UNIVERSITY
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GLASGOW

Studies of Immunoglobulin Receptors and Human Proximal Tubular Cells

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BSc (Hons; K.S.A), MRes (UK)

Submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

University of Glasgow
Division of Cardiovascular & Medical Sciences and FBLS

2007

Acknowledgement I

I wish to express my sincere gratitude to my supervisor ***Professor Alan G. Jardine***, the one who has guided me into the fascinating world of research. I wish to express my thanks, for his contributions, excellent collaboration, valuable discussions, and unreserved support.

I gratefully acknowledge the enthusiastic supervision of ***Dr. Niall MacFarlane***, for his positive and constructive advice and support throughout, and for always being so helpful.

This work would not have been possible without the support and the encouragement of my colleagues ***Dr. Kenneth McDonald*** and ***Ms. Helen Millar***. Very special thanks also to ***Dr. Diane Hillyard*** for her generosity and kindness. In addition, I would like to thank ***Dr. James McLay*** (University of Aberdeen), who kindly provided primary cultures of proximal tubular cells.

Most importantly, thanks and love to my wonderful parents, ***Jamal*** and ***Najat***. They have wished so long for this thesis, waited and supported me throughout these years. To them I dedicate this work.

Manal Jamal Natto

(2007)

Acknowledgement II

<u>(بســـــم الله الرحمـــــن الرحيـــــم)</u>

إلـى قدوتـي فـي حيـاتـي، ومـن أفنـى عمـــره لأكـــون أنـــا .

إلى قرة عيني ، ينبوع الحنان الذي لا ينضب ، ونهر العطاء الخالد .

والدي ووالدتي الأحباء..................

لقد علمتماني أن العلم نبراس في ظلمة الجهل.. أنتما مدرستي الأولى ؛ وجامعتي التي أتعلم في فصولها جميع علوم الحياة بلا مقابل وهيهات أن أستطيع رد جزء من عطائكما.

مهما طال الدرب، لا بد أن يخطر بالبال تلك المحطات التي مرت بي لأقف أمامها إجلالاً واعترافاً بالجميل. اسأل الله العلي القدير أن يقدرني لكي أرد ولو جزءاً من هذا الجميل والعرفان .

وأيضاً............

إلى كل من كان وراء وصولي لهذه المرتبة .....الغالين جميعاً..

وإلى زكي، جيهان، عباس، وإلى جميع أسرتي الكريمة رعاها الله...

أهدي ثمرة جهدي المتواضع هذا بكل الحب الذي عهدته منكم على الدوام ؛ والله من وراء القصد .....

والله يحفظكم ويرعاكم؛؛.

المحبة لكم جميعاً

منـــال جمـــال نتـــو

Author's declaration

I declare that this thesis has been composed by me and is a record of work performed by me, except where stated in the text. The immunohistochemistry of tissue sections in chapter 3 was performed by Dr Barbara Young (Department of Pathology, Western Infirmary, Glasgow). This work has not previously been submitted for a higher degree.

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(2007)

Summary

Glomerulonephritis (GN) is a major cause of progressive chronic renal failure (CRF) and accounts for less than 20% in the UK of patients requiring dialysis and transplantation. There are many forms of GN, of which IgA nephropathy (IgAN) is the most common, affecting up to 2% of the adult population. GN is characterised by irreversible and progressive glomerular and tubulointerstitial fibrosis, reduction in glomerular filtration rate (GFR), and retention of uremic toxins. The rate of progression of GN is increased by various clinical factors including hypertension, and the quantity and specificity of proteinuria. Although albuminuria is most widely studied, higher molecular weight proteins such as immunoglobulins (Ig) are more associated with progression of renal disease. In this thesis I have explored the hypothesis that an interaction between tubular cells and filtered Ig/immuno complex (Ig/IC) may involve specific cellular receptors, and thus provide a novel mechanism for the initiation of tubular injury and fibrosis.

Deposition of Ig and IC is seen in renal diseases such as membranous glomerulonephritis, and IgAN. Ig/IC are regarded as responsible for the initiation of glomerular injury in these diseases. However, whether Ig/IC contribute directly to tubular injury is unknown. We previously identified a novel IgA receptor, the Fcα/μ receptor (Fcα/μR), in mesangial cells which may contribute to IgAN. This receptor binds both IgA and IgM but not IgG. I hypothesised that Ig and IC, filtered at the glomerulus, may act on Ig receptors in human proximal tubular epithelial cells (PTEC) and may play a direct pathophysiological role in the development of interstitial fibrosis. Specifically, that filtered IgA may bind to the Fcα/μR in IgAN. The activation of Ig receptors may stimulate tubular cells to alter cell proliferation and extracellular matrix formation (e.g fibronectin (FN)), which are pathophysiological characteristics of tubulointerstitial fibrosis in GN.

I studied primary and immortalised human PTEC using qualitative RT-PCR and quantitative real-time PCR (qRT-PCR) for expression of candidate Ig receptors. I investigated the expression of Ig receptors and their regulation by cytokines (e.g. IFN-γ, TGF-β1 and IL-1α) known to be implicated in GN. The receptors studied include the polymeric immunoglobulin receptor (pIgR), the neonatal receptor (FcRn), the Fcα/μR, the classical IgG receptors (FcγR1, γIIa, γIIb, γIII and the related FcR γ-chain) and the classical IgA receptor (FcαR). None of the classical IgG receptors were expressed by

PTEC, nor was the classical IgA receptor. However, the FcRn, pIgR and the Fcα/µR were expressed.

Gene expression of these receptors was investigated under cytokine regulation. IFN-γ, IL-1α and TGF-β1 had no effect on FcRn gene expression. The expression of the pIgR gene was up-regulated by IFN-γ and the Fcα/µR transcript level was up-regulated by IL-1α and IFN-γ but down-regulated by TGF-β1. Expression of Fcα/µR protein by tubular cells was confirmed by western blot analysis by using specific Fcα/µR monoclonal antibody.

Immunohistochemistry confirmed the expression of Fcα/µR by PTEC in both normal and diseased kidney and that IgG, IgA and IgM binding varies in parallel with gene expression of related Fc receptors. This binding was associated with increased FN production (assessed by ELISA) and reduced the proliferation of PTEC, demonstrated by [^{3}H]-thymidine uptake assay.

I screened the ability of PTEC to release cytokines under normal conditions and confirmed this by ProteoPlex cytokine array. Among these cytokines there was no significant effect of Ig/IC on IL-6, IL-8 and GM-CSF released by PTEC, and this was confirmed by specific ELISA for each individual cytokine. The phosphotyrosine/phospho-ERK signalling pathway was unaffected by Ig/IC; however, IgM was able to activate the phospho-ERK pathway. These results suggest that there might be alternative signalling pathways activated by Ig/IC in PTEC.

I then studied the effect of calcineurin inhibitor immunosuppressive drugs as a model of tubular toxicity, and statins as immunomodulatory drugs, reputed to protect renal function and known to have immunomodulatory effects. I hypothesised that immunosuppressant agents/statins either alone or in combination with LPS might affect Ig receptors expression, cytokine release, FN production and proliferation by PTEC.

My results inferred that immunosuppressive agents alone have a non-inflammatory response on Ig receptor expression. However, in combination with LPS, the study drugs showed a slight inhibitory effect on the expression of the pIgR on PTEC at the highest concentration only. There was no significant effect of immunosuppressant agents alone or in combination with LPS on IL-6, IL8 and GM-CSF released by PTEC. Cyclosporine, FK506 and sirolimus were shown to inhibit both the production of FN and the growth of PTEC at high doses, thereby exerting an antiproliferative effect.

Both simvastatin and fluvastatin inhibited LPS induced production of IL-6, IL-8 and GM-CSF, FN and proliferation in a dose-dependent manner. 1 micromolar simvastatin/fluvastatin was associated with a 35% reduction in unstimulated FN production, a 51% reduction in proliferation, and reduced production of IL-6 (58%), IL-8 (65%) and GM-CSF (57%). The expression of Ig receptors was increased in a dose-dependent manner in PTEC treated with statins alone or in combination with LPS.

In conclusion, the expression of tubular FcRn, pIgR and Fcα/µR by PTEC is regulated by proinflammatory cytokines. The binding of IgA and IgM, possibly through the Fcα/µR, by PTEC may contribute to immune-mediated nephropathy. The FN production and the proliferation suggest that Ig/IC binding may contribute to the pathophysiology of IC-mediated renal disease. The ability of immunosuppressant agents to reduce FN production, proliferation, and pIgR expression by PTEC may offer potential new strategies for the prevention and treatment of nephrotoxicity, proteinuria and chronic allograft nephropathy (CAN). The inhibitory effect of statins alone or in combination with LPS on FN production, proliferation and cytokines released suggest that statin therapy may have potential benefit in interstitial nephritis and fibrosis. On other hand, statins alone or in combination with LPS increased the expression of Ig receptors by PTEC.

This thesis shows that the novel human Fcα/µR in PTEC may play an important role in the immune mechanisms involved in the tubular response during renal injury associated with tubular Ig/IC deposits in renal disease.

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Abstract presented

Manal J. Natto, Dianne Hillyard, Kenneth McDonald, James McLay, Angus Cameron, Niall MacFarlane, Alan G. Jardine. Fc receptors on human proximal tubular cells and their potential role in glomerulonephritis. *Renal Association, London, September 2004*.

Manal J. Natto, Dianne Hillyard, Kenneth McDonald, James McLay, Angus Cameron, Niall MacFarlane, Alan G. Jardine. Fc receptors on human proximal tubular cells and their potential role in glomerulonephritis. *Scottish Society for Experimental Medicine, Edinburgh, November 2004*. (Oral presentation)

Manal J. Natto, Kenneth McDonald, Dianne Hillyard, Helen Miller, Jackie Thomson, Wei Zhang, Ying Fu, James McLay, Angus Cameron, Niall MacFarlane and Alan G. Jardine. Regulation and function of the $\alpha\mu$ receptor, and other immunoglobulin receptor, in human proximal tubular cells. *American Society of Nephrology 38th Annual Renal Week Meeting, Philadelphia, November 2005*.

Manal J. Natto, Dianne Hillyard, Kenneth McDonald, James McLay, Niall MacFarlane, Alan G. Jardine. Statins inhibit the effects of lipopolysaccharide on human proximal tubular cells. *Newcastle University, Proceeding of the Saudi Innovation Conference (SIC), May 2007*. (Oral presentation)

Publication

Manal J. Natto, Kenneth McDonald, Dianne Hillyard, Angus Cameron, James McLay, Wei Zhang, Ying Fu, Niall MacFarlane, Barbara Young, Alan G. Jardine. Regulation and function of the Fcα/μ receptor and other immunoglobulin receptors in human proximal tubular cells. *Kidney International*. (Submitted)

Manal J. Natto, Dianne Hillyard, Kenneth McDonald, Niall MacFarlane, James McLay, Alan G. Jardine. Statins inhibit effects of lipopolysaccharide on human proximal tubular cells. *Biochem Biophys Res Commun*. (Submitted)

Abbreviations

Ab	Antibodies
ACE	Angiotensin converting enzyme
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADH	Antidiuretic hormone
Ag	Antigen
APCs	Ag-presenting cells
ARF	Acute renal failure
ASGPR	Asialoglycoprotein receptor
CAN	Chronic allograft nephropathy
CD	Cluster of differentiation (antigen)
cDNA	Complementary deoxyribonucleic acid
CHO- K1	Chinese hamster ovary
CIC	Circulating immune complex
CNIs	Calcineurin inhibitors
CRF	Chronic renal failure
C-SFs	Colony-stimulating factors
C_t	Threshold cycle
CTL	Cytotoxic T-lymphocyte
Cys A	Cyclosporine A
dbcAMP	Dibutyryl cyclic adenosine monophosphate
dIgA	Dimeric IgA
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial cell nitric oxide synthase
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESRD	End stage renal disease
ESRF	End stage renal failure
Fab	Antigen binding fragment (of immunoglobulin)
Fcα/μR	Fc alpha/mu receptor
FcγR	Fcγ receptors
FcRn	Fc neonatal receptor
FcRs	Fc receptors, crystallisable fragment (of immunoglobulin)
FCS	Foetal calf serum
FcγR	FcR common γ-chain

FcαRI	Fc alpha receptor I
FK506	Tacrolimus
FKBPs	FK506-binding proteins
Fluva	Fluvastatin
FN	Fibronectin
FPP	Farnesyl-pyrophosphate
Gal	Galactose
GalNAc	*N*-acetyl galactosamine
GBM	Glomerular basement membrane
GFP	Green fluorescent protein
GFR	Glomerular filtration rate
GGPP	Geranylgeranyl-pyrophosphate
GM-CSF	Granulocyte macrophage colony-stimulating factor
GN	Glomerulonephritis
GPI	Glycosylphosphatidylinositol
HAg-IgA	Heat aggregated IgA
HAg-IgG	Heat aggregated IgG
HDL	High density lipoprotein
HK-2	Human transformed PTEC cell line
HLA	Human leukocyte antigen
HMC	Human mesangial cell
HMG-CoA	3-hydroxy, 3-methylglutaryl Coenzyme A
IC	Immune complex
ICAM-I	Intercellular adhesion molecule-I
IFN	Interferons
Ig	Immunoglobulins
IgA	Immunoglobulin A
IgAN	IgA nephropathy
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
J-chain	Joining chain
JNKs	c-jun N-terminal kinases
KIR	Killer cell inhibitory receptors
LDL	Low density lipoprotein
LMWH	Low-molecular-weight heparins
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
MAPK	Mitogen-activated protein kinases

MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage-colony stimulating factor
MGN	Membranous glomerulonephropathy
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MSC	Mesangial cells
NANA	*N*-acetylneuraminic (sialic) acid
NEP	Neutral endopeptidase
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pgp	P-glycoprotein
pIgA	Polymeric immunoglobulin A
pIgR	Polymeric immunoglobulin receptor
PMN	Polymorphonuclear neutrophils
PTEC	Proximal tubular epithelial cells
qRT-PCR	Quantitative real-time PCR
RANTES	Regulated upon activation, normal T cell expressed and secreted
RME	Receptor-mediated endocytosis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide electrophoresis
SIgA	Secretory immunoglobulin A
Simva	Simvastatin
TCR	T cell receptor
TEM	Transepithelial migration
TfR	Transferrin receptor (CD71)
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TOR	Target of rapamycin
VCAM-1	Vascular cell adhesion molecule-I
XL	Cross linker

Chapter 1: Introduction

1.1 Applied anatomy of the kidney

As a vital component of the urinary system, the kidneys have numerous functions and play a crucial role in maintaining equilibrium of water and electrolyte concentrations in the body. These functions include the production and secretion of urine, hormones (erythropoietin) and enzymes (renin, an important enzyme in the control of blood pressure). In this section I will briefly outline the anatomical structure of the kidneys.

1.1.1 The nephron system

Each kidney contains approximately 1 million functional and structural units called nephrons. Each nephron consists of a tubule that opens into a collecting duct at one end and is closed at the other and is responsible for the production of urine in response to secretions from other glands. The closed end of the nephron (the 'glomerular corpuscle') is indented to form Bowman's capsule, a double-layered epithelial cup-shaped structure that almost completely surround the glomerulus, a network of 10–20 arterial capillary loops. Blood flowing through these capillary loops undergoes the first step in the filtration process, from which an initial urine filtrate is produced. This occurs because the walls of the glomerulus are more permeable and blood pressure (BP) is higher than in other capillaries (Mirpuri&Patel, 2000). The initial urine filtrate then passes through the remainder of the nephron: the proximal tubule, the loop of Henle and the distal tubule leading into a collecting duct. The structure and function of each of these components is described in more detail below.
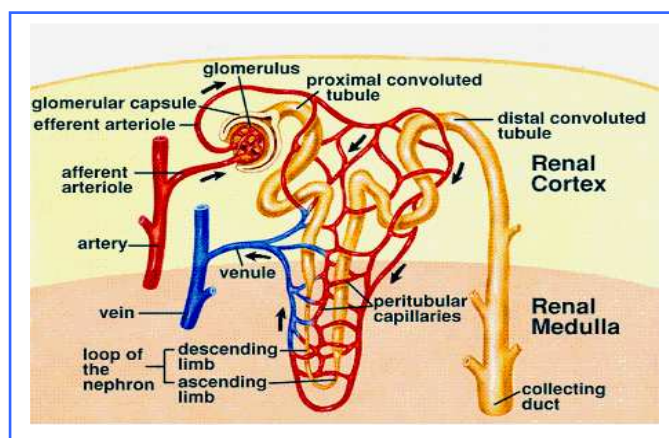
Figure 1-1 Nephron system anatomy

Adapted from: http://www.mhhe.com/biosci/ap/dynamichuman2/content/gifs/0176.gif.

1.1.1.1 The glomerulus

1.1.1.1.1 Podocytes

The visceral layer of Bowman's capsule is lined with podocytes, cells that are a crucial component of the glomerular filtration process. These cells and their pedicles (or 'foot processes') interdigitate with neighbouring cells to cover the thick basal pedicles lamina that joins the endothelium to the podocytes (the glomerular basement membrane (GBM), see below), leaving only narrow slits through which molecules can be filtered. The size and patency of these filtration slits is thought to be regulated by a large number of actin microfilaments and by the presence of a thin membrane that spans the slits in a similar manner to the diaphragm of fenestrated capillaries (Mirpuri&Patel, 2000).

1.1.1.1.2 Glomerular basement membrane (GBM)

The GBM is the principal component of the filtration barrier and contains type IV collagen, sialoglycoproteins and other non-collagenous glycoproteins (e.g. laminin), in addition to proteoglycans and glycosaminoglycans, particularly heparin sulphate. As well as acting as a physical barrier, the GBM is also an ion-selective filter and restricts the movement of particles, usually proteins, larger than approximately 70KDa (Mirpuri&Patel, 2000).

1.1.1.1.3 The mesangium

Like podocytes, mesangial cells (MSC) are found in the outer layers of the GBM and are collectively referred to along with their extracellular matrix (ECM) as the mesangium. Unlike podocytes, MSC can be found outside of the glomerular corpuscle as part of the juxtaglomerular apparatus. Here, they are sometimes also referred to as lacis cells (Ross *et al.*, 1995).

MSC are known to be contractile, phagocytic and, alongside their ECM, to provide structural support for podocytes where the GBM is absent. They help to keep the glomerular filter free of debirs by removing trapped residues and aggregated proteins from the GBM and may also regulate blood flow in the glomerulus. Indeed, MSC have been observed to proliferate in renal diseases that involve excessive protein deposition in the GBM (Moura *et al.*, 2004).

1.1.1.2 The proximal tubule

The initial urine filtrate leaves the glomerular corpuscle through the proximal tubule, a 15-mm long narrow structure lined with a single layer of cuboidal cells. These cells interdigitise tightly and are connected by their luminal surfaces, each one consisting of millions of microvilli that are collectively referred to as an 'apical brush border'. These densely packed structures vastly increase the surface area for absorption of the filtrate and help to distinguish proximal epithelial tubular cells (PTEC) from cells in other tubules. The brush borders of cells in the *pars convolute* (the first, convoluted part of the proximal tubule) tend to have a higher density and a larger number of mitochondria than those of cells in the *pars recta* (the second, straight part of the proximal tubule), suggesting that transport functions in the former are more developed than in the latter (Ross *et al.*, 1995).

The proximal tubule is where most of the reabsorption process takes place and is regarded as the most vulnerable segment of the nephron (Bakoush *et al.*, 2001). As such, it requires sufficient oxygenation to ensure enough metabolic energy is present to aid solute transport. In the *pars convolute*, proteins and peptides in the initial glomerular filtrate are usually reabsorbed by endocytosis and binding to the brush border membrane (Koeppen&Stanton, 2007). Enzymes within the brush border cleave peptides at the amino site of hydrophobic amino acides The brush border contains enzymes that can cleave peptides at the amino site of hydrophobic amino acids, such as membrane metallo-endopeptidase (NEP; neutral endopeptidase, kidney-brush-border neutral proteinase, enkephalinase, EC 3.4.24.11) (Connelly *et al.*, 1985; Inoue *et al.*, 2003).

1.1.1.2.1 Proximal tubular epithelial cells (PTEC)

Like other transporting epithelial cells, PTEC have a basic polarised structure comprising an apical (luminal) membrane and a basolateral membrane. The former contains a number of transmembrane carriers that allow solutes to enter the cell from the tubular lumen (Schnermann, 1998), while the latter contains other transporters (including the Na^+-K^+-ATPase pump) and ion channels that return the filtered solutes back into the circulation (Burrow *et al.*, 1999). The shape and polarity of PTEC is governed by the cytoskeleton, which also plays a vital role in the maintenance of cell:cell and cell:matrix interactions (Khurana, 2000).

1.1.1.3 Loop of Henle

The descending limb of the proximal tubule is linked to the ascending limb of the distal tubule by the medullary loop (or 'loop of Henle'), a thin-walled U-shaped structure with a hairpin turn. In some nephrons, this may be very short and there is an abrupt change between the distal thin and thick ascending segments. Here, the luminal membrane does not contain a brush border and there are few folds in the basal membrane (Ross *et al.*, 1995).

1.1.1.4 Distal tubule

From the loop of Henle, the filtrate proceeds into the distal convoluted tubule. This structure is typically only one-third of the size of the proximal tubule and leads to the collecting ducts. Here, the cells have no brush border and the number of basal infolds gradually decreases along the length of the tubule. This segment plays an important role in the reabsorption of Na^+ (with concomitant secretion of K^+) and the continued reabsorption of biocarbonate ion (with concomitant secretion of H^+). Thus, the urine becomes more acidic and ammonia is converted to ammonium ion; this then enters the urea cycle in order to avoid toxicity (Mirpuri&Patel, 2000).

1.1.1.4.1 Juxtaglomerular apparatus

Where the distal thick segment of the nephron is situated next to the glomerular vascular pole, the tubule is lined with a distinctive cell type collectively referred to as the macula densa. These cells are narrow and often more elongated than other cells in the distal tubule. In this same region, the smooth muscle cells of the afferent (and sometimes the efferent) arteriole are modified. These modified cells (or 'juxtaglomerular cells') synthesise, store and release renin into the blood, and are distinguished by their spherical nuclei and granulated cytoplasm. Currently, it is believed that a reduction in either blood volume or Cl^- concentration in the blood stimulates the release of renin by these cells. Thus, the juxtaglomerular apparatus acts as both a regulator and sensor of blood composition and volume (Koeppen&Stanton, 2007).
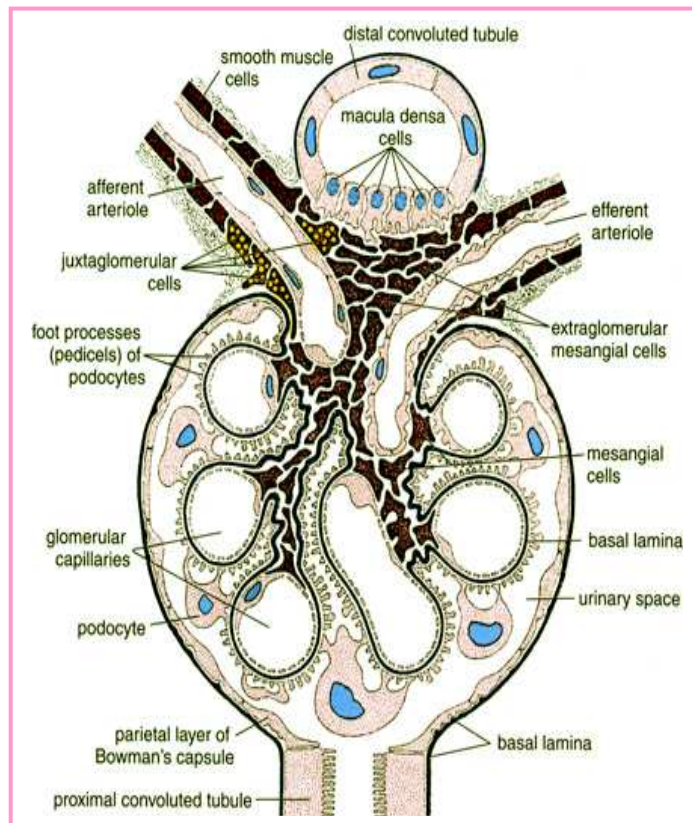
Figure 1-2 Schematic diagram of renal glomerulus

Adapted from:
http://anatomy.iupui.edu/courses/histo_D502/D502f04/lecture.f04/urinaryf04/glom.jpg.

1.1.1.5 Collecting ducts

The final segment of the nephron is the cortical collecting duct. These are approximately 20 mm long and are lined with cuboidal cells, only a small number of which project onto the luminal surface. Before draining the urine into the renal pelvis, the collecting ducts pass through the renal cortex and medulla. In the former, each duct drains approximately six distal tubules, while in the latter the distal tubules combine in pairs to form a duct of Bellini. The urine is subsequently drained into the renal calyx, the renal pelvis, the ureter and into the bladder (Ross *et al.*, 1995).

1.2 Applied physiology of the kidney

The overall composition of urine is first and foremost a reflection of the filtration process, the exchange of molecules between the nephron and the blood that takes place in the glomerulus. However, as filtration is nonselective with regard to small molecules, the selective reabsorption phase is crucial for returning any small molecules that the body needs back into the bloodstream. The final phase in the formation of urine is secretion .

1.2.1 Filtration

Glomerular filtration is a passive transport process that allows macromolecules to pass through the capillaries and into the nephron, depending upon their shape, charge and molecular weight. For instance, blood cells, plasma proteins and other large molecules remain in the capillaries while water and other small molecules <70 kDa (e.g. glucose, amino acids, Na^+, urea, K^+) pass through the filter, some of which are reabsorbed later. Other than the important exception of plasma proteins, the composition of the glomerular filtrate is not hugely different from that of the plasma.

The difference in BP between the glomerulus and Bowman's capsule aids the filtration process. The glomerular filtration rate (GFR) is often used to assess how well the kidneys are working and reflects the volume of filtrate produced by each kidney per minute. Where differences are seen in the volume and concentration of filtrate, this is due to the selective reabsorption of some molecules and tubular secretion of others (Koeppen&Stanton, 2007).

1.2.2 Reabsorption

The process of selective reabsorption returns the small molecules that the body needs from the filtrate to the interstitial fluid and blood plasma by transporting them across the epithelium of the renal tubule. Once the initial filtrate leaves the glomerular corpuscle, reabsorption takes place throughout the rest of the nephron, mainly in the proximal tubule but also in the loop of Henle, the distal tubule and the collecting ducts. Almost all organic nutrients (such as sugar and vitamins) are reabsorbed from the initial filtrate, significantly modifying its composition by increasing concentrations of some substances and reducing the concentrations of others in the excreted urine.

If an unusually high level of a particular substance (e.g. glucose) is in the blood, the reabsorption process may be overloaded. In such cases, the transport maximum (the kidney's highest capacity for reabsorption of a substance) is exceeded and the substance will be detected on urinalysis. Active transport out of the tubules cannot occur because all the carrier sites are occupied; other substances reabsorbed by active transport include Na^+, K^+, PO_4^- and Cl^-. Depending upon the nephron segment, Na^+ and Cl^- may be reabsorbed passively instead (Koeppen&Stanton, 2007).

The transport maximum typically varies according to needs and may be regulated by hormones. For instance, the reabsorption of calcium and phosphate depends upon

parathyroid hormone and calcitonin, while aldosterone increases the reabsorption of sodium and excretion of potassium. The reabsorption of water is influenced by antidiuretic hormone, which increases the permeability of the distal tubule and collecting ducts. Nitrogenous waste products (e.g. urea and uric acid) and substances not normally found in blood (e.g. drugs) are reabsorbed in very small amounts or not at all. If filtration does not sufficiently clean these substances from the blood, they are secreted directly into the tubules and excreted in the urine (Mirpuri&Patel, 2000).

1.2.3 Secretion

The secretion of unwanted substances in plasma most commonly occurs in the proximal and distal convoluted tubules. Where the initial filtration process is relatively non-selective, secretion is a highly selective process that involves both passive and active transport.

In order to maintain the normal pH in plasma, the cells that line the proximal tubule secrete H^+ ions that combine with buffers – bicarbonate (forming carbonic acid), ammonia (forming ammonium ions) and hydrogen phosphate (forming dihydrogen phosphate) – in the filtrate. The normal pH of urine varies from 4.5–8, depending on a wide array of factors, including time of day and diet (Koeppen&Stanton, 2007).

1.3 Kidney diseases

In the previous sections I have described the normal anatomy and physiology of the human kidney. Here, I describe how abnormalities in renal processes and structures can lead to one of several diseases.

1.3.1 Renal failure

1.3.1.1 Acute renal failure (ARF)

Acute renal failure (ARF) is a sudden and dramatic drop in the GFR, reflecting a severe impairment of kidney function that is usually reversible on treatment. ARF may occur as a complication of a number of non-renal conditions; the causes are classified as follows:

- Pre-renal: caused by reduced blood flow, such as in severe and prolonged shock.

- Renal: caused by damage to the kidney itself, for example, due to glomerulonephritis (GN) or acute tubular necrosis.

- Post-renal: caused by an obstruction in urinary outflow, e.g. kidney stones or tumour.

1.3.1.2 Chronic renal failure (CRF)

Chronic renal failure (CRF), defined as irreversible damage to three-quarters of nephrons in the kidney, usually develops over several years, slowly and asymptomatically. It is characterised by a severe impairment in GFR that allows waste products such as urea and creatinine to accumulate in the blood. The signs and symptoms of CRF include acidosis, electrolyte imbalances, anaemia and hypertension, and are collectively referred to as uraemia. Common causes of CRF include hypertension, diabetes mellitus, reflux nephropathy and GN.

1.3.2 IgA nephropathy (IgAN)

First described in 1968, IgA nephropathy (IgAN) (Berger&Hinglais, 1968) is characterised by deposits of IgA1 in the mesangium, often alongside deposits of complement factor C3 and IgG, IgM or both (Bairoch *et al.*, 2005; Khan *et al.*, 2003; Monteiro, 2005; Tomino, 2005). In early studies, IgAN lesions were found in approximately one-quarter of renal biopsies (D'Amico *et al.*, 1987), with later studies from all over the world confirming that IgAN is the most prevalent form of primary GN (Wiwanitkit, 2006).

IgAN may present as a wide variety of glomerular lesions. When making a diagnosis, serum or plasma levels of IgA-containing immune complexes (IC) should be measured (Czerkinsky *et al.*, 1986; Tomana *et al.*, 1997). Approximately 35–50% of patients exhibit an elevated serum IgA; this elevation persists in some patients and declines to normal in others (Jia *et al.*, 2004). An increase in serum IgA levels in itself does not result in IgAN. Although the IgA1 subclass is more common worldwide, African–American patients also show an elevated concentration of IgA2 (in accordance with the finding that over half of African–American patients with IgAN have IgA2 deposits in the mesangium).

High titers of IgA-containing ICs are commonly found in patients with primary IgAN, occurring in 30–70% of patients (Coppo *et al.*, 1995), while high serum concentrations of polymeric IgA (pIgA) have been found in 25% of patients. IgA-containing ICs are seen

both in acute disease and in remission; IgG-containing ICs are much less common and only seem to appear during relapses (Schena *et al.*, 1989).

Proliferation of MSC and fibrosis have been observed even in patients with mild clinical disease, with 30–40% of patients progressing to end-stage renal failure (ESRF) via progressive glomerular and interstitial sclerosis within 20 years of diagnosis (Emancipator, 1998). Although the clinical course and impact of IgAN are well documented, the molecular pathogenesis of IgAN is as yet unknown. There is evidence to suggest that the condition is induced by factors outside of the kidney; IgAN recurs in around 50–60% of patients who receive a renal allograft (Coppo *et al.*, 1995; Odum *et al.*, 1994), while patients with non-IgAN renal disease who receive an inadvertent transplant from a donor with subclinical IgAN are able to clear the immune deposits within a matter of weeks. The risk of recurrence of IgA deposits in transplant recipients does not seem to be affected by the use of immunosuppressants and may be associated with recurrence of GN, which may lead to graft failure (Floege *et al.*, 1998). In addition, the idea of a genetic basis for susceptibility to IgAN is being increasingly supported, with several families having been identified (Hsu *et al.*, 2000).

1.3.2.1 Clinical and histological features of IgAN

The clinical and histological severity of IgAN can vary widely from patient to patient. Although typical cases experience a slow progression from mesangial proliferative GN to glomerulosclerosis via tubulointerstitial fibrosis and atrophy, sometimes microscopic haematuria is the only clinical indication of illness (Smith&Feehally, 2003). Two other small subgroups of patients have been identified: those with ARF in association with crescentic nephritis and those with steroid-responsive nephrotic syndrome.

Immunofluorescence techniques are required for a definitive diagnosis of IgAN. Specific antibodies that recognise IgA are used to highlight the deposits; these are usually found in the glomerular mesangium but may also occur within the walls of the glomerular capillaries (Figure 1-3). Immunofluorescence staining may also show C3, IgG and IgM co-deposits sometimes associated with IgAN. IgA-containing ICs within the mesangium may be identified by granular electron-dense deposits using electron microscopy.
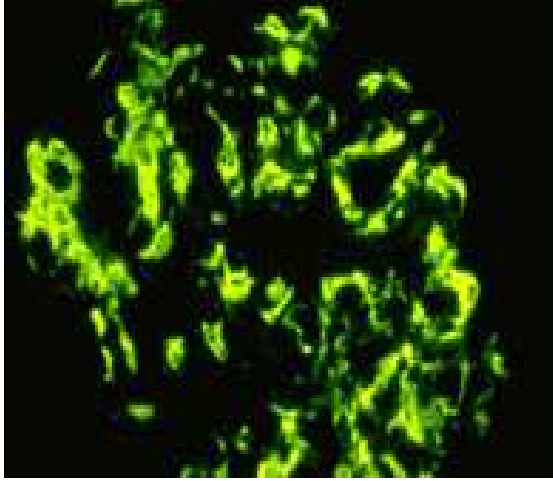
Figure 1-3 A biopsy from a patient with IgA nephropathy (Berger disease)

Positive staining by immunofluorescence shows mesangial distribution of IgA in a glomerulus with IgAN.
Adapted from: http://www.iganbio.com/images/glomerulus-stained.jpg

1.3.2.2 Pathogenesis of IgAN

The molecular pathogenesis of IgAN is not yet fully understood, though various cytokines and growth factors have been implicated (Gomez-Chiarri *et al.*, 1994; Horii *et al.*, 1989; Lovett *et al.*, 1986; Wardle, 1991). Most studies to date have been of limited scope, focusing on no more than a few proinflammatory cytokines at a time, and whilst these molecules may indeed have an important role in the pathogenesis of IgAN, it is difficult to disentangle their influence on the induction and maintainence of the immunological insult. Various animal studies have shown that the blockade of some cytokines can attenuate renal injury.

Experimental studies in different animal models support the role of cytokines in development of renal injury. Transforming growth factor-β (TGF-β) antagonists have been shown to prevent glomerular sclerosis (Border *et al.*, 1992), while interleukin-1 (IL-1) in combination with either IL-6 or interferon-γ (IFN-γ) has been implicated in the upregulation of MSC proliferation in the presence of IgA-containing IC deposits in an experimental model of IgAN (Feehally, 1997; Montinaro *et al.*, 1992; Moura *et al.*, 2004).

Three crucial stages are involved in the pathogenesis of IgAN (Bairoch *et al.*, 2005; Monteiro, 2005):

- Generation of abnormal IgA1 and formation of IgA1 IC.

- Mesangial injury mediated by the interaction of IgA1 IC with (as yet unidentified) MSC IgA1 receptors.

- Progression of IgA-mediated mesangial injury.

A number of immunoregulatory abnormalities in the synthesis of IgA1 have been reported in patients with IgAN. These include overproduction of IgA1 by B-lymphocytes *in vitro*, elevated levels of IgA in serum, IgA-containing IC (Leung *et al.*, 2003). A deficiency in the galactosylation of O-linked glycans in the hinge region of circulating IgA1 molecules in IgAN patients has also been proposed (Allen&Feehally, 1998; Couser, 1999; Floege&Feehally, 2000; Hiki *et al.*, 1995; Tomana *et al.*, 1997).

These hinge-region glycopeptides lead to the formation of circulating ICs via antibodies specific to anti-glycan or anti-hinge region peptides (Kokubo *et al.*, 1999; Tomana *et al.*, 1999). Circulating ICs containing Gal-deficient IgA1 from IgAN patients have been shown to bind more efficiently with MSC than non-multimer IC/IgA (Novak *et al.*, 2005). The same study showed that circulating ICs from IgAN patients bound to MSC in greater numbers than ICs from healthy controls.

The results of Novak et al. therefore support the hypothesis that the pathogenesis of IgAN somehow involves abnormally glycosylated IgA1-containing ICs. Other studies have shown that binding of heat aggregated IgA (HA-IgA) to MSC activates the cells, mobilises intracellular calcium storage and induces both protein phosphorylation (Gomez-Guerrero *et al.*, 1996) and the release of IL-6 and tumour necrosis factor-α (TNF-α) (Gomez-Guerrero *et al.*, 1994). Further studies have shown that ECM components such as fibronectin (FN) and collagen may be increased associated with circulating ICs in the serum of patients with IgAN (Moura *et al.*, 2004; Novak *et al.*, 2005).

In relation to cytokine release, studies in experimental models of IgAN have suggested that T cells and macrophages have a pivotal influence on glomerular and interstitial injury (Lai *et al.*, 1994). Enhanced gene expression of cytokines implicated in IgAN is seen in CD4-positive T cells (Lai *et al.*, 1994), while the degree of localised macrophage proliferation appears to correlate with the severity of the insult. Whilst these mechanisms are certainly feasible, more research is needed to further elucidate the manner in which glomerular deposits of IgA-containing ICs mediate MSC proliferation and tubulointerstitial sclerosis.

1.3.3 Membranous glomerulonephropathy (MGN)

Membranous glomerulonephropathy (MGN) is the most common cause of the nephrotic syndrome, with up to one-half of all patients progressing to ESRF within 10 years (Glassock *et al.*, 1991). It is distinguished by the accumulation of small IC deposits in the lamina rara externa of the GBM that eventually results in large deposits encapsulated in GBM matrix (Bohle *et al.*, 1994) (Figure 1-4). As with IgAN, the pathogenesis of MGN is not yet fully understood. A popular proposed mechanism is that of an autoimmune disease in which autoantibodies specific for visceral epithelial cells develop; these antibodies then permeate through the GBM and deposit subepithelial IC (Schena *et al.*, 1997).

If the above mechanism holds then mesangial or subendothelial deposits are not possible due to ultrafiltration moving away from the lumen. Thus, a secondary mechanism has been proposed in which MGN is caused by IC composed of circulating antigens and antibodies; once permeated through the GBM, some of these antibodies will bind with antigens in the subepithelial zone and some will form IC in subendothelial or mesangial regions. This secondary MGN may be caused by infection, systemic autoimmune disease or tumour.

Figure 1-4 Membranous nephropathy

Electron micrograph illustrates feature of MGN showing thickened glomerular basement membrane (arrow) representing deposits of ICs in the mesangium. Adapted from: www.gamewood.net/rnet/renalpath/ch7.htm

1.3.4 Other forms of glomerulonephritis (GN)

From its first description in the literature by Klebs (Stratta *et al.*, 1999), GN has developed into an umbrella term for any primary renal disorder or a secondary renal complication of systemic disease (Couser, 1999), and is one of the primary causes of ESRF. Although the

term itself implies some degree of inflammation, this is not always the case. Besides glomerular injury caused by ICs, other immune mechanisms have been implicated in GN. Microscopy is necessary to differentiate between different forms of GN, as well as any immunological characteristics and known causes. Microscopy findings distinguish between the different forms of GN by evaluating the extent of the damage (diffuse or focal) and glomerular appearance (e.g. cell proliferation or thickening of the GBM).

1.3.4.1 Immune-complex glomerulonephritis

It is widely accepted that the pathogenesis of many common forms of idiopathic GN (e.g. IgAN, MGN) are related to ICs, whether as circulating multimers or as *in situ* deposits (Cohen *et al.*, 1979). At present, these diseases can only be classified by morphological means; subepithelial deposits indicate primary MGN, while IgA antibodies in deposits defines IgAN. Where GN occurs secondary to infectious diseases, the associated antigenic stimuli are known. Thus, in order to establish a causal link between the infection and GN, the circulating ICs must contain both the specific antigen and the antibody directed against it. In addition, glomerular IC deposits should be both evident and contain the infectious agent (Javaid&Quigg, 2005).

Inflammation within the glomerulus is a fundamental factor of IC-GN. It is unlikely that IC formation can be uncoupled from the inflammatory response due to the complexity of the inflammatory cascade induced by these deposits (Clynes *et al.*, 1998). Studies have shown that the size and blood clearance kinetics of ICs are influenced by the biochemical characteristics of the circulating antigen (Doekes *et al.*, 1984) (Figure 1-5). Small, soluble ICs are generated when excess antigen or oligovalent antigen is present; these ICs are often essentially inert as they are inefficient at activating complement and attaching to Ig receptors. Large, soluble ICs are formed when the ratio of antigen to antibody approaches equivalence and cross-linking is maximised; these ICs are quickly removed from the circulation by phagocytosis as they bind with high affinity to Ig receptors and efficiently activate complement (Nangaku&Couser, 2005).

Thus, it is the medium-sized complexes that cause problems as they can activate complement and bind to Ig receptors but are less easily cleared from the bloodstream; therefore, they tend to deposit in tissues and induce glomerular injury. In MGN, these deposits are found between the GBM and the visceral glomerular epithelial cells (GEC). Consequently, there has been a considerable research effort to identify a specific IgG receptor on the surface of the GEC, with limited success. Although soluble aggregrated

IgG and Fc fragments, but not F(ab'2) fragments, were able to bind GEC both in culture (Mancilla-Jimenez *et al.*, 1984) and in tissue sections (Mizoguchi&Horiuchi, 1982), investigators were unable to identify the receptors responsible for this binding. Further studies also failed to find expression of FcγRI, FcγRII or FcγRIII receptors on the surface of GEC (Aarli *et al.*, 1991; Morcos *et al.*, 1994; Tuijnman *et al.*, 1993), suggesting that alternative receptors may be involved.

The presence of complement components in renal biopsies provide further, indirect evidence of IC-GN, as well as changes in serum complement levels and the presence of circulating complement breakdown products.
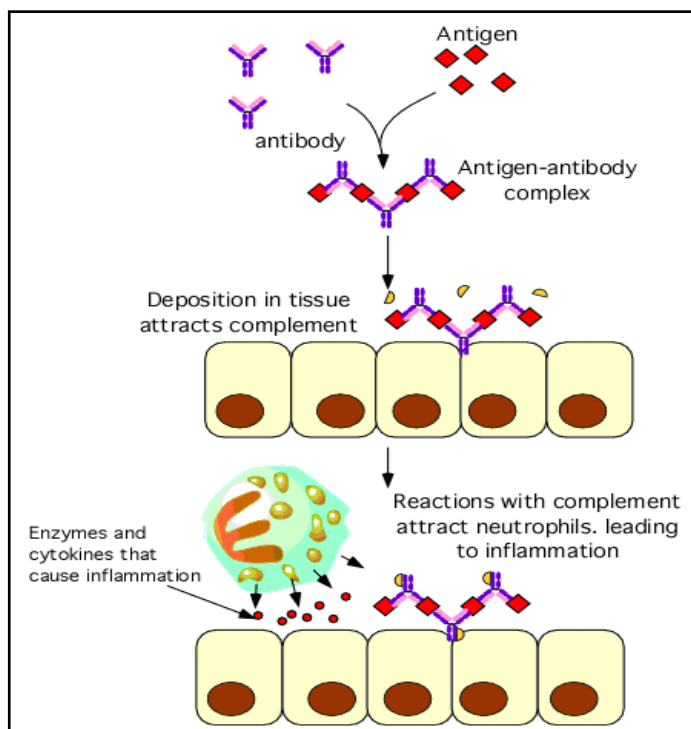
Figure 1-5 Immune complex deposion

This diagram shows that chronic exposure to an antigen causes the formation of ICs between the antigen and antibodies raised against it. These accumulate in specific areas of the kidney and elicit an inflammatory response that damages the surrounding tissue. Adapted from:
http://www.bact.wisc.edu/Microtextbook/images/book_4/chapter_15/15-30.gif.

1.3.5 Tubulointerstitial inflammation

Studies have identified four different mechanisms of tubulointerstitial injury (Barratt *et al.*, 2004; Chan *et al.*, 2005):

- Monocytic/macrophage infiltration.

- Proteinuria.

- Inflammation as a direct consequence of IgA (or other Ig) deposits.

- Glomerulotubular cross-talk.

The infiltration of inflammatory cells into the tubulointerstitium plays an important role in the mediation of renal fibrosis and tubular injury (Wu *et al.*, 2004). Studies have shown that PTEC can induce this infiltration and, when activated as a consequence of this, can contribute to fibrosis by producing an array of inflammatory mediators (van Kooten *et al.*, 1999). A vicious cycle occurs in which mediators released by infiltrating cells directly activate PTEC, which in turn release other proinflammatory mediators that can amplify the inflammatory cascade. This positive feedback loop can lead to excessive production of ECM components and, subsequently, renal fibrosis. It has therefore been proposed that a cytokine 'cross-talk' network exists between PTEC and immunocompetent cells within the interstitium and is the key inducing factor in tubulointerstitial injury.

In most glomerular diseases, proteinuria can be considered as the primary cause of PTEC activation (Tang *et al.*, 2003), and is associated with the release of vasoactive and inflammatory mediators, including endothelin-1, monocyte chemoattractant protein-1 (MCP-1) and RANTES (regulated upon activation, normal T-cell expressed and secreted) (Praga&Morales, 2002). Heavy proteinuria (i.e. urine containing a high content of high-molecular-weight IgM and IgG proteins) has been associated with eventual progression to ESRF (Tencer *et al.*, 2000). Although IgG and complement components are often co-deposited, IgA alone can provoke glomerular injury in some patients.

1.3.6 Tubulointerstitial fibrosis

Whatever the initial renal insult, tubulointerstitial fibrosis is regarded as the common final pathway leading to ESRF (Becker&Hewitson, 2000; Eddy, 2000) and is a better predictor of functional impairment than glomerular damage (Nath, 1992). In CRF patients, the rate of decline in GFR is strongly correlated with the severity of fibrosis (Bohle *et al.*, 1996). In older patients, structural changes in otherwise healthy kidneys involves some degree of tubulointerstitial fibrosis (Ruiz-Torres *et al.*, 1998), while in transplant recipients tubulointerstitial fibrosis is characteristic of chronic allograft nephropathy (CAN), the most common cause of graft failure in the first 10 years (Paul, 1999).

Tubulointerstitial fibrosis is a direct effect of the overproduction and blunted degradation of ECM (Heidland *et al.*, 1997). The accumulation of ECM has been associated with a number of cytokines, which also compound the process by stimulating macrophage infiltration and the proliferation/transdifferentiation of fibroblasts (Eddy, 1996b). Aberrant TGF-β signalling has been identified in various proliferative disorders, including GN, cancer and atherosclerosis (Bottinger *et al.*, 1997; Hu *et al.*, 1998). There is significant evidence to suggest that TGF-β1 expression is closely linked to the progression of tubulointerstitial fibrosis (Border *et al.*, 1996), and that renal PTEC may contribute to this by releasing TGF-β1 when activated (Johnson *et al.*, 1998). By reducing the expression of metalloproteinase enzymes that degrade interstitial proteins (e.g. the ECM components FN and collagen) and by stimulating the production of metalloproteinase inhibitors in tissue, TGF-β effectively promotes the accumulation of these proteins in the interstitium (Grande *et al.*, 2002).

Studies in rats have suggested that, in addition to modulating ECM turnover, TGF-β1 may have an important role in mediating the differentiation of PTEC into α-smooth muscle actin (α-SMA)-positive cells (Tian *et al.*, 2003). Other studies have proposed that TGF-β1 induces changes in the phenotype of PTEC, though the mechanisms by which this occurs remain to be clarified.

1.3.7 Proteinuria

Proteinuria is a well recognised risk factor for disease progression in GN and occurs by two distinct mechanisms: increased permeability of the glomerular filtration slits causing abnormal passage of proteins, and impaired reabsorption by PTEC (D'Amico&Bazzi, 2003b). By the time proteinuria becomes clinically detectable (i.e. urinary excretion of low- and high-molecular-weight proteins is significantly elevated), many patients will already have structural damage to the podocytes in the glomerulus and to the tubulointerstitium. Recent clinical studies have suggested that the urinary excretion of some high-molecular-weight proteins (IgG, IgM) and of some low-molecular-weight proteins (β1- and β2-microglobulin) is a better predictor of the histological severity of disease than the overall quantity of proteinuria. Compared with other forms of GN, IgAN presents a unique complication when assessing the prognostic value of high-grade proteinuria as heavy proteinuria is not common in IgAN and nephrotic syndrome occurs in only 5% of patients (Lai *et al.*, 2005; Tomino&Sakai, 2003).

1.3.8 Nephrotic syndrome

The nephrotic syndrome is not a disease in itself; instead, it is a term applied to a group of abnormalities, including proteinuria, hypoalbuminaemia, generalised oedema and hyperlipidaemia, which occur in several forms of renal disease. Damage to the glomeruli results in increased permeability of the glomerular membrane and plasma proteins are filtered out of the bloodstream. Albumin, the smallest and most common of the plasma proteins, is most affected, and when the daily loss of albumin exceeds the hepatic production, a significant drop in total plasma protein level occurs (D'Amico&Bazzi, 2003a). This is accompanied by widespread oedema and reduced plasma volume, which reduces renal blood flow and stimulates the renin–angiotensin–aldosterone system (RAAS), causing increased reabsorption of water and sodium in the tubules. This increase in water reabsorption further reduces the osmotic pressure and worsens the swelling. This damaging cycle continues as long as albumin is depleted throughout the glomerular membrane. Nitrogenous waste products usually remain at normal levels. The cause of hyperlipidaemia, especially hypercholesterolaemia, in the nephrotic syndrome is unknown.

1.4 Therapeutic strategies

At present, there is no effective or specific treatment for IgAN and intervention has relied upon attempting to slow the rate of disease progression. In order to do this, the inflammatory cascade must be prevented and thus the pro-inflammatory signals generated by cytokines and growth factors are suitable targets. As with other glomerular and progressive renal diseases, attempts must be made to control hypertension and reduce proteinuria using agents that inhibit the RAAS (Russo *et al.*, 2001). Immunosuppressants are often used with the aim of reducing Ig/IC formation, but there is no clear consensus on how effective these are. Commonly used agents include corticosteroids (Katafuchi *et al.*, 2003; Pozzi *et al.*, 1999), azathioprine (AZA) (Yoshikawa *et al.*, 1999), cyclosporine A (Cattran, 1991) and mycophenolate mofetil (MMF). Various studies have shown that while these drugs may effectively reduce proteinuria (Tang *et al.*, 2005), they do not prevent progression to ESRF (Frisch *et al.*, 2005). Two exceptions to this lack of consensus exist: IgAN patients with nephrotic syndrome clearly benefit from corticosteroid therapy, while patients with rapidly progressing IgAN with crescent formation may benefit from the immunomodulatory effects of cyclophosphamide.

Other pharmacological interventions include the use of angiotensin-converting enyme (ACE) inhibitors and low-molecular-weight heparins (LMWH). *In vitro* studies have shown that ACE inhibitors reduce the expression of TGF-β mRNA (which, alongside platelet-derived growth factor [PDGF], is stimulated by angiotensin II) (Kagami *et al.*, 1994; Ruiz-Ortega *et al.*, 1995). Clinical trials of ACE inhibitors and angiotensin receptor blockers (ARBs) have been carried out in patients with either primary or secondary GN with subsequent attenuation of the disease progression (Gansevoort *et al.*, 1995). LMWH have been shown to have several anti-inflammatory properties, including the blockade of TGF-α and fibroblast growth factor (FGF) (Wardle, 1996), and to inhibit the proliferation of mesenchymal cells. They also inhibit complement activation and the secretion of elastane and other lysosomal enzymes from polymorphonuclear (PMN) cells.

Non-pharmacological interventions have also been proposed. First suggested in 1984, the hypothesis that omega-3 fatty acids in fish oils may benefit cell membrane fluidity and slow the rate of disease progression (Hamazaki *et al.*, 1984) has yielded conflicting results. In 1994, a randomised clinical trial supported this effect and suggested that it occurred independently of changes in proteinuria and BP (Donadio *et al.*, 1994). However, in 1997, a meta-analysis concluded that fish oils do not confer a significant benefit in the treatment of IgAN (Dillon, 1997). A protein-restricted diet has long been recommended for the reduction of glomerular hyperfiltration and to slow the progression of renal damage. In addition, a study in rats with MGN recently demonstrated that a low-protein diet significantly reduced TGF-β expression (Okuda *et al.*, 1991).

Although most of these therapeutic options are still in development, they are representative of a new paradigm for slowing the progression to ESRF in IgAN by ameliorating tubulointerstitial injury.

1.5 The immune system

In order to understand the involvement of PTEC in the development of renal disease, it is necessary first to discuss some aspects of the immune system, a complex network that guards against invading pathogenic microorganisms. The immune system network comprises two different branches: innate (non-specific) immunity and adaptive (specific) immunity (Takeda *et al.*, 2003). The former is present from birth and does not develop memory, while the latter requires a previous encounter with invading microbes.

Innate immunity has two components: cellular and soluble. The cellular component includes the phagocytic system, which encompasses and digests invading microorganisms and includes neutrophils and monocytes in serum and macrophages in tissue, and natural killer (NK) cells, which attack certain tumours, microorganisms and virus-infected cells. Complement proteins, acute phase reactants and cytokines make up the soluble component.

When challenged, the innate immune system activates the adaptive immune system, which provides a potent defence against microbial infection by detecting 'non-self' using antigen receptors found on the surface of B and T cells. Once recognised, the adaptive immune system remembers specific pathogens and prepares itself for future challenges so that it can become increasingly effective in attacking the invading microorganism each time it is encountered. The cellular and soluble components of adaptive immunity are lymphocytes and Igs, respectively.

Adaptive immune responses can be further categorised as either humoral or cellular immunity. Humoral immunity is mediated by antibodies produced by B cells, while cellular immunity involves the activation of macrophages and NK cells and the release of cytokines in response to an antigen. The majority of antigens are T cell-dependent and need to be processed by antigen-presenting cells (APCs), which convey the antigens to both T and B cells. The T cells then release cytokines that induce the B cells to generate antibodies.

1.5.1 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS), also known as endotoxin, is the major Gram-negative bacterial surface component in mammalian cells and prompts the release of inflammatory mediators (Hajjar *et al.*, 2002). Owing to their amphiphilic nature, these glycolipids are able to self-aggregate and form membranes (Wiese *et al.*, 2003). LPS is recognised by elements of the innate immune system called toll-like receptors (TLRs) expressed on the surfaces of macrophages, neutrophils and epithelial cells, especially TLR4. When activated, TLRs generate an immune response primarily through gene regulation via the nuclear factor-κB (NF-κB) transcription factor system (Takeda *et al.*, 2003).

In addition to TLR4, the recognition of LPS involves CD14, a glycosylphosphatidylinositol-anchored molecule usually expressed in macrophages and neutrophils. Several studies have suggested that an interaction between CD14 and TLR4 may play an important role in LPS signalling, which may explain why the two molecules

appear to become physically closer following LPS stimulation. This stimulation occurs when LPS binds to LPS-binding protein present in serum and the resulting complex is recognised by CD14 (Hedlund *et al.*, 2001; Hoshino *et al.*, 1999; Lien *et al.*, 2000).

A number of cytokines regulate the expression of TLRs. In renal epithelial cells, the expression of TLR2 and TLR4 is prompted by IFN-γ and TNF-α, inducing inflammation and the detection of bacterial invasion (Wolfs *et al.*, 2002). IFN-γ also enhances surface expression of TLR4 in human monocytes and macrophages, effectively priming phagocytes to respond to LPS (Bosisio *et al.*, 2002). Following a challenge with LPS or infection *in vivo*, colony-stimulating factor-1 (CSF-1) is induced and can prime macrophages to release even more inflammatory cytokines in response to continued LPS stimulation. The expression of the TLR2 gene on macrophages is induced by LPS and inflammatory cytokines, including IL-1, IL-2, IFN-γ and TNF-α (Matsuguchi *et al.*, 2000).

ECM components, including FN, are produced in response to tissue injury and play important roles in tissue remodelling (Hedlund *et al.*, 2001). This dual role of LPS as target and effector makes it a fascinating molecule, which, even though first described more than 100 years ago, still hides many miracles.

1.5.2 Phagocytes

Phagocytes (monocytes and macrophages) are vital to both the innate and adaptive immune systems (Abbas, 2000; Gordon, 1999). The innate functions of these molecules (specifically, the internalisation and digestion of pathogens bound to receptors on the cell surface) represent the first line of defence against invading microorganisms. In the adaptive response, B cells produce antigen-specific antibodies that then either form ICs or lead to the opsonisation of the pathogen. The antibodies are subsequently recognised by Ig receptors and are internalised by either endocytosis (ICs) or phagocytosis (opsonised particles). Macrophages participate in the production, activation and regulation of all immune effector cells, play a key role in tissue homeostasis and are highly heterogeneous.

1.5.3 Natural killer (NK) cells

There are various types of killer cells and these can be neatly dichotomised into those that are restricted by major histocompatibility complex (MHC), for example, cytotoxic T lymphocytes (CTLs), and those that are not. Cells in either category do not require antibodies, complement or phagocytosis to kill their targets, but rather they border the

target cell and deliver the lytic signal through the membrane. NK cells are of the latter type and make up approximately 5–30% of lymphocytes in normal peripheral blood (Trinchieri, 1989). Despite being lymphocytes, NK cells belong to neither the T nor B cell lineage and do not express either surface Ig or T cell receptor/CD3 on their surface. It is possible to increase target cell susceptibility to killing by NK cells by increasing MHC expression, for example, by transfection or by stimulation with IFN-γ (Zompi&Colucci, 2005).

Some tumour cells are killed by NK cells regardless of whether they express MHC, and there is some evidence to suggest that these cells may preferentially kill target cells that do not express class I MHC, or do so in small amounts. This apparent inhibition of NK killing activity by class I MHC expression led to the identification of several class I MHC receptors on the surface of NK cells. These receptors are structurally different from the T cell receptors (TCR) and are generally referred to as killer cell inhibitory receptors (KIRs). While engagement of MHC by the TCR on T cells leads to T cell activation, engagement of the MHC by most KIR leads to inhibition of NK activity, although there are some KIRs that can lead to activation.

NK cells express the Ig receptor FcγRIII and can use this receptor to mediate another kind of MHC-nonrestricted killing. Antibody-dependent cell-mediated cytotoxicity (ADCC) depends on the presence of antibodies that recognise a target cell (ADCC specificity is therefore conferred by the specificity of the antibody). Upon binding its antigen, the Fc region of the antibody is exposed and will bind its receptor on the NK cell to form a bridge. Once the bridge is formed, a poorly understood lytic signal is delivered to the target cell, resulting in release of granzyme and perforine and, ultimately, the demise of the target cell (Perussia, 2000).

Some forms of ADCC may prove useful for targeting tumor cells *in vivo* as a form of immunotherapy.

1.5.4 Lymphocytes

Lymphocytes originate from bone marrow lymphoid stem cells and are the principal cells of adaptive immunity, defending against invading pathogens and to preventing or reducing reinfection (Bradley, 2003). Lymphocytes mature in lymphoid organs: B cells are bone marrow-derived, while T cells migrate from the bone marrow to mature in the thymus. Lymphocytes can be divided into subsets either by function or by surface markers called clusters of differentiation (CD). To date, 166 CD have been identified. Lymphocytes are

clonally distributed and each clone specialises in recognising a specific antigen by means of its antigen receptor.

1.5.4.1 B cells

As discussed above, the principal function of B cells is to make antibodies against soluble antigens. The first interaction between the antigen and the B cell comprises the primary immune response, and the B cells committed to respond to this antigen undergo differentiation and clonal proliferation. Some become memory cells while others differentiate into mature antibody-synthesising plasma cells. The principal characteristics of the primary immune response are a latent period before the appearance of antibodies, the production of only a small amount of antibody, initially IgM, and then a switch of the Ig isotype (with T cell help) to IgG, IgA or IgE (Bradley, 2003). This process leads to the creation of numerous memory cells capable of generating a stronger response to the same antigen in the future.

The secondary immune response takes place on subsequent encounters with the same antigen. The principal characteristics are rapid proliferation of B cells, rapid differentiation into mature plasma cells and prompt production of large amounts of antibody, chiefly IgG, which are released into the blood and other body tissues where they can effectively encounter and react efficiently with the antigen. IgM, IgG and IgA may be generated against the same antigen. B cells derived from a single mature naïve B cell may differentiate into a family of B cells genetically programmed to synthesise antibodies of a single antigenic specificity, with representative clones committed to the production of each Ig class.

1.5.4.2 T cells

Once the TCR comes into contact with an APC, the T cell proliferates quickly and either differentiates in effector cells or matures into memory T cells that are stored in the spleen and at the site of first infection in preparation for a secondary response if and when it happens (Bradley, 2003; Daeron, 1997). Mature T cells can be divided into subclasses, the two most important being helpers (CD4+) and suppressors (CD8+) (Grunwelad, 1996). Studies have shown that there are two distinct subsets of T helper cells, namely Th1 and Th2, which regulate the cellular and humoral aspects of the specific immune response, respectively (Mosmann&Sad, 1996). The type of T helper cell appears to be determined in some way by IL-12 and IL-4 for Th1 and Th2, respectively (Romagnani, 1996). By

secreting proinflammatory cytokines such as IL-2 and IFN-γ, Th1 cells promote phagocytosis of intracellular microbes, whereas Th2 cells secrete cytokines including IL-4, -5, -10 and -13 to stimulate white blood cell/mast cell-mediated immune reactions and to downregulate the damaging effects of Th1 immune responses (Mosmann&Sad, 1996). T suppressor cells such as CTL recognise infected or cancerous cells and destroy them by releasing cytoplasmic granules containing membrane pore-forming proteins and enzymes.

The pro-inflammatory effects of Th1 cells are powerful and occur both within the kidney and in the systemic circulation, activating not only monocytes/macrophages and T cells but also resident renal epithelial cells (Mosmann *et al.*, 1991). Thus, T cells are vitally important to the immunological processes involved in all forms of GN (Nolasco *et al.*, 1987; Schena *et al.*, 1997).

1.5.5 Cytokines

Cytokines are the primary communicator between different immune system cells and also between immune system cells and cells belonging to other types of tissue. Once released in response to an immune stimulus, typically at very low concentrations, they tend to act over short distances and short time spans, binding to specific membrane receptors in the target cell and altering its gene expression. In the activation phase of immune responses, cytokines stimulate lymphocyte growth and differentiation, while in the effector phase they activate different effector cells in order to eradicate antigens. Cytokines also stimulate the development of hematopoietic cells. Different cell types may secrete the same cytokine; similarly, a single cytokine may have pleiotropic effects and act on several different cell types. Moreover, cytokine release often occurs in a cascading manner as one cytokine stimulates the target cell to produce additional cytokines.

Cytokines can be classified according to various subtypes, including TNFs (α, β), ILs (IL-1 to IL-8, IFNs (α, β, γ), TGFs and CSFs. In any given immune response, it is likely that numerous cytokines will be involved and that they may act synergistically or antagonistically. A new family of cytokines, the chemokines, has been described. These induce chemotaxis and migration of leukocytes, acting as magnets for eosinophils and are consequently involved in lymphocyte, granulocyte and monocyte infiltration and activation with the glomerulus and tubulointerstitium (Abbas, 2000).

1.5.5.1 Tumour necrosis factor-α (TNF-α)

LPS-activated mononuclear phagocytes are the major cellular source of TNF where it is initially synthesised as a non-glycosylated transmembrane protein of approximately 25kDa, though antigen-stimulated T cells, activated NK cells and activated mast cells are also able to secrete this cytokine. The synthesis of TNF is augmented by IFN-γ produced by T cells; therefore, TNF can be considered a mediator of both innate and acquired immunity and as such provides an important link between specific immune responses and acute inflammation. TNF acts as a regulator of leukocytes and endothelial cells, and many TNF responses involve upregulation of transcription of specific genes, often through NF-κB activation (Abbas, 2000).

1.5.5.2 Interleukin-1 (IL-1)

Stimulated macrophages are the primary cellular source of IL-1 but neutrophils, endothelial cells, smooth muscle cells, glial cells, astrocytes, B and T cells, fibroblasts and keratinocytes may also secrete this cytokine. Various processes can trigger the release of IL-1, for example, coming into contact with CD4+ T cells or bacterial products such as LPS, TNF or even IL-1 itself. The two IL-1 subtypes, α and β, are products of different genes and are <30% structurally homologous. However, both bind to the same cell surface receptors and have essentially identical biological effects. IL-1 is one of the most important immunomodulatory ILs and, at low concentrations, plays a regulatory role in local inflammation. IL-1 activation of T cells stimulates the release of IL-2, establishing an autocrine loop that further increases T cell activation. In addition, as with TNF-α, IL-1 activation of T cells upregulates the expression of IFN-γ. Another important effect of IL-1 is the induction of proliferation in non-lymphocytes (Abbas, 2000).

1.5.5.3 Interleukin-6 (IL-6)

IL-6 is secreted by several different cell types including macrophages, fibroblasts, endothelial cells and activated TH cells, and affects a number of target cells including resident glomerular and tubular cells (Coleman&Ruef, 1992). The regulation of IL-6 expression in monocytes is governed in part by a functional interaction between IFN-γ and TNF-α, which, while often transient, causes rapid release (Sanceau *et al.*, 1991). Following LPS challenge, IL-6 is detectable in the circulation and appears to be secreted as a secondary response (e.g. to TNF or IL-1) rather than as a consequence of LPS itself. Unlike the cytokines described above, IL-6 does not stimulate the release of more

cytokines and therefore serves mainly to enhance the immune response to other cytokines. IL-6 acts as a second signal for IL-2 release and promotes IL-2-dependent T cell proliferation (Smyth *et al.*, 1990). Moreover, both *in vitro* and *in vivo* studies have shown that IL-6 stimulates the proliferation of MSC (Horii *et al.*, 1989). lL-6 also acts as a co-factor with other cytokines to promote the early growth of haematopoietic stem cells in bone marrow. Furthermore, in the liver, IL-6 is the primary stimulator of the acute phase response. Other effects of IL-6 include enhancing B cell differentation and Ig receptors expression and enhancing the synthesis of glucocorticosteroids.

1.5.5.4 Interleukin-8 (IL-8)

Of the known chemokines, IL-8 is the most studied and is produced by monocytes and other lymphoid cells, endothelial cells, keratinocytes, epithelial cells and various tumours (Baggiolini *et al.*, 1989; Cubitt *et al.*, 1993). The synthesis of IL-8 is stimulated by LPS, TNF-α and IL-1α. As well as promoting the generation of reactive oxygen intermediates, degranulation and an increase in cystolic calcium levels in neutrophils *in vitro* (Peveri *et al.*, 1988), IL-8 acts as a chemoattractant and engineers the migration of neutrophils, basophils and T cells to the site of inflammation. Further *in vitro* studies have shown that IL-8 production occurs locally in glomerular cells, including MSC (Brown *et al.*, 1991) and podocytes (Huber *et al.*, 2002), PTEC (Gerritsma *et al.*, 1996b; Schmouder *et al.*, 1992) and kidney-derived fibroblasts (Lonnemann *et al.*, 1995). *In vivo*, the effects of IL-8 are commonly associated with the localised accumulation of neutrophils at sites of acute or chronic inflammation (Niemir *et al.*, 2004; Swensson *et al.*, 1991).

1.5.5.5 Interferons (IFN)

There are two types of interferons – type I (IFN-α, IFN-β) and type II (IFN-γ) – the latter of which plays an important role in immunity. The major cellular source of IFN-γ is CD8+ T cells, though nearly all cells express receptors for IFN-γ and, after binding with IFN-γ, promote APC interaction with T helper cells by increasing the surface expression of class I MHC (Abbas, 2000). IFN-γ also increases the expression of class II MHC proteins, further enhancing the APC–T cell interaction. The overall affect of IFN-γ is to promote Th1 inflammatory responses, while suppressing Th2 reactions. IFN-γ also can modulate the activity of polymorphonuclear neutrophils (PMN) and preincubation of normal PMN with IFN-γ has been reported to increase phagocytosis (Klebanoff *et al.*, 1992).

1.5.5.6 Transforming growth factor-β (TGF-β)

TGF-β plays a major role in the pathogenesis of progressive renal fibrosis in various types of GN (Schena *et al.*, 1997). When first synthesised, the protein is expressed in a latent form that must be activated by proteases (Tian&Phillips, 2003). Once activated, TGF-β exerts highly pleiotropic actions, inhibiting the growth of several cell types while promoting it in others, including a potent antiproliferative action. *In vitro* studies have shown that TGF-β inhibits renal PTEC proliferation and stimulates the synthesis of ECM components (including FN) as well as promoting apoptosis (Basile *et al.*, 1998; Nowak&Schnellmann, 1996). In addition, TGF-β appears to have an inhibitory effect on immune system activation, antagonising several facets of lymphocyte response to infection. TGF-β also acts on other cells, such as PMN and endothelial cells, primarily in a protective response to pro-inflammatory cytokines and generates signals that downregulate immune responses.

1.5.5.7 Granulocyte macrophage colony-stimulating factor (GM-CSF)

Granulocyte–macrophage CSF (GM-CSF) promotes the proliferation of lymphoid cells and is part of the inflammatory cascade. In humans, GM-CSF promotes the growth of leukocyte precursors such as platelets and erythrocyte progenitors, as well as activating mature leukocytes. It is not detectable in the circulation and is presumably limited to local actions. Accordingly, it has been proposed that the primary function of macrophage-derived GM-CSF is to activate mature leukocytes at the site of the inflammatory immune response. In bone marrow, it has been suggested that GM-CSF produced by T cells, endothelial cells or stromal fibroblasts plays a role in the mediation of haematopoietic effects (Abbas, 2000).

Factor	Size	Principal Source	Primary Activity
TNF-α	14 kDa	Macrophages, T cells mast cells, NK cells	Activation of neutrophils, synthesis of acute-phase proteins and induce apoptosis in several cell types.
IL-1α	17kDa	Monocytes, macrophages, B cells, APCs	Co-stimulates the maturation, proliferation and activation of APCs and T cells; induces inflammation, acute phase response and haematopoiesis.
IL-6	19-26kDa	Monocytes, macrophages, activated Th2 cells, APCs, other somatic cells	Promotes differentiation into plasma cells, antibody secretion, acute phase response and thrombopoiesis; synergistic with IL-1 and TNF on T cells.
IL-8	8.9kDa	Macrophages, endothelial cells, other somatic cells	Chemoattractant for neutrophils and T cells, chemotaxis
IFN-γ	25kDa	Th1 cells, T cells, NK cells	Induces class I and II MHC on APCs and somatic cells; activates macrophages, neutrophils and NK cells; promotes cell-mediated immunity, antiviral state, viral replication, Ig class switch to IgG2a, proliferation and pathogen elimination
TGF-β	25kDa	T cells, monocytes	Anti-inflammatory; promotes chemotaxis, proliferation and the synthesis of IL-1 and IgA; suppresses cytokine production and class II MHC expression; promotes wound healing; inhibits macrophages and lymphocyte proliferation
GM-CSF	14 kDa	Macrophages, Th cells	Stimulates stem cells to produce granulocytes (neutrophils, eosinophils, basophils) and macrophages; promotes the growth and differentiation of monocytes and denteric cells.

Table 1-1 Selected cytokines and their properties.

1.5.6 Immunoglobulins (Ig)

Ig antibodies are large proteins composed of four polypeptide chains (two identical heavy chains and two identical light chains) joined together by disulphide bonds. Each Ig recognises a specific antigen unique to its target and is used by the immune system to locate and destroy invading microorganisms. The family of Igs includes proteins in the IgG, IgA, IgM, IgD and IgE subclasses, all of which share the same basic, monomeric structure but have unique features that determine their specific effects (Fig. 1-6) (Takai, 2002). The heavy chains have a molecular weight of 50–70 kDa, compared with approximately 23 kDa for the light chains. The Y-shaped Ig molecule is divided into variable (V) and constant (C) regions. The V region is located at the distal ends of the Y arms where the diversity of amino acids is high (thereby determining the antigen-binding capacity of the molecule), while the C region, proximal to the antigen-combining site, contains a relatively constant sequence of amino acids that is distinctive for each Ig class.

Each Ig antibody has two functional fragments: Fab, which binds antigen, and Fc, which crystallises when stored at 4 ℃. Only two different amino acid sequences, κ and λ, are found in the constant regions of light chains, while there are five common sequences in the heavy chain constant regions; μ, γ, α, ε, and δ heavy chains are found in IgM, IgG, IgA, IgE, and IgD, respectively. Therefore, it is the constant region of the Ig heavy chain that determines the isotype of the antibody. IgG, IgA and IgD have heavy chains containing a hinge region, a proline-rich domain that allows for greater flexibility of the Fab. With the added advantage of being able to make contact at various angles, there is an increased likelihood of binding to the antigen.

1.5.6.1 IgG

IgG is a large monomeric Ig of approximately 150 kDa and is naturally found in serum at levels of 8–16 g/L. Having two antigen binding sites, IgG binds to a wide variety of pathogens, including bacteria, viruses and fungi, combating them by activating complement, opsonisation for phagocytosis and neutralisation of their toxins (Rojas&Apodaca, 2002). As the only Ig isotype to be able to pass through the placenta, it provides vital protection to the foetus before its own immune system has had time to develop.

In humans, the four IgG subclasses (IgG1, IgG2, IgG3 and IgG4) can be differentiated by the size of their hinge region and by the location and number of disulphide bonds, as well as by function. IgG2 is the only one unable to cross the placenta, IgG3 is the most effective at activating complement and IgG4 cannot activate complement at all. IgG1 and IgG3 are associated with opsonisation as they bind FcRs on phagocytic cells.

1.5.6.2 IgM

In its pentameric form (i.e. with five subunits), IgM is larger than IgG with a molecular mass of approximately 900 kDa and is present in serum at levels of 0.7–3.0 g/L. The five subunits of IgM are linked by an Fc-linked polypeptide called the joining chain (J chain). Due to the size of the molecule, its diffusion is limited. Thus, it is primarily found in serum (accounting for 5–10% of all serum antibodies) and is only seen in very small quantities in the interstitium. Due to the J chain, which is necessary for pentameric IgM to form and is added just prior to secretion, IgM is an important secretory Ig. It is the first isotype to be generated during a primary immune response and is a highly effective activator of

complement. By virtue of its pentameric structure, IgM has 10 antigen binding sites and thus each antibody can bind up to five molecules of antigen.

1.5.6.3 IgA

The human body produces more IgA daily than any other antibody isotype (approximately 70 mg/kg). IgA has an approximate molecular mass of 160 kDa and, after IgG, is the second most prevalent antibody in the bloodsteam at concentrations of approximately 2–4 g/L (Woof&Mestecky, 2005). IgA is found in most external secretions that bathe mucosal surfaces, for example, saliva, tears, mucus and breast milk, and is principally dimeric (i.e. composed of two monomeric IgA subunitys and a J chain) (van Egmond *et al.*, 2001). The total area of these mucosal surfaces is vast and they are vulnerable to exposure to pathogens. As the primary antibody isotype present at these locations, secretory IgA is an important defence mechanism against invading antigens. Serum IgA acts as both a pro- and an anti-inflammatory mediator. By engaging cellular IgA receptors it can deliver antigens to the immune system, while engaging effector leukocytes can result in either protective or pathological inflammatory reactions (Wines&Hogarth, 2006).

Two different gene product subclasses of IgA exist in humans: IgA1 and IgA2. In serum, IgA is mainly composed of the former. There is good homology between the two subclasses, with the exception of a short mucin-like hinge region, unique to primate IgA1, that gives the molecule its unique receptor and lectin-binding properties. Receptors for IgA were first described more than 25 years ago (Lawrence *et al.*, 1975). At present, there are five known types of IgA receptor: FcαRI (CD89), the polymeric Ig receptor (pIgR), Fcα/µR, the transferrin receptor (CD71) and the asialoglycoprotein receptor (Ding *et al.*, 2003).

1.5.6.4 IgD

IgD is a monomeric antibody with a molecular mass of approximately 184 kDa. It circulates in serum at very low concentrations (<80 mg/L) and its function has yet to be determined. Studies have shown that IgD is expressed on the surface of developing B cells and it may therefore play an important role in their growth and development. Experimental models have shown that mice deficient in IgD appear to generate normal immune response and IgD is simply no longer expressed on activated B cells. Further research is needed to determine whether IgD functions as a regulatory antigen receptor or if it is simply redundant.

1.5.6.5 IgE

Like IgD, IgE (molecular mass approximately 188 kDa) is present in serum only at very
low concentrations of roughly 0.1–0.4 mg/L. Unlike IgD, however, IgE is capable of
triggering powerful immune reactions even in small amounts. When two IgE molecules are
bridged by an allergen when interacting with mast cells, degranulation of these cells
follows with the subsequent release of inflammatory mediators that trigger an allergic
reaction.
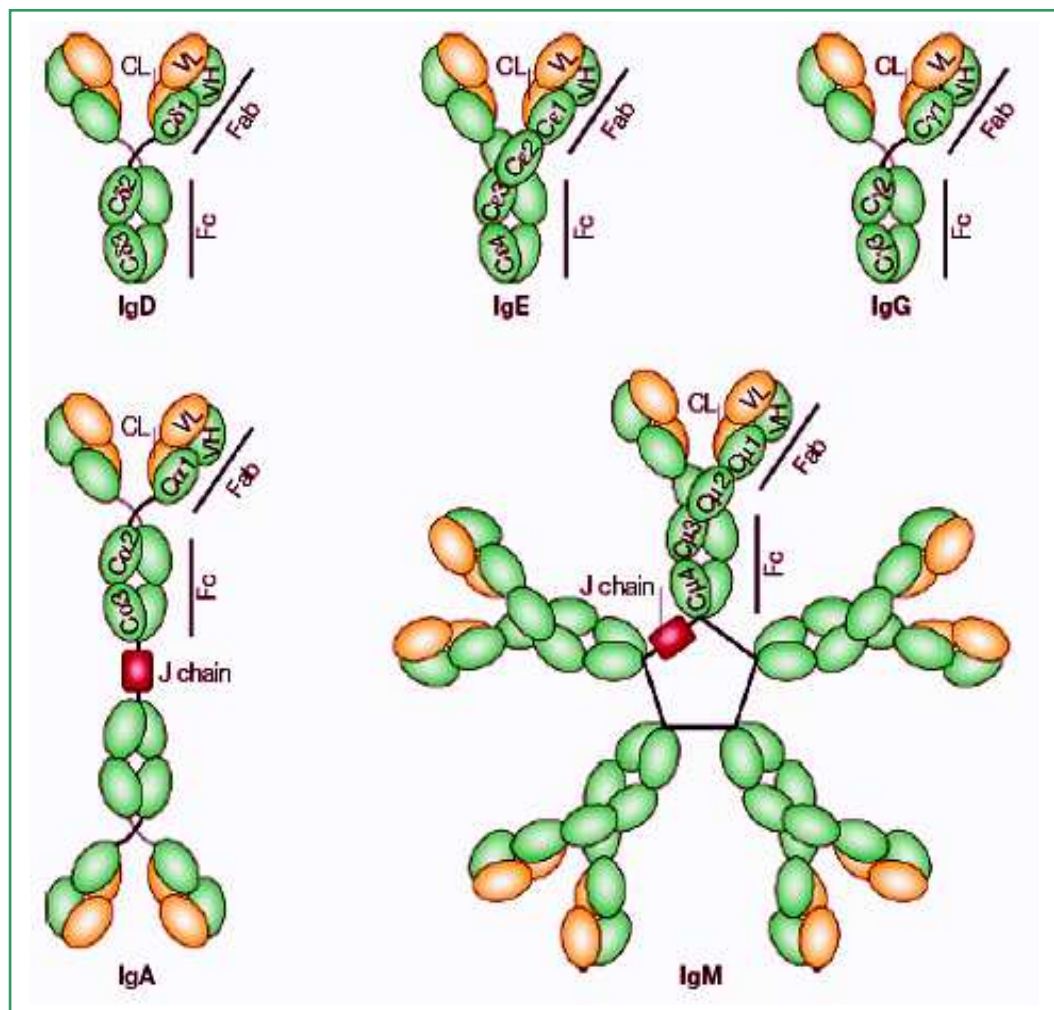
Figure 1-6 Structures of the five immunoglobulin classes

The five classes of immunoglobulin share the same basic, monomeric structure but
feature unique characteristics that allow them to carry out their specific functions
within the immune system. Adapted from (Rojas&Apodaca, 2002).

1.5.7 Fc receptors

FcRs are specialised receptors that recognise and bind to the Fc fragment (constant region)
of Ig heavy chains, providing an important link between the humoral and cellular branches

of the specific immune system. Each Ig isotype binds specifically to at least one FcR, activating the receptor and initiating a range of responses, including antigen presentation, ADCC, phagocytosis, production of reactive oxygen species (ROS) and release of cytokines and other mediators of inflammation (Miller *et al.*, 1996; Phillips-Quagliata *et al.*, 2000). Among the FcRs, the most studied include the high- and low-affinity receptors for IgG (FcγRI, FcγRII and FcγRIII), the medium-affinity receptor for IgA (FcαRI) and the high-affinity receptor for IgE (FcεRI) on mast cells (Raghavan&Bjorkman, 1996).

In addition to these receptors, other FcRs have been identified and each is structurally and functionally distinct. These include the neonatal IgG transporter receptor (FcRn) and the polymeric Ig transporter receptor (pIgR) for IgM and IgA (Daeron, 1997; Raghavan&Bjorkman, 1996; Ravetch&Kinet, 1991). In general, these receptors exist as complexes with numerous subunits and comprise a ligand binding α-chain and associated signalling chains (γ-chains). The FcRγ–chain is a membrane-spanning protein with an immunoreceptor tyrosine-based activation motif (ITAM). Once the receptor is activated, the ITAM is phosphorylated and subsequently promotes the release of additional downstream signalling molecules.

The main focus of this thesis will be on Fcα/μ receptors (Fcα/μR) of IgA and IgM. However, due to some analagous functions and mechanisms of action, FcRn and pIgR will also be discussed in some detail.

1.5.7.1 Fc receptor effector functions

Although Fc-mediated activation and suppression of the immune response has been studied since the 1960s (Sinclair, 2001), the past decade has seen a significant leap forward in our understanding of FcRs at the molecular level (Launay *et al.*, 1998; Takai, 2002). Three main functional categories have been identified: the regulation of immune cell responses, the internalisation and degradation of ICs and the transcellular transfer of antibodies (though this really only applies to pIgR and FcRn).

The major function of FcRs is the regulation of cellular immune responses, such as B cell proliferation, phagocytosis by macrophages and the degranulation of mast cells. Once activated, FcRs can initiate the inflammatory cascades evident in various forms of GN by transcriptionally activating cytokine-encoding genes. The internalisation and elimination of circulating ICs is efficiently carried out by macrophages, which also direct the antigenic peptides along either the MHC class I or class II antigen-presentation pathways

(Amigorena&Bonnerot, 1999). In addition to the three main functions of FcRs, there is growing evidence that soluble FcRs in the blood may also have immunomodulatory properties (Fridman *et al.*, 1992).

The concept of FcRs as a major, if not *the* major, effector of antibody-based inflammation has become more popular over the last decade in particular (Hogarth, 2002). Current thinking favours the idea that the biological response to FcR activation depends more upon the cell type than the receptor itself; indeed, different receptors expressed in the same cell often generate the same response. Accordingly, when expressed in different cell types, FcRs typically respond in a cell-specific manner (Daeron, 1997).

1.5.7.1.1 Phagocytosis

The process of phagocytosis involves the internalisation of large particles ($\geq$0.5 μm) and is mediated by reorganisation of the actin-based cytoskeleton (Silverstein *et al.*, 1989). In humans, phagocytosis is limited to specific phagocytic cells, such as monocytes, macrophages and neutrophils. FcR-mediated phagocytosis involves the internalisation and elimination of Ig opsonised pathogens and is initiated by interactions between specialised cell surface receptors and ligand-coated particles. These interactions cause local actin rearrangements resulting in pseudopod extension and breakdown of the particle into antigenic peptide products that may then be presented to T cells via the MHC class II pathway. Degradation of the internalised phagosomes occurs by exposing them to acidic conditions and hydrolytic enzymes following fusion with endosomes and/or lysozomes.

1.5.7.1.2 Endocytosis

Unlike phagocytosis, endocytosis does not involve actin rearrangements and can be mediated by most FcRs. Small particles and soluble complexes are internalised purely via receptor–ligand interactions, which typically occurs through the formation of clathrin-coated pits in the plasma membrane. These pits contain ligand–receptor complexes and merge into the cytoplasm where the internalised microbes are fused with early endosomes. Once endocytosed, ICs are directed to subcellular compartments where they are either degraded or presented to T cells (Fig. 1-7). It is worth noting that different FcRs may have different requirements for endocytosis to occur (Lauffenburger&Linderman, 1993).
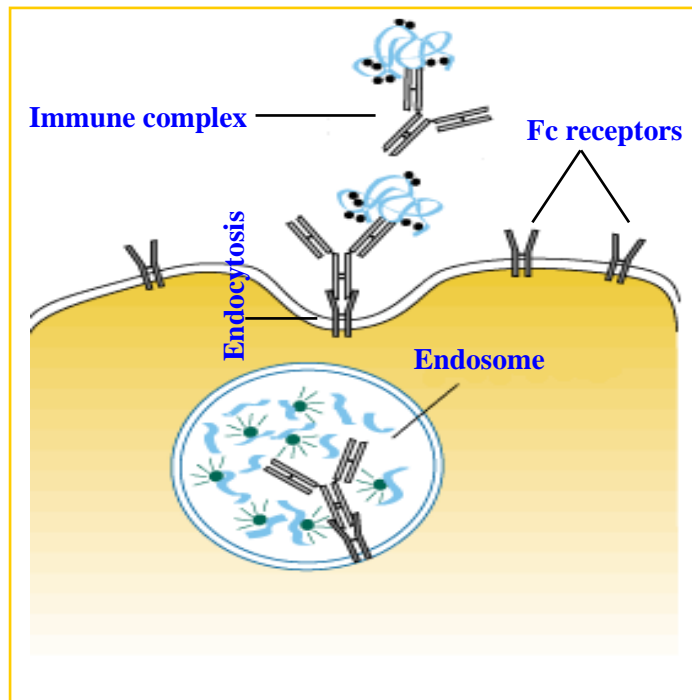
Figure 1-7 A simple model of synthetic IC endocytosis

Synthetic receptors embedded in the cellular plasma membrane rapidly cycle between the cell surface and intracellular endosomes. These receptors become incorporated in cellular plasma membranes, enabling the cellular uptake of cognate ligand, such as Ig, bound to antigen by synthetic receptor-mediated endocytosis to form IC. This results in uptake of IC by endocytosis whereupon the endosomes free the receptor to return to the cell surface. The receptors in the centre of the cell represent the population in intracellular endosomes in dynamic exchange with receptors on the cell surface. Adapted from http://probes.invitrogen.com/handbook/figures/1534.html.

1.5.7.2 Fcγ receptors (FcγR)

At least eight separate genes for the FcγRs have been identified so far, all of which map to chromosome 1 and have multiple splice variants (Hulett&Hogarth, 1994; Ravetch, 1994), making them the most diverse group of FcRs. Each of the FcγRs is composed of an extracellular region, a membrane-spanning region and a cytoplasmic region. The FcγR consists of three Ig superfamily domains, namely FcγRI, FcγRII and FcγRIII.

1.5.7.2.1 FcγRI (CD64)

FcγRI has the unique property of binding monomeric IgG with high affinity (association constant $[K_a]$ 10^8–10^9 M^{-1}) and is constitutively expressed on monocytes and macrophages (Flesch&Neppert, 2000; Hulett&Hogarth, 1994). Furthermore, FcγRI expression on neutrophils and eosinophils may be enhanced by IFN-γ or GM-CSF. The ability to bind monomeric IgG implies a potential role for FcγRI in initiating effector functions even at

low concentrations of IgG (Shen *et al.*, 1987). Moreover, studies have proposed that this ability is associated with the presence of a third extracellular domain that is lacking in all other human FcγRI (Allen&Seed, 1989). At present, three FcγRI genes have been identified in humans and mapped to chromosome 1q21.1, namely FcγRIA, FcγRIB and FcγRIC (Hulett&Hogarth, 1994). So far only FcγRIA protein has been detected on haematopoietic cells. It is thought that FcγRIB and FcγRIC exist purely as soluble proteins due to a stop codon in the exon encoding the third extracellular domain preventing the development of a transmembrane region (Flesch&Neppert, 2000).

FcγR signalling acts along a cascade of events common to several other immune system receptors. However, no signalling motif has yet been found in the cytoplasmic tail of FcγRI (Allen&Seed, 1989), meaning that it must recruit accessory molecules that contain these sequences in order to activate intracellular signalling molecules such as soluble tyrosine kinases. In order to join the signalling pathways, FcγRI associates at the cell surface with γ-chain homodimers (Ernst *et al.*, 1993). The γ-chain, initially found in association with FcεRI, contains a cytoplasmic ITAM responsible for the recruitment of tyrosine kinase-dependent signalling pathways (Cambier, 1995).

1.5.7.2.2 FcγRII (CD32)

Of all the FcγR, FcγRII is expressed in the widest range of cell types, including monocytes, macrophages, neutrophils, eosinophils, basophils, B cells, platelets, and dendritic cells (Ravetch&Kinet, 1991; van de Winkel&Capel, 1993). Unlike FcγRI, it has low affinity for IgG binding ($K_a < 10^7$ M^{-1}) and even then this occurs only with complexes and not monomeric IgG (van de Winkel&Capel, 1993). IgG binding directly involves the second extracellular domain, whereas the first domain plays an important role in optimal ligand binding (Hulett&Hogarth, 1994; Hulett *et al.*, 1995).

In humans, three different FcγRII genes (FcγRIIA, FcγRIIB and FcγRIIC) have been located on chromosome 1q23-24, each with multiple splice variants (Ravetch&Kinet, 1991; van de Winkel&Anderson, 1991). The FcγRIIA gene encodes FcγRIIA1, a transmembrane receptor molecule with cytoplasmic portion, while FcγRIIA2 lacks a transmembrane region and is presumed to be soluble (Brooks *et al.*, 1989). FcγRIIB molecules are preferentially expressed on monocytes and macrophages but can also be found on B cells (Cassel *et al.*, 1993), while the FcγRIIC gene codes for a protein that is intracellularly homologous to FcγRIIA but extracellularly resembles the FcγRIIB form,

due to an unequal crossover between the FcγRIIA and IIB genes (Warmerdam *et al.*, 1993). Although the cellular expression of FcγRIIC has yet to be studied in detail, gene transcripts have been found on the membranes of B cells and myelomonocytic cells (Cassel *et al.*, 1993).

Each of the FcγRII isoforms consists of two extracellular domains, and all but FcγRIIA2 have a cell membrane and a cytoplasmic region (Stuart *et al.*, 1989). Unlike the other FcγRs, FcγRII encode signalling motifs within their cytoplasmic tails and are therefore able to initiate a signalling cascade (Van den Herik-Oudijk *et al.*, 1995). Both the FcγRIIA1 and FcγRIIC isoforms contain a unique ITAM copy in their cytoplasmic tail that is critical for phagocytosis and tyrosine phosphorylation, but not for the internalisation of ICs (Indik *et al.*, 1995; Van den Herik-Oudijk *et al.*, 1995). It has been proposed that the association of FcγRIIA with an FcR γ-chain unit may mediate this and other functions (e.g. cytokine release and antigen presentation) (Masuda *et al.*, 1993). Although the FcR γ-chain ITAM is much more effective at triggering cytokine release and antigen presentation than the FcRIIA ITAM, the latter appears to exert a bigger influence on phagocytosis (Van den Herik-Oudijk *et al.*, 1995). The FcγRIIB1 and FcγRIIB2 isoforms are the only receptors with an immunoreceptor tyrosine-based inhibition motif (ITIM), which recruits an inhibitory signalling pathway (Daeron, 1997; Van den Herik-Oudijk *et al.*, 1995). Reports have suggested that FcγRIIC and FcγRIIIA are co-expressed by NK cells (Metes *et al.*, 1994; Metes *et al.*, 1999).

1.5.7.2.3 Fcγ*RIII (CD16)*

Two genes encoding FcγRIII have been discovered in humans – FcγRIIIA and FcγRIIIB – both of which are located on chromosome 1q23-24 (Qiu *et al.*, 1990; Ravetch&Kinet, 1991). FcγRIIIA is expressed on macrophages, NK cells and mast cells, while FcγRIIIB expression is limited to neutrophils alone. Both gene products have two extracellular domains and are highly homologous in these regions; elsewhere, however, they are completely different (Ravetch&Perussia, 1989). FcγRIIIB has no transmembrane or cytoplasmic region and thus cannot associate with either γ-chain homodimers or the related ζ-chain. There is no evidence that a deficiency of FcγRIIIB causes disease. Similarly to FcγRI, FcγRIIIA associates with the γ-chain. Interestingly, however, it can also associate with the ζ -chain (in NK cells) (Lanier *et al.*, 1989).

1.5.7.2.4 The γ-chain

The FcR γ-chain is a 7 kDa, type I membrane-spanning polypeptide normally found as a homodimer in the membrane, linked with the α-chain by a single disulphide bridge (Takai, 2002). The α-chain binds IgE and was first identified as a component of the multi-subunit, high-affinity IgE receptor on mast cells (Perez-Montfort *et al.*, 1983). The γ-chain belongs to a family of membrane-associated signal transduction molecules that also includes the ζ-chain component of the TCR (Orloff *et al.*, 1990). Accordingly, it shares the distinct structural characteristics associated with these molecules; the membrane-spanning region is a single α-helix through which the γ-chain associates with FcγRI via non-covalent interactions (Ernst *et al.*, 1993; Harrison *et al.*, 1995), while the intracellular domain contains a classic ITAM signalling motif (Samelson&Klausner, 1992).

1.5.7.3 FcαR (CD89)

First cloned in 1990, FcαRI (CD89) is the only known receptor for IgA (Daeron, 1997; Maliszewski *et al.*, 1990) and is constitutively expressed on a wide variety of cells, including neutrophils (the largest source of FcαRI in blood and tissues (Hamre *et al.*, 2003)), monocytes, eosinophils, some macrophages and Kupffer cells (Woof&Kerr, 2006). As with most FcRs, FcαRI contains two extracellular regions, a transmembrane region (associated with the FcRγ-chain) and a cytoplasmic tail that regulates cytoskeletal association of the receptor (Bracke *et al.*, 2001). Overall, the extracellular regions contain 206 amino acids, the transmembrane domain contains 19 and the cytoplasmic tail contains 41 (Ding *et al.*, 2003; Maliszewski *et al.*, 1990).

In contrast to FcγR and FcεRI, whose α-chain genes map to a cluster on chromosome 1, the FcαRI α-chain genes map to chromosome 19 (Ding *et al.*, 2003; Woof&Kerr, 2006). When the FcαRI α-chain is engaged by ligand and effectively becomes cross-linked, the γ-chain ITAM tyrosines are phosphorylated, representing the first step in a signalling cascade that ultimately leads to the assembly of lipid rafts within the plasma membrane that serve as platforms for the initiation of signal transduction (Lang *et al.*, 1999; Lang *et al.*, 2002). Cross-linking of FcαRI on myeloid cells has been shown to initiate a wide range of functional processes, including phagocytosis, ROS generation, ADCC and the release of inflammatory mediators (Morton *et al.*, 1996; van Zandbergen *et al.*, 1999). Moreover, the cell surface expression of FcαRI is upregulated by cytokines (TNF-α, GM-CSF, IFN-γ, IL-1b, IL-4, IL-5, IL-6, IL-8), LPS and HAg-IgA (Hostoffer *et al.*, 1994; Monteiro *et al.*, 1993; Shen *et al.*, 1994), accompanied by an increase in FcαRI transcript level and

enhanced IgA-mediated phagocytosis. In contrast, TGF-β appears to downregulate the expression of FcαRI in cultured monocytes (Reterink *et al.*, 1996).

Similarly to the observed actions of many other FcRs, FcαRI has been shown to associate with γ-chain homodimers (Morton *et al.*, 1995). Researchers have described two spliced variant messages encoding smaller variants of the receptor protein, but the biological characteristics of these is as yet unclear (Monteiro&Van De Winkel, 2003). Ligand binding by FcαRI differs from that by FcεRI and FcγRs since binding occurs via the first extracellular region (Morton&Brandtzaeg, 2001; Wines *et al.*, 1999) and not the second or the linking region. Thus, altered FcαRI expression may have a direct effect on the effector function of FcαRI-expressing cells. Furthermore, although there is no available data on the existence or otherwise of soluble forms of FcαR, should they exist it has been proposed that these soluble receptors may have a pathophysiological role in several diseases (Huizinga *et al.*, 1990; Koene *et al.*, 1998).

FcαRI receptor possesses medium-to-high affinity for IgA ($K_a \sim 1 \times 10^7$ M^{-1}) and can therefore bind either the monomeric or polymeric forms of both IgA1 and IgA2 (Monteiro *et al.*, 1990). FcαRI binds the IgA Fc region at the hydrophobic interface. Studies of the binding reaction have found that the monomeric receptor binds IgA with low micromolar affinity, as befits a receptor that functions as a sensor for IgA ICs by avidly binding aggregates of IgA (Wines&Hogarth, 2006). This affinity switch appears to involve a cytokine-triggered serine dephosphorylation in the cytoplasmic domain of FcαRI. Consequently, the receptor is released from its cytoskeletal base, presumably allowing bivalent ligand binding to occur (Bracke *et al.*, 2001). Moreover, mutations in the alanine residues of the apical loops in the first extracellular region inhibit receptor binding, even when cytokines are present (Wines *et al.*, 2001). It has thus been proposed that cytokine activation alters the receptor organisation leading to an increase in IgA binding without altering the overall number of receptors (Weisbart *et al.*, 1988).

1.5.7.4 Fc neonatal receptor (FcRn)

FcRn was first isolated from the intestine of foetal rats (Simister&Rees, 1985) and, in humans, is found in epithelial cells, some vascular endothelial cells (Junghans&Anderson, 1996), in the placenta (Simister&Story, 1997) and in both foetal and adult intestine (Israel *et al.*, 1997). The gene encoding FcRn has been mapped to chromosome 19q13.3 (Junghans&Anderson, 1996). FcRn is a heterodimer comprising a glycosylated heavy α-chain that associates non-covalently with β2-microglobulin on a species-dependent basis

(Claypool *et al.*, 2002). The extracellular region of the α-chain is composed of three Ig-like domains (homologous to MHC class I), a transmembrane region and a cytoplasmic region. The site of MHC peptide binding is distinct from the site of IgG Fc binding (Burmeister *et al.*, 1994). Thus, FcRn is structurally very different from the FcγRs and the functional receptor is likely to be a dimer of heterodimers that binds a single IgG molecule (Burmeister *et al.*, 1994; Kim *et al.*, 1994).

A key role of FcRn in immunity is the absorption of maternal IgG and the subsequent bi-directional trafficking of the bound complex across the epithelial cells that line the lumen of various tissues (Claypool *et al.*, 2004; Kobayashi *et al.*, 2002; McCarthy *et al.*, 2001). These tissues include the human placenta, the yolk sac in rabbits and rodents and the proximal small intestine in neonatal rodents (Firan *et al.*, 2001; Medesan *et al.*, 1996).

FcRn also regulates IgG levels in plasma and is sometimes referred to as the specific IgG 'protection receptor' (Flesch&Neppert, 2000; Ghetie&Ward, 1997). Once antigen-bound IgG is internalised by the plasma membrane the complex is uncoupled, leaving IgG free to bind to FcRn which then redirects it back to the circulation so that only the antigen is catabolised in lysosomes. FcRn binds IgG at acidic pH (≤ 6.5) but not at or above neutral pH (≥ 7.0); the IgG and the receptor uncouple at neutral pH (Claypool *et al.*, 2004). When IgG levels in plasma are high there is greater competition for FcRn binding, the capacity for IgG transport back to the circulation is overstretched and so more IgG is degraded by lysosomes. When IgG levels in plasma are low, a higher proportion of IgG is returned to the circulation. Ligand binding to FcRn occurs both within the acidic endosome and at the cell surface (Claypool *et al.*, 2004; Kobayashi *et al.*, 2002), though the bulk of FcRn is stored intracellularly at steady state (Dickinson *et al.*, 1999; Ober *et al.*, 2001).

Studies in rats have shown that the trafficking of FcRn is dependent upon the sorting motifs found in the cytoplasmic tail (McCarthy *et al.*, 2001; Wu&Simister, 2001). A mutation and a tryptophan residue in the cytoplasmic tail of rat FcRn that impairs endocytosis has been identified (Wu&Simister, 2001). This affects both the apical and basolateral membrane domains, with a more severe reduction in endocytosis in the former. Removal of the cytoplasmic tails significantly impairs the transport of IgG in the basolateral direction but not in the apical direction (Wu&Simister, 2001). However, there are conflicting data on whether or not the FcRn cytoplasmic tail has an impact on sorting in the biosynthetic pathway.

1.5.7.5 Polymeric immunoglobulin receptor (pIgR)

pIgR is an important transmembrane glycoprotein expressed on the basolateral surface of most human secretory epithelial cells, including those in the intestine, bronchus, salivary glands, gallbladder, renal tubules, pancreas, lactating mammary glands and the uterus (Brandtzaeg P. *et al.*, 1994). As such, pIgR is crucial to the first line of defence at mucosal surfaces, neutralising both extra- and intracellular pathogens in these membranes by the epithelial transport of pIgA–pathogen ICs that are mucosally secreted via epithelial transcytosis (Mostov *et al.*, 1984; Rojas&Apodaca, 2002).

pIgRs are formed when IgA and IgM are oligomerised by the J-chain cross-linking the homodimeric Fc regions of multiple molecules (two for IgA and five for IgM) (Mestecky *et al.*, 1971). After synthesis in the endoplasmic reticulum, pIgR is delivered directly from the trans-Golgi network to the basolateral surface where it binds dIgA. The pIgR–dIgA complex undergoes endocytosis and is then transported through a series of endosomal compartments across the cell to the apical surface (Rojas&Apodaca, 2002). The secretory component (i.e. the ectoplasmic domain) of pIgR is secreted luminally in its bound (with pIgA) or unbound forms (Piskurich *et al.*, 1997). Thus, pIgR is not only capable of delivering pIgA into mucosal secretions, but also aids the transportation of locally-formed ICs containing pIgA across the epithelium from the lamina propria (Robinson *et al.*, 2001; Woof&Kerr, 2006).

Studies have shown that the pIgR transcription gene is upregulated by proinflammatory cytokines (e.g. IFN-γ, TNF-α, IL-1 and IL-4), bacterial products (e.g. LPS) and hormones (Schneeman *et al.*, 2005). Moreover, the neutralisation of LPS by secretory IgA controls pIgR expression levels via a negative feedback cycle. These findings suggest that transcriptional regulatory mechanisms may be involved in the function of pIgR and that IFN-γ may induce transcription factor synthesis in epithelial cells.

1.5.7.5.1 J-chain

As discussed above, the binding of human pIgR to pIgA or IgM is dependent on the presence of the J-chain (Vaerman *et al.*, 1998a), a polypeptide sequence of approximately 137 amino acid residues with a total molecular mass of 15–16 kDa that links the Fc fragments of antibodies by disulphide bridges (Koshland, 1985; Mestecky *et al.*, 2005). In humans, a single N-linked glycan accounts for approximately 8% of the molecular mass of the J-chain (Baenziger, 1979). This carbohydrate appears to be crucial to the formation of

polymers between the J-chain and monomeric IgA (mIgA) subunits (Krugmann *et al.*, 1997; Woof&Mestecky, 2005).

1.5.7.6 Fc alpha/mu receptor (Fcα/μR)

The novel receptor Fcα/μR was first isolated on B cells and macrophages in murine (Shibuya *et al.*, 2000) and in human homologues and is capable of binding both IgA and IgM. Mapped to chromosome 1q32.3, adjacent to the pIgR, FcγR and FcεRI genes, the Fcα/μR gene encodes a single glycopolypeptide with a molecular mass of approximately 70 kDa and was isolated using an expression library from the T-cell line and selection of an IgM-binding clone (Shibuya *et al.*, 2000). Structurally, Fcα/μR consists of a single Ig superfamily domain (most similar to the first extracellular region of pIgR), an extended mucin-like membrane proximal region, a transmembrane region and a small cytoplasmic tail. It is preferentially expressed on most B cells and macrophages but not on T cells, NK cells or granulocytes (Sakamoto *et al.*, 2001; Shibuya *et al.*, 2000). As such, Fcα/μR is widely expressed in non-haematopoietic tissues, mainly in the kidney and intestine, but also in the lung, liver and heart at lower levels. It is also expressed in lymphoid cells, including the lymph nodes, spleen and appendix (Sakamoto *et al.*, 2001). Fcα/μR binds IgM with high affinity ($K_a \sim 10^9$ M^{-1}) and IgA with lower affinity ($K_a \sim 10^8$ M^{-1}) (Wines&Hogarth, 2006).

The functions of Fcα/μR *in vivo* have yet to be fully determined, though the generation of Fcα/μR knockout mice will ensure that research in this area will continue to develop. The relatively high binding affinities of Fcα/μR suggests a role in the early stages of a primary immune response. Studies in mice have shown that Fcα/μR mediates B-cell endocytosis of IgM opsonised bacterial targets. A similar uptake of IgA-coated targets has been proposed. In addition, a role for Fcα/μR in IgM-mediated antigen presentation to T cells in the early stages of immune response has been proposed; mice deficient in secretory IgM display impaired T-cell-dependent antibody responses (Sakamoto *et al.*, 2001). Alternatively, deficiencies in either IgA or IgM have been linked with the production of autoantibodies; therefore, there may be a role for an IgA/IgM receptor that passively removes ICs without significant presentation of antigens.

Following the initial finding of Fcα/μR expression in the kidney (Sakamoto *et al.*, 2001), it was reported that human MSC express this receptor and that this expression is upregulated by IL-1α (McDonald *et al.*, 2002). It was therefore hypothesised that Fcα/μR may be

responsible for the deposition of pIgA-containing ICs in IgAN; however, other studies conducted in MSC have indicated that Fcα/μR may not bind IgM in these cells (Moura *et al.*, 2001; Moura *et al.*, 2004; Wines&Hogarth, 2006).

1.5.7.7 Transferrin receptor (CD71)

First described as a receptor for transferrin, CD71 is an IgA receptor comprising a 180 kDa disulphide-linked homodimer that binds polymeric human IgA1 (rather than monomeric) but not IgA2 (Monteiro *et al.*, 2002; van der Boog *et al.*, 2004; Woof&Mestecky, 2005). The receptor is expressed on mononuclear haemopoietic cells in the foetus but is often undetectable on either adult mononuclear or PMN cells. Expression of CD71 has also been found on certain lymphocytic and myeloid cells (Woof&Kerr, 2006). The expression of CD71 on human MSC is upregulated in IgAN, leading to suggestions that the receptor may be linked to IgA deposition in the tubulointerstitium (Moura *et al.*, 2001). A recent study showed that interstitial dendritic cells also express CD71 and that the receptor mediates endocytosis of IgA-containing ICs in immature but not in activated monocyte-derived cells (Pasquier *et al.*, 2004).

1.5.7.8 Asialoglycoprotein receptor (ASGPR)

The ASGPR is a C-type lectin involved in the endocytosis of serum glycoproteins due to its ability to recognise galactose and N-acetylgalactosamine residues of desialylated glycoproteins (Stockert, 1995). Thus, ASGPR may represent a degradative pathway with an important role in maintaining the homeostasis of glycoprotein levels. Whilst ASGPR has long been associated with hepatic cells, studies in rats have shown that ASGPR mRNA is widely expressed in various tissues and cell lines (Leung *et al.*, 2000; Park *et al.*, 1998a).
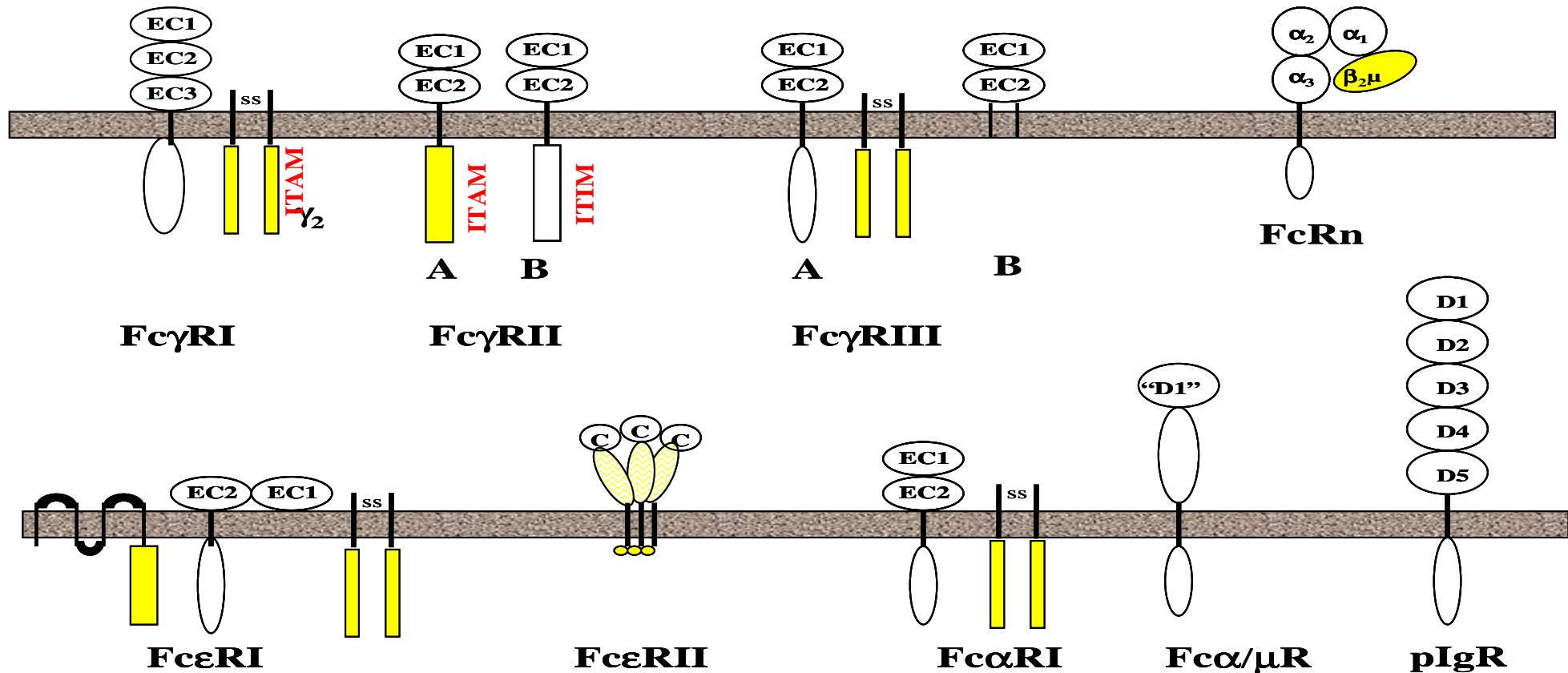
Figure 1-8 Fc receptors structure

Immunoglobulin receptors: the upper panel shows receptors for IgG, and the lower panel receptors for other Ig isotype. Each Ig-like extracellular domain (EC, or D) is shown as a circle, and the domain are numbered from the amino terminus of the receptor molecule. Note that FcγRIIB is linked to the membrane via a glycophosphotidylinositol (GPI) anchor. ITAM and ITIM intracellular domains are represented as filled and opened rectangles, respectively. Intracellular domains with no or undefined signals functions are shown as open ovals. FcεRI is depicted in a 'lying-down' orientation for FcεRII, the C-type lectin domain is depicted as 'C' and the oval domain represent the stalk region. Adapted from (Cushley, 2005)

Ig bound	Receptor	CD	Gene	Chromosome	Transcript	Proteins KDa	K_a (M^{-1})	Isotype Preference	Signalling	Complex
IgM	**Fcα/μR**			**1q.32.3**		**70**	3×10^9 **(IgM)** 3×10^8 **(IgA)**	**IgM > IgA**		
IgA	FcαR	CD89		19		55-75	5×10^7	1, 2	γ	α, αγ$_2$
IgM, IgA	**pIgR**			**1q31-41**		**100-105**	2×10^9 **(IgM)** 10^8 **(IgA)**	**IgM > IgA**		
IgG	FcγRI	CD64	FcγRIA FcγRIB FcγRIC	1q21.1	FcγRIA FcγRIB1 FcγRIB2 FcγRIC	60-70 " " "	10^7-10^9 " " "	3≥1>4>> 2	γ	αγ$_2$
	FcγRII	CD32	FcγRIIA	1q23-24	FcγRIIA1 FcγRIIB2	40-60 "	10^7 "	3≥1,2>>4, 2	ITAM	
			FcγRIIB		FcγRIIB1 FcγRIIB2 FcγRIIB3	" " "	" " "		ITIM	
			FcγRIIC		FcγRIIC	"	"		ITAM	
	FcγRIII	CD16	FcγRIIIA FcγRIIIB	1q23-24	FcγRIIIA FcγRIIIB	50-80 50	$1\text{-}3\times10^7$	1,3>>2,4	γ (or ζ)	αγ$_2$, αζ$_2$, αζγ
	FcRn			**19**		**45-50**	$2\text{-}5\times10^8$	**1,3>>2,4**		β2m[a]
IgD	FcδR					70				
IgE	FcεRI			1q23		45-65	10^{10}		α,β,γ	αβγ$_2$, αγ$_2$
	FcεRII	**CD23**	**FcεRIIA**		**FcεRIIA**	**45**	10^7			

Table 1-2 Molecular properties of human Fc receptors

Adapted from (Cushley, 2005) and (Hulett&Hogarth, 1994)

1.6 PTEC functions and immune mechanism in renal disease

1.6.1 Fc receptors expression

The important functional effects of FcRs in immunity include interacting with ICs and mediating antigen presentation, ADCC and release of inflammatory mediators (Hulett&Hogarth, 1994). The reabsorption of filtered ICs in the proximal tubule is primarily a receptor-mediated process. FcRs appear to be somehow involved in the IC-mediated activation of PTEC and a common binding site for low-molecular-weight proteins in these cells has been suggested. When renal conditions are impaired, higher levels of Ig are observed in the urine and some studies have proposed that, like other proteins, Igs themselves may damage the tubules (Abbate *et al.*, 1998; Zoja *et al.*, 1995). However, the mechanisms by which this process may occur are not yet clear.

FcRn-mediated IgG transcytosis appears to be localised to the brush border of PTECs (Haymann *et al.*, 2000; Kobayashi *et al.*, 2002) and so any injury resulting in the loss of PTEC will impair IgG reabsorption because of insufficient endocytosis. When PTEC are exposed to higher levels of IgG, an upregulation of ROS and NF-κB activation has been reported (Ronda *et al.*, 2005). In patients with GN, the cationic fragment of the IgG molecule is preferentially reabsorbed from the filtrate, suggesting that FcRn-mediated endocytosis may not be the only mechanism involved in IgG transcytosis and that IgG reabsorption in PTEC is also charge-induced (Takahashi *et al.*, 2004).

Although there were several early reports of FcαRI expression in MSC (Gomez-Guerrero *et al.*, 1993; Kashem *et al.*, 1997), it is now generally accepted that this is not the case (Barratt *et al.*, 2000; Westerhuis *et al.*, 1999) and a number of other receptors have been proposed. MSC expression of ASGPR has been reported, although this finding was later refuted in an important study which also showed that MSC do not express pIgR under normal conditions or even when exposed to proinflammatory cytokines known to upregulate the transcription of pIgR (including IFN-γ, TNF-α, IL-1 and IL-4 (Leung *et al.*, 2000; Schjerven *et al.*, 2004). These findings also extended to include FcαRI and the ASGPR, despite the known upregulation of MSC IgA binding when exposed to proinflammatory cytokines (Barratt *et al.*, 2000; Leung *et al.*, 2000). Subsequently, IL-lα, IFN-γ and TGF-β were used in my study as proinflammatory cytokines to demonstrate the expression of Ig receptors in PTEC that may have a specific role in IgAN.

Although Fcα/µR-mediated endocytosis has been proposed, the mechanism by which this occurs is not yet clear. MSC cultured *in vitro* have been shown to bind both monomeric IgA and pIgA. They express pIgR and Fcα/µR, but not the IgA receptor FcαR (McDonald *et al.*, 2002; Smith&Feehally, 2003; Westerhuis *et al.*, 1999). A human homologue of Fcα/µR was recently identified in MSC on the basis of sequence similarity with murine Fcα/µR (McDonald *et al.*, 2002). However, whilst the Fcα/µR is an interesting candidate for IgA binding in MSC, additional receptors may be required as some studies have shown that the IgA binding is Fcα-specific and cannot be blocked by excess IgM (Barratt *et al.*, 2000).

CD71 is one such candidate and has been shown to bind IgA1 in MSC. Renal biopsies from IgAN patients have indicated CD71 expression (Moura *et al.*, 2001). However, as this receptor is IgA1 selective and binds to polymeric forms more efficiently than monomeric forms, it also cannot fully account for the specific FcαR binding of MSC. However, it is possible that MSC and PTEC contain a novel Fcα/µR isotype that has yet to be characterised. Other FcR families have multiple members and the possibility that others await discovery cannot be excluded.

1.6.2 ECM production

1.6.2.1 ECM overview

ECM is a complex aggregate structure composed of four different classes of macromolecules: collagens, proteoglycans, structural glycoproteins (including FN) and elastin (Johansson *et al.*, 1997). Abnormalities in ECM biosynthesis and catabolism occur in both inherited and acquired diseases, and are sometimes seen in wound healing. The maintenance of normal tissue architecture and the integrity of other tissue-specific functions such as formation and remodelling depend upon ECM. It is also involved in the recirculation of lymphocytes, blood clot formation and tumour growth and metastasis. To date, two major families of cell surface receptors have been identified: the integrins and the syndecans (Johansson *et al.*, 1997). These receptors appear to mediate the influence of ECM and its components on cell growth, differentiation, development and metabolic response. Specific cell surface receptors for ECM components have been identified, providing a link between ECM and cell biology.

The integrin receptors are a family of heterodimeric transmembrane glycoproteins that mediate the adhesion and interaction of cells with ECM, modulating changes in cell shape

(Hornberger *et al.*, 2000). In the absence of growth factors, integrins have been shown to mediate cell signalling (Moro *et al.*, 1998). Even more importantly, these glycoproteins appear to both directly (Jones *et al.*, 1997; Miyamoto *et al.*, 1996) and indirectly (via modulation of focal adhesions (Cary&Guan, 1999) enhance the cell response to growth factors.

Over the past two decades, changes in the ECM have come to be recognised as critical events for the initiation and progression of many renal diseases, including GN, glomerulosclerosis and tubulointerstitial fibrosis. Much more than simply providing a structural support for cells, ECM should be considered a dynamic complex with several important functions in both normal and injured kidneys.

1.6.2.2 Fibronectin (FN)

FN is one of the major structural glycoproteins of ECM, alongside laminin, entactin, tenascin, thrombospondin, vitronectin and fibrillin, and promotes cell adhesion (Hynes *et al.*, 1992). As well as being present in ECM, FN is also found in plasma and is known to be involved in cell differentiation, wound repair, thrombosis and cytoskeletal reorganisation. FN contains two subunits, each with a molecular mass of approximately 250 kDa, and binding domains for cells and fibrin (Haralson&Hassell, 1995).

1.6.2.3 PTEC and FN

In IgAN, the release of inflammatory cytokines from MSC after binding to IgA activates PTEC, which, in turn, may release even more pro-inflammatory molecules (including IL-6, TNF-α, soluble intercellular adhesion molecule-1 [ICAM-1] and angiotensin II) that attract further inflammatory competent cells (Arrizabalaga *et al.*, 1997; Ranieri *et al.*, 1996). This positive feedback loop in the renal tubules leads to the excessive production of ECM components, including FN and various collagens, resulting in fibrosis and, ultimately, tubulointerstitial scarring.

The accumulation of FN and other proteins in the interstitium of the kidney, due to increased synthesis, reduced degradation or both, is fundamental to tubulointerstitial fibrosis (Norman&Fine, 1999). The increased production of interstitial proteins is largely mediated by fibroblasts, which proliferate during fibrosis. Interestingly, *in vitro* studies have shown that fibroblasts from injured kidneys appear to synthesise more FN and collagens than do fibroblasts from healthy kidneys (Rodemann&Muller, 1991). As

tubulointerstitial fibrosis has a peritubular organisation, it has been suggested that PTEC themselves may have an important pathogenic role. Primary insults to the PTEC (e.g. hypoxia) or sustained exposure to factors in the glomerular ultrafiltrate (e.g. proteins, cytokines, glucose) or complement proteins and their activation products in the glomerular filtrate (e.g. during states of non-selective proteinuria (Zhou *et al.*, 2001)) may lead to the release of chemoattractants that promote the infiltration of monocytes into the interstitium, releasing yet more inflammatory mediators, stimulating fibroblast proliferation and upregulating the production of interstitial proteins (Peake *et al.*, 2002; Tian *et al.*, 2003).

As high-grade proteinuria is infrequent in IgAN and it remains to be confirmed whether there is a specific binding of IgA to PTEC, an alternative pathway for tubulointerstitial atrophy and fibrosis dependent upon IgA-containing ICs in the mesangium has been hypothesised. In this thesis, I examined the possibility that these Ig/ICs cause accumulation of FN in the proximal tubule.

1.6.3 Proliferation

1.6.3.1 The cell cycle

The cell cycle consists of four distinct phases, G1 (gap 1), S (synthesis), G2 (gap 2) and M (mitosis), the first three of which are collectively known as the 'interphase' (Fig. 1-9). An additional phase, G0, is reserved for cells that have temporarily or reversibly stopped dividing; senescent cells are those that have permanently stopped dividing due to age or accumulated DNA damage. In mature organisms, some cell types enter the G0 phase semi-permanently (e.g. parenchymal cells in the liver and kidney) and only re-enter the cell cycle under highly specific conditions. The G1 and G2 phases are characterised by protein and RNA synthesis and S by DNA synthesis. The M phase comprises two distinct but tightly coupled processes: mitosis (where the cell nucleus divides and its chromosomes are allocated to the two daughter cells) and cytokinesis (the physical division of the cytoplasm). The timing of the cell cycle is cell-specific and also depends upon local conditions.
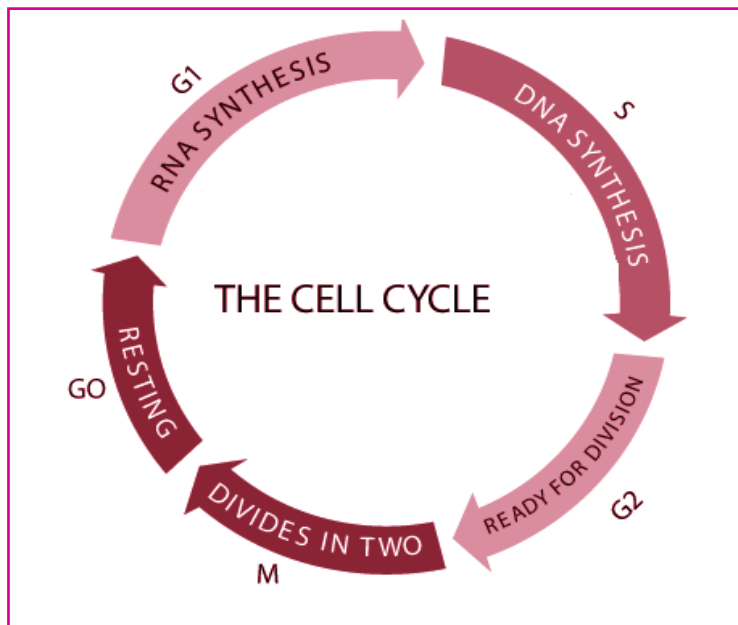
Figure 1-9 The cell cycle

1.6.3.2 Cellular proliferation

The proliferation and dedifferentiation of renal PTEC is crucial to the regulation of renal repair, particularly early on in the injury recovery process where the denuded tubular basement membrane begins to be replenished. However, the exact nature of the relationship between cellular proliferation and dedifferentiation is unclear, for example, the timing of PTEC reversion and how this 'remodelling' process relates temporally to the initial insult, cellular proliferation and the eventual redifferentiation of the cell are unconfirmed (Bard, 2002; Bonventre, 2003). Renal repair within the proximal tubule also depends on cell migration across the basement membrane, hypertrophy and apoptosis of hyperplastic PTEC in order to restore normal morphology, cell–cell contact and transport capacity (Bonventre, 1993). Current data estimate that complete structural and functional recovery may take 4–6 weeks (Basile *et al.*, 1998; Witzgall *et al.*, 1994).

In vitro studies have shown that MSC proliferation in an experimental model of MGN is accompanied by a reduction in the accumulation of ECM proteins and an improvement in renal function (Pippin *et al.*, 1997). Although this thesis focuses primarily on the proliferation of PTEC, some renal diseases (e.g. IgAN (Moeller *et al.*, 2004)) also involve the proliferation MSC. Induction of proliferation is a receptor-mediated process that involves the stimulation of the cells to re-enter the cell cycle (Kurogi, 2003). Proliferation may be limited by blockading specific growth factors or, more generally, by targeting angiotensin II receptors, calcium channels and 3-hydroxy, 3-methylglutaryl Coenzyme A (HMG-CoA) (Guan&Breyer, 2001; Javaid&Quigg, 2005). The clinical relevance of these

potential therapeutic strategies is only just becoming apparent and further research is needed.

1.6.4 Cytokine production

At present, few data exist on the specific effects of inflammatory cytokines on PTEC. PTEC are known to release a wide variety of inflammatory mediators, including ECM components, chemokines and cytokines, and that this process occurs in response to various stimuli including cytokines (particularly IL-1α, TNF-α and IFN-γ (Boswell *et al.*, 1994; Gerritsma *et al.*, 1996a; Gerritsma *et al.*, 1996b; Schmouder *et al.*, 1992), hypoxia, proteinuria and LPS (Frank *et al.*, 1993; Glynne *et al.*, 2001). The release of these mediators is thought to worsen interstitial disease by recruiting and activating various inflammatory competent cells, including macrophages and lymphocytes (Jevnikar *et al.*, 1990), though this has yet to be rigorously proven. Moreover, only some of the mediators released by PTEC have pro-inflammatory signals, while others are anti-inflammatory. So while the effects of some single mediators (including TNF-α, IL-6, IL-8 and RANTES) are well studied (Chen&Lin, 1994; Wang *et al.*, 2004), the net effects of activated PTEC require further study.

In various forms of GN and tubulointerstitial nephritis, both the infiltrating cells and resident PTEC appear to have upregulated levels of TNF-α and IL-6 (Leonard *et al.*, 1999). *In vitro* studies conducted in MSC and PTEC have demonstrated that IL-6 production is increased in these cells in response to stimulation by various stimuli, including LPS, TNF-α and IL-1 (Abbott *et al.*, 1991; Boswell *et al.*, 1994). Infection with Gram-negative bacteria causes LPS toxicity, which is widely assumed to upregulate the generation of TNF-α and may subsequently initiate an inflammatory cascade that may ultimately lead to either apoptotic or necrotic cell death (Jones *et al.*, 2000; Yamaoka *et al.*, 2002; Zager, 1986). A logical hypothesis, therefore, is that if acute injury to PTEC increases LPS-mediated TNF-α production, the severity of the injury may worsen. Moreover, if renal TNF-α production leads to systemic TNF-α release, additional damage may occur in extrarenal tissues.

Expression of the IL-8 gene and protein has been detected throughout the glomeruli in some proliferative forms of GN (Cockwell *et al.*, 1999), though this is sometimes limited to mesangial areas (Lim *et al.*, 2003; Mezzano *et al.*, 1997). IL-8 caused sequestration of neutrophils within the microvasculature and failed trafficking of neutrophils to sites of

extravascular IL-8 and other neutrophils and chemoattractants (Hechtman *et al.*, 1991). IL-1 and TNF-α are among the most potent inducers of IL-8 (Gerritsma *et al.*, 1996b; Kusner *et al.*, 1991; Lonnemann *et al.*, 1995; Niemir *et al.*, 2004), while IFN-γ has been shown to downregulate IL-8 production. Both spontaneous and LPS-induced release of IL-8 is significantly higher in patients with either proliferative or non-proliferative GN than in healthy controls (Matsumoto, 1995). Thus, it seems likely that human PTEC are a potential renal source of IL-8 and that various inflammatory mediators can induce the release of IL-8 in the proximal tubule.

Whilst the above cytokines are thought to play a role in both the initiation and amplification phases of the immune response, RANTES appears to be involved only in the latter. Studies have shown that the release of RANTES by PTEC is more pronounced in the presence of T-cell-derived IFN-γ (in combination with either IL-lα or TNF-α) (Deckers *et al.*, 1998).

1.6.5 Fc receptor signalling

Our understanding of FcR and antigen receptor signals have developed at a comparable rate as their respective multiple signal transduction pathways are remarkably similar (Ravetch, 1997). The initiation of FcR and antigen receptor signalling depends upon phosphotyrosine and extracellular signal-regulated kinase (ERK). Mitogen-activated protein kinases (MAPKs), which include ERK and c-jun N-terminal kinase (JNK), mediate signal transduction from the cell surface to the nucleus (Cano&Mahadevan, 1995; Su&Karin, 1996). Phosphotyrosine is important in the early stages of signalling, while the Ras–Raf–MAPK/ERK kinase–ERK pathway influences cellular proliferation, differentiation and survival (Kolch, 2000). The identification of the signalling pathways involved in the regulation of cytokine gene expression by PTEC may lead to novel therapeutic interventions.

MAPK activation involves the dual phosphorylation of conserved threonine and tyrosine residues, leading to the downstream phosphorylation and activation of target proteins, which may subsequently lead to altered gene expression. In renal diseases, MAPK activation has been shown to occur in response to both growth factors and pro-inflammatory cytokines (Schramek *et al.*, 1996; Schramek *et al.*, 1997). A study investigating how MAPK activation regulates the release of IL-6 in cultured human PTEC in response to IL-1, TNF-α, IL-17 and CD40 ligand (a leukocyte-derived growth factor)

suggested that the JNK pathway may interfere with other signalling pathways involving NF-κB and possibly ERK (Javaid&Quigg, 2005).

Ligand binding of TGF-β with its cell surface receptors generates a heteromeric complex that results in the activation of signalling intermediates and initiates the transcriptional activation of various target genes (Tian *et al.*, 2003). Owing to its highly pleiotropic nature, TGF-β1 is known to activate other signalling pathways in addition to the Smad proteins (Edlund *et al.*, 2002; Hocevar *et al.*, 1999). In a study by Zhang et al., IL-1β-induced transdifferentiation of renal PTEC is characterised by an upregulation of α-SMA and of cell migration, in addition to increased activity of ERK, JNK and p38 MAPK, suggesting that the JNK and p38 MAPK signalling pathways may mediate this process (Zhang *et al.*, 2005). The MAPK signalling pathway depends upon the early activation of PTEC by IgG and the subsequent expression and activation of ERK. Studies in experimental animal models have reported that bovine IgG induces endothelin-1 secretion in rabbit PTEC (Zoja *et al.*, 1995) and RANTES production and NF-κB activation in pigs (Zoja *et al.*, 1998). A recent study in humans reported increased ROS and NF-κB activation in PTEC stimulated with human IgG and albumin (Morigi *et al.*, 2002). Studies of renal biopsies from patients with IgG proteinuria have suggested that *in vivo* events appear to correspond with those observed *in vitro*.

1.7 Aim of the study

The main aim of this thesis was to investigate the expression of the Ig receptors involved in the pathogenesis of renal disease involving PTEC-induced primary GN and whether the expression of these receptors were stimulated by pro-inflammatory cytokines. I hypothesised that renal tubular cells might be exposed to, and in turn activated by ICs, and thus may contribute to tubular damage, fibrosis and enhance GN. Depending on whether the Igs bind to their specific receptor, I sought to elucidate the effect of binding on cytokine production, FN production, signalling pathways and proliferation of PTECs. In particular, this project aimed to expand our knowledge about the novel IgA receptor, Fcα/μR. In addition, I examined two different models of nephrotoxicity in PTEC: (a) the effect of immunosuppressive agents, and (b) the effect of HMG-CoA inhibitors (statins), which have known immunomodulatory effects. I hypothesised that immunosuppressant agents/statins either alone or in combination with LPS might affect Ig receptors expression, cytokine release, FN production and proliferation by PTEC (Figure 1-10).
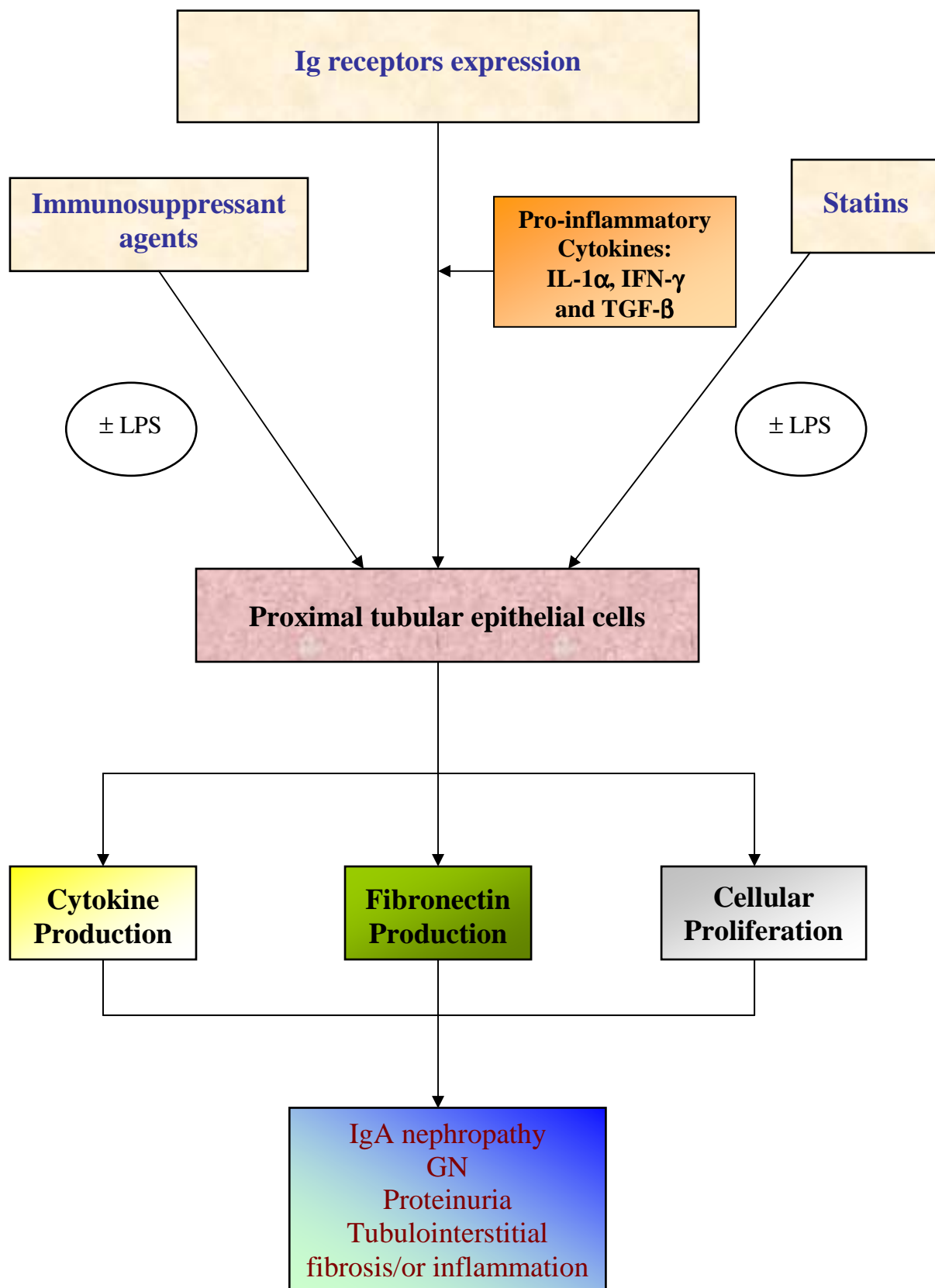
Figure 1-10 Summary of the experiments undertaken and hypotheses made relating to this thesis.

Chapter 2: Material and Methods

2 Outline of experimental plan

In GN and experimental models of renal disease, the deposition of Ig and ICs in the glomeruli and tubulointerstitium of the kidney is an important factor in determining the severity and progression of disease. For example, in IgAN, mesangial IgA deposition is associated with mesangial proliferation and matrix production, in some cases leading to glomerulosclerosis and renal failure. However, the mechanism by which this occurs is not fully understood. Our group discovered a novel IgA receptor, the Fcα/μR, on mesangial cells, which might be involved in IgAN. This receptor binds both IgA and IgM (the latter more strongly) but not IgG (McDonald *et al.*, 2002). Rather than proteinuria being a non-specific effect of renal injury we have developed a hypothesis that Ig and IC, filtered at the glomerulus, may act on Ig receptors in human PTEC, which are a pathological characteristic of glomerular and tubular damage.

This project aimed to unravel the Fc receptors (FcγRI, FcγRIIA, FcγRIIB and FcγRIII, FcαR, γ-chain, FcRn, pIgR, Fcα/μR) expressed by primary and immortalised (HK-2) human PTEC, using techniques like semi qualitative reverse transcriptase-PCR (RT-PCR), quantitative real-time PCR (qRT-PCR), immunoblotting and immunohistochemistry (IHC) to characterise the receptors present in these cells.

Receptor expression and their regulation by cytokines implicated in primary renal disease and the effect of receptor engagement to PTEC was studied using RT-PCR, qRT-PCR and IHC followed by which, the role of cytokines (e.g. IL1-α, TGF-β1, IFN-γ) in GN was then investigated.

Finally functional effects of Ig/IC binding may alter cell proliferation, ECM (e.g. FN) formation and cytokine release, which are a pathological characteristic of glomerular and tubular damage on PTEC and were assessed by ELISA and [^{3}H]-thymidine incorporation respectively.

I also sought to determine the effect of immunomodulators such as immuosuppressive agents and statin therapy alone or in combination with the role of proinflammatory LPS action to determine Ig receptors expression, proliferation, cytokines release and FN production on PTEC. This was studied *in vitro* by techniques such as qRT-PCR, [^{3}H]-thymidine incorporation and ELISA.

2.1 Material

2.1.1 Biochemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Irvine, UK). Tissue culture products were purchased from Gibco™ Invitrogen Corporation (Paisley, UK).

2.1.2 Immunochemicals

A full list of all commercial antibodies and other immunochemicals used in this study is given in Table 2-1.

2.2 Cell biology techniques

2.2.1 Cell culture

All cells were grown at 37°C in a water-saturated atmosphere containing 5% CO_2 and were typically maintained in 75 cm^2 tissue culture flasks in a 20 ml volume.

2.2.1.1 Human Proximal Tubular cells (PTEC)

Human PTEC were derived from the normal pole of human nephrectomy specimens, characterised and cultured as described by Mistry et al. (2001) and kindly provided as a gift by Dr. James McLay (Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK). PTEC were cultured in Dulbecco's DMEM/NUT mixed with F12 Ham's media in a 1:1 ratio, supplemented with 5% foetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (10 μg/ml). The human transformed PTEC cell line HK-2 (American Type Culture Collection [ATCC], Manassas, VA, USA) was cultured in keratinocyte-serum free medium supplemented with epidermal growth factor (0.1–0.2 ng/ml), bovine pituitary extract (20–30 μg/ml), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (10 μg/ml). Cells were routinely subpassaged twice weekly, during which they were centrifuged for 5 min at (400 g) and re-suspended in fresh complete medium.

2.2.1.2 U937 cell line

U937 cells (Sundstrom&Nilsson, 1976) were routinely cultured in RPMI 1640 medium (JRH Biosciences, Andover, UK) supplemented with 10% FCS, glutamine (2 mM), penicillin (10 U/ml) and streptomycin (10 µg/ml). Cells were maintained at a density of between 500,000–2,000,000 cells/ml, as measured using a Neubauer haemocytometer, and were routinely subpassaged twice weekly by the method described above.

2.2.1.3 NK-92 cell line

The NK-92 cell line (Gong *et al.*, 1994) were routinely cultured in α-minimum essential media without ribonucleoside and deoxyribonucleoside but containing glutamine (2 mM), sodium bicarbonate (1.5 g/L), inositol (2 mM), 2-mercaptoethanol (0.1 mM), folic acid (0.02 mM), 12.5% horse serum, 12.5% foetal bovine serum, penicillin (100 U/ml) and streptomycin (10 µg/ml).

2.2.1.4 COS-7 cell line and CHO cells

The COS-7 human fibroblast cell line (ATCC, Manassas, VA, USA) was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (10 µg/ml). Chinese hamster ovary (CHO-K1) cells (European Collection of Cell Cultures, Salisbury, UK) were cultured in RPMI 1640 media and supplemented as above.

2.3 Molecular biology

The work presented in this chapter focuses on attempting to quantify the levels of Ig receptors in immortalised HK-2 cell lines and primary PTEC. Cells were cultured in 6-well plates and stimulated with the IFN-γ (at concentrations of 20 and 200 ng/ml), TGF-β1 (at concentrations of 5 and 50 ng/ml) and IL-1α (at a concentration of 50 ng/ml) for 24 hrs using a RT-PCR assay and qRT-PCR.

2.3.1 Reverse transcription-PCR (RT-PCR)

RT-PCR rather than PCR alone is often used in expression mapping, isolating and identifying a specific sequence, and is essentially normal PCR with a preceding step during which the enzyme reverse transcriptase is added to mature, fully-spliced messenger RNA

(mRNA) to convert it back to complementary DNA (cDNA). Operating on a single strand of mRNA, reverse transcriptase generates cDNA by combining the RNA base pairs (A, U, G, C) with their DNA complements (T, A, C, G). As this cDNA cannot be degraded by the RNase enzymes found in most reagents and on most surfaces in the laboratory, it is considerably more stable than RNA.

2.3.1.1 RNA extraction for RT-PCR

Total cellular RNA was isolated from the cultured HK-2 cells using TRIzol® LS Reagent (Invitrogen Life Technologies, Paisley, UK) extraction. During this process, cells were lysed by resuspending with 1 ml TRIzol® LS Reagent through pipetting. Samples were then incubated for 5 min at room temperature (15–30°C) to permit the complete dissociation of nucleoprotein complexes before the addition of 0.2 ml chloroform per 1 ml of TRIzol® LS Reagent. Tubes were shaken vigorously by hand for 15 sec then incubated for 3 min at room temperature to ensure good chloroform phase separation. Tubes were centrifuged at 13,000 rpm (11,000 g) for 15 min at 4°C. The aqueous layer, which contains the RNA, was then transferred to a fresh tube and 0.5 ml of isopropanol was added to precipitate RNA followed by centrifugation (11,000 g for 10 min) at 4°C. The samples were incubated at room temperature for 10 min followed by centrifugation (13,000 rpm [11,000 g] for 10 min) at 4°C. The RNA pellet was then washed with 1 ml of 75% ethanol in diethylpyrocarbonate (DEPC)-treated water followed by vortexing and centrifugation at 7,500 g for 5 min at 4°C. The RNA pellet was allowed to dry in air for 5–10 min then resuspended in 30 µl of DEPC-treated water and stored at –70°C.

2.3.1.2 RNA concentration measurement

The concentration of a known volume of RNA was determined by measuring absorbance at 260 nm and 280 nm as estimated using a Beckman DU640B spectrophotometer blanked against DEPC water. RNA quality was estimated by looking at the absorbance ratio A_{260}/A_{280}. Pure RNA has an absorbance ratio of approximately 2. RNA samples were stored at –70°C.

2.3.1.3 cDNA synthesis for RT-PCR

For RT-PCR, first-strand cDNA synthesis was prepared using oligo(dT), a Superscript™ First-Strand synthesis system for RT-PCR (Invitrogen Life Technologies, Paisley, UK) kit.

Less than 5 μg of total RNA was incubated with 1 μl of 10 mM dNTP mix and 1 μl of oligo (dT) (0.5 μg/μl) in a total volume of 10 μl for 5 min at 65°C, before being placed on ice for 1 min. A reaction mixture containing 10× RT buffer (2 μl), 25 mM MgCl₂ (4 μl), 0.1M DTT (2 μl) and RNaseOut™ Recombinant RNase Inhibitor (1 μl) per sample was prepared and added. The samples were then incubated at 42°C for 2 min before 1 μl (50 units) of Superscript™ II RT was added. The reactions were continued at 42°C for 50 min and terminated after reacting at 70°C for 15 min. The samples then were placed on ice before being diluted by adding 80 μl of distilled water to give a final volume of 100 μl. They were then stored at –20°C.

2.3.1.4 RT-PCR reaction

The cDNA generated in the first phase of RT-PCR can be used as a template for the second phase, the PCR reaction. This reaction also relies on a non-mammalian enzyme, in this case a bacteria-derived DNA polymerase called Taq, the thermostable properties of which means it can resist being denatured even at the very high temperatures at which DNA strands can be separated. In the PCR reaction, the length of the amplified DNA sequence can be extended using forward and reverse primers. In doing this, millions of copies of the original sequence can be produced.

PCR was performed using a PTC-100 programmable Thermal Controller or Minicycler™ (MJ Research Inc). PCR reactions were carried out in a 50 μl volume. A mastermix containing 10× PCR buffer, 2.5mM MgCl₂, dNTP mix (final concentration 200 μM of each dNTP) and 20 pM of each primer was prepared and added to each tube (Table 2-2). Template cDNA in a 5 μl volume (or distilled water as a control) was then added before the addition of *Taq*. *Taq* DNA polymerase was added at a volume of 1 μl per reaction and the samples were subsequently kept on ice before being placed in the cycler with the block already heated to a denaturing temperature.

The reaction was conducted according to the following programme: samples were denatured at 94°C for 90 sec, followed by 30–36 cycles of denaturation at 94°C for 30 sec, primer annealing for 60 sec at a temperature determined by the specific primer combination (Table 2.3) and DNA extension at 72°C for at least 60 sec. A final extension at 72°C was then performed for 5–10 min.

2.3.1.5 Agarose gel and electrophoresis of DNA

PCR products were typically electrophoresed at 70 V through 1–2% (w/v) agarose gels, depending on the product size required in the 1× tris-acetate (TAE) electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8). For this, 20 µl of the sample were mixed with 6× blue/orange loading buffer (Promega, UK) and loaded on the agarose gels. The gels were then stained for 15–20 min in a solution containing 2–3 µl/ml ethidium bromide in distilled water. Bands were visualised on an ultraviolet transilluminator and photographed with a Fluro-S® MultiImager using Multi-analyst software (Bio-RAD Laboratories, Hertfordshire, UK).

2.3.2 Quantitative Real Time PCR (qRT-PCR)

Quantitative real-time PCR is used to determine the presence and the number of copies of a specific DNA sequence in a given sample. The 'real-time' aspect of this process refers to the fact that, unlike in normal PCR, the DNA is quantified after each amplification cycle. Combining real-time PCR with RT-PCR enables us to quantify low-abundance mRNA using fluourescent dyes and to estimate relative gene expression in a particular cell or tissue type at a particular time.

2.3.2.1 RNA extraction for Real time PCR

The relative levels of mRNA expression for a range of Ig receptors were assessed by fluorescent real-time PCR. Total RNA was extracted using a Qiagen column extraction system (Table 2-1) in order to obtain a purer RNA than is possible to achieve using the TRIzol protocol. When used for normal extraction, the TRIzol protocol and ethanol can affect the fluorescein SYBER green in gene amplification and may give erroneous results.

As previously stated, HK-2 cells were grown on 6-well plate to confluence before the addition of the proinflammatory cytokines IFN-γ, TGF-β and IL-1α to each well for 24 hrs. The samples were disrupted and lysed by adding 300 µl of lysis buffer (β-mercaptoethanol added to GITC-containing buffer RLT). The samples were then homogenised in order to shear genomic DNA and reduce the viscosity of the lysate by passing it through a 20-gauge needle fitted to a syringe for at least 5–10 min. RNase-free water (590 µl) and proteinase K (10 µl) were then added to digest and remove any proteins, and the whole solution was thoroughly mixed by pipetting. Following a 10 min

incubation in the water bath at 55°C, the samples were centrifuged (10,000 g for 3 min) at 20–22°C to ensure that the debris was pelleted. The supernatant (approximately 900 μl) was then pipetted into a new 1.5-ml tube before 450 μl of ethanol was added to the cleared lysate and mixed well by pipetting.

Of this sample, 700 μl was pipetted into an RNase Mini Spin Column in a 2 ml collecting tube and the samples centrifuged (8500 g for 15 sec) at 20–22°C. In doing this, the RNA bound to the RNeasy silica gel membrane. 350 μl of RW1 buffer was added to the column and centrifuged (8500 g for 15 sec) at 20–22°C before being applied to the RNeasy spin column to aid absorption of RNA to membrane. A stock solution (10 μl DNase I to 70 μl buffer RDD) was prepared and then the total volume pipetted directly onto the RNase silica gel membrane so that any traces of DNA that may copurify were removed. Following incubation at room temperature for 15 min, the column was washed clean of DNase and other contaminants using a 350 μl RW1 buffer centrifuged for 15 sec at 8500 g at 20–22°C. The column was then transferred into a new 2-ml collecting tube and, by adding 500 μl buffer RPE, was washed by centrifuging (8500 g for 2 min) at 20–22°C. This step was repeated twice. The column was then transferred to a new 1.5-ml collecting tube by pipetting 30 μl RNase-free water directly onto the RNase silica gel membrane. Finally, the samples were centrifuged (8500 g for 1 min) at 20–22°C to elute ready-to-use RNA in RNase-free water (Fig. 2-1).
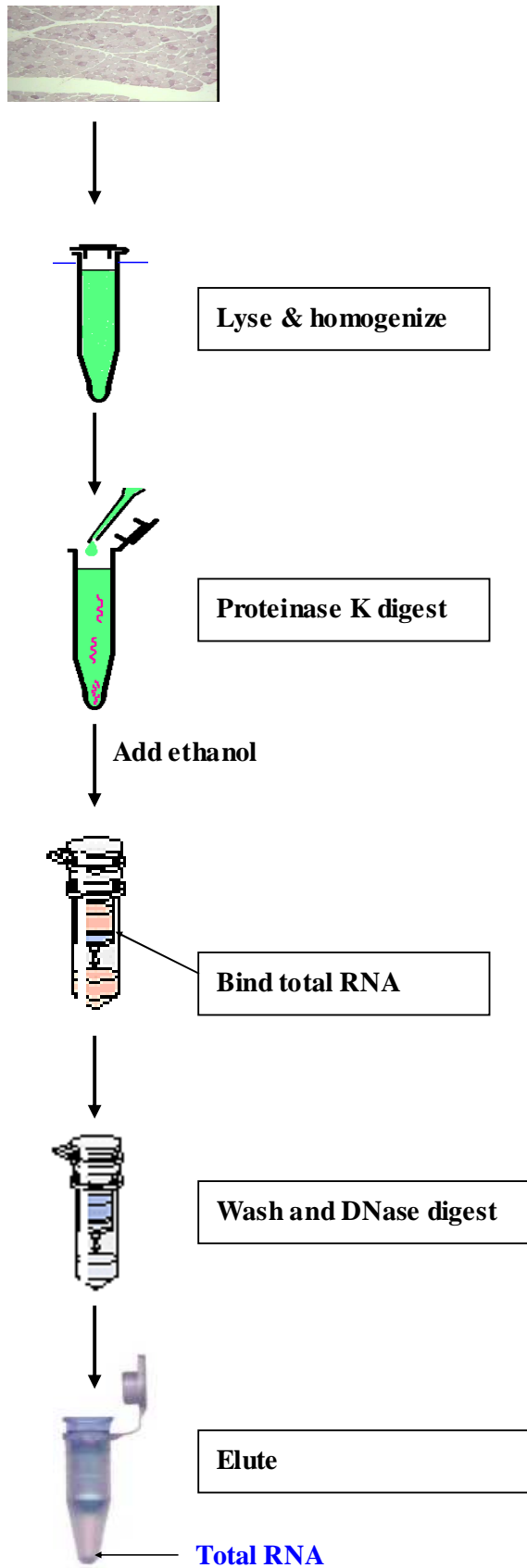
Figure 2-1 The principles of RNA extraction.

2.3.2.2 cDNA synthesis for real-time PCR

For cDNA synthesis, a machine manufactured by iGene Legends was used. Approximately 300 ng was reverse transcribed to cDNA using the Iscrip^{TM} cDNA synthesis kit according to the manufacturer's protocol (Bio-RAD Laboratories, Hertfordshire, UK).

2.3.2.3 Real-time PCR

Identification of Ig receptor genes differentially expressed in immortalised and primary PTEC was quantitatively verified by RT-PCR. The relative quantities of the Ig receptor gene transcripts in PTEC cells were measured before and after stimulation with proinflammatory cytokines for 24 hrs by semi-qRT-PCR. Total RNA was extracted as described in section 2.3.2.1 and was processed directly to cDNA synthesis as described in section 2.3.2.2. The primer for Ig receptors and the housekeeping gene β-actin were designed (http://frodo.wi.mit.edu/cgi-bin/primer3) and synthesised by TAGN Ltd. showed in table 2-4. The primers were designed to be across different exons to completely abolish amplification from genomic contamination. The house-keeping gene β-actin is widely used as standard because this protein, essential for maintenance of cell function, is generally constitutively expressed at similar level in all cell types and tissues. cDNA was amplified using qRT-PCR with fluorescence using SYBR Green which fluoresces on binding to all newly synthesized double stranded DNA (ds)DNA. An increase in fluorescence intensity is measured, thus allowing initial concentrations to be determined. In this system primers are designed to generate a small DNA product to maximise reaction efficiency and the reaction takes place in the presence of SYBR Green dye. However, SYBR Green will label all dsDNA including any unexpected PCR products, leading to potential complications.

Fluorescence is measured at the end of each PCR cycle and increases exponentially as the reaction progresses. For each reaction the threshold cycles (C_t) is calculated based on the point at which a statistically significant increase in the amount of PCR product is detected. The C_t inversely reflects the number of target sequences present in each sample prior to amplification (Higuchi *et al.*, 1993).

Five reactions were performed in triplicate, and the mean of five C_t were determined. To determine sensitivity of the expression, the amplification of known mRNA concentration in serial dilutions was measured. PCR efficiency was assessed for all primer pairs by

generating an mRNA titration curve showing C_t values of these amplifications; these were plotted against the logarithm of the relative initial amount of cDNA. The slope of the straight line obtained provided a measure of PCR efficiency. The transcription efficiency was between 80–100%, thus, at different concentrations of proinflammatory cytokines the Ig receptor genes present at low expression levels would be reliably quantified. The correlation coefficients obtained were all in excess of 0.999.

Fluorescent real-time PCR was performed using an iQ SYBER green supermix (Bio-RAD) on a Bio-Rad iQ light cycler. Amplification of cDNA aliquots was carried out in 25 µl reaction volumes containing 3µl cDNA, and 0.5 µl of each appropriate primer added to 12.5 µl of the Supermix and brought to a final reaction volume of 25 µl with RNase-free water. The thermal cycler conditions included an initial denaturation step at 95°C for 3 min followed by 60 cycles at 95°C for 30 s and 60°C for 30 s. In order to assess specificity of amplified product and avoid spurious results from "primer-dimer" formation and amplification, dissociation curves showing product melting points were assessed. PCR products were run on agarose gels to confirm that their size corresponded to the expected length.

2.4 Biochemical assay and methods

2.4.1 Signalling pathway or Ig-IC cross-linking

HK-2 cells were grown overnight on a Petri dish and incubated at 37°C. To cross-link specific receptors, cells were washed and incubated with specific antibodies (Table 2-1) for 45 min at 4°C. Cells were washed three times with fresh media to remove unbound antibodies and the receptors were cross-linked by the addition specific of anti-Igs (1:50 dilution; Sigma). Cells were then warmed to 37°C for the specified durations (0, 2, 5, 10 and 30 min).

2.4.2 Cell lysate preparation

In general, cells were lysed by the addition of an ice-cold lysis buffer (1% (v/v) Triton X-100, 50mM Tris-HCl pH 7.5, 0.25% (w/v) sodium deoxycholate, 150mM sodium chloride, 1mM EDTA, 1mM vanadate and 1mM NaF) containing protease inhibitors (1mM phenylmethylsulfonyl fluoride and 1 µg/ml each of chymostatin, leupeptin, antipain and pepstatin). Cells in suspension were pelleted by centrifugation at 13,000 rpm (11,000 g) for

15 min to remove insoluble cell debris. Any medium was removed prior to the addition of the lysis buffer. After 30 min of tumbling motion at 4°C, lysates were either used immediately or stored at –20 °C prior to electrophoresis.

2.4.3 Protein quantification (Bradford assay)

Protein concentrations were determined using the Bio-RAD Protein Assay System for all cell lysates, which is based on the Bradford Coomassie brilliant blue dye binding system. A range of bovine serum albumin concentrations (0, 2, 5, 10 and 15 μg/ml) was used to construct a standard curve and samples were suitably diluted to allow their protein level to fall within the linear range of this curve. Light absorbance at 595 nM was measured on a Beckman DU640B spectrophotometer. All samples were measured in duplicate.

2.4.4 Sodium dodecyl sulphate–polyacrylamide electrophoresis (SDS–PAGE)

SDS–PAGE was carried out using the NuPAGE™ Pre-Cast Gel System. The NuPAGE™ system works with bis-tris buffered (pH 6.4) polyacrylamide gels, available at three different acrylamide concentrations (10% w/v, 12% and 4–12% gradient gels) allowing different separation ranges to be selected. The running buffer used was 20x NuPAGE™ MOPS SDS. After heating to 70°C for 10 min, protein samples were denatured in 4× NuPAGE™ LDS (lithium dodecyl sulphate) sample buffer. For reduced proteins, 50 mM dithiothreitol was added prior to sample denaturing.

2.4.5 Western blot analysis

In Western blot analysis, gel electrophoresis is used to separate denatured proteins by mass before they are transferred onto nitrocellulose membrane where they are investigated using antibodies specific to the protein. Phosphotyrosine, phospho-ERK and Fcα/μR were detected by western blot using a monoclonal antibody as described in Table 2-1. Using the standard blotting profile outlined in section 2.4.4, the antibody gave a low signal and high background and it was therefore necessary to develop a modified protocol.

The SDS–PAGE gel was prepared and cleaned as per the manufacturer's instructions and a running buffer was used to clean the individual wells of non-polymerised gel solution to get rid of air bubbles. The gel was then immersed in a tank containing MOPS SDS running

buffer (20×). Samples prepared in SDS sample buffer (as described in section 2.4.4) were loaded into wells and the ladder was pipetted into the first well. Proteins were then electrophoresed using a Bio-RAD power PAC 300 gel unit at a constant voltage of 200 V for 1 hr until the bromophenol blue tracking dye reached the bottom of the gel.

For Western blotting, proteins were transferred to nitrocellulose membrane using the Novex XCell II Surelock™ blotting apparatus. To confirm adequate transfer and equal loading of lanes, membrane was washed with Panceau S solution for 5 min to visualise the proteins, followed by washing with dH$_2$O and TBS (20 mM Tris.HCl [pH 7.6] and 136 mM NaCl) containing 0.1% (v/v) Tween 20 (TBST). A nitrocellulose membrane was blocked in TBST with 4% non-fat milk (Marvel) for 1 hr at room temperature and washed in wash buffer three times. Typically, blots were incubated with primary antibodies (Table 2.1) diluted 1:200 in 4% milk-TBST overnight at 4°C. Membrane was washed three times for 10 min each in wash buffer at room temperature in a shaker and incubated with the appropriate horseradish peroxidase conjugated secondary antibody diluted 1:2000 in 4% milk-TBST for 2 hrs at room temperature with shaking. Blots were then washed extensively with large volumes of TBST (typically 5×5 min washes) followed by a final wash in dH$_2$O prior to developing. For development using the enhanced chemiluminescence detection system ECL-Detection, equal volumes of reagent 1 with reagent 2 were used. Under red light in a dark room the X-ray film was developed using a Kodak X-Omat 2000 Processor. For quantification of expressed protein, radiographs were scanned and densitometry of expressed band was performed using Kodak ID software.

2.4.6 Anti-Fcα/µ receptor antibodies purification

Fcα/µR antibody was provided by the Chinese Academy of Medical Sciences, Beijing, China (Wei Zhang, Ying Fu). An IgG1 monoclonal antibody (clone 10C4) against human Fcα/µR was produced using cDNA coding for the EC2 exon (McDonald *et al.*, 2002) ligated into a pET-30a vector (Novagen) and expressed in E. coli. The expressed protein was used for immunization of BALB/c mice. The first immunization used 30 µg of protein in complete Freund's adjuvant and subsequent injections were given at 3-week intervals using antigen in incomplete Freund's adjuvant. Five weeks after the last injection, one mouse was challenged intravenously with 30 µg of antigen and fusion was done three days later using mouse spleen cells and myeloma Sp2/0 cells. Successfully fused cells were screened for antibody production against Fcα/µR and positive cells were further sub-

cloned. Antibodies secreted in culture supernatants were purified using a protein A column.

2.4.7 Fc receptor aggregation

2.4.7.1 Preparation of heat-aggregated IgG (HAg-IgG)

IgG was heat aggregated by heating human IgG at a concentration of 50 mg/ml to 65°C for 30 min. The aggregated IgG was pelleted by micro centrifugation at 13,000 rpm (11,000 g) for 30 min, and then washed three times with phosphate buffered saline (1×PBS) (0.07M NaCl, 75 mM disodium hydrogen orthophosphate and 5 mM sodium dihydrogen orthophosphate, pH 7.2). The protein concentration was measured by Bradford's assay (as described in section 2.4.3). The measured protein concentration of 28 mg/ml was equivalent to actual concentration of 50 mg/ml of IgG. The concentration of HAg–IgG used on HK-2 cells was 100 µg/ml.

2.4.7.2 Preparation of heat-aggregated IgA (HAg–IgA)

IgA was heat aggregated by heating human IgA at a concentration of 2.4 mg/ml to 65°C for 1 hr. The aggregated IgA was pelleted by microcentrifugation at 13,000 rpm (11,000 g) for 30 min, and then washed once with 1×PBS. Again, the protein concentration was measured by Bradford's assay. The measured protein concentration of 1.4 mg/ml was equivalent to actual concentration of 2.4 mg/ml of IgA. The concentration of HAg-IgA used on HK-2 cells was 100 µg/ml.

2.4.8 Immunofluorescence and laser scanning confocal microscopy

Cells stained with fluorescent dyes are said to contain immunofluorescently labelled antibodies and antigens and are studied either by using a fluorescence microscope or by confocal microscopy. Human monozygotic U937 cells primed in fresh medium with 1 mM dibutyryl cyclic AMP (dbcAMP) for 48 hrs prior to experiments as per Cameron et al. (Cameron *et al.*, 2002) were used as a positive control for the experiments. Differentiation of these cells to a macrophage phenotype with dbcAMP induces both mRNA and protein expression of the inhibitory IgG (FcγRIIA) receptor. Typically, one million U937 cells were washed with ice-cold PBS and resuspended in 100 µl PBS with 1 µg the appropriate monoclonal antibody. In order to restrict any non-specific interactions of monoclonal

antibodies with the Fc receptors, 3 µM monomeric human IgG was added if needed. After being incubated on ice for 45 min, the cells were washed three times with PBS and incubated for a further 45 min with a fluorescrin isothiocyanate conjugated goat anti-mouse IgG secondary antibody (1:100 dilution). Nonspecific isotype control antibodies were used to control for nonspecific labelling. Cells were harvested by centrifugation (400 g for 5 min) at 4°C onto chrome/alum coated glass slides.

HK-2 cells were grown on glass coverslips for 24 hrs. The cells were washed with 0.2% bovine serum albumin (BSA) in PBS and incubated at 4°C with 100 µg/ml of biotin-conjugated IgG, monomeric IgA or IgM (Table 2-1). Cells were also incubated with biotin-conjugated IgM Fc-µ fragment in 0.2% BSA for 45 min. Washed cells were then incubated with 90 µg/ml of fluorescein (DTAF)-conjugated streptavidin (Jackson Immuno Research Laboratories) at 4°C for 45 min and subsequently lightly stained with propidium iodide (0.1 mg/ml, for 10–15 mins at 4°C) to heavily stain the nuclei, which also lightly stained the cytoplasm. The cells were then washed once with PBS. The coverslips were mounted using Vectorshield mounting medium.

For specific Fcα/µR staining, washed cells were incubated in a 1:50 dilution of mouse anti-Fcα/µR antibody (Table 2-1) at 4°C for 45 min. After further washing, a FITC-labelled goat anti-mouse antibody was added for 45 min at 4°C before visualisation. Nonspecific isotype control antibodies were used to control for nonspecific labelling. Ig binding was analyzed using a laser scanning confocal microscopy imaging system (Bio-RAD, Hertfordshire, UK; excitation: 488 nm, emission: 515 nm).

2.4.9 Immunohistochemistry for the Fcα/µ (DAB) staining

Immunohistochemistry is a widely used technique that allows us to visualise binding between antibodies and antigens by using antibodies conjugated to enzyme (e.g. peroxidase) or fluorophore (e.g. FITC). This technique was kindly performed by Ian Downie, a pathologist at the Royal Infirmary Glasgow. Immunohistochemistry was performed on isolated cells grown on glass coverslips or on sections of human kidney extracted from the normal pole of tumour nephrectomy specimens (the latter was used for staining with human anti-Fcα/µR, diluted 1:2000). Three-micron paraffin sections were dewaxed for 10 min in xylene and then dipped in graded alcohols. Prior to immunostaining, heat-mediated antigen retrieval was performed using Tris buffer/EDTA

(pH 8) in a pressure cooker. The solution was brought to boil before immersing slides for 5 min at full pressure. The slides were allowed to cool for 20 min and then washed in running water.

The sections were immunostained in a Dako Autostainer using Envision Peroxidase. The slides were washed with TBS/10% Tween buffer for 30 s before treatment with blocking serum (1:20 normal goat serum) for 20 min and subsequently anti-Fcα/μR primary antibody. After rinsing, slides were incubated in Dako Envision for 30 min and visualised using 3,3-diaminobenzidine (DAB substrate KIT for peroxidase) for 10 min.

Finally, after 30-s rinses with TBS/Tween buffer, the location of the antigen–antibody complex was visualised using 3,3-diaminobenzidine. The slides were then washed with water, counterstained using haematoxylin (nuclear stain), placed in copper sulphate for 5 min and then dehydrated, cleared and coverslipped.

2.4.10 PTEC proliferation assay

The first indirect use of [^{3}H]-thymidine uptake assay in biochemical research was the identification of dividing cells by incorporation of radiolabelled thymidine and subsequent measurement of the radioactivity to identify the dividing cells. For this purpose, tritiated thymidine is included in the growth medium. In spite of errors in the technique it is still used to determine the growth rate of the cells. Thymidine (or deoxythymidine) is a DNA nucleoside with a key function in the synthesis of DNA and therefore in cell division. Deoxythymidine is present in the body fluids as a result of degradation of DNA from live and from dead cells.

The total number of cells was calculated using a standard haemocytometer. Suspension cells were diluted in trypan blue 1:1 (v:v) and injected under a coverslip on a haemocytometer. Cells were counted in four defined areas and the mean calculated. Next, cells were seeded onto 96-well plates in replicates of six at a confluency of 5×10^4 cells/ml for 48 hrs. Proliferation was measured by [^{3}H]-thymidine uptake using standard methodology. For the final 18 hrs, 1 μCi [^{3}H]-thymidine was added to each well. Cells were harvested onto filter paper using an automated cell harvester and the amount of [^{3}H]-thymidine incorporated into replicating DNA counted by Betaplate (1205) flatbed liquid scintillation counter.

2.4.10.1 Morphology of PTEC

The cells were grown in four chambers mounted on a glass slide with cover (Lab-Tek$^®$II Chamber Slide™ Systemat, USA) at a confluency of 5×10^4 cells/ml. The pictures were taken at magnification $\times100$.

2.4.10.2 H & E staining

This technique was kindly performed by Dr Barbra Young in the pathology department at Glasgow University using standard protocol for H & E staining.

2.5 Manufactured kits

2.5.1 FN measurement

The human FN ELISA kit is a 96-well system that allows the easy measurement of FN from most samples. There are two incubation steps: the first with specific primary antiserum (rabbit polyclonal) raised against highly purified human FN and the second with an alkaline phosphatase human FN conjugate. The wells are pre-coated with antibody that causes a separation of bound and free fractions and permits the estimation of the amount of bound enzyme by absorption at 400–410 nm after incubation. This information is desirable as it is known to be inversely proportional to FN concentration, which ranges from 25–2000 ng/ml as standard.

FN production by PTEC was measured in response to Ig cross-linking or immunomodulatory agents using ELISA. PTEC were grown to confluency in 6-well plates. In studies of matrix production, TGF-β (50 ng/ml) was used as a positive control. Cells were incubated at 37°C for 72 hrs, followed by three washes with 0.05M Tris Cl in 15M NaCl at pH 7.5. Matrix was removed from the plate using the same buffer (50 mM Tris HCl in 150mM NaCl at pH 7.5). Cell pellets were collected by centrifugation at 13,000 rpm (11,000 g) for 15 min and resuspendend in the above buffer with the addition of aprotinin 5 µg/ml, phenylmethylsulfonyl fluoride 0.5 mM and urea in a 0.6 g/ml suspension. The samples were mixed by vortexing and incubated in a heat block at 37°C for 2 hrs to dissolve the urea before being transferred to a dialysis tube. Dialysis against 0.01M 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), pH 11, at room temperature for 24 hrs purified the FN. Next, the samples were neutralised by the addition of 1/10

volume of 0.5M Tris Cl, pH 7. The ELISA was performed according to the manufacture's instructions.

2.5.2 Measurement of cytokine release

Cytokine release was studied using ELISA techniques with the aim of quantifying the amount of protein in cell culture media after 24-hrs stimulation with IgG, XL-IgG, HAg-IgG, IgA, IgA-XL, HAg-IgA and IgM.

2.5.2.1 IL-6, IL-8, GM-CSF ELISA

HK-2 cells were seeded onto a 24-well plate in 0.5 ml complete medium to confluency. Fresh media was supplemented with either a variety of Ig (with and without cross-linking), HA-Ig, growth factor or immunomodulatory agents or statins (Table 2-1). Cells treated with 10 μg/ml LPS alone were used as a positive control and untreated HK-2 cells served as a negative control. Cells were incubated at 37°C for 24 hrs. Supernatants were collected by centrifugation and assessed for IL-6, IL-8, GM-CSF, TNF-α and IFN-γ in triplicate using sandwich ELISA. The cells were lysed as described in section 2.4.2 and the concentration of protein measured as described in section 2.4.3 in order to calculate the ratio of cytokine release to the supernatant (stored at –20°C for each individual).

The cytokine ELISA assay was a "sandwich" enzyme immunoassay employing two monoclonal antibodies. Anti-capture antibody is a monoclonal antibody specific for human IL-6, IL-8 and GM-CSF. This was diluted in ELISA coating buffer and 100 μl added to each well of a 96-well ELISA plate. Plates were sealed with wax paper and incubated for 18 hrs at 4°C before being warmed to room temperature. Excess capture antibody was removed by washing with 2×PBS with 0.05% Tween-20. Non-specific peptide binding was blocked by incubating each well for 2 hrs at room temperature with 100 μl ELISA blocking buffer. Blocking buffer was removed by three washes with PBS with 0.05% Tween-20.

The samples and standards were prepared in assay diluent (PBS with 10% FCS). Standards were prepared in multiple dilutions with concentrations up to 120 ng for IL-6 and GM-CSF to 100 ng for IL-8. 100 μl samples and standards were pipetted into wells where the capture antibody bound any cytokine protein. The plate was then sealed with wax paper

and incubation was allowed to continue for a further 2 hrs. Unbound material was washed away five times with PBS with 0.05% Tween-20.

Biotinylated anti-IL-6, IL-8, and GM-CSF with avidin-horseradish peroxidase (HRP)-conjugated was diluted 1:250 in assay diluent, and 100 µl was added to each well. Incubation was allowed to continue for 1 hr at room temperature and plates were washed seven times with PBS with 0.05% Tween-20. Following incubation and washing, the chromogenic substrate solution tetra-methylbenzidine (TMB) and hydrogen peroxide, was added 100 µl per well and incubated at room temperature in the dark until catalysed from a colourless solution to blue by the HRP, then yellow by adding the stop solution of 1M sulphuric acid (H_3PO_4). The intensity of colour was proportional to the amount of the cytokine protein present. Absorbance was measured at 450 nm within 30 min, standard curves were calculated and samples were measured in reference to these.

2.5.2.2 Cytokine array kit (ProteoPlex™)

The ProteoPlex 16-well human cytokine array is designed for multiplex detection and measurement of 12 human cytokines in parallel from up to 16 experimental samples. The cytokines measured in the kit are pro- and anti-inflammatory cytokines important for the study of immune system regulation and diseases, namely IL-1α, IL1-β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL12, GM-CSF, IFN-γ and TNF-α. Each well on the slide contains a microarray of spotted antibodies with four 'spots' for each of the 12 cytokines, plus additional spots for positive and negative controls. The replicate spots provide reliable quantitative data from a single sample (Fig. 1-2A, B).

The protocol for these experiments was identical to that described in section 2.5.2.1 for the incubation of Ig and cross-linking with HK-2 cell lines for 24 hrs. The cytokine array was performed according to the manufacturer's instruction. The detection range was 5–2500 pg/ml and the standard curve range from 15–800 pg/ml. The slides were scanned and analysed by Merck Biosciences by using SensiLight™PBXL-3 (Cyc5 wavelengths 633 nm excitation; 660 nm emission).

A-
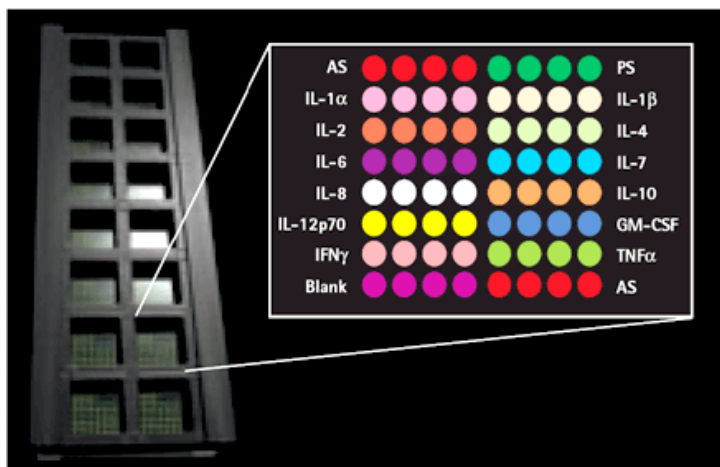
Figure 2-2 ProteoPlex slide map

(a) This figure provides a map of each microarray, showing the arrangement of the 12 capture antibodies, alignment spots (AS), positive control spots (PS), and negative control spots (blank).
Adapted from: http://www.genpromag.com/images/0505/arr3_lrg.jpg

B-
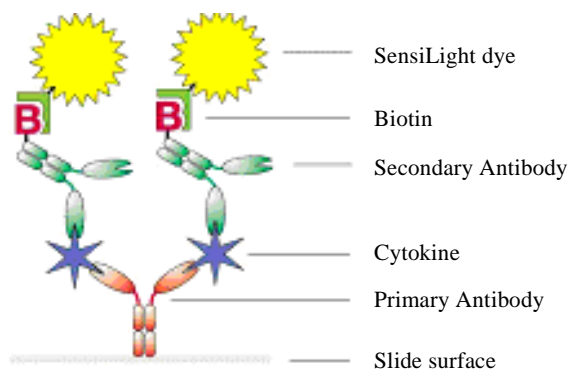
(B) Strategy of sandwich antibody–antigen binding

In the assay represented in this figure the detection strategy is a standard "sandwich" immunoassay that relies on biotinylated detection antibodies and a streptavidin-conjugated fluorophore for detection. Adapted from: http://www.emdbiosciences.com/SharedImages/Novagen/EMD_USD_71414

2.6 Statistics

All statistical analyses were conducted using Microsoft Excel 2000. The statistical analysis for qRT-PCR was calculated by taking the average of five experiments with three replicates in each assay for all of the samples. Each of the experiments had six conditions: control (unstimulated PTEC), cells treated with IL-1α, TGF-β and IFN-γ. In some cases a positive control was used, e.g. U937 or NK92 cells. In each experiment, the house-keeping gene (β-actin) was used to calculate the Ct. The mean Ct in the five experiments was calculated for β-actin and for each of the six conditions. The first step was to calculate the difference in average Ct between the expression of β-actin and the gene of interest (e.g. FcRn, pIgR) for the control. Secondly, the difference in mean Ct was calculated between the expression of the gene of interest for the treatment condition and the control. The relative expression of the Ig receptor gene to the control gene was calculated by squaring the difference in mean Ct.

For the FN, cytokine and proliferation assays, I conducted 3–4 experiments, each with 2–3 replicates on each plate. In the control condition (unstimulated PTEC), I set the variables of interest (FN production, cytokine release and thymidine uptake) to 100% and expressed the results of each treatment condition as a percentage of this by calculating the ratio between the treatment condition and the control. For each sample, I then calculated the average percentage across all experiments. Using MINITAB I confirmed that the spread of my data followed a Normal distribution. Then, in Excel, I calculated the standard deviation (SD) of each variable and plotted the results. Each graph shows the mean $\pm$ SD and statistically significant differences are indicated where they exist. Significance at the 95% level was determined using two-sided t-tests comparing the treatment conditions to the control. A value of $p<0.05$ was considered to be significant.

As my experiments involved making multiple comparisons, it was appropriate to apply a Bonferroni correction to the results of the statistical tests. The Bonferroni correction is calculated by dividing the statistical significance of one experiment (ie 0.05) by the number of hypothesis being compared. For example, for experiments conducted five times, the significance level was adjusted accordingly to 0.01. For experiments conducted three times, the significance level was 0.017. For experiments conducted only twice, the significance level was 0.025. The Bonferroni correction is a conservative way of ensuring that the number of false-positives (i.e. significant associations that occur by chance) is kept to a minimum.

Specificity	Usage	Product Provenance
(ERK) Phospho-p44/42 Map Kinase (Thr202/Tyr204) (rabbit)	WB	Cell Signalling Technology, U.K
$[^3H]$-thymidine	Proliferation	Amersham PharmicaBiotech, Little Chalfont, U.K
1205 Betaplate scintillation counter	Proliferation	Wallace Oy, Turku, Finland
20x NuPAGE™ MOPS SDS	WB	Invitrogen, Paisley, UK
Anti-human IgG (rabbit)	WB, FN, Cytokine production, Proliferation	Jackson Immuno Research Laboratories, West Grove, PA, USA
Anti-human IgM Fc5μ. F(ab')2 (goat)	WB, FN, Cytokine production Proliferation	Jackson Immuno Research Laboratories, West Grove, PA, USA
Anti-human serum IgA [α chain specific] (rabbit)	WB, FN, Cytokine production, Proliferation	Jackson Immuno Research Laboratories, West Grove, PA, USA
Anti-mouse IgG:HRP (sheep)	WB secondary Ab	ECL™ Amersham Biosciences, U.K
Anti-Phosphotyrosine, mouse monolocolonal $IgG_{2b\kappa}$ (4G10®)	WB	Upstate, U.K
Bio-Rad iQ light cycler	Real Time PCR	Bio-RAD Laboratories, Hertfordshire, U.K
Bio-Rad Protein Assay	WB, Cytokine Production	Bio-RAD Laboratories, Hertfordshire, U.K
Biotin-conjugated IgA	IHC	Jackson Immuno Research Laboratories, West Grove, PA, USA
Biotin-conjugated IgG	IHC	Jackson Immuno Research Laboratories, West Grove, PA, USA
Biotin-conjugated IgM	IHC	Jackson Immuno Research Laboratories, West Grove, PA, USA
Biotin-conjugated IgM Fc-μ fragment	IHC	Jackson Immuno Research Laboratories, West Grove, PA, USA
Blocking serum (goat)	IHC	Novocastra, UK
Bovine serum albumin (BSA)	IHC	Sigma-Aldrich, Irvine, U.K
CAPS	FN Production	Sigma-Aldrich, Irvine, U.K
Cell harvester	Proliferation	Wallace Oy, Turku, Finland
Cyclosporin A	Real Time PCR, Cytokine Production, FN, Proliferation	Novartis, Basel, Switzerland
DAB substrate KIT	IHC	Dako Ltd., Ely, U.K
Dialysis Tubing	FN Production	Medicall International Ltd., London, U.K

ECL-Detection	WB	Amersham Biosciences, Buckinghamshire, UK
Envision Peroxidase	IHC	Dako Ltd., Ely, U.K
Ethidium bromide	RT-PCR	Invitrogen, Paisley, UK
Fcα/μR (clone 10C4 anti-EC2) (mouse)	IHC	Chinese Academy of Medical Sciences, Beijing, China (Wei Zhang, Ying Fu)
Fcα/μR (clone 10E8 anti-EC3) (mouse)	WB	Chinese Academy of Medical Sciences, Beijing, China (Wei Zhang, Ying Fu)
FITC-labelled goat anti-mouse	IHC	Sigma-Aldrich, Irvine, U.K
Fluorescein (DTAF)-conjugated streptavidin	IHC	Jackson Immuno Research Laboratories, West Grove, PA, USA
Fluvastatin	Real Time PCR, Cytokine Production, FN, Proliferation	Novartis, Basel, Switzerland
FN ELISA kit	FN Production	Biomedical Technologies Inc, Stoughton, USA
Haematoxylin	IHC	Cellpath, UK
Human GM-CSF ELISA kit	Cytokine Production	BD OptEIA™Biosciences, Oxford, U.K
Human IFN-γ ELISA kit	Cytokine Production	BD OptEIA™Biosciences, Oxford, U.K
Human IgA	WB, FN, Cytokine production, Proliferation, IHC	Jackson Immuno Research Laboratories, West Grove, PA, USA
Human IgG	WB, FN, Cytokine production, Proliferation	Jackson Immuno Research Laboratories, West Grove, PA, USA
Human IgM	WB, FN, Cytokine production, Proliferation	Jackson Immuno Research Laboratories, West Grove, PA, USA
Human IL-6 ELISA kit	Cytokine Production	BD OptEIA™Biosciences, Oxford, U.K
Human IL-8 ELISA kit	Cytokine Production	BD OptEIA™Biosciences, Oxford, U.K
Human TNF-α ELISA kit	Cytokine Production	BD OptEIA™Biosciences, Oxford, U.K
IFN-γ	RT-PCR, Real Time PCR	Serotec, Oxford, UK
IL-1α	RT-PCR, Real Time PCR	Sigma-Aldrich, Irvine, U.K
iQ SYBER green supermix	Real Time PCR	Bio-RAD Laboratories, Hertfordshire, U.K
IscripTM cDNA synthesis kit	Real Time PCR	Bio-RAD Laboratories, Hertfordshire, U.K
LPS (from *Escherichia coli* 055:B5)	Real Time PCR, Cytokine Production, FN, Proliferation	Sigma-Aldrich, Irvine, U.K

Nitrocellulose membrane	WB	Schleicher & Schuell BioScience , Dassel, Germany
NuPAGE™ Pre-Cast Gel System	WB	Invitrogen, Paisley, UK
Oligonucleotide primers	Real Time PCR	TAGN Ltd., Newcastle, UK
Oligonucleotide primers	RT-PCR	MWG-Biotech, Ebersberg, Germany
ProteoPlex™ 16-well human cytokine array kit	Cytokine Production	Merck Biosciences Ltd, Nottingham, U.K
Rapamycin	Real Time PCR, Cytokine Production, FN, Proliferation	Wyeth Pharmaceuticals, U.S.A
Rnase free water	RT PCR, Real Time PCR	Invitrogen, Paisley, UK
RNeasy® Fibrous Tissue kit	Real Time PCR	QIAGEN Ltd., Crawley, U.K
SDS-PAGE	WB	Invitrogen, Paisley, UK
Simvastatin	Real Time PCR, Cytokine Production, FN, Proliferation	Merck, USA
Tacrolimus	Real Time PCR, Cytokine Production, FN, Proliferation	Fujisawa, Munich, Germany
Taq DNA polymerase	RT-PCR	Promega, Madison, WI, USA
TGF-β	RT-PCR, Real Time PCR, FN	Serotec, Oxford, UK
TMB substrate system for ELISA	Cytokine Production	Sigma-Aldrich, Irvine, U.K
Trypan blue	Proliferation	Invitrogen, Paisley, UK
Vectorshield mounting medium	IHC	Vector Labs, Peterborough, U.K
X-ray film	WB	Kodak, UK

Table 2-1 Materials, Antibodies, Cytokines, Immunomodulatory

WB; Western blotting, IHC; Immunohistochemistry, RT-PCR; reverse transcriptase-PCR, FN; Fibronectin, ELISA; Enzyme linked immunosorbent assay.

Target	Name	Sequence (5' → 3')	Product size
β actin	β actin-for	GGG GTA TGC CCT CCC CCA TGC CAT CCT GCG	482bp
	β actin-rev	TTG GCG TAC AGG TCT TTG CGG ATG TCC ACG	
FcγRI	I-for	CAT GTG GTT CTT GAC AAC TCT GCT CC	876bp
	I-rev	TGA AAC CAG ACA GGA GTT GGT AAC TGG	
FcγRIIa	II-for	GCA CAG GAA ACA TAG GCT ACA CG	717 + 594bp
	IIa-rev	GGT ATC TTC TTA GAA AGT CCC	
FcγRIIb	II-for	GCA CAG GAA ACA TAG GCT ACA CG	287bp
	IIb-rev	GGT GAT TGT GTT CTC AGC CCC	
FcγRIII	III-for	ATG TGG CAG CTG CTC CTC CCA ACT G	567bp
	III-rev	GGT GAT GTT CAC AGT CTC TGA AGA CAC	
FcαR	CD89-F2	CGC TTA AGA TGG ACC CCA AAC AGA CCA C	880bp
	CD89-R2	GCT CTA GAT TAC TTG CAG ACA CTT GGT G	
Fcα/μR	amr-for1	GAC AAC TAC CAA GGC TGA TAG G	702bp
	amr-rev	TCT GTC CCT CAG GGT CCT GGA T	
pIgR	PIGR-for	GCC CGA GCT GGT TTA TGA AG	694bp
	PIGR-rev	AGC CGT GAC ATT CCC TGG TA	
FcRn	FcRn-for	CAA AGC TTT GGG GGG AAA AG	359bp
	FcRn-rev	TGC AGG TAA GCA CGG AAA AG	
Gamma-chain	GAM3-for	GCC CAA GAT GAT TCC AGC AG	458bp
	GAM3-rev	CCG TAA ACA GCA TCT GAG C	

Table 2-2 Primer (forward and reverse) sequences used in RT-PCR

Primer	Annealing Temperature °C
β actin	60
FcγRI	57
FcγRIIa	49
FcγRIIb	60
FcγRIII	60
FcαR	60
Fcα/μR	60
pIgR	60
FcRn	59
γ-chain	60

Table 2-3 Primers annealing temperatures

Target	Name	Sequence (5' → 3')	Product size
β actin	β actin-for	GAC AGG ATG CAG AAG GAG ATT ACT	100bp
	β actin-rev	TGA TCC ACA TCT GCT GGA AGG T	
FcγRI	I-for	AAT GGC ACC TAC CAT TGC TC	100bp
	I-rev	TGT CAC AGA TGC ATT CAG CA	
FcγRIIa	II-for	GGG CAC CTA CTG ACG ATG AT	100bp
	IIa-rev	TTG TCA TCC ACT CAG CAA GC	
FcγRIIb	II-for	TCC ATT CAG TGG TTC CAC AA	100bp
	IIb-rev	GTG TAC TCC CCG CTG TCA TT	
FcγRIII	III-for	GAC AAA CCT CTC CAC CCT CA	100bp
	III-rev	CTT CCA GCT GTG ACA CCT CA	
FcαR	CD89-F	TCA CAG ACT CCA TCC ACC AA	136bp
	CD89-R	GAC GCT TCC TTG TTC AGT GC	
Fcα/μR	amr-for	TGC TAC CTC TGC GGC ATT G	100bp
	amr-rev	AGA CGC TGT TCC ATA GGA TCT CA	
pIgR	PIGR-for	CTG GTA CTG GTG TGG AGT GA	120bp
	PIGR-rev	GCA CCT TCT CAT CAG GAG CA	
FcRn	FcRn-for	GTC AAA AGT GGC GAT GAG CA	100bp
	FcRn-rev	TAG CAA GAC ACC GAT GAC GA	

Table 2-4 Primer (forward and reverse) sequences used in Real Time PCR

Chapter 3: Ig Receptors Expression and Function in Proximal Tubular Cells

3.1 Introduction

Expression of Ig receptors on PTEC may play a role in the pathogenesis of human GN. However, little is known about which receptors are expressed, the regulation of Ig receptors, the relationship of Ig binding and the consequences of the receptor-Ig binding on PTEC functions.

3.2 Study objectives

In this study I have set out to answer the following questions:

1. Are Ig receptors FcγRI, FcγRIIA, FcγRIIB, FcγRIII, γ-chain, FcαR, FcRn, pIgR and the novel Fcα/μR expressed by immortalised HK-2 cell lines and by primary PTEC?

2. Is the expression of Ig receptors regulated by proinflammatory cytokines?

3. Does the presence of Ig receptors correlate to Ig binding in human PTEC and how is this related to Ig receptor expression?

4. Does the novel Fcα/μR antibody specifically bind and localise in biopsy samples in kidney and other tissues that express Fcα/μR?

5. What is the effect of Ig/IC on FN production, proliferation, signalling and cytokine release by PTEC, thus leading to tubular interstitial fibrosis?

6. Do Igs affect the morphology of human PTEC?

3.3 Results

3.3.1 Gene expression and regulation of Ig receptors by IL-1α, TGF-β, and IFN-γ using qualitative RT-PCR

3.3.1.1 Are FcγR or FcαR genes expressed by human PTEC?

I investigated whether the mRNA of Ig receptors was expressed by PTEC and whether the modulation of these receptors by proinflammatory cytokines involved gene expression for classical FcRs using both immortalised HK-2 cells and multiple primary human cells from nephrectomy specimens, as described in Section 2.2.1.1. In this study, unstimulated HK-2 cell lines and cells stimulated with 20ng/ml and 200ng/ml IFN-γ, 5ng/ml and 50ng/ml TGF-β1 for 24 hrs were studied using RT-PCR. I assessed the mRNA for the classical IgG receptors FcγRI, RγIIa, RγIIb, RγIII and γ-chain, the related FcRγ chain, or the classical IgA receptor (FcαRI) as described in Section 2.3.1.3. The primers sequences are shown in Table 2-2. The U937 cell line, known to express FcγRI, RγIIA, RγIIB and γ-chain, was used as the positive control and NK-92 served as positive control for FcγRIII.

U937 cells were treated with 1mM dbcAMP for 48 hrs to induce expression of FcαRI. β-actin was used as the house-keeping gene in this study. Expressed mRNA for the classical IgG receptors FcγRI, RγIIA, RγIIB, RγIII, the related γ-chain, and the classical IgA receptor (FcαRI) could not be detected in immortalised HK-2 cells (Fig. 3-1), nor could FcγRIII shown in (Fig. 3-2).

3.3.1.2 Are FcRn, pIgR and Fcα/μR expressed by human PTEC?

The expression of FcRn, pIgR and Fcα/μR by PTEC was also studied by RT-PCR. Unstimulated HK-2 cell lines and stimulated cells with 50 ng/ml IL1α, 20 ng/ml, 200 ng/ml IFN-γ, and 5 ng/ml, 50 ng/ml TGF-β were studied. Stimulated cells were incubated for 24 hrs.

The FcRn mRNA showed no significant difference in expression in cells treated with either IFN-γ or TGF-β1 compared with the U937 positive control. The band shown in Fig. 3-3A is of expected size, as shown in Table 2-2.

Cells treated with 20 ng/ml and 200 ng/ml IFN-γ for 24 hrs showed qualitative up-regulation in the expression of pIgR mRNA, while the expression was down regulated by 5 ng/ml and 50 ng/ml TGF-β1 respectively. The band was at the expected size of ~694 bp (Fig. 3-3B).

I investigated whether the Fcα/μR could be expressed in PTEC. Untreated cells expressing mRNA for Fcα/μR were highly up-regulated by 50 ng/ml IL-1α and moderately up-regulated by IFN-γ. However, the expression was totally inhibited by TGF-β as shown in Fig. 3-4. The bands were at the expected size of 702 bp. This was comparable to the results obtained by McDonald *et al*., 2002 in which IL-1α increased the expression of Fcα/μR mRNA in human mesangial cells. The primers used in this study were described in Table 2-2. The expressions of all Ig receptors in primary PTEC are shown in Fig. 3-5.

3.3.2 Expression of FcRn, pIgR and Fcα/μR using qRT-PCR

The identification of Ig receptors differentially expressed in immortalised and primary PTEC was quantitatively verified by RT-PCR. The relative quantities of the Ig receptor gene transcripts in PTEC cells were measured before and after proinflammatory cytokines stimulation for 24 hrs by semi-qRT-PCR. Total RNA was extracted as described in section 2.3.2.1 and was processed directly to cDNA as described in section 2.3.2.2. The primers for Ig receptors and the house-keeping gene (β-actin) were designed (Primer 3 software) and synthesised by TAGN Ltd. are shown in Table 2-4. The primers were designed to cross different exons to completely abolish amplification from genomic contaminations. The house-keeping gene β-actin is widely used as a standard because this protein is essential for maintenance of cell function and is generally constitutively expressed at a similar level in all cell types and tissues. cDNA was amplified using qRT-PCR with SYBER green as described in section 2.3.2.3.

The relative expression of FcRn showed no significant or unaffected fold differences relative to β-actin in unstimulated cells or cells up-regulated with proinflammatory cytokines at the concentration previously used in RT-PCR in both HK-2 cells and primary PTEC. cDNA for NK92 cells was used as positive control and the fold differences in expression is shown in HK-2, as shown in Figure 3-6, and primary PTEC, as shown in Figure 3-7.

Amplification of pIgR showed up-regulation with the various concentration of IFN-γ while no regulation or expression with either concentration of TGF-β1. The fold differences of pIgR expression are greater in primary PTEC than seen in HK-2 cells. The cDNA for U937 cells was used as negative control as shown in Fig. 3-6, 3-7.

The expression of Fcα/μR in both cell lines and primary PTEC increased by 50 ng/ml IL1α compared with the unstimulated cells. It was moderately up-regulated with either concentration of IFN-γ, while only slightly regulated or reduced in response to TGF-β1. The fold differences in expression are showed in Fig. 3-6, Fig. 3-7 relative to β-actin. Each reaction was performed in triplicate and the mean of five Ct was determined. To determine sensitivity of the expression, the amplification of known mRNA concentration in serial dilutions was measured. Thus, at different concentrations of proinflammatory cytokines, the Ig receptor genes present at low expression levels could be reliably quantified. PCR efficiency was assessed for all primer pairs by generating mRNA titration curves as shown in Fig. 3-8A, shows Ct values of these amplifications plotted against the logarithm of the relative initial amount of cDNA. The slope of the straight line obtained provided a measure of PCR efficiency. Efficiency was 100%, ensuring the doubling of the amplified target gene at each cycle. Figures 3-8B and C show the Ct values and melting curves for Fcα/μR and β-actin, respectively.

These results showed significant effects in expression of mRNA for FcRn, pIgR and Fcα/μR after stimulation with proinflammatory cytokines in HK-2 cells and primary PTEC induced an expression at biologically significant levels.

Fig. 3-9 shows that there were no fold differences in expression of FcγRI, FcγRIIB and FcγRIII relative to the control U937 cells in HK-2 cells and primary PTEC.

3.3.3 Does the protein of Fcα/μR express in human PTEC?

The cloned Fcα/μR cDNA was used to identify the protein expression on HK-2 cells and lysates were made after treating the cells with 5 and 50 ng/ml TGF-β1 and 50 ng/ml IL-1α (see section 2.4.2). Immunoblotting was carried out for 48 hrs as described in section 2.4.4. In immunoblots Fig. 3-10A the expression of the Fcα/μR is increased by IL-1α, and induction inhibited by co-incubation with TGF-β1. These findings were also confirmed using densitometry (Fig. 3-10B). However, TGF-β1 alone did not induce any expression. CHO cell lysate transfected with Fcα/μR were used as positive control. The molecular

weight of the Fcα/μR protein was approximately 70 kDa. The expression of protein for any of the classical IgG or IgA receptors was not detected in this study.

3.3.4 Are Fcα/μR receptors expressed in human PTEC?

I tested the expression of the Fcα/μR in PTEC by using murine antibodies against the Fcα/μR, which were previously used for immunoblotting. Immunofluorescence laser scanning confocal microscopy was used as described in section 2.4.8 to find out whether there was evidence of expression of the Fcα/μR on PTEC. Fig. 3-11 identifies the Fcα/μR by specific antibody. An isotype control (anti-mouse IgG) was used to visualise any non-specific binding (Fig. 3-11A). Fig. 3-11B shows the specific binding of Fcα/μR antibody to Fcα/μR on HK-2 cells. Fig. 3-11C shows decreased binding of antibody to the receptor due to TGF-β1 treatment, which decreased the receptor expression (Fig. 3-4). Fig. 3-11D shows an increase in the binding of antibody to the Fcα/μR due to increased expression induced by the 50 ng/ml IL-1α treatment.

3.3.5 Is the Fcα/μR expressed in vivo in human kidney?

IHC was used to confirm the expression of Fcα/μR in normal kidney and other tissues. Fig. 3-12 shows the expression Fcα/μR in various tissues including normal kidney (Fig. 3-12A), kidney for patients with IgAN (Fig. 3-12B), small intestine (Fig. 3-12C) and tonsil (Fig. 3-12D). IHC was prepared by Ian Downie at the Royal Glasgow Infirmary as described in Section 2.4.9. IHC was performed using the same murine antibodies against Fcα/μR. Fig. 3-12A shows the brown-coloured, positively stained PTEC, distal tubular cells and glomerular podocytes in the normal kidney, in IgAN, and also the B-lymphocytes in the small intestine and tonsil. This pattern of staining confirms the expression of Fcα/μR in various tissues from human body, which indicates that Fcα/μR expression to human kidney and expression on the B-lymphocytes in other tissues.

3.3.6 Do IgG, IgA and IgM bind to human PTEC?

Confocal laser scanning microscopy was used to visualise the binding of IgG, IgA and IgM to HK-2 cells and primary PTEC. The effect of cytokine regulation on the pattern of binding was also studied. U937 cells (positive control) were incubated with biotinylated-IgG and fluorescent streptavidin as described in section 2.4.8 to show the viability of the technique (Fig. 3-13A). IgG, IgA and IgM bind to PTEC which is shown as green

fluorescence in (Fig. 3-13B–D) respectively. PTEC cells bind to IgG, possibly by FcRn, and the cells could bind to IgA and IgM, possibly by pIgR or Fcα/μR via the J-chain that is present in polymeric IgA and IgM. The Fcμ fragment of IgM with PTEC showed binding (green fluorescence which could be due to pIgR) is shown in Fig. 3-13E.

IFN-γ increased and TGF-β1 decreased the binding of IgM to the PTEC, and this was confirmed by gene expression (Fig. 3-13F, G). The same was observed for IgA binding (Fig. 3-13J, K). Fig. 3-13L shows the binding of IgA to Fcα/μR. This was acheived by incubating 50-fold excess unlabelled monomeric IgA to saturate the Fcα/μR on the cells with 1-fold of labelled IgA. The ability of unlabelled monomeric IgA to displace the majority of IgM binding is consistent with a significant proportion of IgM binding being to the Fcα/μR. COS-7 cells were used as negative controls and were incubated with biotinylated-IgA and biotinylated-IgM followed with fluorescent streptavidin. The results showed no binding of IgA or IgM to COS-7 cells (Fig. 3-13H, I).

3.3.7 Does IgM bind to Fcα/μR?

In Fig. 3-14A, it is shown that IgM binds to Fcα/μR. However, to determine the specificity of the binding, 50-fold unlabelled IgA was added with 1-fold labelled IgM, which was overlayed on to the HK-2 cells. IgA displaced the binding of IgM to Fcα/μR, indicating that IgM binds to Fcα/μR (Fig. 3-14B). IFN-γ and TGF-β1 treatment did not affect the binding of IgM to Fcα/μR as the 50-fold unlabelled IgA displaced IgM (Fig. 3-14C, D).

3.3.8 Do the Igs have any affect on human PTEC to produce FN?

FN production was assessed in the cells as described in section 2.5.1 using a specific ELISA. FN production by HK-2 cells was measured under various treatments with Ig/IC for 72 hrs at 37°C. TGF-β1 was used as a positive control to stimulate FN production. Fig. 3-15 shows that TGF-β1 (50 ng/ml) increased the FN by ~1.8-fold. Native IgG and IgA had no effect. Cross-linked (XL)-IgG increased FN production by ~1.9-fold compared with untreated cells. In contrast, XL-IgA showed a 2-fold increase in FN production. HAg-IgG showed a 1.1-fold increase in FN production, whereas HAg-IgA showed a 1.4-fold increase in FN production. Native IgM was increased in FN by 1.5fold compared with the control.

3.3.9 Do Igs affect the proliferation of human PTEC?

Proliferation studies were undertaken in order to assess the significance of FN production. A decrease in proliferation of PTEC is indicative of an increase in FN production. Fig. 3-16 shows the proliferation of HK-2 cells by using a [^{3}H]-thymidine uptake assay, as described in section 2.4.10, for 24 hrs after various Ig/IC treatments at 37°C. It was compared with untreated and TGF-β1 treated cells (positive control, 50 ng/ml) which has an antiproliferative effect. Native IgG showed no effect on proliferation whereas native IgA decreased the proliferation by 80%. XL-IgG, XL-IgA and native IgM have the same antiproliferative effect as TGF-β1. HAg-IgG and HAg-IgA showed similar levels of antiproliferative effect when compared with TGF-β1. None of the XL-Ig antibodies alone had any independent antiproliferative effect. The differences in antiproliferative effect of various treatments were statistically significant.

3.3.10 Does the binding of IgG, IgA or IgM affect the signalling of PTEC?

HK-2 cells were incubated with Ig with or without (±) XL over a time course (0, 2, 5, 10, 20, 30 mins) (Fig. 3-17) and lysates were prepared as described in Section 2.4.2. Immunoblotting was performed after determining protein concentration, as described in sections 2.4.3 and 2.4.4, and the samples were normalised for equal protein concentration (10–20 μg). Normalisation involved incubation at 4°C for the primary antibody to prevent internalisation of the Ig receptors, and at 37°C to allow the XL to work. Many proteins were phosphorylated when blotted by anti-phosphotyrosine antibody. These blots did not show any change in protein phosphorylation during the time course treated with IgG ± XL and IgA ± XL. The same pattern was shown for cells when blotted with ERK. This indicates that no specific ERK signalling pathways were involved in the binding of IgG and IgA to PTEC in any of the conditions investigated. However, IgM activated phosphotyrosine and ERK signalling during the time course at 2 and 5 minutes.

3.3.11 Do PTEC release cytokines when stimulated with Ig?

The Proteoplex™ 16-well human cytokine array system, as described in section 2.5.2.2 was used to screen the ability of PTEC to release each of the following twelve cytokines: IL-1α, IL-1β, IL-2 IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, GM-CSF, IFN-γ and TNF-α. I investigated the effect of Ig ± XL on cytokine release by PTEC compared to unstimulated

cells. TGF-β1 (50 ng/ml) was used as positive control and the supernatant media was assayed for each of the twelve cytokines. After 24 hrs stimulation, PTEC released the cytokines IL-6, IL-7, IL-8, GM-CSF and TNF-α, but in general the results were very inconsistent (Fig. 3-18A).

To compensate for this inconsistency, I used a specific ELISA as described in Section 2.5.2.1 for IL-6, IL-8, GM-CSF, and TNF-α individually, as these were the cytokines whose levels were shown to be altered in the Proteoplex™ studies. In Fig. 3-18B, TGF-β1 increased IL-6, IL-8 and GM-CSF secretion. However, the stimulation with IgG alone, HAg-IgG, IgA alone, XL-IgA, HAg-IgA and IgM alone did not show any statistical significant changes in cytokine release. XL-IgG elevated cytokine release.

TNF-α was released in very low levels (data not shown). I also tested IFN-γ release under the same conditions with results showing no release in unstimulated cells in all cases except XL-IgG treatment where it was elevated (data not shown).

3.3.12 Do Igs affect the morphology of human PTEC?

A confluent monolayer of PTEC was taken and stimulated by various treatments for 72 hrs at 37°C with TGF-β1 (50 ng/ml) and Ig ± XL to study the effect of stimulation on the morphology of PTEC. The H&E method (as described in section 2.4.10.2) showed that neither TGF-β1 nor Ig ± XL affected the morphology of the PTEC (Fig. 3-19). Surprisingly, TGF-β1 resulted in a slight elongation, though this was nonsignificant. These results confirm those shown in Fig. 3-16 that TGF-β1 up-regulated the FN production by 80%. The lack of any significant change in morphology was confirmed by pathology expert Dr Barbara Young.

3.4 Discussion:

Patients with GN commonly have deposits of Ig/IC in the glomeruli and tubules, but the means by which Ig/IC may mediate tubulointerstitial injury has not yet been investigated fully (Chan *et al.*, 2005; Takai, 2002). Although albuminuria has been more extensively examined, the role of high molecular weight proteins (e.g. transferrin) in renal damage has been clearly established (Birn&Christensen, 2006). Proteinuria is associated with more rapid disease progression in patients with renal disease (Nath, 1992). I hypothesised that renal tubular cells might be exposed to, and in turn be activated by, these IC, and thus may contribute to tubular damage, fibrosis and enhance CRF. However, the mechanism by which Ig/IC contribute directly to tubular injury is unknown.

Cell membrane receptors specific for the Fc portion of Ig that play an important role in immunity interact with the IC and mediate several immune processes including antigen presentation, ADCC and cytokine/chemokine production (Hulett&Hogarth, 1994). These receptors are potentially involved in IC-mediated activation of PTECs. Using qualitative RT-PCR and qRT-PCR in primary PTEC and immortalised HK-2 cell lines, this study has shown that the expression of Ig receptors is regulated by proinflammatory cytokines like IL-1α, IFN-γ and TGF-β1. Moreover, Ig binds to the receptors, leading to changes in cell functions.

In my thesis, I focused mainly on the novel IgA receptor Fcα/μR. I was unable to identify mRNA expression in primary PTEC and HK-2 cells, using either PCR technique, for any of the classical IgG receptors FcγRI, FcγRIIA, FcγRIIB and FcγRIII, or the IgA receptor FcαR. The ability of FcαR to bind both human IgA1 and IgA2 subclasses has been reported in other studies (Monteiro&Van De Winkel, 2003). This receptor can be expressed on neutrophils and monocyte/macrophages due to increased glycosylation (Westerhuis *et al.*, 1999). U937 cells have been used as a positive control in this study because Cameron *et al.*, (2001) showed that treatment with dbcAMP enhanced the expression of FcαR after 48 hrs.

The novel Fcα/μR present is a type 1 transmembrane protein that, unlike FcαR, has a single Ig-like domain that is expressed on the majority of murine B-lymphocytes and macrophages (Shibuya *et al.*, 2000). Shibuya et al., (2000) and Mantis *et al.*, (2002) reported that human and murine mature B cells express an Fcα/μR that can mediate

endocytosis of both IgA- and IgM-containing ICs. In this project, I showed that human primary PTEC and HK-2 cells express mRNA for the novel IgA and IgM receptor Fcα/μR, a candidate receptor for the pathogenesis of IgAN. The mRNA for Fcα/μR expression is increased by IL-1α, and to a lesser extent with IFN-γ, and is reduced by TGF-β1. These findings are in agreement with those of Yano *et al.*, (1997) and Yoshioka *et al.*, (1993), who showed that the expression of these pro-inflammatory mediators may have a role in the pathogenesis of IgAN. Fcα/μR upregulation by IL-1α is of significance as increased internal expression of this inflammatory cytokine has been described in IgAN (McDonald *et al.*, 2002). The regulation of the expression of Fcα/μR by these cytokines corroborates the studies in MSC by McDonald, Cameron *et al.,* (2002) where they showed that Fcα/μR is one of the major IgA receptor expressed in MSC. Nakamura *et al.*, (1993) showed that the FcμR is expressed in non-haematopoietic cells (e.g. PTEC and central nervous system tissue), suggesting an important role for this receptor in the immune response of these tissues.

It has been shown that various cells such as monocytes, macrophages and T cells infiltrate into the kidney during inflammation (Gerritsma *et al.*, 1996a; Kooijmans-Coutinho *et al.*, 1995; Passwell *et al.*, 1988). Infiltrated immune cells secrete IL1-α, which might induce synthesis of complement components by tubular cells. IL-1α produced by infiltrating monocytes or by local intraglomerular cells could result in the upregulation of the PTEC Fcα/μR expression and promote IgA deposition. Apart from immune cells, MSC are also a source of IL1-α (Lovett *et al.*, 1986; Sedor *et al.*, 1992; Werber *et al.*, 1987; Zoja *et al.*, 1993), which may also indirectly lead to the production of complement proteins by tubular cells. In IgAN, the correlation between the influence of IL1-α on the expression of Fcα/μR in the kidney and the degree of tubulointerstital damage is unknown. Rincheval-Arnold *et al.*, (2002) showed that the IFN-γ receptor is expressed in a wide range of cell types, suggesting that IFN-γ has an array of functions. It has been hypothesised that IFN-γ produced by T cells may act directly via the IFN-γ receptor present on the PTEC to enhance not only MHC expression but also that of complement component and other mediators such as chemokines (Gerritsma *et al.*, 1997).

Although IL1-α and IFN-γ enhanced the expression of Fcα/μR, TGF-β1 showed opposite results, suggesting antagonistic functions of this cytokine. Indeed, TGF-β1 has been hypothesised to possess an inhibitory effect on immune system activation (Tian&Phillips, 2003). As it is a novel receptor, there is very little information available regarding the signal transduction mechanism controlling Fcα/μR following the stimulation of pro-

inflammatory cytokines. However, it is known that IL1-α-mediated upregulation of Fcα/μR expression is dependent on the activation of the promoter region, which contains all the essential transcription regulatory components.

According to Chan *et al.*, (2005), IgAN is the most common form of GN, and is one of the leading causes of renal failure. This disease is characterised by the mesangial deposition of pathogenic pIgA1. In the kidney, receptor-mediated transport of Igs has been studied in the pIgR, which transports pIgA and pentameric IgM from the basolateral to the apical surface. The pIgR neutralises extracellular and intracellular pathogens in mucous membranes by epithelial transport of pIgA pathogen complex, which is then excreted via epithelial transcytosis (Leung *et al.*, 2000). pIgR plays an important role in the mucosal immunity of the urinary tract (Rojas&Apodaca, 2002). Cultured primary PTEC and HK-2 cells express mRNA for the pIgR and this expression is increased by IFN-γ. Phillips-Quagliata *et al.*, (2000) reported pIgR in epithelial cells that bind IgM with higher affinity than IgA. The J-chain is essential for the assembly of pIgR and epithelial transport, but is not known to play a role in inflammation. Vaerman *et al.*, (1998b) established the presence of the J-chain in human pIgA, in agreement with the notion that the J-chain is required in pIg for binding to SC/pIgR. The J-chain is also present in human pIgM (Wines *et al.*, 1999). Hempen *et al.*, (2002) developed a model of transcriptional regulation of the human pIgR gene that involves cooperative binding among multiple transcription factors.

Numerous studies have highlighted the preponderant effect of IFN-γ on pIgR gene expression in human MSC. Rincheval-Arnold *et al.*, (2002) showed that IFN-γ strongly increased pIgR mRNA levels through a direct effect on PTEC. Piskurich *et al.*, (1997) showed that the pIgR promoter can be modulated by IFN-γ in MSC. Since both pIgR and Fcα/μR are capable of binding IgM, it can be speculated that IFN-γ might utilise similar mechanisms in regulating the expression of Fcα/μR.

A recent study argued that neither FcαμR nor pIgR are expressed in PTEC (Chan *et al.*, 2005). In this study, the authors investigated the binding of IgA receptors by primary PTEC and MSC from patients with IgAN to cultured PTEC, compared with the binding of IgA from healthy controls. My results are in contrast with these data with regard to the lack of expression of the Fcα/μR. This could be because of the experimental conditions or culture condition altering the cell phenotype. I hypothesised that the expression of Fcα/μR would be lost as the number of cell passages increased, and that varying culture conditions may lead to loss of the receptor from the cell surface.

Raghavan & Bjorkman, (1996) reported that FcRn binds IgG in the lumen and then transports the IC across the intestinal barrier into the lamina propria for processing by dendritic cells and presentation to CD4+ T cells. MHC class I-related FcRn plays a central role in delivering IgG within and across the cells. Kobayashi *et al.*, (2002) showed that a specific IgG receptor, FcRn, mediates IgG transcytosis and is localised to the proximal tubular brush border. Any injury to proximal tubules resulting in loss of PTEC will lead to ablation of IgG reabsorption because of insufficient endocytosis. FcRn is associated with β2-microglobulin and binds IgG in a pH-dependent manner with higher affinity binding at an acidic pH (6.0) and low or no affinity at neutral pH (8.0). I demonstrated that primary cultured PTEC and immortalised HK-2 cells expressed mRNA for FcRn, but there was no synergistic effect by various proinflammatory cytokines in the upregulation of FcRn mRNA. In this thesis, I confirmed the expression of Ig receptors on PTEC using sensitive qRT-PCR analysis. Fold differences in the expression of these receptors between unstimulated and stimulated cells indicated that the expression of FcRn, pIgR and Fcα/μR mRNA is biologically significant relative to β-actin mRNA.

After checking the expression of mRNA for the Fcα/μR in PTEC, I checked the expression of proteins of the same receptors using a mAB specific for Fcα/μR. This mAB has only recently become available and so previous research in this area has been limited. Protein expression of the Fcα/μR was regulated in the same way as its mRNA by the proinflammatory cytokine IL-1α. In PTEC, the product of the tagged Fcα/μR gene was approximately 70 kDa. In unstimulated HK-2 cells, there was no visible expression of Fcα/μR protein. This could be for a number of reasons. Cherayil *et al.*, (1993) suggested that, after synthesis, the receptor protein in the endoplasmic reticulum lacked the signal peptide for its transport to the membrane. Another possible explanation is that PTEC may typically express the mRNA of Fcα/μR and not transcribe its protein. If this were the case, patients with IgAN might be able to express Fcα/μR either by abnormally glycosylated IgA-containing ICs or by inflammatory mediators. The expression of this functional receptor may be determined by genetic or other factors, and these may enable us to predict the subset of patients who are susceptible to disease and who subsequently develop progressive renal failure.

IL-1α-treated cells showed increased expression of the Fcα/μR protein, as detected by immunoblotting. This expression was markedly reduced by TGF-β1 in combination with IL-1α. These differences were confirmed by densitometric analysis of Western blot bands. CHO cells transfected with Fcα/μR were used as a positive control, as reported by

McDonald et al. (2002), who showed that Fcα/μR transfected into CHO cells can bind IgA and IgM but not IgG. Recently, Cho *et al.*, (2006) successfully developed an anti-FcαμR mAB, which should prove useful for the molecular and functional characterisation of IgA and IgM interactions with Fcα/μR, an area that needs further investigation.

In several experiments, I attempted to detect Fcα/μR in PTEC using FACS analysis with no positive results. However, using an immunofluorescence technique applying anti-Fcα/μR directly to the cells, I was able to show that there is binding of Fcα/μR in PTEC. The binding increased in cells stimulated with IL-1α and was reduced in cells stimulated with TGF-β1. From this result, I proposed that the surface protein was detected for Fcα/μR suggesting that Fcα/μR transcript is essential for the translation or membrane transportation of Fcα/μR.

I have shown that Fcα/μR is expressed on the normal kidney and in some patients with IgAN. This was demonstrated by using IHC in normal kidney, IgAN and B lymphocytes in tonsil and small intestine sections incubated with mAB against Fcα/μR. Positive staining in both proximal and distal tubular cells, and also the podocyte, (shown in brown colour in Fig. 3-12) confirmed the expression of Fcα/μR in the human kidney. This finding is important and implicates Fcα/μR as a candidate for mediating IC deposition in IgAN and suggests that it is regulated by cytokines implicated in progressive renal disease and may lead to ESRF.

I identified the binding specificity of IgA, IgM and IgG in PTEC incubated with fluorescein-labelled IgA, IgM or IgG antibodies, as well as unlabelled monomeric IgA. The binding pattern of Ig to PTEC was then examined using confocal laser microscopy. The IHC results showed that both IgA and IgM bind to PTEC and that binding to IgA might occur through the Fcα/μR as binding was seen with the monomeric IgA antibody, which does not contain the J-chain. The J-chain is required for binding to pIgR. Binding of PTEC to IgM may occur through either the Fcα/μR or pIgR and is stronger than that of IgA, possibly due to the presence of the J-chain. I also showed that PTEC were able to bind IgG, possibly through the FcRn.

The displacement of labelled IgA (1-fold) and IgM (1-fold) with monomeric unlabelled IgA (50-fold excess) showed relatively less binding of labelled IgA and IgM to Fcα/μR which suggests that monomeric IgA saturates Fcα/μR. Binding of IgA and IgM was increased by IFN-γ and reduced by TGF-β1 as shown in Fig. 3-13F/G for IgM and Fig. 3-

13J/K for IgA, which correlates with the gene expression data. Overall, these data show that the expression of Fcα/μR on the surface of PTEC is regulated by pro-inflammatory cytokines implicated in the pathogenesis of progressive renal disease. The mechanism by which this regulation occurs needs further investigation.

Despite the implication of Ig/IC in progressive renal disease, there is little evidence of a direct effect of these complexes on PTEC. In order to examine this further, I looked at the consequence of Ig/IC binding to PTEC in terms of FN production (as a marker of ECM), proliferation, signalling pathways and cytokines released. FN production has been shown in renal disease, to play an important role in the fibrogenesis described as a modulator of the synthesis of ECM proteins (Gomez-Guerrero *et al.*, 2005) leading to ESRF (Roopenian *et al.*, 2003), and is known to be involved in GN. TGF-β1 was used to confirm FN production in PTEC. The results showed that XL-IgG and XL-IgA increased FN production, while HAg-IgG, HAg-IgA and IgM did not significantly change the rate of FN production. IgG alone had no effect on FN production. From these results it can be hypothesised that Ig/IC do have an effect on production of FN in PTEC. Thus, Ig/IC may contribute to tubulointerstitial injury since the change in FN by PTEC can possibly alter their proliferative function.

I further studied the effect of Ig/IC on proliferation of PTEC. My result shows that XL-IgG, XL-IgA, HAg-IgG, HAg-IgA and IgM caused inhibition of proliferation relative to the FN result, whereas IgG and IgA alone had no effect on proliferation. This is in agreement with the finding of Chan (2005), who saw that IgA binding did not enhance the proliferation of PTEC. TGF-β1 was used as a positive control in this study as it has been shown to have antiproliferative effects on some cell types, as reported by Vesey *et al.* (2002). These authors also reported that IL-1β exerted potent TGF-β-dependent fibrogenic and antiproliferative actions on PTEC, which could possibly play an important role in the pathogenesis of tubulointerstitial fibrosis and tubular atrophy. Furthermore, TGF-β inhibits the proliferation of MSC (Basile *et al.*, 1998; Jaffer *et al.*, 1989; Kuncio *et al.*, 1996; Massague, 1990) and has been shown to increase the mRNA expression of FN and collagen IV in cultured PTEC. Thus, TGF-β influences redifferentiation of renal PTEC either directly or indirectly through the production of FN. However, the mechanism of this effect has not yet been identified, though it is unlikely to be wholly due to a single receptor. In other experiments, I found no significant changes in cell morphology with Ig/IC stimulation and fewer significant changes in the morphology with TGF-β1.

A broad range of signalling pathway mechanisms were examined. PTEC were stimulated with Ig/IC and the lysates Western blotted and probed for anti-phosphotyrosine and ERK. The results show phosphorylated proteins at 64 kDa and 97 kDa, all of which remained unaltered over a period of time after treatment with IgG and IgA, with or without XL. IgM activated the ERK and phosphotyrosine signalling pathways, though this may have occurred through pIgR rather than Fcα/μR. However, further investigation into the signalling pathway mechanisms of PTEC may be worthwhile. These cells can probably be stimulated with Ig/IC with cytokine treatment and the lysate used to perform proteomic studies such as diffuse immune gel electrophoresis and mass spectroscopy.

Some studies have shown that TGF-β binds to factors such as ET-1 and ET-2 and inhibits the activation of the p42/44 pathway (Yard *et al.*, 2001). TNF-α and IL-1β also activate ERK1/2 in PTEC, indicating that this pathway plays an important role in matrix metalloproteinase-9 upregulation. Moreover, both TNF-α and IL-1β were shown to increase the phosphorylation of p38 in PTEC (Nee *et al.*, 2004).

Ronda *et al.* (2005) reported that MAP kinases are an important mechanism of transduction of extracellular signals into cellular responses. They reported the activation of a MAP cascade in human PTEC stimulated for 1hr with normal IgG showing the early activation of ERK, which is the final product of translocating to the nucleus. They reported normal and pathological IgG but not transferrin or albumin induced an early significant increase in IL-6 release by PTEC, suggesting that IgG filtered during nonselective proteinuria may play a specific role in tubulointerstitial diseases. The signalling pathways of Fcα/μR have not been extensively studied to date and there is little data in the literature. I suggested that potential signalling mechanisms for Fcα/μR could exist.

Using a ProteoPlex™ assay I screened PTEC under non-stimulated conditions and after stimulation with Ig ± XL to see which cytokines are released. Three cytokines – IL-6, IL-8, and GM-CSF – were then individually examined using a specific ELISA technique. I used TGF-β1 (50 ng/ml) as positive control; this caused an increase in the release of IL-6, IL-8 and GM-CSF, as shown in Fig. 3-18. PTEC did not show any increase in the release of these cytokines after stimulation with IgA ± XL and IgM. Interestingly, XL-IgG in culture caused an increase in the release of the cytokines. However, this may be an artefact of the experimental conditions, for example the intensity of stimulation concentrations of Ig, XL incubation time or temperature. Moreover, even a very low level of contamination (e.g. LPS) in the antibody preparation could stimulate the cells to release the cytokines. This is

in agreement with (Brauner *et al.*, 2001; de Haij *et al.*, 2002; Gerritsma *et al.*, 1998b), who showed that PTEC releases cytokines when stimulated with LPS. In addition, PTEC are known to express HLA, ICAM-1 and VCAM-1, thus indicating their involvement in inflammatory processes.

Several studies have shown that human MSC are able to produce IL-8 *in vitro* in response to LPS, IL-1 and TNF-α (Brown *et al.*, 1991; Kusner *et al.*, 1991; Schmouder *et al.*, 1992). Therefore, the production of IL-8 may not only result in the recruitment of neutrophils but also in their activation, which leads to local inflammation. Other studies indicate that IL-8 staining in the tubule is associated with renal inflammation (Gerritsma *et al.*, 1996b; Schmouder *et al.*, 1992). Frank *et al.,* (1993) reported that IL-6 and GM-CSF are mitogenic for lymphocytes and macrophages and can be expressed in human PTEC. Other researchers have evaluated the production of IL-6 and GM-CSF by tubular epithelial cells. These studies reported that both proinflammatory mediators are able to induce the recruitment and activation of mononuclear cells. Daha & van Kooten, (2000) reported that mediators released by the inflammatory cells are directly responsible for the activation of PTEC, which in turn join in the inflammatory cascade by the local production of various inflammatory mediators.

In conclusion, these results suggest that Fcα/μR is likely to be expressed on cultured PTEC, that PTEC bind IgA and IgM through the Fcα/μR and that this binding may contribute to immune-mediated nephropathy. The expression of Fcα/μR by PTEC was increased by the proinflammatory cytokines IL-1α and IFN-γ, but reduced by TGF-β1. I demonstrated that IgA and IgM increase the production of FN by PTEC, and that this may contribute to tubulointerstitial fibrosis. IgM and IgA were also shown to modulate proliferation, suggesting that Ig binding to PTEC may contribute to the pathophysiology of IC-mediated renal disease. Further studies are required to investigate these and other effects of IC and Ig on PTEC. Although this thesis focused on the novel Fcα/μR expression on PTEC and its potential implication in IgAN, further investigations could be done by using specific antibodies against human pIgR and human FcRn to study their protein expression and regulation by proinflammatory cytokines. Binding of Ig/IC to PTEC and the activation of the Fcα/μR may contribute to the factors leading to ESRF and could therefore represent a future target for the treatment of progressive renal disease.
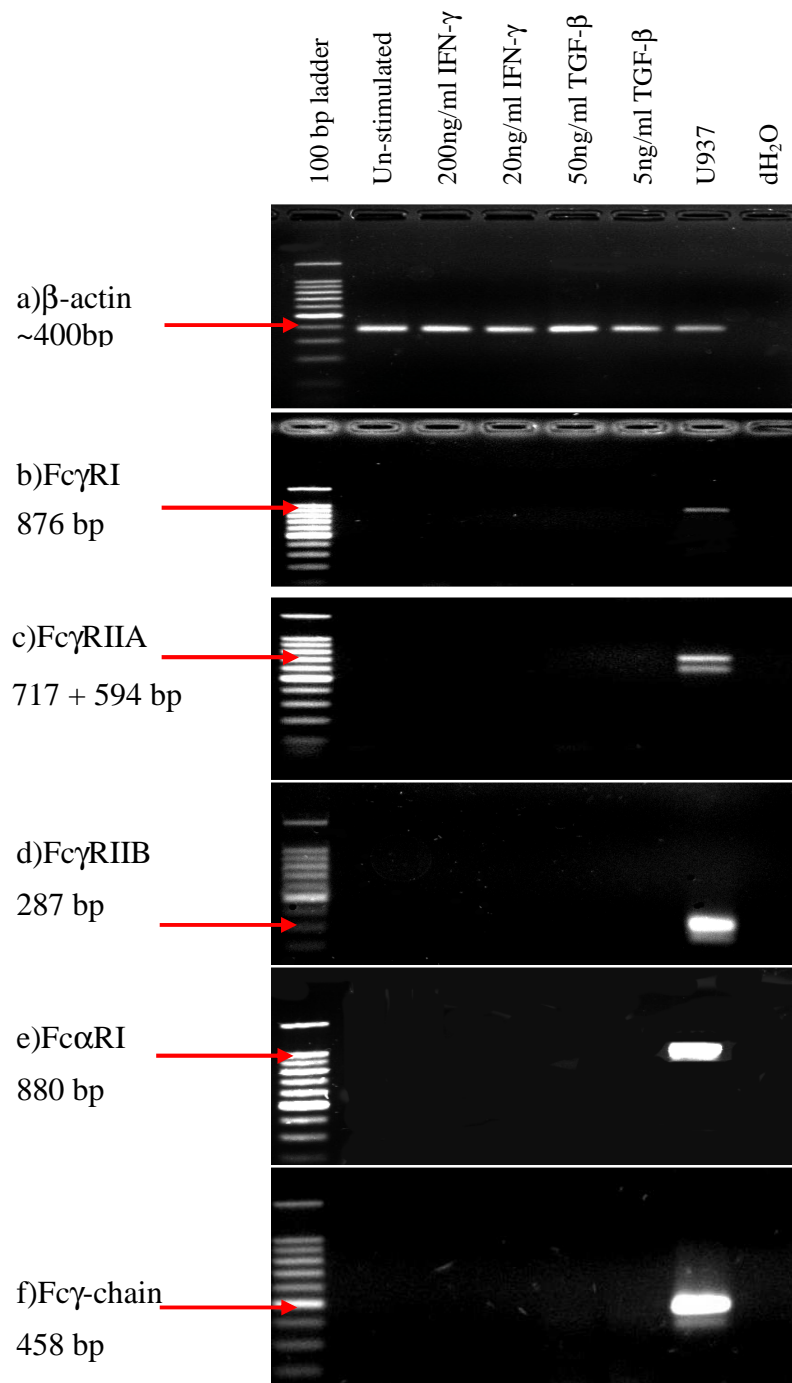
Figure 3-1 The expression of mRNA for FcγRI, γIIA, γIIB, γ-chain, FcαR and β-actin by RT-PCR in HK-2 and U937 cells

RT-PCR was performed with specific primers to confirm the expression of FcγRI, FcRγIIa, FcRγIIb, Fcγ-chain and FcαRI using cDNA extracted from unstimulated cells and from cells treated for 24 hrs with (20, 200 ng/ml) IFN-γ. In addition, cDNA from cells treated with (5, 50 ng/ml) TGF-β1 was used. A 100bp ladder was used as a marker (lane 1). U937 cells were used as positive control (lane 7) and dH2O was used as a template (negative control lane 8). The above results were confirmed after comparing to a positive control β-actin at expected size. The band size for each receptor is shown in Table 2-2, as well as in each figure.
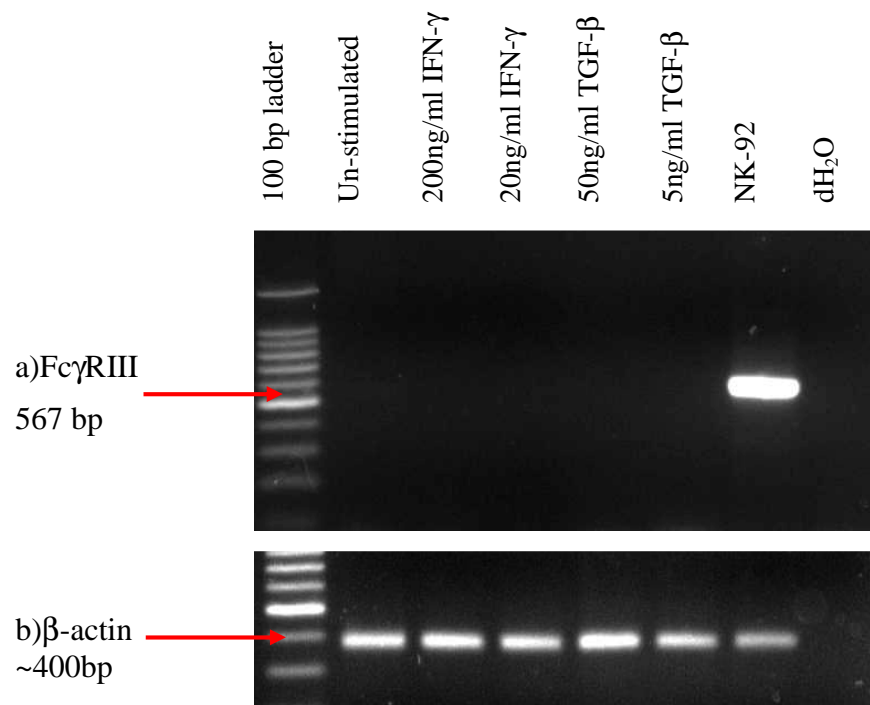
Figure 3-2 The expression of mRNA for FcRγIII and β-actin by RT-PCR in HK-2 cells

RT-PCR was performed with specific primer to confirm the expression of FcRγIII. RT-PCR was carried out with cDNA extracted from unstimulated cells and from cells treated for 24 hrs with (20, 200 ng/ml) IFN-γ. A 100bp ladder was used as a marker (lane 1). In addition, cDNA from cells treated with (5, 50 ng/ml) TGF-β1 was used. For FcRγIII, NK-92 cells were used as a positive control (lane 7) and dH2O was used as a template (negative control lane 8). The above results were confirmed after comparing to a positive control β-actin at expected size. The band size for this receptor is shown in Table 2-2, as well as in each figure.
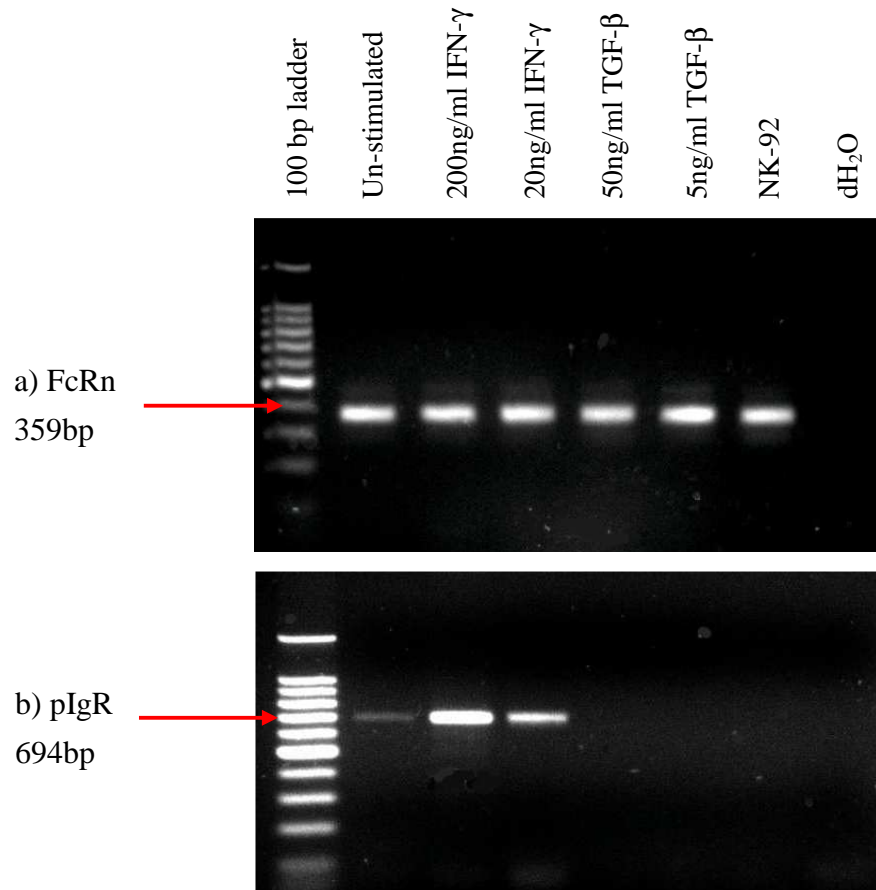
Figure 3-3 The expression of mRNA for FcRn and pIgR by RT-PCR in HK-2 cells

(A) RT-PCR was used to investigate the expression of FcRn in PTEC. Expression was unaltered by various concentrations of IFN-γ and TGF-β1 respectively for 24 hrs (lanes 3–6), as compared with untreated cells. (B) The expression of pIgR was upregulated by treating the cells with IFN-γ in a dose-dependent manner (lanes 3, 4), compared with untreated cells. However, treatment of cells with TGF-β1 (5, 50ng/ml) inhibited the expression of the receptor (lane 5, 6). A 100bp ladder was used as a marker (lane 1). U937 cell cDNA was used as a positive control (lane 7) and dH2O (lane 8) was used as a template.
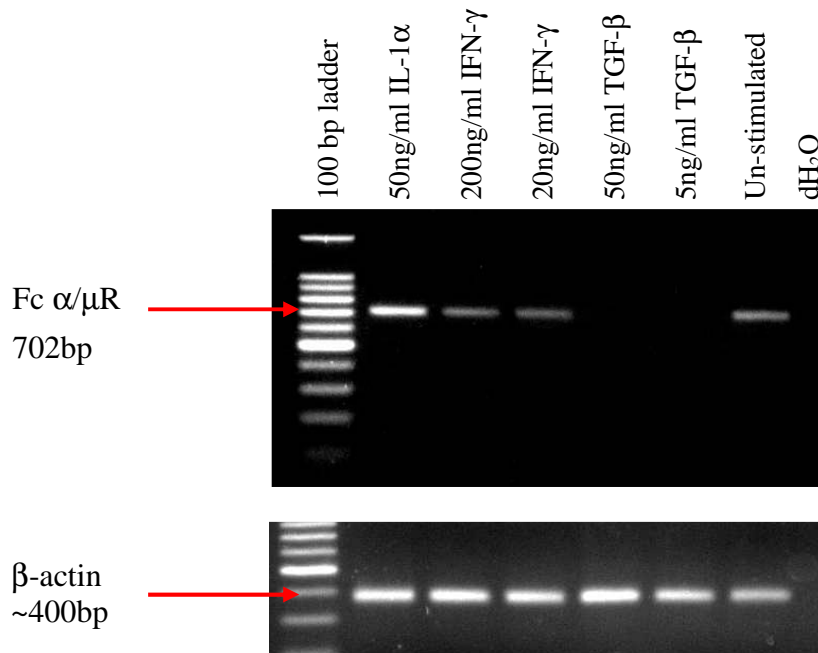
Figure 3-4 The expression of mRNA for Fcα/μR expression by RT-PCR in HK-2 cells

The expression of Fcα/μR was upregulated by 50ng/ml IL-1α (lane 2) in contrast with (20, 200ng/ml) IFN-γ, which only slightly increased the expression of the receptor (lane 3, 4). However, TGF-β1 in various concentrations (lanes 5, 6) completely inhibited the expression of the receptor. Unstimulated cells expressed the receptor at low levels (lane 7). A 100bp ladder was used as a marker (lane 1) and dH2O was used as a template (lane 8). β-actin was used as a loading control in the RT-PCR experiments.
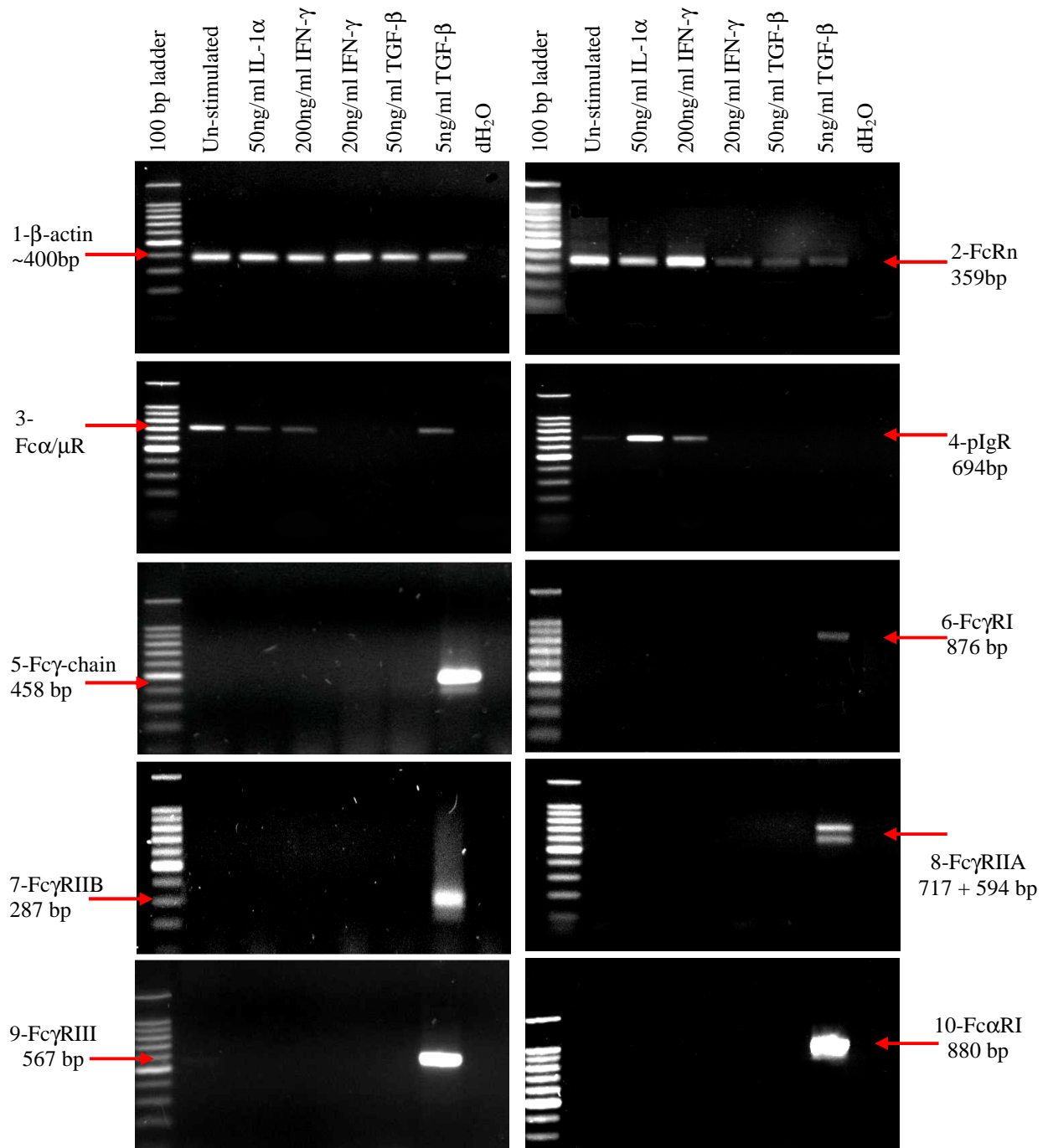
Figure 3-5 The expression of Ig receptors in primary PTEC

RT-PCR showed the same pattern of Ig receptor expression in primary PTEC as is seen in immortalised HK-2 cells. FcRn is expressed at the same level as is seen in cells treated with IFN-γ and TGF-β1, which did not show any effect. Fcα/μR is expressed in untreated cells and is increased when stimulated with 200ng/ml IFN-γ or 50 ng/ml IL-1α. In contrast, stimulation with TGF-β1 reduced the expression of the receptor. pIgR was expressed normally in untreated cells. IFN-γ altered the expression of pIgR in a dose-dependent manner. FcγR, γ-chain and FcαR were not expressed in either untreated cells or cytokine-stimulated cells. A 100bp ladder was used as a marker (lane 1). U937 cells were used as a positive control for all receptors, except FcγRIII for which NK-92 cells were used as a positive control. The housekeeping gene β-actin was used as a loading control and dH2O was used as a template.
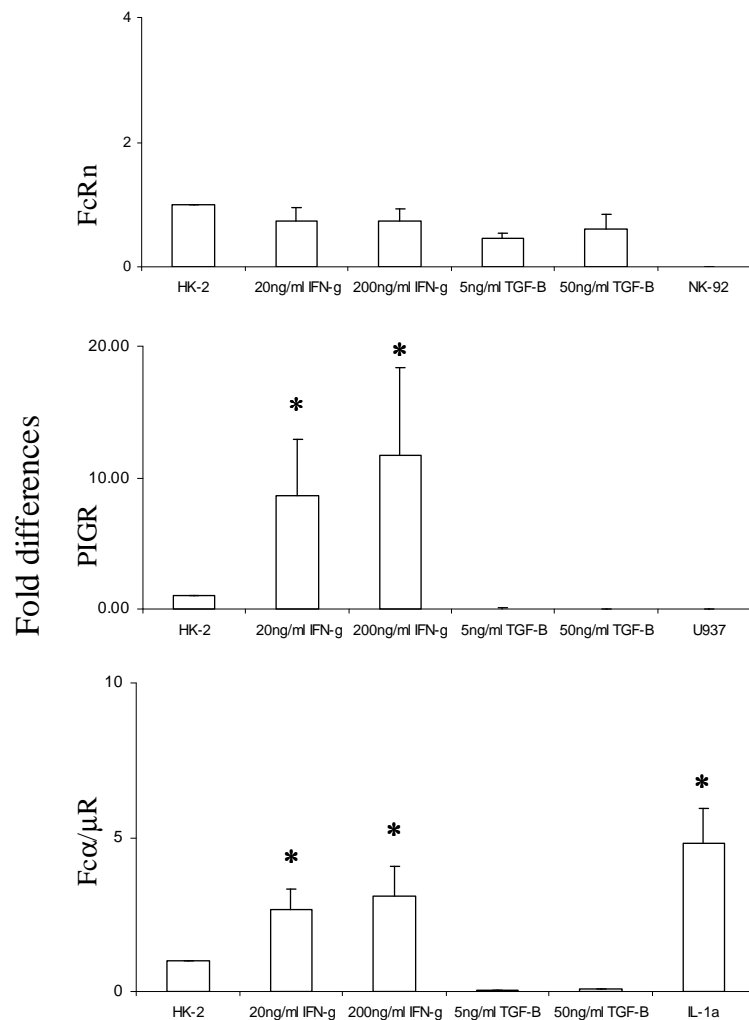
Figure 3-6 Fold differences of mRNA amplification for FcRn, pIgR, Fcα/μR by semi-qRT-PCR in HK-2 cells after 24 hrs stimulation.

For FcRn, no significant change in IFN-γ (20ng/ml 0.73±0.22; 200ng/ml 0.73±0.20) or TGF-β1 (5ng/ml 0.46±0.07; 50ng/ml 0.60±0.25) was seen. Results were compared to the expression of the receptor in NK-92 cells (negative control). pIgR was upregulated by IFN-γ (20ng/ml IFN-γ 8.7±4.2; 200ng/ml IFN-γ 11.7±6.7 fold; P<0.01). Fcα/μR was expressed in immortalised HK-2 cells, upregulated by IFN-γ (20ng/ml IFN-γ 2.7±0.7, 200ng/ml IFN-γ 3.1±0.9 fold) and inhibited by TGF-β. 50ng/ml IL-1α produced greater Fcα/μR upregulation (4.8±1.2 fold; P<0.01). This graph was representative of five experiments and data are shown as mean ± SD.
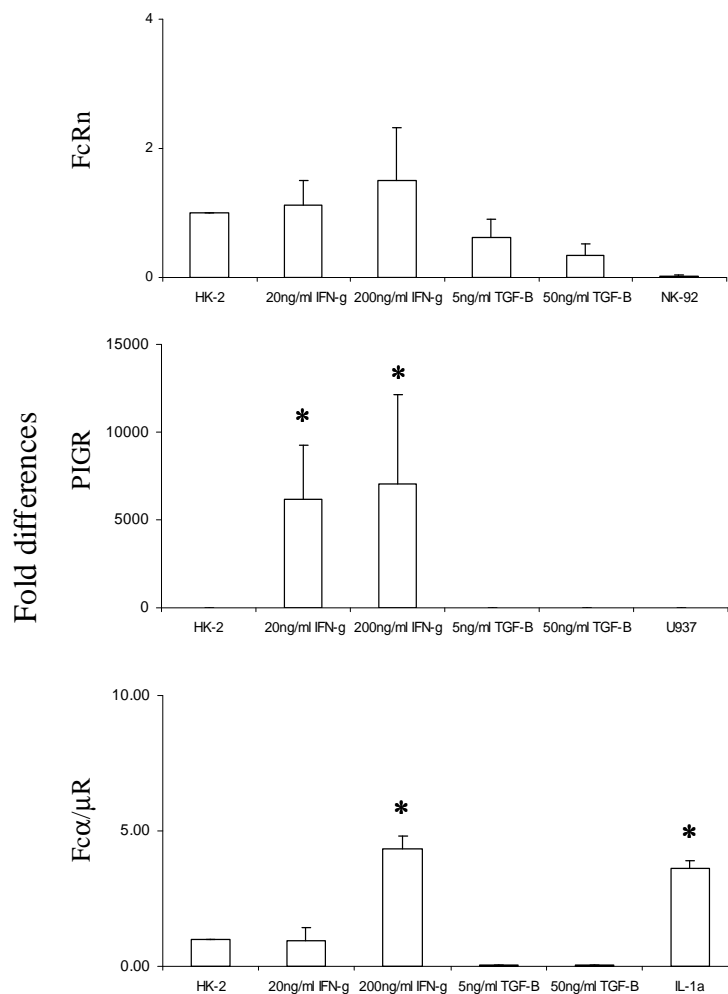
Figure 3-7 The fold differences of mRNA amplification for FcRn, pIgR, Fcα/µR by semi-qRT-PCR in primary PTEC after 24 hrs stimulation.

For FcRn, no significant change in IFN-γ (20ng/ml 1.13±0.37; 200ng/ml 1.50±0.82) or TGF-β1 (5ng/ml 0.61±0.27; 50ng/ml 0.34±0.18) was seen. Results were compared to the expression of the receptor in NK-92 cells (negative control). pIgR was upregulated by IFN-γ (20ng/ml IFN-γ 6170.98±3113.6; 200ng/ml IFN-γ 7086.44±5030.22 fold; P<0.01). Fcα/µR was expressed in immortalised HK-2 cells, upregulated by IFN-γ (20ng/ml IFN-γ 0.94±0.46, 200ng/ml IFN-γ 3.01±1.87 fold) and inhibited by TGF-β. 50ng/ml IL-1α produced greater Fcα/µR upregulation (3.0±0.91 fold; P<0.01). Results were compared with the expression of the receptor in U937 cells, which were also used as a negative control. This graph was representative of five experiments and the data are shown as mean ± SD.

A-Standard curve graph for Fc α/μR by SYBR-490

Correlation Coefficient: 0.996   Slope: -3.325   Intercept: 16.785   Y = -3.325 X + 16.785
PCR Efficiency: 99.9 %

□ Unknowns
○ Standards

Threshold Cycle

Log Starting Quantity, micrograms
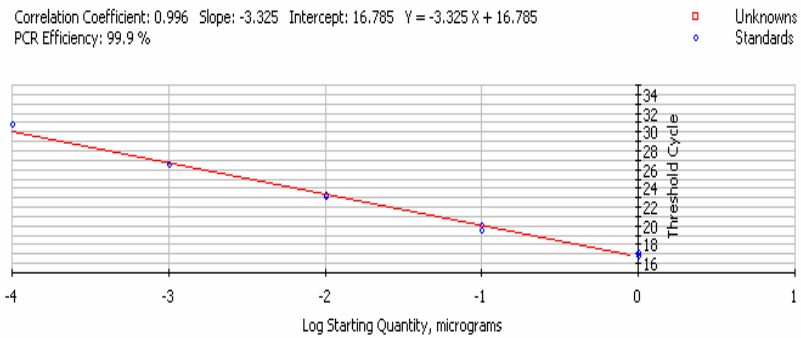
B- PCR Amp/Cycle graph for Fc α/μR by SYBR-490

PCR Base Line Subtracted CF RFU

β-actin

Fc α/μR

Cycle

C- Melt curve graph for Fc α/μR by SYBR-490

-d(RFU) / dT

Fc α/μR

β-actin

Temperature, Celsius
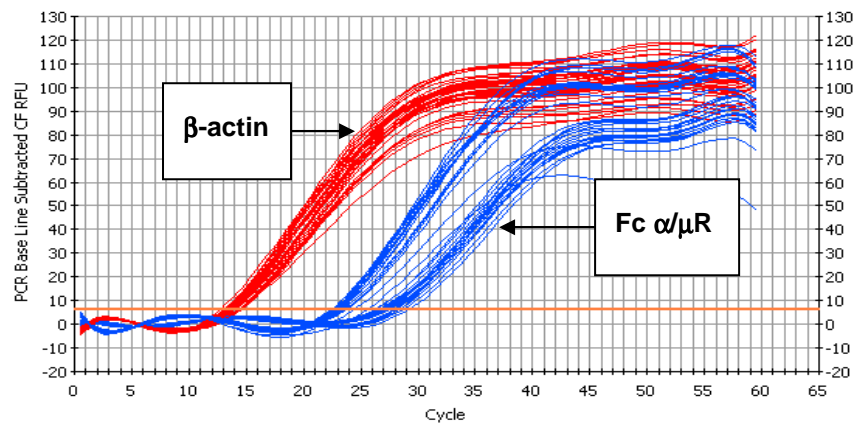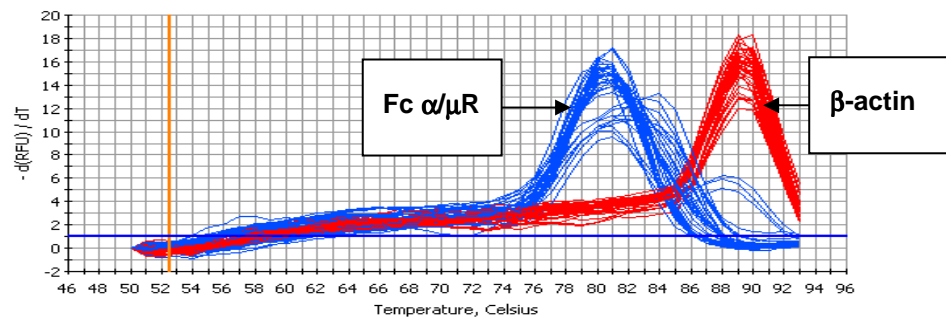
Figure 3-8 Amplification of Ig receptors by semi-qRT-PCR

(A) Dynamic range of β-actin mRNA quantitation assay measuring the amplification of β-actin in 10-fold serial dilutions of a known cDNA sample. Ct of β-actin mRNA amplification has been plotted against the log of the relative initial amount of the pooled cDNA. The slope was −3.325 and the correlation coefficient was 0.996. PCR efficiency was 100.0%. (B) Representative curves of SYBER green fluorescence Ct for Fcα/μR receptor (blue) and the relative expression of the housekeeping gene β-actin (red). (C) Melting curves for primers of Fc receptors (blue) and β-actin (red). The two peaks confirm the absence of gene dimerisation.
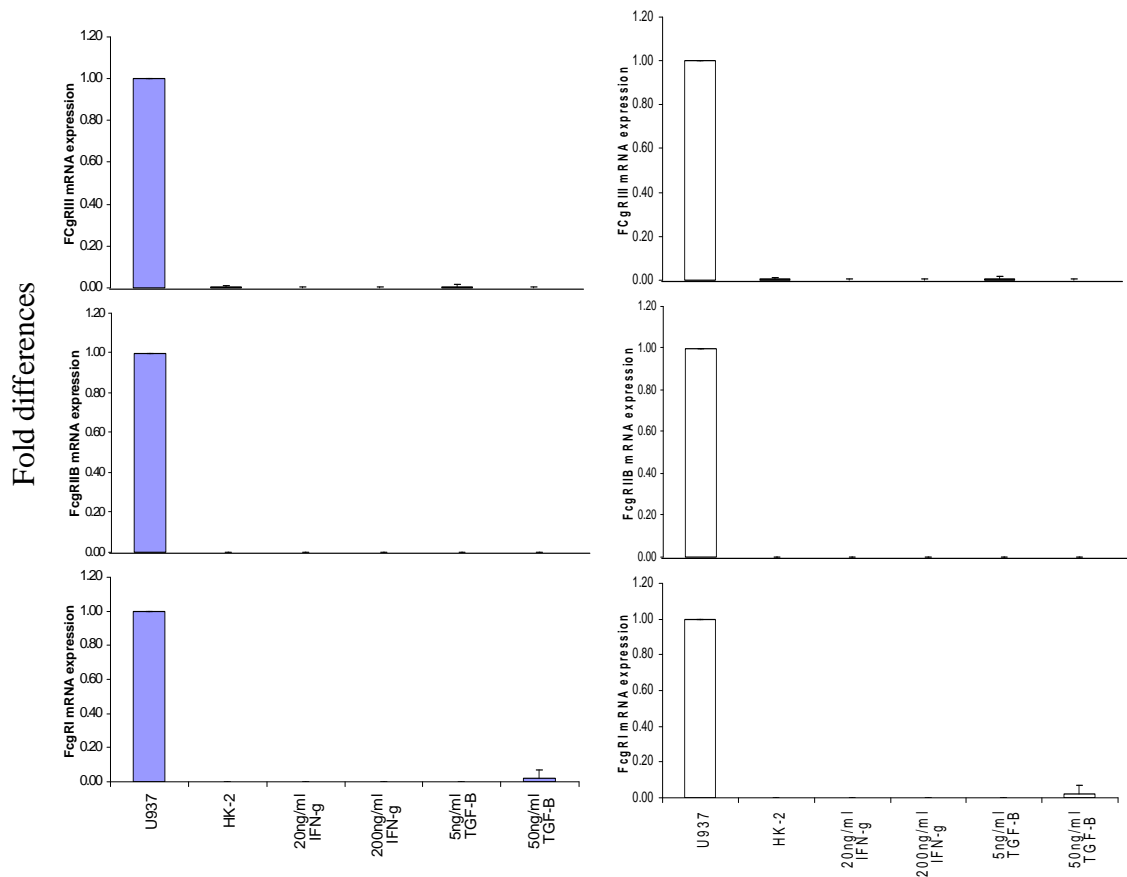
Figure 3-9 Expression of FcγRI,γRIIB,γRIII in HK-2 and primary PTEC

These graphs show the amplification of cDNA using a specific primer of FcγRI, FcγRIIB and FcγRIII in immortalised HK-2 cells (right panel) and in primary PTEC (left panel). U937 cells were used as positive control, except for FcγRIII for which NK-92 cells were used as positive control. None of the receptors were expressed in PTEC. The amplification of the receptor was relative to the housekeeping gene β-actin. Triplicate reactions at each condition were amplified along the iCycler iQ real-time PCR system. The correlation coefficient of the standard curve was 0.999, the slope was −3.284 and the PCR efficiency was 100.0%.

A-

~70KDa Fcα/μ

B-
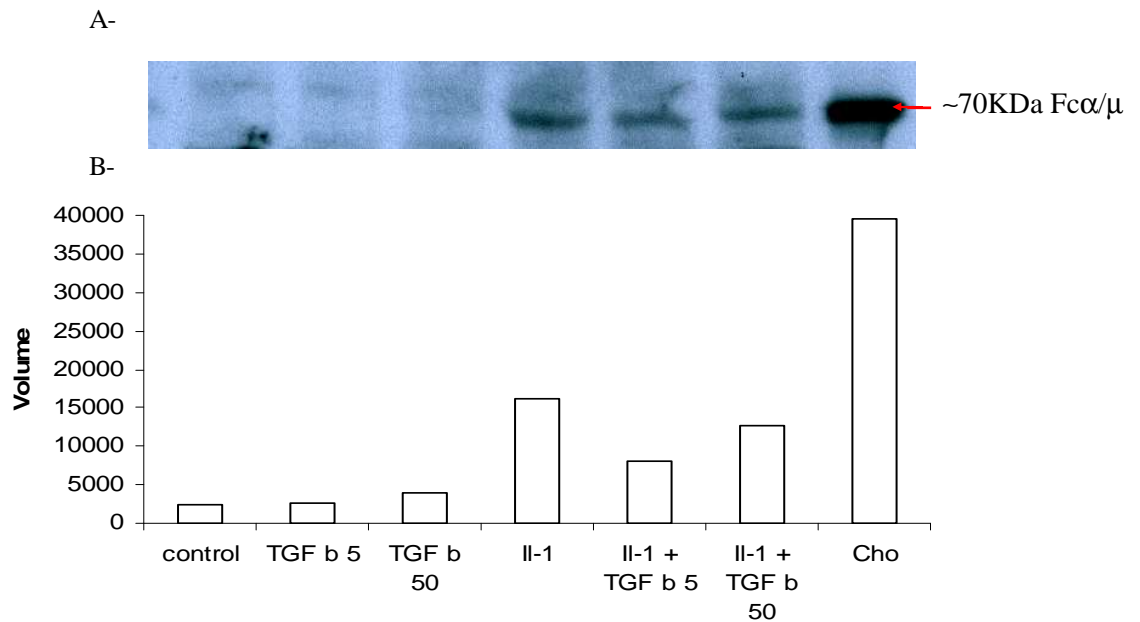
Figure 3-10 Immunoblotting for Fcα/μR antibody in HK-2 cells with densitometry for Fcα/μR protein band expression

Stimulation of HK-2 cells with IL-1α (50 ng/ml), but not TGF-β (5, 50 ng/ml), was able to induce protein expression of Fcα/μR. The expression of the Fcα/μR protein induced by IL-1α was reduced when additionally treated with TGF-β, in a dose-dependent manner, compared with the positive control (CHO cells transfected with Fcα/μR). The band size is ~70 kDa, as seen in Fig. 3-10A. These results were confirmed by densitometry, as seen in Fig. 3-10B. These data are representative of three experiments.
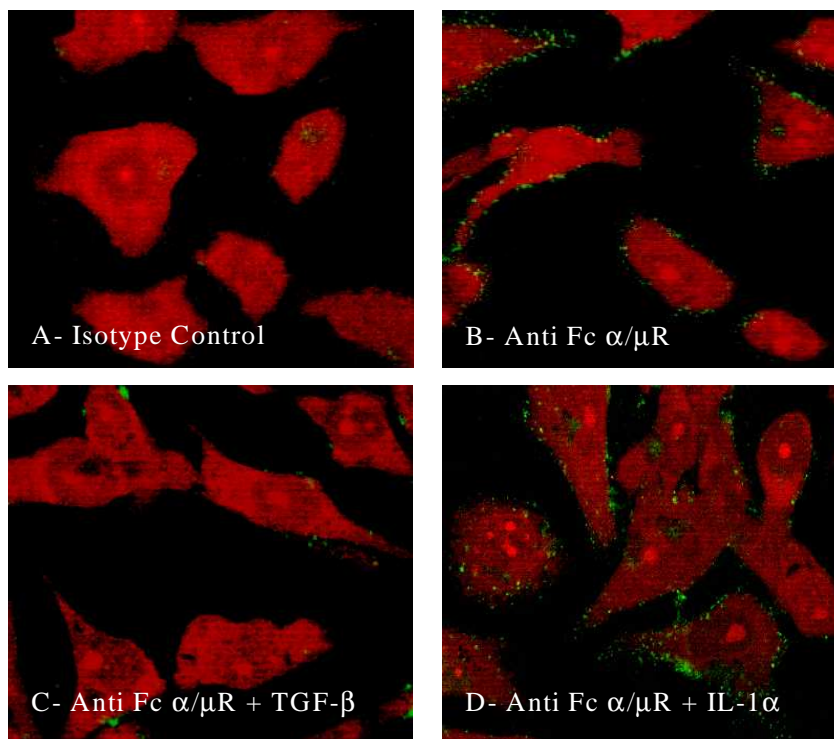
Figure 3-11 Binding of Fcα/μR to HK-2 cells, as seen by confocal microscopy

Immunofluorescence staining with Fcα/μR antibody on immortalised HK-2 cells. (A) Isotype control was used to determine the specificity of the antibody; (B) Specific binding of the Fcα/μR antibody to the receptor can be seen. The regulation of the receptor expression was increased by IL-1α (D) and reduced by TGF-β1 (C). Green fluorescence indicates the binding and red shows the cells and nuclei.
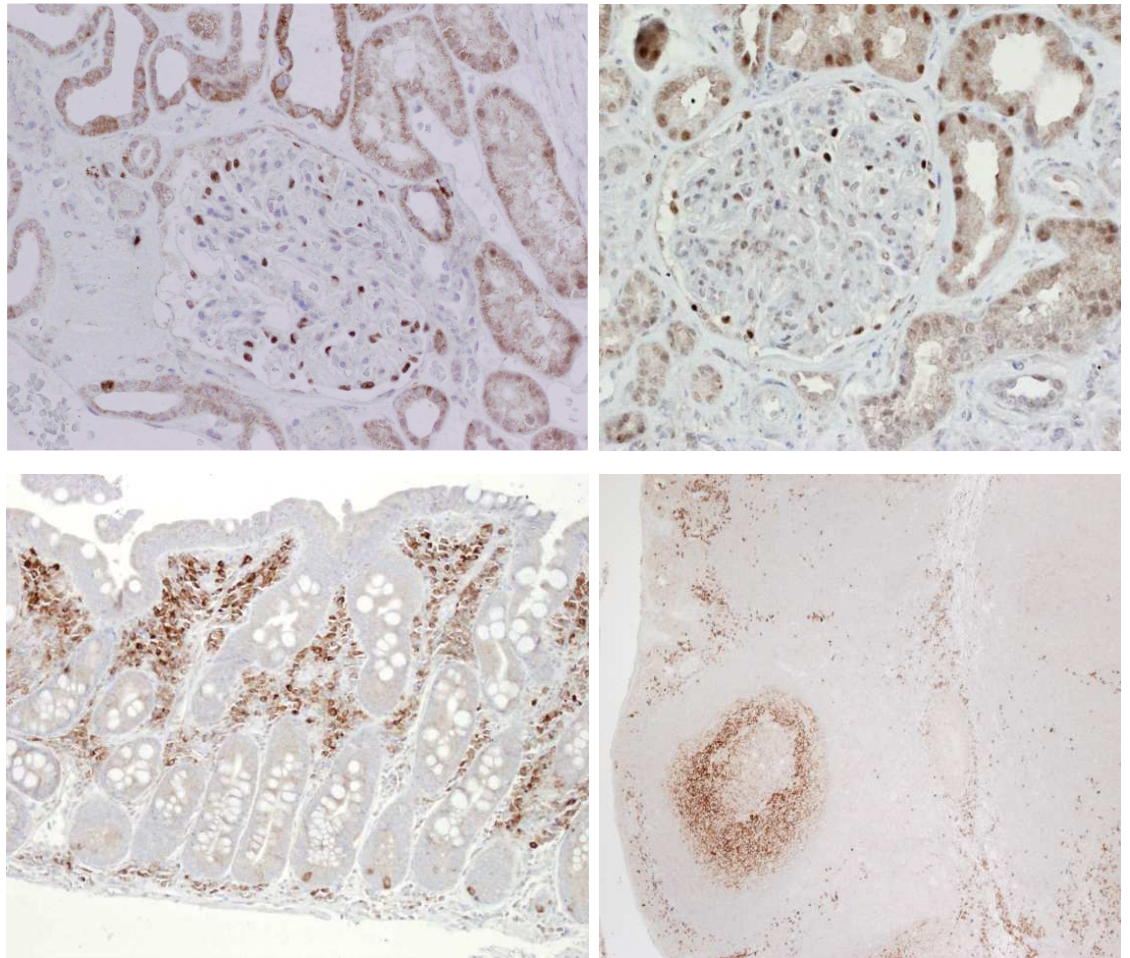
Figure 3-12 Immunocytochemistry for Fcα/μR

Expression of Fcα/μR in (A) normal kidney; (B) kidney of patients suffering from IgA nephropathy; (C) B-lymphocytes of small intestine; and (D) B-lymphocytes of tonsil.
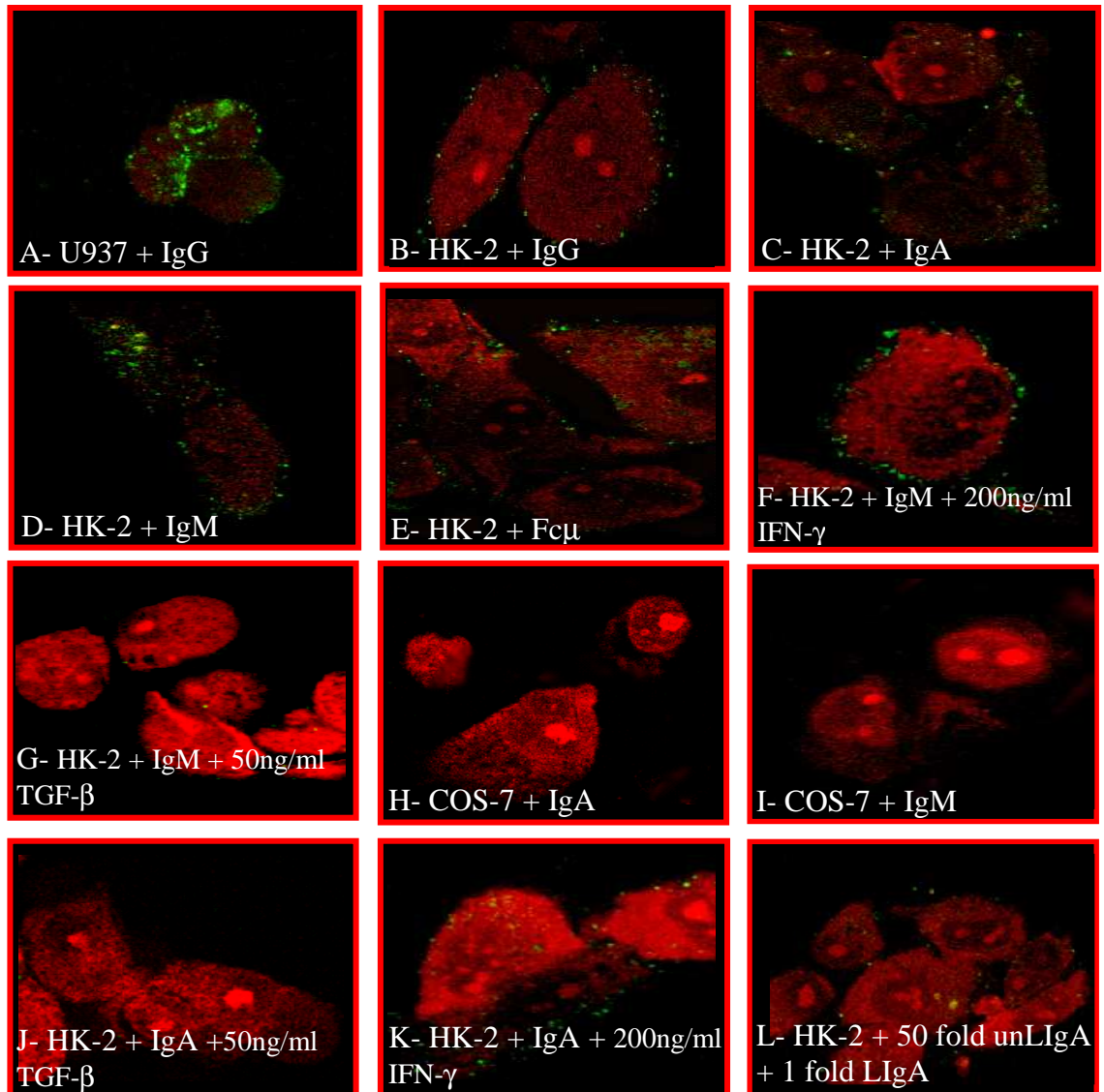
Figure 3-13 Binding of Igs, as seen by confocal microscopy

Confocal scanning laser microscopy showing the binding pattern of various Igs to PTEC. (A) U937 cells incubated with IgG followed by streptavidin were used as positive control. (B, C, D) Cells incubated with Biotin-SP-conjugated IgG, IgA and IgM and then fluorescein-conjugated streptavidin. Potentially, IgA and IgM may bind to the pIgR or Fcα/μR. The former binds Ig through the J-chain, whereas the latter is believed to bind the Fc portion of Ig. (E) Binding with Biotin-Fcμ fragment IgM. (F, G, J, K) The incubation of Biotin-IgA and IgM with IFN-γ and TGF-β1 treated cells. There is binding of IgA or IgM with IFN-γ while there is no binding with TGF-β1. (H, I) Cos-7 Cells incubated with IgA and IgM followed by straptavidin were used as negative controls. (L) To displace IgA and IgM binding to PTEC, there is competition between unlabelled mIgA and biotinylated-mIgA binding to PTEC. The competition was induced by incubation of 50-fold unlabelled IgA together with 1-fold Biotin-IgA in PTEC. There is less binding of IgA in the presence of the unlabelled IgA, suggesting that binding is through the Fcα/μR. Green fluorescence indicates the binding and red shows the cells and nuclei.
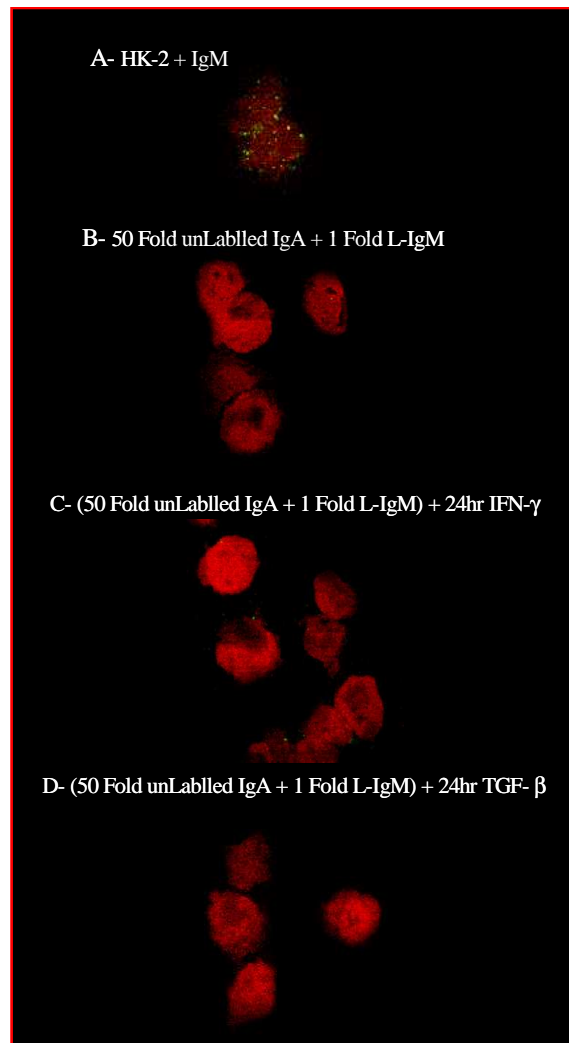
Figure 3-14 Binding of IgM by competition with 50-fold IgA by confocal microscopy

Confocal scanning laser microscopy showing the binding pattern of IgM to PTEC. (A) The binding of Biotin-SP-conjugated IgM to HK-2 cells and then fluorescein-conjugated streptavidin. (B) The specificity of IgM binding to Fcα/µR and its displacement by 50-fold unlablled IgA followed by 1-fold Biotin-IgM. (C,D) The regulation of Fcα/µR expression and its subsequent binding to the antibody by IFN-γ and TGF-β1, respectively. Green fluorescence indicates the binding and red shows the cells and nuclei.
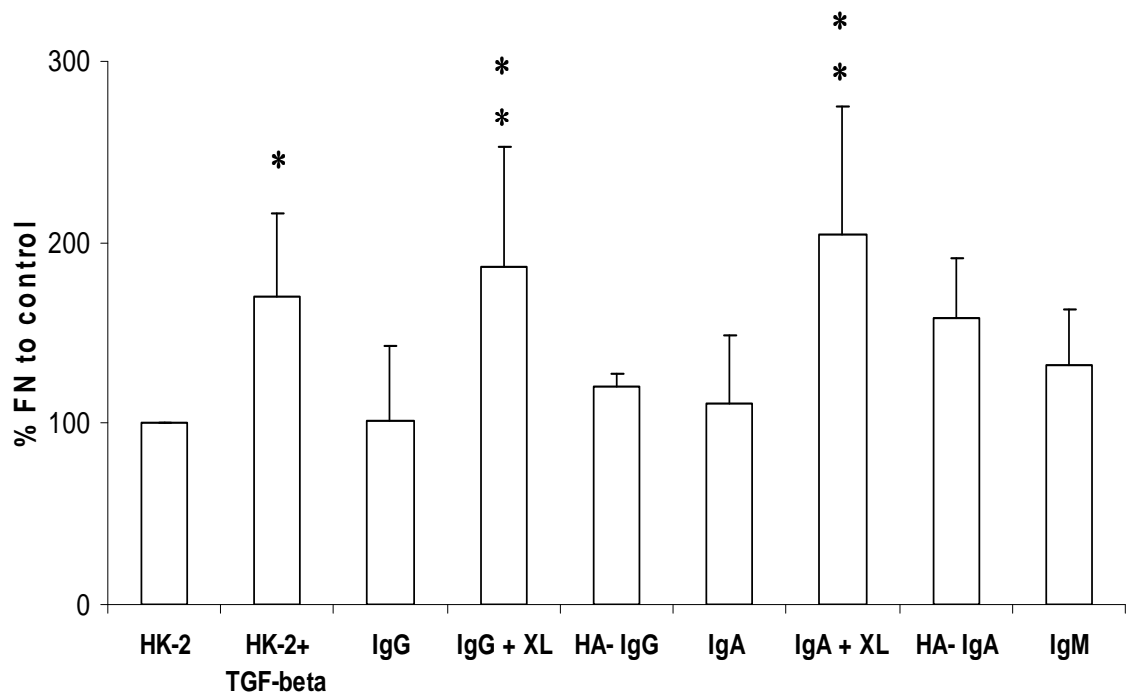
Figure 3-15 Percentage of FN production by HK-2 cells stimulated with Ig ± XL

The first bar represents the immortalised HK-2 cells, which were used as a control. The second bar represents the positive control TGF-β1 (50 ng/ml) and shows an increase in FN production (p<0.01). Bars 3–5 show the effect of IgG, XL-IgG and HA-IgG stimulation of HK-2 cells, XL-IgG shows increasing FN production (p<0.002). Bars 6–8 shows the effect of IgA, XL-IgA and HA-IgA stimulation of HK-2 cells, also XL-IgA shows increasing FN production (p<0.002). Bars 9 show the effect of IgM stimulation of HK-2, increasing FN production compared with the control HK-2 cell line (p<0.01). The addition of Ig without XL had an effect on FN production. This result is representative of nine individual experiments and data are shown as mean ± SD.
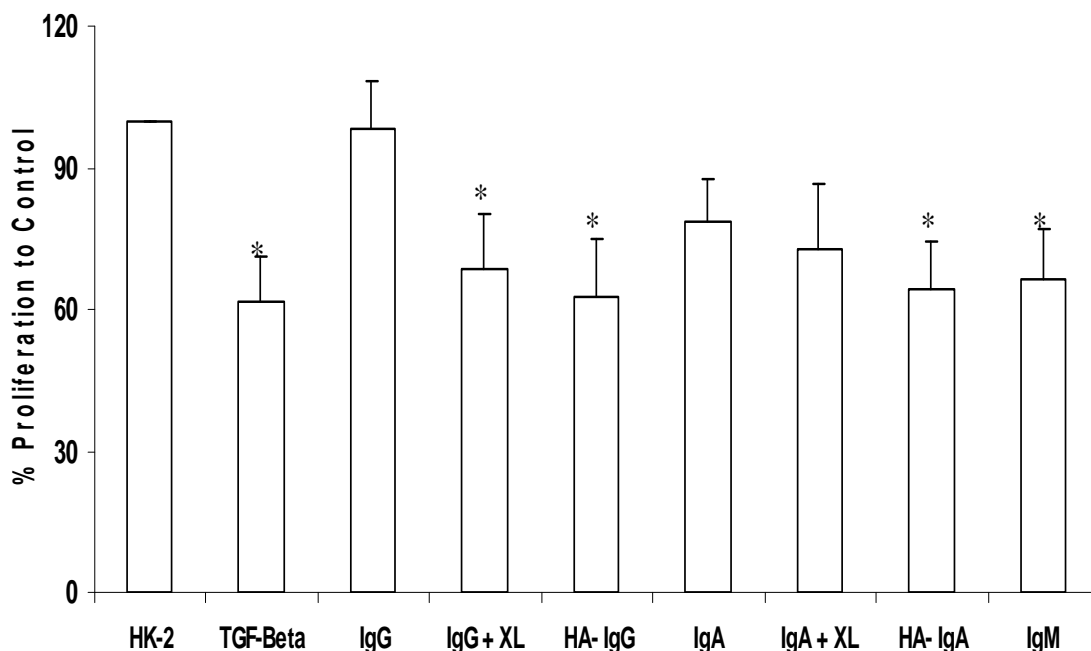
Figure 3-16 Percentage of the proliferation of HK-2 cells stimulated with Ig

Effect of Ig/IC and TGF-β1 on the proliferation of immortalised HK-2 cells, as assessed by [3H]-thymidine uptake. The first bar represents the untreated HK-2 cell line as control. The second bar represents the positive control TGF-β1 (50 ng/ml) and shows inhibition of the proliferation of PTEC. IgG had no effect on proliferation compared with the control. However, incubation with IgA, IgM, XL-IgG, XL-IgA, HA-IgG and HA-IgA inhibited the proliferation of PTEC. This result is representative of five individual experiments of 12 values and data are shown as mean ± SD.
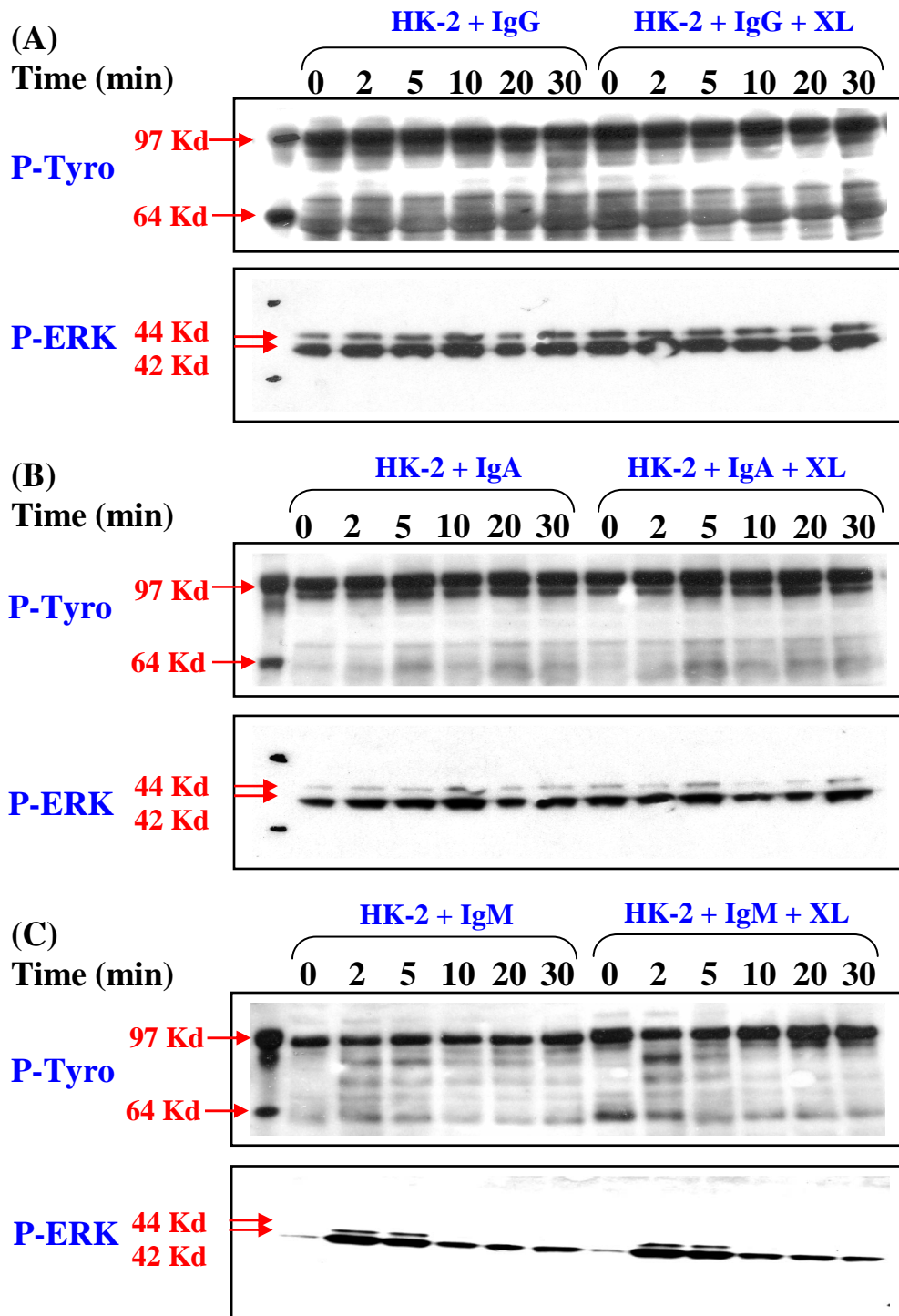
Figure 3-17 Immunoblotting for IgG, IgA, IgM ±XL on PTEC with anti-phosphotyrosine and ERK over time

Immunoblot showing the binding of Ig to PTEC in the presence and absence of XL. Cells were treated with Ig at 4°C and Ig + XL complex at 37°C and immunoblotted for anti-phosphotyrosine (top panel) and ERK (bottom panel). The results suggested no change in the signalling component of phosphotyrosine and ERK with IgG and IgA at all timepoints. However, IgM activated phosphotyrosine and ERK at 2 and 5 mins. (A) IgG ± XL; (B) IgA ± XL; (C) IgM ± XL. This blot was representative of three experiments.

A-

Well Label	HK2	TGF-b	IgG	IgGXL	IgG+XL	HA-IgG	IgA	IgAXL	IgA+XL	IgM
Diln. Fact.	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
IL-1a	*	*	*	330.0	355.1	*	*	*	3.9	*
IL-1b	*	*	*	1.2	602.1	*	*	*	*	*
IL-2	*	*	*	280.8	376.2	*	*	*	3.4	*
IL-4	*	5.2	*	*	175.9	*	*	*	*	*
IL-6	33.0	412.1	22.5	212.2	350.2	30.1	26.1	33.6	40.4	36.1
IL-7	18.7	10.9	18.8	28.6	694.8	12.9	25.8	18.5	22.8	15.7
IL-8	2112.6	2111.5	2112.9	1837.7	1955.3	1761.0	2112.1	2112.1	2112.5	2110.0
IL-10	*	*	*	124.6	207.8	*	*	*	*	*
IL-12	*	*	*	497.8	667.3	*	*	*	3.0	*
GMCSF	935.3	370.4	862.0	42.1	1042.0	778.0	789.4	721.7	1091.0	957.5
IFN-g	*	*	*	3162.6	2926.9	*	*	*	*	*
TNF-a	52.2	*	40.6	115.8	495.4	*	46.1	*	59.6	36.1

B-
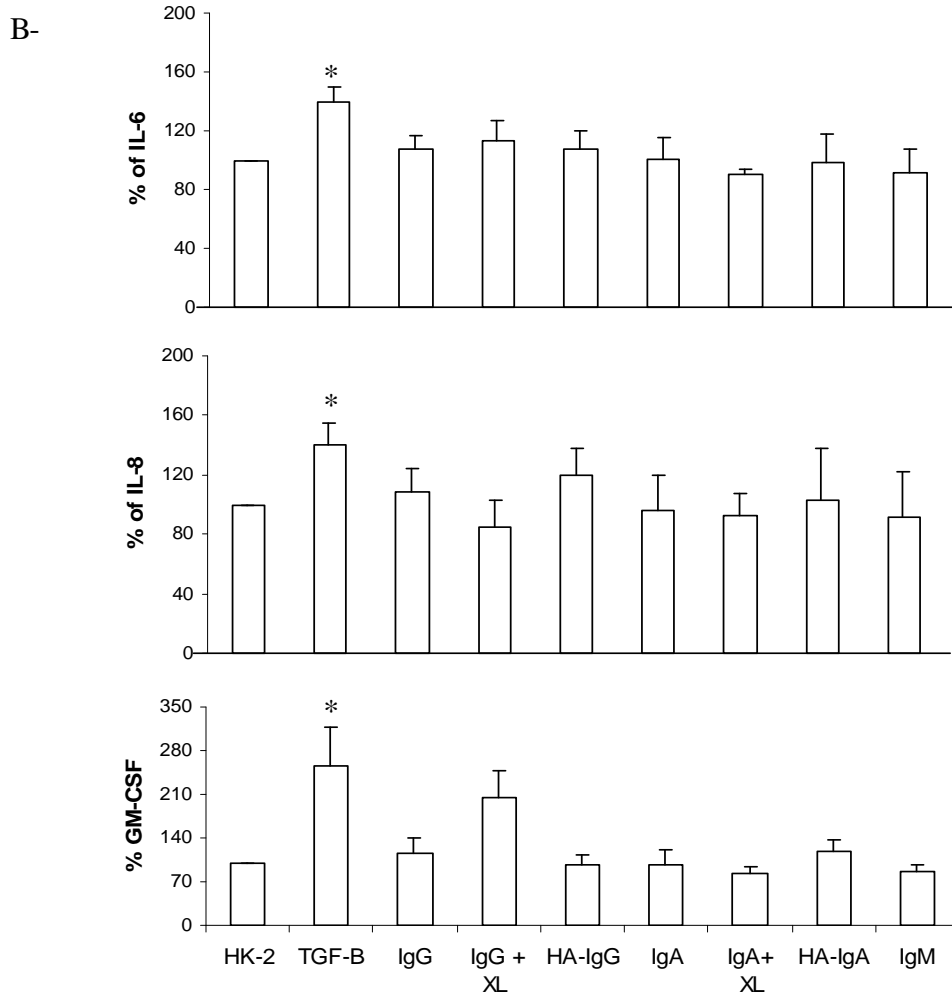
Figure 3-18 Effect of Ig on PTEC to release IL-6, IL-8 and GM-CSF

(A) Proteoplex™ analyser data showing cytokine release by PTEC in culture supernatants measured after 24 hrs. (B) Percentage of IL-6 (upper panel), IL-8 (middle) and GM-CSF (lower) released by untreated HK-2 cells and by cells treated with Ig ± XL for 24 hrs by specific ELISA. TGF-β1 (50ng/ml) was used as a positive control and increased the release of the cytokines. IL-6, IL-8 and GM-CSF were released by HK-2 cells. Ig ± XL stimulation did not show a significant increase compared with the unstimulated cells. Data are mean ± SD of three measurements in three separate experiments. * $p < 0.01$.
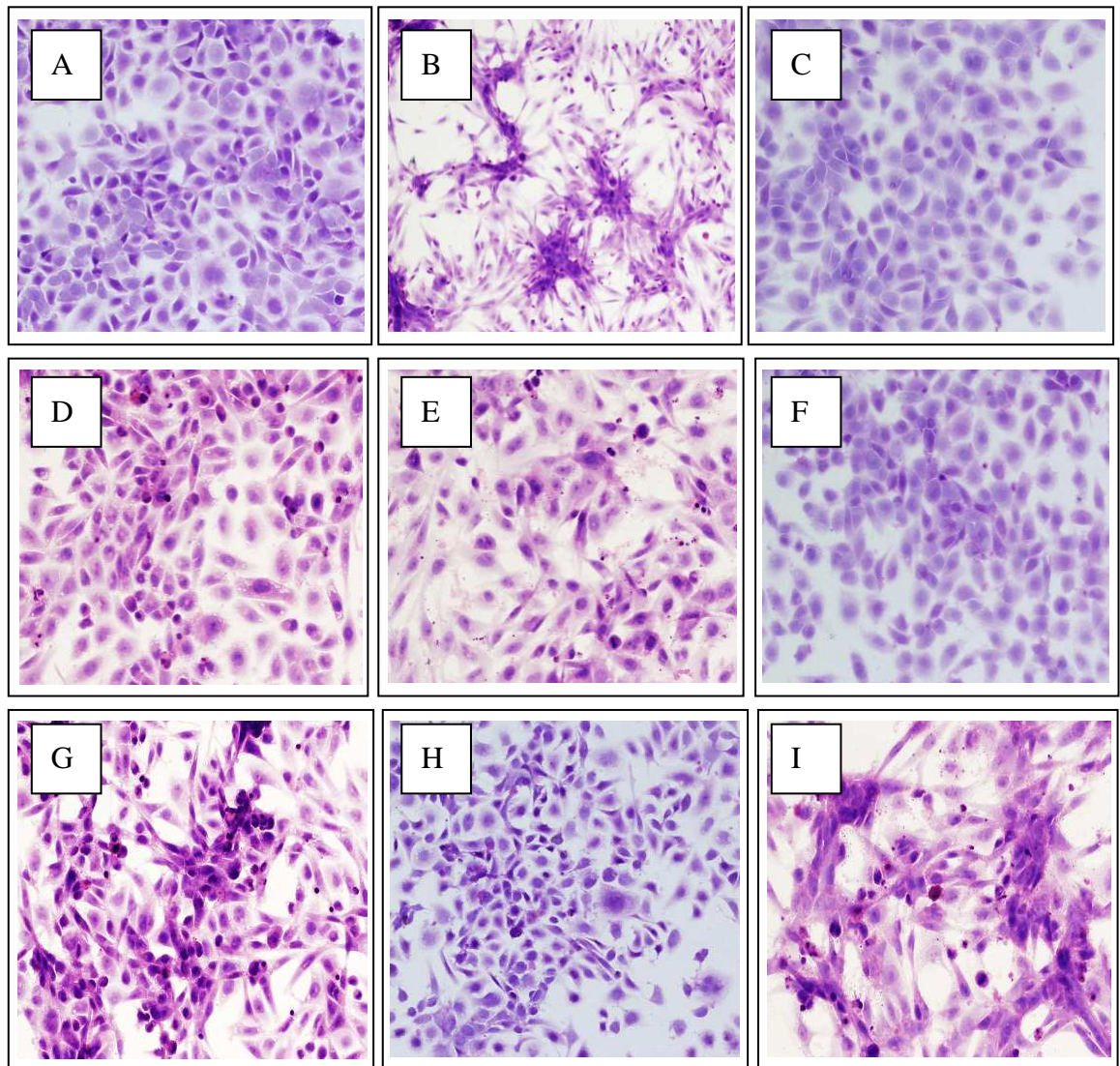
Figure 3-19 Effect of Ig ± XL and TGF-β1 on the cell morphology of PTEC

Morphology of cultured PTEC stimulated with Ig ± XL and TGF-β1 for 72 hrs at 37°C. (A) unstimulated HK-2 cells; (B) TGF-β1; (C) IgG; (D) IgG + XL; (E) HA-IgG; (F) IgA; (G) IgA + XL; (H) HA-IgA; (I) IgM. Photographs are shown at the original magnification × 100.

Chapter 4: Effect of immunosuppressive agents on human PTEC

4.1 Introduction:

4.1.1 Background

For patients with ESRD, transplantation of the kidneys has, in recent years, become the first-line treatment of choice (Wolfe *et al.*, 1999). Over the last two decades, patient outcomes, post-transplantation, have improved dramatically, in the short-term at least, due to the introduction of several new immunosuppressive drugs. Immunosuppressive drugs are classified into antimetabolites (e.g. azathioprine), biological immunosuppressants (antibodies), non-biological immunosuppressants and immunophilin binders. The last group include calcineurin inhibitors (CNIs), such as cyclosporine and FK506 (tacrolimus), and non-CNIs, such as sirolimus (rapamycin).

The introduction of these agents has significantly reduced the likelihood of acute rejection and improved the rate of graft survival at 1 year. However, these treatment advances have not had the same effect on long-term patient outcomes (Morales *et al.*, 2001). This is perhaps due to the serious side effects that may arise from the long-term use of some immunosuppressants, including nephrotoxicity and arterial hypertension (Meier-Kriesche *et al.*, 2004).

The two major therapeutic goals after renal transplantation are to avoid acute rejection episodes and to limit the side effects of immunosuppression. This is particularly important as immunosuppressive drugs are often used in combination with each other, rather than alone (Waller&Nicholson, 2001).

In this chapter, I concentrate on the effects of the immunophilin binders as this class of drugs has been shown to have nephrotoxic effects when used over a long period of time.

4.1.1.1 Cyclosporine

Of the three immunosuppressive drugs under study, cyclosporine is the oldest. When introduced in the early 1980s, it was the most specific immunosuppressant available and dramatically benefited not just renal transplantation patients but also those receiving other donor organs. Today, cyclosporine continues to be an important part of most maintenance immunosuppression treatment regimens for almost all types of organ transplant. Cyclosporine inhibits the activity of calcineurin through binding with its cytoplasmic receptor protein, cyclophilin, and this inhibition leads to a suppression in T cell activation

by impairing the expression of a number of critical activation genes, most importantly the one for IL-2 (Fig. 4.1) (Elliott *et al.*, 1984; Hess *et al.*, 1982; Shevach, 1985).

The action of cyclosporine molecules on the cellular membranes of PTEC causes rapid injury, within hours or days, when the drug reaches trough concentrations of >300 ng/ml in the blood (Cavallo&Roy, 1998). Therapeutic doses of cyclosporine (5–10 mg/kg) lead to trough concentrations in the whole blood of 200–500 ng/ml (0.166–0.416µM) or 20–60 ng/ml (0.0166- 0.050µM) in serum. In a model using immortalised rat PTEC, even short-term use of cyclosporine caused loss of the brush border (leading to a reduction in renal reabsorption) and dilation of the proximal tubules (Burdmann *et al.*, 2003).

4.1.1.2 FK506 (tacrolimus)

Like cyclosporine, FK506 is a CNI commonly used in immunosuppressive treatment regimens and indeed the two agents have a very similar mechanism of action. Whereas cyclosporine acts by binding cyclophilins, FK506 acts by binding FK506-binding proteins (FKBPs). By inhibiting calcineurin, FK506 prevents the activation of transcription factors in response to the elevated levels of intracellular calcium seen with stimulation of TCR. As with cyclosporine, this prevents the synthesis of IL-2 and other important cytokines (Fig. 4.1). In terms of potency, FK506 differs considerably from cyclosporine and is approximately 100 times more potent on a molar basis (Kaplan *et al.*, 1995). Moreover, whereas cyclosporine is ineffective in the treatment of steroid-resistant allograft rejection, FK506 has been shown to be able to reverse it (Almawi&Melemedjian, 2000). Thus, it is possible that FK506 inhibits T-cell activation at additional stages to that of cyclosporine.

FK506, a metabolite of the Japanese soil fungus *Streptomyces tesukubaensi*, was first approved in 1994 for use as an immunosuppressant in liver transplantation. In renal transplantation, FK506 is typically given in doses of 0.03–0.05 mg/kg leading to trough concentrations of 5–20 ng/ml (0.0062–0.02 µM) in blood (Limbird, 2001). After transplantation, FK506 should not exceed a maximum trough concentration of 40 ng/ml (0.050µM) (Japanese, 1991; Nashan *et al.*, 1988; Sano *et al.*, 1995). FK506 significantly inhibits the production of GM-CSF at concentrations of ≥10 ng/ml (0.01 µM), compared with cyclosporine concentrations of 0.1 µg/ml (0.083 µM) or higher.

4.1.1.3 Sirolimus

Derived from a soil actinomycete isolated from *Streptomyces hygroscopicus* originally found on Easter Island (whose native title of Rapa Nui led to the drug first being named rapamycin), sirolimus is a macrolide lactone antibiotic with immunosuppressant properties. Although sirolimus is structurally similar to FK506 and binds to the same immunophilin (FKBP), it has no effect on calcineurin and does not block the calcium-dependent activation of cytokine genes. Instead, the active sirolimus–FKBP complex inhibits cell division by reducing expression of the enzyme P7056 kinase through binding to target of rapamycin (TOR) proteins. In doing so, the cell cycle cannot progress from the G1 to the S phase and the cells do not divide (Fig. 4.1) (Gummert *et al.*, 1999).

To date, sirolimus has been used in a variety of combinations and situations. It is most commonly used in conjunction with one of the CNIs, usually to help withdraw or avoid completely the use of corticosteroids in maintenance immunosuppressive treatment regimens (Lee&Chapman, 2005).

In adults, sirolimus is given at a dose of 2 mg/day following a loading dose of 6 mg. At trough levels of >6 ng/ml (0.006μM), sirolimus is effective at reducing the risk of acute rejection, while levels >15 ng/ml (0.019μM) were associated with more frequent adverse events (Davison, 2005).

4.1.2 The role of immunosuppressant agents in renal disease

In the last 10 years, PTEC have become a key focus for investigators interested in the pathogenesis of renal injury and disease. *In vitro* and *in vivo* studies have shown that PTEC play an important role in tubulointerstitial injury by mediating the production of proinflammatory cytokines and other markers of inflammation (Daha&van Kooten, 2000; Kuroiwa *et al.*, 2000; Zhou *et al.*, 2001). Further studies revealed that PTEC is a major source of complement C3 (an important inflammatory trigger normally derived from hepatocytes (Alper *et al.*, 1969)) in the injured kidney (Ludwig *et al.*, 2001; Zhou *et al.*, 2001); indeed, one study found that, in inflammatory conditions, PTEC expression of C3 constituted up to 15% of the total circulating levels of the complement (Tang *et al.*, 1999). In addition, PTEC are able to function as APCs as they can express MHC-II, adhesion molecules and co-stimulatory molecules such as CD40 and CD40 ligand, enabling them to interact with T-cells (Memoli *et al.*, 2000; Shu *et al.*, 2000; van Kooten *et al.*, 1997).

The location of PTEC in the renal system also makes these cells an interesting target for researchers. Because these cells are found between the urinary space, the renal interstitium and the peritubular capillaries, they react to both blood-borne substances and to substances not normally found in the urine, such as filtered proteins and inflammatory reactants.

There have been few reports on how human PTEC react to therapeutic agents (Hughes *et al.*, 1998; Johnson *et al.*, 2000) and it is not yet clear whether immunosuppressive drugs directly inhibit the ability of PTEC to secrete inflammatory markers. Research *in vivo* has shown that renal PTEC are targeted by several nephrotoxins (Baliga *et al.*, 1999; Eckert *et al.*, 1989; Yamauchi *et al.*, 1998), potentially leading to acute renal damage and chronic tubulointerstitial nephropathy characterised by tubular atrophy, loss of tubular cells and interstitial fibrosis.

In this study, I examined the effects of CNI (cyclosporine, FK506) and non-CNI (sirolimus) as a model of nephrotoxicity as measured by various markers of progressive renal damages. Four mechanisms of renal damage were looked at: the expression of Fc receptors, the release of cytokines, the production of FN and the proliferation of PTEC leading to tubulointerstitial inflammation/fibrosis and ESRF. By focusing on the immunosuppressants already known to have nephrotoxic side effects of their own, this study aims to contribute important new data on the responsiveness and selectivity of human PTEC to these agents. In section 3.3.2, I showed that the Ig receptors FcRn, pIgR and Fcα/μR are expressed in immortalised HK-2 cells and primary PTEC and may play a role in renal disease.

First, I will provide a more general overview of how these agents may cause nephrotoxicity.

4.1.3 Immunosuppressant-induced nephrotoxicity

Nephrotoxicity is recognised as the major adverse effect of cyclosporine and FK506; however, the mechanism for this remains unclear. In the case of cyclosporine, therapeutic doses lead to impaired renal function in almost every patient; hypertension is seen in up to 50% of patients, serum creatinine increases by approximately 30% and both GFR and renal plasma flow are reduced. In addition, cyclosporine may cause early post-transplant graft dysfunction or worsen existing poor graft function due to its calcium-dependent potentiation of vasoconstriction of the renal vasculature. Fortunately, this vasoconstriction is likely to be a transient, reversible and dose-dependent phenomenon. In a study by

Mourad *et al.*, (1998) chronic cyclosporine nephrotoxicity was estimated to be the sole cause of chronic allograft dysfunction in up to 6% of all renal transplant patients.

Researchers have proposed a number of different nephrotoxic effects leading to renal failure. Firstly, it has been suggested that tubulointerstitial damage occurs as a consequence of chronic renal ischaemia. In addition to the release of factors such as TGF-β, endothelin-1 and plasminogen activator inhibitor type 1, fibroblast proliferation and matrix synthesis may occur and GFR are reduced (Anglicheau *et al.*, 2006).

Second, it is significant that cyclosporine enhances the expression of TGF-β, a potent fibrogenic and angiogenic cytokine released after tissue injury, as heightened expression of TGF-β1 may contribute to the antiproliferative/immunosuppressive activity of cyclosporine. Indeed, blockade of cytokine gene activation does not fully account for the antiproliferative effect of cyclosporine (Wolf *et al.*, 1995).

A third potential cause of nephrotoxicity is the involvement of P-glycoprotein (Pgp), a protein expressed on the brush border of PTEC and on the distal tubule. It has been suggested that Pgp has an important role in cyclosporine nephrotoxicity. Interestingly, sirolimus is a Pgp substrate and an interaction between cyclosporine and sirolimus has been observed. However, the significance of these findings has yet to be fully explored (Anglicheau *et al.*, 2006).

Long-term administration of cyclosporine and FK506 is known to produce histological changes similar to those of allograft fibrosis. If cyclosporine/FK506-induced nephrotoxicity remains untreated, it can contribute to CAN, the most common cause of late graft loss by causing irreversible renal dysfunction in association with tubular atrophy, interstitial fibrosis of the renal parenchyma.

Sirolimus may be used as an alternative to cyclosporine or FK506 as part of a calcineurin-sparing protocol. To date, both preclinical and human studies of sirolimus have revealed that the agent has few long-term nephrotoxic effects. Using a calcineurin-sparing protocol may also reduce hypertension, hyperlipidaemia, glucose intolerance and the overall risk of cardiovascular disease. Hence, sirolimus may prove to be better than cyclosporine or FK506 for the long-term preservation of renal function in transplant recipients.

4.1.4 The role of proteinuria in nephrotoxicity

Although cyclosporine, FK506 and sirolimus have been shown to slow the progression of renal disease in the short-term, loss of renal function continues and proteinuria is rarely completely normalised. Working against the drugs, studies have shown that persistent proteinuria can cause progression of tubulointerstitial disease by inducing inflammation and fibrotic changes through the stimulation of cytokines, chemokines and growth factors such as IL-8, RANTES, MCP-1 and TGF-β (Bonegio *et al.*, 2005). The development of new therapeutic agents that either slow or prevent renal injury due to proteinuria is a key research focus but the mechanisms of proteinuria-induced tubulointerstitial disease have proved difficult to study and are beyond the scope of this project.

4.1.5 The effect of immunosuppressive agents on the expression of FcRs

The family of Fc receptors include the FcRn, pIgR and Fcα/μR. FcRn are responsible for the transportation of IgG from mother to neonate and are thought to maintain the long plasma half-life of IgG. Research has also shown that FcRn are able to activate complement. pIgR are transporters found on the surface of epithelial cells and hepatocytes; they are thought to mediate transcytosis of polymeric IgM and IgA. Fcα/μR is a novel receptor that binds both IgM and IgA.

As discussed in Chapter 3, my early experiments showed that FcRn, pIgR and Fcα/μR are expressed in PTEC. These findings prompted me to investigate how the expression of these receptors in PTEC can be affected by the use of immunosuppressive agents with known nephrotoxicity. At present, the published literature contains very little information on this topic. Therefore, this study may provide new insights.

4.1.6 The effect of immunosuppressive agents on cytokine production

The macrophage-derived cytokines known to have proinflammatory properties include IL-1β, IL-6, IL-8 and TNF-α and these may play a role in the mechanisms of renal inflammation in tubulointerstitial diseases (Losa Garcia *et al.*, 1999). It has been reported that cyclosporine inhibits the production of IL-1β, TNF-α and IL-6 (Rofe *et al.*, 1992; Tipton *et al.*, 1990), but the mechanism by which this occurs is not clear. Moreover,

cyclosporine has been shown to upregulate the production of GM-CSF, but it is not yet clear whether this is simply part of a more general increase in cytokine production (Nguyen *et al.*, 1990). Several reports have documented that cyclosporine inhibits the production of IL-2 by T-cells by inhibiting specific mRNA synthesis. Furthermore, additional work has shown that cyclosporine downregulates IL-4 and IFN-γ, also both at the protein and mRNA level. The aim of the present study was to investigate the effect of cyclosporine, FK506 and sirolimus on the secretion of GM-CSF, IL-6 and IL-8 by human PTEC.

4.1.7 The effect of immunosuppressive agents on FN production

The long-term administration of CNIs is known to produce histological changes similar to those seen in allograft fibrosis (Palestine *et al.*, 1984; Shihab, 1996). Cyclosporine directly stimulates renal scarring by increasing collagen production (Ghiggeri *et al.*, 1994) and by increasing the synthesis of transcription factors that promote TGF-β gene transcription (Khanna *et al.*, 1997). In various rodent models, studies of the molecular events that lead to renal fibrosis have uncovered three recurrent themes: a marked infiltration of monocyte/macrophage-derived cells, upregulated TGF-β1 expression and disturbances in ECM turnover caused by increased fibrogenesis and/or decreased fibrolysis (Eddy, 1996a). Distinct differences have been noted in the immunohistochemical localisation of TGF-β1 in PTEC with FK506 and sirolimus, suggesting that these agents may have different mechanisms of nephrotoxicity (Ninova *et al.*, 2004). This hypothesis requires further study.

Until recently, most research into cyclosporine-induced renal fibrosis has focused on ECM disturbances. In this thesis, I investigated the effects of cyclosporine, FK506 and sirolimus on FN production (as a marker of ECM) by PTEC.

4.1.8 The effect of immunosuppressive agents on the proliferation of PTEC

Under normal conditions, the proliferation of PTEC is uncommon. During acute renal failure, however, the severity of tubulointerstitial injury and the recovery of tubular function is subject to the ability of viable PTEC to enter the cell cycle and proliferate. The proliferation of PTEC, as measured by [^{3}H]-thymidine uptake into cell DNA, has previously been studied in relation to both cyclosporine and FK506 that inhibited cell

growth by 50% at 1μg/ml, but pharmacological doses were not toxic to renal cells (Blaehr *et al.*, 1993; Rezzani *et al.*, 2002). In this study, I sought to extend these findings to include sirolimus and to confirm these earlier results.

4.1.9 The use of lipopolysaccharide

LPS is a bacterial cell wall product that acts as an important mediator in the development of acute renal injury. Secondary to infection, by expressing high-output nitric oxide synthase, LPS exerts a cytotoxic effect that may cause tubular cell injury and, in some cases, acute renal allograft rejection. As discussed in Chapter 1, previous research has shown that LPS stimulates PTEC and provides a pathophysiological model of kidney injury.

4.1.10   Study objectives

In general, nephrotoxicity is the major concern regarding adverse effects of immunosuppressive drugs, especially the effects on PTEC. In this study I have set out to answer the following questions:

1. Do cyclosporine, FK506 or sirolimus alone, or in combination with LPS, affect Ig receptor expression on human PTEC?

2. Do cyclosporine, FK506 or sirolimus alone, or in combination with LPS, affect the release of cytokines by human PTEC?

3. Do cyclosporine, FK506 or sirolimus alone, or in combination with LPS, affect the production of FN by human PTEC?

4. Do cyclosporine, FK506 or sirolimus alone, or in combination with LPS, affect the proliferation of human PTEC?
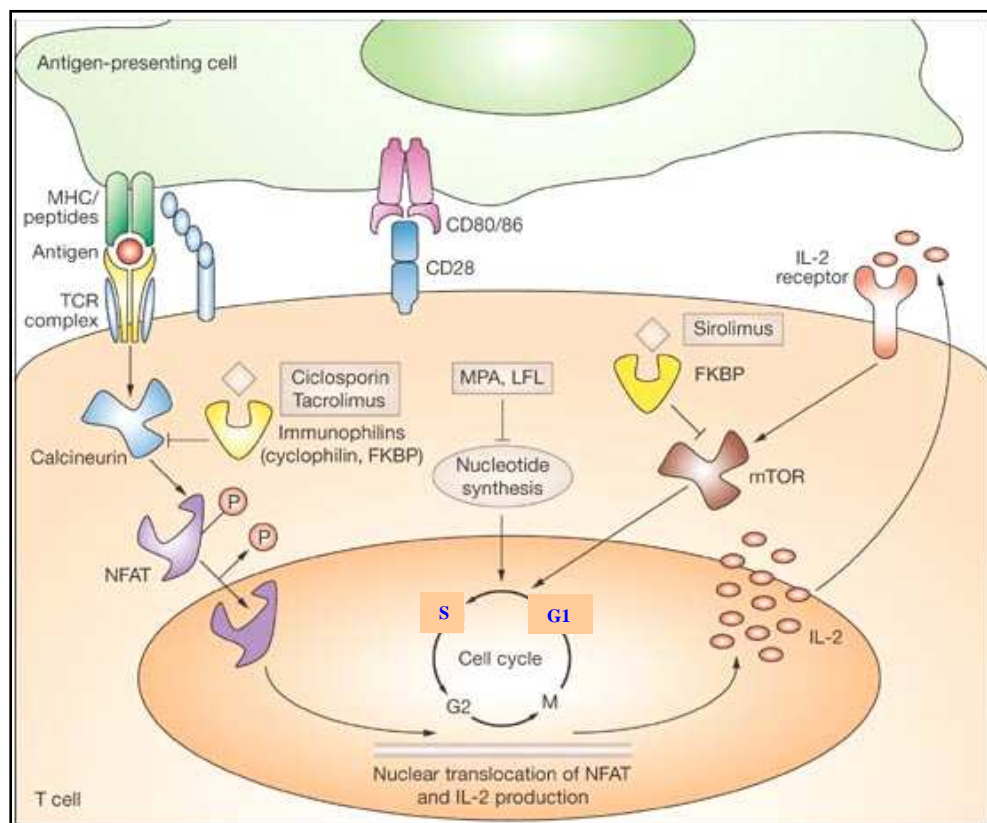
Figure 4-1 Mechanisms of action of maintenance immunosuppressive agents

Cyclosporine and tacrolimus bind to their respective immunophilins, and inhibit calcineurin. Calcineurin is then unable to dephosphorylate NFAT, which will prevent translocation of NFAT to the nucleus and thereby production of IL-2. Sirolimus is an mTOR inhibitor. It binds to FKBP and inhibits mTOR, which in turn inhibits transition of the cell cycle from G1 to S phase. MPA and LFL are also cell-cycle inhibitors, and act via inhibition of nucleotide synthesis. Abbreviations: CNI, calcineurin inhibitor; FKBP, FK506-binding protein; IL-2, interleukin-2; LFL, leflunomide; MHC, major histocompatibility complex; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; TCR, T cell receptor, Adapted from (Samaniego *et al.*, 2006).

4.2 Results

4.2.1 Do immunosuppressants or LPS, alone or in combination, affect Ig receptor expression on human PTEC?

In order to assess the extent to which immunosuppressive agents affect the mRNA expression of these receptors, I carried out a series of experiments with semi-qRT-PCR (as described in section 2.3.2) using specific forward and reverse primers for each receptor (Table 2-4). HK-2 cells were incubated for 24 hrs after treatment with one of the three study agents – cyclosporine, FK506 or sirolimus – with or without additional treatment of 10 µg/mL LPS. Doses were tested in serial ten-fold dilution ($0.01 \rightarrow 0.1 \rightarrow 1$ µM).

Treating the HK-2 cells with LPS alone led to a significant increase in the mRNA expression of all three of the Ig receptors studied FcRn, pIgR and Fcα/µR relative to the housekeeping gene β-actin, versus the untreated (control) HK-2 cells. The upregulation of pIgR mRNA was much greater than that of FcRn and Fcα/µR; pIgR expression was increased by 7.86-fold versus 1.55 and 1.95-fold for FcRn and Fcα/µR, respectively (Fig. 4-2–4).

When treated with immunosuppressants alone (i.e. without LPS), there were no significant changes in the mRNA expression of any of the Ig receptors studied, relative to β-actin, compared with the untreated controls. However, when LPS was applied alongside the immunosuppressants, a significant reduction was seen in the levels of pIgR mRNA expression, relative to β-actin, versus the untreated controls at the highest concentration only ($P<0.025$). The expression of FcRn and Fcα/µR mRNA was not significantly altered.

In these experiments I used U937 cells as a positive control when measuring FcRn expression, 200 ng/ml IFN-γ when measuring pIgR and 50 ng/ml IL-1α when measuring Fcα/µR. As expected, a significant increase was seen in all three conditions. U937 cells upregulated FcRN expression by 3.16-fold, IFN-γ upregulated pIgR expression by 11.24-fold and IL-1α increased Fcα/µR expression by 9.76-fold, relative to β-actin, versus the untreated controls ($P<0.005$ in all cases).

4.2.2 Do immunosuppressants or LPS, alone or in combination, affect the release of cytokines by human PTEC?

In section 3.3.11, I discussed how PTEC release the cytokines IL-6, IL-8 and GM-CSF in the supernatant of cultured HK-2 cells in basal conditions. Using the specific ELISA described in section 2.5.2.1, I screened the release of these cytokines under the same conditions following treatment with the immunosuppressants cyclosporine, FK506 and sirolimus at three different concentrations in a 10-fold serial dilution, as before. These experiments were conducted with and without LPS (10 μg/ml) stimulation after 24 hrs incubation in the supernatant.

When the HK-2 cells were treated with LPS alone, the release of IL-6, IL-8 and GM-CSF increased by approximately 1.5-, 1.8- and 2.0-fold, respectively ($P<0.017$ in all cases). Conversely, treating with immunosuppressants alone had no effect on the release of the above cytokines (Fig. 4-5–7).

Even at the highest concentration (1 μM), none of the three immunosuppressive agents (in combination with LPS) significantly were not inhibited the release of IL-6, IL-8 and GM-CSF. Any variation in cell number was corrected by assessing cellular protein as described in section 2.4.3.

4.2.3 Do immunosuppressants or LPS, alone or in combination, affect the production of FN by human PTEC?

Previously, in section 3.3.8, I demonstrated the way in which PTEC produce FN. In a series of experiments, I investigated whether applying cyclosporine, FK506 or sirolimus, with or without LPS, had an effect on the production of FN. Using a specific ELISA, as described in section 2.5.1. I measured the levels of FN after 72-hrs incubation with immunosuppressant agents either alone or in combination with LPS. TGF-β was used as a positive control.

My results show that LPS and TGF-β both increased the production of FN by PTEC compared with the untreated HK-2 cells. Treatment with immunosuppressants both alone and in combination with LPS attenuated the production of FN in a dose-dependent manner. In cells treated with immunosuppressants alone, an effect was seen only at the highest concentration (1 μM) (Fig. 4-8).

4.2.4 Do immunosuppressants or LPS, alone or in combination, affect the proliferation of human PTEC?

As described in section 2.4.10, I measured the proliferation of HK-2 cells according to their uptake of $[^3H]$-thymidine. After 48-hrs incubation in the supernatant, I investigated whether cyclosporine, FK506 and sirolimus, alone and in combination with LPS, had an effect on the proliferation of HK-2 cells.

As predicted in section 3.3.9, LPS alone showed an antiproliferative effect when compared with TGF-β1, which was used as a positive control. With or without LPS, the immunosuppressants significantly decreased the proliferation of treated HK-2 cells compared with untreated HK-2 cells. This reduction occurred in a dose-dependent manner (Fig. 4-9).

4.3 Discussion:

The importance of PTEC in the pathogenesis of kidney disease and injuries sustained during renal transplantation is widely accepted. Many studies have shown that PTEC release a number of inflammatory mediators that can lead to tubulointerstitial injury and, in some cases, to CAN (Daha&van Kooten, 2000; Hong *et al.*, 2002; Kuroiwa *et al.*, 2000; Zhou *et al.*, 2001). In combination with immunosuppressant-induced nephrotoxicity, the proinflammatory and profibrotic products of PTEC can ultimately lead to acute rejection episodes that may be fatal (Healy *et al.*, 1998; Hortelano *et al.*, 2000; Markewitz *et al.*, 1993; Ortiz *et al.*, 1998).

In this study, I investigated the *in vitro* effects of the immunosuppressants cyclosporine A, FK506 and sirolimus alone and in combination with LPS, as well as LPS alone, on FcRn, pIgR and Fcα/μR expression, cytokine release, FN production and the proliferation of PTEC. From a review of the literature, I observed that the variance in the concentrations of immunosuppressants used was large, ranging from 0.1–30 μM. Although it was generally acknowledged that concentrations higher than 10 μM were too toxic, as confirmed by my early experiments [data not shown], there was considerable disagreement on the optimal concentration for efficacy without nephrotoxicity. This variance may be explained by different study protocols, taking into account such factors as incubation time and cell culture, as well as experimental error. Based on this evidence, I opted to use a serial ten-fold dilution with a maximum concentration of 1 μM (0.01→0.1→1.0 μM) when applying the immunosuppressive agents to the cells.

The expression of the FcRn, pIgR and Fcα/μR by human PTEC was demonstrated previously in Chapter 3, which has important implications for the pathogenesis of IgAN. The development of renal injury could be accelerated or contributed to by IgA present in the glomerular filtrate that interact with and activate tubular cells, leading to local cytokine production and tubulointerstitial fibrosis. Using semi-qRT PCR, I was unable to show an effect of cyclosporine, FK506 or sirolimus on the expression of Ig receptor mRNA by PTEC. In observing that the expression of FcRn and Fcα/μR by PTEC did not differ when exposed to immunosuppressive agents with or without LPS, these findings favour a non-inflammatory role for these receptors in renal disease. However, when LPS was administered on its own, the expression of the above receptors was slightly increased.

A recent study by Kobayashi et al. confirmed the presence of FcRn on renal PTEC and proposed a functional role for the receptor in the reabsorption of IgG from tubular filtrate (Kobayashi *et al.*, 2002). In accordance with my findings, the investigators were unable to show a significant enhancement of FcRn transcript levels when cultured human PTEC were treated with known nephrotoxic immunosuppressants.

In comparison with FcRn, much less is known about the function of the novel receptor Fcα/μR in PTEC. A study by Macdonald et al. (2002) found that the expression of Fcα/μR by MSC was upregulated by treatment with IL-1α in a similar way to that seen in human PTEC. The enhanced expression of Fcα/μR in the tubules may be of significance in the pathogenesis of proteinuric IgAN where PTEC are exposed to filtered IgA.

In addition, there is evidence to suggest that LPS significantly increases the upregulation of Fc receptor expression (specifically FcγR and FcαR) in certain cells, for example PMN and monocytes (Lazaron *et al.*, 2001; Palermo *et al.*, 1997; Refici *et al.*, 2001; Shen *et al.*, 1994). This evidence appears to confirm my finding that LPS increases the expression of FcRn, pIgR and FcαμR in PTEC. The interactions between LPS-binding protein and Fc receptor at the cell surface results in inflammatory cytokine release and internalisation and detoxification of LPS (Lazaron *et al.*, 2001).

My investigations into the expression of Ig receptors by PTEC in response to treatment with immunosuppressive agents were limited as I was unable to determine whether the receptors had an effect at the cell surface in PTEC. Failure to detect an effect on the expression of Ig receptors may be due to an altered cell phenotype; it is possible that the cells differentiated at a different stage and so would not have responded to treatment. Alternatively, it is possible that only a subgroup of patients, differentiated by genetic and/or other factors, functionally express the Ig receptors. By identifying this subgroup, it may be possible to determine which patients will develop progressive disease and which patients are susceptible. In addition, the use of immortalised cell lines *in vitro* may give different results to those of *in vivo* experiments. Using primary cultures of human PTEC may be vital in order to improve our understanding of the molecular mechanisms of progressive tubulointerstitial diseases and lead to more specific treatments (Border&Noble, 1997; Lipkowitz *et al.*, 1996). The possibility of unfavourable experimental conditions must also be considered, e.g. the incubation period, temperature and concentrations of the drugs.

There is little available data on the nephrotoxic effect of immunosuppressive agents on the expression of Ig receptors by PTEC, and their molecular mechanisms are incompletely defined. As I was unable to show a significant effect of cyclosporine, FK506 or sirolimus on Ig receptors, further research is needed to develop a greater understanding of how these receptors are expressed on PTEC and to develop new immunosuppressants that may offer potential new strategies for the prevention and treatment of nephrotoxicity. Clarification of the ways in which tubular cells express Ig receptors should contribute to new hypotheses regarding the physiological and pathological renal response to kidney disease and transplantation injury.

Cyclosporine, FK506 and sirolimus have been described to exert a strong inhibitory effect on the immune response to renal allografts (Jain&Ridker, 2005; Kino *et al.*, 1987; Liu *et al.*, 1991). Using a specific ELISA, my early experiments in Chapter 3 showed that the cytokines IL-6, IL-8 and GM-CSF were released by PTEC in significantly large quantities to warrant further, more detailed investigations. My results show that the amounts of IL-6, IL-8 and GM-CSF produced by unstimulated PTEC and PTEC stimulated by immunosuppressive agents alone do not significantly differ, despite a slight reduction in the production of IL-8 and GM-CSF at high doses (1 µM). The absence of a significant reduction in the release of IL-6, IL-8 and GM-CSF by PTEC following treatment with immunosuppressive agents is in contrast to the findings of earlier studies (Halloran, 1996; Kunz&Hall, 1993). In some studies, an impressive reduction of >50% in the secretion of IL-6 by U937 cells was observed when treated with high-concentration cyclosporine (200 ng/ml or ~0.166 µM) (Garcia *et al.*, 1999; Moutabarrik *et al.*, 1993).

LPS is known to produce a global, nonspecific activation signal. In my experiments, I used LPS as a model of kidney injury in an attempt to evaluate the full potential of PTEC (Bank *et al.*, 1998; Bian *et al.*, 1999; Brauner *et al.*, 2001). The response to LPS has been shown to be important to the innate immune response. Several reports have indicated regulation of LPS by urinary proteins and various cytokines, of which PTEC can produce a large number, and these observations have been linked to a pathogenetic role of LPS in various diseases (Vink *et al.*, 2002; Xu *et al.*, 2001).

Following stimulation of PTEC by LPS, there was a large increase in the secretion of all three cytokines. None of the three immunosuppressive drugs significantly inhibited cytokine release, despite a slightly greater reduction with FK506 and sirolimus than with cyclosporine. FK506 is estimated to be approximately 100 times more potent than cyclosporine in suppressing cytokine production by PTEC through inhibition of the

calcineurin-dependent pathway, which may explain the lack of any effect with cyclosporine, even with the addition of LPS. These findings are in contrast with those of (Kino *et al.*, 1987; Liu *et al.*, 1991; Sasakawa *et al.*, 2000).

In vivo, PTEC are exposed to a wide variety of cytokines and other mediators, which may prime the cells for enhanced function (Sean Eardley&Cockwell, 2005; Zheng *et al.*, 2005). If the elevated expression of certain cytokines by PTEC from stimulated subjects with immunosuppressive agents is due to *in vivo* priming, then the priming mechanism may be selective for certain cytokines. The lack of a significant reduction in cytokines seen in my results is in disagreement with the observations of previous studies suggesting that any interruption in PTEC activation by immunosuppressive agents leads to downstream inhibition of cytokine expression and T-cell proliferation (Halloran, 1996; Kunz&Hall, 1993). However, the pathogenetic factors of chronic nephrotoxicity are not well understood. More research is needed to examine in further detail the impact of immunosuppressive drugs on the release of cytokines, which is important in the development of nephrotoxicity and the risk of CAN.

Evidence suggesting a possible link between fibrosis and proliferation was first put forward by Kuze et al. (Kuze *et al.*, 1997). According to the methods described in Chapter 2, I assessed the synthesis of FN by PTEC and the proliferation of these cells *in vitro*. My results show that both the production of FN and the proliferation by PTEC stimulated with immunosuppressive agents, either alone or in combination with LPS, are inhibited in a dose-dependent manner. However, it is important to notice that while the proliferation of PTEC is affected by the use of immunosuppressants at all concentrations, the production of FN is only significantly reduced at the highest concentration (1 μM). At this level, it is likely that the cytotoxicity in the cell was enough to prevent the production of FN.

Alternatively, the reduction in FN seen in my results may be due to the loss of epithelial adhesion, disruption of the tubular basement membrane and enhanced cell migration and invasion due to immunosuppressant-induced epithelial-mesenchymal transition (EMT) (Liu, 2004; McMorrow *et al.*, 2005; Yang&Liu, 2001). Rastaldi *et al.*, (2002) and Slattery *et al.*, (2005) reported that, at therapeutic doses, cyclosporine is known to provoke EMT in PTEC and this causes large amounts of ECM to be released by myofibroblasts that migrate into the interstitium. As a result, fibrosis may develop and this in turn may provoke further EMTs in neighbouring epithelial cells. The migration of myofibroblasts caused by cyclosporine has been shown *in vitro* and *in vivo* to be reduced by antibodies that target TGF-β1 (Healy *et al.*, 1998).

My *in vitro* experiments clearly showed that clinically relevant concentrations of the study drugs were directly toxic to human PTEC, as evidenced by reduced cellular FN production and growth function. TGF-β is a potent stimulus for the synthesis of ECM proteins (including collagen, FN and proteoglycans (Ignotz *et al.*, 1987)) in various cell types (Border&Noble, 1994) and appears to play a key role in this *in vitro* model of tubulointerstitial diseases, raising the possibility that more specifically targeted therapies to reduce fibrogenic cytokine activity within the tubulointerstitium may improve the ability to prevent disease progression following nephrotoxic injury (Johnson *et al.*, 1999).

An important study by Wolf et al. suggested that the endogenous stimulation of TGF-β1 by cyclosporine mediates the antiproliferative effect of the immunosuppressant (Wolf *et al.*, 1995). The authors proposed that cyclosporine-mediated intrarenal synthesis and release of TGF-β1 may play a role in the cyclosporine-induced growth arrest and might therefore be relevant in the development of chronic nephrotoxicity (AyanlarBatuman *et al.*, 1991; Li *et al.*, 1991; Wolf *et al.*, 1995). Perhaps it would have been useful to extend the results of Wolf et al. to include LPS alone and in combination with FK506 and sirolimus. In my experiments, LPS alone increased the production of FN by PTEC, indicating that the ability of bacterial cell wall products to alter FN production may account for renal fibrosis (Adachi *et al.*, 1996; Kitamura *et al.*, 1995).

In my experiments, cyclosporine, FK506 and sirolimus were shown to have an antiproliferative effect on PTEC. These observations indirectly suggested that the treatment with the study drugs caused significant reductions in DNA synthesis in a dose-dependent manner, as indicated by lower [^{3}H]-thymidine uptake. This concurs with the findings of other *in vitro* studies that have shown that immunosuppressive agents inhibit growth factor-stimulated cell progression, prevent cell division and reduce DNA synthesis, thereby exerting an antiproliferative effect in PTEC and MSC (Blaehr *et al.*, 1993; Sehgal, 1998; Sun&Wang, 1997; Wann *et al.*, 1992).

I observed that cyclosporine and FK506 reduced [^{3}H]-thymidine uptake into renal cells in a dose-dependent manner, whereas sirolimus gave a stronger inhibition of PTEC responses. Whether this response was a specific effect of the immunosuppressants or a result of cytotoxicity is still unknown. This observation is in agreement with that of Kim et al. (Kim *et al.*, 2000) and with studies performed on the tubular cell line LLC-PK1 (Moutabarrik *et al.*, 1991). In my study, the dose-dependent reduction of proliferation was similar to the results reported by (Radeke *et al.*, 1993). However, the mechanism by which this occurs is still unknown.

There are two possible mechanisms by which immunosuppressive agents may inhibit $[^3H]$-thymidine uptake. The first is that the drugs may cause a blockade in the G0/G1 phases of the cell cycle, thereby inhibiting the proliferation of PTEC and slowing the recovery of the kidneys after acute ischaemia; the second possibility is that PTEC lose their brush border *in vitro*. In a model using immortalised rat PTEC, even short-term use of cyclosporine caused loss of the brush border and dilation of the proximal tubules, causing a reduction in tubular reabsorption rate (Burdmann *et al.*, 2003).

In reference to the first possible mechanism, Healy et al. (Healy&Brady, 1998) observed dose-dependent elevations in the proportion of cells in the G0/G1 phases of the cell cycle using flow cytometry following exposure to cyclosporine. In addition, cyclosporine induced glomerular synthesis of ROS, such as hydrogen peroxide, which damages DNA (Parra *et al.*, 1998). Enhanced generation of intracellular ROS may represent an alternative explanation for the growth-inhibitory mechanism of immunosuppressive agents. A recent study by (Lieberthal *et al.*, 2001) used an experimental model of acute ischaemic renal failure to show that even though the use of sirolimus delays recovery of glomerular filtration rate and the regeneration of PTEC, renal function does eventually return, even if the patient continues treatment with sirolimus. The authors propose that this recovery of function against the odds is indicative of an "adaptive" response by PTEC characterised by the development of resistance to sirolimus. They also speculate that this resistance is limited to the renal tubular cells and does not involve the immune system, thereby providing a potential explanation for the relative lack of nephrotoxicity associated with sirolimus (Lieberthal *et al.*, 2006). In my study, LPS was shown to reduce the proliferation of PTEC at the effective concentration of 10 μg/ml and, when given in combination with immunosuppressive agents, reduces the proliferation further. Thus, it can be concluded that LPS in combination with immunosuppressive agents had a more potent effect on the reduction of PTEC proliferation than LPS alone. Further experimental and clinical studies should be conducted to determine the exact relationship between FN production, TGF-β1 secretion and the proliferation of PTEC cells.

Although it was beyond the scope of this project, it would have been interesting to observe the effect of cyclosporine, FK506 and sirolimus on apoptotic cell death of PTEC. However, in addition to the many papers on PTEC apoptosis already in the published literature, a large amount of research is currently underway in this area. The main limitation of the present study is the inherent difficulties in extrapolating the results of an *in vitro* study to *in vivo* effects in treated patients. As cyclosporine and sirolimus are lipophilic compounds, their respective concentration might be higher in the cellular

membrane than in blood. It is therefore difficult to ensure that *in vitro* concentrations we used have an *in vivo* relevance.

In summary, I have investigated the role of cyclosporine, FK506 and sirolimus on susceptibility to nephrotoxicity in cultured human PTEC. My results inferred that immunosuppressive agents alone have a non-inflammatory response on Ig receptor expression. However, in combination with LPS, the study drugs showed a slight inhibitory effect on the expression of the pIgR on human PTEC at the highest concentration. This finding may offer potential new strategies for the prevention and treatment of drug-induced nephrotoxicity. My results failed to show any significant inhibition of cytokine release with the study drugs, even at the highest concentration. However, the pathogenetic factors of chronic nephrotoxicity are not yet fully understood. More research is needed to examine in further detail the impact of immunosuppressants on the release of cytokines, which has important implications for inflammation, the development of nephrotoxicity and the risk of CAN. Cyclosporine, FK506 and sirolimus were shown to inhibit both the production of FN and the growth of PTEC, thereby exerting an antiproliferative effect. However, the inhibition achieved at the concentrations used in this study did not reach statistical significance. As always, a major limitation of *in vitro* studies such as this is that the relevance of the findings may not extend to the *in vivo* condition in treated patients.
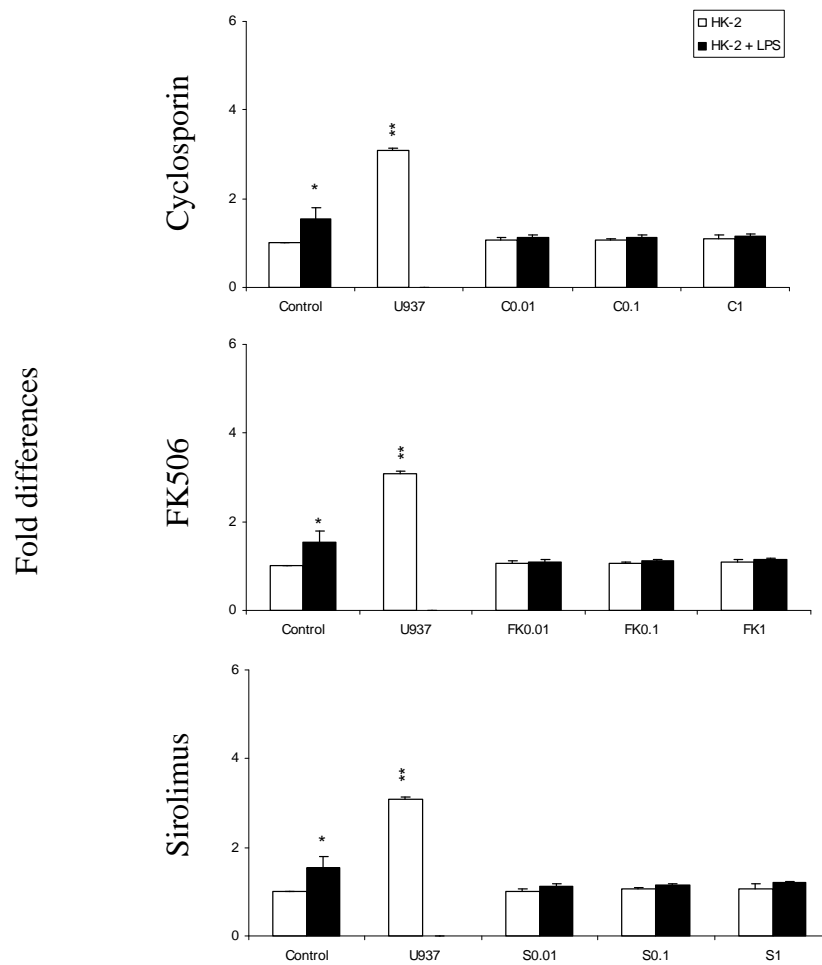
Figure 4-2 Effect of immunosuppressive agents on FcRn expression on PTEC

The fold differences of expression of FcRn receptor measured by semi-qRT-PCR in relation to control treatment with immunosuppressive agents, with and without LPS for 24 hrs in HK-2 cells. The amplification of FcRn was relative to β-actin gene, which was used as a house-keeping gene. LPS alone shows a slight increase in expression 1.55 ± 0.252 fold ($*P<0.025$). The result showed there is no significant change in expression with immunosuppressive agents alone or in combination with LPS in a dose-dependent manner. The positive control used to compare the expression were U937 cells, which were up-regulated 3.16 ± 0.042 fold ($**P<0.005$). This graph was representative of two experiments and triplicate reactions at each condition, which were amplified along the iCycler iQ Real-time PCR system. Error bars show the ($\pm$ SD). Standard curve had r = 0.990, slope= -3.452, PCR efficiency = 94.8%. ($*$) Comparison between control and U937 cells, and also with LPS).
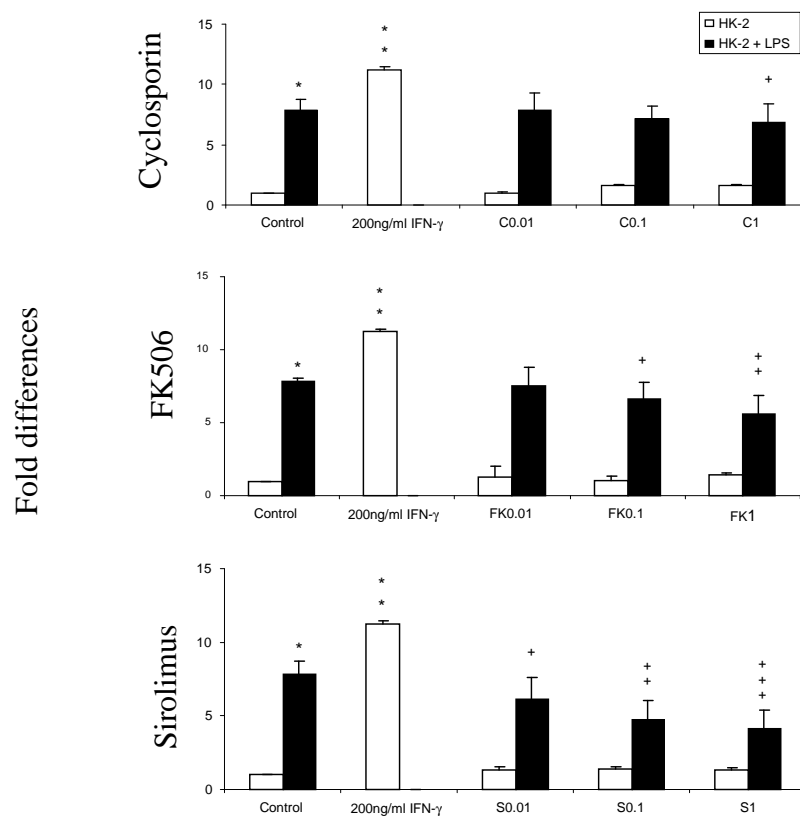
Figure 4-3 Effect of immunosuppressive agents on pIgR expression on PTEC

The fold differences of expression of pIgR receptor measured by semi- qRT-PCR in relation to control treatment with immunosuppressive agents, with and without LPS for 24 hrs in HK-2 cells. The amplification of the pIgR was relative to β-actin gene which was used as a house-keeping gene. LPS alone shows an increase in expression 7.86±0.858 fold (∗P<0.025). The result showed there is no significant change in expression with immunosuppressive agents alone. In combination with LPS/immunosuppressive agents that was showed a significant decrease in expression with FK506 and sirolimus and cyclosporine A at the highest dose of 1 µM. The positive control was used to compare the expression of pIgR in HK-2 cell line and was up-regulated by 200 ng/ml IFN-γ 11.24±0.195 fold (∗∗P<0.005). This graph was representative of two experiments and triplicate reactions at each condition, which were amplified along the iCycler iQ Real-time PCR system. Error bars show the (± SD). Standard curve had r = 0.997, slope= -3.772, PCR efficiency = 100.5%. (∗) Comparison between control and HK-2 cell lines treated with either 200 ng/ml IFN-γ or LPS). (+) Comparison between LPS alone and LPS in combination with immunosuppressive agents: + P<0.025).
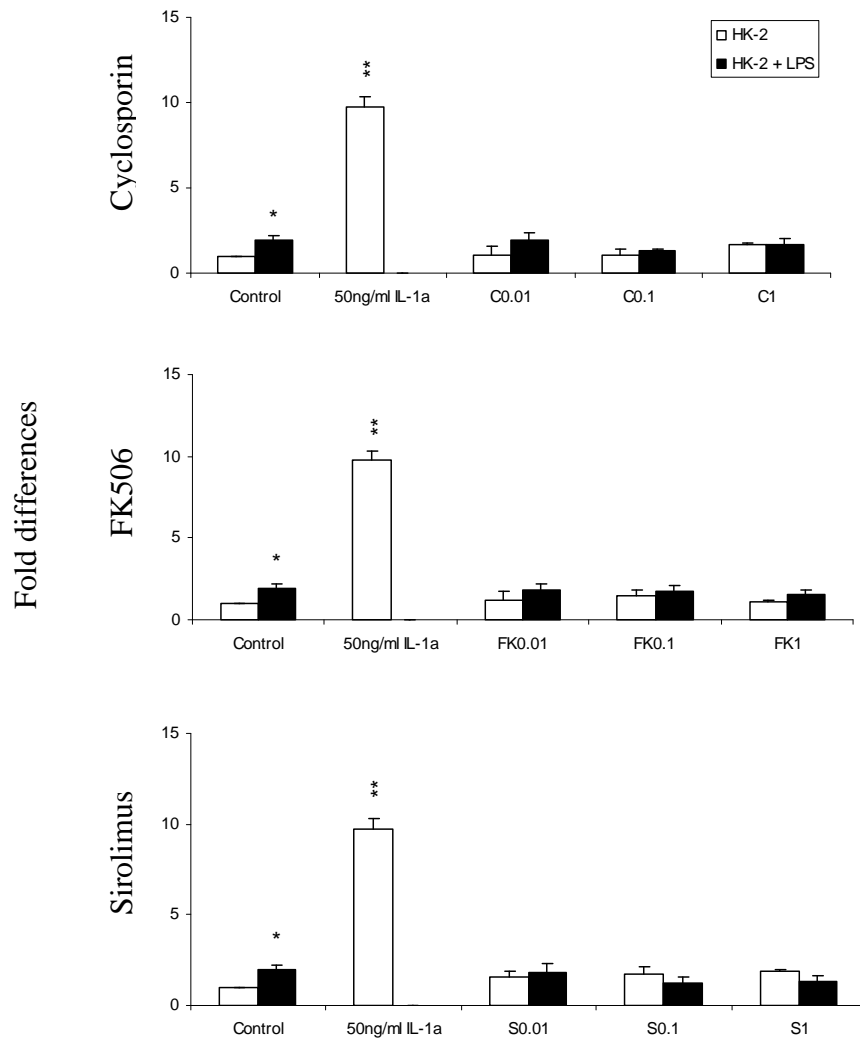
Figure 4-4 Effect of immunosuppressive agents on Fcα/μR expression on PTEC

The fold differences of expression of Fcα/μR receptor measured by semi- qRT-PCR in relation to control treatment with immunosuppressive agents with and without LPS for 24 hrs in HK-2 cells. The amplification of Fcα/μR was relative to β-actin gene, which was used as a house-keeping gene. LPS alone showed a slight increase in expression 1.95±0.252 fold (∗P<0.025). The result showed that there is no significant change in expression with immunosuppressive agents alone or in combination with LPS in a dose-dependent manner. The positive control was used to compare the expression of Fcα/μR in HK-2 cell line and were up-regulated 50 ng/ml IL-1α 9.76±0.563 fold; (∗∗P<0.005). This graph was representative of two experiments and triplicate reactions at each condition, which were amplified along the iCycler iQ Real-time PCR system. Error bars show the (± SD). Standard curve had r = 0.988, slope= -2.928, PCR efficiency = 119.6%. (∗) Comparison between control and HK-2 cell lines treated with either 50 ng/ml IL1-α or LPS).
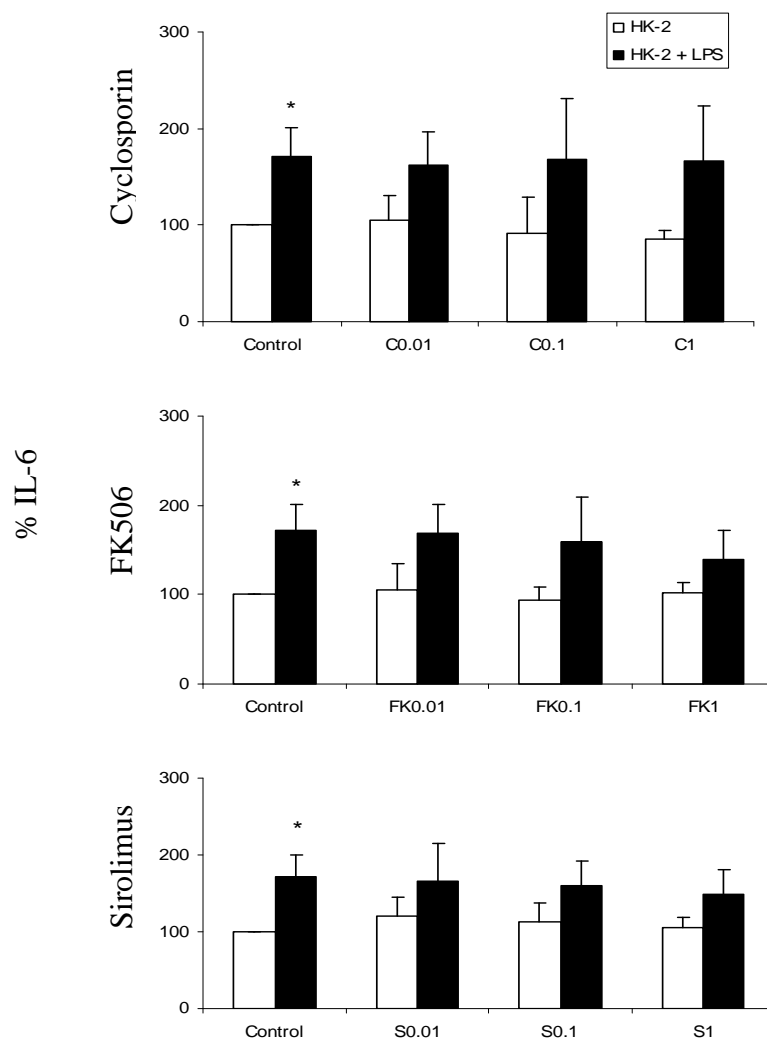
Figure 4-5 Effect of immunosuppressive agents on IL-6 production by PTEC

Dose-dependent induction of IL-6 by immunosuppressive agents with and without LPS in cultured supernatant HK-2 cells for 24 hrs expressed as a percentage of control. Level of IL-6 was measured by ELISA. None of the three immunosuppressive agents showed significant changes in IL-6 production. LPS stimulated cytokine production but this was not significantly inhibited. Media alone was used as a negative control and LPS as a positive control. These data represent three measurements in three separate experiments (n=9). Error bars show the (± SD) (∗P<0.017). (∗) Comparison between control treated and untreated with LPS.
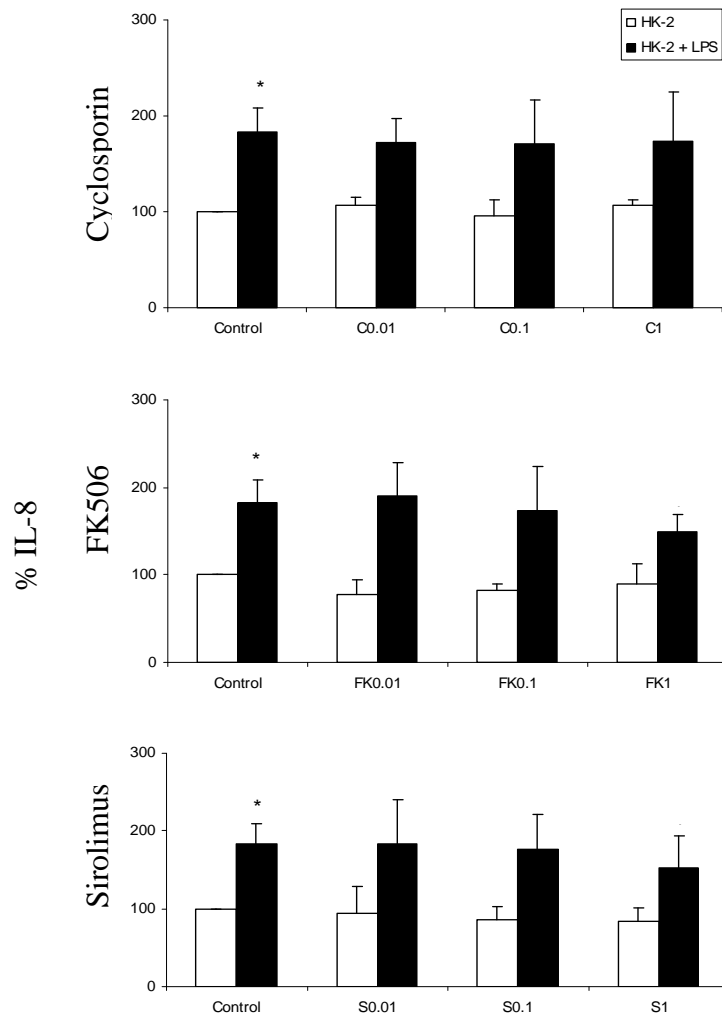
Figure 4-6 Effect of immunosuppressive agents on IL-8 production by PTEC

Dose-dependent induction of IL-8 by immunosuppressive agents with and without LPS in cultured supernatant HK-2 cells for 24 hrs expressed as a percentage of control. Level of IL-8 was measured by ELISA. None of the three immunosuppressive agents significantly reduced IL-8 production. LPS stimulated cytokine production but this was not significantly inhibited. Media alone was used as a negative control and LPS as a positive control. These data represent three measurements in three separate experiments (n=9). Error bars show the (± SD) (∗ P<0.017). (∗) Comparison between control treated and untreated with LPS.
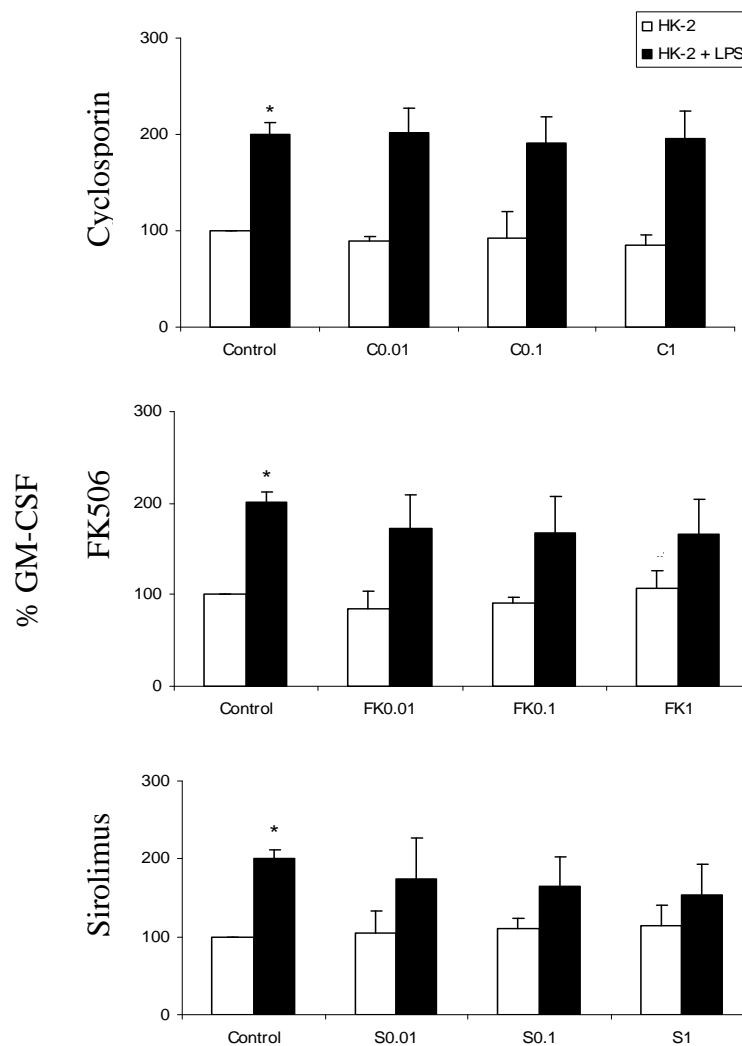
Figure 4-7 Effect of immunosuppressive agents on GM-CSF production by PTEC

Dose-dependent induction of GM-CSF by immunosuppressive agents with and without LPS in cultured supernatant HK-2 cells for 24 hrs expressed as a percentage of control. Level of GM-CSF was measured by ELISA. None of the three immunosuppressive agents significantly reduced GM-CSF production. LPS stimulated cytokine production but this was not significantly inhibited. Media alone was used as a negative control and LPS as a positive control. These data represent 3 measurements in three separate experiments (n=9). Error bars show the (± SD) (∗P<0.017). (∗) Comparison between control treated and untreated with LPS.
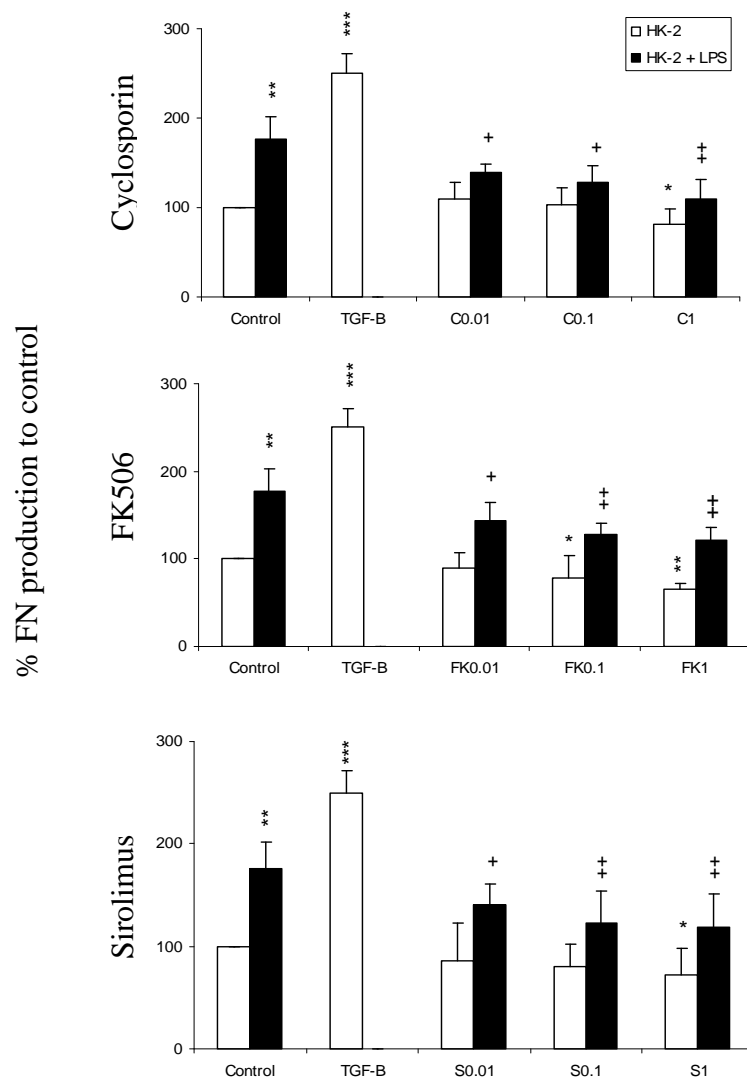
Figure 4-8 Effect of immunosuppressive agents on FN production by PTEC

Effect of immunosuppressive agents with and without LPS on FN production. Cells were treated with LPS and immunosuppressive agents respectively in a dose-dependent manner for 72 hrs and expressed as a percentage of control. FN production was measured by using specific ELISA. TGF-β1 was used as a positive control. LPS and TGF-β1 alone increased FN production, whereas immunosuppressive agents alone or incombination with LPS reduced FN production in a dose-dependent manner. Data are shown as the mean of four experiments performed in duplicate (n=8). Error bars show the ($\pm$ SD) ($*P<0.0125$, $**P<0.0025$, $***P<0.00025$; $+P<0.0125$, $++P<0.0025$). ($*$) Comparison between control and cells treated with either TGF-β or LPS. (+) Comparison between LPS alone and LPS in combination with immunosuppressive agents).
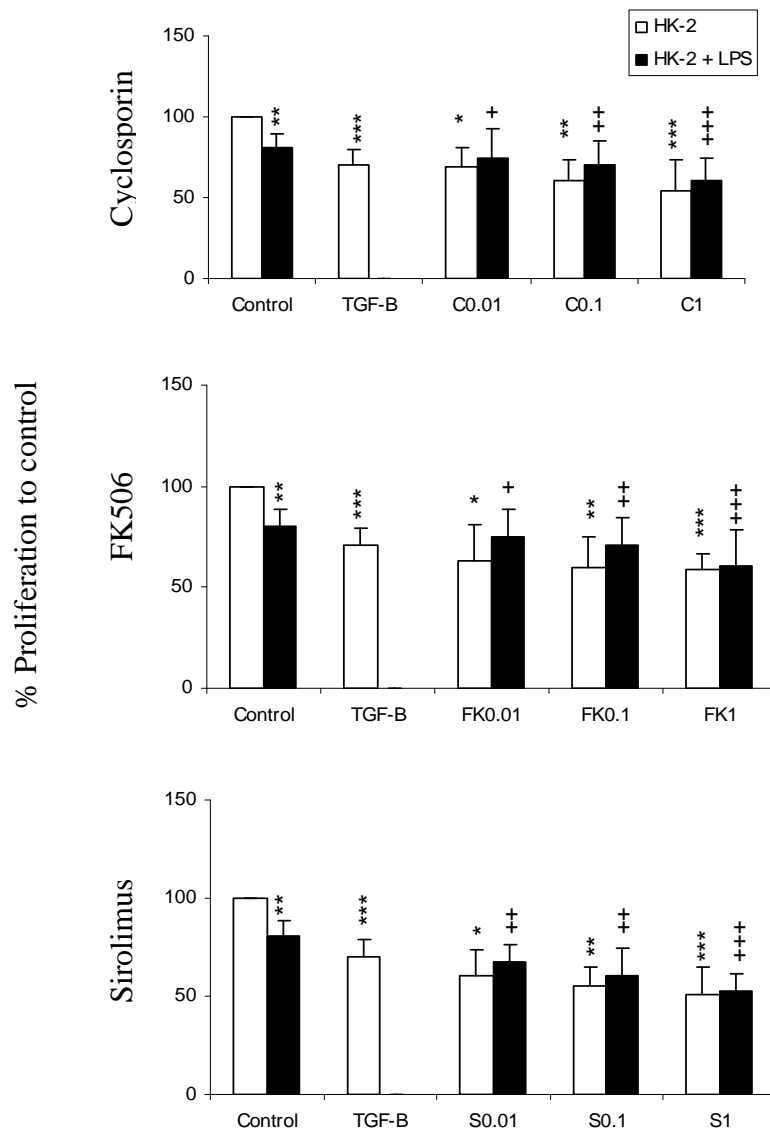
Figure 4-9 Effect of immunosuppressive agents on proliferation of PTEC

Effect of immunosuppressive agents alone and in combination with LPS on HK-2 cell proliferation measured using [3H]-thymidine uptake for 24 hrs, expressed as a percentage of control. Cells treated with immunosuppressive agents with and without LPS showed an inhibitory effect on proliferation in a dose-dependent manner. HK-2 culture media was used as a negative control and TGF-β1 as a positive control. Data shows the mean of two experiments performed in replicates of 12 (n=24). Error bars show the (± SD) (∗ $P<0.025$, ∗∗ $P<0.005$, ∗∗∗ $P<0.0005$: + $P<0.025$, ++ $P<0.005$, +++ $P<0.0005$). (∗) Comparison between control treated and untreated with TGF-β and/or LPS. (+) Comparison between LPS alone and LPS in combination with immunosuppressive agents).

Chapter 5: Effects of Statins on Human PTEC

5.1 Introduction

5.1.1 Background

As seen in previous chapters, the degree of tubulointerstitial injury in human GN is associated with progression to ESRF, and that the underlying mechanisms behind this are not yet fully understood. We know that it involves infiltration of the nephron by inflammatory mediators and the overproduction of ECM, and we have seen that PTEC have a potential role in the development of tubular injury, not only as a target in themselves but also through their ability to release cytokines and produce ECM components such as FN.

In this chapter I will discuss the properties and efficacy in kidney disease of 3-hydroxy, 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, otherwise known as statins. Statins have been described as the principal and the most effective agents for reducing serum cholesterol levels (LaRosa, 2002). Moreover, statins have been shown to have diverse effects on the immune system, and because of this they have significant potential in the treatment of autoimmune disease (Gurevich *et al.*, 2005; Sherer&Shoenfeld, 2002; Steffens&Mach, 2004; Zamvil&Steinman, 2002). Currently, these agents are best known for their use in preventing atherosclerosis. They work by lowering plasma lipids in patients at increased cardiovascular risk, and by doing so reduce both mortality and morbidity rates (Baigent *et al.*, 2005). Indeed, so popular have they become in this therapeutic area that they are now prescribed to more than 25 million people worldwide, and this number appears to be constantly increasing.

Due to their relatively good safety profile and oral administration, statins are viewed in an attractive light and much research has been conducted to discern the properties of these agents. Extensive clinical and laboratory studies have provided compelling evidence that statins are able to modulate a significant number of pathophysiological pathways beyond lipid metabolism (Jain&Ridker, 2005; Laufs&Liao, 2003; Palinski&Tsimikas, 2002; Takemoto&Liao, 2001). These so-called 'pleiotropic' properties of statins include anti-inflammatory, anti-proliferative and immunomodulatory processes that may be beneficial in treating (predominantly slowing) progressive renal disease (Agarwal&Curley, 2005; Buemi *et al.*, 2002; D'Amico, 2006). However, whilst the ability of statins to attenuate experimental inflammatory disease is impressive, it has yet to be determined which statin-modified pathway is most important in yielding improved clinical outcomes (Greenwood

et al., 2006). In experimental models, the anti-lipaemic effects of statins have been shown to be independent of all other actions of these agents (Muso *et al.*, 1994; Phanish *et al.*, 2006). In humans, however, proving the dissociation of the effects of statins on lipid metabolism from their other actions has proven difficult (Heusinger-Ribeiro *et al.*, 2004).

Whilst there is certainly a large body of evidence to recommend the use of statins in kidney disease, reports have emerged that statins can (indirectly) cause renal damage. However, in these cases, the damage is typically due to rhabdomyolysis-associated acute tubular necrosis and not a direct effect of the statins themselves. In one case, however, the severity of disease appeared to be dose-related (van Zyl-Smit *et al.*, 2004). Although tubular injury may be slight and hard to detect, cumulative injury may result in significant renal damage over a period of years.

In my thesis, I examined the effects of simvastatin 0.01–10 µM and fluvastatin 0.01–10 µM on PTEC in response to activation by LPS 10 µg/ml. In particular, I looked at the effect of statins on the expression of Ig receptors on PTEC, the release of cytokines by PTEC, FN production by PTEC and the proliferation of PTEC.

5.1.1.1 Cholesterol pathway

Cholesterol is necessary for the synthesis of steroid hormones, vitamin D, bile acids and lipoproteins, and the maintenance of intracellular levels is important. In patients with hypercholesterolaemia, the reduction of intracellular cholesterol upregulates low density lipoprotein (LDL) receptors and aids the clearance of LDL from plasma.

The inhibition of HMG-CoA reductase intervenes at the rate-limiting step in cholesterol synthesis, namely the conversion of HMG-CoA into mevalonate (Massy&Guijarro, 2001). The mevalonate pathway branches out before the synthesis of squalene and cholesterol (Figure 5-1), leading to other biologically important products such as dolichols (involved in the synthesis of lipoproteins), ubiquinone (or Coenzyme Q) and the isoprenoids, farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP) (Palinski&Tsimikas, 2002) Non-sterol pathway enzymes have a higher affinity for mevalonate substrates than sterol pathway enzymes and are thus favoured in the presence of sterols, which suppress the production of squalene synthetase and limit the incorporation of mevalonate into further sterols.

5.1.1.2 Chemistry and (structural/functional) properties of statins

There are two types of statin: fungal-derived (simvastatin, lovastatin and pravastatin) and fully synthetic (fluvastatin, atorvastatin, cerivastatin, pitavastatin and rosuvastation) (Davidson, 2002).

Statins may also be defined according to whether they are lipophilic (fluvastatin, simvastatin, atorvastatin and lovastatin) or hydrophilic (pravastatin and rosuvastatin) (McTaggart *et al.*, 2001; McTavish&Sorkin, 1991). In contrast with their hydrophilic counterparts, which have been shown to substantially reduce the risk of cardiovascular disease (Sacks *et al.*, 1996; Shepherd *et al.*, 1995), studies with the lipophilic statins have shown a modest reduction in cardiac events, despite aggressive lowering of cholesterol (Herd *et al.*, 1997; Waters *et al.*, 1994).

5.1.1.3 Pharmacokinetic properties of statins

As stated above, I concentrated on simvastatin and fluvastatin in my experiments. In the clinical setting, simvastatin is given as a lactone pro-drug that is then hydrolysed *in vivo* into the active hydroxy acid form (Corsini *et al.*, 1995), while fluvastatin is administered in its active form directly (Corsini *et al.*, 1999; McTaggart *et al.*, 2001). All statins are absorbed rapidly following administration, reaching peak plasma concentration within 4 h (Cilla *et al.*, 1996; Tse *et al.*, 1992). With respect to inhibition of HMG-CoA reductase, all statins are relatively hepatoselective (the extent of which is determined by their solubility). This is particularly important as most endogenous production of cholesterol takes place in the liver.

Researchers have shown that hydrophilic statins are distributed more selectively in hepatic cells than lipophilic ones. Because the membrane of extrahepatic cells consists of lipid bilayers, hydrophilic statins cannot penetrate it, and thus cannot reach the intracellular enzyme; however, the hepatic cell membrane contains organic anion transporters, which take hydrophilic substances into the cell. Cellular entry of such molecules is therefore dependent on active uptake by these transporters (Inui *et al.*, 2000; Kusuhara&Sugiyama, 2002), which, *in vivo*, are mainly present on the basolateral side of the renal epithelium (Inui *et al.*, 2000). Conversely, lipophilic statins (e.g. simvastatin, fluvastatin) can enter extrahepatic and hepatic cells. Thus, lipophilic statins could inhibit not only cholesterol synthesis but also production of essential substances via the HMG-CoA reductase reaction in many extrahepatic tissues (Hamelin&Turgeon, 1998; Ichihara&Satoh, 2002).

In a large clinical trial programme, 2.9% of patients receiving either simvastatin 10–80 mg or fluvastatin 10–40 mg developed side effects necessitating withdrawal from the study (Brewer, 2003; Schachter, 2005). Only a small number of patients developed proteinuria, and this was usually transient and reversible and not associated with long-term renal damage (Brewer, 2003). Treatment-associated serious adverse events were rare and were reported in less than 1% of patients. There were no treatment-related deaths.

Other studies have reported an apparent higher propensity for statins to cause tubular injury at high doses. Statins may reduce the reabsorption of proteins *in vivo* when prenylation of proteins involved in endocytosis is reduced, and when HMG-CoA reductase is sufficiently inhibited in PTEC, leading to proteinuria (Verhulst *et al.*, 2004).

5.1.1.4 Immunomodulatory effects/mechanisms of action of statins

The lipid-independent anti-inflammatory effects of statins appear to be regulated by interference in the synthesis of mevalonate metabolites and by inhibition of the NF-κB-dependent regulation of inflammation (Steiner *et al.*, 2005). Many of the pleiotropic effects of statins have been linked to the isoprenylation of regulatory proteins (Hall, 1998); by covalently binding to these proteins, isoprenoids create lipid-binding sites that regulate the intracellular trafficking and membrane binding of these proteins (Kimoto-Kinoshita *et al.*, 2000). For example, blocking the synthesis of GGPP with statins upregulates the expression of endothelial cell nitric oxide synthase (eNOS) and reduces geranylation of Rho (Laufs&Liao, 2003; Laufs *et al.*, 1998).

Much more than simply cholesterol by-products, isoprenoids have an important role in the post-translational modification of regulatory proteins, including the Ras superfamily guanosine triphosphate hydrolase enzymes (GTPases, or 'small G proteins') Ras, Rho and Rab (Hillyard *et al.*, 2004a; Khwaja *et al.*, 2006), which play a critical role in regulating cell proliferation and differentiation (Barbacid, 1987; Kocher *et al.*, 2005). Considering the central role these proteins have in determining cell function, it follows that they can modify the immune response independently of their anti-lipaemic effects.

One of the key determinants of effective treatment is the inhibition of macrophage recruitment and function by reducing the expression of proinflammatory mediators, including NF-κB and MCP-1 (Park *et al.*, 1998b; Sukhova *et al.*, 2002; Yokota *et al.*, 2003). By blocking the NF-κB signalling pathway, statins prevent the release of downstream proinflammatory mediators (Dichtl *et al.*, 2003) and the upregulation of the

MCP-1 gene (Eddy&Giachelli, 1995; Klahr&Morrissey, 2002). NF-κB is typically activated when the endoplasmic reticulum of cells becomes congested with viral proteins, initiating a fast and effective antiviral response. A more general mechanism of activation has been proposed, namely that protein stress in the endothelium upregulates NF-κB target genes, such as MCP-1, and may involve other gene products (Pahl&Baeuerle, 1995). The chemokine RANTES, a NF-κB-dependent gene, is known to be expressed by renal PTEC and is known to be produced following the stimulation of PTEC by TNF-α, IL-1α, LPS or aggregated IgG (Heeger *et al.*, 1992; Satriano *et al.*, 1997; Wolf *et al.*, 1993).

Recent reports have suggested that statins are also able to inhibit the expression of MHC-II on PTEC co-stimulated by IFN-γ (Palinski, 2000). The ability of statins to downregulate MHC-II expression may lead to reduced Th1 cell proliferation and inhibition of proinflammatory cytokine release (Kwak *et al.*, 2000). However, it has also been proposed that statins may induce the opposite effect by reducing Th2 proliferation, causing a shift towards a Th1 immune response (Figure 5-2) (Palinski, 2000).

5.1.1.5 Lipid rafts

The concept of lipid rafts was first introduced in 1972 by Singer and Nicolson, whose seminal review proposed that the outer layers of plasma membranes contain specialised lipid microdomains ('rafts') within which proteins are randomly interspersed and are free to move about (Lopez *et al.*, 2005). These lipid membranes are primarily composed of cholesterol and sphingolipids, and are consequently thicker and less fluid than the other layers (Brown&London, 2000) (Figure 5-3). There are two types of lipid raft – caveolar and non-caveolar – and these co-exist in distinct areas of the membrane (Wang&Paller, 2006). Both caveolar and non-caveolar rafts are enriched in cholesterol. Thus, depletion of cholesterol from cell membranes is an effective means of breaking down rafts (Li&Nord, 2004).

Lipid rafts play a role in a number of important biological processes; they help to modulate signal transduction, membrane trafficking, cytoskeletal organisation and motility, polarisation and pathogen entry (including viruses, pathogenic bacteria and parasites) (Pike, 2005; Rajendran&Simons, 2005). With regard to signalling, rafts are associated with a large number of membrane receptors, particularly Fc receptors and T and B cell antigen receptors (Pierce, 2002; Xavier *et al.*, 1998). In addition, rafts play important roles in nearly every stage of haemostasis and thrombosis.

5.1.2 The effects of statins on renal disease progression

Although the bulk of statin research has focused on their actions in cardiovascular indications, experimental models have shown that these agents also possess a number of beneficial effects in renal disease (Park *et al.*, 2000; Vieira *et al.*, 2005). The concept of lipid nephrotoxicity first emerged in 1982 and appears to share many of the characteristics of atherosclerosis (Moorhead *et al.*, 1982). Progressive renal failure occurs once some critical threshold of injury has been reached, characterised by proliferative and sclerotic processes that worsen glomerular and tubulointerstitial injury, regardless of the initial insult (Avram, 1989). Thus, there is potential for considerable renal benefits deriving from the anti-lipaemic properties of statins.

However, to fully understand the mechanisms of the renal benefits of statins, we must also look at their lipid-independent actions. As well as reducing oxidative stress, some studies have suggested that statins are able to inhibit the proliferation of PTEC. Moreover, the immunomodulatory and anti-inflammatory properties of statins may also modify the course of renal disease, independent of cholesterol reduction (Chang *et al.*, 2002; Palinski&Tsimikas, 2002; Park *et al.*, 2000). Clinical and pharmacokinetic studies have demonstrated that statins are well tolerated and effective, even in patients who have CRF and are on maintenance dialysis (Lins *et al.*, 2003; Saltissi *et al.*, 2002).

A protective effect of statins has been reported in experimental models of renal ischaemia–reperfusion injury (Gueler *et al.*, 2002; Yokota *et al.*, 2003). In one such study, the anti-inflammatory effects of statin therapy directly improved post-ischemic ARF, reducing cell infiltration and inhibiting the activation of NF-κB and MAP kinase (Gueler *et al.*, 2002). In a typical response to ischaemia–reperfusion injury, FN is overproduced in the renal interstitium (Zuk *et al.*, 2001); however, statin pre-treatment has been shown to modulate this process and inhibit the subsequent assembly of ECM.

The susceptibility to injury of renal PTEC is significantly increased when the cells are cholesterol depleted (Beutler, 2004). In various *in vivo* experimental models of renal injury, PTEC cholesterol content was shown to increase by 20–25%. This phenomenon suggests that cholesterol protects the renal tubules from superimposed attack (sometimes referred to as 'acquired cytoresistance'). Normalising PTEC cholesterol levels reverses this cytoresistance, suggesting that intracellular changes in cholesterol levels are a spontaneous correlate of *de novo* ischaemic and toxic renal injury.

Overall, statins appear to have important potential in the treatment of progressive renal diseases, such as IgAN, although further studies are required to confirm this in humans (Buemi *et al.*, 2002). Clinical trials indicate that the pleiotropic effects of statins may make them useful as immunosuppressive agents in transplant recipients (Masterson *et al.*, 2005; Steffens&Mach, 2006; Tuncer *et al.*, 2000). However, the impact of statin treatment on renal graft outcome needs to be examined in greater detail.

5.1.3 The effects of statins on proteinuria

As described previously, it is pertinent to remember that statins have been linked to renal damage as well as renal benefit. In high doses, all statins have the potential to cause tubular degeneration and proteinuria, especially in the short-term (Agarwal, 2004).

As seen in previous chapters, proteinuria is an important factor in progressive renal disease; protein reabsorption triggers pro-fibrogenic and pro-inflammatory genes in the kidney, possibly through the activation of PTEC. When challenged with albumin and IgG, PTEC basolaterally release the chemokine RANTES, which may contribute to the infiltration of inflammatory cells seen in CKD. Albumin uptake in PTEC is governed by receptor-mediated endocytosis (RME) (Agarwal, 2004). Provided that prenylated GTPases are present, RME of proteins in the proximal tubules occurs through megalin and cubulin receptors. By decreasing the activity of mevalonate, and thereby inhibiting the synthesis of FPP and GGPP, statins reduce the availability of prenylated GTPases and, consequently, impair RME.

Sidaway et al. confirmed the hypothesis that statins impair RME by reducing prenylation of GTP binding proteins in cell cultures of PTEC derived from the opossum kidney (Sidaway *et al.*, 2004). Not only was RME reduced in a dose-dependent manner, this reduction was not related to binding of albumin to the receptor but receptor-mediated albumin endocytosis (which was shared by other proteins such as β2-microglobulin). Moreover, the extent to which RME was inhibited was related to the *in vitro* inhibition of HMG-CoA reductase, which is dependent upon the lipophilicity of the statin. Thus, statins reduce albumin uptake by RME and therefore lead to proteinuria.

Exposure of cultured PTEC to albumin is also reported to cause cellular activation via the JAK/STAT signalling pathways (Nakajima *et al.*, 2004). The STAT family currently consists of seven known members (in mammalian cells) (Darnell, 1997), and can be activated by a large number of cytokines, growth factors, ROS and more (Herrington *et al.*,

2000; Ihle, 2001; Simon *et al.*, 1998). Activation of the JAK/STAT pathway appears to be mediated by PTEC production or ROS in response to albumin challenge.

These observations support my hypothesis that PTEC respond to the presence of excess or abnormal filtered protein by activating specific cellular signal transduction pathways that lead, in turn, to the local production of inflammatory mediators and contribute to tubulointerstitial injury.

Statins are unique in that they have the potential to increase proteinuria in the short term, but reduce proteinuria (as well as inflammation, fibrosis and renal disease progression) in the long term. Whether or not these effects are renoprotective or toxic cannot yet be determined.

5.1.4 The effects of statins on Ig receptor expression

Following on from Chapter 3 in which I showed how LPS activation could be used as a model of tubular injury, I investigated the potential beneficial effects of statins with regard to Ig receptor gene expression on PTEC. In Chapter 3, I showed that PTEC express FcRn, PIgR and Fcα/µR, all of which are relevant to the pathogenesis of renal injury, and that the gene expression of these receptors is upregulated in renal cells by proinflammatory cytokines (IL-1α, IFNγ, TGF-β1) and bacterial LPS. There do not appear to be any published studies on the effect of statins on Ig receptor gene expression on PTEC.

5.1.5 The effects of statins on cytokine release

In the present study, I examined the effects of statins on cytokine release by PTEC in response to activation by bacterial LPS. Statins have previously been reported to inhibit monocyte production of TNF-α and IL-8 (Grip *et al.*, 2000; Romano *et al.*, 2000), while a recent study showed that fluvastatin and simvastatin inhibited the FcγR-coupled release of IL-6, IL-8, IL-10 and MMP-1, with associated inhibition of the ERK and p38 (Hillyard *et al.*, 2004a). An anti-inflammatory effect was also indicated by increased IL-6 expression in an experimental model of renal ischaemia–reperfusion injury after statin therapy, which correlated with a protective effect (Deslypere *et al.*, 1990).

5.1.6 The effects of statins on FN production

Tubulointerstitial fibrosis is caused by unbalanced ECM production and proteolysis and is recognised as one of the main prognostic factors for renal outcome in GN and tubulointerstitial nephropathy (Risdon *et al.*, 1968). TGF-β1 is known to play a key role in the development of renal fibrosis (Border&Noble, 1997) by stimulating the proliferation of interstitial fibroblasts and signalling them to secrete ECM (Border&Noble, 1994; Sharma&Ziyadeh, 1994). As well as their marked anti-inflammatory properties, statins are anti-fibrotic, perhaps as a result of interference in the renal fibrosis process. In an animal study, statins were shown to reduce progressive glomerulosclerosis and tubulointerstitial fibrosis in different models of renal injury (Vrtovsnik *et al.*, 1999). Indeed, further studies are necessary to clarify the role of statins on fibrosis in cultured PTEC and on how this activity relates to cell proliferation.

5.1.7 The effects of statins on proliferation of PTEC

Cell number abnormalities resulting from an imbalance between cell birth and death can lead to various disorders with either too many cells (e.g. proliferative GN) or too few (e.g. renal atrophy) (Ortiz *et al.*, 2001). *In vitro* studies have shown that statins can inhibit proliferation in smooth muscle cells, MSC and PTEC, independent of their anti-lipaemic effects (Corsini *et al.*, 1993; Lan, 2003; Massy *et al.*, 1997). The addition of mevalonate or its isoprenoid metabolites was able to at least partially reverse this inhibition. *In vivo* models of GN have also shown that statin therapy attenuates neointimal thickening and MSC proliferation, independent of cholesterol levels, suggesting that the *in vitro* findings may extend to animal models (Nabeshima *et al.*, 1997; Soma *et al.*, 1993).

Some of the benefits of statin therapy may be accounted for by a reduction in renal interstitial inflammation and tubular activation. Isoprenylated proteins are necessary for cell cycle progression and for controlling cell growth (Agarwal&Curley, 2005); however, a study of simvastatin showed a significant antiproliferative action on PTEC (Vieira *et al.*, 2005), while another study showed that simvastatin was able to block high density lipoprotein (HDL) stimulation of proteins and monolayer resistance in PTEC (Buemi *et al.*, 2002). Thus, there is a clear need for more research in this area.

Earlier in this chapter, I discussed how statins interact with Ras monomeric GTPases. Recent studies have shown that these small G proteins play a critical role in cell

proliferation and may therefore represent potential targets in renal therapies (Hendry&Sharpe, 2003; Khwaja *et al.*, 2000; Pruitt&Der, 2001; Sharpe *et al.*, 2000).

5.1.8 Uses of LPS with statins

As we have seen, PTEC are capable of producing a large number of pro-inflammatory mediators, including chemoattractants, cytokines and ECM components. Several reports have indicated the regulation of LPS by various cytokines, urinary proteins and molecules in PTEC, and these observations have been linked to a pathogenetic role of LPS in various renal diseases (Vink *et al.*, 2002; Xu *et al.*, 2001). Renal inflammation increases circulating cytokine and chemokine levels, leading to nephrotoxic and post-ischaemic ARF (Bonventre&Zuk, 2004; Friedewald&Rabb, 2004; Ramesh&Reeves, 2002), and potentially to ARF-associated multi-organ failure (Zager *et al.*, 2007). As discussed in Chapter 1, LPS is an appropriate model for the renal inflammation involved in ARF.

Recent studies have identified TLR4 as the signalling receptor for LPS (Kawai&Akira, 2005; Wolfs *et al.*, 2002). However, despite good evidence to suggest that, in ARF, the kidney over-responds to TLR ligands (Zager *et al.*, 2006), the manner in which tubular cell injury directly impacts on the expression of TLR proteins is as yet unclear. When incubated with both statins and LPS together, an inhibition of LPS-mediated activation of human peripheral mononuclear cells and endothelial cells has been noted (Rice *et al.*, 2003; Zeuke *et al.*, 2002). This observation suggested that statins attenuated LPS effect.

5.1.9 Study objective

In my experiments with statins, I set out to answer the following questions:

1. Do statins alone or in combination with LPS affect Ig receptor expression on human PTEC? If yes, do they act synergistically or do they antagonise each other?

2. Do statins alone or in combination with LPS affect the release of cytokines by human PTEC? If yes, do they affect each other's influence when used together?

3. Do statins alone or in combination with LPS affect the production of FN by human PTEC?

4. Do statins alone or in combination with LPS affect the proliferation of human PTEC?
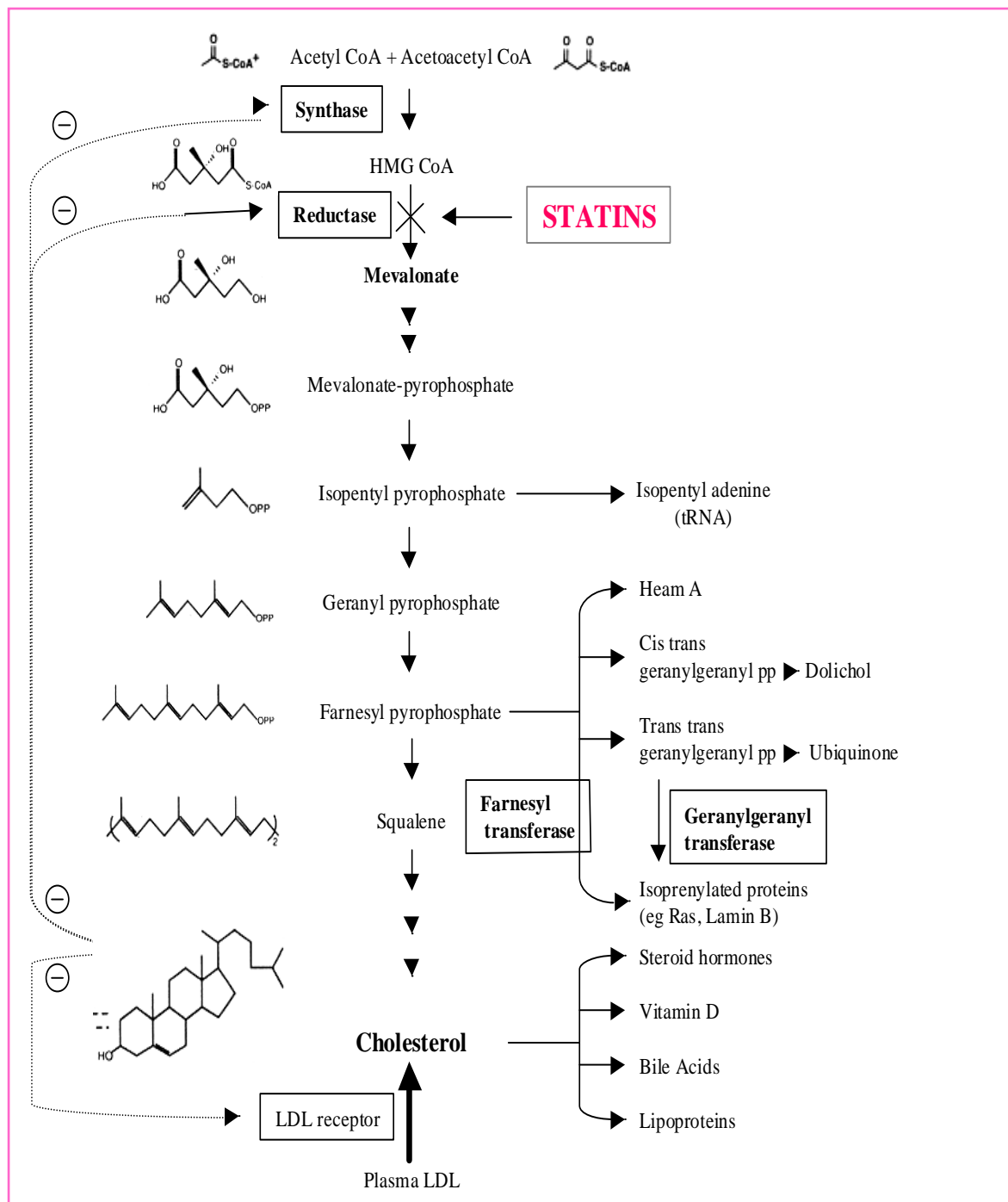
Figure 5-1 Cholesterol metabolic pathway.

The schematic representation of the cholesterol biosynthetic pathway includes a number of cholesterol by-products including dolicholphosphate and ubiquinone. Adapted from (Corsini *et al.*, 1999).
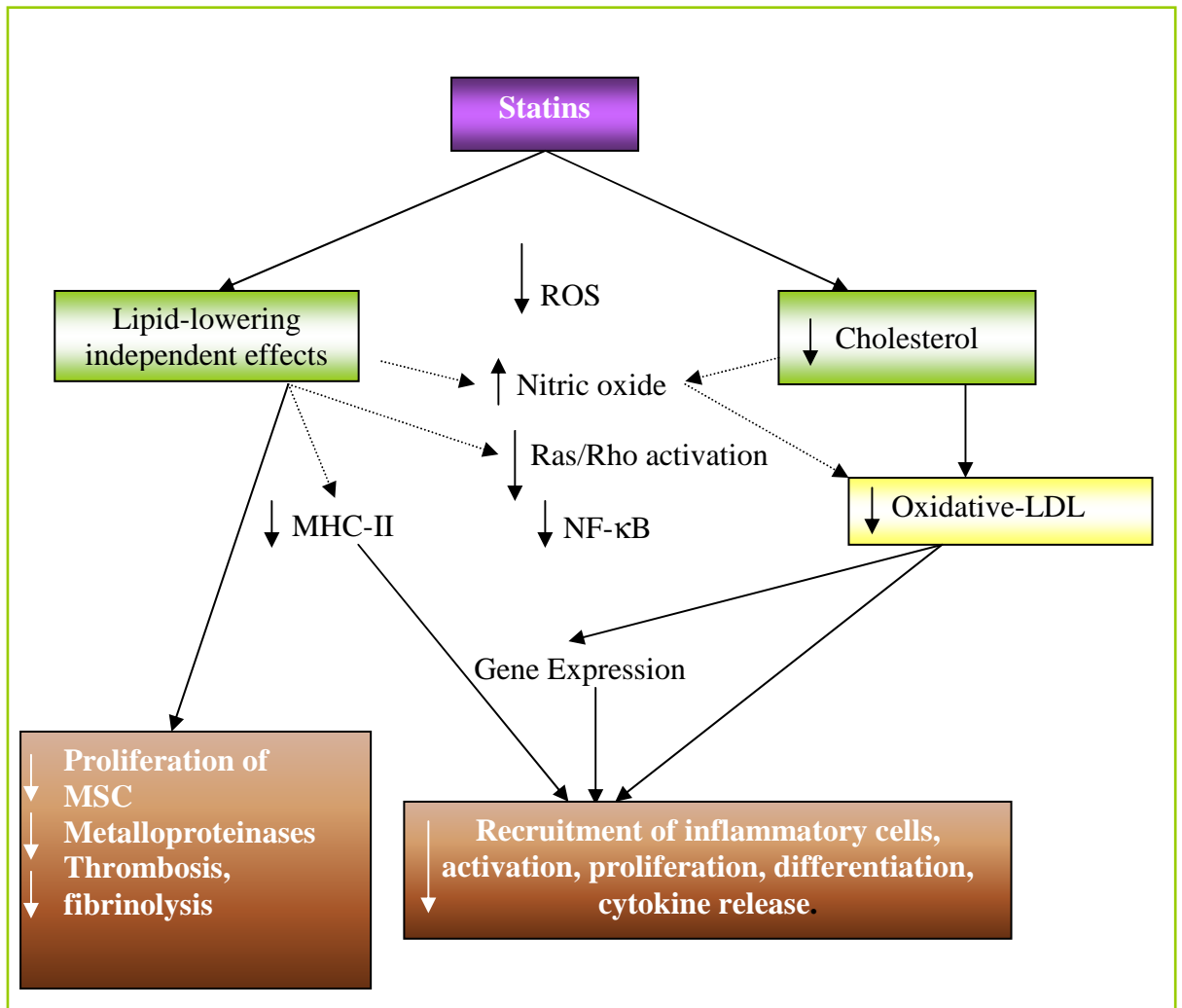
Figure 5-2 Pleiotropic effects of HMG-CoA reductase inhibitors.

Adapted from (Blanco-Colio *et al.*, 2003).
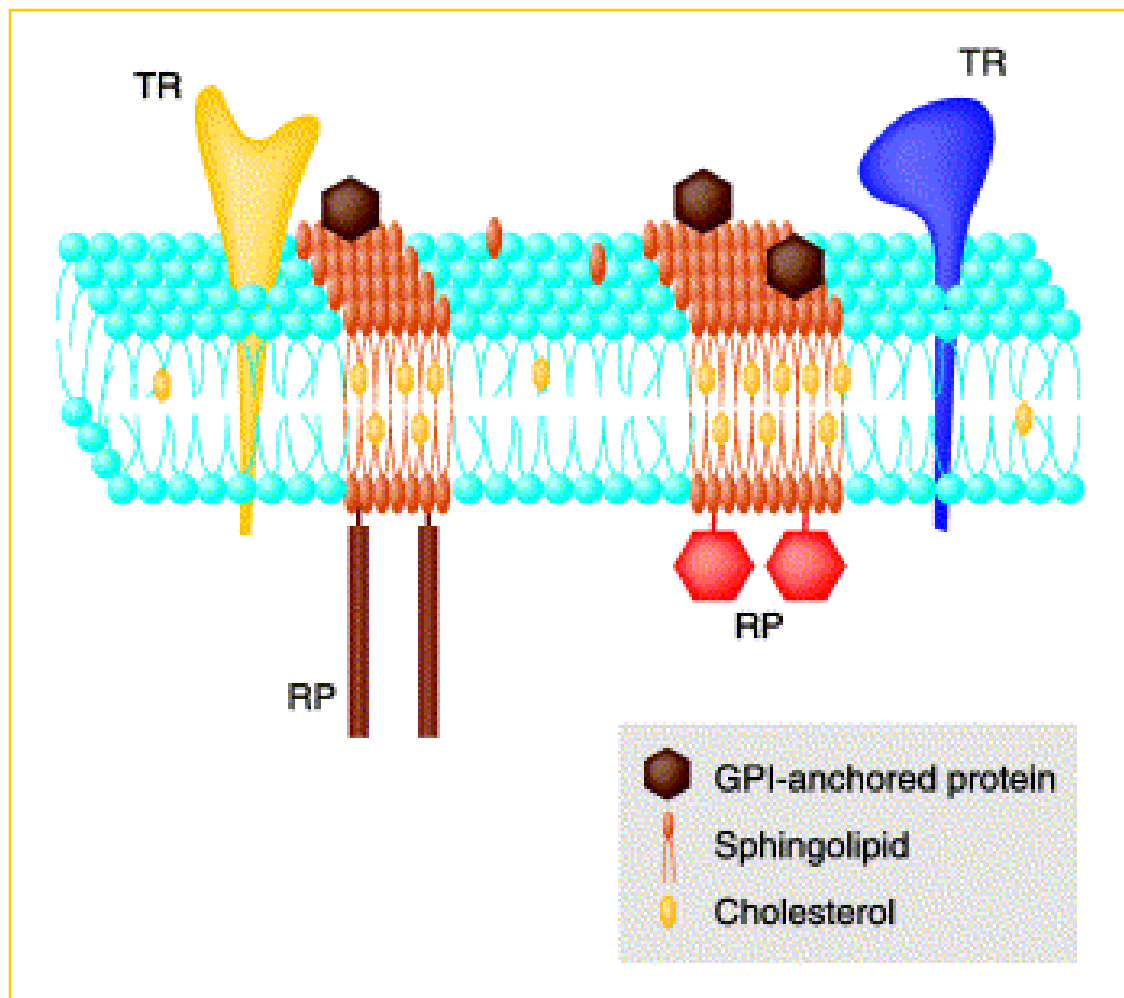
Figure 5-3 A model of lipid raft organisation

Lipid rafts present at the plasma membrane outer leaflet are lipid membrane microdomains that are highly enriched in tightly packed sphingolipids and cholesterol. The composition of the raft present at the plasma membrane inner leaflet is still not known. Among the raft-associated proteins (RPs), there are GPI-anchored proteins (such as CD52 and CD59) and proteins endowed with a lipid anchor composed of saturated acyl chains (such as Lck and Lyn in T and B cells, respectively). Transmembrane receptors (TRs) are usually raft-excluded. Adapted from (Pizzo&Viola, 2003).

5.2 Results

5.2.1 Do statins or LPS alone or statins in combination with LPS affect Ig receptor expression on human PTEC?

In section 3.3.2 I showed that Ig receptors FcRn, pIgR and Fcα/μR are expressed in immortalised HK-2 cells and primary PTEC. Therefore, I investigated the effect of simvastatin and fluvastatin, with and without LPS, on the mRNA expression of Ig receptors.

I investigated the mRNA expression levels of Ig receptors in immortalised HK-2 cells using semi-qRT-PCR (as described in section 2.3.2.1) with specific forward and reverse primers for each receptor, as shown in Table 2-4. HK-2 cells were incubated for 24 hrs following treatment with either statins or 10 μg/ml LPS, or a combination of statins with LPS, in a dose-dependent manner (0.01 μM, 0.1 μM, 1 μM and 10 μM). Cells treated with statins showed a dose-dependent increase in fold differences of the levels of FcRn, pIgR and Fcα/μR mRNA expression relative to β-actin, versus the untreated HK-2 cells.

Whilst treating with LPS alone led to an increase in the expression of mRNA for FcRn, pIgR and Fcα/μR, additional treatment with statins led to a further increase in the expression of these receptors, in a dose-dependent manner, so that the fold differences at higher concentrations were significant. U937 cells were treated as a positive control for the expression of FcRn. HK-2 treated with IFN-γ (200 ng/ml) and IL-1α (50 ng/ml) were used as positive controls for the expression of pIgR and Fcα/μR, respectively. The observed fold differences of Ig receptor expression were compared with untreated cells (control) relative to the housekeeping gene β-actin (Fig. 5-4).

5.2.2 Do statins or LPS alone, or statins in combination with LPS, affect the release of cytokines by human PTEC?

I showed in section 3.3.11 that PTEC release IL-6, IL-8, GM-CSF and TNF-α into the supernatant of cultured HK-2 cells. I assessed the levels of the IL-6, IL-8 and GM-CSF released by PTEC after treatment with simvastatin or fluvastatin, with and without LPS stimulation after 24 hrs, in the supernatant.

Using specific ELISAs for individual cytokines, as described in section 2.5.2.1, I showed that LPS alone increased the release of IL-6, IL-8 and GM-CSF by approximately two-fold. Conversely, statins inhibited the release of these cytokines in a dose-dependent manner. Furthermore, statins attenuated the effect of LPS on cytokine release in comparison with untreated cells (Fig. 5-5). The doses used are of clinical relevance, with peak plasma levels of approximately 1 μmol/L, and below the concentrations that are associated with apoptosis (Hillyard *et al.*, 2004a). The variation in cell number was corrected by assessing cellular protein, as described in section 2.4.3.

5.2.3 Do statins or LPS alone, or statins in combination with LPS, affect the production of FN by human PTEC?

I investigated the effect of simvastatin or fluvastatin, with and without LPS, and LPS alone, on FN production by PTEC. FN was measured after 72-hrs incubation of cultured HK-2 cells treated with statins, with and without LPS, and also after treating with LPS alone, using an ELISA as described in section 2.5.1. LPS increased FN production, though to a lesser extent than TGF-β1, which was used as a positive control (as described in section 3.3.8). Statins inhibited FN production and attenuated the affect of LPS on FN production in a dose-dependent manner. The percentage of FN production to untreated HK-2 cells is shown in Fig. 5-6.

5.2.4 Do statins or LPS alone, or statins in combination with LPS, affect the proliferation of human PTEC?

HK-2 cell proliferation was assessed using [^{3}H]-thymidine uptake, as described in Section 2.4.10. I investigated the effect of statins and LPS alone, and statins in combination with LPS, on cell proliferation after 48-hrs incubation. LPS showed an antiproliferative effect, though to a lesser extent when compared with TGF-β1.

Statins also showed an antiproliferative effect on HK-2 cells. This effect occurred in a dose-dependent manner in keeping with the pleiotropic effects of statins in other cells. LPS in combination with statins led to a dose-dependent reduction in cell proliferation in comparison with unstimulated HK-2 cells (Fig. 5-7). This result is in parallel with the earlier observations relating to cytokine release and FN production.

5.3 Discussion

The clinical benefit of statins is largely mediated by their effects on lipid metabolism, although there is growing evidence to suggest that these agents have pleiotropic effects that include anti-inflammatory, anti-fibrotic and immunomodulatory mechanisms of action. All four of these effects have potential to improve outcomes in renal disease and may even be renoprotective, though more research is needed to confirm this.

Currently, there are two proposed mechanisms by which these pleiotropic effects are mediated. First, the reduction of the isoprenoids FPP and GGPP in the cholesterol biosynthetic pathway; by decreasing the levels of these mevalonate derivatives, statins interfere with the isoprenylation of important signalling molecules, such as proteins of the Rho and Ras families (Boguski&McCormick, 1993; Laufs&Liao, 2003; Porter *et al.*, 2004; Scita *et al.*, 2000; Seabra&James, 1998; Sharpe&Hendry, 2003; Zerial&McBride, 2001), and therefore affect multiple cellular functions (Blanco-Colio *et al.*, 2003). Second, the disruption, or reduction, of cholesterol-rich lipid rafts (Dykstra *et al.*, 2001; Hillyard *et al.*, 2004a; Kono *et al.*, 2002; Lopez *et al.*, 2005); by reducing the density of these microdomains, statins limit the functions of localised receptors (Hillyard *et al.*, 2004a). The latter hypothesis, however, has received relatively little attention.

In addition to their complex phenotype, the actions of PTEC are numerous and include the enzymatic degradation of filtered proteins, the processing and expression of antigen and the expression of various transporters (Healy&Brady, 1998). Any small proteins or polypeptides reabsorbed from the tubular fluid may be catabolised by membrane digestion at the surface of the brush border membranes. Along with other cells in the renal tubules, PTEC are a known target for specific injury, including infection (both ascending and systemic) and drug toxicity leading to interstitial fibrosis and progressive renal disease (Healy&Brady, 1998; Nangaku, 2004; Ong&Fine, 1994). Although tubular injury may be caused by a number of agents, CNIs (cyclosporine A and tacrolimus) are associated with the greatest clinical impact.

Several factors complicate the evaluation of the immunomodulatory effects of statins, not least the fact that immune cells and their secretory products have a complex role in kidney disease and can sometimes exert renoprotective effects (Palinski&Tsimikas, 2002). There is a clear need for extensive *in vivo* studies to investigate the immune effects of statins and how they impact on renal diseases before we can expand the indication of these agents to chronic inflammatory and autoimmune diseases. The immunosuppressive actions of statins

have been proposed to extend to a reduction in the severity and frequency of acute allograft rejection episodes following kidney transplantation (Kwak *et al.*, 2000; O'Donnell *et al.*, 1995). Fluvastatin in particular has potential benefits in transplant recipients being treated with CNIs as these drugs are metabolised via different cytochrome enzyme pathways (Holdaas *et al.*, 2001; Jardine&Holdaas, 1999). However, there is a chance that, even when given at therapeutic doses, statins may increase or induce the effects of other drugs that affect cellular function (e.g. immunosuppressives) (Kakkis *et al.*, 1997; Kobashigawa *et al.*, 1995).

There is a growing body evidence to suggest that statins attenuate progressive renal disease (Baigent *et al.*, 2005; Jain&Ridker, 2005; Palinski&Tsimikas, 2002; Takemoto&Liao, 2001). For example, studies have shown that the anti-inflammatory effects of statins improved renal outcomes in a model of diabetes (Li *et al.*, 2004) and attenuated the fibrosis associated with cyclosporine nephrotoxicity (Usui *et al.*, 2003). In a mouse model of ARF, simvastatin was able to limit the severity of nephritis when given either before or at the time of disease induction (Christensen *et al.*, 2006). The course of established nephritis, however, remained unchanged.

Recent studies suggested that statins have the potential to reduce protein reabsorption *in vivo* providing there is sufficient inhibition of HMG-CoA reductase inhibitor in the PTEC and reduced prenylation of proteins involved in endocytosis (Verhulst *et al.*, 2004). This, in turn, may result in tubular proteinuria in some patients treated with high-dose statins. However, long-term statin treatment was associated with a reduction in proteinuria. No studies reported a statistically significant increase in proteinuria. Thus, statins have the unique potential to increase proteinuria in the short term, but causes less inflammation, less progression and less proteinuria over the long term. This is achieved by reducing RME, particularly in high doses, and may exert a renoprotective benefit (Verhulst *et al.*, 2004). Studies in humans are being conducted in order to verify the findings of the *in vitro* experiments of Sidaway et al and Verhulst et al. It has been recommended that renal function and protein and red blood cell excretion be monitored throughout statin therapy, especially at high doses and in patients with CKD (Agarwal, 2004).

Studies have shown that LPS is an important pathogenic factor in both acute and chronic renal disease, and so I chose this agent to stimulate PTEC in my experiments. It has been shown previously that bacteria can attach to PTEC and produce LPS, which stimulates immune host mechanisms. LPS binds to TLRs, and the upregulation of these receptors and their downstream signalling in monocytes are inhibited by simvastatin (Hillyard *et al.*,

2006; Methe *et al.*, 2005). In ARF, the loss of TLR4 either during or after injury may be considered a beneficial response as it restricts activity in the overstimulated TLR4–ligand pathway (Zager *et al.*, 2005; Zager *et al.*, 2006). As this response is unlikely to be specific, the benefit of statins seen in ARF may extend to other types of kidney disease, particularly those with recurrent episodes of inflammation, such as IgAN. In patients with IgAN, statins have the potential to limit the severity and number of disease exacerbations, and thereby slow the progressive loss of renal function.

It is important to note that although LPS primarily binds to serum proteins that stimulate proinflammatory pathways, the endotoxin is also bound by triglyceride-rich lipoproteins. Because statins expand LDL receptors, thereby promoting the internalisation of lipoprotein-bound endotoxin, an alternative hypothesis is provided for the anti-inflammatory actions of these agents (Spitzer&Harris, 2006); once internalised, fragments of lipoprotein-bound endotoxin affect proinflammatory cytokine signalling pathways and ameliorate the systemic inflammatory response.

To investigate the effects of statins on the inflammatory response, I conducted a series of experiments to assess how these agents affect the expression of Ig receptors on PTEC. As was previously shown in Chapter 3 using semi-qRT-PCR, PTEC activation increases the expression of FcRn, pIgR and Fcα/μR. Interactions between LPS-binding proteins and Fc receptors at the cell surface are known to cause the release of proinflammatory cytokines and the internalisation and detoxification of LPS (Lazaron *et al.*, 2001).

Some studies have reported that LPS upregulates the expression of pIgR mRNA and cell surface protein in intestinal epithelial cells, thus enhancing IgA-mediated homeostasis (Schneeman *et al.*, 2005), while other studies have shown that LPS affects FcγR and FcαR by significantly increasing Fc receptor expression in certain cells (e.g. PMN and monocytes) (Lazaron *et al.*, 2001; Palermo *et al.*, 1997; Refici *et al.*, 2001; Shen *et al.*, 1994). Following on from this, in Chapter 4 I showed that stimulation of PTEC by LPS increased the expression of FcRn, pIgR and FcαμR in these cells. I suggested that the upregulation of the transcription factor of LPS may be important in innate immune triggers, synergistically activating several cytokines and chemokines, including IL-6, IL-8, TNF-α and MCP-1 (Shen *et al.*, 1994).

In an investigation of the effects of simvastatin on FcγR expression and signalling, Hillyard found that the changes in lipid raft content failed to reflect either the total cellular or cell supernatant cholesterol levels (Hillyard *et al.*, 2004a). She therefore proposed that

the inhibition of cholesterol biosynthesis might reduce lipid levels within specific cellular compartments, independently of extracellular cholesterol. Moreover, it was suggested that this novel effect of fluvastatin might also be relevant for transmembrane receptors in tubular injury (Christensen *et al.*, 2006).

Using semi-qRT-PCR, I investigated the expression of FcRn, pIgR and FcαμR in cultured PTEC treated with statins (fluvastatin or simvastatin) and/or LPS 10 μg/ml. As expected, and in concordance with those of previous studies, my results showed that LPS alone increased the cellular expression of FcRn, pIgR and FcαμR. The mRNA levels of all three receptors increased in a dose-dependent manner in PTEC treated with statins alone or in combination with LPS, compared with unstimulated cells. These increases were only slightly greater than those seen when PTEC were treated with LPS alone. Simvastatin and fluvastatin increase receptor gene transcriptional activity either directly or via some transcriptional factors independent of the blockade of HMG-CoA reductase. This observation suggests that statin-mediated regulation of the gene expression of Ig receptors is a reflection of cell survival mechanisms in PTEC.

Previous studies have shown that statins are able to inhibit the expression of several cytokines, chemokines and acute-phase reactants (Diomede *et al.*, 2001; Gueler *et al.*, 2002; Ortego *et al.*, 1999; Park *et al.*, 1998b; Sukhova *et al.*, 2002; Yokota *et al.*, 2003). The majority of the effects of LPS on PTEC are mediated by cytokines produced by mononuclear phagocytes (Ballou&Lozanski, 1992). Recently, proinflammatory cytokines have been linked with the stimulation of transpithelial migration (TEM) of PMN through monolayers of PTEC, a phenomenon that occurs mainly through changes in the density of receptors on the cell surface of PTEC (Bijuklic *et al.*, 2006).

In Chapter 3, I showed that stimulated PTEC release a significant amount of IL-6, IL-8 and GM-CSF, in agreement with several existing studies (Ashizawa *et al.*, 2003; Brauner *et al.*, 2001; Gerritsma *et al.*, 1998a; Huang *et al.*, 2005; Leonard *et al.*, 1999; Li&Nord, 2002; Li&Nord, 2004). Moreover, it seems that these cytokines play a crucial role in the recruitment and activation of leukocytes in kidney disease. IL-6 is considered to be one of the most important proinflammatory cytokines with multiple effects in a wide range of conditions (Van Snick, 1990). IL-8 is considered to be the plasma expression of the immune response to acute-phase reactants, is implicated in several inflammatory diseases and is thought to be a direct pathogenetic mediator of renal disease (Panichi *et al.*, 2006). GM-CSF mRNA has been shown to be upregulated in diseased renal cells using quantitative analysis of the mRNA transcripts of PTEC-released cytokines (Frank *et al.*,

1993). In a study of patients with IgAN, both GM-CSF and MCP-1 were upregulated in the glomerulus and interstitium (Ashizawa *et al.*, 2003). Moreover, this effect correlated with the progression of tissue injury. In addition, the progression of tissue injury appears to be influence by the induction of transcriptionally regulated genes in IgAN and their association with NF-κB.

In this chapter, I showed that stimulation of cultured PTEC with simvastatin and fluvastatin induced a dose-dependent inhibition of IL-6, IL-8 and GM-CSF release. When compared with the cytokine release in response to LPS alone, both drugs significantly inhibited LPS-induced release of IL-6, IL-8 and GM-CSF by approximately 40%. Statins alone also inhibited PTEC release of all three cytokines by 58%, 65% and 57%, respectively, with a maximal effect at the 10 μM concentration. However, because different concentrations were used, it is difficult to decide whether all effects must be considered pharmacological or whether some of them only occurred at much higher concentrations and are reflective of toxicity. No significant differences were observed between simvastatin and fluvastatin. These data may provide an insight into the mechanisms of the anti-inflammatory action of statins, suggesting as they do that these agents may reduce macrophage accumulation. Whether or not this anti-inflammatory effect can be generalised to include other statin agents remains to be seen.

A literature review suggests that isoprenylated proteins may be implicated in the release of IL-6, IL-8 and GM-CSF through the dependence of MAPK on these molecules (Andrews *et al.*, 1994; Hillyard *et al.*, 2002; Palinski, 2000; Takemoto&Liao, 2001). Moreover, there is evidence that MAPK proteins – including ERK, JNK and p38 (Li&Nord, 2004) – play a key role in the production of IL-6, IL-8 and GM-CSF in PTEC (Leonard *et al.*, 1999; Li&Nord, 2002; Li&Nord, 2004). Importantly, lipid rafts and caveolae also appear to be involved in the release of IL-8 (Li&Nord, 2004). Caveolin-1, which exerts its effects through the SAPK/JNK, P38 and ERK1/ERK2 signalling pathways, has been implicated in the activation of proinflammatory cytokines through its association with CD40 in unstimulated, but not stimulated, PTEC. Elimination of caveolin-1 with filipin (the collective name given to four isomeric polyene macrolides that bind to cholesterol in membranes) reduced all signalling activity and, consequently, IL-8 release in human PTEC.

These observations lend further weight to the hypothesis that the overall effects of statins are reflective of a dual action on lipid rafts and isoprenylated proteins (Hillyard *et al.*, 2006). From my results, it seems likely that neither simvastatin nor fluvastatin directly

inhibit the release of IL-8 by PTEC, but that they interfere in the signalling pathways of caveolin-1 by disrupting rafts and caveolae. This mechanism might also explain the inhibition of IL-6 and GM-CSF release in statin-treated PTEC.

Given that nearly all cytokines activate the JAK/STAT signalling pathway, it seems highly likely that STATs are crucial to the inflammatory process. Indeed, there is some evidence that albumin activates PTEC in a similar manner to cytokines following ROS-mediated STAT activation (Nakajima *et al.*, 2004). The activation of this signalling pathway may subsequently lead to formation of ECM and cell proliferation, as well as the induction of immune responses involved in progressive renal disease.

In Chapter 3, I showed that stimulated PTEC produce FN, one of the major components of ECM and an important mediator in the pathogenesis of renal disease by virtue of its role in tissue remodelling associated with inflammation, such as that seen in interstitial fibrosis, the final common pathway of all progressive renal diseases. Recent research has shown that the mediation of proinflammatory molecules by PTEC from the glomerulus to the interstitium is vitally important to the progression to ESRF, and that interstitial inflammation and fibrosis has a central role in this process (Sean Eardley&Cockwell, 2005).

Previous studies have proven inconclusive with regard to whether or not statins have a direct effect on ECM accumulation in PTEC (Laufs&Liao, 2003; Takemoto&Liao, 2001). However, there is some evidence to suggest that the benefits of statins in renal diseases are due, in part, to the enhancement of matrix protein degradation (Nogaki *et al.*, 1999). Several studies in different cell types have reported that FN production is significantly increased following stimulation with LPS 0.1–1 µg/ml, potentially increasing the ability of bacterial products to modify FN production and lead to chronic infections (Adachi *et al.*, 1996; Kubo *et al.*, 1996; Liang *et al.*, 2001; Mahler *et al.*, 1997; Nooteboom *et al.*, 2000; Okamura *et al.*, 2001; Sandstrom *et al.*, 1994; Schmid-Kotsas *et al.*, 1999).

Previous studies have shown that the progression of glomerulosclerosis and tubulointerstitial fibrosis is closely dependent upon the increased expression of TGF-β (Basile, 1999; Zhang *et al.*, 2004). Thus, it can be assumed that TGF-β has a profibrotic action, stimulating matrix production of fibroblasts (Healy&Brady, 1998; Massague, 1990), and there is evidence to suggest that it also has anti-proliferative effects (Franklin, 1997; Lawrence, 1995).

In my experiments relating to FN production and cell proliferation, TGF-β1 was used as a positive control. Both simvastatin and fluvastatin inhibited FN production in a dose-dependent manner, effective at the 0.1 µM concentration, compared with TGF-β1. At the 1-µM concentration, both drugs were associated with a 35% reduction in unstimulated FN production; LPS-stimulated production was also inhibited. Importantly, the observed reduction in FN production seen at 10 µM is probably a reflection of excessive toxicity and apoptosis and is therefore unlikely to be of pathophysiological relevance. This is in accordance with previously published research in mice suggesting that, in the proximal tubules, statins induce apoptosis via inhibition of isoprenoid production and that this may be related to the disruption of actin filaments (Iimura *et al.*, 1997). Therefore, it is difficult to determine whether the pleiotropic effects of the statins all occurred at the same time and within the same concentration range.

In vitro experiments in several different cell types (including PTEC, MSC, immune mediator cells and vascular smooth muscle cells) have shown that statins inhibit cell proliferation (Buemi *et al.*, 2002; Guijarro *et al.*, 1998; Iimura *et al.*, 1997; Ishikawa *et al.*, 1995; Jain&Ridker, 2005; Palinski&Tsimikas, 2002; Takemoto&Liao, 2001). This generally accepted phenomenon concurs with the mechanism described above in the mouse study of statin-induced apoptosis (Iimura *et al.*, 1997). The effective concentration of statins is approximately 0.1 µM, with around 50% inhibition of proliferation seen at the 1 µM concentration, which corresponds to the peak plasma levels of simvastatin or fluvastatin *in vivo* (Hillyard *et al.*, 2002; Hillyard *et al.*, 2004b; Park *et al.*, 2001).

In concordance with these results, I found that both simvastatin and fluvastatin treatment were associated with a 51% reduction in cell proliferation at the 1 µM concentration. Overall, the results observed in the FN production experiments were in parallel with those seen in the proliferation experiments. PTEC were treated over 24 hrs with statins using [³H]-thymidine incorporation in a dose-dependent manner in the micromolar range in agreement with Vrtovsnik et al (Vrtovsnik *et al.*, 1999). The inhibition of PTEC proliferation was associated with blockade of the cell cycle in the G1 phase, preventing transition to the S phase. In a recent study, Khwaja et al showed that, at high concentrations, pravastatin arrested the cell cycle in the G1 phase (Khwaja *et al.*, 2006).

In addition, I showed that PTEC expressed Ig receptors, cytokines release, produced FN and proliferated and that these functions were increased when the cells were stimulated with LPS, which may contribute to the influx of inflammatory cells in bacterial infection and to the development of interstitial fibrosis. *In vitro*, statins were able to suppress these

actions and attenuate the effect of LPS on cytokine release, FN production and proliferation by PTEC. The significant anti-inflammatory effects of statins are certainly partly attributable to the downregulation of FN production, leading to a reduction in interstitial fibrosis, less severe tubular phenotypical changes, less myofibroblast expression and downregulation of TGF-β, while the control of PTEC proliferation may represent a novel strategy to slow the progression of renal disease. By increasing the proteolytic activity of PTEC, it may be possible to reduce or prevent ECM deposition and interstitial fibrosis.

In conclusion, I demonstrated that LPS can induce the release of IL-6, IL-8 and GM-CSF in PTEC and statins can inhibit these responses in a dose-dependent manner, which may provide an insight into the mechanisms of anti-inflammatory actions of these agents. In concordance with my results in Chapter 4, I again showed that PTEC stimulated with LPS upregulated the expression of Ig receptors, cytokine release, production of FN and cell proliferation; these functions may contribute to the influx of inflammatory cells in bacterial infection and also to the development of interstitial fibrosis. The control of PTEC proliferation may represent a promising strategy to attenuate the progression of renal diseases. Currently, quality clinical trial data to suggest that statin therapy is able to ameliorate the deterioration of function in patients with mild renal disease are scarce. There is a clear need for further research to investigate the therapeutic advantages of the lipid-independent pleiotropic effects of these agents. In particular, it needs to be established whether the anti-inflammatory properties of statins is related to the ability of these agents to attenuate tubulointerstitial inflammation, especially if this frequently recurs (as in IgAN), and whether or not the beneficial effects of statins are seen in the early stages of progressive disease. The results of *in vitro* and animal experiments may only provide proof in principle for improved patient outcomes in renal disease, but these urgently need to be verified in humans.
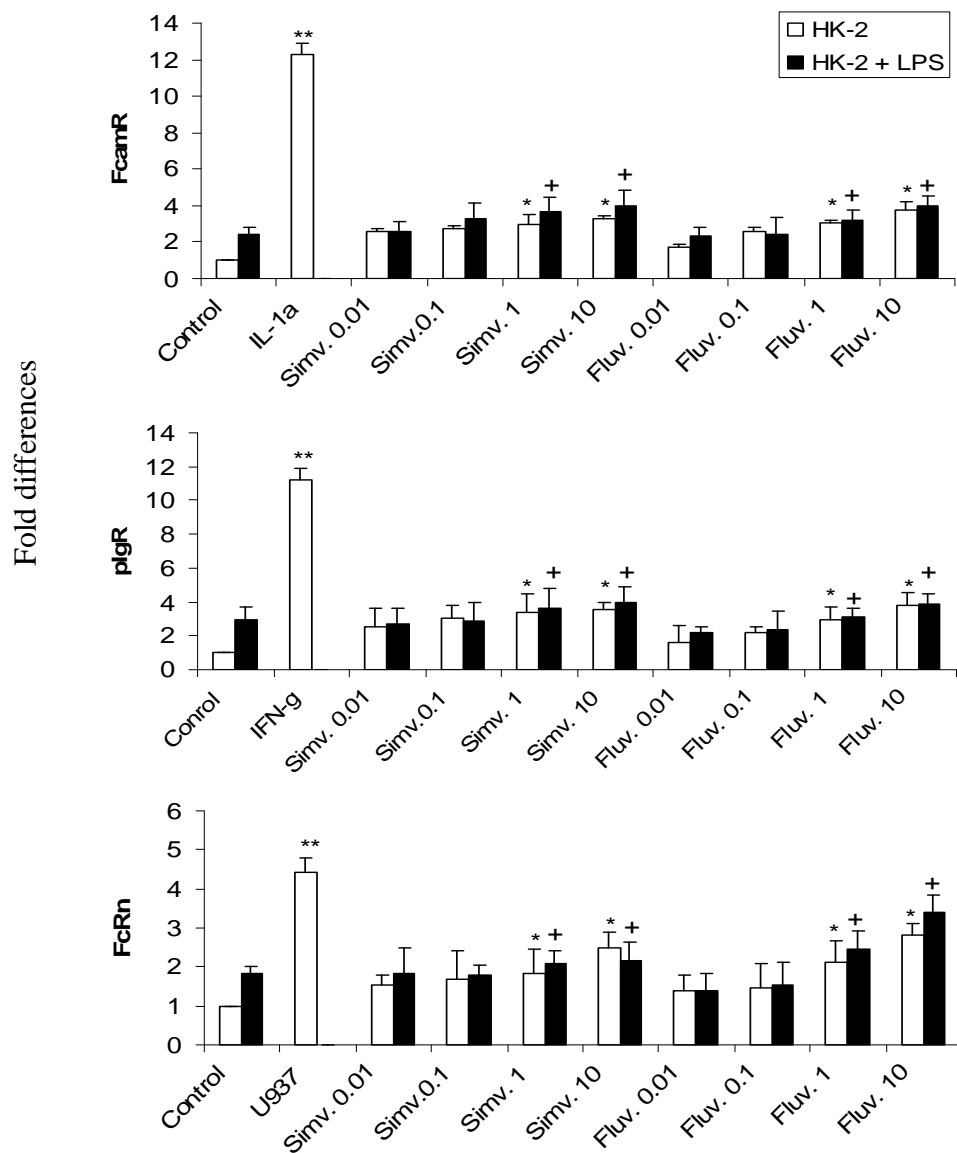
Figure 5-4 Dose–response curve of the effect of statins ± LPS on IgR expression by PTEC

The fold differences of expression of FcRn, pIgR and Fcα/μR was measured by semi-qRT-PCR in relation to control treatment with statins, with and without LPS, for 24 hrs in HK-2 cells. The amplification of each of these Ig receptors was relative to the housekeeping gene, β-actin. All of the receptors showed a significant increase in expression following treatment with SIM or FLU alone or in combination with LPS in a dose-dependent manner. LPS alone resulted in a slight increase in expression. FcRn expression in U937 cells was upregulated by 4.43±0.36-fold (p<0.005); pIgR was upregulated by 200 ng/ml IFN-γ by 11.18±0.67-fold (p<0.005); and Fcα/μR was upregulated by 50 ng/ml IL-1α by 12.26±0.61-fold (p<0.005). This graph is representative of two experiments. Triplicate reactions at each condition were amplified along the iCycler iQ RT-PCR system. Error bars show the (± SD). Standard curve had r = 0.999, slope = −3.272 and PCR efficiency = 100%. (*) Significant comparison between HK-2 cells treated and untreated with LPS alone (*p<0.025, **p<0.005). (+) Significant comparison between LPS alone and LPS in combination with statins (+p<0.025).
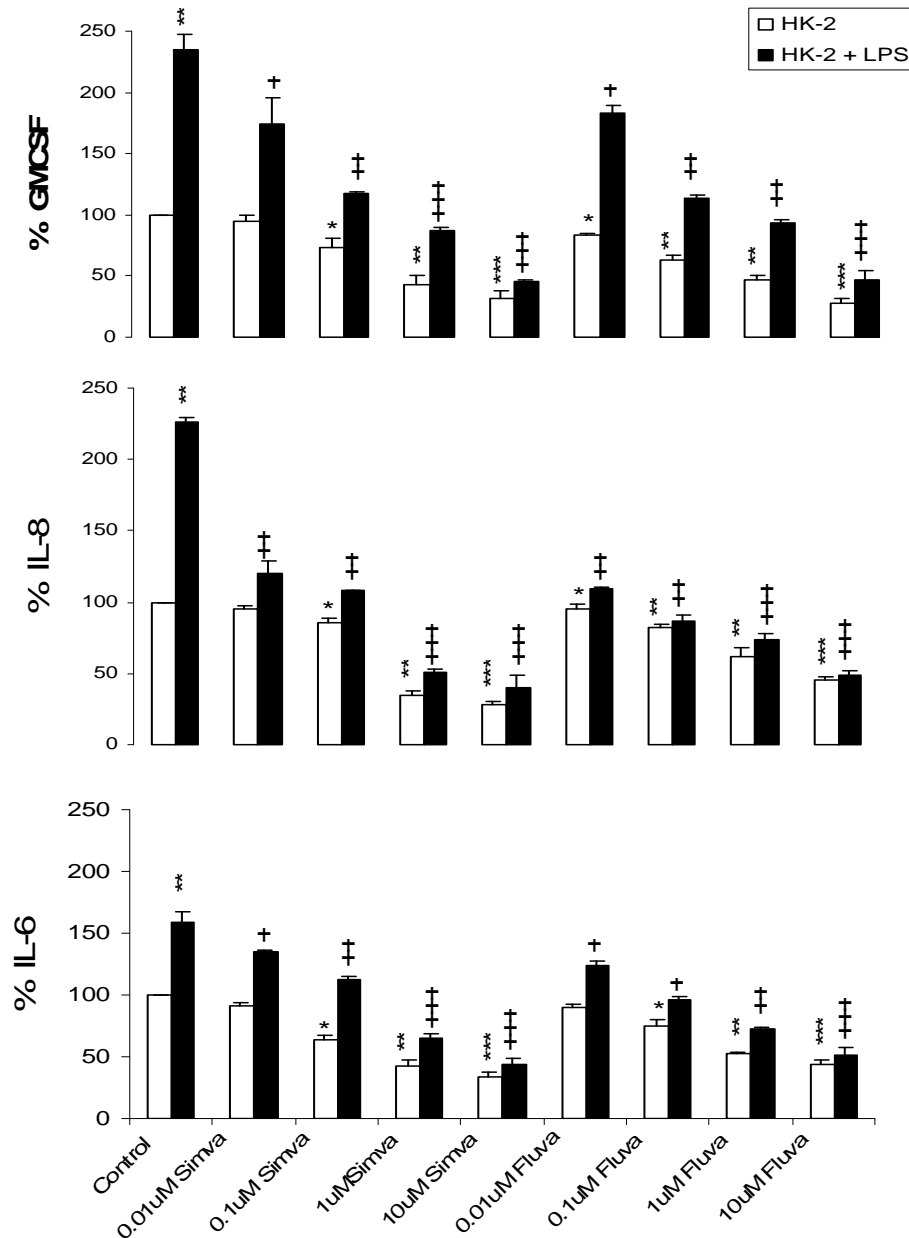
Figure 5-5 Dose–response curve of the effect of statins ± LPS on cytokine release by PTEC

These graphs show a dose-dependent stimulation of IL-6, IL-8 and GM-CSF release by cultured supernatant HK-2 cells following treatment with SIM or FLU with and without LPS and 24 hr incubation, expressed as a percentage of control. Levels of IL-6, IL-8 and GM-CSF were measured by ELISA. Statins alone inhibited cytokine release in a dose-dependent manner. LPS-stimulated cytokine release was also inhibited in a dose-dependent manner when additionally treated with SIM or FLU. Media alone was used as negative control and LPS as positive control. These data represent three measurements in three separate experiments (n=9). Error bars show the (± SD). (*) Significant comparison between HK-2 cells treated and untreated with LPS alone (*p<0.017, **p<0.003, ***p<0.0003). (+) Significant comparison between LPS alone and LPS in combination with statins (+p<0.017, ++p<0.003, +++p<0.0003).
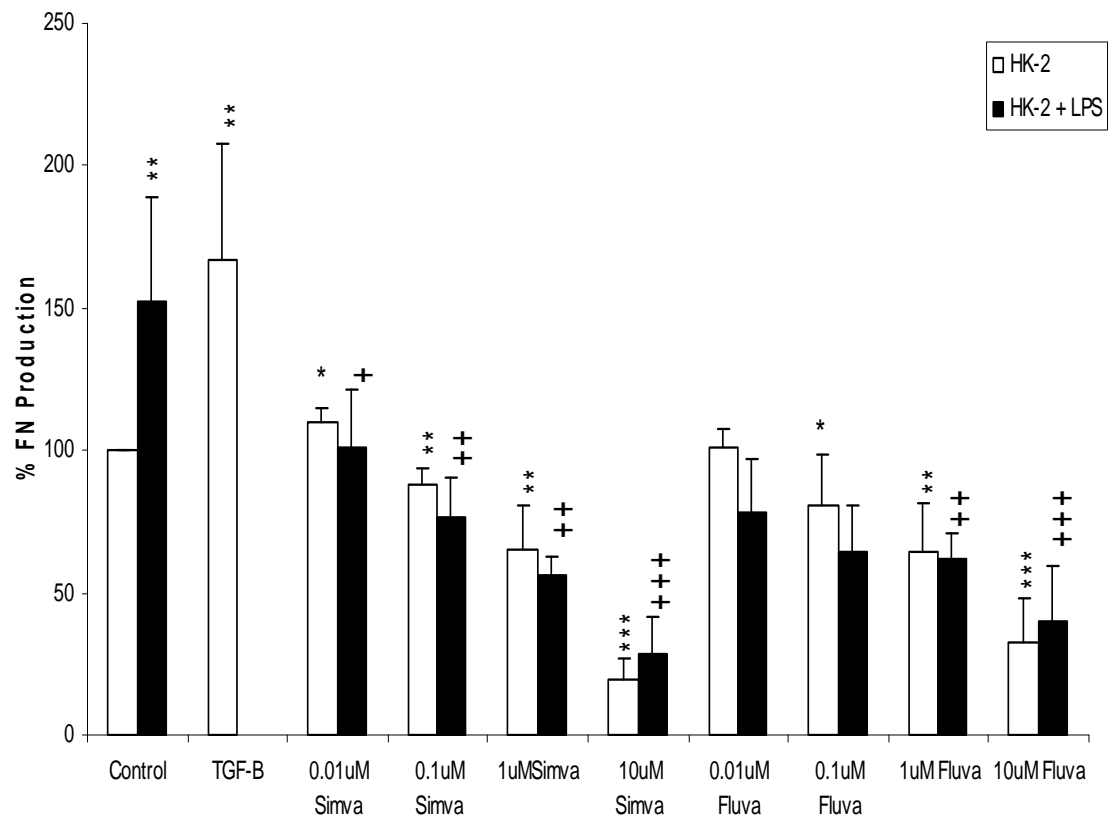
Figure 5-6 Dose–response curve of the effect of statins ± LPS on FN production by PTEC

Cells were treated with statins alone and in combination with LPS and then incubated for 72 hrs. FN production was measured by using specific ELISA and expressed as a percentage of control. TGF-β1 was used as a positive control. LPS alone increased FN production, whereas statins alone or in combination with LPS inhibited FN production in a dose-dependent manner. Data are shown as the mean of four experiments performed in duplicate (n=8). Error bars show the (± SD). (*) Significant comparison between HK-2 cells treated and untreated with LPS alone (*$p<0.0125$, **$p<0.002$, ***$p<0.0002$). (+) Significant comparison between LPS alone and LPS in combination with statins (+$p<0.0125$, ++$p<0.002$, +++$p<0.0002$).
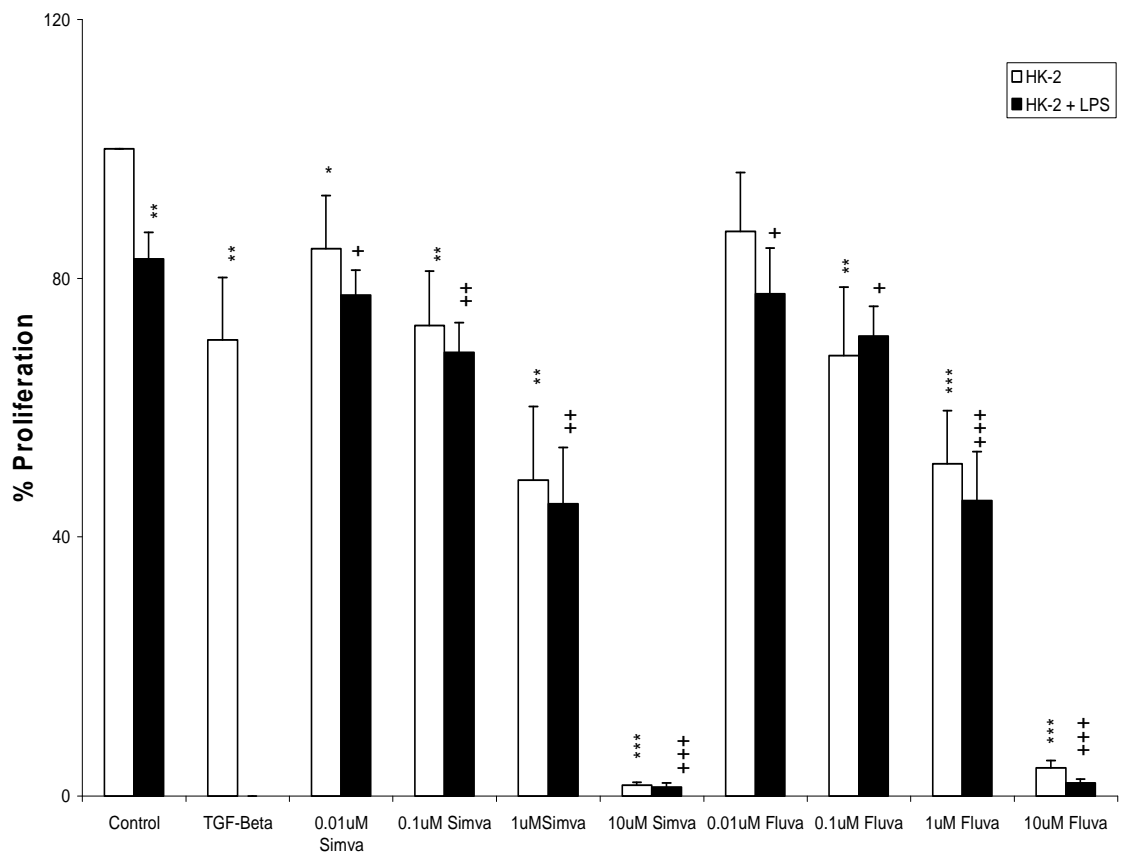
Figure 5-7 Dose–response curve of the effect of statins ± LPS on proliferation of PTEC

The effect of statins alone and in combination with LPS on HK-2 cell proliferation was measured by 24-hr [3H]-thymidine incorporation, expressed as a percentage of control. Cells treated with statins with and without LPS showed an inhibitory effect on proliferation in a dose-dependent manner. HK-2 culture media was used as a negative control and TGF-β1 as a positive control. These data represent the mean of 2 experiments performed in replicates of 12 (n=24). Error bars show the (± SD). (*) Significant comparison between HK-2 cells treated and untreated with LPS alone (*p<0.025, **p<0.005, ***p<0.0005). (+) Significant comparison between LPS alone and LPS in combination with statins (+p<0.025, ++p<0.005, +++p<0.0005).

# Chapter 6: Discussion

6.1 General discussion

GN is a major cause of progressive CRF and accounts for less than 20% in the UK of all patients requiring dialysis or kidney transplantation. There are several forms of GN, of which the most common is IgAN. In most forms of GN, kidney injury is a consequence of the deposition of ICs within the glomerulus. These ICs contain Ig, complement and other proteins, can vary in composition and may directly contribute to progression to ESRF (Nangaku&Couser, 2005). Despite the large volume of research that exists on this disease pathway, few studies implicate pre-formed ICs (which enter the kidney via the circulation) in the development of significant tissue injury where the complexes are localised, including the glomerulus (Couser&Salant, 1980). Once glomerular damage occurs, additional plasma proteins are filtrated into the lumen of the proximal renal tubule. As the concentration of protein increases, specific PTEC receptors stimulate increased protein uptake into the cells, eventually triggering interstitial fibrosis and inflammation.

In proteinuric renal disease, progression to ESRF leads to glomerulosclerosis and tubulointerstitial fibrosis/inflammation in the late stages, independently of the nature of original injury (Tang *et al.*, 2002). The mechanisms by which proteinuria causes GN and tubulointerstitial injury are not completely understood. Current evidence suggests that proteinuria contributes to progressive renal disease by initiating signals that increase inflammation and fibrosis in the kidney (Remuzzi&Bertani, 1998). One theory is that, as the proximal tubular epithelium is strategically located between the urinary space and the surrounding interstitium, proteinuric injury may be mediated by the activation of PTEC. This thesis describes the role of Ig receptors on PTEC when stimulated with pro-inflammatory cytokines, which are a key feature of the immune system. Fig. 6.1 provides an overview of my results.

It is clear that proteinuria has numerous effects within the proximal tubule and a number of questions arise from this finding. Firstly, which Ig receptors are expressed on tubular cells? Receptors for the Fc region of Igs are expressed on many different cell types within the immune system and kidney. PTEC have been shown to express Fcα/μR, pIgR and FcRn and these receptors may play a role in protein endocytosis. Whether or not these three receptors are primarily responsible for the initiation of pro-inflammatory responses by PTEC in the presence of proteinuria is not yet clear. Indeed, these responses may not be receptor-mediated at all. One of the causes of IgAN may be abnormal glycosylation of serum IgA1, which may help to explain why the presence of IC deposits is associated with

renal damage in only a subset of patients. However, this abnormal glycosylation alone would most likely be insufficient to fully explain the pathology of IgAN, but may be a necessary factor for the disease to develop.

In this thesis, I have demonstrated mRNA expression of the novel Fcα/µR by PTEC. Any IgA receptor expressed by PTEC is a potential mediator of IgA deposition in IgAN and may contribute to PTEC activation by IgA ICs, in agreement with the findings of McDonald et al., (2002). I have shown that the expression of Fcα/µR transcript by PTEC is increased by the pro-inflammatory cytokines IL-1α and IFN-γ, and inhibited by TGF-β1. These results confirm those of Yoshioka et al., (1993) and Yano et al., (1997), who considered the array of inflammatory mediators found within the kidneys of IgAN patients.

Secondly, which Igs activate tubular cells, and how? In this thesis, I reported that PTEC in culture bind IgG, IgA and IgM. I have also shown that human Fcα/µR was able to bind IgA and IgM with a specific monoclonal Fcα/µR antibody, generated using IHC of normal and IgAN kidney showing Fcα/µR expression by renal PTEC. Using this antibody, I was able to determine whether or not the receptor was expressed at the cell surface in cultured PTEC. Recently, Cho et al., (2006) developed a new anti-Fcα/µR molecule that will prove useful for future experiments of this type.

The major implications for the pathogenesis of IgAN inferred by the expression of Ig receptors by PTEC is that any IgA found in the glomerular filtrate might interact with and activate these cells, leading to tubulointerstitial injury. I was unable to confirm Fcα/µR protein expression by PTEC, as discussed in Section 3.3. However, Fcα/µR may still be able to mediate the binding of IgA and IgM by activated PTEC *in vivo*. Restricted expression of Fcα/µR under certain conditions may be associated with a specific response, potentially leading to renal damage.

Thirdly, do Ig/IC deposits increase proteinuria as implicated in the early stages of tubulointerstitial fibrosis? PTEC synthesis of FN and other ECM proteins occurs as a direct response to proteinuria, especially TGF-β (Eddy, 2004), and contributes to progressive renal injury by activating the pro-apoptotic pathways that lead to PTEC death (Erkan *et al.*, 2001; Thomas *et al.*, 1999). Following injury by Ig/IC deposits, PTEC proliferate and release high levels of cytokines and growth factors, and increase FN production, as seen in IgAN (Eitner *et al.*, 1994; Lai *et al.*, 1996). However, the pathogenic role of FN in IgAN is still unknown. Similarly, the pathogenesis of PTEC proliferation and its role in the

development of glomerulosclerosis is poorly understood. A recent study by Chan et al., (2005) showed that the binding of IgA to PTEC had no effect on the proliferation of PTEC. In contrast, my results showed that XL- or HAg-IgA may have an antiproliferative effect on PTEC. As such, the role of PTEC proliferation in glomerulosclerosis and tubulointerstitial fibrosis requires further investigation. The cross-linking of Fc receptors by Ig/IC in PTEC may cause inflammation and tubular injury, and this is subsequently worsened by pro-inflammatory cytokines and activated complement components. Antagonism of these receptors may prove to be a useful strategy for therapeutic intervention.

I have also demonstrated that PTEC are able to synthesise and release several cytokines, in agreement with several other *in vitro* studies which found that certain urinary proteins led to the release of IL-6, IL-8 and GM-CSF (Donadelli *et al.*, 2003; Tang *et al.*, 2003; Zoja *et al.*, 1998). IL-8 and IL-6 are increased in patients with various forms of glomerular diseases, such as IgAN and membranoproliferative GN (Wang *et al.*, 1997). That human PTEC are able to release these molecules suggests a similar pathway *in vivo*, thereby providing a plausible mechanistic link between proteinuria and interstitial inflammation.

As part of the research project resulting in this thesis, I planned to do clinical research on patients suffering from IgAN or GN to measure Ig/IC concentrations in the urine, to work on biopsy samples of patients with these diseases to localise tubular ICs by immunofluorescence or electron microscopy stained with the specific monoclonal antibody to Fcα/µR, and to find out the expression and binding of Igs. Unfortunately, there was not enough time to conduct this research.

The second part of this thesis examined the response of PTEC to CNIs (cyclosporine A and FK506) and the non-CNI sirolimus, in addition to the immunomodulatory effects of statins (simvastatin and fluvastatin). Previous studies have shown that each of the immunosuppressive agents studied substantially reduces proteinuria, tubulointerstitial inflammation, cortical fibrosis, renal expression of pro-inflammatory and profibrotic genes and glomerular hypertrophy. Interestingly, sirolimus alone has been shown to effectively halt disease progression altogether. However, long-term treatment with these immunosuppressive agents may result in nephrotoxicity and hypertension in renal transplant recipients.

My results showed that LPS alone stimulated PTEC to increase the release of cytokines, in agreement with (Brauner *et al.*, 2001; Gerritsma *et al.*, 1998b; Hajjar *et al.*, 2002). I found

no significant effect of immunosuppressant agents alone on Ig receptor expression, cytokine release, FN production and proliferation of PTEC. However, when treated in combination with LPS, each of these immunosuppressive agents reduced the expression of pIgR at highest concentration, FN production and proliferation in PTEC when treated at the highest dose. This variance might be explained by differences in the experimental protocol and further work is needed to clarify these observations.

Patients with CKD have a significantly increased risk of cardiovascular morbidity and mortality (Levy, 2006), and several studies have shown that increased proteinuria and reduced renal function are associated with more rapid progression to ESRF and a higher incidence of cardiovascular problems. HMG-CoA reductase inhibitors (statins) have dramatically improved cardiovascular outcomes in patients without significant kidney disease and may prove to have similar effects in patients with kidney disease. Alongside the primary cardiovascular benefit of statin therapy (a reduction in cholesterol synthesis), there is evidence to suggest that these agents have a renoprotective effect through reducing proteinuria. However, high doses of statins may induce proteinuria (Agarwal, 2004), although the nature of the proteinuria is not clear.

To confirm the appealing hypothesis that statins reduce proteinuria and the rate of kidney function loss, I demonstrated a reduction in cytokine release by PTEC stimulated by simvastatin and fluvastatin, alone or in combination with LPS. FN production and proliferation were also reduced in a dose-dependent manner, with or without LPS, and, importantly, without altering cell viability. These findings concur with those of Methe et al., (2005) and Hillyard et al., (2006). The expression of Ig receptors by PTEC was increased in a dose-dependent manner, with or without LPS. This hypothesis may be supported by further work investigating the effects of statins on PTEC *in vivo*.

In conclusion, the main focus of this study was to determine the potential role of FcRn, pIgR and the novel Fcα/μR, all of which have been identified in PTEC, and their potential implications for IgAN.

- Using primary and immortalised human PTEC, I was able to show Fcα/μR gene expression and surface receptor detectable by immunoblot and IHC.

- My findings support the hypothesis that tubular binding of filtered Ig and activation of FcRs may contribute to progression of renal disease and provide a potential therapeutic target.

- I also investigated the role of cyclosporine A, FK506 and sirolimus on susceptibility to nephrotoxicity in cultured human PTEC.

- My results inferred that immunosuppressive agents alone have a non-inflammatory response on Ig receptor expression. However, in combination with LPS, the study drugs showed a slight inhibitory effect on the expression of pIgR, on FN production and on the proliferation of human PTEC.

- In addition, I have shown that human PTEC release IL-6, IL-8 and GM-CSF in response to LPS. These cytokines/chemokines may contribute to the influx of inflammatory cells in bacterial infection and also to the development of interstitial fibrosis. However, the immunosuppressive agents were unable significantly alter cytokine release.

- Statins inhibit these actions at biologically relevant *in vitro* concentrations, and may be of clinical significance. There are limited clinical trial data to suggest that statins may slow the decline of renal function in patients with mild renal impairment. My results provide a possible mechanism for this effect, and is worthy of further investigation.

- Moreover, my *in vitro* findings may help to explain why GN is frequently encountered in patients with chronic tubular proteinuria, as well as providing an explanation for the development of GN in diseases that primarily affect the tubulointerstitial compartment. This finding may offer potential new strategies for the prevention and treatment of drug-induced nephrotoxicity.

- As always, a major limitation of *in vitro* studies such as this, is that the relevance of the findings may not extend to the *in vivo* condition in treated patients.

Proteinuric glomerulopathies
Circulating ICs
Antigen stimuli
Infectious diseases
Generation of abnormal
glycosylated IgA
Unknown mechanisms

IFN-γ
IL-1α

\+

\-

TGF-β

**Expression of tubular
Fcα/μR, pIgR and FcRn by PTEC**

**Non-receptor
mediated**

Tubular Ig/IC deposits

\-

Cellular
proliferation

\-

\+

Fibronectin
production

\-

**Immunosupp-
ressive agents
± LPS**

LPS

\+

Cytokine
release

\-

Statins ± LPS

Tubulointerstitial inflammation/fibrosis
Glomerulonephritis
IgA nephropathy
End-stage renal failure
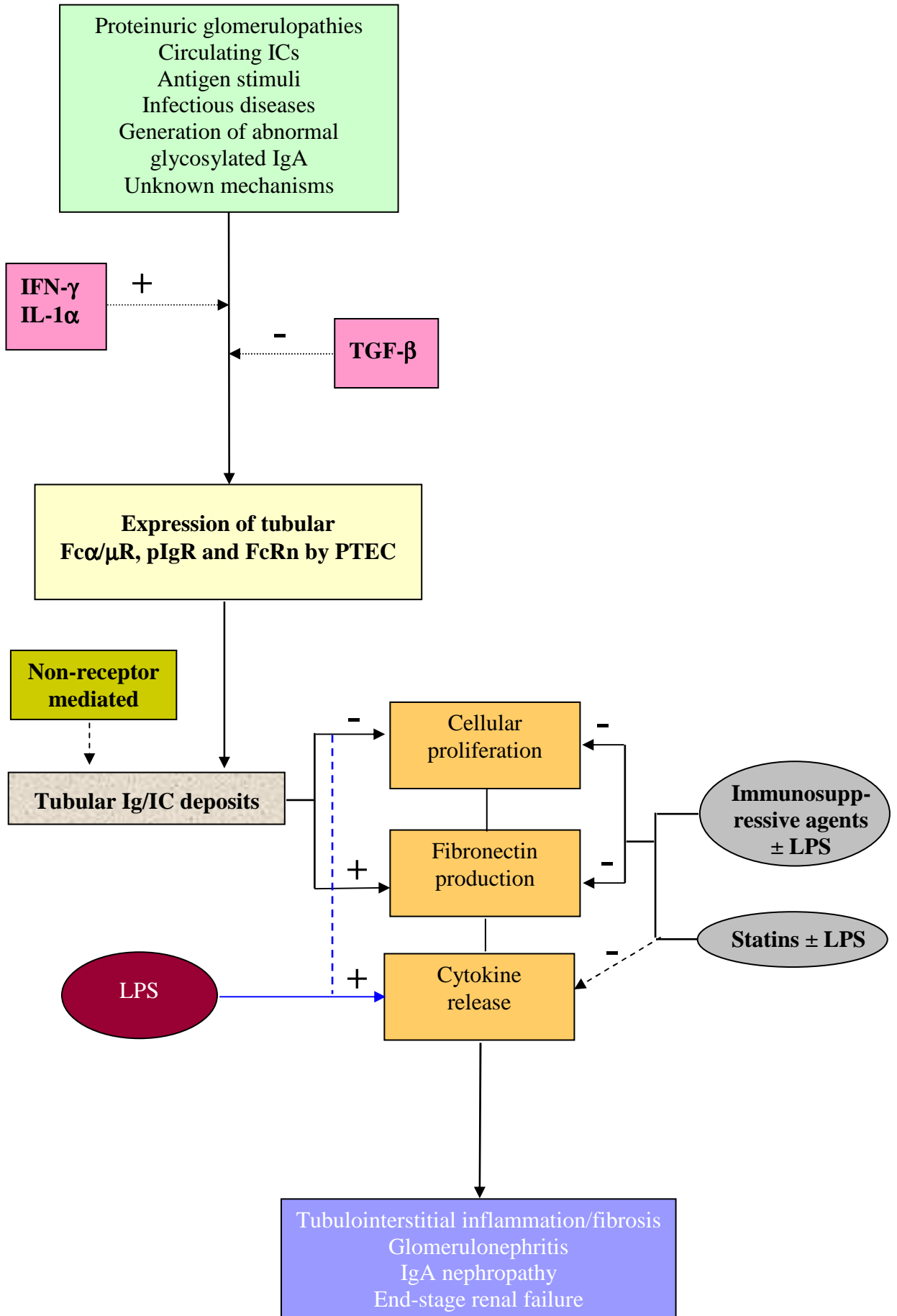
Figure 6-1 Summary study of PTEC in renal disease

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