, since intra-peritoneal injection of IL-1 receptor antagonist between days 3-9 of pregnancy has been shown to almost completely block embryo implantation (Simon et al. 1994).

The human cervix has also been identified as a site of constitutive IL-6 production as well as secretion into the cervical mucous (Woodworth et al. 1993). In addition, very little variation in cervical IL-6 production has been observed throughout the menstrual cycle and therefore it does not appear to be hormonally regulated (Kutteh et al. 1988). Exposure to semen was found to induce a mean 2.6-fold increase in cervical IL-6 mRNA expression in the repeat biopsies of women who engaged in unprotected intercourse. In contrast, IL-6 mRNA synthesis tended to decline following condom-protected intercourse, and was not different with abstinence. In vitro studies have also shown that seminal plasma stimulates the up-regulation of IL-6 mRNA within primary endometrial epithelial cells (Gutsche et al. 2003), however whether similar changes are induced within the endometrium following insemination in vivo remains to be examined. This finding is consistent with studies in mice which have shown that exposure to semen triggers an increase in uterine IL-6 production (Robertson et al. 1992). In rodents, it has been suggested that the surge in IL-6 production following mating may serve to enhance the maternal immune response to paternal antigens and pathogens introduced at the time of mating, since intra-uterine administration of IL-6 to ovariectomised rats has been reported to increase antigen presentation and antibody production by the uterine mucosa (Prabhala et al. 1995). It is therefore reasonable to speculate that the up-regulation of IL-6 mRNA within the cervix of women following intercourse may act to stimulate antigen presentation by macrophages, dendritic cells as well as cervical epithelial cells. This suggestion is supported by the findings in chapter 3, which showed that the macrophages and dendritic cells recruited into the cervical tissue following intercourse are of an activated phenotype. Furthermore, studies in other laboratories have shown that cervical epithelial cells themselves are also capable of presenting antigens and therefore these cells may also contribute to the induction of pregnancy-associated immune responses (Ljunggren et al. 1998, Mota et al. 1999). A secondary potential role for the increase in cervical IL-6 production may be in enhancing the fertilising
capacity of human sperm, since IL-6 has been shown to trigger sperm capacitation in vitro (Naz et al., 1994, Naz et al. 1996, Laflamme et al. 2005).

The human female reproductive tract has been shown to be a rich source of IL-8, with functional protein being detected within the vaginal (Fichorova et al. 1999, Yamada et al. 2002, Donders et al. 2003), ectocervical (Barclay et al. 1993, Fichorova et al. 1999) and endocervical epithelium (Fichorova et al. 1999) as well as cervical mucous (Sakai et al. 2004). Interestingly, seminal plasma has been shown to induce a marked increase in IL-8 production by cervical tissue explants in vitro (Denison et al. 1999). The experiments described in this chapter have identified a strong trend towards increased IL-8 mRNA expression within the cervix of women following exposure to semen. Despite a mean 17-fold increase in IL-8 mRNA expression, the large amount of variation observed between individuals in the magnitude of this induction meant this increase was not significant. Little change in IL-8 mRNA was observed following condom-protected intercourse or abstinence, further implicating semen in providing the activating stimulus for induction of this response. This finding is consistent with studies in the mouse where KC (the murine equivalent of IL-8) production by uterine epithelial cells is strongly induced following exposure to seminal plasma (Robertson and Glynn, unpublished data). The precise role of this increase in IL-8 within the cervical tissues has not been determined, however it is likely that IL-8 acts to stimulate the recruitment of neutrophils into the upper vagina, cervical mucous and epithelium where they would be ideally positioned to phagocytose non-fertilising spermatozoa (Denison et al. 1999) and aid in the defence against infection by potentially pathogenic bacteria (Parr et al. 1985). This suggestion is supported by the observation that exposure to semen following donor insemination stimulates the recruitment of neutrophils into the cervical mucous and superficial layers of the epithelium (Pandya et al. 1985, Thompson et al. 1992). These results are however in direct contrast to our immunohistochemical findings, which identified neutrophils as accounting for only a small proportion of the total cell population within the cervix both before and after intercourse. Given that IL-8 is potently chemoattractive for neutrophils, the dramatic increase in IL-8 mRNA would certainly suggest that a large number of neutrophils would be expected to have migrated into the cervix following intercourse. As mentioned in chapter 3, a possible explanation for the lack of neutrophils observed in this study may be that the neutrophils had already migrated from the stroma to the epithelium and into the lumen, before the 'repeat' biopsy was taken (approximately 12 hours after intercourse). Other investigators have shown that the post-coital associated influx of neutrophils is short-lived, occurring as early as 4 hours after intercourse and is resolved within 24 hours (Thompson et al. 1992). Therefore, cervical biopsies collected at an earlier time point, for example 4-8 hours after intercourse, may reveal an influx of neutrophils within the tissues comparable to that seen in other studies. A second possibility
is that the neutrophils were still present in the superficial cell layers and cervical mucous at the time of biopsy, but were not detected due to washing of the cervix prior to biopsy to confirm compliance.

TNFα is produced by the cervical and endometrial epithelium throughout the menstrual cycle, with its expression under the control of ovarian steroid hormones (Hunt 1992, Mota et al. 1999, Tabibzadeh et al. 1999). The results described in this chapter indicate that exposure to semen has little effect on TNFα mRNA expression, since the pattern of synthesis was similar following unprotected or condom-protected intercourse and abstinence. Therefore it is likely that the small mean increase in TNFα mRNA expression observed in the repeat biopsies of each of the groups is due to hormone-regulated fluctuations through the menstrual cycle. While this response was not investigated within the endometrial tissues, these findings are consistent with studies which have shown that seminal plasma does not affect TNFα mRNA expression by primary endometrial epithelial cells in vitro (Gutsche et al. 2003). These results are however in contrast to studies in the mouse, where TNFα mRNA expression is transiently increased following mating (Sanford et al. 1992). The precise role of TNFα within the cervical tissue of humans remains largely unknown, but a role in contributing to the prevention of infection is likely (Wira et al. 1998, Ahmed et al. 2001).

IFNγ mRNA expression has been detected within the cervical epithelium of women where it has been largely attributed to the large populations of T lymphocytes within this tissue (El-Sherif et al. 2001). In our experiment, a trend towards decreased IFNγ mRNA expression was observed following unprotected intercourse, condom-protected intercourse and abstinence suggesting that IFNγ mRNA expression is not responsive to semen exposure, but rather its expression may be under the influence of ovarian steroid hormones.

The amount of variation between individuals with respect to the magnitude of the induction of cytokine and chemokine mRNA expression observed following exposure to semen was far greater than that observed for immunohistochemical analysis of duplicate biopsies. There are a number of potential factors which may contribute to this, including differences in the size or composition of cervical tissue collected during the biopsy process. The relative content of epithelial and stromal tissue in the collected biopsy would influence the individual cytokine mRNA content detected, since cytokines such as GM-CSF, IL-6 and IL-8 are more highly expressed by cervical epithelial cells than by stromal cells (Woodworth et al. 1993, Shen et al. 2004). Secondly, variation could be influenced by differences in the number and relative abundance of resident microflora present within the lower female reproductive tract at the time of biopsy, since several of the cytokines examined are regulated by signals mediated by
Toll-like receptors, or would be influenced by background immune responses to microflora. Finally, another contributing factor may be genetic variation between individuals. For example, a number of cytokine gene polymorphisms have been described which result in an individual constitutively expressing a particular cytokine at higher or lower levels.

Our investigation of CCR5 and CXCR4 mRNA expression is relevant to the significance of semen in affecting transmission of HIV, since these chemokine receptors have been implicated in the sexual transmission of the HIV virus by providing cell surface receptors for HIV binding and entry. Heterosexual transmission of HIV is now the leading mode of transmission of the virus world-wide. Therefore, the acquisition of a better understanding of the mechanisms involved in male to female transmission of the virus is critical to the development of effective prevention strategies. Male to female transmission of HIV involves the deposition of HIV-infected semen within the vagina in close proximity to the cervix (Stratton et al. 1993). The sexual transmission of HIV requires the CD4 cell surface molecule as well as a chemokine co-receptor. The two main chemokine co-receptors involved are CCR5, which is utilised by macrophage tropic isolates of HIV-1 (Dragic et al. 1996, Feng et al. 1996) and the second is CXCR4, which is utilised by lymphotropic isolates (Bleul et al. 1997). A number of recent studies have investigated the expression of these receptors within the lower female reproductive tract, particularly within the cervical tissues, since this region is often the first site of viral exposure during heterosexual transmission. CCR5 but not CXCR4 is expressed by ecto- and endocervical epithelial cells (Yeaman et al. 1998, Prakash et al. 2004b, Yeaman et al. 2004), while both CCR5 and CXCR4 are expressed by leukocytes within these tissues (Yeaman et al. 1998, Prakash et al. 2004a, Prakash et al. 2004b, Yeaman et al. 2004). The leukocytes that express these receptors include macrophages, dendritic cells and CD4+ T lymphocytes (Sallusto et al. 1998, Juffermans et al. 2002, Prakash et al. 2004a, Prakash et al. 2004b), all of which are present in relatively high numbers within the cervix. The studies described in this chapter aimed to investigate whether exposure to semen following intercourse is capable of altering the expression of CCR5 and CXCR4. Our results demonstrate for the first time that exposure to semen following sexual intercourse is capable of stimulating a small increase in both CCR5 and CXCR4 receptor mRNA expression relative to condom-protected intercourse and abstinence. This shows that the increase following unprotected intercourse is mediated by specific factors present in semen. These findings together with the observation that exposure to semen following intercourse elicits a marked increase in MHC class II positive antigen presenting cells such as macrophages and dendritic cells, as well as the relative abundance of CD4+ T lymphocytes within the ectocervical epithelium and stroma (refer to chapter 3), suggests that semen exposure and the resultant inflammatory response may indeed act to increase female susceptibility to
HIV transmission. These results concur with studies in other laboratories clearly implicating the cervix as an important target site for infection to occur.

The results described in this chapter highlight the importance of specific factors present in semen in stimulating the post-coital inflammatory response observed within the female reproductive tract following insemination. These data provide experimental evidence for the first time that chemotactic and pro-inflammatory cytokines mediate the post-coital influx of inflammatory leukocytes into the cervical tissues of women, with exposure to semen, as opposed to mechanical trauma, being responsible for generating this response. It is likely that this response is important in eliciting immune responses to sperm antigens and micro-organisms contained within the ejaculate. The results also further confirm that several similarities exist between mice and humans in the molecular mechanisms through which the post-coital response is regulated.

It is interesting to speculate that the semen-induced induction of pro-inflammatory cytokine expression and the resultant leukocyte recruitment observed within the cervical tissue may also be mimicked within the endometrial tissues following intercourse. As mentioned previously, in vitro studies have shown that endometrial epithelial cells are in fact responsive to seminal plasma, therefore it would be useful to investigate whether this response extends to the endometrial tissue. This could be achieved using a variation of the experimental protocol utilised in this current study, where the only alteration required would be the collection of endometrial tissue biopsies before and after unprotected intercourse, condom-protected intercourse or abstinence. If it was found that the effects of semen do extend to the endometrium then it may be that the resultant induction of pro-inflammatory cytokine and chemokine expression and subsequent leukocyte recruitment could then impact on the local cytokine environment of the endometrium, potentially influencing microenvironmental factors that facilitate implantation and optimal pregnancy outcome.

The main limitation encountered during this study related to the amount of cervical tissue obtained using the tissue biopsy technique. The small amount of tissue obtained restricted the repertoire of cytokines and chemokines that could be examined. If the amount of tissue collected had been greater, it would have been desirable to measure additional molecules that have demonstrated immune-deviating activities such as TGFβ, IL-10, IL-12 and IL-13 as well as additional chemotactic factors influencing leukocyte recruitment such as MCP-1, MIP-1α and MIP-1β.
Chapter 5

Pro-inflammatory activity of seminal plasma constituents
In women, the lower reproductive tract consists of three morphologically and functionally different compartments; the vagina, ectocervix, and endocervix. The vagina and ectocervix are covered by stratified squamous non-keratinizing epithelium, forming an effective physical barrier acting as a first line of defence against invasion by foreign pathogens (Quayle 2002). The lining of the endocervical canal consists of a single layer of columnar epithelial cells, with these cells synthesizing mucus and other substances, which are packaged into vacuoles for secretion (Fichorova et al. 1999). The endocervical epithelium is believed to play an important role in mucosal defence of the lower female genital tract primarily because of its role in polymeric immunoglobulin transport (Quayle 2002). There is a multitude of evidence emerging in many species, including humans, to suggest epithelial cells are crucial in generating immune responses within female reproductive tissues. These cells have a pivotal role in the defence against pathogens, and would be expected to also be important in the generation of appropriate immune responses towards male antigens contained within the ejaculate.

Historically, seminal plasma was thought to act purely as a transport medium for the survival of spermatozoa traversing the female reproductive tissues (Ventura et al. 1973). It is now becoming increasingly evident that seminal plasma plays a broader role in human reproduction, extending beyond sperm support. Emerging studies indicate that this complex fluid contains an array of cytokines and other active molecules that can interact with epithelial cells lining the female reproductive tract, and activate changes in the number and behaviour of leukocytes in such a way as to 'condition' the tract environment for implantation (Robertson et al. 1997). Studies in mice show this 'conditioning' effect is mediated via changes in the female immune response which facilitate tolerance of the conceptus in an ensuing pregnancy, as well as tissue remodelling and secretion of embryotrophic cytokines (Robertson et al. 2001).

The ability of semen exposure to initiate an inflammatory response within the cervix of women was clearly demonstrated in previous chapters. We found exposure to semen following natural intercourse results in a dramatic influx of leukocytes into the cervical tissue of peri-ovulatory women, with striking increases in the number of macrophages, dendritic cells and lymphocytes observed. This leukocyte recruitment requires contact between seminal fluid and the female reproductive tract tissues since no inflammatory response was observed following condom-protected intercourse. Moreover, we have also demonstrated that the changes in leukocyte populations observed following intercourse are
regulated by the semen-induced activation of pro-inflammatory cytokines such as GM-CSF, IL-6 and IL-8.

To date, very little is known as to the identity of the active constituents within human semen responsible for generating this response in humans. Two previous in vivo studies in humans have investigated the fraction of the ejaculate required to activate neutrophil exocytosis either following sexual intercourse or artificial insemination and both reported that sperm, but not seminal plasma, is required (Pandya et al. 1985; Thompson et al. 1992). However, these studies only measured the ability of sperm or seminal plasma to promote an infiltration of leukocytes into the cervical mucus, but did not evaluate leukocyte infiltration into the deeper epithelium and stromal layers of the cervix as was done in our current study. Furthermore, data published more recently from in vitro studies clearly demonstrate that as in mice, human female reproductive tract cells are responsive to seminal plasma, with several studies demonstrating the ability of seminal plasma to stimulate cytokine production from both cervical explants and endometrial cells (Kelly et al. 1997; Gutsche et al. 2003).

A similar influx in leukocytes, together with an induction of pro-inflammatory cytokine expression has also been identified after semen exposure in mice and pigs (Robertson et al. 1996; O’Leary et al. 2004). This response is best characterised in the mouse, where studies in our laboratory have identified the key molecular agents in seminal plasma that mediate activation of female responses. In the mouse, the use of protein chromatography and neutralising antibodies led to TGFβ1 being identified as the predominant factor responsible for initiating the post-mating inflammatory response (Tremellen et al. 1998). Neutralisation of TGFβ1 bioactivity was found to reduce the ability of seminal plasma to induce GM-CSF production from uterine epithelial cells by more than 75%.

Given the similarities that exist between mice and humans in the nature of the inflammatory response generated following copulation, we proposed that seminal plasma constituents may also be responsible for generating the post-coital inflammatory response in humans. Furthermore, we postulated that since TGFβ is also present in very high concentrations within human seminal plasma (total TGFβ content of approximately 400 ng/ml), that this molecule may also be responsible in humans.

The studies described in this chapter aimed to identify the predominant active moieties in human seminal plasma responsible for generating the post-coital inflammatory response in the cervix. To investigate this, we obtained immortalised ectocervical (Ect1), endocervical (End1) and vaginal (Vk2)
epithelial cell lines. These cell lines had been characterised previously (Fichorova et al. 1999) and were shown to have retained the morphological and immunocytochemical characteristics of their relative tissues of origin and behaved similarly in vitro to corresponding primary cell cultures. To investigate whether these cells were responsive to seminal plasma, cells were cultured in the presence of seminal plasma and evaluated for synthesis of GM-CSF, IL-6, IL-8 and IL-10. We also investigated whether the addition of recombinant human TGFβ1, TGFβ2, and TGFβ3 to these cells could mimic the effects of seminal plasma on pro-inflammatory cytokine production. Similar experiments were then undertaken using primary ectocervical epithelial cells prepared from cervical tissue obtained from healthy women undergoing hysterectomy for benign, non-gynaecological indications, as a means of confirming the results obtained using cell lines. To determine the relative contribution of each of the isoforms of TGFβ to the generation of this response, immortalised cells were incubated in the presence of seminal plasma and individual TGFβ isoform-specific neutralising antibodies. Finally, the actions of other seminal immune regulators were examined. These included 19-hydroxy prostaglandin E1 (19-OH PGE1), the most abundant prostaglandin present in human seminal plasma, and IFNγ, identified in mouse studies as being inhibitory to TGFβ actions in uterine epithelial cells (Robertson 1992).
5.2 THE EFFECT OF SEMINAL PLASMA ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY HUMAN FEMALE REPRODUCTIVE TRACT CELL LINES

To investigate whether human Ect1, End1 and Vk2 cells are responsive to human seminal plasma, cells were incubated in the presence of diluted seminal plasma, and their production of cytokines including GM-CSF, IL-6 and IL-8 was assessed.

Briefly, cell lines were propagated to approximately 80% confluence using the protocols described by Fichorova et al (Fichorova et al. 1997) (refer to chapter 2 for more detailed description). Cells were then plated in fresh keratinocyte serum-free media (KSFM) (1 x 10^6 cells in 500 µl medium) in 1.5 ml culture wells and incubated for 2-3 days to generate a confluent monolayer. At this time, the culture supernatant was carefully removed and exchanged for 500 µl of fresh KSFM. Twenty-four hours later, this 'pre-treatment' supernatant was collected and replaced with 500 µl of fresh media containing culture media alone (control) or alternatively, 10% (v/v) seminal plasma, which in turn was collected 24 hrs later as the '0 – 24 hr post-treatment' supernatant. The supernatants were then centrifuged to remove cellular debris and the GM-CSF, IL-6, IL-8 content measured by commercial ELISA. Comparison of the GM-CSF, IL-6 and IL-8 content within pre-treatment and post-treatment supernatants from each individual culture allowed the calculation of any increase in GM-CSF, IL-6 and IL-8 in response to seminal plasma.

Ect1 cells were found to have the highest baseline levels of expression of GM-CSF, IL-6 and IL-8, while End1 and Vk2 cells were found to secrete less cytokine when unstimulated and when exposed to seminal plasma (Figure 5.1 A, B and C respectively). For example, End1 cells cultured without seminal plasma secreted only 9%, 36% and 94% of the GM-CSF, IL-6 and IL-8 per pg / 10^5 cells / 12hr, secreted by Ect1 cells. Vk2 cells cultured without seminal plasma secreted only 6%, 20% and 9% per pg / 10^5 cells / 12hr, secreted by Ect1 cells.

Ect1 cells were also found to be more responsive to stimulation by seminal plasma, with a 2.9-fold increase in GM-CSF production observed following treatment compared to much smaller increases of just 70% and 12% observed in End1 and Vk2 cells respectively (Figure 5.1 A). Similar results were also observed for IL-6, where incubation with seminal plasma was found to induce a 7.4-fold increase in IL-6 production by Ect1 cells, whereas increases of 60% and 6% were observed in End1 and Vk2 cells respectively (Figure 5.1 B). In contrast to the results obtained for GM-CSF and IL-6 production, Ect1 and End1 cells exhibited increases in IL-8 production of comparable magnitude, with 6.8 and 7.4-fold
increases respectively, while Vk2 cell production of IL-8 was found to increase by approximately 40-fold following incubation with seminal plasma (Figure 5.1 C).

The observation that End1 and Vk2 cells secrete lower levels of cytokines than Ect1 cells when unstimulated, together with the observation that Ect1 cells were more responsive to stimulation by seminal plasma led to the decision not to continue using the End1 and Vk2 cell lines in future experiments. This decision was also influenced by additional in vitro experiments in our laboratory which found primary endocervical epithelial cells to be less responsive to seminal plasma than cells from the ectocervix (Tremellen 1998).
FIGURE 5.1 Effect of seminal plasma on Ect1, End1 and Vk2 epithelial cell line GM-CSF, IL-6 and IL-8 production in vitro. Ect1, End1 and Vk2 cells were cultured to confluence before adding either culture media (control) or 10% (v/v) seminal plasma. The GM-CSF, IL-6 and IL-8 content was then measured in the 24-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in the control. All measurements are the mean ± SD of triplicate wells in each experimental group. These data are representative of three replicate experiments.
5.3 THE DOSE RESPONSE TO SEMINAL PLASMA INDUCED GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO

To investigate whether human Ect1 cells are responsive to human seminal plasma in a dose-dependant manner, cells were incubated in the presence of varying concentrations of seminal plasma (0.01, 0.1, 1.0 and 10%), and their production of cytokines including GM-CSF, IL-6 and IL-8 was assessed.

Exposure to seminal plasma was found to markedly increase GM-CSF production by Ect1 cells, when compared to untreated control cells (Figure 5.2 A). Ect1 cells responded to seminal plasma in a dose-dependent manner with cells incubated with 0.1, 1.0 and 10% seminal plasma showing 1.2, 3.2 and 3.5-fold increases respectively in GM-CSF production compared to the control wells.

Seminal plasma was also found to be a potent stimulator of IL-6 production by Ect1 cells, with cells exposed to as little as 0.01% seminal plasma exhibiting a 2.1-fold increase in IL-6 production (Figure 5.2 B). More marked increases were observed following incubation with higher concentrations of seminal plasma. Treatment with 0.1% seminal plasma resulted in a 2.7-fold increase and incubation with 1.0 and 10% seminal plasma resulted in 4.1 and 9.5-fold increases compared to the controls.

Incubation of Ect1 cells in the presence of low doses of seminal plasma failed to elicit any significant increase in IL-8 production. At concentrations of 1.0 and 10% seminal plasma, seminal plasma stimulated a 3.2 and 12.5-fold increase respectively in IL-8 production (Figure 5.2 C).

Regulation of Ect1 cell production of IL-10 was also investigated, however we failed to detect IL-10 in any of the cell culture supernatants collected from Ect1 cells.
FIGURE 5.2 Effect of seminal plasma on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro. Ect1 cells were cultured to confluence before adding either culture media (control) or diluted seminal plasma at concentrations of 0.01, 0.1, 1.0 or 10% (v/v). The GM-CSF, IL-6 and IL-8 content was then measured in the 24-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in the control. All measurements are the mean ± SD of triplicate wells in each experimental group. These data are representative of three replicate experiments.
5.4 THE EFFECT OF SEMINAL PLASMA ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY PRIMARY CERVICAL EPITHELIAL CELLS IN VITRO

The results of the previous experiment identified Ect1 cells as being very responsive to factors contained within seminal plasma, with marked increases in GM-CSF, IL-6 and IL-8 production by these cells observed following incubation with diluted seminal plasma. To substantiate the physiological relevance of these findings, it was important to determine whether seminal plasma could induce similar changes in cytokine production in primary ectocervical epithelial cell cultures.

Briefly, primary ectocervical epithelial cells were prepared by enzymatically digesting ectocervical biopsies collected from consenting pre-menopausal women undergoing routine vaginal or total abdominal hysterectomy for benign, non-cervical pathologies (refer to Appendix A for patient information sheet and consent form). Cervical epithelial cells were suspended in ectocervical culture media and layered over mitogenically inactive murine 3T3 fibroblast cells. Cells were incubated for 5 – 7 days to enable the majority of epithelial cells to attach to the culture well and displace the 3T3 fibroblasts. The culture supernatant containing desquamated epithelial cells and 3T3 cells was carefully removed once good attachment was observed, and exchanged for fresh media. This media was collected 12 hours later as the ‘pre-treatment’ supernatant and replaced with either culture media alone (control), or media containing either 0.1, 1.0 or 10% seminal plasma. Cervical epithelial cells were then incubated for 12 hours, after which the supernatant was collected as the 0 - 12 hour ‘post-treatment’. Immediately following collection, all supernatants were centrifuged to remove cellular debris. Comparison of the GM-CSF, IL-6 and IL-8 content within pre-treatment and post-treatment supernatants from each individual culture allowed the calculation of any increase in GM-CSF, IL-6 and IL-8 production by cervical epithelial cells in response to varying doses of seminal plasma.

The small amount of tissue obtained following hysterectomy meant that the experiments undertaken with Ect1 cells could not be fully replicated in primary cells in a single experiment. Sufficient sample was obtained to allow us to use three different concentrations of seminal plasma, as opposed to the four dilutions used with cell lines. The concentration of 0.01% seminal plasma was omitted since this treatment elicited the least stimulatory ability in cell line experiments.

Exposure to seminal plasma was found to markedly increase GM-CSF production by primary ectocervical epithelial cells in a dose dependent manner (Figure 5.3 A). Primary cells incubated in the presence of 0.1, 1.0 and 10% seminal plasma showed 2.1, 2.5 and 3.0-fold increases in GM-CSF production.
production respectively, compared to the control wells. The increases in seminal plasma-induced GM-CSF were of a similar magnitude to those observed in Ect1 cells.

Seminal plasma was also found to be a strong stimulator of IL-6 production in primary ectocervical epithelial cells. Seminal plasma was found to stimulate dose-dependent increases in IL-6, with cells exposed to 0.1, 1.0 and 10% seminal plasma exhibiting 2.4, 2.7 and 3.3-fold increases in IL-6 production respectively (Figure 5.3 B).

As was the case with Ect1 cells, seminal plasma was also found to be a very powerful stimulator of IL-8 production by primary ectocervical epithelial cells. Seminal plasma was found to stimulate dose-dependent increases in IL-8 production, with cells exposed to 0.1, 1.0 and 10% seminal plasma exhibiting 3.2, 6.5 and 12-fold increases in IL-8 production respectively (Figure 5.3 C).

In general, there were considerable similarities between Ect1 and primary ectocervical epithelial cells in the way in which they responded to incubation with seminal plasma. Similar increases in GM-CSF, IL-6 and IL-8 were observed following incubation with 1.0% and 10% doses of seminal plasma. Both cell types showed dose responsive increases in all three cytokines after incubation with seminal plasma. However, there were some differences between the immortalised and primary cells. Primary ectocervical epithelial cells had consistently higher basal levels of GM-CSF, IL-6 and IL-8 production than Ect1 cells when unstimulated. For example, Ect1 cells cultured without seminal plasma secreted only 72%, 16% and 4% of the GM-CSF, IL-6 and IL-8 per pg / 10^5 cells / 12hr, secreted by primary ectocervical epithelial cells.

Primary ectocervical epithelial cells were also more responsive to seminal factors than Ect1 cells. In contrast to Ect1 cells where the lower doses of seminal plasma (0.01 and 0.1%) failed to elicit significant responses other than in IL-6 production, primary ectocervical epithelial cells responded to low dilutions of seminal plasma. For example, 0.1% seminal plasma elicited approximately 2-fold increases in GM-CSF and IL-6 production compared to the control. Primary epithelial cells were more responsive to seminal plasma particularly in regard to IL-8 production, with a low dose (0.1%) of seminal plasma stimulating a 3.2-fold increase in IL-8, while the equivalent dose of seminal plasma in Ect1 cells elicited only a 50% increase in IL-8 synthesis.
FIGURE 5.3 Effect of seminal plasma on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro. Primary cervical epithelial cells were cultured in combination with murine fibroblasts for 7 days before adding either culture media (control) or diluted seminal plasma at concentrations of 0.1, 1.0 or 10% (v/v). The GM-CSF, IL-6 and IL-8 content was then measured in the 12-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in each of their respective controls. All measurements are the mean ± SD of triplicate wells in each experimental group. These data are representative of two replicate experiments.
5.5 THE EFFECT OF RECOMBINANT TGFβ1, TGFβ2 AND TGFβ3 ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO

Studies in our laboratory have identified seminal plasma-derived TGFβ1 as being the predominant molecule responsible for eliciting the GM-CSF and IL-6 surge observed within the murine uterine epithelium following mating (Tremellen et al. 1998). Given that GM-CSF, IL-6 and IL-8 are also up-regulated within the human cervix following intercourse (chapter 4), and also that TGFβ1 is present in abundance in human seminal plasma (approximately 250 ng/ml) (chapter 6), we postulated that TGFβ1 or other TGFβ isoforms may be the major active factor in semen in triggering the release of GM-CSF, IL-6 and IL-8 from human cervical epithelial cells. To investigate whether TGFβ can mimic the seminal plasma-induced increases in GM-CSF, IL-6 and IL-8, Ect1 cells were incubated in the presence of recombinant TGFβ1, TGFβ2 and TGFβ3 at concentrations of 0.5, 5.0 and 50 ng/ml.

Addition of TGFβ1 to the culture media of Ect1 cells markedly increased their GM-CSF production in a dose dependent manner, when compared to the control (Figure 5.4 A). Ect1 cells exposed to 0.5 ng/ml TGFβ1 exhibited a 2.8-fold increase in GM-CSF production while cells incubated with 5.0 or 50 ng/ml, showed 6.8 and 7.0-fold increases respectively compared to the control wells. TGFβ1 was also found to be a potent stimulator of IL-6 production, with 2.1, 2.4 and 2.7-fold increases compared to the control when cells were incubated in the presence of 0.5, 5.0 and 50 ng/ml TGFβ1 compared to the controls (Figure 5.4 B). The magnitudes of these increases are comparable to those observed following treatment with seminal plasma in Ect1 and primary cells. However, in contrast to the dramatic increases in IL-8 production observed following treatment with seminal plasma, TGFβ1 was unable to stimulate the production of IL-8 in these cells at any of the concentrations tested (Figure 5.4 C).

Addition of TGFβ2 to the culture media of Ect1 cells also markedly increased their GM-CSF production, when compared to the control (Figure 5.4 A). Ect1 cells exposed to 0.5 ng/ml TGFβ2 exhibited a 2.3-fold increase in GM-CSF production, while cells incubated with 5.0 or 50 ng/ml showed 5.4 and 4.9-fold increases respectively compared to the control wells. Maximal stimulation occurred at a concentration of 5 ng/ml. TGFβ2 was also found to be a potent stimulator of IL-6 production, with 70%, 2.2 and 3.0-fold increases observed when cells were incubated in the presence of 0.5, 5.0 and 50 ng/ml TGFβ2 compared to the controls (Figure 5.4 B). The magnitudes of these increases were comparable.
or slightly less than those observed following incubation with TGFβ1. TGFβ2 did not stimulate any significant increase in IL-8 production in Ect1 cells at any of the concentrations tested (Figure 5.4 C).

Finally, addition of TGFβ3 to the culture media resulted in a marked increase in GM-CSF production by Ect1 cells compared to the control (Figure 5.4 A). Again this increase was in a dose dependent manner, with cells exposed to 0.5 ng/ml TGFβ3 exhibiting a 4.9-fold increase in GM-CSF production, while cells incubated with 5.0 or 50 ng/ml, showed 5.5 and 7.8-fold increases respectively compared to the control wells. TGFβ3, like TGFβ1 and TGFβ2, was also found to be a potent stimulator of IL-6 production, with 2.1, 2.8 and 6.4-fold increases compared to the control when cells were incubated in the presence of 0.5, 5.0 and 50 ng/ml TGFβ3 (Figure 5.4 B). The magnitude of these increases is comparable to those observed following treatment with seminal plasma in Ect1 and primary cells. TGFβ3 was also unable to mimic the dramatic seminal plasma-induced increase in IL-8 production at any of the concentrations tested (Figure 5.4 C). Unlike TGFβ1 and TGFβ2, TGFβ3 did appear to stimulate a small dose-dependent increase in IL-8 in Ect1 cells (a maximum increase of approximately 50% above the controls occurred at a concentration of 50 ng/ml TGFβ3), however this was a considerably lesser response than the increase observed following incubation with even low doses of seminal plasma. Overall, TGFβ3 appeared to be just as potent as TGFβ1 and slightly more potent than TGFβ2 in stimulating GM-CSF and IL-6 from Ect1 cells, and was the only isoform of TGFβ to elicit any increase in IL-8 from Ect1 cells.
FIGURE 5.4 Effect of recombinant TGFβ on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro.

Ect1 cells were cultured to confluence before adding either culture media (control) or recombinant TGFβ1, TGFβ2 or TGFβ3 at concentrations of 0.5, 5.0 or 50 ng/ml. The GM-CSF, IL-6 and IL-8 content was then measured in the 24-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in the control. All measurements are the mean ± SD of triplicate wells in each experimental group. These data are representative of three replicate experiments.
5.6 THE EFFECT OF RECOMBINANT TGFβ1, TGFβ2 AND TGFβ3 ON GM-CSF, IL-6 AND IL-8 CYTOKINE PRODUCTION BY PRIMARY CERVICAL EPITHELIAL CELLS IN VITRO

Having demonstrated that Ect1 cells can respond to TGFβ isoforms, it was investigated whether primary ectocervical epithelial cells responded similarly when exposed to recombinant TGFβ. To investigate this, primary epithelial cells were incubated in the presence of recombinant TGFβ1, TGFβ2 and TGFβ3 at a concentration of 5.0 ng/ml. Difficulties in obtaining ectocervical tissue along with the small sample size meant that we could only investigate each isoform of TGFβ at a single concentration. A concentration of 5.0 ng/ml was chosen as the preferred dose as it mimics the physiological concentrations that cervical epithelial cells would be expected to be encountering following intercourse. Also, pilot studies in primary cells and the results from the Ect1 cells above confirmed that a concentration of 5.0 ng/ml was sufficient to induce marked increases in cytokine production from these cells.

The addition of TGFβ1 to the culture media of primary ectocervical epithelial cells markedly increased GM-CSF, when compared to the control (Figure 5.5 A). Ectocervical epithelial cells exposed to 5.0 ng/ml TGFβ1 exhibited a 2.6-fold increase in GM-CSF production, an increase comparable with that seen in Ect1 cells. TGFβ1 was also found to be a potent stimulator of IL-6 production, with a 3.3-fold increase compared to the control (Figure 5.5 B), a slightly higher increase than observed in Ect1 cells. TGFβ1 was unable to replicate the increase in IL-8 elicited by seminal plasma. Interestingly, the addition of TGFβ1 to these primary cultures instead resulted in a 35% decrease in IL-8 production compared to the control (Figure 5.5 C), whereas the same treatment in Ect1 cells had no effect on IL-8 production.

The addition of TGFβ2 to the culture media of primary ectocervical epithelial cells also triggered a marked increase in GM-CSF production, compared to the control (Figure 5.5 A). Ectocervical epithelial cells exposed to 5.0 ng/ml TGFβ1 exhibited a 2.2-fold increase in GM-CSF production. TGFβ2 was also found to be a potent stimulator of IL-6 production, with a 2.4-fold increase observed in the presence of 5.0 ng/ml TGFβ1 (Figure 5.5 B). The increases in GM-CSF and IL-6 were slightly lower than those observed using the same concentration of TGFβ2 in Ect1 cells. As was the case with Ect1 cells, TGFβ2 was unable to replicate the increase in IL-8 elicited by seminal plasma. Interestingly the addition of TGFβ2, like TGFβ1, resulted in a small decrease (37%) in IL-8 production by primary
ectocervical epithelial cells compared to the control (Figure 5.5 C), whereas this was not evident in Ect1 cells.

Addition of TGFβ3 to the culture media also increased GM-CSF production by primary ectocervical epithelial cells (Figure 5.5 A). Ectocervical epithelial cells exposed to 5.0 ng/ml TGFβ3 exhibited a 2.4-fold increase in GM-CSF production. As was the case for TGFβ1 and TGFβ2, TGFβ3 was also found to be a potent stimulator of IL-6 production, with a 2.8-fold increase observed (Figure 5.5 B). The increases in GM-CSF and IL-6 using the same concentration of TGFβ3 were lower than that observed in Ect1 cells. As was the case with Ect1 cells, TGFβ3 was unable to replicate the increase in IL-8 elicited by seminal plasma. Addition of TGFβ3 to primary cells resulted in a 45% decrease in IL-8 production (Figure 5.5 C), whereas the same treatment in Ect1 cells elicited a small increase in IL-8 production compared to the control.

Overall, TGFβ1 and TGFβ3 appeared to exhibit similar potencies in stimulating GM-CSF and IL-6 from these cells, while TGFβ2 consistently elicited somewhat lower levels of stimulation.
FIGURE 5.5 Effect of recombinant TGFβ on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro. Primary cervical epithelial cells were cultured in combination with murine fibroblasts for 7 days before adding either culture media (control) or recombinant TGFβ1, TGFβ2 or TGFβ3 at a concentration of 5 ng/ml. The GM-CSF, IL-6 and IL-8 content was then measured in the 12-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in each of their respective controls. All measurements are the mean ± SD of triplicate wells in each experimental group. These data are representative of two replicate experiments.
5.7 THE EFFECT OF IFNγ ON GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO

IFNγ is present in human seminal plasma (Paradisi et al. 1996; Fujisawa et al. 1998; Maegawa et al. 2002), and has been demonstrated to antagonise the action of TGFβ in various cell types (Eickelberg et al. 2001; McCartney-Francis et al. 2002; Higashi et al. 2003; Ishida et al. 2004). In mouse uterine epithelial cells, IFNγ inhibits GM-CSF production (Robertson 1992). Furthermore, in mouse uterine epithelial cells, IFNγ inhibits the GM-CSF promoting effects of TGFβ (Glynn and Robertson, unpublished data). To investigate whether IFNγ can alter pro-inflammatory cytokine production induced by TGFβ in Ect1 human cervical epithelial cells, Ect1 cells were incubated with either recombinant TGFβ1 alone, IFNγ alone, or a combination of TGFβ1 and IFNγ. Of the different TGFβ isoforms, TGFβ1 was chosen to investigate this interaction as it was observed to be the most powerful stimulator of GM-CSF and IL-6 production in both Ect1 and primary ectocervical epithelial cells.

The addition of TGFβ1 to the culture media markedly increased GM-CSF production by Ect1 cells in a dose dependent manner, when compared to the control (Figure 5.6 A), with increases in GM-CSF production of 80%, 2.8 and 3.1-fold observed in cells incubated with 0.5, 5.0 or 50 ng/ml respectively compared to the control wells. As observed in earlier experiments, TGFβ1 was again found to be a potent stimulator of IL-6 production, with increases of 30%, 80% and 2.7-fold observed when incubated in the presence of 0.5, 5.0 and 50 ng/ml TGFβ1 compared to the controls (Figure 5.6 B), but no effect on IL-8 production was seen (Figure 5.6 C).

Treatment of these cells with IFNγ alone (5 ng/ml) was found to decrease GM-CSF production by approximately 25% compared to the controls (Figure 5.6 A). IFNγ was found to have little impact on IL-6 production (Figure 5.6 B) and did not affect baseline IL-8 production (Figure 5.6 C).

Incubation of Ect1 cells with combinations of both TGFβ1 and IFNγ showed an interaction between the two molecules. Ect1 cells exposed to 5 ng/ml IFNγ and 0.5 ng/ml TGFβ1 produced approximately 40% less GM-CSF compared to cells treated with 0.5 ng/ml TGFβ1 alone (Figure 5.6 A). Cells incubated with IFNγ and 5.0 ng/ml TGFβ1 showed a 60% reduction in GM-CSF production, compared to cells exposed to an equivalent amount of TGFβ1 alone, with IFNγ almost completely blocking the ability of TGFβ1 to induce GM-CSF. Addition of 50 ng/ml of TGFβ1 in the presence of IFNγ
was able to almost completely overcome the inhibitory effect of IFNγ. Ect1 cells incubated under these conditions were found to produce just 20% less GM-CSF than cells incubated with 50 ng/ml of TGFβ1 alone.

Addition of IFNγ to cultures in combination with TGFβ1 at concentrations of either 0.5, 5.0 and 50 ng/ml had no effect on IL-6 production by these cells (Figure 5.6 B), with increases in IL-6 comparable to those observed following incubation with TGFβ1 alone.

Addition of either TGFβ1 or IFNγ to Ect1 cells resulted in a decrease in IL-8 production. The amount of inhibition observed when cells were incubated with IFNγ in the presence of either 0.5 or 5.0 ng/ml TGFβ1 was comparable to that seen when these cells are incubated with either cytokine alone. Interestingly, when cells were incubated with IFNγ and high concentrations of TGFβ1 (50 ng/ml), the effect on IL-8 production observed appeared to be additive, with greater inhibition observed than when cells were incubated with either IFNγ or TGFβ1 alone (Figure 5.6 C).
FIGURE 5.6 Effect of recombinant TGFβ1 and IFNγ on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro. Ect1 cells were cultured to confluence before adding either culture media (control), recombinant TGFβ1 (0.5, 5.0 or 50 ng/ml), IFNγ (5 ng/ml) or TGFβ1 (0.5, 5.0 or 50 ng/ml) in combination with IFNγ (5 ng/ml). The GM-CSF, IL-6 and IL-8 content was then measured in the 24-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in the control. All measurements are the mean ± SD of triplicate wells in each experimental group. These data are representative of three replicate experiments.
THE EFFECT OF RECOMBINANT TGFβ1 AND IFNγ ON GM-CSF, IL-6 AND IL-8 PRODUCTION BY PRIMARY CERVICAL EPITHELIAL CELLS IN VITRO

The experiments described in the previous section identified IFNγ as being an inhibitor of the stimulatory effect of TGFβ1 in immortalised Ect1 cells. To investigate whether IFNγ can also alter pro-inflammatory cytokine production induced by exposure to recombinant TGFβ1 in primary cells, these experiments were repeated using primary ectocervical epithelial cells incubated with either recombinant TGFβ1 alone, IFNγ alone, or a combination of TGFβ1 and IFNγ.

Addition of TGFβ1 to the culture media again led to increased GM-CSF production by primary cervical epithelial cells in a dose dependent manner, when compared to the control (Figure 5.7 A). Cells exposed to 0.5 ng/ml TGFβ1 exhibited a small increase in GM-CSF production of 15%, while cells incubated with 5.0 or 50 ng/ml, showed 50% and 70% increases respectively compared to the control wells. As observed in earlier experiments, TGFβ1 was a strong stimulator of IL-6 production, with increases of 80%, 2.9-fold and 4.3-fold observed after incubation with 0.5, 5.0 and 50 ng/ml TGFβ1 (Figure 5.7 B). No significant effect of TGFβ1 was evident on IL-8 production except at a high concentration (50 ng/ml), where 30% inhibition of IL-8 production was seen (Figure 5.7 C).

Treatment of primary ectocervical epithelial cells with IFNγ alone (5 ng/ml) was found to decrease GM-CSF production by approximately 40% compared to the controls (Figure 5.7 A). IFNγ was found to have very little impact on IL-6 production (Figure 5.7 B), and addition of IFNγ inhibited IL-8 production by primary epithelial cells to a greater extent than observed in Ect1 cells, with a decrease in IL-8 production of approximately 70% compared to the controls (Figure 5.7 C).

Incubation of primary epithelial cells with combinations of both TGFβ1 and IFNγ again showed interaction between the two molecules. Primary cells exposed to 5 ng/ml IFNγ and 0.5 ng/ml TGFβ1 showed a 20% decrease in GM-CSF production compared to the controls (Figure 5.7 A). These results indicate that IFNγ completely ablates the ability of 0.5 ng/ml TGFβ1 to induce GM-CSF production and indeed results in a decrease of approximately 35% compared to cells treated with 0.5 ng/ml TGFβ1 alone. Cells incubated with IFNγ and 5.0 ng/ml TGFβ1 showed a 30% reduction in GM-CSF production induced by IFNγ, compared to cells exposed to an equivalent amount of TGFβ1 alone. Addition of 50 ng/ml of TGFβ1 in the presence of IFNγ was able to almost completely overcome the inhibitory effect of
IFNγ. Primary cells incubated under these conditions were found to produce just 20% less GM-CSF than cells incubated with 50 ng/ml of TGFβ1 alone.

Addition of IFNγ to cultures in combination with TGFβ1 at concentrations of either 0.5, 5.0 and 50 ng/ml had little effect on IL-6 production (Figure 5.7 B), with increases in IL-6 comparable to those observed following incubation with TGFβ1 alone.

Addition of either TGFβ1 or IFNγ to primary ectocervical epithelial cells resulted in a decrease in IL-8 production. The amount of inhibition observed in primary cells incubated with IFNγ in the presence of either 0.5 or 5.0 ng/ml was greater than that observed in Ect1 cells, with decreases of 60% and 70% respectively at these concentrations. When cells were incubated with IFNγ and high concentrations of TGFβ1 (50 ng/ml), IL-8 inhibition was even greater, with an approximately 75% reduction observed compared to the controls (Figure 5.7 C).

These results suggest that primary epithelial cells are more susceptible to the potent inhibitory effect of IFNγ on TGFβ1-induced pro-inflammatory cytokine production than Ect1 cells.
FIGURE 5.7 Effect of recombinant TGFβ1 on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro. Primary cervical epithelial cells were cultured in combination with murine fibroblasts for 7 days before adding either culture media (control) or recombinant TGFβ1 (0.5, 5.0 or 50 ng/ml), IFNγ (5 ng/ml) or TGFβ1 (0.5, 5.0 or 50 ng/ml) in combination with IFNγ (5 ng/ml). The GM-CSF, IL-6 and IL-8 content was then measured in the 12-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in each of their respective controls. All measurements are the mean ± SD of triplicate wells in each experimental group. These data are representative of two replicate experiments.
5.9 THE EFFECT OF TGFβ ISOFORM-SPECIFIC NEUTRALISING ANTIBODIES ON SEMINAL PLASMA-INDUCED INCREASE IN GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO

The results described in previous sections clearly demonstrate the ability of recombinant TGFβ to at least partially mimic seminal plasma in induction of pro-inflammatory cytokine production by cervical epithelial cells. The purpose of this experiment was use TGFβ isoform-specific neutralising antibodies to determine the amount of activity within seminal plasma attributable to TGFβ and evaluate the relative contribution of each of the isoforms of TGFβ present in seminal plasma. To investigate this, Ect1 cells were incubated in the presence of 10% seminal plasma alone (positive control), 10% seminal plasma plus anti-TGFβ1, anti-TGFβ2 or anti-TGFβ3 neutralising antibodies, or finally 10% seminal plasma plus all three neutralising antibodies in combination. Recombinant TGFβ1, TGFβ2 and TGFβ3 at a concentration of 5 ng/ml were also included in the experiment as additional positive controls. The concentration of each of the individual TGFβ isoform-specific neutralising antibodies required to completely neutralise concentrations of TGFβ1, TGFβ2 and TGFβ3 comparable to their content in seminal plasma was determined in preliminary experiments (data not shown).

Addition of recombinant TGFβ1, TGFβ2 or TGFβ3 to Ect1 cells stimulated cytokine production to a similar extent seen in previous sections, with each isoform stimulating considerable increases in GM-CSF and IL-6 production whilst having little to no effect on IL-8 production (Figure 5.8 A, B and C respectively).

Addition of a TGFβ1-specific neutralising antibody to Ect1 cells also exposed to 10% seminal plasma was found to result in a 30% decrease in GM-CSF production compared to cells incubated with seminal plasma alone (Figure 5.8 A). Neutralisation of TGFβ1 was also found to decrease IL-6 production by approximately 12%, whilst having no effect on IL-8 production by these cells (Figure 5.8 B and C respectively).

The addition of a TGFβ2-specific neutralising antibody to Ect1 cells also exposed to 10% seminal plasma was found to result in a 30% decrease in GM-CSF production compared to cells incubated with seminal plasma alone (Figure 5.8 A). Neutralisation of TGFβ2 was also found to decrease IL-6 production by approximately 20%, whilst also having no effect on IL-8 production by these cells (Figure 5.8 B and C respectively).
The addition of a TGFβ3-specific neutralising antibody to Ect1 cells also exposed to 10% seminal plasma was found to result in a 60% decrease in GM-CSF production compared to cells incubated with seminal plasma alone (Figure 5.8 A). Neutralisation of TGFβ3 was also found to result in a marked decrease (53%) in IL-6 production, whilst again having no effect on IL-8 production by these cells (Figure 5.8 B and C respectively).

Neutralisation of seminal plasma-derived TGFβ1, TGFβ2 and TGFβ3 activity using all three TGFβ isoform-specific neutralising antibodies together resulted in a 40% decrease in GM-CSF production compared to cells incubated with seminal plasma alone. Neutralisation of all isoforms of TGFβ in seminal plasma was found to result in a dramatic decrease of 77% in IL-6 production, whilst having no effect on IL-8 production by these cells (Figure 5.8 B and C respectively).

**FIGURE 5.8** Effect of TGFβ isoform-specific neutralising antibodies on seminal plasma-induced increase in GM-CSF, IL-6 and IL-8 production by Ect1 cervical epithelial cells in vitro. Ect1 cells were cultured to confluence before adding either culture media (control), recombinant TGFβ1, TGFβ2, or TGFβ3 (5.0 ng/ml), 10% SP (v/v), or 10% SP (v/v) plus neutralising antibodies specific for either TGFβ1 (15 µg/ml), TGFβ2 (15 µg/ml), TGFβ3 (15 µg/ml) or a combination of all three. The GM-CSF, IL-6 and IL-8 content was then measured in the 24-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in the control. All measurements are the mean ± SD of triplicate wells in each experimental group.
Graph A: GM-CSF Output (% of Control)
- Control
- TGFβ1
- TGFβ2
- TGFβ3
- 10% SP
- + αTGFβ1
- + αTGFβ2
- + αTGFβ3
- + αTGFβ1+2+3
- + 5 ng/ml αTGFβ
- + 10% seminal plasma

Graph B: IL-6 Output (% of Control)
- Control
- TGFβ1
- TGFβ2
- TGFβ3
- 10% SP
- + αTGFβ1
- + αTGFβ2
- + αTGFβ3
- + αTGFβ1+2+3

Graph C: IL-8 Output (% of Control)
- Control
- TGFβ1
- TGFβ2
- TGFβ3
- 10% SP
- + αTGFβ1
- + αTGFβ2
- + αTGFβ3
- + αTGFβ1+2+3
5.10 THE EFFECT OF 19-HYDROXY PROSTAGLANDIN E₁ ON GM-CSF, IL-6 AND IL-8 PRODUCTION BY IMMORTALISED CERVICAL EPITHELIAL CELLS IN VITRO

The results described in previous sections clearly demonstrate the ability of recombinant and seminal plasma-derived TGFβ to mimic the semen-induced induction of GM-CSF and IL-6 cytokine production in cervical epithelial cells in vitro. TGFβ was unable however to replicate the major surge in IL-8 production observed following exposure to seminal plasma. The observation of this differential regulation of pro-inflammatory cytokine production led us to investigate whether additional molecules present in human seminal plasma could stimulate an increase in IL-8 from Ect1 cells. We postulated that 19-OH PGE₁ was a likely candidate molecule for a number of reasons. 19-OH PGE₁ was chosen firstly because it is the most abundant prostaglandin found in human semen (µg/ml concentrations) and secondly because this molecule has previously been shown to induce IL-8 production from the human monocytic U937 cell line (Denison et al. 1999).

The purpose of this experiment therefore was to investigate whether 19-OH PGE₁ could influence cytokine production from immortalised cervical epithelial cells in vitro. To investigate this, Ect1 cells were incubated in the presence of varying concentrations of 19-OH PGE₁ (starting concentration of 2.5 µg/ml, followed by serial 1:10 dilutions to 0.25 ng/ml) and cell culture supernatants were assayed for GM-CSF, IL-6 and IL-8 content.

In contrast to TGFβ and seminal plasma, addition of 19-OH PGE₁ to the culture media of Ect1 cells led to only marginal increases in GM-CSF production at any of the concentrations tested, with maximal stimulation (20% increase) observed at a concentration of 250 ng/ml, when compared to the control (Figure 5.9 A). 19-OH PGE₁ was found to be a strong stimulator of IL-6 production, with increases comparable in magnitude to those observed following treatment with TGFβ. Interestingly, the increase in IL-6 was greatest at the lowest dose of 0.25 ng/ml 19-OH PGE₁ which elicited a 6.5-fold increase compared to the control, whilst a dose of 2.5 µg/ml, which approximates physiological concentrations within seminal plasma, elicited a 5.5-fold increase (Figure 5.9 B). Finally, it was found that unlike TGFβ, 19-OH PGE₁ was able to stimulate IL-8 production from Ect1 cells. The increase in IL-8 appeared to be independent of dose, as no differences were observed in the stimulatory ability of 19-OH PGE₁ at the range of concentrations tested (Figure 5.9 C). The mean increase in IL-8 observed was 3.2-fold, with less than 6% variation in mean increase detected across the range of 19-OH PGE₁ concentrations. Despite the increases observed, the extent of IL-8 induction remained considerably lower than that observed following incubation with seminal plasma.
FIGURE 5.9 Effect of 19-OH PGE on Ect1 cervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro. Ect1 cells were cultured to confluence before adding either culture media (control) or 19-Hydroxy PGE1 at concentrations of 0.25, 2.5, 25, 250, 2500 ng/ml. The GM-CSF, IL-6 and IL-8 content was then measured in the 24-hour pre- and post-treatment supernatants by ELISA. Post-treatment data was normalised to the pre-treatment values and expressed as a percentage of the (A) GM-CSF, (B) IL-6 or (C) IL-8 output in the control. All measurements are the mean ± SD of triplicate wells in each experimental group.

Sharkey Chapter 5
5.11 DISCUSSION

The experiments described in this chapter aimed to identify the predominant active moieties in human seminal plasma responsible for generating the post-coital inflammatory response in the human cervix. These results have shown that stimulation of pro-inflammatory cytokine production in cervical epithelial cells is differentially regulated, with multiple factors contributing to the overall cytokine profile observed following incubation with seminal plasma (summarised in Table 5.1). These experiments identified the three mammalian isoforms of TGFβ as being the predominant active constituents in seminal plasma, with TGFβ1, TGFβ2 and TGFβ3 all being capable of stimulating marked increases in GM-CSF and IL-6 production by ectocervical epithelial cells in vitro. These results are consistent with observations in murine species, where TGFβ1 has also been identified as being the predominant active constituent within murine seminal plasma (Tremellen et al. 1998).

<table>
<thead>
<tr>
<th></th>
<th>Seminal plasma</th>
<th>TGFβ1, 3</th>
<th>19-OH PGE1</th>
<th>IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>IL-6</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>IL-8</td>
<td>↑</td>
<td></td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

TABLE 5.1 The effect of seminal plasma, TGFβ, 19-OH PGE1 and IFNγ on pro-inflammatory cytokine expression from cervical epithelial cells in vitro.

The addition of dilute seminal plasma to immortalised and primary ectocervical epithelial cells was found to induce a marked increase in GM-CSF production, with both cell types yielding comparable results. This finding is consistent with studies in the mouse, where GM-CSF production by uterine epithelial cells is also up-regulated following exposure to seminal plasma (Robertson et al. 1992). The addition of recombinant TGFβ to ectocervical epithelial cells was found to induce changes in GM-CSF production comparable to those observed following treatment with dilute seminal plasma, with all three isoforms of TGFβ capable of inducing GM-CSF production. Recombinant TGFβ1 and TGFβ3 were found to have a slightly higher stimulatory ability on GM-CSF production than TGFβ2 when tested at the same dose. This difference in stimulatory ability may be due in part to differences in the relative
concentrations of specific TGFβ receptors present on ectocervical epithelial cells, since TGFβ1 and TGFβ3 have a high affinity for the Tβ-RII receptor, whereas TGFβ2 has only a very low affinity for this receptor and requires the co-expression of the Tβ-RI receptor for successful signal transduction to occur (Laiho et al. 1991; Massague 1998). Although TGFβ receptor expression was not investigated in these experiments, other investigators have shown that Tβ-RII receptors are highly expressed in the cervix and endometrium, whereas Tβ-RI receptors are present at much lower concentrations (Chegini et al. 1994; Piestrzeniewicz-Ulanska et al. 2002). Studies have also shown that TGFβ receptor expression within the endometrium is regulated by ovarian steroid hormones and fluctuate throughout the menstrual cycle (Chegini et al. 1994), though very little is known about their regulation in cervical tissue.

The relative contribution that each of the isoforms of TGFβ contained within human seminal plasma makes to GM-CSF production was determined using immortalised ectocervical epithelial cells incubated with dilute seminal plasma and TGFβ isoform-specific neutralising antibodies. Neutralisation of TGFβ1 and TGFβ2 bioactivity were both found to reduce GM-CSF production by 30%, while neutralisation of TGFβ3 resulted in a 60% reduction. Neutralisation of all three isoforms resulted in a 40% decrease in GM-CSF production. These experiments have demonstrated that TGFβ plays a crucial role in generating the surge in cervical epithelial cell GM-CSF production observed following exposure to seminal plasma. These results are consistent with findings in the mouse, where approximately 75% of the increase in GM-CSF production by murine uterine epithelial cells following incubation with seminal plasma was attributable to TGFβ1 (Tremellen et al. 1998). It is also possible that that additional members of the TGFβ family present in human seminal plasma, such as activin may play a role in generating this increase in GM-CSF production (Tremellen 1998).

In our study, primary ectocervical epithelial cells were prepared from cervical tissue where no distinction was made as to the stage of the menstrual cycle at the time of tissue collection. Therefore, it would be interesting to investigate whether there are any differences in the ability of cervical epithelial cells to respond to seminal plasma and recombinant TGFβ throughout the different stages of the menstrual cycle. Our observations indicate there is little variation between individuals in their responsiveness to either seminal plasma and TGFβ and therefore suggest that it is unlikely that the stage of the menstrual cycle would have a dramatic impact on ectocervical epithelial cell responsiveness. However differential regulation of Tβ-RI and RII across the cycle (Chegini et al. 1994) suggest variation should occur so additional experiments are required to investigate this.
IFNγ is also present in semen and is notable for its potent ability to inhibit the female tract response to stimulation by TGFβ. Incubation of immortalised and primary ectocervical epithelial cells in the presence of IFNγ was found to reduce GM-CSF production by approximately 40% compared to the controls. We also found that the ability of low doses of TGFβ1 (0.5 ng/ml) to stimulate GM-CSF production, especially from primary ectocervical epithelial cells, was completely inhibited by the presence of IFNγ. At a concentration of 5 ng/ml TGFβ1, the addition of IFNγ resulted in a 30% reduction in the amount of GM-CSF produced by these cells compared to cells treated with TGFβ1 alone. These results are consistent with observations in the mouse, where IFNγ has also been shown to be a potent inhibitor of TGFβ activity in murine uterine epithelial cells (Robertson 1992). Interestingly, high doses of TGFβ1 (50 ng/ml) were able to almost completely overcome the inhibitory effect of IFNγ.

IFNγ is believed to exert its inhibitory effect through the activation of SMAD7, an inhibitory SMAD which has the ability to form stable associations with the TGFβ receptors Tβ-RI and Tβ-RII, which in turn inhibits TGFβ signalling through receptors (Schiller et al. 2004). More recently, SMAD7 has been shown to interact with the E3 ubiquitin ligases smurf1 and smurf2, recruiting them to the TGFβ receptor complexes and triggering the degradation of activated Tβ-RI (Ebisawa et al. 2001). In rodent species, the administration of exogenous IFNγ at the time of insemination inhibits the semen-induced activation of the inflammatory cascade observed following mating (Robertson and Glynn, unpublished data), and has detrimental effects on implantation and fetal growth trajectory (Chaouat et al, 1990). These observations are thought to result from both the TGFβ-inhibitory effects of IFNγ and its related activity as an immune-deviating agent promoting type-1 cell-mediated immunity (Weiner 1997).

The addition of dilute seminal plasma to immortalised and primary ectocervical epithelial cells was also found to induce a marked dose-dependent increase in IL-6 production, with both cell types again yielding comparable results. This finding is consistent with studies in the mouse, where IL-6 production by uterine epithelial cells is also up-regulated following exposure to seminal plasma (Robertson et al. 1992). The addition of recombinant TGFβ to ectocervical epithelial cells was found to induce changes in IL-6 production comparable to those observed following treatment with dilute seminal plasma, with all three isoforms of TGFβ capable of inducing IL-6 production. As was the case with GM-CSF production, a more dramatic increase in IL-6 production was observed following incubation with recombinant TGFβ1 and TGFβ3 than with TGFβ2 when tested at the same dose.
The relative contribution that each of the isoforms of TGFβ contained within human seminal plasma makes to IL-6 production was also determined using immortalised ectocervical epithelial cells incubated with dilute seminal plasma and TGFβ isoform-specific neutralising antibodies. Neutralisation of TGFβ1, TGFβ2 and TGFβ3 bioactivity was found to reduce IL-6 production by 16%, 20% and 53%, while neutralisation of all three isoforms of TGFβ resulted in a 77% decrease in IL-6 production. The observation that TGFβ accounts for approximately 80% of the increase in IL-6 production in response to seminal plasma further highlights the important role TGFβ plays in eliciting the increase in pro-inflammatory cytokine production observed following exposure to seminal plasma in vitro, or following intercourse in vivo.

Incubation of immortalised and primary ectocervical epithelial cells in the presence of IFNγ was found to have no effect on IL-6 production. Also, IFNγ did not affect the ability of TGFβ1 to stimulate IL-6 production when added in combination with any of the concentrations of TGFβ1 tested. These results are consistent with observations in the mouse, where IFNγ also failed to inhibit the ability of TGFβ to stimulate IL-6 production (Robertson and Glynn, unpublished data). It is unclear how it is that given the effects of IFNγ on TGFβ signal transduction, the consequence of TGFβ signalling can be impervious to IFNγ inhibition.

The addition of dilute seminal plasma to immortalised and primary ectocervical epithelial cells was also found to induce a dramatic dose-dependent increase in IL-8 production, with both cell types again producing very similar results. This finding is consistent with studies in the mouse where KC (the murine equivalent of IL-8) production by uterine epithelial cells is also strongly induced following exposure to seminal plasma (Robertson and Glynn, unpublished data). Also, studies in other laboratories have demonstrated that human seminal plasma stimulates IL-8 production by cervical tissue explants (Denison et al. 1999). Addition of recombinant TGFβ to immortalised ectocervical epithelial cells was found to have little to no impact on IL-8 production. Primary cervical epithelial cells incubated with each of the three isoforms of TGFβ resulted in a 30–40% reduction in IL-8 production compared to the control, indicating that TGFβ was unable to mimic the seminal plasma-induced stimulation of IL-8 production. Not surprisingly, neutralisation of seminal plasma TGFβ1, TGFβ2 and TGFβ3 bioactivity had no impact on IL-8 production by immortalised ectocervical epithelial cells.

Interestingly, TGFβ1 has been shown to stimulate or inhibit IL-8 production depending on the cell type on which it is exerting its effect. For example, TGFβ1 has been shown to stimulate IL-8 mRNA and
immunoreactive protein production by endometrial stromal cells by increasing the stability of the IL-8 protein via a process that is dependent on protein synthesis (Arici et al. 1996). Conversely, TGFβ1 has also been shown to inhibit the production of IL-8 by endothelial cells not by altering the mRNA stability, but by altering the level of transcription thereby inhibiting the expression of IL-8 (Smith et al. 1996). Whether TGFβ1 inhibits IL-8 production via a similar mechanism operating in the cervical tissue of women has not been investigated.

The observation that TGFβ was unable to stimulate the marked increase in IL-8 observed following exposure to dilute seminal plasma suggests that there are additional active molecules in seminal plasma responsible for stimulating IL-8. It also demonstrates the complex nature of this differential regulation of the immune response within the female tissues following exposure to seminal plasma.

Interestingly, incubation of immortalised and primary ectocervical epithelial cells in the presence of IFNγ was found to reduce IL-8 production, with primary cells appearing to be more susceptible to the inhibitory effects of IFNγ. IL-8 production by primary ectocervical epithelial cells was reduced by approximately 70% in the presence of IFNγ alone, and even greater reductions were observed in the presence of IFNγ and increasing concentrations of TGFβ1. These results are consistent with observations in the mouse, where IFNγ has also been demonstrated as having the ability to inhibit KC production by murine uterine epithelial cells (Robertson and Glynn, unpublished data). IFNγ may exert this inhibitory effect on IL-8 production at the level of transcription since studies have demonstrated that IFNγ can selectively down-regulate the constitutive expression of IL-8 mRNA and the release of active protein by human polymorphonuclear cells in vitro (Cassatella et al. 1993). Whether IFNγ acts through similar mechanisms in human ectocervical epithelial cells to inhibit IL-8 production has not been examined.

The emerging picture of the differential regulation of cervical cytokine production by factors contained within seminal plasma, together with the inability of TGFβ to induce IL-8 production led us to attempt to identify other active molecules in seminal plasma. Since human seminal plasma contains extraordinarily high concentrations of prostaglandins, the most abundant of which is 19-OH PGE1 (Kelly et al. 1997), we decided to investigate whether 19-OH PGE1 was capable of stimulating pro-inflammatory cytokine production in immortalised ectocervical epithelial cells. An additional reason for choosing this molecule was because studies by other investigators had identified 19-OH PGE1 as being capable of stimulating IL-8 production in a human monocytic cell line in vitro (Denison et al. 1999). In
contrast to three isoforms of TGFβ, incubation of cervical epithelial cells in the presence of 19-OH PGE1 failed to elicit any increase in GM-CSF production by these cells. 19-OH PGE1 was however capable of inducing a strong increase in IL-6 production, comparable in magnitude to that observed following treatment with TGFβ. Interestingly, 19-OH PGE1 was capable of stimulating an increase in ectocervical epithelial cell IL-8 production, though to a lesser than observed following incubation with seminal plasma. It would also be interesting to investigate the ability of other molecules present in seminal plasma to stimulate IL-8 production, since bacterial LPS has been demonstrated to stimulate KC production in murine uterine epithelial cells (Robertson and Glynn, unpublished data) and has been recently shown to be present in human seminal plasma (refer to chapter 6).

The results described in this chapter highlight the importance of seminal plasma components in stimulating the post-coital inflammatory response observed within the female reproductive tract following insemination. These results continue to confirm that a number of similarities exist between mice and humans regarding the mechanisms by which this response is regulated, with TGFβ also being identified as the predominant active moiety in human seminal plasma. Some differences must also exist however as 19-OH PGE1 also contributes strongly to this response in humans, and this molecule is not present at all in murine seminal plasma. The apparent differential regulation of this response may indicate that the relative abundance of each of these agents in seminal plasma is likely to be important in generating an optimal maternal immune environment for successful pregnancy. Therefore, it would be useful to identify and evaluate other inflammation inducing moieties present in semen that are likely to contribute to the molecular regulation of this response. These may include molecules that are capable of enhancing, or inhibiting the stimulatory ability of TGFβ on female reproductive tract cells. Potential candidate molecules could be identified using either immortalised Ect1 or primary cervical epithelial cell cultures (as described in chapter 5) with the aid of neutralising antibodies, then their presence and concentration in human seminal plasma could be determined using commercially available assays. Whether differences in the abundance of these molecules exist in the seminal plasma of fertile individuals compared to individuals experiencing impaired fertility is addressed in chapter 6.

Limitations encountered during this study were the frequency at which we could acquire cervical tissue from women undergoing hysterectomy and the amount of tissue collected. Firstly, the number of experiments which could be performed using primary cervical epithelial cells was restricted by the frequency at which cervical tissue samples were obtained. This meant that some experiments such as the neutralisation of TGFβ activity within seminal plasma could not be replicated in primary cells. This
will however be done in the future and would be expected to yield similar results to the immortalised cells given the general similarity existing in their responsiveness to seminal plasma. Also, the small amount of tissue collected limited both the size of the experiments that were performed and the number of treatments that were applied in any single experiment. These limitations were partially overcome by the acquisition of the immortalised ectocervical epithelial cell line as it facilitated investigation of the regulation of this response without reliance on the availability of cervical tissue.
Chapter 6

The relationship between seminal plasma cytokine content and fertility status in men
6.1 INTRODUCTION

The previous chapters describe studies which show that deposition of semen into the female reproductive tract following natural intercourse in peri-ovulatory women induces marked changes in cytokine expression and in resident leukocyte populations within the cervix (as described in Chapters 4 and 3 respectively) comparable to those observed in other mammalian species. This inflammatory cascade initiated following intercourse reaches across the full thickness of the cervical epithelium and subjacent stromal tissue with a striking infiltration of macrophages, dendritic cells and lymphocytes in both compartments. As described previously (in Chapter 3), this influx of leukocytes requires direct contact between seminal fluid and the female reproductive tract tissues since no inflammatory response was observed following condom-protected intercourse invoking the actions of specific factors present in seminal fluid. Regulation of the cervical leukocytic infiltrate occurs by activation of the pro-inflammatory cytokines GM-CSF, IL-6 and IL-8 (in Chapter 4).

Other studies in our laboratory have identified the key molecular agents in rodent seminal plasma that mediate activation of female responses. In the mouse, the use of protein chromatography and neutralising antibodies led to TGFβ1 being identified as the predominant initiating factor responsible for initiating the post-mating inflammatory response (Tremellen et al. 1998). In chapter 5 it was demonstrated that members of the TGFβ family including TGFβ1, TGFβ2 and TGFβ3, are key molecules capable of acting independently or in concert to activate cytokine expression in female reproductive tract cells. Experiments using primary ectocervical epithelial and immortalised Ect-1 cells have shown that these isoforms of TGFβ can closely mimic the semen-induced expression of GM-CSF, IL-6 and IL-8 that occurs during the in vivo response of human cervical cells.

Seminal plasma is well recognised for its potent immunomodulatory abilities and is known to contain a diverse range of cytokines including TGFβ1, TGFβ2 and TGFβ3 which are present at concentrations similar to those found in colostrum which is the most abundant biological source of TGFβ known. Addition of TGFβ-specific neutralising antibodies to cultured cervical epithelial cells has also identified that the various isoforms of TGFβ, particularly TGFβ1 and TGFβ3, account for the majority of the seminal plasma activity in humans. Seminal plasma also contains molecules such as IL-8 and bacterial endotoxin that are capable of synergising with TGFβ to enhance stimulation of cytokine production in cervical epithelial cells, along with molecules such as IFNγ, which is a potent inhibitor of the stimulatory activity of TGFβ in these cells (as discussed in chapter 5).
To date, there have been several studies investigating the presence of an array of cytokines in human seminal plasma and the relationship between seminal plasma cytokine content and semen quality or male fertility (Naz et al. 1994, Gruschwitz et al. 1996, Srivastava et al. 1996, Naz et al. 1998, Loras et al. 1999, Pannekoek et al. 2000, Eggert-Kruse et al. 2001, Maegawa et al. 2002, Miller et al. 2002, Friebe et al. 2003). Most of these studies attempted to examine differences in seminal plasma cytokine content between fertile and infertile men (as defined by semen parameters defined as abnormal according to WHO criteria), or in men where urinary tract infection or presence of sexually transmitted pathogens was present. However, it has not been investigated whether altered cytokines have a relationship with fertility independent of interaction with sperm parameters, i.e. a potential role in the female reproductive tract. To investigate this, we have collected semen samples from proven fertile men and male partners of couples experiencing various forms of infertility including male infertility, female infertility, combined male / female infertility, multiple IVF failure, recurrent miscarriage and infertility of unknown origin, to determine whether a relationship exists between fertility status and seminal plasma cytokine content.
6.2 STUDY DESIGN

All couples approached to be involved in this study were undergoing investigation for fertility problems at the University of Adelaide’s reproductive medicine unit (Repromed Pty Ltd.). All potential participants were given an information sheet outlining the purpose of the study and given sufficient time (approximately 48 hours) to consider participation in the study. Written informed consent was then obtained from interested couples before their enrolment in the study (refer to Appendix B for patient information sheet and consent form). Patients were also made aware that they could decline to be involved in the study without affecting their current treatment.

In order to be successfully recruited into the study, male partners who would be providing semen samples had to satisfy a number of selection criteria including:

1. good general health
2. aged 18 - 50 years of age
3. not taking any medications with the potential to modify semen parameters (ie. non-steroidal anti-inflammatory drugs such as aspirin, immune modifying agents such as steroids etc)

A number of exclusion criteria also existed and couples were unable to participate in this study if any of the exclusion criteria were present before or developed during the course of the study. Participants were informed that they could withdraw from the study at any time at the request of either partner without their current treatment being affected. Reasons for exclusion from this study included:

1. Either partner unable to give informed consent.
2. Male partner had symptoms suggestive of a urinary or genital tract infection (dysuria, penile discharge).
3. Male partner was taking medication that may affect semen parameters (immune modifying medication such as methotrexate, NSAIDs).
4. Female patients experiencing recurrent miscarriage in which a potential cause has been found (uterine abnormality, chromosomal abnormality, thrombophilia, hyperhomocysteinaemia, diabetic).
After having met the selection criteria described above and after obtaining informed consent, participants were allocated (by clinical collaborator, Dr Kelton Tremellen) into one of seven fertility classifications depending on their reproductive history and according to the following descriptions:

1. Proven fertile - This group contained men with normal semen characteristics as defined by WHO criteria who had either fathered a child naturally in their current relationship and / or had fathered a child through Repromed's donor insemination program. Only males who had fathered children from normal pregnancies, defined as the delivery (normal vaginal delivery, instrumental or caesarean section) of an infant at term (37 completed weeks) in which there was no evidence of preeclampsia or intra uterine growth restriction during the antenatal course of the pregnancy were included.

2. Male infertility - This group consisted of men with any defect in their semen parameters that resulted in values below the reference ranges according to WHO standards and where there was no evidence of a female problem as the cause of infertility.

3. Female infertility - This group contained men who had normal semen analyses but whose female partners had been identified as having a condition such as anovulation, tubal blockage or endometriosis as their likely cause of infertility.

4. Combined male / female infertility - This group was comprised of male partners of couples where both male and female factors had been identified as the likely cause of infertility.

5. Multiple IVF Failure - This group contained male partners of couples who failed to have a successful pregnancy despite the transfer of 10 good quality embryos during in-vitro fertilization treatment, even if they had a male or female factor as well.

6. Recurrent miscarriage - This group contained male partners of couples who had experienced the successive loss of three or more fetuses in the first trimester of pregnancy, in the absence of an identifiable cause (normal maternal and paternal karyotype; normal uterine cavity according to hysteroscopy / ultrasound or HSG; negative lupus anticoagulant, anticardiolipin antibody and ANA; normal thyroid and glucose function tests; no evidence of hyperhomocystinaemia; normal protein C, protein S and Activated Protein C activity). Couples experiencing recurrent miscarriages
where a cause had been identified (ie. genetic, thrombophilia, severe maternal disease such as diabetes etc) were not included.

7. Infertility of unknown origin - This group was made up of male partners of couples who had been unable to conceive spontaneously, and where tests on both the male (semen defect ruled out) and female (anovulation and tubal blockage excluded) partners failed to identify a potential cause for their infertility.

Semen samples were produced by masturbation, without the use of lubricants or condoms, following a period of at least 48 hours abstinence. The samples were produced at Repromed or were delivered to the Andrology Laboratory at Repromed within 30 min of production. The volume of semen was measured and divided equally into two portions. Semen analysis was performed according to World Health Organisation (WHO) standards (World Health Organisation, 1999) on one portion with parameters such as sperm concentration, motility, morphology, pH etc being recorded. The other portion was centrifuged for 15 min at 14 000 rpm (13 000g) to remove sperm and other cellular debris. The supernatant was then removed, transferred to a fresh tube and stored at - 70°C. Once the seminal plasma samples were received at the medical school they were immediately thawed on ice, aliquoted into 100μl volumes (to prevent repeated freeze thaw cycles) and then refrozen and stored at - 70°C until assayed by ELISA for cytokine content.

6.3 GENERAL SEMEN CHARACTERISTICS OF PARTICIPANTS IN THIS STUDY

The characteristics of participants in each of the individual fertility groups are summarised in Table 6.1. No significant differences in mean age, days abstinence prior to semen sample production, volume of semen produced or the pH of semen samples were identified between any of the fertility groups. Significant differences were observed between proven fertile individuals and those in some of the other fertility groups in regards to semen parameters such as sperm concentration, motility and morphology (Table 6.1). The presence of leukocytes within semen samples were assigned a numerical value of either 0, 1 or 2 depending on the number of leukocytes observed (0 = no leukocytes, 1 = low numbers, 2 = moderate numbers). The mean leukocyte score was then calculated as being the average score obtained for samples in each of the fertility groups.
<table>
<thead>
<tr>
<th>Fertility Group</th>
<th>N</th>
<th>Average Age (years)</th>
<th>Abstinence (days)</th>
<th>Volume (ml)</th>
<th>pH</th>
<th>Sperm Concentration (million/ml)</th>
<th>Motility (% progressive)</th>
<th>Morphology (% Normal)</th>
<th>Mean Leukocyte Score¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proven Fertile</td>
<td>30</td>
<td>X (x-x)</td>
<td>4 (1-7)</td>
<td>3.5 (2-6.6)</td>
<td>8.3</td>
<td>129.9 (14-220)</td>
<td>53.3 (33-61)</td>
<td>27.5 (13-43)</td>
<td>0.9</td>
</tr>
<tr>
<td>Male Infertility</td>
<td>19</td>
<td>34 (26-41)</td>
<td>5 (1-14)</td>
<td>4.1 (1.7-12.0)</td>
<td>8.4</td>
<td>49.2 (0-208)</td>
<td>36.6 (0-60)</td>
<td>10.9 (0-30)</td>
<td>0.6</td>
</tr>
<tr>
<td>Female Infertility</td>
<td>4</td>
<td>35 (29-39)</td>
<td>6 (4-7)</td>
<td>4.4 (1.6-4.7)</td>
<td>8.5</td>
<td>64.5 (58-74)</td>
<td>57.8 (55-62)</td>
<td>26.3 (21-34)</td>
<td>0.0</td>
</tr>
<tr>
<td>Combined Male/Female Infertility</td>
<td>20</td>
<td>35 (27-43)</td>
<td>4 (2-8)</td>
<td>4.1 (1.6-8.0)</td>
<td>8.3</td>
<td>50.4 (2.4-113)</td>
<td>41.3 (0-58)</td>
<td>10.3 (3-19)</td>
<td>0.9</td>
</tr>
<tr>
<td>Multiple IVF Failure</td>
<td>13</td>
<td>36 (28-51)</td>
<td>5 (1-21)</td>
<td>3.6 (1.8-6.5)</td>
<td>8.4</td>
<td>77.6 (4.3-230)</td>
<td>45 (13-60)</td>
<td>16.5 (4-32)</td>
<td>1.1</td>
</tr>
<tr>
<td>Recurrent Miscarriage</td>
<td>16</td>
<td>37 (32-42)</td>
<td>5 (1-14)</td>
<td>3.2 (1.4-6.3)</td>
<td>8.3</td>
<td>101.3 (6.6-288)</td>
<td>44.9 (7-57)</td>
<td>19.9 (1-37)</td>
<td>0.9</td>
</tr>
<tr>
<td>Infertility of Unknown Origin</td>
<td>3</td>
<td>40 (32-45)</td>
<td>4 (2-6)</td>
<td>2.6 (1.4-4.2)</td>
<td>8.4</td>
<td>75.0 (40-95)</td>
<td>52 (45-56)</td>
<td>22 (17-31)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**TABLE 6.1** Characteristics of human semen samples included in this study. Semen samples were produced by masturbation and semen parameters were measured according to World Health Organisation (WHO) standards (WorldHealthOrganisation 1999). The average age of proven fertile individuals included in this study was not available for reasons of confidentiality. Data are presented as means with observed ranges in parentheses. Data was compared initially by a Kruskal-Wallis one-way ANOVA, followed by Mann-Whitney U Tests with statistical significance being inferred when $p < 0.05$. Superscript a = significantly different to proven fertile group, $p < 0.05$ (Mann-Whitney). ¹ = The presence of leukocytes within semen samples were assigned a numerical value of either 0, 1 or 2 depending on the number of leukocytes observed (0 = no leukocytes, 1 = low numbers, 2 = moderate numbers). The mean leukocyte score was then calculated as being the average score obtained for samples in each of the fertility groups.
6.4 THE RELATIONSHIP BETWEEN SEMINAL PLASMA CYTOKINE CONTENT AND FERTILITY STATUS IN MEN

6.4.1 Validation and optimisation of ELISA assays for measuring total TGFβ1 content in human seminal plasma samples

As in any assay system, a number of important criteria must be met to ensure that the results obtained from them are accurate, reliable and reproducible. Since some investigators had identified difficulties in accurately measuring TGFβ content in human blood (Grainger et al. 1995), it was important to investigate whether TGFβ content in human seminal plasma samples could be accurately measure.

Initially the immunoassay for TGFβ1 was validated. It became apparent early on that seminal plasma samples needed to be vigorously centrifuged to remove as much particulate matter as possible, as the presence of remaining cellular debris severely affected the performance of the assay.

To examine the linearity of the assay over a range of seminal plasma dilutions, acid activated seminal plasma samples were serially diluted 1:2, beginning at 1:100 and ending at 1:6400. This starting dilution was chosen based on the approximate TGFβ1 content expected to be present based on previous experiments by other investigators (Nocera et al. 1995, Srivastava et al. 1996, Loras et al. 1999). The concentration of total TGFβ1 was measured at each individual dilution and then final concentration obtained by adjusting for dilution. The corrected concentrations were then compared using regression analysis. Regression analysis showed that the TGFβ1 present in seminal plasma titrated linearly, as evidenced by a regression line with slope equal to 0 (data not shown). A slope other than zero would have indicated an effect of dilution, possibly caused by the presence of interfering compounds within seminal plasma.

The next step was to assess whether TGFβ1 within seminal plasma performed in the assay in a similar manner to the recombinant TGFβ1 standard provided by the assay manufacturer. The test of similarity was performed by plotting the log (absorbance - Y axis) versus log (concentration of recombinant standard - X2 axis), then plotting the log (absorbance - Y axis) versus log (dilution of seminal plasma - X1 axis) (Figure 6.1). Regression analysis was then performed on both sets of data and the resultant regression line for recombinant TGFβ1 and seminal plasma were also plotted.

Sharkey Chapter 6
results of these experiments indicated that the recombinant standard and seminal plasma TGFβ1 reacted similarly in the ELISA assay, since the slope of their regression lines were not significantly different.

![Graph showing absorbance vs. dilution of seminal plasma](image)

**FIGURE 6.1 Test of similarity between recombinant TGFβ1 standard and seminal plasma TGFβ1 content in a TGFβ1-specific ELISA assay.** The test of similarity was performed by plotting the log (absorbance - Y axis) versus log (concentration of recombinant standard - X2 axis) (blue squares) then plotting the log (absorbance - Y axis) versus log (dilution of seminal plasma - X1 axis) (red squares). Regression analysis was then performed on both sets of data and the resultant regression line for recombinant TGFβ1 and seminal plasma were also plotted, indicated by blue and red dashed lines respectively. Similar slopes in regression lines show that the recombinant standard and the seminal plasma react similarly in the ELISA assay.

Once the correct performance of the assay was confirmed, the optimal dilution at which to test seminal plasma samples was then determined. The concentration of total TGFβ1 was measured at a range of dilutions (1:100 - 1:6400) and this experiment was repeated a total of 20 times in order to assess intra- and inter-assay variation. Intra-assay variation was determined by calculating the amount of variation in TGFβ1 content between replicate wells for each dilution tested on each plate individually (ie. each sample was tested in quadruplicate at each dilution in each separate run, repeated a total of 20 times). Dilutions ranging from between 1:100 and 1:3200 were found to have levels of variation below that of the widely accepted level of < 10% (Figure 6.2 A), with mean intra-assay coefficients of
variation ranging from 3 - 8.5%. Inter-assay variation was determined from the same series of experiments by comparing the concentration of TGFβ1 detected at each dilution with that obtained in each of the 20 replicate experiments, then calculating the amount of variation in TGFβ1 concentration between assays (Figure 6.2 B). These experiments identified dilutions ranging from 1:200 - 1:800 as having the least amount of variation between experiments, with inter-assay coefficients of variation ranging from 6.5 - 8.0%. Since dilutions ranging from 1:200 and 1:800 were observed to have the least amount of intra- and inter-assay variation, it was decided that each of the seminal plasma samples would be tested at dilutions that fell within this range. Also, due to the amount of variation other investigators had observed in total TGFβ1 concentrations between individuals, we decided to test each of the samples in duplicate, and at two different dilutions.

FIGURE 6.2 The effect of dilution on TGFβ1 inter-assay coefficient of variation. Multiple acid activated seminal plasma samples were serially diluted 1:2, beginning at a dilution of 1:100 and their total TGFβ1 content determined. These experiments were repeated a total of 20 times and the amount of variation in total TGFβ1 concentration within and between each run was determined for each dilution in each separate experiment. A) Intra-assay variation and B) Inter-assay variation. Data are mean coefficient of variation (%) versus each of the dilutions tested.
6.4.2 Validation and optimisation of ELISA assays for measuring biologically active TGFβ₁, and biologically active and total TGFβ₂

These validation experiments were also performed for biologically active TGFβ₁ and total and biologically active TGFβ₂. Optimal dilutions and intra- and inter-assay coefficients of variation were also determined for total TGFβ₃, IFNγ, IL-8 and endotoxin (Table 6.2). Additionally, positive control samples were included as a quality control standard in every assay.

<table>
<thead>
<tr>
<th></th>
<th>Mean coefficient of variation (%)</th>
<th>Mean coefficient of correlation</th>
<th>Optimal dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-assay</td>
<td>Inter-assay</td>
<td></td>
</tr>
<tr>
<td>Bioactive TGFβ₁</td>
<td>3.8</td>
<td>7.0</td>
<td>0.999</td>
</tr>
<tr>
<td>Total TGFβ₁</td>
<td>3.7</td>
<td>6.5</td>
<td>0.998</td>
</tr>
<tr>
<td>Bioactive TGFβ₂</td>
<td>4.1</td>
<td>7.8</td>
<td>0.987</td>
</tr>
<tr>
<td>Total TGFβ₂</td>
<td>4.3</td>
<td>7.2</td>
<td>0.996</td>
</tr>
<tr>
<td>Total TGFβ₃</td>
<td>3.4</td>
<td>6.1</td>
<td>0.999</td>
</tr>
<tr>
<td>IFNγ</td>
<td>2.5</td>
<td>5.1</td>
<td>0.999</td>
</tr>
<tr>
<td>IL-8</td>
<td>3.6</td>
<td>6.7</td>
<td>0.999</td>
</tr>
<tr>
<td>Endotoxin (LPS)</td>
<td>4.2</td>
<td>8.4</td>
<td>0.985</td>
</tr>
</tbody>
</table>

**TABLE 6.2** Typical performance characteristics of human cytokine assay kits used in this study. Intra- and inter-assay variation for each of the cytokines measured are shown, together with the dilutions at which seminal plasma samples were tested.
To investigate whether a relationship existed between seminal plasma TGFβ1 content and fertility status, semen samples were obtained from proven fertile men and male partners of couples experiencing various forms of infertility as defined in section 6.2. The majority of TGFβ in seminal plasma is present in a latent inactive form and therefore seminal plasma samples were transiently acidified, using the method described in chapter 2, to release biologically active TGFβ from its latent form, prior to being assayed for TGFβ1 content using a TGFβ1-specific ELISA. A separate aliquot of untreated seminal plasma was used for measuring the amount of naturally active TGFβ1 within each of the samples.

Considerable variation was observed between all individuals regarding their total TGFβ1 content, with total TGFβ1 concentrations ranging from approximately 100 ng/ml to 400 ng/ml (5th and 95th percentiles respectively) and a mean concentration of 243 ng/ml within the proven fertile group (Figure 6.1A). The amount of biologically active TGFβ1 in each of the samples was also measured by assaying seminal plasma samples without prior acid treatment (Figure 6.1B). Biologically active TGFβ1 accounted for only approximately 1.3% of the total amount of TGFβ1 detected with no differences in the proportion of active to total TGFβ1 being observed between any of the fertility groups. Again, considerable variation was observed between individuals regarding their biologically active TGFβ1 content, with concentrations ranging from 0.3 ng/ml to 5.2 ng/ml (5th and 95th percentiles respectively) and a mean concentration of 1.9 ng/ml within the proven fertile group.

No differences were observed between the proven fertile group and any of the other fertility groups when comparing the concentration of biologically active or total TGFβ1 in seminal plasma. This was also the case when the total content of active and total TGFβ1 from each of the samples was calculated (ie. concentration of active or total TGFβ1 x volume of semen sample = total TGFβ1 content).
FIGURE 6.3 Concentration of total and biologically active TGFβ1 in human seminal plasma. A) To measure the total amount of TGFβ1 contained in human seminal plasma, samples were transiently acidified using 1M HCl to release biologically active TGFβ1 from its latent complex followed by neutralisation. Following activation, samples were diluted to a final dilution of 1:300 and 1:600 in TGFβ sample buffer immediately prior to assay. B) To measure biologically active TGFβ1, neat seminal plasma samples were diluted in TGFβ sample buffer to a dilution of 1:10 and 1:20 prior to assay. All seminal plasma samples were measured in duplicate and at two concentrations. The box and whisker plots show the 25th percentile (lower edge of box), the median (solid line within the box), the 75th percentile (upper edge of box), and the mean (dotted line). Error bars above and below the box indicate the 95th and 5th percentiles respectively while closed circles show outlying points, defined as falling > 2 SD from the mean. The number of individuals in each group are shown in parentheses. Data was analysed by Kruskal-Wallis test and no effect of fertility classification on biologically active or total TGFβ1 concentration was observed.
To investigate whether a relationship exists between seminal plasma TGFβ2 content and fertility status, semen samples were obtained from proven fertile men and male partners of couples experiencing various forms of infertility as defined in section 6.2. Again, acid treated seminal plasma was used for determining TGFβ2 content using a TGFβ2-specific ELISA. A separate aliquot of untreated seminal plasma was used to measure the amount of naturally active TGFβ2 in each sample.

Total TGFβ2 concentrations were detected at levels approximately 50 times lower than that of total TGFβ1. As was the case with the TGFβ1 results, considerable variation was observed between all individuals regarding their total TGFβ2 content, with total TGFβ2 concentrations ranging from approximately 2.3 ng/ml to 10.1 ng/ml (5th and 95th percentiles respectively) and a mean concentration of 5.1 ng/ml within the proven fertile group (Figure 6.2 A). The amount of biologically active TGFβ2 in each of the samples was measured by assaying seminal plasma samples that had not been acidified (Figure 6.2 B). Biologically active TGFβ2 was present at concentrations almost 8 times less than that of active TGFβ1. The concentration of biologically active TGFβ2 in seminal plasma accounted for approximately 5% of the total amount of TGFβ2 detected, with no differences in the proportion of active to total TGFβ2 observed between any of the fertility groups. Again, considerable variation was observed between individuals regarding their biologically active TGFβ2 content, with concentrations ranging from 0.08 ng/ml to 0.6 ng/ml (5th and 95th percentiles respectively) and a mean concentration of 0.25 ng/ml within the proven fertile group.

No differences were observed between the proven fertile group and any of the other fertility groups in the concentration of biologically active or total TGFβ2 in seminal plasma samples. This was also the case when the total content of active and total TGFβ2 in each sample was calculated (ie. concentration of active or total TGFβ2 x volume of semen sample = total TGFβ2 content).
FIGURE 6.4 Concentration of total and biologically active TGFβ2 in human seminal plasma. To measure the total amount of TGFβ2 contained in human seminal plasma, samples were transiently acidified to release biologically active TGFβ2 from its latent complex followed by neutralisation. Following activation, samples were diluted to a final dilution of 1:10 and 1:20 in TGFβ sample buffer immediately prior to assay. To measure biologically active TGFβ2, seminal plasma samples were tested neat and also diluted in TGFβ sample buffer to a dilution of 1:2 prior to assay. All seminal plasma samples were measured in duplicate and at two concentrations. The box and whisker plots show the 25th percentile (lower edge of box), the median (solid line within the box), the 75th percentile (upper edge of box), and the mean (dotted line). Error bars above and below the box indicate the 95th and 5th percentiles respectively while closed circles show outlying points, defined as falling > 2 SD from the mean. The number of individuals in each group are shown in parentheses. Data was analysed by Kruskal-Wallis test and no effect of fertility classification on biologically active or total TGFβ2 concentration was observed.
6.4.5  The relationship between seminal plasma TGFβ3 content and fertility status

To investigate whether a relationship exists between seminal plasma TGFβ3 content and fertility status, semen samples were obtained from proven fertile men and male partners of couples experiencing various forms of infertility as defined in section 6.2. Acid treated seminal plasma was used for determining TGFβ3 content using a TGFβ3-specific ELISA.

This study was the first to measure TGFβ3 content within human seminal plasma samples using a TGFβ3-specific ELISA kit which has only recently become available commercially. TGFβ3 like the other isoforms of TGFβ mentioned in previous sections above was detectable in all of the samples tested. Total TGFβ3 concentrations were relatively high, with a mean level in the fertile group just 40% lower than that observed for total TGFβ1 and 30 times higher than that observed for total TGFβ2. Greater variation in total TGFβ3 concentration was observed than those seen for both the total TGFβ1 and TGFβ2 results, with total TGFβ3 concentrations ranging from approximately 26 ng/ml to 460 ng/ml (5th and 95th percentiles respectively) and a mean concentration of 146.5 ng/ml within the proven fertile group (Figure 6.2 A). Unfortunately we were unable to measure the amount of biologically active TGFβ3 in these samples due to insufficient sample. Obtaining total TGFβ3 content allowed us to determine the potential amount of TGFβ3 that could be activated once deposited into the female reproductive tract.

No differences were observed between the proven fertile group and any of the other fertility groups when comparing the concentration of total TGFβ3 within seminal plasma samples. This was also the case when the total content of total TGFβ3 in each sample was calculated (ie. concentration of total TGFβ3 x volume of semen sample = total TGFβ3 content).
FIGURE 6.5 Concentration of total TGFβ3 in human seminal plasma. To measure the total amount of TGFβ3 contained in human seminal plasma, samples were transiently acidified to release biologically active TGFβ3 from its latent complex followed by neutralisation. Following activation, samples were diluted to a final dilution of 1:150 and 1:300 in TGFβ sample buffer immediately prior to assay. All seminal plasma samples were measured in duplicate and at duplicate concentrations. Due to a lack of sample remaining, biologically active TGFβ3 was not able to be measured. All seminal plasma samples were measured in duplicate and at two concentrations. The box and whisker plots show the 25th percentile (lower edge of box), the median (solid line within the box), the 75th percentile (upper edge of box), and the mean (dotted line). Error bars above and below the box indicate the 95th and 5th percentiles respectively while closed circles show outlying points, defined as falling > 2 SD from the mean. The number of individuals in each group are shown in parentheses. Data was analysed by Kruskal-Wallis test and no effect of fertility classification on total TGFβ3 concentration was observed.

6.4.6 The relationship between seminal plasma total TGFβ content and fertility status

To investigate whether a relationship exists between total TGFβ content within human seminal plasma samples and fertility status in men, the individual concentrations of total TGFβ1, TGFβ2 and TGFβ3 were added together to calculate total TGFβ (ie. total TGFβ1 + β2 + β3 = total TGFβ).

A high degree of variation between individual total TGFβ values was observed, with total TGFβ concentrations ranging from 186 ng/ml to 683 ng/ml (5th and 95th percentiles respectively) and a mean concentration of 394 ng/ml within the proven fertile group (Figure 6.6). The total amount of biologically active TGFβ in each of the samples was unable to be calculated because the level of bioactive TGFβ3 was no determined.
No difference in the total amount of TGFβ was observed in any of the fertility groups compared to the proven fertile group (Figure 6.6). This was also the case when the total TGFβ content was calculated (ie. concentration of total TGFβ₁ + TGFβ₂ + TGFβ₃ x volume of semen sample = total TGFβ content).

**FIGURE 6.6** Concentration of total TGFβ in human seminal plasma. The total TGFβ content was calculated by combining the concentrations of total TGFβ₁, TGFβ₂ and TGFβ₃ obtained individually. The box and whisker plots show the 25th percentile (lower edge of box), the median (solid line within the box), the 75th percentile (upper edge of box), and the mean (dotted line). Error bars above and below the box indicate the 95th and 5th percentiles respectively while closed circles show outlying points, defined as falling > 2 SD from the mean. The number of individuals in each group are shown in parentheses. Data was analysed by Kruskal-Wallis test and no effect of fertility classification on total TGFβ concentration was observed.
6.5 THE RELATIONSHIP BETWEEN IL-8 CONTENT IN SEMINAL PLASMA AND FERTILITY STATUS IN MEN

To investigate whether a relationship existed between seminal plasma IL-8 content and fertility status, semen samples were obtained from proven fertile men and male partners of couples experiencing various forms of infertility as defined in section 6.2. The concentration of IL-8 in each of the samples was determined using an IL-8-specific ELISA.

Variation was observed between individuals in their seminal plasma IL-8 content, with IL-8 concentrations ranging from 1.3 ng/ml to 4.4 ng/ml (5th and 95th percentiles respectively) and a mean concentration of 2.5 ng/ml within the proven fertile group (Figure 6.7).

No differences were observed between the proven fertile group and any of the other fertility groups when comparing the concentration of IL-8 within seminal plasma samples. This was also the case when the total content of IL-8 from each of the samples was calculated (ie. concentration of IL-8 x volume of semen sample = total IL-8 content).

FIGURE 6.7 The effect of IL-8 concentration on pregnancy outcome. IL-8 was measured by diluting neat seminal plasma samples in sample buffer to a final dilution of 1:2 and 1:4 prior to assay. All seminal plasma samples were measured in duplicate and at two concentrations. The box and whisker plots show the 25th percentile (lower edge of box), the median (solid line within the box), the 75th percentile (upper edge of box), and the mean (dotted line). Error bars above and below the box indicate the 95th and 5th percentiles respectively while closed circles show outlying points, defined as falling > 2 SD from the mean. The number of individuals in each group are shown in parentheses. Data was analysed by Kruskal-Wallis test and no effect of fertility classification on IL-8 concentration was observed.
6.6 THE RELATIONSHIP BETWEEN IFNγ CONTENT AND FERTILITY STATUS IN MEN

To investigate whether a relationship exists between seminal plasma IFNγ content and fertility status in men, the concentration of IFNγ was measured in each of the seminal plasma samples. Initially, IFNγ content was measured using a standard ELISA where the lower limit of detection was determined to be approximately 5 pg/ml. The results of these experiments showed that the concentration of IFNγ in seminal plasma was relatively low, with detectable levels of IFNγ above this threshold observed in only 5% of the samples tested (data not shown). To more accurately measure IFNγ content we acquired a highly sensitive IFNγ ELISA with a lower level of detection of 0.1 pg/ml. The use of this kit allowed us to detect IFNγ in more than 90% of the samples tested and gave us an increased ability to detect small differences in concentrations between individuals. For the purpose of data analysis, any sample with a concentration of IFNγ below that of the lower level of detection was assigned a value of 0.1 pg/ml.

Variation was evident between all individuals in their seminal plasma IFNγ content, with concentrations ranging from approximately 0.4 pg/ml to 4.6 pg/ml (5th and 95th percentiles respectively) and a mean concentration of 1.4 pg/ml within the proven fertile group (Figure 6.8).
No relationship between fertility classification and IFNγ content was seen when data was compared by Kruskall-Wallis test. This was also the case when the total content of IFNγ from each of the samples was calculated (ie. concentration IFNγ x volume of semen sample = total IFNγ content).

![Graph showing IFNγ concentration on fertility status](Image)

**FIGURE 6.8 The effect of IFNγ concentration on fertility status.** The concentration of IFNγ within human seminal plasma samples was measured using a high sensitivity IFNγ-specific ELISA with a lower level of detection limit of 0.1 pg/ml. This scatter plot shows the concentration of IFNγ (pg/ml) obtained from each individual included in the study. Men whose concentration of IFNγ was below the lower level of detection of the assay were assigned a concentration of 0.1 pg/ml on the graph. The horizontal dotted line depicts an arbitrary threshold of 3 pg/ml (refer to text for details).

Observation of the IFNγ data set suggested the existence of two subpopulations, with the majority of samples containing low levels of IFNγ, and a smaller population of samples containing high IFNγ. When a threshold of 3 pg/ml is applied, it can be seen that 3 / 30 (10%) of proven fertile men fall into the 'high IFNγ' category, compared to 13 / 73 (18%) of male partners of infertile couples (Figure 6.8). In particular high IFNγ was prevalent in male partners of women experiencing recurrent miscarriage (refer to Table 6.3) where 5 / 14 (36%) scored high ($p = 0.005$, $\chi^2$ test versus proven fertile group).
Since there is also evidence indicating that cytokines are able to exert their immunological functions in concert with each other, it was postulated that the relative abundance of each of the individual cytokines within seminal plasma may be important to its overall biological function. To investigate this, IFNγ content was evaluated relative to the total TGFβ concentration (total TGFβ : IFNγ ratio). Observation of the data showed that when a total TGFβ : IFNγ ratio threshold of $200 \times 10^3$ was applied, the discriminating value of IFNγ was increased as demonstrated by the finding that only 5 / 30 (17%) of proven fertile individuals have a ratio below this threshold whereas 6 / 14 (43%) of male partners of couples experiencing recurrent miscarriage have a ratio below this threshold ($p = 0.003$, $\chi^2$ test versus proven fertile group) (Table 6.3).

<table>
<thead>
<tr>
<th>Fertility Status</th>
<th>N</th>
<th>IFNγ Concentration (pg/ml)</th>
<th>IFNγ (&gt; 3 pg/ml)</th>
<th>Total TGFβ:IFNγ (&lt; 200 x 10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proven Fertile</td>
<td>30</td>
<td>1.4 ± 0.3</td>
<td>3 / 30 (10%)</td>
<td>5 / 30 (17%)</td>
</tr>
<tr>
<td>Male Infertility</td>
<td>19</td>
<td>2.1 ± 0.4</td>
<td>3 / 19 (16%)</td>
<td>5 / 17 (29%)</td>
</tr>
<tr>
<td>Female Infertility</td>
<td>4</td>
<td>0.8 ± 0.3</td>
<td>0 / 4 (0%)</td>
<td>0 / 4 (0%)</td>
</tr>
<tr>
<td>Combined Male / Female Infertility</td>
<td>20</td>
<td>2.1 ± 0.7</td>
<td>3 / 20 (15%)</td>
<td>4 / 16 (25%)</td>
</tr>
<tr>
<td>Multiple IVF Failure</td>
<td>13</td>
<td>2.6 ± 1.1</td>
<td>2 / 13 (15%)</td>
<td>4 / 13 (31%)</td>
</tr>
<tr>
<td>Recurrent Miscarriage</td>
<td>14</td>
<td>3.7 ± 1.3</td>
<td>5 / 14* (36%)†</td>
<td>6 / 14** (43%)</td>
</tr>
<tr>
<td>Infertility of unknown origin</td>
<td>3</td>
<td>0.1 ± 0.03</td>
<td>0 / 3 (0%)</td>
<td>0 / 3 (0%)</td>
</tr>
</tbody>
</table>

TABLE 6.3 Comparison of seminal plasma IFNγ concentrations between proven fertile men and male partners of couples experiencing infertility. Data are expressed as mean concentration (pg/ml) +/- SEM. Proportion data was analysed by $\chi^2$ test. * $p = 0.005$, ** $p = 0.003$, or Fischer’s exact test † $p = 0.08$ compared with proven fertile group.

6.7 EFFECT OF SEMINAL PLASMA BACTERIAL ENDOTOXIN CONCENTRATION ON PREGNANCY OUTCOME

To investigate whether a relationship exists between bacterial endotoxin content and fertility status, semen samples were obtained from proven fertile men and male partners of couples experiencing various forms of infertility as defined in section 6.2. The concentration of endotoxin in each of the samples was determined using the limulus amebocyte lysate assay.
Variation was observed between individuals in their seminal plasma endotoxin content, with concentrations ranging from 1.5 to 60 EU/ml (equivalent units / ml) (5th and 95th percentiles respectively) and a mean concentration of 15.8 EU/ml within the proven fertile group (Figure 6.9).

However, using Kruskall-Wallis U-test, no difference in seminal plasma endotoxin concentrations was observed between any of the fertility groups when compared to the proven fertile group. This was also the case when the concentration of endotoxin was adjusted for the total volume of the ejaculate.

**FIGURE 6.9 Concentration of bacterial endotoxin within human seminal plasma samples.** Bacterial endotoxin was measured by diluting neat seminal plasma samples in sterile pyrogen-free water to a final dilution of 1:5 and 1:10 prior to assay. All seminal plasma samples were measured in duplicate and at two concentrations. The box and whisker plots show the 25th percentile (lower edge of box), the median (solid line within the box), the 75th percentile (upper edge of box), and the mean (dotted line). Error bars above and below the box indicate the 95th and 5th percentiles respectively while closed circles show outlying points, defined as falling > 2 SD from the mean. The number of individuals in each group are shown in parentheses. Data was analysed by Kruskal-Wallis test and no effect of fertility classification on endotoxin concentration was observed.
ASSOCIATIONS BETWEEN CYTOKINE AND SPERM PARAMETERS IN SEMEN

Correlation between individual cytokine content

In order to assess whether associations existed between the abundance of any of the individual cytokines analysed, bi-variate analysis (Pearson's correlation) was performed on the entire seminal plasma data set.

Firstly we explored the relationship between total TGFβ1, total TGFβ2 and total TGFβ3 concentrations, however none were identified. Weak negative associations between biologically active TGFβ1 concentration and both total TGFβ2 \((r = -0.244, p = 0.014)\) and total TGFβ3 \((r = -0.206, p = 0.04)\) were seen. Biologically active TGFβ3 was found to be strongly positively correlated with total TGFβ3 concentration \((r = 0.829, p < 0.00)\). A positive relationship was also observed between biologically active TGFβ1 and biologically active TGFβ2 \((r = 0.301, p = 0.016)\).

Next we investigated the association between IFNγ content and each of the other cytokines measured. IFNγ was found to be positively correlate with IL-8 concentration \((r = 0.305, p = 0.002)\) but no other associations were identified. IL-8 concentration was positively correlated with both total TGFβ1 \((r = 0.278, p = 0.006)\) and total TGFβ3 concentrations \((r = 0.243, p = 0.015)\).

Bacterial endotoxin was identified as having a negative correlation with biologically active TGFβ1 \((r = -0.192, p = 0.05)\) and a negative correlation with biologically active TGFβ2 \((r = 0.289, p = 0.023)\). Endotoxin concentrations failed to correlate with any of the other cytokines measured.
6.8.2 Correlation between cytokine content and sperm parameters

Bi-variate analysis was also performed to investigate whether any associations existed between seminal plasma cytokine content and sperm parameters.

No correlations were identified between the concentrations of total TGFβ1, TGFβ2 or TGFβ3 and any of the semen parameters analysed. A negative correlation was identified between the concentration of biologically active TGFβ1 and sperm motility (r = -0.274, p = 0.005), while biologically active TGFβ2 and sperm morphology were positively correlated (r = 0.256, p = 0.04).

IL-8 did not correlate with any of the sperm parameters measured however it was found to have a positive correlation with volume of ejaculate (r = 0.302, p = 0.002), suggesting the greater the volume of ejaculate, the higher the IL-8 content.

No correlations were identified between IFNγ content and any of the semen parameters measured, as was also the case for bacterial endotoxin.

6.8.3 Correlation between cytokine content and leukocytes

Finally we wished to investigate whether there was any association between individual cytokine content and the relative abundance of leukocytes within semen samples.

A positive correlation was identified between IFNγ content and the presence of leukocytes within semen (r = 0.288, p = 0.007), with positive correlations also observed for total TGFβ1 concentration (r = 0.283, p = 0.011) and total TGFβ (total TGFβ1 + β2 + β3) concentrations (r = 0.272, p = 0.015).
6.9 VARIATION IN SEMINAL PLASMA TGFβ CONTENT WITHIN INDIVIDUALS OVER TIME

Multiple seminal plasma samples were obtained from a number of individuals participating in this study, with samples collected at approximately 2 month intervals over a period of 12 months. The collection of these samples allowed us to assess variation in TGFβ cytokine content within individuals over time.

Differences in the extent of variation within individuals over time was observed in total TGFβ₁ content (Figure 6.10 A). Total TGFβ₁ concentrations were found to fluctuate within seminal plasma samples by between 9% and 26% from the mean concentration for each individual, with an average variability of 19%. Biologically active TGFβ₁ was found to be more variable than total TGFβ₁ (Figure 6.10 B), with variation ranging from 29% - 52%, with an average variability of 36%.

Total TGFβ₂ concentration was more variable within individuals than total TGFβ₁, with some individuals exhibiting variations as low as 11%, while others were considerably higher at 60% (Figure 6.10 C). The average variability from mean total TGFβ₂ concentrations in repeat seminal plasma samples was 30%. Within individuals, biologically active TGFβ₂ was found to vary at levels comparable to that of total TGFβ₂ and biologically active TGFβ₁, with variation ranging from 10% - 61%, with an average variability of 36% (Figure 6.10 D).

Total TGFβ₃ concentrations were found to be slightly more variable within individuals than observed for total TGFβ₁ and lower than for total TGFβ₂, with some individuals exhibiting variations as low as 10%, while others were higher at 29% (Figure 6.10 E). The average variability from mean total TGFβ₃ concentrations in repeat seminal plasma samples was found to be 24%.

When total TGFβ concentrations were compared within individuals, the extent of the variation was lower than that observed for each of the individual TGFβ₁, TGFβ₂ and TGFβ₃ concentrations. Total TGFβ concentrations in seminal plasma were found to fluctuate by between 7% and 22% from the mean concentration for each individual, with an average variability of 16% (Figure 6.10 F).
FIGURE 6.10 Variation in seminal plasma TGFβ content within individuals over time. Multiple seminal plasma samples were collected from individuals at approximately 2 monthly intervals over a 12 month period in order to assess variation in cytokine concentrations over time. Variation in total TGFβ1 (A) bioactive TGFβ1 (B) total TGFβ2 (C) bioactive TGFβ2 (D) total TGFβ3 (E) and total TGFβ (F) are shown, with each plot showing the individual concentrations of cytokines over a 12 month period. Each same coloured line on each of the plots represents results from the same individual.
6.10 VARIATION IN SEMINAL PLASMA IFN\textsubscript{\(\gamma\)}, IL-8 AND ENDOTOXIN CONTENT WITHIN INDIVIDUALS OVER TIME

Variation in each of the individual cytokines was also examined using the multiple repeat seminal plasma samples collected from subjects participating in this study.

There was considerable variation within individuals in IFN\textsubscript{\(\gamma\)} content over time (Figure 6.11 A). IFN\textsubscript{\(\gamma\)} concentrations were found to fluctuate in seminal plasma by between 23% and 91% from the mean concentration for each individual, with an average variability of 51%.

Less variation was observed when comparing IL-8 content within repeat seminal plasma samples from the same individual than observed for IFN\textsubscript{\(\gamma\)} (Figure 6.11 B). Seminal plasma IL-8 concentrations were found to fluctuate from the mean concentration by between 9% and 33% within individuals, with an average variability of 25%.

Bacterial endotoxin content was by far the most variable factor measured within seminal plasma samples (Figure 6.11 C). Endotoxin concentrations were found to fluctuate from their mean concentration by between 21% and 187% within individuals, with an average variability of 83%.
FIGURE 6.11 Variation in individual cytokine content within individuals over time. Multiple seminal plasma samples were collected from individuals at approximately 2 monthly intervals over a 12 month period in order to assess variation in cytokine concentrations over time. Variation in IFNγ (A) IL-6 (B) and endotoxin (C) are shown, with each line and scatter plot showing the individual concentrations of cytokines over a 12 month period. Each same coloured line on each of the plots represents results from the same individual.
6.11 DISCUSSION

The experiments described in this chapter aimed to investigate whether seminal cytokine content is associated with fertility in men, either linked with or independent of the conventional parameters. Our results suggest there is no relationship between seminal plasma TGFβ1, TGFβ2 or TGFβ3 concentrations and fertility status in men, since comparable concentrations were detected within each of the impaired fertility groups when compared to the proven fertile group. Nor are they associated with semen parameters, with no correlation between cytokine content and sperm number, motility or morphology. This finding was consistent with other studies who failed to detect any difference in TGFβ1 concentrations between proven fertile and men with male factor infertility (Srivastava et al. 1996, Loras et al. 1999).

The levels of total TGFβ1 reported in our study (mean 243 ng/ml, proven fertile group) were comparable to those observed by Nocera and Chu (mean 238 ng/ml in pooled seminal plasma samples) (Nocera et al. 1995), but were considerably higher than those found by other investigators (Srivastava et al. 1996, Loras et al. 1999). These differences in the amount of total TGFβ1 detected may be due in part to differences in the nature of the assays used for detection, since early measurements were performed by adding seminal plasma to TGFβ sensitive CCL-64 mink lung cells and testing for inhibition of cell proliferation (Lokeshwar et al. 1992, Nocera et al. 1993). Also, those who reported lower concentrations may not have adequately activated all of the TGFβ in seminal plasma samples. Investigators stated that the activation process was carried out according to the assay manufacturer’s instructions, however we found that these protocols resulted in incomplete activation when tested on our seminal plasma samples. In our study, we confirmed that all seminal plasma samples were acidified to a pH of 3.0 - 4.0, a pH reported to induce maximal activation (Nocera et al. 1995). pH testing was not reported as being performed in any other study except for Nocera and Chu, where comparable concentrations of total TGFβ1 were observed.

Furthermore, we did not find any effect of fertility status on total TGFβ2 concentrations. The concentrations observed were comparable to those obtained in the only other study to measure TGFβ2 content in seminal plasma (Nocera et al. 1995).

Importantly, this study is the first to actually identify and measure TGFβ3 content in human seminal plasma samples. Until now, investigators had only speculated that TGFβ3 may also be present...
in seminal plasma, a suggestion which became more likely when recently it was found present within Leydig cells of human testes where it is believed to play a role in regulating spermatogenesis (Zhang et al. 2004). Total TGFβ3 was detected at high concentrations, just 40% lower than that of TGFβ1, and more than 30 times greater than TGFβ2. With a mean concentration of 146 ng/ml in the proven fertile group, total TGFβ3 content was not associated with fertility status.

Whilst considerable variation in total TGFβ1, TGFβ2 and TGFβ3 was observed between individuals, there was relatively little (<30%) variation within individuals over time as assessed by analysing repeat seminal plasma samples collected over a period of approximately 12 months. Interestingly, while the relative abundance of each of these isoforms fluctuated within individuals, the total TGFβ concentration, as determined by combining the concentrations of total TGFβ1, TGFβ2 and TGFβ3, exhibited the least amount of variation, averaging 17%. The low level of variation detected indicates the representative value of measurement at a single time point.

We also found that while there was no relationship between fertility status and the content of biologically active TGFβ1 or TGFβ2 (mean, 1.9 ng/ml and 0.25 ng/ml respectively, proven fertile group). Concentrations of biologically active TGFβ1 and TGFβ2 in proven fertile controls were comparable to previous studies (Nocera et al. 1995, Loras et al. 1999), and representing less than 2% and 5% of the total TGFβ1 and TGFβ2 respectively. The presence of TGFβ in a predominantly latent form may have a number of physiological advantages, including preventing TGFβ from having a biological effect at its site of production, an important consideration given the extremely diverse range of cell types on which it can exert its effects (Moses et al. 1987). Presumably, mechanisms must exist within the female reproductive tract to release active TGFβ from its latent precursor form as has been observed in the mouse (Tremellen et al. 1998). The acidic pH of the vagina, as well as the presence of numerous enzymes (plasmin, TSP-1 etc) implicated in the release of active TGFβ from its latent complex have been identified as being present within the female reproductive tract (Chu et al. 1998, Khalil 1999) (discussed in more detail in chapter 1). This stringently controlled activation and release of TGFβ within the female reproductive tract may contribute to a sustained release of active TGFβ, thereby allowing it to have a more prolonged biological effect.

Importantly, this current study was the first of its kind to investigate whether seminal plasma IFNγ content is associated with fertility status in men. While we did not observe a relationship between IFNγ content and fertility status in men when directly comparing IFNγ concentrations it was observed
that there is an increased likelihood of high IFNγ content in the male partners of couples experiencing infertility. The proportion of individuals scoring above an arbitrary threshold value of 3 pg/ml was 3 / 30 (10%) in the proven fertile group, versus 13 / 73 (18%) in male partners of infertile couples. In particular, the proportion of individuals with high IFNγ was greatest in male partners of women experiencing recurrent miscarriage, where 5 / 14 (36%) scored > 3 pg/ml. Interestingly, the discriminating value of IFNγ was increased when evaluated as a ratio of total TGFβ content.

Importantly, raised IFNγ was found to be independent of sperm parameters, with no increase in men exhibiting male factor infertility as defined by WHO criteria and no correlation with sperm number, morphology or motility. This finding was in direct contrast to a previous study which detected elevated IFNγ in men with male factor infertility compared to proven fertile men, along with a negative correlation between IFNγ concentration and sperm parameters (Paradisi et al. 1996). Conversely, another study reported no difference in IFNγ content between fertile men and men with sperm parameter-related infertility, and no correlation with sperm parameters (Fujisawa et al. 1998). We did however identify positive correlations between IFNγ content and leukocyte score (discussed in more detail below), as well as IL-8 concentration.

IL-8 is predominantly produced by macrophages in response to foreign antigen or infection and is responsible for recruiting and activating neutrophils. We found IL-8 to be constitutively expressed in all seminal plasma samples (mean 2.5 ng/ml, proven fertile group), with no relationship between IL-8 content and fertility status and unlike previous studies (Eggert-Kruse et al. 2001, Maegawa et al. 2002, Friebe et al. 2003), we failed to observe any correlation between IL-8 and leukocyte score. This discrepancy may be due to the populations from which the other studies sourced their participants. Specifically, their studies compared IL-8 content from normal proven fertile men with men who had being diagnosed with leukocytospermia, a sign of male tract infection, whereas our study precluded individuals with any overt signs of infection. IL-8 was also not correlated with semen parameters, but was positively correlated with IFNγ content. The coincident elevation of these two pro-inflammatory cytokines raises the possibility of a previously undiagnosed infection or some other immune pathology in some of these individuals.

This study was also the first to measure bacterial endotoxin in human seminal plasma samples and investigate whether there was a relationship with fertility status in men. Endotoxin was detected in all samples, however no relationship with fertility status was identified. Interestingly, endotoxin has been shown to elicit many of the same pro-inflammatory cytokines from female reproductive tissues as seminal plasma, including IL-8 synthesis in humans (Watari et al. 2003) and GM-CSF production in...
mice (Glynn and Robertson, Unpublished data). Endotoxin may therefore contribute to female responsiveness to TGFβ present in seminal plasma (Robertson et al. 1991). The effects of endotoxin are elicited through binding to toll-like receptors (TLR) on epithelial cells (Wolfs et al. 2002, Backhed et al. 2003, Samuelsson et al. 2004, Schaefer et al. 2004, Hirata et al. 2005). Whether there is any physiological significance of the source of endotoxin, for example whether probiotic Lactobacilli elicit the same cytokine response as pathogenic bacteria associated with sexually transmitted infections, remains to be examined.

To date we have no indication of the determinants of seminal plasma cytokine status, particularly the reason for elevated IFNγ content in some men. IFNγ is characteristically synthesised by lymphocytes and natural killer cells in response to viral and bacterial challenge. Our seminal plasma samples were sourced from individuals without diagnosed infection or overt symptoms. However we observed a positive correlation between IFNγ content and seminal leukocyte score, and have witnessed increased viscosity in seminal plasma samples from infertile men, both markers of immune pathologies. On the basis of its known role in the immune response, we postulate that IFNγ synthesis in male reproductive tract tissues is associated with the presence of viral or bacterial infection. This suggestion is supported by the recent observation that the addition of Sendai virus, a virus similar to mumps with a tropism for testicular cells, to cultures of human Leydig cells causes a dramatic increase in the amount of IFNγ produced by these cells (Le Goffic et al. 2002). Furthermore, many common sexually transmitted infections in the male tract are asymptomatic, and therefore may not produce any overt symptoms, with high incidences depending on the reference population ranging from approximately 10 - 50% for Herpes Simplex Virus (HSV), 6 - 7% for Cytomegalovirus (CMV), 15% for Epstein Barr Virus (EBV) and 15 - 23% for Human Papilloma Virus (HPV) (Dejucq et al. 2001, Aynaud et al. 2002, Rintala et al. 2004). Male genital tract infections have also been reported as often not altering the quality of semen as assessed by WHO criteria (Dejucq et al. 2001, Rintala et al. 2004), and therefore is not commonly regarded as having functional consequences for fertility.

Alternatively, it is reasonable to speculate that immune pathologies independent of any infectious agent, for example auto-immune mediated inflammatory activity, might result in elevated IFNγ. Interestingly, altered abundance and viscosity of secretions are evident in other mucosal tissues in the event of auto-immune pathology, i.e. in Sjogren’s syndrome where low saliva and dry eyes occur (Kassan 2001, Streckts et al. 2001, Soto-Rojas et al. 2002, Chambers 2004).
Sperm-reactive antibodies (SpAbs) are found attached to sperm, or present in serum or seminal plasma, in approximately 6% of men experiencing infertility (Baker et al. 1983). There are a number of potential causes for SpAbs with the most likely causes being obstruction or herniation (de Kretser et al. 1998), blunt testicular trauma, acute epididymitis and genital tract infection (Witkin et al. 1992). The diverse range of antigens to which these antibodies are generated means that only a relatively small proportion of individuals are diagnosed, perhaps allowing their presence to go undetected. Interestingly, men with SpAbs have been identified as having T lymphocytes with reduced inhibitory activity within their seminal plasma (Imade et al. 1997), with investigators implicating these cells as contributing to the generation of these antibodies. The presence of these antibodies clearly alters the function of sperm (reviewed by McLachlan, 2002) (McLachlan 2002), though whether they also alter the local cytokine environment has not been investigated.

Although infection is postulated as being the most likely explanation for elevated IFNγ in seminal plasma, it is also possible that other factors may contribute to its presence. Besides infection, we can speculate that factors such as smoking and nutrition may also play a role. Cigarette smoking has been associated with altered systemic cytokine production by peripheral leukocytes (van der Vaart et al. 2004) and therefore it is possible that seminal cytokine production may be affected as a result of this exposure. Whether cigarette smoking or nutrition can influence seminal cytokine production are yet to be examined.

The results described in this chapter together with those in chapter 5 highlight the potential diagnostic value of measuring cytokines and related immune mediators in seminal plasma. It is becoming increasingly apparent that the relative abundance of these agents are likely to be important in generating an optimal maternal immune environment for successful pregnancy. Therefore, it would be useful to identify and evaluate other inflammation inducing moieties present in semen that are likely to play a role in determining fertility status. These may include molecules that are capable of enhancing, or inhibiting the stimulatory ability of TGFβ on female reproductive tract cells. Potential candidate molecules could be identified using either immortalised Ect1 or primary cervical epithelial cell cultures (as described in chapter 5) with the aid of neutralising antibodies, then their presence and concentration in human seminal plasma would be determined using commercially available assays. This would be advantageous over untargeted ‘trawling’ of our seminal plasma tissue bank as it would preserve precious sample, ensuring that the molecules tested had proven biological effects in the female reproductive tract and would allow the evaluation of potential interactions between these molecules. Of particular interest are agents such as Activin, 19-hydroxy PGE, IL-6, IL-12 and TNFα, all
of which have been shown to be present in human seminal plasma (Hussenet et al. 1993, Kelly et al. 1994, Naz et al. 1994, Anderson et al. 1998, Naz et al. 1998), and most of which have already been identified as being able to interact with either cervical or endometrial epithelial cells in our preliminary experiments and studies in other laboratories.

The major limitations encountered during this study related to the volume of seminal plasma collected and the time taken to identify and recruit suitable study participants. Firstly, the volume of semen produced by each individual varied greatly. Therefore, for those individuals from whom we received only a very small volume of sample (approximately 500 μl), the number of cytokines that we could investigate was limited. This problem was compounded by the fact that immediately following collection, half of the sample was removed for semen analysis. The second problem we had was the amount of time taken to recruit subjects into the study. Our stringent criteria for inclusion in the study and our strict definitions of each of the fertility groupings made recruitment a time consuming and slow process. To some extent this was unavoidable and might be addressed by taking a multi-centre approach to future studies.
Chapter 7

General discussion and conclusions
7.1 DISCUSSION AND CONCLUSION

It is well established that the deposition of semen into the female reproductive tract at insemination evokes cellular and molecular changes that closely resemble a classical inflammatory response in a number of species including mice, rats, pigs and sheep. At the outset of these studies, very little was known of the nature of this response in humans with the two previous studies into this response reporting an influx of leukocytes, predominantly neutrophils, into the cervical mucous following intercourse or artificial insemination. These studies were deficient however as they examined only the superficial surface of the tissue and did not investigate the cellular and molecular events that occur deeper within the cervical epithelium and stroma where the more important changes would be expected to occur. Furthermore, these studies provided conflicting evidence pertaining to the factor(s) responsible for initiating this response with one group suggesting seminal plasma was responsible, while the other group suggested that sperm and not seminal plasma was in fact the triggering factor. Thus the source of the factors responsible for initiating this response in humans had not been determined, let alone the precise identity of the initiating factors.

The studies described in this thesis therefore aimed to examine a number of research questions, beginning with an investigation into whether similarities exist between mice and humans in the ability of semen exposure following intercourse to induce changes in leukocyte distribution and activation status within the tissues of the female reproductive tract. The molecular regulation of the inflammatory response following insemination was also investigated.

In chapter 3, immunohistochemical analysis of cervical tissue biopsies collected before and after unprotected intercourse revealed that an inflammatory response does occur within the cervical tissues of women following exposure to semen. The inflammatory response was detected in all women following intercourse and was characterised by a marked increase in dendritic cells and macrophages, predominantly of an activated phenotype given their elevated MHC class II expression, along with a smaller contingent of CD8+ and memory T lymphocytes. Furthermore, this response was found to require intimate contact between semen and cervical epithelial cells, invoking the actions of specific factors in seminal fluid, since only marginal increases in leukocyte numbers were observed following condom-protected intercourse. The current study failed to observe a significant influx of neutrophils into the cervical tissues as reported in previous studies. However it is likely that the timing of our sampling protocol accounts for this, with neutrophils likely to have already traversed the cervical epithelium when the repeat biopsy procedure was performed. The two previous studies identified neutrophils as the...
predominant leukocytes present in cervical mucous samples within 4 hours following intercourse or artificial insemination (Pandya et al. 1985, Thompson et al. 1992), whereas in this study tissue was recovered 12 hours following intercourse.

In chapter 4, quantitative real-time PCR analysis of cervical tissue biopsies revealed the human post-coital inflammatory response to be regulated by the semen-induced expression of pro-inflammatory cytokine expression by cervical epithelial cells. In particular, GM-CSF, IL-6 and IL-8 mRNA expression were identified as being the key regulators of leukocyte recruitment, a finding consistent with observations in the murine and porcine species (Robertson et al. 1998, O'Leary et al. 2004). It would be of interest to examine other chemokines and inflammatory mediators in an effort to more fully define this molecular regulation. The current study was limited in the number of mediators we could examine by the quantity of tissue recovered. Also, no attempt to separate epithelial from stromal tissue was made prior to RNA preparation. It would be interesting to use techniques such as laser capture dissection to evaluate the cellular origin of individual mediators.

Another research question addressed in this thesis was whether seminal plasma components were responsible for triggering the post-coital inflammatory response. The experiments in chapter 5 aimed to identify the predominant active moieties in human seminal plasma responsible for interacting with the cervical tissue. Previous studies in our laboratory had identified the seminal vesicle-derived molecule, TGFβ1, as the predominant active molecule within murine seminal plasma (Tremellen et al. 1998). This, together with the numerous similarities evident between mice and humans in the nature of the inflammatory response induced following insemination, and the observation that TGFβ is present in very high concentrations within human seminal plasma (Nocera et al. 1993, Srivastava et al. 1996), led to the postulate that TGFβ may also be responsible for triggering this response in humans. The use of in vitro cultures using primary and immortalised female reproductive tract cells found that stimulation of pro-inflammatory cytokine production in cervical epithelial cells was differentially regulated, with multiple factors contributing to the overall cytokine profile observed following incubation with seminal plasma. The three mammalian isoforms of TGFβ were identified as the predominant active constituents in seminal plasma, with TGFβ1, TGFβ2 and TGFβ3 all demonstrating the ability to induce marked increases in GM-CSF and IL-6 production by ectocervical epithelial cells in vitro. 19-OH PGE1, the most abundant prostaglandin in human seminal plasma, was also identified as strongly contributing to the post-coital inflammatory response in the cervix by stimulating increased IL-6 and IL-8 production.
These results further demonstrate the similarities between mice and humans regarding the mechanisms by which this response is regulated, though differences between the species do exist since 19-OH PGE₁ is undetectable in murine seminal plasma. The observation that IFNγ, also present in human seminal plasma, was able to interact with TGFβ and potently reduce its activity in cervical epithelial cells further emphasizes the complex nature of this response. Therefore, it would be useful to identify and evaluate other inflammation-inducing moieties present in semen that are likely to contribute to the molecular regulation of this response. These may include molecules that are capable of enhancing, or inhibiting the stimulatory ability of TGFβ on female reproductive tract cells. The apparent differential regulation of the different female tract cytokines indicates that the relative abundance of each of these inducing agents in seminal plasma is likely to be important in generating an optimal maternal immune environment for successful pregnancy.

The final research question examined in these studies aimed to determine whether seminal cytokine content was associated with fertility in men, either linked with or independent of conventional sperm parameters for male fertility. In chapter 6, seminal plasma samples were collected from proven fertile men and male partners of couples experiencing various forms of infertility and their individual concentrations of selected cytokines were examined. No relationships were identified between seminal plasma TGFβ₁, TGFβ₂ or TGFβ₃ content and fertility status in men, nor were they associated with sperm abundance, motility or morphometry. This was the first study to detect high concentrations of TGFβ₃ contained in human seminal plasma with concentrations just 40% lower than TGFβ₁, and more than 30 times greater than TGFβ₂. Considerable variation in individual TGFβ isoform concentrations was observed within individuals, though total TGFβ concentration, as determined by combining the concentrations of total TGFβ₁, TGFβ₂ and TGFβ₃, exhibited the least amount of variation, averaging just 17% of the mean value. Furthermore, replicate ejaculates collected from the same individual over several months showed very little variation over time, thereby indicating the representative value of measurement at a single time point. The majority of TGFβ present in seminal plasma is in the latent form which may have a number of physiological advantages, including preventing TGFβ from having a biological effect at its site of production in the male accessory glands.

Importantly, this study was the first to investigate whether seminal plasma IFNγ content was associated with fertility status in men. There was an increased likelihood of high IFNγ content in the male partners of couples experiencing infertility. In particular, the proportion of individuals with high IFNγ was greatest in male partners of women experiencing recurrent miscarriage, where 5 / 14 (36%)
scored above an arbitrary threshold. Interestingly, the discriminating value of IFNγ was increased when evaluated as a ratio of total TGFβ content.

This study was also the first to measure bacterial endotoxin in human seminal plasma samples and while endotoxin was detected in all samples, no relationship with fertility status was identified. This was also the case when IL-8 content was measured.

The concept in which different seminal constituents act in concert to activate pro-inflammatory cytokines and thereby recruit inflammatory cells in the cervix is schematically illustrated in Figure 7.1 below.

**FIGURE 7.1 Schematic illustration of the cellular and molecular events that occur within the female reproductive tract following intercourse.** Factors present in seminal plasma including TGFβ, 19-OH PGE and IFNγ act to target cervical and potentially uterine epithelial cells to activate expression of the pro-inflammatory cytokines GM-CSF, IL-6 and IL-8, leading to the recruitment and activation of inflammatory leukocytes. The relative abundance of macrophages, dendritic cells and neutrophils and their activation phenotypes would be influenced by the pattern of cytokines induced, and therefore by the balance of activating signals present in seminal plasma.
The precise functions of the post-coital inflammatory response observed within the cervix of peri-ovulatory women following intercourse were not investigated, however it is likely to play a role in various reproductive processes due to the vast array of actions leukocytes could exert when recruited into the cervical tissues. Firstly, an influx of leukocytes into the cervical epithelium and mucous may assist in the maintenance of sterility within the higher female reproductive tract by assisting in the phagocytic removal of potentially pathogenic bacteria and viruses introduced into the vagina following intercourse. The importance of cervical immunity is highlighted by the observation that women who smoke and therefore have reduced cervical immunity, are at an increased risk of contracting a Human Papilloma Virus infection, compared to non-smokers (Poppe et al. 1995). Secondly, the influx of leukocytes within the cervical tissue may facilitate the removal of non-fertilising spermatozoa and other seminal debris from the female tract. It has been postulated that the phagocytic clearance of sperm by leukocytes may act as an immunological filter, thereby removing morphologically abnormal sperm (Tomlinson et al. 1992). However, apparently viable and morphologically normal sperm are also targeted, suggesting that factors other than fertilisation competence may be involved in this selection. This raises the interesting question of whether the female tissues can distinguish and select sperm on the basis of genetic or antigenic factors that may have implications for the quality of pregnancy and health or the characteristics of resulting children.

It is currently unknown whether seminal plasma exposure and the subsequent post-coital inflammatory response has a beneficial effect on human pregnancy, however emerging evidence in a number of species suggests such a benefit may exist (schematically illustrated in Figure 7.2). The practise of artificial insemination clearly demonstrates that pregnancies can be initiated in the absence of seminal plasma, though experimental evidence particularly from rodent species, suggests that the success and quality of the pregnancy are compromised if females are not exposed to seminal plasma. In mice, embryos transferred to pseudo-pregnant recipients in the absence of exposure to male fluids display a higher rate of fetal loss and abnormality compared to recipients exposed to seminal plasma by mating to vasectomised males (Watson et al. 1983). Recent studies in our laboratory have further implicated seminal plasma as having a role in promoting receptivity in the female reproductive tract by demonstrating that embryos transferred into females which are mated with vasectomised males give rise to fetuses with retarded growth trajectories and placental development (Bromfield et al. 2004). Similar results have also been observed in pigs, where the use of diluted semen during artificial insemination results in reduced litter sizes, but mating with a vasectomised male or interuterine infusion of heat-killed semen restores litter size and improves farrowing rate (Murray et al. 1983, Mah et al. 1985).
Evidence supporting both acute and cumulative benefits of seminal plasma exposure in human reproduction comes from epidemiological studies. Women who engaged in sexual intercourse and were therefore exposed to semen prior to IVF or GIFT treatment were found to exhibit significantly improved implantation and live birth rates (Bellinge et al. 1986, Marconi et al. 1989, Tremellen et al. 2000). Furthermore, treatment of female partners of couples experiencing recurrent miscarriage with seminal plasma pessaries has also been reported to improve pregnancy success (Coulam et al. 1993). Also, there appear to be cumulative benefits of seminal exposure in humans, since limited sexual experience or the use of barrier methods of contraception are associated with increased risk of implantation failure, spontaneous miscarriage and pre-eclampsia (Klonoff-Cohen et al. 1989, Robillard et al. 1995).

Interestingly, there is some evidence to suggest that the beneficial effect of semen exposure on pregnancy is partner-specific, with a change of male partner or pregnancy resulting from donor gametes leading to a markedly increased risk of developing hypertensive disorders such as pre-eclampsia (Dekker et al. 1998).

The precise mechanisms governing how semen exposure exerts its beneficial effects on the quality of a pregnancy in humans are yet to be determined, though experimental evidence from rodent species and humans have allowed potential mechanisms to be proposed. Exposure to semen and the subsequent recruitment of leukocytes into the female reproductive tract may act to prime the maternal immune system to paternal antigens and subsequent induction of maternal immune tolerance. This is likely to have relevance to pregnancy by virtue of the shared antigens contained within the ejaculate and later expressed by the fetus and placenta and may therefore impact on the quality of the ensuing pregnancy. Whilst the majority of information presented below comes from studies in rodent species, similarities in the nature in which the human female reproductive tract responds to semen exposure may infer that similar mechanisms exist in women.

Macrophages and dendritic cells are the predominant leukocytes recruited into the endometrium in mice during the post-mating inflammatory response. These are professional antigen presenting cells which possess the ability to process and present paternal antigens contained within the ejaculate. In mice, macrophages and dendritic cells engulf and transport seminal antigens to draining lymph nodes, resulting in the activation of immune responses to paternal MHC and other antigens in semen. This immune response is characterised by the hypertrophy of lymph nodes draining the uterus along with evidence of lymphocyte activation (Beer et al. 1974, Piazzon et al. 1985, Johansson et al. 2004). Matings with vasectomised males of tubally-ligated females indicates that this immune activation occurs independently of sperm and the embryo (Chambers et al. 1979, Johansson et al. 2004). Furthermore,
males from which the seminal vesicle has been surgically removed fail to induce lymphocyte activation and proliferation, further highlighting the absolute requirement for seminal plasma in activating this immune response. Given that macrophages and dendritic cells are also the predominant cells recruited into the cervical tissue of women following intercourse, it is possible that similar mechanisms for the generation of immune tolerance to paternal antigens may also exist in the female tract.

Obviously, any form of hostile response to seminal antigens would grossly affect fertility, thereby preventing the female reproductive tract from tolerating any future semen exposures. The resultant immune response would also be detrimental to pregnancy, since the conceptus shares the same paternally-derived antigens with those in semen (Thaler 1989). The type of immune response activated by semen prevents the rejection of male antigens since seminal plasma contains an abundant amount of extremely powerful immuno-regulatory molecules such as TGFβ and PGE, both of which have the ability to prevent destructive type-1 or cell-mediated immune responses (Letterio et al. 1998). In fact, rather than a general suppression of the immune response within the female reproductive tract, semen appears to instead induce a dynamic and functional state of immune tolerance to male antigens. Supporting this is the observation that female mice exposed to seminal plasma even without the presence of sperm or a conceptus become transiently tolerant to male MHC antigens and fail to reject challenge with male tumour cells (Robertson et al. 1997). The protection of male antigens from rejection by the maternal immune system is afforded by MHC antigens and the minor histocompatibility antigen H-Y, but only when sperm is delivered in the presence of seminal plasma (Beer et al. 1974, Hancock et al. 1986).

It is currently unknown whether the human post-coital inflammatory response extends to the endometrium in vivo, though this could be examined in future experiments by collecting endometrial biopsies before and after exposure to semen using the same protocols employed in this current study. The distribution of seminal material within the female reproductive tract after intercourse would limit the tissues infiltrated by inflammatory cells and thus the range of downstream effects in a species-specific manner. In rodents and pigs the ejaculate fills the uterine lumen where it gains direct access to the implantation site, but in humans the ejaculate is deposited at the external os of the cervical canal. In vitro studies provide evidence suggesting that the effects of seminal plasma are likely to extend to the uterus since endometrial epithelial and stromal cells are responsive to stimulation by seminal plasma (Tremellen et al. 1997, Gutsche et al. 2003). In vivo studies have also demonstrated that active seminal constituents, including TGFβ, are present on the post-acrosomal region of the sperm head and are thereby transported into the uterine environment (Chu et al. 1996). This movement into the higher
female tract is facilitated by rapid and sustained peristaltic uterine contractions capable of transporting macromolecular material as high as the fallopian tube (Kunz et al. 2002). The transport of progesterone and other mediators from the cervix to the endometrial tissues are also facilitated by unique vascular connections which may also allow the transportation of inflammatory mediators (Bulletti et al. 1997).

Studies in mice have demonstrated that the influx of leukocytes within the endometrial tissues and lumen can exert effects other than by activating immune responses. There is evidence to suggest that these cells also produce an array of soluble signalling molecules and potent enzymes likely to affect the turnover of the ECM along with altering the characteristics of endothelial cells in the endometrium, a process referred to as tissue remodelling. Macrophages have been described as the major leukocyte population responsible for the regulation of endometrial remodelling to facilitate implantation and placental development (Robertson 2005). Part of this endometrial restructuring involves the process of angiogenesis, a process which is tightly regulated and driven by macrophages. Activated macrophages have been observed to play crucial roles in all aspects of the angiogenic process, including alterations to the extracellular matrix, induction of endothelial cell migration and proliferation, and formation of capillaries (Sunderkotter et al. 1994). Macrophage-secreted cellular products have also been suggested to target the ECM of endometrial stroma, which is known to be remodelled prior to and during decidualisation (Aplin et al. 2002), with MMPs being key regulators of this process (Curry et al. 2003). MMPs exert this influence after coordinated increases in their transcription, secretion and proteolytic activation, along with their regulatory proteins, the tissue inhibitors of metalloproteinases (TIMPs).

In addition to roles in tissue remodelling, macrophages may also play a role in facilitating implantation, by selectively targeting luminal epithelial cells involved in embryo attachment during the early stages of implantation. The fluctuating expression of adhesion and anti-adhesion molecules throughout the cycle provides a physical barrier, preventing embryo attachment until the window of implantation when selective changes in the expression of integrins and mucins by epithelial cells (Aplin 1997) allow the close apposition and then attachment between the blastocyst and the luminal surface, just prior to invasion. In vitro studies using uterine epithelial cells have demonstrated that leukocytes are also capable of regulating epithelial adhesive properties in humans (Kosaka et al. 2003). Macrophages may further contribute to implantation success by virtue of their ability to alter transport properties and epithelial cell barrier integrity, thereby facilitating trophoblast invasion through the epithelial surface (Robertson 2005). Whether exposure to semen following intercourse and the resultant post-coital inflammatory response within the human cervix is capable of influencing endometrial tissue...
remodelling or embryo implantation in humans remains to be examined, but seems warranted particularly in view of emerging evidence linking altered endometrial leukocyte populations and cytokine synthesis with infertility (Laird et al. 2003).

A further potential role for the cytokines produced by the female reproductive tract following insemination may be in communicating with the developing embryo as it traverses the oviduct and uterus. Several cytokines induced by semen are amongst those attributed with regulating proliferation, viability and differentiation in blastomeres in embryos (Pampfer et al. 1991). GM-CSF, a principal cytokine in the murine and human post-coital inflammatory response, has been shown to target the pre-implantation embryo to promote blastocyst formation through increasing the number of viable blastomeres in both species (Sjoblom et al. 1999, Robertson et al. 2001). In humans, embryos cultured in GM-CSF are twice as likely to continue to the blastocyst stage of development, blastulate earlier and have increased cell numbers both in the inner cell mass and trophoderm (Sjoblom et al. 1999). Additional cytokines targeting the developing blastocyst including IL-6 and LIF are also induced after exposure to semen (Robertson et al. 1992, Gutsche et al. 2003). Perturbations in the growth factor environment experienced by the pre-implantation embryo impairs normal development of the placenta and fetus, with long term consequences for post-natal health and metabolic programming in progeny (Sjoblom et al. 2005).
FIGURE 7.2 Schematic illustration of the potential consequences of the post-coital inflammatory response in the human cervix. Exposure to semen following intercourse induces pro-inflammatory cytokine production by female reproductive tract epithelial cells triggering the recruitment of leukocytes into the female tissues. This inflammatory-like response is thought to impact on numerous reproductive processes including the removal of potentially harmful pathogens introduced into the female tract following insemination and clearance of seminal debris. It is also reasonable to postulate that this response contributes to maternal immune regulation, tissue remodelling, embryo development and may even facilitate sperm selection (refer to text for details). DC, dendritic cell; Mφ, Macrophage.

Of emerging interest is the impact of the relative abundance and identity of bacteria within the female and male reproductive tract flora. The studies described in chapter 6 demonstrated that bacterial endotoxin was present in human seminal plasma. Interestingly, endotoxin has been shown to elicit many of the same pro-inflammatory cytokines from female reproductive tissues as seminal plasma, including IL-8 synthesis in humans (Watari et al. 2003) and GM-CSF production in mice (Glynn and Robertson, unpublished data). It is therefore possible that endotoxin may contribute to the female
responsiveness to TGFβ present in seminal plasma (Robertson et al. 1991). The effects of endotoxin are presumably elicited through binding to toll-like receptors (TLR) on epithelial cells (Wolfs et al. 2002, Backhed et al. 2003, Samuelsson et al. 2004, Schaefer et al. 2004, Hirata et al. 2005). Whether there is any physiological significance of the source of endotoxin, for example whether probiotic Lactobacilli elicit the same cytokine response as pathogenic bacteria associated with sexually transmitted infections, remains to be examined.

The studies described in this thesis provide a mechanistic basis for the observations linking exposure to semen with pregnancy success in humans and have greatly expanded our current knowledge of the cellular and molecular events that occur within the female reproductive tract following insemination. Seminal plasma can therefore no longer be thought of as merely a transport medium for spermatozoa, rather as a means for communication between the male and female reproductive tissues, required for optimal pregnancy success. This function of seminal plasma provides new insight into the processes influencing reproduction and fertility. From a female perspective, the ability to activate the immune response prior to implantation may facilitate sperm selection and the identification of competent embryos.

In addition, much of the research in this area has been performed in rodents and livestock species and the obvious difficulties in obtaining human samples have meant that the significance of seminal factors in humans has been extremely difficult to explore. While it is not possible to directly extrapolate from rodents to humans, the emerging picture justifies closer examination of the relationship between seminal exposure and the incidence of infertility and subfertility in human pregnancy. It is speculated that conditions such as pre-eclampsia, where shallow placentation occurs, and recurrent miscarriage may well have an immune basis, with insufficient or inappropriate immune responses to seminal antigens being implicated (Dekker et al. 1998). If proven to be the case, it may be that these conditions are influenced by the interaction between semen and the female tissues, arising through mechanisms such as partner incompatibility, seminal plasma cytokine deficiency or a failure in the ability of the female tract to respond to semen (Dekker et al. 1998). There may also be implications for artificial reproductive technologies, where pregnancies are routinely initiated in the absence of seminal plasma.
A better understanding of the physiological significance of semen in human reproduction requires a further detailed understanding of the cellular and molecular events within the female reproductive tract following insemination. This may eventually lead to the development of novel therapies for infertility and pathologies of pregnancy that mimic or amplify the physiological events normally occurring at insemination to promote and assist the ‘conditioning’ effects of semen in preparation for pregnancy. Furthermore, it is becoming increasingly apparent that the presence of important immuno-regulatory cytokines and other related immune mediators within seminal plasma can influence the quality of the female reproductive tract environment and hence the likelihood of a successful pregnancy. A more detailed understanding of the importance of the relative abundance of these molecules within human seminal plasma may eventually lead to the development of novel diagnostic tools for predicting fertility status in men.

With this endpoint in mind, it would be useful to identify and evaluate other inflammation inducing moieties present in semen that are likely to play a role in determining fertility status. These may include molecules that are capable of enhancing, or inhibiting the stimulatory ability of TGFβ in female reproductive tract cells.

Finally, it will be of great interest to understand how the expression of TGFβ and other active seminal moieties are regulated in the male. It seems reasonable that endogenous factors such as sex steroids, infection and inflammatory status should be involved, as well as environmental determinants such as nutrition, stress, and environmental chemicals. With the dramatic and rapid increase in human infertility over the past century, an increased effort in this area of research is definitely warranted.
Patient information sheet - CIRT study

Thank you for considering your involvement in the CIRT study. The aim of this study is to investigate the potential of a new drug called transforming growth factor beta (TGFβ) for the treatment of infertility. Earlier studies in mice have shown that this drug can cause white blood cells to move into the uterus (womb), where in turn they release chemicals called cytokines that stimulate the growth of the baby and its placenta. It is believed that some women are unable to have children because their uterus does not contain enough white blood cells producing these vital growth-promoting cytokines. It is hoped that by giving these women TGFβ medication we may increase the number of white blood cells in the uterus, thereby allowing the placenta and baby to grow normally. However, before we embark on a clinical trial of TGFβ as a treatment of infertility we must first make sure that TGFβ can attract white blood cells into the human uterus.

If you agree to be involved in this study we will take a small sample of tissue from your cervix (neck of the womb) and grow the cells in a test-tube in our laboratory. Next we will add the drug TGFβ to these cells and see if it can stimulate the production of chemicals that attract white blood cells. You should be aware that:

- You will not be personally exposed to the drug TGFβ at any stage of this study. All testing of the drug will be done in test-tubes in the laboratory once the cervix cells have been removed from your body by hysterectomy.

- This study will in no way extend the length of your operation or change the manner in which the surgeon will perform your hysterectomy.

- The amount of tissue removed from the cervix is only a small proportion of the total cervix area (about 20%). A pathologist will still examine the remaining cervix and uterus under a microscope, as is usual practice following all hysterectomies.

This research project has been approved by the Human Ethics Research Committee of the North Western Adelaide Health Service. As part of its professional responsibility, this committee may need to access medical records from time to time. Any such access will be made in a manner that respects and protects your privacy. You do not have to be involved in this study. Your declining to be involved will in no way affect your ongoing treatment at this hospital.

If you have any questions concerning this study please contact Dr Kelton Tremellen on [phone number]. Should you wish to speak to a person not involved with the project, you can contact Paul Miller, Executive Officer, Ethics of Human Research Committee on [phone number]. Thankyou for taking the time to consider your involvement in this study.

Kind regards,

Dr Kelton Tremellen
Registrar in Obstetrics and Gynaecology
Lyell McEwin Health Service
NORTH WESTERN ADELAIDE HEALTH SERVICE

STANDARD CONSENT FORM

1. I, the undersigned, hereby consent to my involvement in the research project titled
   Cervical Immune Response to Transforming growth factor - beta (CIRT study)

2. I have read the information sheet, and I understand the reasons for this study. The ways in which it will affect me have been explained by the research worker. My questions have been answered to my satisfaction. My consent is given voluntarily.

3. The details of the research project have been explained to me, including:
   - the expected time it will take
   - the nature of any procedures being performed, and the number of times they will be performed
   - the nature of any medication I may be given
   - any risks/discomforts which I may experience

4. I understand that the purpose of this research project is to improve the quality of medical care, but my involvement may not be of benefit to me.

5. I have been given the opportunity to have a member of family or a friend present while the project was explained to me.

6. No information about my medical history will be taken from the hospital without the researcher being present. My identity will be kept confidential, and nothing will be published which could possibly reveal my identity.

7. My involvement in the project will not affect my relationship with my medical advisers. I understand I am free to withdraw from the project at any stage without having to give any reasons, and that if I do withdraw from the project it will not affect my treatment at this hospital in the future.

SIGNED ........................................................................................................................................
ADDRESS ......................................................................................................................................
(please print) ......................................................................................................................................
WITNESS .........................................................................................................................................
RESEARCH WORKER ....................................................................................................................
DATE ...............................................................................................................................................
Appendix B
Thank you for considering your involvement in the SEPO study. The main aim of this study is to investigate whether there is a link between women experiencing recurrent miscarriage and low levels of immune substances in their partners' semen.

Previous studies have found that a significant proportion of women experience recurrent miscarriage because their bodies' immune system destroys the developing placenta. The mother's immune system attacks the placenta because it contains antigens from the father and therefore is recognised as being foreign. However, in successful pregnancy it is believed that immune substances within semen may actually prevent the mother's immune system from attacking the placenta. A lack of these immune protective substances may leave the placenta exposed to immune attack and result in recurrent pregnancy failure.

In order to achieve our study aim we need to collect semen samples from men whose partners have experienced recurrent miscarriage and compare them with samples taken from men whose partners have had a normal pregnancy. Semen samples will need to be produced by masturbation, as outlined in the accompanying information sheet. Since it appears that the number of times a woman is exposed to her partner's semen is important, we would like to know how often you had intercourse while trying to fall pregnant and when you stopped using condoms for contraception. If you feel these questions are too embarrassing you can decide not to answer them but may still be involved in the study.

We would like to stress that you are not compelled to be involved in this study. If you refuse to be involved it will not affect your future treatment by the staff of the Maternity unit. Total confidentiality will be maintained at all times and if you do agree to be involved, you may withdraw from the study at any stage. Each participant will be paid $20 to cover the transport costs of delivering the semen sample to our laboratory. In addition, participants will be able to request a copy of their sperm count if they would like access to this information.

The research described in this paper has been approved by the Human Ethics Research Committee of the North Western Adelaide Health Service. As part of its professional responsibility, this committee may need to access medical records from time to time. Any such access will be made in a manner that respects and protects your privacy.

If you have any questions concerning this study please contact Dr. Tremellen on .

Appointments for the collection of semen are made through the Andrology Laboratory at the Queen Elizabeth Hospital on . Should you wish to speak to a person not involved with the project, you can contact Paul Miller, Executive Officer, Ethics of Human Research Committee on 8.

Thank you for taking the time to consider your involvement in this study.

Kind regards

Prof G Dekker
Professor of Obstetrics
University of Adelaide and NWAHS

Dr K Tremellen
Registrar in Obstetrics
NWAHS
Semen Exposure Pregnancy Outcome (SEPO) Study

1. I, the undersigned .................................................................
   hereby consent to my involvement in the research project titled:
   Semen Exposure Pregnancy Outcome (SEPO) Study.

2. I have read the information sheet, and I understand the reasons for this study. The ways in which it will affect
   me have been explained by the research worker. My questions have been answered to my satisfaction. My
   consent is given voluntarily.

3. The details of the research project have been explained to me, including:-
   • the expected time it will take
   • the nature of any procedures being performed, and the number of times they will be performed
   • the nature of any medication I may be given
   • any discomfort which I may experience

4. I understand that the purpose of this research project is to improve the quality of medical care, but my
   involvement may not be of benefit to me.

5. I have been given the opportunity to have a member of family or a friend present while the project was
   explained to me.

6. No information about my medical history will be taken from the hospital without the researcher being present.
   My identity will be kept confidential, and nothing will be published which could possibly reveal my identity.

7. My involvement in the project will not affect my relationship with my medical advisers. I understand I am free
   to withdraw from the project at any stage without having to give any reasons, and that if I do withdraw from
   the project it will not affect my treatment at this hospital in the future.

SIGNED
ADDRESS
(please print)
WITNESS
RESEARCH WORKER
DATE

Sharkey

Appendix B
8.1 REFERENCES


Sharkey Chapter 8 221


Sharkey Chapter 8


Sharkey Chapter 8


Sharkey Chapter 8


Sharkey Chapter 8


proliferation, phenotypic heterogeneity, and extrathymic differentiation." Journal of Immunology 159(7); 3266-3277.


Sharkey Chapter 8 237


Sharkey Chapter 8 243


Yie, S.-m., Li, L.-h., Li, Y.-m. and Librach, C. (2004). "HLA-G protein concentrations in maternal serum and placental tissue are decreased in preeclampsia." 191(2): 525.


## Errata

<table>
<thead>
<tr>
<th>Page</th>
<th>Line</th>
<th>Correction</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>17</td>
<td>Replace “cells” with “cells’ “</td>
</tr>
<tr>
<td>39</td>
<td>31</td>
<td>Replace “membrane-bound” with “membrane-bound”</td>
</tr>
<tr>
<td>41</td>
<td>2</td>
<td>Insert “to be” between “suggested” and “associated”</td>
</tr>
<tr>
<td>99</td>
<td>4</td>
<td>Figure 3.3 legend should include the statement “All photomicrographs were taken at 20x magnification”</td>
</tr>
<tr>
<td>106</td>
<td>13</td>
<td>Delete “be” from between “also” and “mimicked”</td>
</tr>
<tr>
<td>132</td>
<td></td>
<td>Insert “(data not shown)” at the end of sentence</td>
</tr>
<tr>
<td>143</td>
<td>1</td>
<td>Replace title “5.7 The effect of IFNγ on GM-CSF, IL-6 and IL-8 production by immortalised cervical epithelial cells in vitro” with “5.7 The effect of recombinant TGFβ1 and IFNγ on GM-CSF, IL-6 and IL-8 production by immortalised cervical epithelial cells in vitro”</td>
</tr>
<tr>
<td>148</td>
<td>1</td>
<td>Replace Figure 5.7 legend “Effect of recombinant TGFβ1 on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro” with “Effect of recombinant TGFβ1 and IFNγ on primary ectocervical epithelial cell GM-CSF, IL-6 and IL-8 production in vitro”</td>
</tr>
<tr>
<td>178</td>
<td></td>
<td>Replace “The total amount of biologically active TGFβ in each of the samples was unable to be calculated because the level of bioactive TGFβ3 was not determined.” with “It was not possible to calculate the total amount of biologically active TGFβ in each of the samples because the levels of bioactive TGFβ3 was not determined”</td>
</tr>
<tr>
<td>195</td>
<td></td>
<td>Delete duplicate reference (McLachlan 2002)</td>
</tr>
</tbody>
</table>