The Role of T-cells in Peritoneal Dialysis Related Inflammation

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Dedication

To Resh, Maia and Nieve

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Summary

Despite advances in treatment, peritoneal infection remains one of the main causes of technique failure in Peritoneal Dialysis (PD) patients. Understanding the nature of the peritoneum's response to inflammation is vital if we wish to reduce the negative impact of inflammation and infection on the peritoneal membrane. Given that aberrant leukocyte recruitment and activation appears to be a feature of these responses it is particularly important that we understand the contributions made by both resident and recruited peritoneal leukocytes in controlling this process as well as their direct contribution to membrane damaging events.

Data presented in this thesis has shown that the peritoneal T-cell pool is a heterogeneous mixture of specialized T-cell subsets. In contrast to previous observations, we have shown that (under appropriate conditions) these cells are capable of being activated, though their effector function appears to be tightly regulated by peritoneal dendritic cells. The phenotype and activation threshold of peritoneal T-cells differs markedly from that of peripheral blood T-cells in that the peritoneal cavity is enriched in both effector memory and regulatory T-cells. These cells exist in a delicate equilibrium to both maintain homeostasis and ensure prompt removal of invading pathogens. During episodes of acute inflammation (peritonitis) further specialized T-cell subsets (TH17 and $V\gamma9\delta2$ T-cells) are recruited into the peritoneal cavity. These cells form part of the early immune response and are a pivotal link between the innate and adaptive arms of the immune response.

Recurrent peritonitis has a detrimental impact on the function of peritoneal T-cells, impairing both the proliferation and effector function of these cells. This peritoneal T-cell dysfunction contributes to a state of immuno-suppression thus potentially rendering the patients more susceptible to further episodes of peritonitis. It is also likely to have a negative impact on immuno-resolution and contribute to neutrophil retention, chronic inflammation and membrane fibrosis.

Oral presentations arising from this thesis

Welsh Association of Renal Physicians, Saundersfoot June 2006
"Peritoneal T-cell phenotype and function"

8th I3-IRG Meeting Millenium stadium, Cardiff July 2006
"Peritoneal T-cells, bedside to bench"

Cardiff University Postgraduate Research Day, November 2006
"The regulation of peritoneal T-cell proliferation"

Euro PD 2007, Helsinki, Finland
"The impact of recurrent infection on peritoneal T-cell phenotype and function"
Awarded prize for best oral presentation

Cardiff University Postgraduate Research Day, November 2007
"The role of peritoneal T-cells in peritoneal dialysis related inflammation"
Awarded prize for best oral presentation

Poster presentations arising from this thesis

American Society of Nephrology, San Fransisco, November 2007
"The Impact of recurrent peritonitis on peritoneal T-cell effector function"

American Society of Nephrology, San Fransisco, November 2007
"Regulatory T-cells isolated from the peritoneal cavity of Peritoneal Dialysis (PD) patients possess efficient suppressor cell function"

Abbreviations

AICD	Activation induced cell death
AGE	Advanced glycation end products
APC	Allophycocyanin
APCs	Antigen presenting cells
APD	Automated Peritoneal dialysis
CAPD	Continuous ambulatory peritoneal dialysis
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen induced arthritis
ConA	Concanavalin A
CRP	C-reactive protein
CTLA	Cytotoxic T-Lymphocyte Antigen
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylenediaminetetra-acetic acid
ESRF	End stage renal failure
FACS	Flow activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GDP	Glucose degradation products
GFR	Glomerular filtration rate
GITR	Gluccocorticoid-induced tumor necrosis factor receptor family related protein
GPCR	G-protein coupled receptors
HA	Haemaglutinin
HD	Haemodialysis
HMB-PP	E-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HPMC	Human peritoneal mesothelial cell
ICAM	Intercellular adhesion molecule

IFN-γ	Interferon gamma
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X linked syndrome
IPP	Isopentenyl pyrophosphate
LAG	Lymphocyte activated gene
Mbq	Megabecquerel
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
ml	millilitre
µl	microilitre
µM	micromolar
ng	nanogram
nm	nanometre
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Peritoneal Dialysis
PE	Phycoerythrin
PET	Peritoneal equilibration test
pg	Picogrammes
PHA	Phytohaemagluttinin
PI	Propidium iodide
PMA	Phorbol myristate acetate
PMØ	Peritoneal macrophage
PPD	Purified protein derivative
PRR	Pattern recognition receptors
Q-FISH	Quantitative fluorescence *in situ* hybrization
RAG	Recombination-activating gene
RANTES	Regulated upon activation, normal T-cell expressed and secreted
rpm	Revolutions per minute
RRF	Residual renal function
RRT	Renal replacement therapy
SIL-6R	Soluble interleukin-6 receptor

SSC	Side scatter
STELA	Single telomere length analysis
TB	Tuberculosis
T_{CM}	Central memory T-cells
TCR	T-cell receptor
T_{EM}	Effector memory T-cells
TEMRA	Terminally differentiated effector memory cells
TGF	Transforming growth factor
TH	T helper cells
TH17	IL-17 producing T-cells
TLR	Toll-like receptors
TNF	Tumor necrosis factor
T-reg	Regulatory T-cells
TRF	Terminal restriction fragment
TT	Tetanus toxoid
UF	Ultrafiltration
WBC	White blood cells

Contents **Page**

Figures

Figures

Tables

Chapter 3: Peritoneal T-cells: Phenotype, Activation Status, Proliferation and Cytokine Production

Figures

Chapter 4: Characterisation of Specialised Peritoneal T-cell Sub-Populations

Figures

Chapter 1
Introduction

1.1 Renal replacement therapy

1.1.1 End Stage Renal Failure

End-stage renal failure (ESRF) is a condition in which a person's kidneys cease to function on a permanent basis. This necessitates a form of renal replacement therapy (regular dialysis or a kidney transplant) to maintain life. At present the number of available donor kidneys falls woefully short of the demand, so that the majority of ESRF patients are dependant on some form of dialysis.

1.1.2 Haemodialysis vs. Peritoneal Dialysis

Two major forms of dialysis are currently available: Haemodialysis (HD) and peritoneal dialysis (PD). HD is most frequently carried out at a hospital dialysis unit. Blood is pumped in an extracorporeal manner through an artificial kidney. PD is carried out by patients at home, in this case dialysis fluid is instilled into the peritoneal cavity and dialysis takes place across the peritoneal membrane. PD has a number of advantages over HD including better preservation of residual renal function, maintenance of higher haemoglobin levels, and lower cost. PD patients also benefit from the more flexible nature of the treatment, which allows them to work and travel, thus considerably improving their quality of life [1, 2].

Prevalence of patients on renal replacement therapy increases by 7-8% per annum in a compound manner and is therefore expected to double over the next ten years. In view of this, it is essential to maximise the choice available to patients and to provide the most cost effective type of dialysis option. It is established that the most benefit is obtained when patients commence dialysis with PD and that this delivers a better-integrated programme. Currently however the median time on PD is 2.5-3.5 years. Limitations include peritonitis, loss of residual renal function and loss of effective membrane function [1, 2].

1.2 Peritoneal Dialysis

1.2.1 PD technique

The peritoneal cavity is a coelomic cavity within which the abdominal viscera are located. The peritoneal membrane that lines the abdominal organs is the visceral layer; the membrane that lines the abdominal wall is the parietal layer. The peritoneal cavity normally contains only a small amount of viscous lubricating fluid that enables unrestricted movement of the gut.

During a PD exchange, 2-3 litres of dialysis fluid is instilled into the peritoneal cavity via a percutaneous catheter. Uraemic toxins and solutes diffuse across the peritoneal membrane from blood into the dialysis fluid, which is then drained and discarded. The commonest PD regimen uses 3 daytime exchanges (each of 4h duration) and one overnight exchange (8-12h). An alternative PD regimen using shorter but more frequent dwells is Automated Peritoneal Dialysis (APD); in this case a mechanised cycler infuses and drains peritoneal dialysate at night allowing for more rapid fluid exchange.

1.2.2 Ultrafiltration

In addition to removing waste products, PD also removes excess body water - a process known as ultrafiltration (UF). In order to achieve UF, PD fluid must be hypertonic as compared to blood. This is achieved by the addition of an osmotically active agent (usually glucose) to the PD fluid. During the course of a dwell, this osmotic gradient dissipates as glucose is absorbed. If the glucose is absorbed too rapidly, the concentration gradient is lost and the UF achieved may be inadequate.

1.2.3 Peritoneal membrane transport kinetics

The rate at which solutes are transferred across the membrane varies, both from patient to patient and (in an individual patient) with time on PD. A better understanding of a patients membrane transport characteristics enables a physician to prescribe the appropriate dialysis regimen for that individual. A

Peritoneal Equilibration Test (PET) is used to measure the rate at which solutes pass from the bloodstream across the membrane into the dialysis fluid. If solutes move quickly, the person is termed a "high transporter"; if the solutes move slowly, the person is termed a "low transporter". A "high transporter" will have excellent solute transfer across the membrane but will have limited ultrafiltration capacity as the osmotic gradient dissipates too rapidly. These patients may benefit from more frequent exchanges with shorter dwell times e.g. APD. They may also benefit from the use of a different solution (such as one containing a glucose polymer) where the osmotic agent is larger and less easily absorbed [3].

1.2.4 PD adequacy

To determine whether a given PD regimen is providing "adequate dialysis" a clearance test is performed. This involves collecting all the PD effluent and urine from a 24-hour period and comparing the amount of urea and creatinine in the solution to the amount in the blood. From these figures a weekly urea clearance (termed Kt/V) and creatinine clearance (CrCl) is calculated. Current guidelines recommend a minimum target of 1.7 for Kt/V and 60 L/wk for CrCl (KDOQI 2006). Clearance can be improved by increasing either the volume or the number of exchanges. Total clearance is significantly enhanced by the maintenance of residual renal function.

1.2.5 PD solution biocompatibility

Standard PD solutions have a number of characteristics that can potentially have an adverse effect on the homeostasis of the peritoneal cavity. These characteristics include low pH (necessary to prevent caramelization of glucose during sterilization) and a high concentration of lactate, glucose and glucose degradation products (GDP). These conventional PD fluids are referred to as being "bioincompatible". There is abundant evidence to show that these fluids have detrimental effects on the cells of the peritoneal cavity. These findings have prompted the development of more biocompatible PD fluids which have a physiological pH, use bicarbonate as a buffer and have a low GDP content. Although laboratory studies confirm that these newer fluids are less cytotoxic, there remains a dearth of clinical studies showing any

improvement in patient or treatment outcomes. Consequently many health care providers are (at present) reluctant to fund the prescribing of these newer, more expensive products [4-13].

1.2.6 PD Peritonitis

The most significant problem associated with PD therapy is peritonitis. Peritonitis can occur in a relatively high number of patients ~ one episode per 15 patient-months (although these figures vary considerably from unit to unit and from country to country) and carries both short and long-term consequences for the patient. (See table.1.1)

Short-term	**Long–term**
Hospitalisation Transfer to haemodialysis Mortality	Membrane failure Cardiovascular morbidity

Table 1.1 Complications of peritonitis [4, 5]

PD peritonitis most frequently occurs when skin commensals are introduced into the peritoneal cavity during the bag exchange. Patients usually present with abdominal pain and "cloudy" dialysis fluid. The diagnosis is supported by the finding of >100 white blood cells (WBC) /mm^3 of dialysate (of which at least 50% are neutrophils). As soon as a provisional diagnosis of peritonitis is made, symptomatic patients are treated with empirical intra-peritoneal broad-spectrum antibiotic therapy. If a patient is asymptomatic (cloudy fluid, no pain or fever), therapy may be delayed until the Gram stain results are available (as long as this takes no more than 2-3hrs). Treatment is subsequently refined according to culture and sensitivity results.

Culture results should become available for most patients within 2-3 days, however in 2-20% of cases no organism is grown (culture negative peritonitis). Culture negative peritonitis is more likely to occur if a patient is already on antibiotics, if culture techniques are inadequate or if the organism

is unusual e.g. Mycobacterium spp. The commonest organisms cultured during peritonitis are skin commensals such as Coagulase negative Staphylococcus (including *Staphylococcus epidermidis* or *Staphylococcus aureus*). Gram-negative infections are less common, however they are associated with a more severe and protracted clinical and inflammatory course. A mixed growth or a growth of anaerobic organisms is suggestive of bowel perforation [6, 7].

1.3 The Peritoneal membrane

1.3.1 Membrane structure

Critical to the success of PD is the ability of the peritoneal membrane to function as a selective, semi-permeable dialysis membrane. As shown in Figure 1.1, the peritoneal membrane has a multi-layered structure. The mesothelial layer is an epithelial monolayer of mesothelial cells packed together via tight junctions and communicating junctions. The mesothelial cells express microvilli, which increase the surface area of the membrane thus allowing greater absorption. An underlying basement membrane provides support for the mesothelial cell layer. The Compact Zone is a thin connective tissue matrix that lies beneath the mesothelial layer. The compact zone is made up of loosely arranged collagen fibres (Type I and III) interwoven with occasional elastin fibres. The compact zone provides strength and elasticity to the membrane. Though occasional blood vessels are seen to run through the compact zone, the vascular bed is maximal at the junction of the compact zone with the underlying adipose tissue [8].

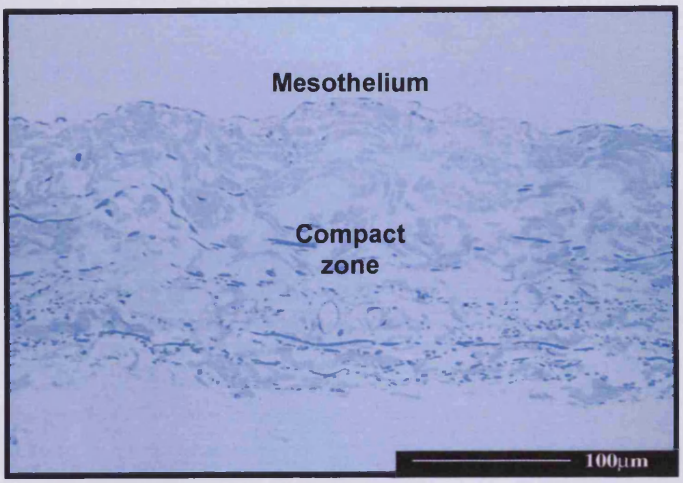
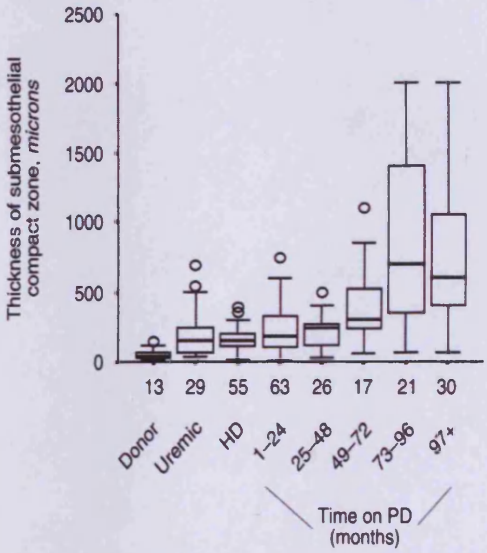


Figure 1.1 Structure of the normal peritoneal membrane [19].

A



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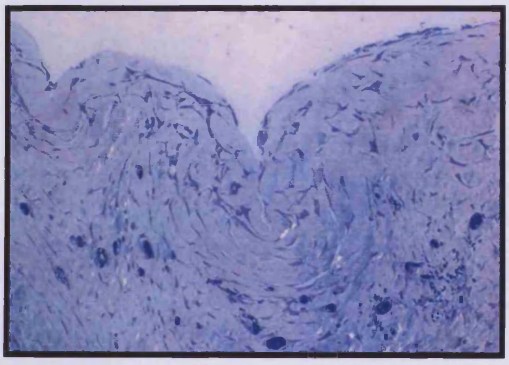


Figure 1.2 Changes in the peritoneal membrane with time on PD. (A) Long term PD is associated with an increased compact zone thickness. (B) A parietal peritoneal biopsy showing characteristic mesothelial loss and collagen deposition in the sub-mesothelial compact zone [18,19].

1.3.2 Changes in the peritoneal membrane with time on PD

Long term PD is associated with characteristic membrane changes (Figure 1.2). Loss of mesothelial cells is a well-described, almost universal feature. The remaining mesothelium is often abnormal, displaying degenerative changes with loss of cytoplasmic organisation [9]. An increase in thickness of the compact zone occurs, broadly related to time on dialysis. This is characterised by changes in extracellular matrix turnover and deposition that leads to irreparable deposition of a dense collagenous matrix [8-10]. PD associated vascular changes include sub-endothelial hyalinization, intimal distortion and eventual venule obliteration. These have been described as being associated with advanced glycation end products (AGE) accumulation [8-11]. These alterations in membrane structure are collectively termed membrane fibrosis. They are associated with detrimental functional changes including an increase in solute transport and the loss of ultrafiltration capacity. Ultimately this can lead to technique failure and transfer to HD [8, 10, 12].

1.3.3 The aetiology of membrane fibrosis

Though the aetiology of membrane fibrosis is multi-factorial, the two most important factors have been identified as exposure of the membrane to the bioincompatbile components of PD solutions and episodes of peritonitis.

1.3.3.1 Exposure to bioincompatible PD fluids

Studies have suggested that cumulative exposure to the bioincompatible components of PD fluid is associated with membrane fibrosis [13-15]. Glucose itself is a potent activator of human peritoneal mesothelial cells (HPMC), inducing production of fibronectin and transforming growth factor beta (TGFβ). PD fluid may also contribute to membrane damage via the presence of GDP and the formation of advanced glycation end products (AGE). *In vitro* studies have shown that certain GDP inhibit mesothelial wound healing while *in vivo* murine studies suggest that they contribute to angiogenesis and fibrosis. AGE are thought to disrupt numerous processes including chemotaxis, matrix cross-linking and inflammation. Increased AGE deposition has been correlated with increased membrane fibrosis suggesting a causal relationship. Additionally, both glucose and lactate together drive

production of intracellular sorbitol via the polyol pathway. Blocking the polyol pathway in the rat model has been shown to reduce membrane fibrosis and angiogenesis suggesting that intracellular sorbitol is detrimental to membrane survival [9, 13, 19-21, 25-31].

1.3.3.2 Recurrent peritonitis

There is a strong association between peritonitis (frequency and severity) and the loss of membrane function. Peritoneal biopsy data shows that progressive fibrosis, vasculopathy and UF failure are far more likely to occur in patients who suffer from peritonitis (see Figure 1.3). These biopsy findings are mirrored by observations in the murine model which have shown that repeated episodes of peritoneal inflammation result in membrane fibrosis [8-10, 12, 16].

1.4 Immunobiology of the peritoneal Cavity

1.4.1 Peritoneal macrophages (PMØ)

The peritoneal cavity contains a specialised population of resident leukocytes (macrophages, lymphocytes and dendritic cells), which together with the mesothelial cells constitute the cellular arm of the peritoneal immune system (see Figure 1.4). The PMØ are the most abundant (70-80%) and the most widely studied of the peritoneal resident leukocyte population. During episodes of peritonitis, their two primary functions are phagocytosis of invading bacteria and the production of inflammatory mediators such as IL-1β and TNFα. With time on PD, PMØ display a progressively more immature phenotype, increasing the expression of CD15 and CD71 (both markers of immaturity) and down regulating expression of CD11c (a marker of maturity). These phenotypic changes are associated with functional deficiencies including decreased bactericidal activity, decreased phagocytic activity and impaired cytokine production. Recurrent episodes of peritonitis appear to accelerate the progression of these detrimental functional changes [17-22].

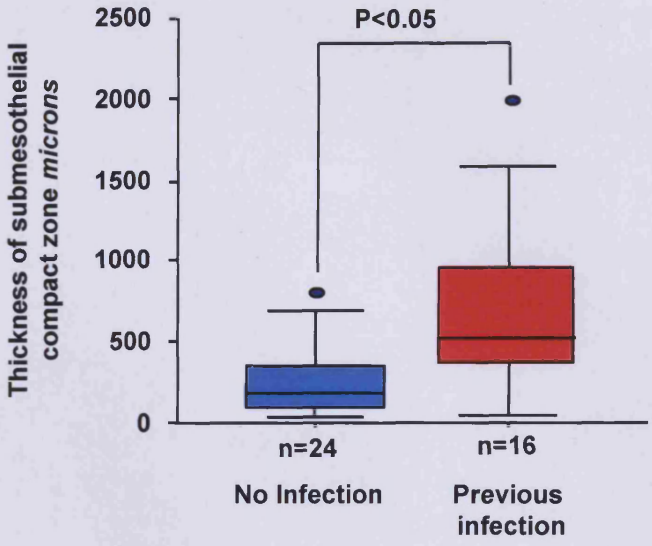


Figure 1.3 Effect of recurrent peritonitis on membrane thickness. Previous peritonitis is associated with increased sub-mesothelial compact zone thickness [18].

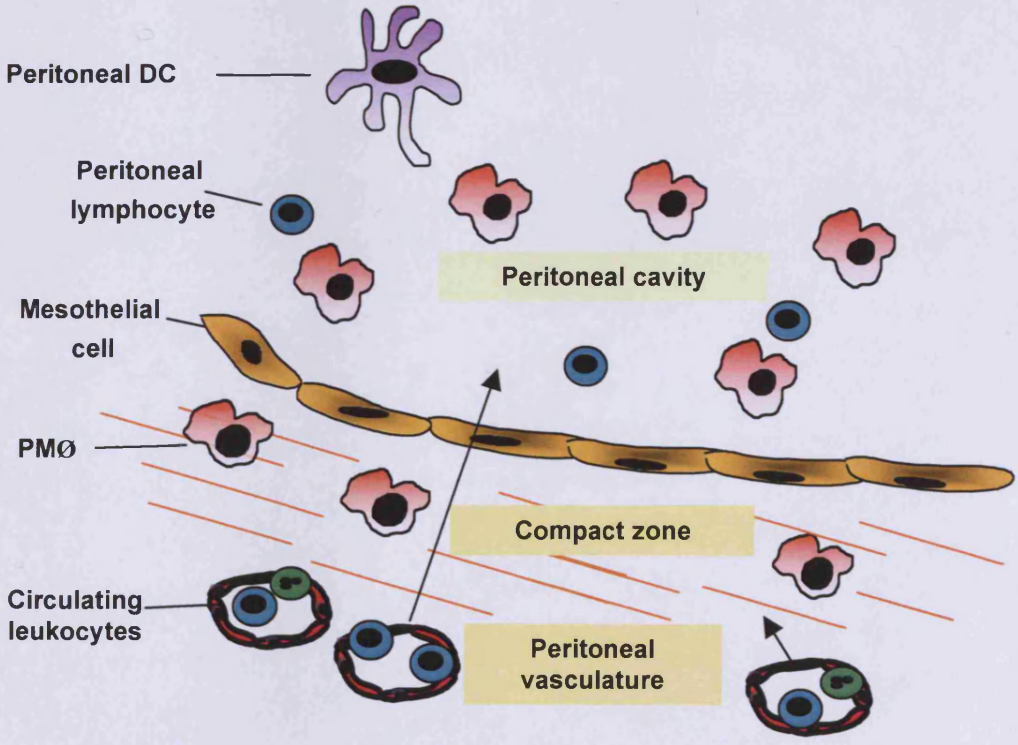


Figure 1.4 Immunobiology of the peritoneal cavity. Circulating leukocytes are selectively recruited from the systemic circulation into the peritoneal cavity.

1.4.2 Human peritoneal mesothelial cells (HPMC)

Mesothelial cells were once regarded as a passive lining to the peritoneal cavity, however we now realise that this is not the case. These cells are an integral part of the peritoneal immune response; indeed during peritonitis the HPMC are the main source of many pivotal chemokines (chemotactic cytokines) such as interleukin 8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1). In addition to their secretory function, *in vitro* evidence suggests that mesothelial cells can also become potent antigen presenting cells (APC). Once activated they express MHC class 2 molecules on their surface, thus allowing them to present processed antigen to T-cells. This function is aided by constitutive expression of intercellular adhesion molecule-1 (ICAM-1, the major accessory molecule expressed by non-professional APC) on their surface [22-25].

Long term PD therapy is associated with a reduction in total number of mesothelial cells; additionally the remaining cells adopt an abnormal morphological appearance suggesting that PD therapy may be detrimental to their function and survival [8, 10].

1.4.3 Peritoneal dendritic cells (DC)

Immature DC are found in peripheral tissues where they constantly sample the external environment via receptor-mediated endocytosis. They possess specialised pattern recognition receptors (PRR), which recognise highly conserved microbial antigens known as pathogen associated molecular patterns (PAMP). One group of PRR are the Toll like receptors (TLR), which can activate cell signalling and DC activation. Once activated, DC upregulate expression of antigen loaded MHC molecules and express lymph node homing signals such as the chemokine receptor CCR7. DC maturation is accompanied by up-regulation of T-cell co-stimulatory molecules such as CD80 and CD86 [26, 27].

Very little was known about the precise phenotype and function of peritoneal DC until recently when investigators characterised a small subset (5-6%) of

$CD14^+CD4^+$ cells, which fulfil the definition of DC pre-cursors. As compared to PMØ, these pre-DC1 cells express higher levels of co-stimulatory molecules, and are potent stimulators of allogenic T cells. Under appropriate conditions the pre-DC1 cells have the capacity to differentiate further, acquiring the morphological characteristics of mature DC [26, 27]. The number of pre-DC1 cells increases during the course of peritonitis suggesting that these cells are specifically recruited into the inflamed peritoneal cavity. Given that they express both PRR and MHC class II molecules, it is likely that they form a pivotal link between the innate and adaptive arms of the peritoneal immune response (see later). The impact that PD therapy has on the function of these cells is, at present unknown [17, 27, 28].

1.4.4 Peritoneal lymphocytes

There is relatively little data available regarding the phenotype and function of human peripheral tissue lymphocytes. This is due in part to the logistical difficulties involved with sample collection and the (usually) low yield of cells obtained from peripheral sites. The natural corollary of this is that most of our knowledge regarding human lymphocyte biology has been gained from animal models, cell lines and studies of peripheral blood lymphocytes.

A few studies have attempted to characterise the resident peritoneal lymphocytes. Most have focused on the surface phenotype of these cells with very little functional analysis. From these studies we can conclude that peritoneal lymphocytes represent between 10-30% of the resident peritoneal cell population. The majority of these are peritoneal T-cells with a smaller population of peritoneal B-cells (3-5%) and Natural killer (NK) cells (1-2%). When compared to peripheral blood lymphocytes, a large proportion of resident peritoneal T-cells express surface markers of activation such as CD69 and HLA-DR. They also display increased expression of CD45R0, which has been associated with memory cells [29-33]. The phenotype and function of peritoneal NK and B-cells remain poorly defined in the PD population. NK cell numbers appear to increase with time on PD, however their recruitment profile and effector function during peritonitis is not well documented [34, 35]. Given that NK cells are a pivotal part of the innate

immune response, it is likely that they are recruited early in the course of peritonitis along with neutrophils and inflammatory monocytes [36].

1.4.5 Peritoneal Mast Cells

Despite representing only a small proportion of the total PD effluent leukocytes (<1%), mast cells are frequently observed in peritoneal membrane biopsy specimens. In addition to their well documented role in allergic responses and immune response to parasites, more recent evidence suggests that these tissue resident mast cells play an important role in the early phase of the immune response. Mast cell depletion attenuated both KC production and neutrophil accumulation in the murine model of peritonitis. The number of resident peritoneal mast cells decreases with time on PD, which may in turn compromise the host "innate" immune response. [37, 38]

1.4.6 Peritoneal milky spots

Peritoneal milky spots were first described by Recklinghausen in 1863 and have since been identified as a pivotal component of the peritoneal immune defence. Milky spots are found within the omentum and appear as dense corpuscles to the naked eye. They are devoid of a capsule and contain aggregates of macrophages, B-cells, T-cells and mast cells. During bacterial peritonitis, milky spots increase dramatically in both size and number becoming easily visible to the naked eye as "cotton wool-like" masses [39, 40].

Numerous studies have highlighted the importance of peritoneal milky spots in orchestrating the host defence against invading pathogens. Peritoneal macrophages have been shown to undergo proliferation, differentiation and maturation locally within the milky spots. In addition they serve as a "relay station", regulating transit of B-cells from the circulation into the peritoneal cavity. Peritoneal milky spots are clearly an active site of leukocyte migration and are thus an important source of "primed" effector cells during bacterial peritonitis [41, 42].

The impact of PD therapy on peritoneal milky spots has been studied in both animals and humans. In a rat model of PD, exposure of the peritoneum to some of the more bio-incompatible components of PD solution (low pH, lactate and GDPs) resulted in a significant increase in both the number and diameter of milky spots [42, 43]. Analysis of human peritoneal biopsies suggests that with time on PD, milky spots appear to increase significantly in size. Collectively these studies suggest that PD therapy in itself may trigger a localized inflammatory response within the peritoneal cavity [40, 44].

1.5 The peritoneal immune response

1.5.1 Early vs. late phase

During an episode of peritonitis the various cellular components of the peritoneal immune system must work in conjunction to mount an effective peritoneal defence. The readily accessible nature of PD has allowed detailed analysis of the temporal changes in leukocyte phenotype that occur during an episode of peritonitis. The immune response is broadly divided into early and late phases. The early phase is characterised by a rapid influx of neutrophills, which are rapidly cleared and replaced by a slower, more sustained influx of mononuclear cells (Figure 1.5) [45-47].

The terms "innate" and "adaptive" are often used when referring to the early and late phases of the immune response. This is not strictly correct and may lead to confusion since the terms innate and adaptive refer to the specific nature of the response rather than to its temporal profile. The innate response describes a non-specific, highly stereotypical response to pathogen invasion, which is devoid of a memory component. In contrast to this, the adaptive immune response is a highly antigen-specific response characterised by long-term immunological memory. It is clear that some cells participating in the later phase of the immune response are in fact part of the innate immune system whereas some T-cells (the hallmark "adaptive" cells)

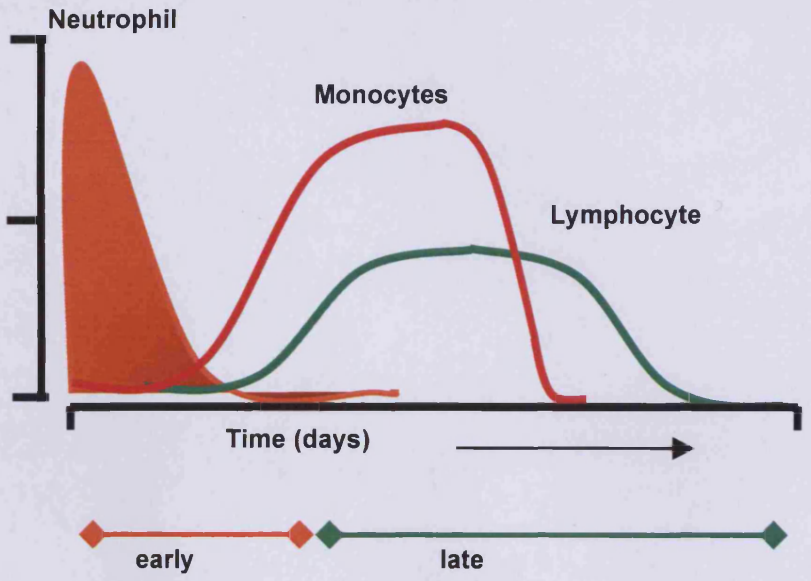


Figure 1.5 The peritoneal immune response; early vs. late. Recruited neutrophils are rapidly cleared and replaced by a slower, more sustained influx of mononuclear cells [50-52].

may play an important role in the very early phase of the immune response [45-47].

1.5.2 Peritoneal leukocyte recruitment

Peritonitis triggers a multifaceted, co-ordinated series of events, involving both innate and adaptive arms of the immune response. This tightly controlled response to infection serves to eradicate the invading organism whilst minimising host damage. Critical to the success of this process is the controlled migration of leukocytes from the peripheral blood into the peritoneal cavity [46, 47].

Leukocyte recruitment is a multi-step process mediated by chemokines, selectins and integrins. PMØ derived inflammatory signals induce up-regulation of vascular endothelial adhesion molecules such as selectins and ICAM-1. Concurrently mesothelial derived chemokines are transduced across the vascular endothelium and are displayed to circulating leukocytes. Peripheral blood leukocytes that express the appropriate chemokine receptor repertoire undergo a series of adhesion steps (tethering, rolling and arrest), which are mediated via selectins, integrins and GPCR (G-protein coupled receptors). Once a leukocyte is bound tightly to the endothelial surface, transmigration (diapedesis) is initiated [48].

The peritoneal chemokines are subdivided into 2 main groups. CC-Chemokines attract mononuclear cells to sites of inflammation. The best-characterised CC Chemokine is Monocyte Chemoattactant Molecule 1 (MCP-1). The second group are termed CXC chemokines (since they have a single amino acid between the first two cysteines). A sub-family of CXC chemokines carry a characteristic glutamate-leucine-Arginine (ELR) motif. A key function of the ELR+ CXC chemokines is to attract neutrophills to sites of inflammation. IL8 (CXCL8) is the prototype CXC chemokine [49].

Chemokine production and the associated leukocyte migration follows a tightly regulated temporal pattern. ELR^+ chemokines are produced on day 1, leading to recruitment and intra-peritoneal accumulation of neutrophils.

Mesothelial cells then switch production from CXC-chemokines to CC-chemokines, resulting in the recruitment of mononuclear cells (lymphocytes/monocytes/DC) from the blood [50]. Recently it has become evident that IL-6 signalling is pivotal in regulating this recruitment process. Two pathways of IL-6 signalling have been described (Figure1.6).

IL-6 trans-signalling confers IL-6 responsiveness in cell types lacking expression of the cognate IL-6 receptor e.g. HPMC. During peritonitis, sIL-6R levels rise dramatically (due in part to the shedding of membrane bound cognate IL-6R from the recruited neutrophils) thus conferring IL-6 responsiveness to HPMC. Both *in vivo* and *in vitro* studies have shown that IL-6 trans-signalling down regulates HPMC IL-8 production and increases MCP-1 expression, hence attenuating neutrophil recruitment and promoting MNC recruitment. IFN-γ has also been shown to regulate neutrophil recruitment, due in part to its ability to act in synergy with IL-1β and TNFα to promote IL-6 production [46, 47, 50-53].

1.5.3 Immuno-resolution

In addition to appropriate recruitment, a successful immune response also requires prompt removal of inflammatory leukocytes. This ensures minimal host damage and prevents the development of chronic inflammation. Within the peritoneal cavity an elegant IL-6-dependant control mechanism exists to ensure that activated neutrophils are efficiently cleared. Firstly, IL-6 trans-signalling leads to decreased recruitment (as mesothelial cell ELR^+ CXC chemokine production is down-regulated). Secondly, IL-6 trans-signalling (driven by IFN-γ induced IL-6 production) have been shown to promote neutrophil apoptosis. This ensures prompt resolution of the early inflammatory response [46, 47, 50-52]. A schematic representation of the peritoneal response to infection is shown in Figure 1.7.

1.5.4 Chronic inflammation and membrane fibrosis

There is growing evidence from both human studies and the murine model of peritoneal inflammation that repeated or severe episodes of peritonitis disrupt

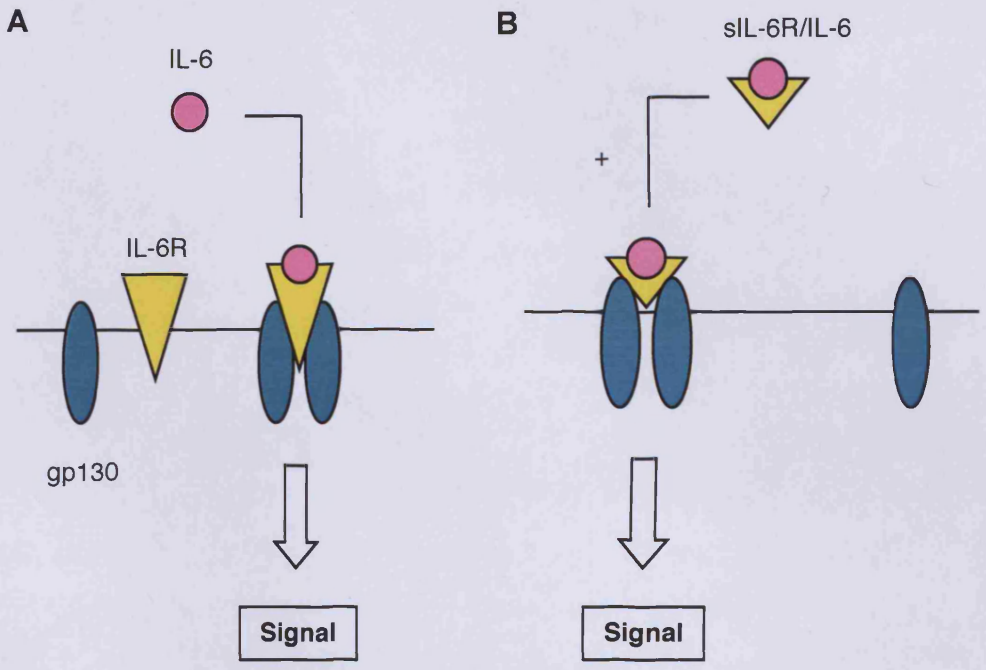


Figure 1.6 IL-6 signalling pathways. (A) classic pathway: IL-6 signalling binds its cognate receptor on the cell surface. (B) IL-6 trans–signalling: IL-6 binds a soluble receptor (sIL-6R) to form a ligand-receptor complex, which can activate cells by binding, to the ubiquitously expressed signal transducing element gp130 [51,52,57].

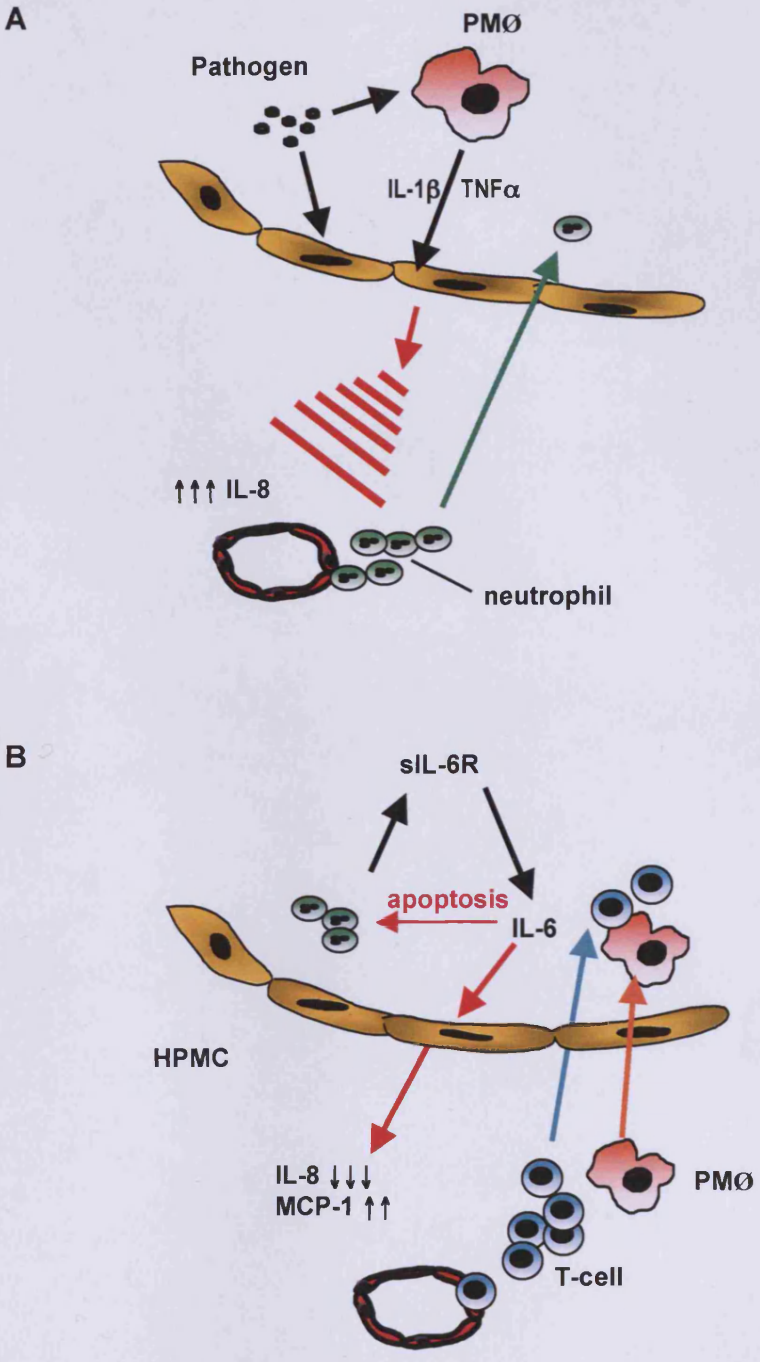


Figure 1.7 The peritoneal immune response. (A) Peritoneal inflammation is initiated at the HPMC/pathogen interface. Activated neutrophils exit the circulation and follow a gradient of HPMC derived IL-8 to enter the peritoneal cavity.(B) Apoptotic neutrophils shed their IL-6R which in turn binds intraperitoneal IL-6. This allows HPMC to respond to IL-6 via trans-signalling. HPMC chemokine production switches from IL-8 to MCP-1, which abrogates neutrophil recruitment and attracts mononuclear cells from the circulation (adapted from *McLoughlin et al* [50]).

the intricate control mechanisms that regulate the peritoneal immune response. This leads to dysregulated inflammation characterised by neutrophil retention and on-going production of pro-inflammatory chemokines (even in the absence of on-going infection). These detrimental changes herald the onset of chronic peritoneal inflammation, a process that is likely to initiate or perpetuate the development of peritoneal membrane fibrosis (Figure 1.8).

1.6 An emerging role for peritoneal T-cells

Most of our understanding of the human peritoneal immune response is based on *in-vitro* analysis of PMØ and HPMC. The role of the peritoneal T-cell in this process has been largely ignored. There is however evidence to suggest that peritoneal T-cells play a key role in orchestrating the peritoneal immune response. Firstly, elevated levels of T-cell derived cytokines such as IFN-γ have been detected during episodes of peritonitis suggesting that significant effector T-cell activation is occurring [23, 54, 55]. Secondly, data derived from knockout mice studies have shown that T-cell derived cytokines (in particular IFN-γ) are critical in regulating leukocyte recruitment during peritonitis. Finally, more recent data emerging from the mouse "repeat hit model" model of peritonitis suggests that repeated episodes of peritonitis alter resident peritoneal T-cell phenotype and function, and that this may contribute to chronic inflammation [Topley, Jones, unpublished data]. In spite of this, many questions remain regarding both the nature and activation status of human peritoneal T-cells and their precise contribution to the regulation of the immune responses in PD patients.

In trying to define their role in peritoneal inflammation, we have to take into account that the field of T-cell biology has moved on significantly over the last 5 years, giving us fresh insight into the dynamics of T-cell development, activation and differentiation. The most relevant discoveries made will now be summarised.

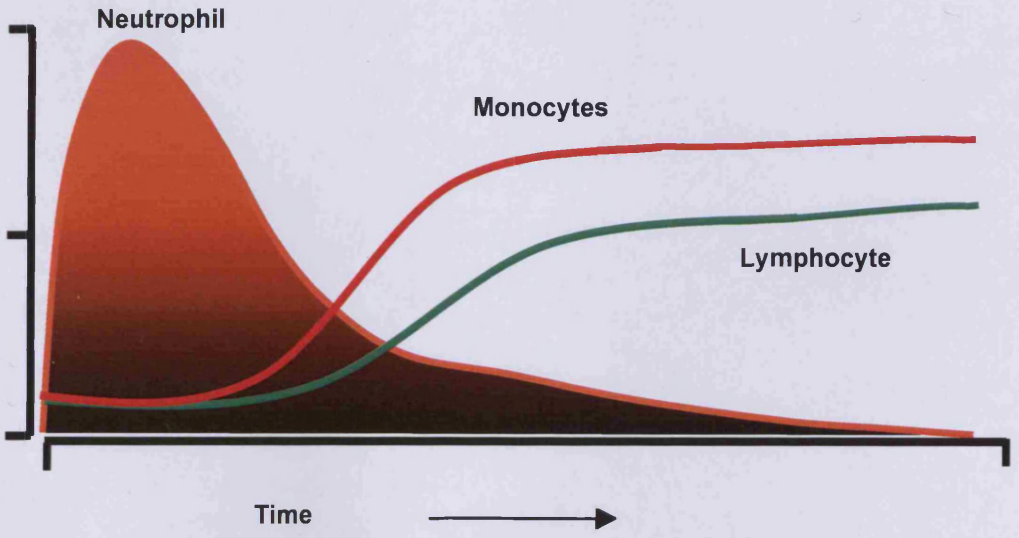


Figure 1.8 Dysregulated leukocyte trafficking. Chronic inflammation is characterised by aberrant recruitment and retention of activated leukocytes.

1.6.1 T-cell development and activation

Immature T-cells generated in the bone marrow migrate to the thymus where they undergo a series of maturation steps including positive (for cells that recognise self-MHC molecules) and negative (against self-reactive T-cells) selection. The minority of cells that survive this maturation process (less than 2%) exit the thymus to join the peripheral T-cell pool. These cells are known as naïve T-cells; they are yet to encounter antigenic stimulation and display minimal effector function [56]. Each naïve T-cell expresses a clonal, antigen-specific T-cell receptor (TCR). The TCR gene locus contains constant (C), variable (V), diversity (D) and joining (J) segments each of which exist in multiple copies. The vast TCR structural diversity (necessary to deal with millions of potential antigens) is generated by the combinatorial joining of these gene segments [56].

T-cell activation and differentiation is initiated when the T-cell receptor (TCR) of a naïve T-cell interacts with the peptide-MHC (major histocompatibility complex) displayed on the surface of antigen-presenting cells (APC). A three-signal model has now emerged for T-cell activation. The first signal is provided by the recognition of peptide:MHC complex by the TCR, the second signal is provided by co-stimulatory molecules present on the surface of APC (e.g. CD80, CD86 and CD40) and signal three refers to the cytokines (e.g. IL-12) that modulate T-cell differentiation [27].

1.6.2 TH1 vs. TH2 differentiation

Following activation, naïve T-cells proliferate and differentiate into a variety of effector cell fates that help determine the shape and effector capacity of the immune response. The TH1 vs. TH2 theory describes two separate T-cell effector subsets. TH1 cells drive cell mediated immune responses and are essential for the eradication of intracellular pathogens such as bacteria and viruses. Cytokines produced by TH1 cells stimulate macrophages to produce reactive oxygen species and nitric oxide. In addition, they increase macrophage phagocytic function and enhance MHC class II presentation.

The hallmark TH1 cytokine is IFN-γ [56, 57]. TH2 cells produce IL-4, IL-5 and IL-13 all of which can activate mast cells and eosinophills. TH2 cells also provide B-cell help and aid immunoglobulin class switching to the IgE subtype. TH2 cells predominate during nematode and helminth infection and have also been implicated in the pathogenesis of allergy and atopic reactions [56, 58, 59].

The major inducers of TH1 and TH2 subset development are DC derived cytokines. IL-12 is a strong driving force for a TH1 type response while production of IL-4 early in an immune response directs the development of TH2 type cells. A reciprocal control mechanism exits whereby development of one subset is promoted at the expense of another e.g. IFN-γ up-regulates the expression of the IL-12 receptor but inhibits the growth of TH2 cells, conversely IL-4 antagonizes TH1 development (Figure 1.9). This regulatory mechanism streamlines the immune response by promoting the development of one TH subset at the expense of the other. The main transcription factors involved in TH1 vs. TH2 responses have been identified as T-bet and GATA–3 respectively [55, 60, 61].

1.6.3 IL-17 producing T helper cells (TH17)

One of the most significant challenges to the TH1/TH2 model came with the observation that cell-mediated tissue damage in experimental allergic encephalomyelitis (EAE) was not mediated by IFN-γ, in fact IFN-γ appeared to be protective against disease. This observation was surprising at the time since EAE (an animal model of multiple sclerosis) was thought to be the archetypal TH1-mediated disease [61, 62].

Their discovery stemmed from work done on IL12/IL-23 double-deficient mice. Both IL-12 and IL-23 are heterodimers containing a p40 sub-unit in combination with a second sub-unit. In the case of IL-12 this is p35 whereas for IL-23 the second sub unit is p19. This subtle difference allowed investigators to manipulate IL-12/IL-23 signalling pathways by creating mice deficient in either the p40, p35 or p19 sub units. Experiments with these mice

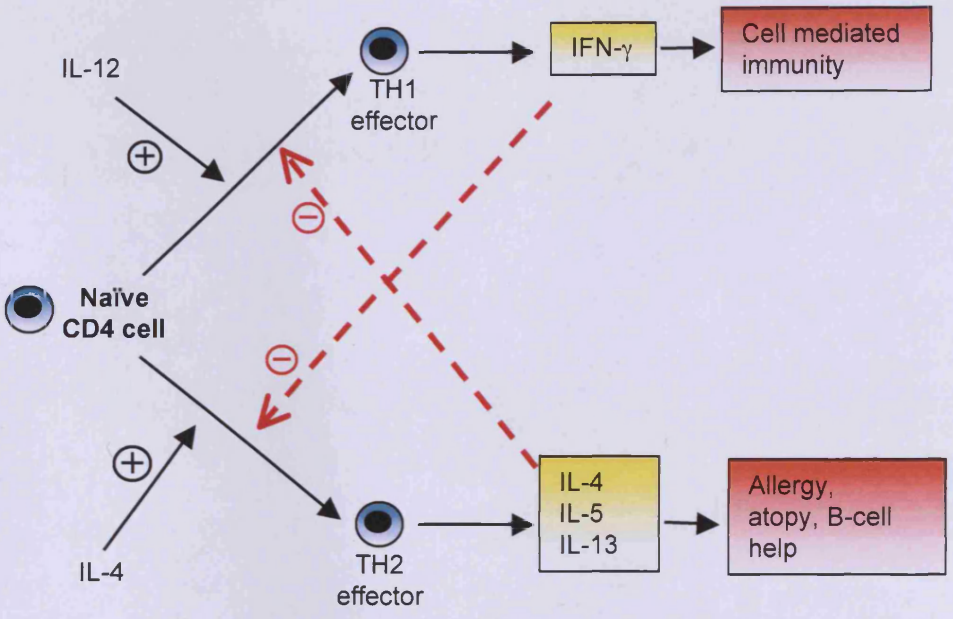


Figure 1.9 TH1 vs. TH2 effector T-cell development [60-65].

revealed that both $p19^{-/-}$ mice (deficient in IL-23 signalling) and $p40^{-/-}$ mice (deficient in both IL-23 and IL-12 signalling) are protected against EAE whereas $p35^{-/-}$ mice (deficient in IL-12 signalling) still develop the disease. These findings were the exact opposite of what would have been predicted from the TH1 model of EAE pathogenesis and suggested that the disease was not in fact driven by the IL-12/IFN-γ axis. Further experiments delineating the role of IL-23 in this process led to the discovery of a unique, highly pro-inflammatory IL-17 producing T-cell subset (TH17) [61, 62].

Since their discovery, TH17 cells have been implicated in the pathogenesis of numerous murine models of disease such as EAE, myocarditis, inflammatory colitis, collagen induced arthritis and airway hypersensitivity. There is also a growing body of evidence suggesting that IL-17 plays a role in human disease such as multiple sclerosis, rheumatoid arthritis, graft rejection and psoriasis (indeed anti-p40 treatment is currently being investigated as a potential therapy for psoriasis) [61].

The precise cytokine milieu necessary to drive TH17 development remains an area of controversy. Initial observations in the EAE model suggested that IL-23 signalling was essential, however this was challenged by series of experiments revealing that transforming growth factor beta (TGFβ) in the presence of IL-6 is sufficient. It appears that both findings are correct, since although IL-23 is not necessary for TH17 development, it is essential for their survival and effector function. In addition to IL-17, TGFβ driven TH17 cells also produce IL-10, so that IL-23 is required to overcome the anti–inflammatory effects of IL-10 [62-65].

Similar to TH1/TH2 regulation, a reciprocal control mechanism exists between TH1 and TH17 subsets with one subset preventing development of the other. The key transcription factor necessary for TH17 development has now been recognised as the orphan nuclear receptor RORγt [62-65].

1.6.4 Regulatory T cells (T-regs)

A key feature of the immune system is the ability to discriminate between self and non-self. This ensures effective clearance of microbial pathogens but prevents the development of autoimmune disease. Thymic deletion or inactivation of self-reactive T-cells (clonal deletion/anergy) helps to maintain self-antigen tolerance. This mechanism is not fail-safe and significant numbers of self-reactive T-cells avoid deletion and enter the peripheral circulation. Additional protection against autoimmunity is provided by the presence of a population of circulating (and local) regulatory T-cells (T-regs).

T-regs are able to prevent autoimmune disease by suppressing the effector function of potentially self reactive T-cells. In addition to their well documented role in preventing autoimmunity more recent evidence suggest that these cells play a role in regulating the host response to malignancy, infection and allogenic organ transplants [66, 67].

Although initially described in the 1970's, a phenotypic description of T-regs did not become available until the mid 1990's. Murine experiments performed at that time showed that $CD4^+$ T-cells expressing the IL-2 receptor ($CD4^+/CD25^+$ T-cells) were able to suppress the development of experimentally induced autoimmune disease. Unfortunately, this early phenotypic definition fails to distinguish T-regs from recently activated effector CD4 cells (as both express CD25). Similarly, the specificity of other T-reg surface markers such as LAG-3, CTL-4 and GITR (Glucocorticoid-induced Tumour necrosis factor receptor family related protein) are limited by the fact that recently activated effector and memory T cells also express these markers. The more recent discovery of Foxp3 (a member of the *forkhead* family of transcription factors) has allowed a more refined phenotypic description of T-regs [68-71].

Foxp3 is thought to be the first specific marker of T-reg cells, discriminating between T-regs ($CD4^+/CD25^+/Foxp3^+$) and activated effector T-cells ($CD4^+/CD25^+$ $/Foxp3^-$). The importance of Foxp3 in T-reg development and function is highlighted by observations in both the murine and human system. Mice deficient in Foxp3 lack T-reg cells and develop multi-organ autoimmune

pathology, whereas in humans Foxp3 mutations cause the autoimmune phenomenon known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X linked syndrome) [68-71].

A recent sub-classification divides T-regs into natural T-regs (generated within the thymus during the process of T-cell maturation) and inducer T-regs (which develop from $CD4^+CD25^-$ naïve T-cells in the periphery). As compared to natural T-regs, inducer T-regs express lower levels of CD25 and are more dependant on TGFβ for their development and effector function [72-74]. Inducer T-regs appear to have close ties with the TH17 subset since both are believed to differentiate from the same precursor cell and a model has been proposed whereby they work in conjunction to restrict or elicit tissue inflammation (Figure 1.10).

The T-reg/TH17 development hypothesis has challenged many theories regarding pro- and anti-inflammatory response by showing that a TH1 response (by preventing TH17) can in fact be anti-inflammatory while T-regs (via production of TGFβ) may sometimes have a pro-inflammatory effect [61].

1.6.5 Gamma delta T-cells (γδ T-cells)

Whilst the majority of circulating T-cells express the TCRαβ (αβT-cells) on their surface, a minority (2-20%) express the TCRγδ. In humans the most abundant γδ T-cells is the subset expressing the Vγ9δ2 TCR. There is emerging evidence that these cells orchestrate the early phase of the immune response [75]. Vγ9δ2 T-cells differ fundamentally from αβ T-cells with respect to the type of antigen recognised, mode of antigen recognition and chemokine receptor profile. With regards to antigenic stimulation, Vγ9δ2 T-cells recognise low molecular weight non-peptide compounds in an MHC independent manner. This is in stark contrast to αβ T-cells that recognise peptides presented on MHC molecules by APC [75-78].

The most potent stimulant of Vγ9δ2 T-cells is E-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the microbial non-mevalonate

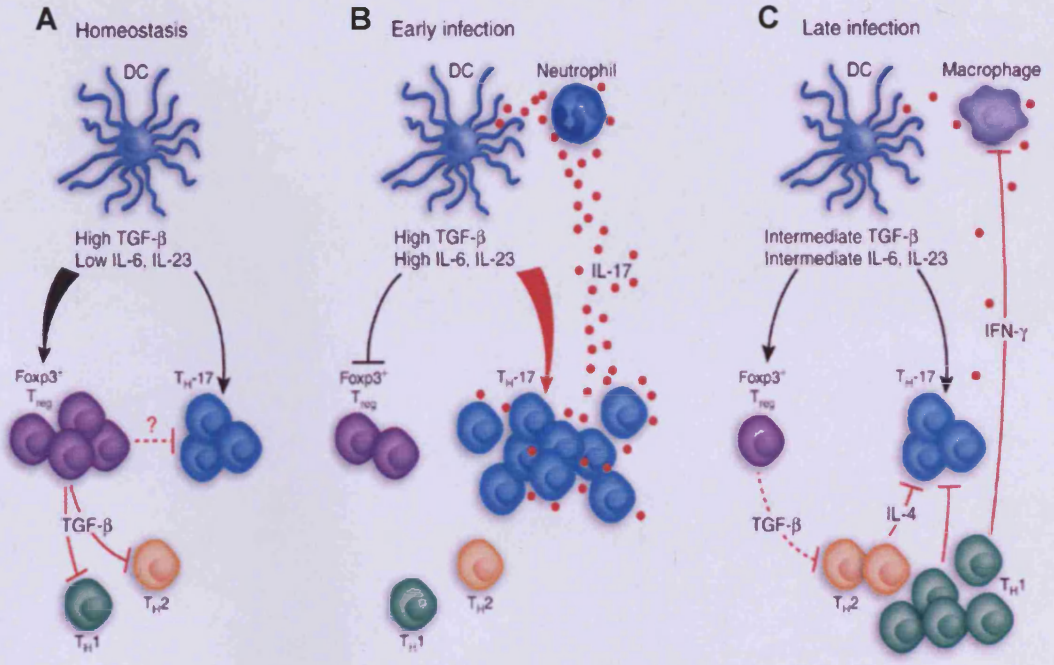


Figure 1.10 The balance between T-regs and TH17 cell development (A) During the steady (non-infected) state T-regs dominate and suppress TH1 and TH2 cell effector function. (B) During infection, microbial products trigger DC (via Toll-like receptors) to produce large amounts of IL-6 and IL-23, which together with TGFβ increase the rate of TH17 cell differentiation. IL-23 promotes the proliferation and inflammatory function of these cells. (C) Later in the course of infection, TH1 and TH2 cells pre-dominate and (via IFN-γ and IL-4), inhibit TH17 cell differentiation and function. Finally, as infection subsides inflammatory T-cells are cleared, T-regs pre-dominate and homeostasis is restored (reproduced from *Cua et al* [70]).

pathway of isopentenyl pyrophosphate (IPP) biosynthesis. The ability to recognise a by-product of a key metabolic pathway shared by numerous organisms (but absent in all higher eukaryotes) offers a simple means for Vγ9δ2 T-cells to respond to a host of different pathogens. Common infective agents that utilise the non-mevalonate pathway include, *Mycobacterium tuberculosis*, *Plasmodium falciporum* and most Gram-negative organism species including *Pseudomonas spp.* and *Escherichia coli.* Interestingly, *Streptococci* and *staphylococcal* species (like eukaryotes) utilise the classical pathway of IPP biosynthesis, hence do not produce HMB-PP and do not stimulate Vγ9δ2 T-cells *in vitro* [75].

In contrast to peripheral blood αβ T-cells most Vγ9δ2 T-cells express an inflammatory chemokine receptor profile characterised by increased expression of CCR5 (and decreased expression of CCR7). This "inflammatory phenotype" is shared by neutrophils, monocytes and immature DC and suggests that Vγ9δ2 T-cells respond early during the course of an inflammatory episode. Once at the site of infection, Vγ9δ2 T-cells up-regulate lymph node homing receptors and develop potent antigen processing capabilities (similar to mature DC). This suggests that they have the potential to activate αβ T-cells, thus triggering the adaptive immune response (see later) [75-78].

1.6.6 T cell memory

Following prolonged stimulation of the TCR, most effector T-cells undergo activation-induced cell death (AICD). A small percentage of those that survive persist as long-lived memory T-cells, providing recall responses to the primary antigenic stimulus. A classification of T-cell memory subsets has been proposed based on the expression of the lymph node homing signals CCR7, CD62L and the memory cell marker CD45R0 (Figure 1.11)[59, 79].

Effector memory (T_{EM}) cells (CD45R0/CD62L^{Lo} /CCR7^{-}) express receptors that allow them to migrate to inflamed areas. Upon stimulation, T_{EM} display immediate effector function [59, 80]. Central memory (T_{CM}) cells

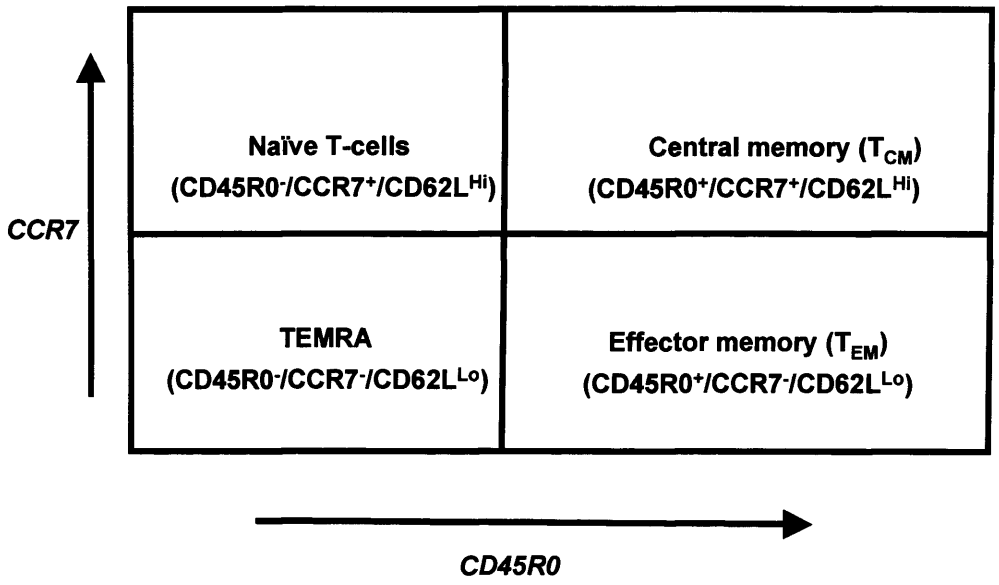


Figure 1.11 T-cell memory subsets. Memory subsets are defined by their expression of CCR7, CD62L and CD45R0 (64,83).

(CD45R0/CD62L^{Hi} /CCR7^{+}) express receptors for lymph node migration and lack immediate effector function. They retain their proliferative capacity and efficiently differentiate into effector cells [59, 80]. Terminally Differentiated Effector memory cells (TEMRA) (CD45RA/CD62^{Lo}/CCR7^{-}) are found in the CD8 population. These are thought to be T_{EM} cells which express CD45 RA rather than CD45R0 [59, 80].

The existence of such functionally distinct memory subsets provide an effective defence mechanism against infection as T_{EM} provide immediate protection while T_{CM} provide a secondary response and long term protection. Two different pathways of T-cell memory differentiation have been postulated, these are outlined in Figure 1.12 [59, 79, 80].

Unlike short lived effector T-cells, memory cells can persist for a lifetime even in the absence of antigen. This longevity is dependant on the presence of cytokines such as IL-7 and IL-15, which maintains homeostatic proliferation, and prevents cell removal by apoptotic mechanisms. IL-7R expression has recently been identified as a useful phenotypic memory cell marker, which may distinguish between memory cells and short-lived effector cells [80].

1.6.7 A current paradigm of αβT-cell diversity

The advances in the field of T-cell biology (summarised in Figure 1.13) serve to highlight how little we know regarding the phenotype and function of the peritoneal T-cell population. Since most peritoneal T-cell studies are over 10 years old, they pre-date the recognition of most of the subsets described above. Consequently it is hardly surprising that none of these cells have been identified in the peritoneal cavity of PD patients. Moreover since most human studies have focussed on peripheral blood, there is a general paucity of data describing these cells in any peripheral tissue.

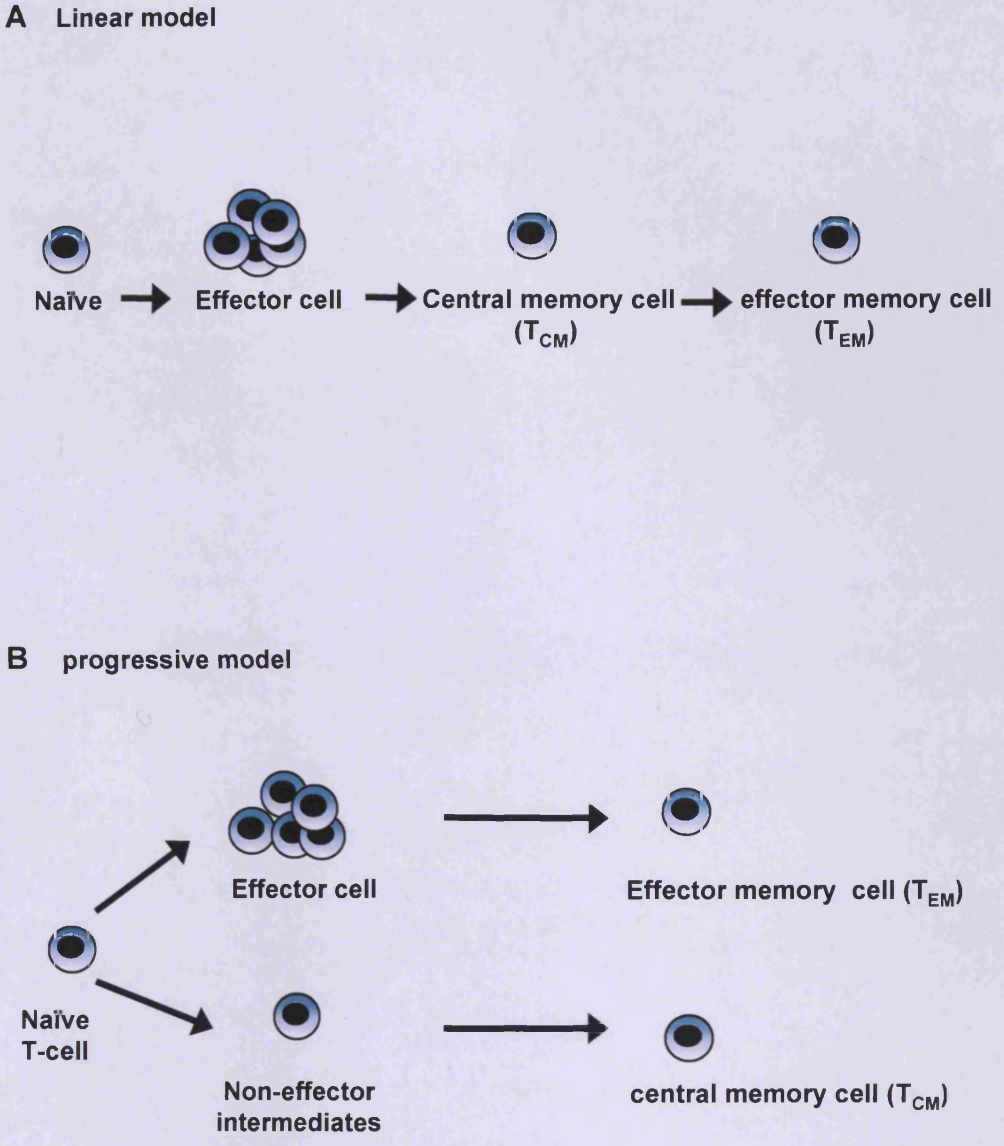


Figure 1.12 Memory T-cell differentiation. (A) According to the linear model, memory cells arise directly from effector cells which have reverted to a quiescent state. The T_{CM} serve as a pool of memory cells capable of replenishing the T_{EM} pool. (B) According to the progressive model, the fate of the naïve cells depends on the duration and strength of the antigenic stimulus. A strong, prolonged stimulus leads to the generation of effector cells. A weaker stimulus leads to the production of non-effector intermediates which serve as a pool of highly sensitive cells that can be recruited in the secondary immune response. One type of cell migrates to peripheral tissue and displays immediate effector function, the other type migrates to lymph nodes and lacks immediate effector function [83]

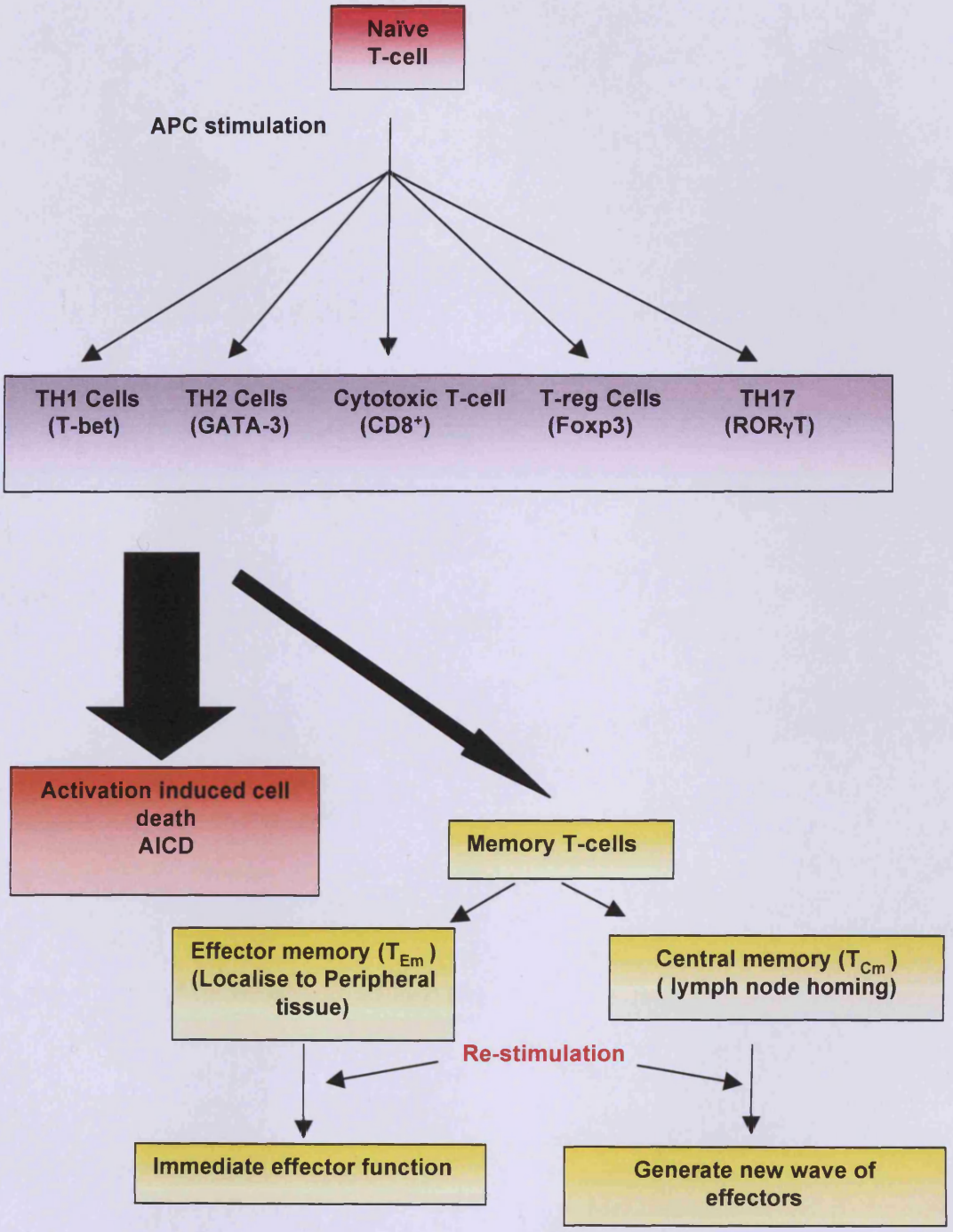


Figure 1.13 A current paradigm of αβT-cell differentiation encompassing some of the more recent developments

1.7 Scope of this thesis

Appropriate regulation of leukocytes and the mediators responsible for their recruitment and clearance is a hallmark of competent host defence and the resolution of inflammation. Understanding and modifying the potentially detrimental pathways of the immune response is vital if we wish to limit the exposure of the peritoneal membrane to prolonged inflammation. To this extent, information about resident peritoneal T-cells during prolonged periods of PD is sparse, and their impact on the development of membrane fibrosis may have been severely underestimated. Observations from the mouse "repeat hit model" suggest that dysregulation of the resident T-cell population may initiate chronic inflammation and membrane fibrosis. We feel that a contemporary phenotypic and functional analysis of the peritoneal T-cell population is essential if we are to understand the basic disease mechanisms that trigger immune dysregulation, chronic inflammation and membrane fibrosis.

The specific aims of each investigative chapter are as follows:

Chapter 3 has characterised the basic phenotype, activation status and function TH1/TH2-polarisation, proliferation of resident peritoneal T-cells.

Chapter 4 identifies specialised T-cell subsets (including memory, T-regs, TH17 and $V\gamma9\delta2$ subsets) within the resident peritoneal T-cell population.

Chapter 5 identifies the impact of recurrent infection on resident peritoneal T-cell phenotype and function.

Chapter 2

Materials and Methods

2.1 Patient selection

Ethical approval was obtained from the South East Wales Ethics Committee (*04WSE04/27*). Consenting patients were recruited from the University Hospital of Wales PD Unit. Unless specifically stated, all patients were receiving a standard peritoneal dialysis therapy of four exchanges per day with 2 litres of dialysis fluid (Dianeal ± Extraneal; see appendix section for further details on PD prescriptions).

2.2 Leukocyte isolation from PD fluid

PD effluent (mean drained volume 2187ml, range 1585-3120ml) was collected from patients after an overnight 8-10h dwell. Samples were collected on ice to reduce adhesion of cells to the PD bag [81]. Following collection the PD effluent was aliquoted into 50ml tubes (Sarstedt AG & Co, Nümbrecht, Germany) and centrifuged at 3000 rpm (2133xg), at 4°C for 35mins (Minifuge T, Heraeus/Kendro Laboratory products, Stevenage UK). The cell pellets were re-suspended in 50ml phosphate buffered saline (PBS, Sigma-Aldrich, Kent, UK). The cell suspension was centrifuged at 4°C for 20 mins at 3000 rpm (2133xg). The supernatant was discarded and the cell pellet was re-suspended in lysis buffer (see section 2.15) and allowed to stand for 5 mins at room temperature. The suspension was re-centrifuged at 3000 rpm (2133xg) at 4°C for 20mins and the pellet re-suspended in PBS at $1x10^6$ cells/ml (mean number of leukocytes/bag was $6.4x10^6$, range $1.0\text{-}25x10^6$). Leukocyte numbers obtained from the overnight pd effluent of an individual (stable) patient remained constant over a 3/4 month collection period.

2.3 Leukocyte isolation from peripheral blood

Venous blood was collected from consenting healthy laboratory volunteers/ patients in an ethylene-diamine-tetra-acetic (EDTA) vacutainer tube (Becton

Dickinson Ltd., Oxfordshire, UK) and centrifuged at 1299 rpm (400xg) for 12mins. (Minifuge T, Heraeus/Kendro Laboratory products, Stevenage UK). After discarding the plasma, the remaining blood cells were re-suspended in an equal volume of PBS. The cell suspension was layered onto Ficoll-paque (Amersham Biosciences Ltd, Bucks, UK) and centrifuged at 1299 rpm (400xg), at 10°C for 35 mins. The mononuclear cell layer (formed at the interface) was aspirated into a 50ml tube and suspended in 10ml of PBS at 10°C. The cell suspension was centrifuged at 10°C for 10mins at 1299 rpm (400xg). The supernatant was discarded and the cell pellet re-suspended in lysis buffer (see section 2.15) and allowed to stand for 5mins at room temperature. The suspension was re-centrifuged at 1299 rpm (400xg) at 10°C for 10mins and the pellet was washed (x3) prior to suspension in PBS at $1x10^6$cells/ml.

2.4 Total cell count

Total cell count was determined using a Coulter Counter (Z2, Beckman Coulter, Buckinghamshire, UK). One hundred µl of the cell suspension was diluted in 20ml of Isoton II solution (Beckman Coulter, Buckinghamshire, UK) in a counting vial and placed in the machine. The coulter Counter analyzed 0.5ml of sample.

The number of cells per ml was calculated

$$\frac{(A1 - B1) + (A2 - B2)}{2} \times 2 \times 2000 = \text{Total cell number}$$

A = Total cell count of the sample

B = Total background count of clean Isoton II in a counting vial

2.5 Differential cell count

Cytospin preparations were prepared to assess the differential cell counts. Samples of cell suspension were added to the cytocentrifuge (Thermo Shandon cytospin4, Shandon Scientific Ltd., Cheshire, UK). The cells were adhered to microscope slides (Blue Star Micro Slides, West Midlands, UK) by centrifuging at 1000 rpm for 10 minutes. The slides were air-dried overnight and stained using a Quick III Haematology stain kit (Astral Diagnostics Inc., West Deptford, NJ, USA). The slides were fixed in methanol for 60 seconds then dipped sequentially into an eosin (eosinophilic staining) solution and azure solution (basophilic staining) for 60 seconds each. The slides were then rinsed with de-ionised water and allowed to air dry. They were examined by light microscopy (Carl Zeiss Ltd, Hertfordshire, UK) 200 cells were counted and the percentage of each cell type present recorded based on the assessment of at least 200 cells (see figure 2.1).

2.6 T-cell isolation

2.6.1 Magnetic column negative selection

Cells were re-suspended in 40µl of MACS buffer per 10^7 cells. Biotin antibody cocktail containing biotin conjugated anti - CD14, CD16, CD19, CD36, CD56, CD123, and CD235 (Miltenyi Biotech Ltd, Surrey, UK) was added (10µl/10^7 cells) and the cells were incubated at 4°C for 10mins in the dark (see table 2.1). Following incubation a further 20µl per 10^6 cells of MACS buffer and 20µl/10^7 cells of anti biotin antibody (Miltenyi Biotech Ltd, Surrey, UK) were added. The cells were incubated for a further 15mins at 4°C in the dark. Following incubation the cells were washed with 200µl MACS buffer then centrifuged at 1299 rpm (400xg) for 15min at 6°C. The cells were re-suspended in 500µl MACS buffer (at this point samples were taken for FACS analysis). The cells were passed through a mini MACS magnetic separator column (Miltenyi Biotech Ltd, Surrey, UK). The column was then washed (x3) with 500µl MACS buffer. All the effluent was collected and represented the enriched T-cell population. T-cell purity by

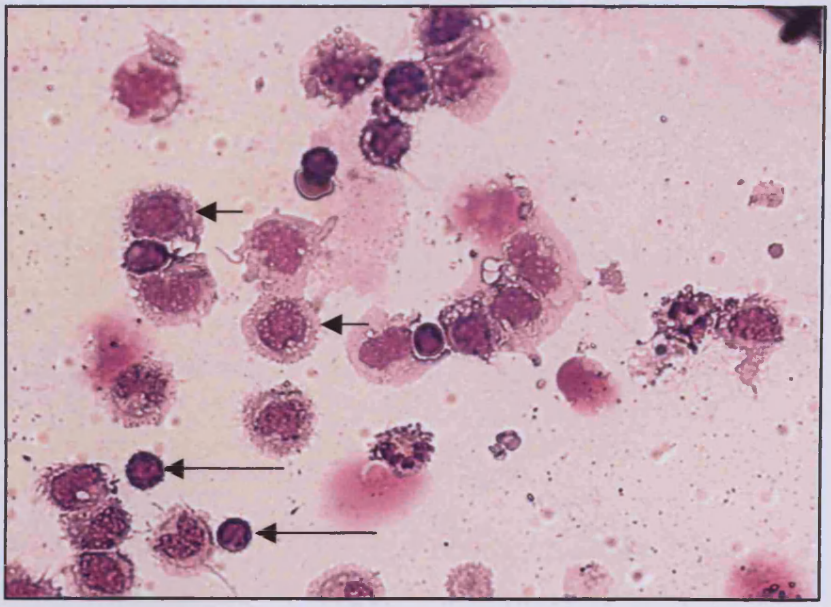


Figure 2.1 Cytospin preparation of peritoneal leukocytes showing lymphocytes (long arrows) and peritoneal macrophages (short arrows).

this method was >85% as assessed by flow cytometric analysis of $CD3^+$ lymphocytes (see Figure 2.2). The majority of "non-$CD3^+$" contaminants were CD14+ Monocyte/Macrophages or $CD19^+$ B-cells. T-cell purity and the nature of contaminating cells varied from patient to patient and may be a confounding factor when comparing *in-vitro* T-cell activation between patient groups. It should also be noted that though CD56 is a NK cell marker it is also expressed by a subset of activated T-cells which are thus depleted by the negative selection process.

.

Antigen	Cells expressing antigen
CD14	Monocytes/Macrophages
CD16	Neutrophills/macrophages/NK cells
CD19	B-cells/plasma cells
CD36	Platelets/RBC/Monocytes
CD56	NK cells/activated T-cells
CD123	Plasmacytoid DCs
CD235	RBC

Table 2.1 Cells depleted by the negative selection process

2.7 Isolation of BDCA-1 dendritic cells from blood

Peripheral blood mononuclear cells (PBMC) were isolated from 150ml of blood by ficol density gradient centrifugation. Cells were re-suspended in 200µl of MACS buffer per 10^8 cells. One hundred µl of FcR blocking reagent (Miltenyi Biotech Ltd, Surrey, UK) was added per 10^8 cells. One hundred µl of CD19 (Miltenyi Biotech Ltd, Surrey, UK) micro beads per 10^8 total cells was added followed by 100µl of anti-BDCA-1-biotin antibody (Miltenyi Biotech Ltd, Surrey, UK) per 10^8 cells. The cells were then incubated for 15mins, washed in 20x labelling volume of MACS buffer and centrifuged for 12 mins at 1299 rpm (400xg). The supernatant was removed completely and the cells re-suspended in a final volume of 500µl/10^8 cells of MACS buffer. The cell suspension was passed down the magnetic separation column. The column

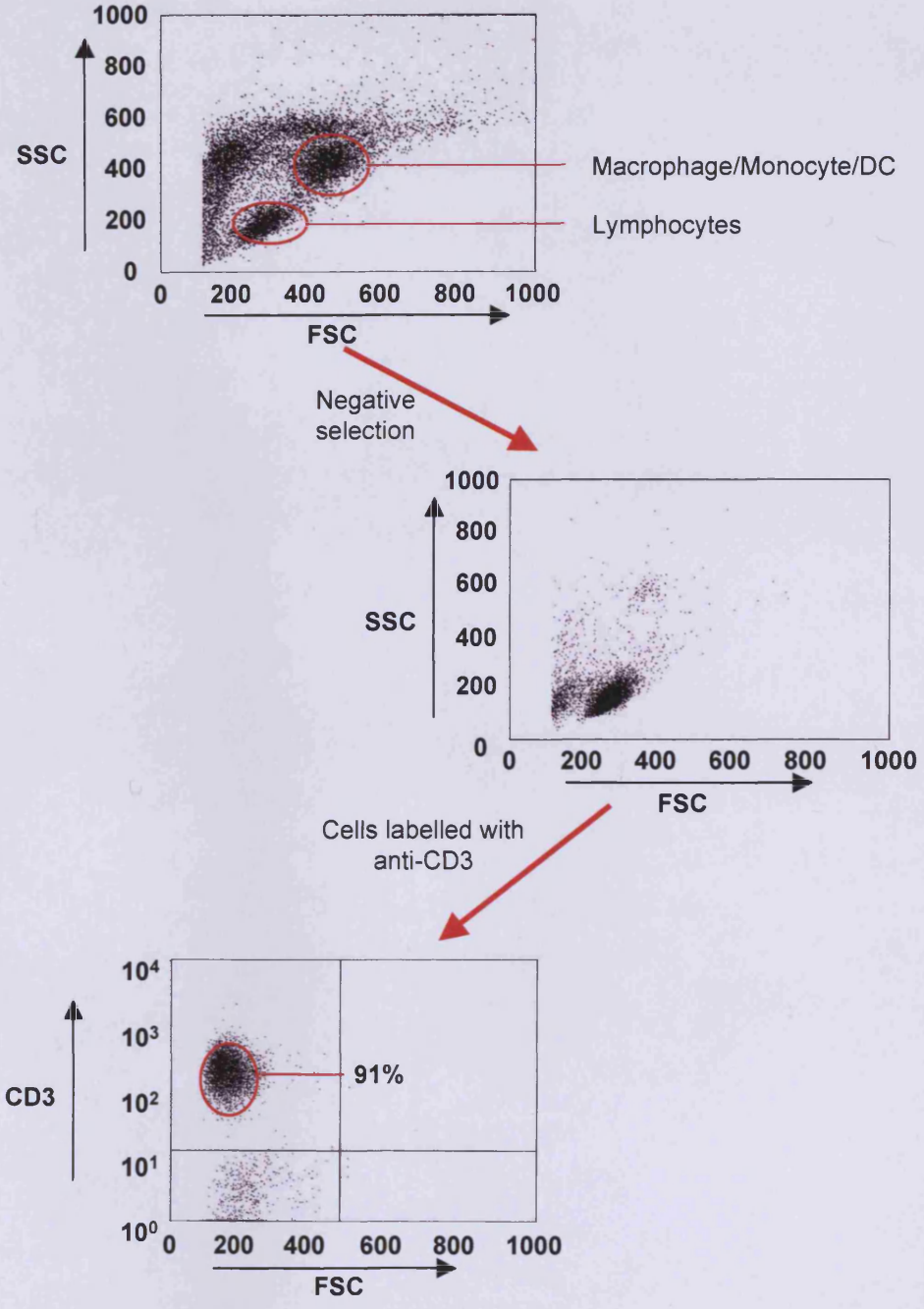


Figure 2.2 Peritoneal T-cell magnetic column negative selection. PD effluent was collected from a timed overnight exchange and leukocytes were isolated by centrifugation. The non T-cell fraction were labelled with biotin conjugated anti-CD14, CD16, CD19, CD36, CD56, CD123, and CD235. Following incubation magnetically labelled anti-biotin antibody was added and the cells were passed through a mini MACS magnetic separator column (Miltenyi Biotec Ltd, Surrey, UK). To asses purity, cells were labelled with fluorescently conjugated monoclonal antibody directed against CD3. T-cell purity by this method was consistently >85% as assessed by flow cytometric analysis of CD3+ lymphocyte (see appendix for patient details).

was rinsed (x3) with 500µl of MACS buffer and the non-labelled, B-cell deplete fraction collected.

The B-cell depleted cell suspension was centrifuged for 12mins at 1299 rpm (400xg), the supernatant was removed completely and the cell pellet re-suspended in 400µl of MACS buffer per 10^8 cells. On hundred µl of anti-biotin micro beads per 10^8 cells was added and the mixture was incubated for 15mins at 6°C. Cells were washed in 5ml MACS buffer per 10^8 cells and centrifuged for 12mins at 1299rpm (400xg) and the supernatant removed completely. The cells were re-suspended in MACS buffer (500µl/10^8 cells) and passed down a magnetic separator column. The column was washed (x3) with 500µl of MACS buffer. The column was removed from the separator and the BDCA-1 positive cells removed by adding 1ml of MACS buffer and flushing it through the column with the plunger. Cells were washed (x3) with PBS.

2.8 Cell culture

2.8.1 T-cell culture

T-cells (isolated by MACS separation) were re-suspended ($1x10^6$ cells/ml) in RPMI 1640 (Sigma-Aldrich, Kent UK) containing 10% foetal calf serum (Hyclone-Perbio, Northumberland, UK), 2mM L-Glutamine (Gibco/invitrogen, Renfrewshire, UK), 100U/ml of Penicillin, 100µg/ml of Streptomycin (both from Gibco/Invitrogen, Renfrewshire, UK) and 25 U/ml IL-2 (Roche, Mannheim Germany). Cells were cultured in U-bottomed 96 well plates (Sigma-Aldrich, Kent, UK) at $2x10^5$ cells/well. The cells were incubated at 37°C in a humidified incubator with 5% CO_2.

2.8.2 Preparation of APC coated plates

APC coated plates were prepared by incubating unfractionated peritoneal leukocytes ($0.25\text{-}2x10^6$/ml) at 37°C in U-bottomed 96 well plates. After 1 hour, non-adherent cells were removed, counted and the number of adherent cells calculated (mean percentage adherence 35%, range 22-47% n=20). Flow

cytometry performed pre- and post-adherence showed that adherent cells were derived from the cells in the monocyte/macrophage/DC gate (Figure 2.3), however the exact phenotype of these adherent cells was not determined in these experiments.

2.8.3 Trans-well experiments

Trans-well experiments were performed using 24-well inserts, [pore size 0.3μM] (BD Falcon 24-Multiwell Insert System, BD Biosciences, Oxfordshire, UK).

2.8.4 Mesothelial cell culture

Human peritoneal mesothelial cell isolation and culture was performed with the assistance of Dr. Ann Kift-Morgan (department of Medical Biochemistry and Immunology, Cardiff University) as previously described [82]. Briefly, omental tissue was collected from surgery in a sterile plastic receptacle containing approximately 50ml of sterile PBS solution and stored at 4°C. The tissue was gently manipulated to remove as much blood and unwanted surface material as possible. Twenty ml of freshly made trypsin-EDTA (Sigma-Aldrich Kent, UK; see 2.15) solution was aliquoted into 50ml tubes. Using two scalpels the tissue was dissected into pieces using a crossover motion. Each piece of tissue was placed into a tube with 20ml of trypsin-EDTA (0.25% trypsin, 0.1% EDTA) solution. The tube was sealed with a screw cap and placed on a rotating wheel (Stuart Scientific, Staffordshire, UK) in the 37°C incubator for 15 mins. The tubes were then centrifuged at 1600 rpm (500xg) for 6 minutes at 15°C.

Meanwhile 20ml of trypsin-EDTA solution was aliquoted into the required number of tubes for the second digest. Tissue from the initial digest was transferred to the second digest tube and placed on rotating wheel in the 37°C incubator for a further 15 mins. The tubes were centrifuged at 1600 rpm (500xg) for 6 minutes at 15°C. Supernatant was carefully removed using a syringe and the bottom of the tube was tapped vigorously on the floor of the hood to loosen the pellet and make re-suspension easier and more efficient.

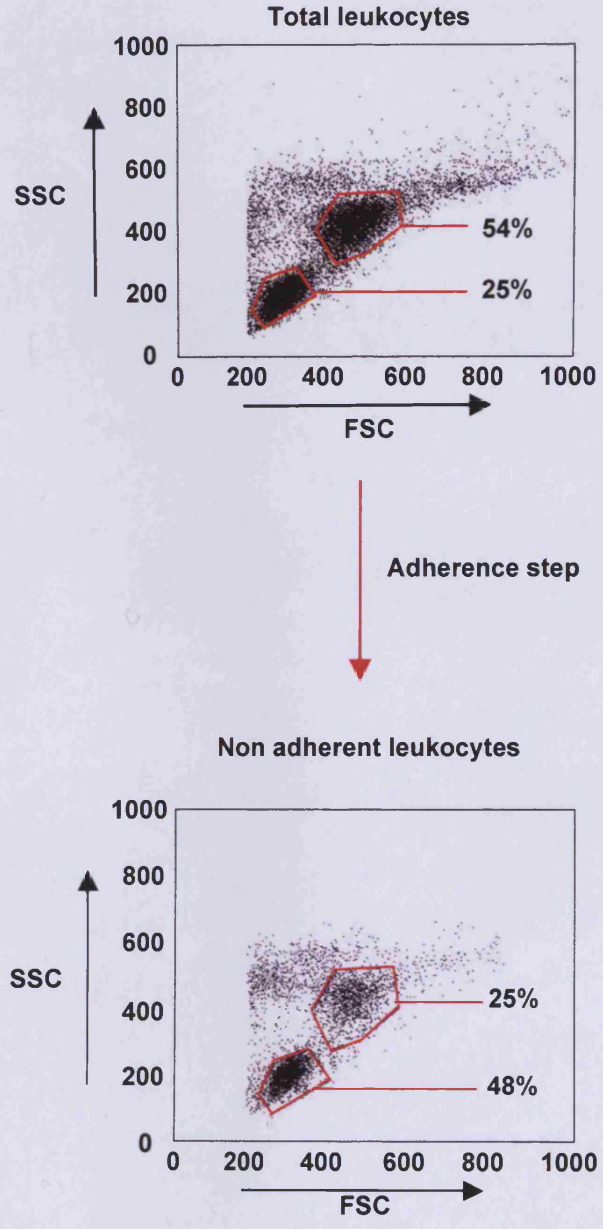


Figure 2.3 Cells within the Monocyte/Macrophage/DC gate adhere to the cell culture well. Peritoneal leukocytes were collected from a timed overnight exchange by centrifugation. Leukocytes were incubated in 96 well tissue culture plates for 1h at 37˚C. Non-adherent cells were removed by gentle aspiration. Cells were analysed by flow cytometry both pre and post adherence (percentage of total cells show; see appendix for patient demographics).

Five ml of 10%v/v FCS supplemented M-199 (Gibco/Invitrogen, Renfrewshire, UK) was added and rinsed around the tube to re-suspend and wash all the pelleted cells. The tubes were centrifuged at 1600 rpm (500xg) for 6 mins at 15°C. The supernatants were removed and replaced with an appropriate volume of fresh 10% serum supplemented M-199. Cells were detached and re-suspended gently using a syringe. Five ml was syringed into a fresh T25 culture bottle (Becton Dickinson, Oxfordshire, UK) and left to stand for 1 min. Cells were incubated at 37°C and observed frequently. After 1-2 days cells were pooled into a T75 flask (Becton Dickinson, Oxfordshire, UK). Medium was replaced every 3 days. Prior to transmigration assays HPMC from the 2° passage were transferred to 24 well plates and growth arrested as previously described [83]. Previous work in our lab has shown that mesothelial cell purity by this method is consistently >97% (with mesothelial cells identified by their unique cobblestone appearance, the presence of cytokeratin and the absence of factor VIII) [84, 85].

2.9 Flow cytometry

2.9.1 Preparation for fluorescence activated cell sorting analysis (FACS)

Prior to analysis, cells were re-suspended in PBS at 5 x 10^5 cells / 50µl. Fifty µl of cell suspension was added to individual wells of round-bottomed 96 well plates (BD Falcon, BD Biosciences, Oxfordshire, UK). Prior to flow cytometry, cells were incubated for 10 mins with 50µl blocking buffer containing 25% normal rabbit serum (DakoCytomation, Cambridgeshire, UK), 25% Human AB serum (HD supplies, Buckinghamshire, UK) and 50% PBS (to prevent non-specific binding). Dilution experiments for all fluorochrome-conjugated antibodies were performed in order to determine optimal concentration for use in all subsequent assays. Following addition of antibody, the cells were incubated for 30 mins at 4°C in the dark. Cells were subsequently washed (x3) with PBS, then re-suspended in 250µl FACS buffer (section 2.15.2) in

5ml round bottomed tubes (BD Falcon, BD Biosciences, Oxfordshire, UK) and analysed.

A Becton Dickinson FACS Calibur (Becton Dickinson, San Jose, CA, USA) was used to analyse the fluorescently labelled cells. T-cells were defined by their characteristic forward/side scatter profiles and by their co-expression of the T-cell marker CD3. Quadrant labels were placed on FACS plots so that 99% of cells in the isotype control sample were within the lower left quadrant.

2.9.2 Isolation of T-regs by FACS

For mechanistic studies we defined T-regs as $CD3^{+}/CD4^{+}CD25^{Hi}$ cells. This phenotype does not discriminate between T-regs and recently activated effector T-cells, however we were unable to utilise Foxp3 staining since this requires the cells to be fixed, permeabilsed and hence non-viable. Peritoneal T-cells were labelled with anti-CD3, CD4 and CD25 as per labelling protocol (section 2.9.1). A MoFlo high-speed cell sorter (DakoCytomation, Cambridge shire, UK) was used to isolate T-regs ($CD3^{+}/CD4^{+}/CD25^{Hi}$; Figure 2.4). Cells were sorted at between 5000 and 10,000 events per second and purity was assessed by re-analysing the sorted populations. Target cell purities >98% were achieved in every case.

2.10 Cell proliferation assays

2.10.1 ^{3}H-Thymidine incorporation

Following stimulation for 96h, stimulated cell cultures were incubated with 1µl of ^{3}H-Thymidine (37MBq/ml Amersham Biosciences Ltd, Buckinghamshire, UK) for 18hrs. The cells were then harvested onto filter plates using a cell harvester (Tomtec, Hamden, UK), and counts were analysed using a scintillation counter (Wallac, Turku, Finland).

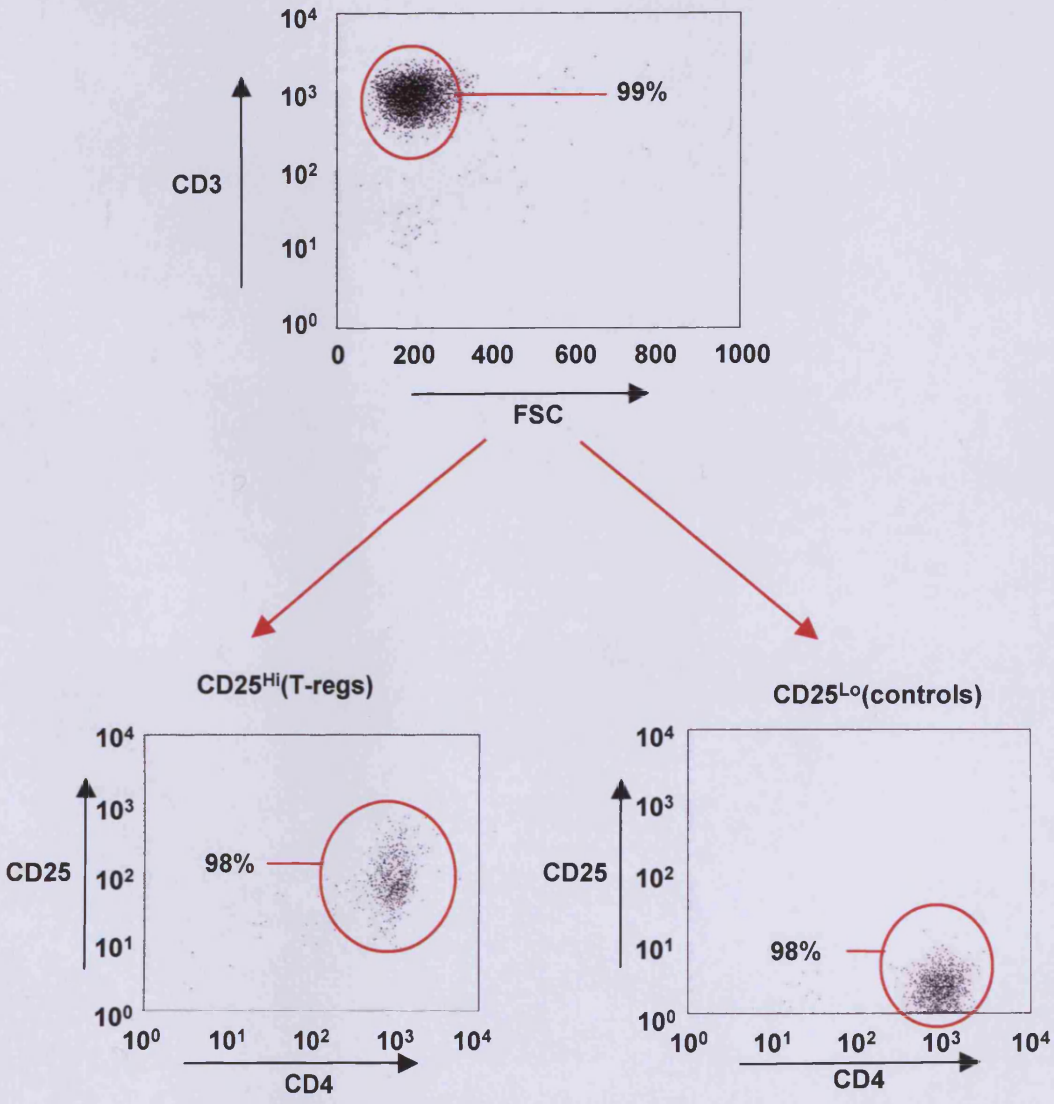


Figure 2.4 Isolation of peritoneal T-regs by fluorescence activated cell sorting analysis (FACS). PD effluent was collected from a timed overnight exchange and leukocytes were isolated by centrifugation. Cells were labelled with fluorescently conjugated anti-CD3, anti-CD4 and anti-CD25. Cells were sorted into T-regs and non-T-regs depending on their expression of CD25.

2.10.2 CFSE Labelling of T-cells

T-cells were suspended in PBS to a final concentration of $0.5x10^6$ cells/ml. A 5mM stock solution of CFSE in DMSO (Gibco/Invitrogen, Renfrewshire, UK) was prepared. This solution was added to the cells to make a final concentration of 3µM. Cells were subsequently incubated in the dark for 10 mins at 37°C. FCS was added to a final concentration of 10%v/v and cultures further incubated for 5 mins at room temperature. Five volumes of ice-cold FCS were added to quench staining and the cells were washed (x3) with PBS. The cells were subsequently cultured and stimulated. CFSE labelling was analysed by flow cytometry (CFSE detected in the FL-1 channel).

2.11 Detection of apoptosis and T-cell viability

Cell viability was assessed by using propidium iodide (PI) staining (Calbiochem, San Diego, USA). Prior to analysis, cells were re-suspended in 100µl of PBS ($1x10^6$ cells/ml). Two µl of PI was added to the cell suspension and PI staining was detected in the FL-3 channel (figure 2.5A).

T-cell apoptosis was assessed with the Annexin V apoptosis detection kit (Calbiochem, San Diego, Ca, USA) [86]. Cells were washed with PBS (x3) and re-suspended in 100µl ($1x10^6$ cells/ml) of binding buffer (Calbiochem, San Diego, Ca,USA). Subsequently 1.25µl of Annexin V-FITC was added to each well for 10 mins. Cells were washed with PBS (x3) then analysed by flow cytometry (Figure 2.5B).

2.12 Cytokine detection assays

2.12.1 Intracellular flow cytometry

To stimulate cytokine production, cells were stimulated with Phorbol Myristate Acetate (PMA, 500ng/ml) and Ionomycin (50ng/ml) (both from Sigma St Louis, Mo, USA) in the presence of monensin (2µM) (eBiosceience, San Diego, Ca,

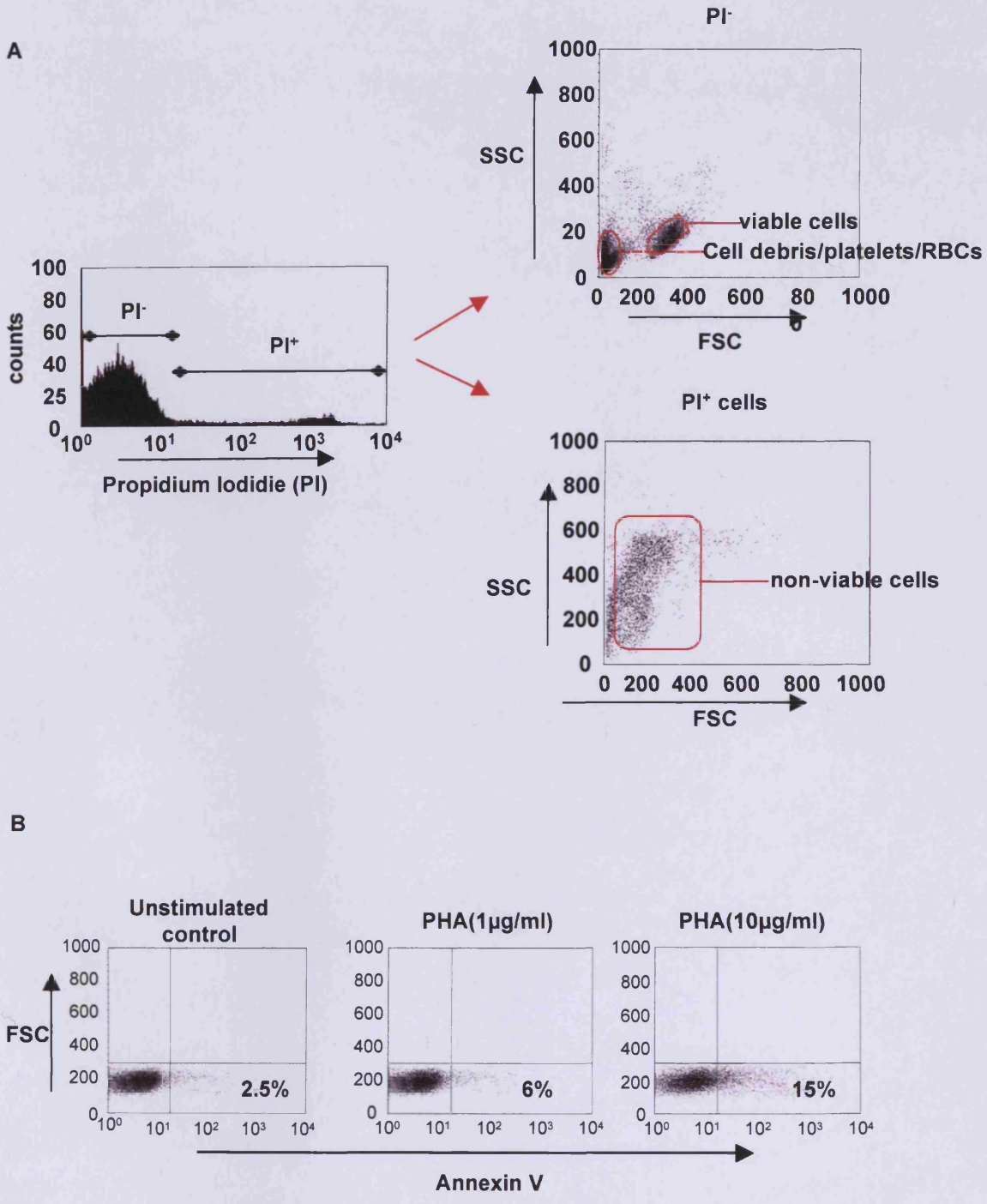


Figure 2.5 Assessment of peritoneal leukocyte viability (A) T-cells were isolated from overnight PD effluent by magnetic column negative selection. T-cells were re-suspended in 100 µl PBS ($1x10^6$ cells/ml). Two µl of Propidium Iodide was added and cells were analysed by flow cytometry (gates set according to isotype control). (B) Peritoneal T-cells were isolated by magnetic column negative separation. Cells were stimulated with increasing concentrations of PHA. Following 24h of stimulation, cells were stained with fluorescently labelled monoclonal antibody directed against CD3 and Annexin V. A combined gate was set on T-cells based on their CD3 and FSC:SSC profiles. (Numbers indicate the percentage of CD3+ T-cells expressing Annexin V; see appendix for patient demographics)

USA) for 4h at 37°C. Cells were then stained for surface marker expression (following the FACS staining protocol above). After washing in PBS (x3) cells were incubated in 100µl fixation/permeabilisation buffer (eBiosceience, San Diego, Ca, USA) for 30 mins at 4°C in the dark. Cells were subsequently washed (x3) with permeabilisation buffer (eBiosceience, San Diego, Ca, USA), and then re-suspended in 50µl of permeabilisation buffer containing optimal concentrations of fluorochrome-conjugated antibody (Table 2.1). Cells were incubated with antibody for 30 mins at 4°C in the dark, then washed (x3) with permeabilisation buffer, re-suspended in 200µl of PBS and analysed by flow cytometry (FACS Calibur, BD Biosciences, Oxfordshire, UK). It should be noted that the degree of cell permeabilisation achieved with this technique was not assessed. Consequently some differences in intracellular staining may reflect differences in the adequacy of permeabilisation rather than changes in cytokine production.

2.12.2 ELISpot assay

Anti human IFN-γ antibody (Mabtech, Dorset, UK) was diluted in PBS to a final concentration of 10µg/ml. Fifty µl was added to each well of an ELISpot plate (Millipore, Hertfordshire, UK). Plates were incubated at 37°C for 3h then washed (x5) with PBS. One hundred µl of FCS-RPMI (section 2.15.6) was added to each well for 1 hour at room temperature as a blocking step. Blocking media was discarded and the responder cells (either PBMC or peritoneal leukocytes) were added to the wells in 50µl FCS-RPMI ($2x10^5$ cells per well). A further 50µl of FCS-RPMI containing 10µg/ml of either purified protein derivative (PPD), Tetanus toxoid (TT) or Haemaglutinin (HA) were added to each well (control wells that did not require peptide received 50µl of the media alone). Plates were then wrapped in foil and incubated at 37°C for 18h. Following incubation, wells were washed (x5) with PBS to remove non-adherent cells. Adherent cells were lysed by the addition of distilled water (100 µl /well for 10 mins at room temperature).

The biotinylated secondary antibody 7-B6-1-Biotin (Mabtech, Dorset, UK) was diluted 1:1000 in PBS and 50µl was added to each well. Plates were

subsequently placed in the dark room for 2h at room temperature. The plates were then washed x5 with PBS and 50µl of Streptavadin-Alkaline phosphatase (Bio-Rad, Hertfordshire, UK) (diluted 1:1000 with PBS) was added to each well. Plates were subsequently placed in the dark room for 2h at room temperature. Fifty µl of developing solution (Bio-Rad, Hertfordshire, UK) was added to each well and plates were left to develop in the dark for a minimum of 15 mins until the spots were clearly visible. The plates were washed (x5) with tap water to stop the reaction. Plates were then air-dried in the dark before the spots were counted.

2.12.3 Multiplex bead-based cytokine assay

Cytokine levels were analysed using the Bio-Plex cytokine assay system as per manufacturer's instructions (Bio-Rad, Hertfordshire, UK). Wells were pre-wetted with Bio-Plex assay buffer (Bio-Rad, Hertfordshire, UK). Buffer was removed by vacuum filtration. A working solution of multiplex beads (Bio-Rad, Hertfordshire, UK) was prepared by adding 240µl of stock bead solution to 2800µl of Bio-Plex assay buffer. Fifty µl of multiplex bead working solution was added to each well and buffer was removed by vacuum filtration. Each well was washed twice with Bio-Plex wash buffer (Bio-Rad, Hertfordshire, UK) and 50µl of the PD effluent to be analysed was added to each well. Plates were covered with sealant tape (Bio-Rad, Hertfordshire, UK) and aluminium foil, then left to incubate for 30 mins at room temperature. Wells were washed with Bio-Plex wash buffer (x3) and 25µl of detection antibody (Bio-Rad, Hertfordshire, UK) was added to each well. Plates were covered with sealant tape and aluminium foil then incubated at room temperature for a further 30 mins. Wells were further washed (x3) and 50µl of Streptavadin PE (Bio-Rad, Hertfordshire, UK) was added to each well. Plates were covered with sealant tape and aluminium foil then incubated at room temperature for 10 minutes followed by washing (x3) with 100µl of wash buffer. Beads were re-suspended in 125µl of Bio-Plex assay buffer (Bio-Rad, Hertfordshire, UK) and the plate was read on the Bio-Plex array reader.

2.13 Single telomere length analysis (STELA)

STELA (Single telomere length analysis) was performed on our behalf by Dr. Duncan Baird, Department of Pathology, School of Medicine, Cardiff University. This PCR based technique allows the accurate measurement of single telomere molecules. The methodology is described in detail elsewhere [87, 88].

2.14 Statistical analysis

Statistical analysis was carried out using the SPSS statistical programme (SPSS for Mac, Rel. 10.0.0. 1999. Chicago: SPSS Inc). Differences between experimental groups were tested non-parametrically with the Mann Whitney or Kruksal Wallis test for unpaired samples and the Wilcoxan Signed Ranks test for paired samples. In proliferation studies, the mean was analysed using repeated measures analysis of variance. A *p* value of less than 0.05 was considered statistically significant.

2.15 Reagents

2.15.1 RBC Lysis buffer

155mM Ammonium Chloride (4.145g), 7mM Potassium Carbonate (0.48g), 0.1mM EDTA (0.0185g) (Gibco/Invitrogen Ltd., Renfrewshire UK) were dissolved in 500ml distilled water at pH 7.2 and stored at 4°C.

2.15.2 FACS buffer

10mM EDTA (1.85g), 15mM Sodium Azide (0.05g) (Fisher Scientific UK, Leicestershire, UK) and 1% w/v Bovine Serum Albumin (BSA) (5g) (Fisher Scientific UK, Leicestershire, UK) dissolved in 500ml PBS at pH 7.3 and stored at 4°C.

2.15.3 MACS buffer

EDTA 2mM (0.372g), 1%w/v BSA (5g) dissolved in 500ml PBS and stored at 4°C.

2.15.4 EDTA free MACS buffer

1% BSA (5g) dissolved in 500ml PBS and stored at 4°C.

2.15.5 Trypsin – EDTA Solution

5 mls of x10 Trypsin – EDTA stock solution (Sigma-Aldrich, Kent, UK) was added to 45ml of sterile PBS

2.15.6 FCS-RPMI

50mls of FCS was added to 450mls of RPMI.

2.16 Soluble mediators and blocking antibodies

	Supplier
Interleukin-2	Roche, Hertfordshire, UK
Interleukin-6	BD Pharmingen, Oxfordshire, UK
Soluble gp130	R&D Systems, Oxfordshire, UK
HMB-PP	A gift from Dr. Armin Reichenberg Universitäts klinikum Giessen und MarburgInstitut für Klinische Chemie und Pathobiochemie Gaffkystrasse, Giessen, Germany
Anti-TGFβ	R&D Systems, Oxfordshire, UK
Anti-IL-10	R&D Systems, Oxfordshire, UK
Concanavalin A	Sigma-Aldrich, Kent, UK
OKT3 (anti-CD3)	A gift from Dr E.C. Wang, Cardiff University Department of Medical Biochemistry and Immunology.

Table 2.2 Soluble mediators and blocking antibodies

Antibody	Supplier	Clone	Isotope	Dilution
FITC mouse IgG isotype	eBioscience, San Diego, USA	P3	IgG1	1:50
PE mouse IgG isotype	BD pharmingen, Oxfordshire, UK	MOPC-21	IgG1	1:100
APC mouse IgG isotype	eBioscience, San Diego, USA	P3	IgG1	1:25
PE-CY5.5 mouse IgG isotype	eBioscience, San Diego, USA	P2	IgG2a	1:100
CD25 APC	eBioscience, San Diego, USA	BC96	IgG1	1:25
CD69 FITC	BD pharmingen, Oxfordshire, UK	FN50	IgG1	1:100
IL-6R PE	BD pharmingen, Oxfordshire, UK San Diego, Ca	M5	IgG1	1:100
CCR5 FITC	BD pharmingen, Oxfordshire, UK	2D7/CCR5	IgG2a	1:100
CXCR3 PE	BD pharmingen, Oxfordshire, UK	IC6/CXCR3	IgG1	1:50
CD8 PE	DakoCytomation, Cambridgeshire, UK	DK25	IgG1	1:100
CD4Cy5.5	Caltag Labs San Francisco, Ca	2212	IgG2a	1:250
CD127 (IL-7R)	eBioscience, San Diego, USA	eBioRDR5	IgG1	
CD45R0 FITC	BD pharmingen, Oxfordshire, UK	UCHL1	MsIgG2a	1:50
CXCR1 PE	BD pharmingen, Oxfordshire, UK	42705.111	IgG2a	1:100
CD3 PE	BD pharmingen, Oxfordshire, UK	UCHT1	IgG1	1:100
CD14 FITC	BD pharmingen, Oxfordshire, UK	M5E2	IgG2a	1:50
Foxp3 FITC	eBioscience, San Diego,USA	236A/E7	IgG1	1:50
IFN-γ PE	eBioscience, San Diego,USA	4S.B3	IgG1	1:100
IL-4 PE	eBioscience, San Diego,USA	8D4-8	IgG1	1:50
Vγ9 FITC	Immunotech A Coulter Company	1463 Lot 17	IgG1	1:100
Vγ9 Cy5.5	Immunotech A Coulter	4G	IgG1	1:250
TCRVβ	IO test, BetaMark, Beckman Coulter, High Wycombe,UK).			1:10

Table 2.3 Fluorochrome conjugated antibodies.

Chapter 3

Peritoneal T-cells: Phenotype, Activation Status, Proliferation and Cytokine Production

3.1 Introduction

Peritonitis remains a major cause of morbidity in patients on PD. Many patients suffer only a single episode in the early years of treatment, but in some patients there is relapse or frequent recurrence. Degree of severity is linked to the pathogenesis of the organism as well as the source of infection. Incidence and severity have also been linked to detrimental structural and functional changes of the peritoneal membrane [8, 9, 12]. Over the past 20 years our understanding of the peritoneal inflammatory process has increased significantly. The vast majority of these studies have focused on the contribution of either the peritoneal macrophage or the peritoneal mesothelial cell in regulating inflammatory processes. Despite representing up to 25% of the resident peritoneal leukocyte population and forming a significant proportion of the cell population in resolving peritonitis, the role of peritoneal T-cells has received significantly less attention [33-41].

Previous studies have suggested that resident peritoneal T-cells display an activated phenotype characterised by increased expression of CD69, CD25 and HLA-DR. This "increased activation" has been interpreted differently by different authors with some suggesting that the T-cells are "primed" for subsequent activation whilst others have suggested that they may be in a hypo-responsive state [29-33]. Similarly, although some have suggested that peritoneal T-cells are unlikely to proliferate following activation, this has yet to be been formally assessed *in-vitro* [89].

We now have evidence from the mouse model of peritonitis [Topley, Jones, unpublished data] to suggest that T-cell activation is a pivotal event in the acute response, the transition from innate to adaptive immunity and immuno-resolution. It is also apparent that T–cell dysregulation is one of the triggers that drive chronic inflammation in the mouse peritoneum. In light of these findings it is essential that we gain a better understanding of the role of human T-cells in the peritoneal inflammatory pathways and inflammation induced membrane damage.

The specific aims of this chapter are to:-

a) Define the basic phenotype (including activation status) of resting peritoneal T-cells.

b) Determine the cytokine profile of peritoneal T-cells (TH1/TH2 balance).

c) Asses the proliferative capacity of peritoneal T-cells following stimulation.

3.2 Results

3.2.1 Peritoneal lymphocytes - basic phenotype

Based on data from leukocytes isolated from 20 stable (non-infected) patients, lymphocytes represent 18 (±5.4%) of the total peritoneal leukocyte population in patients on PD. Within this peritoneal lymphocyte population the vast majority of the cells are CD3 positive (mean 88±8%). This group of T-cells can be further sub-divided into CD4 positive (mean 36±3%) and CD8 positive (mean 44±2.66%). A representative flow cytometry scatter plot is shown in Figure 3.1.

3.2.2 Peritoneal T-cell activation status

In order to quantitate the proportion of activated T-cells, we analysed the expression of CD69 (an early activation marker) and CD25 (a late activation marker) on both peripheral blood and peritoneal T-cells isolated from stable (no infection in the last 3 months) patients on PD.

The results show that when compared to peripheral blood T-cells, peritoneal T-cells express much higher levels of CD69 (Figure 3.2A) and marginally higher levels of CD25 (Figure 3.2B). The precise mechanism responsible for

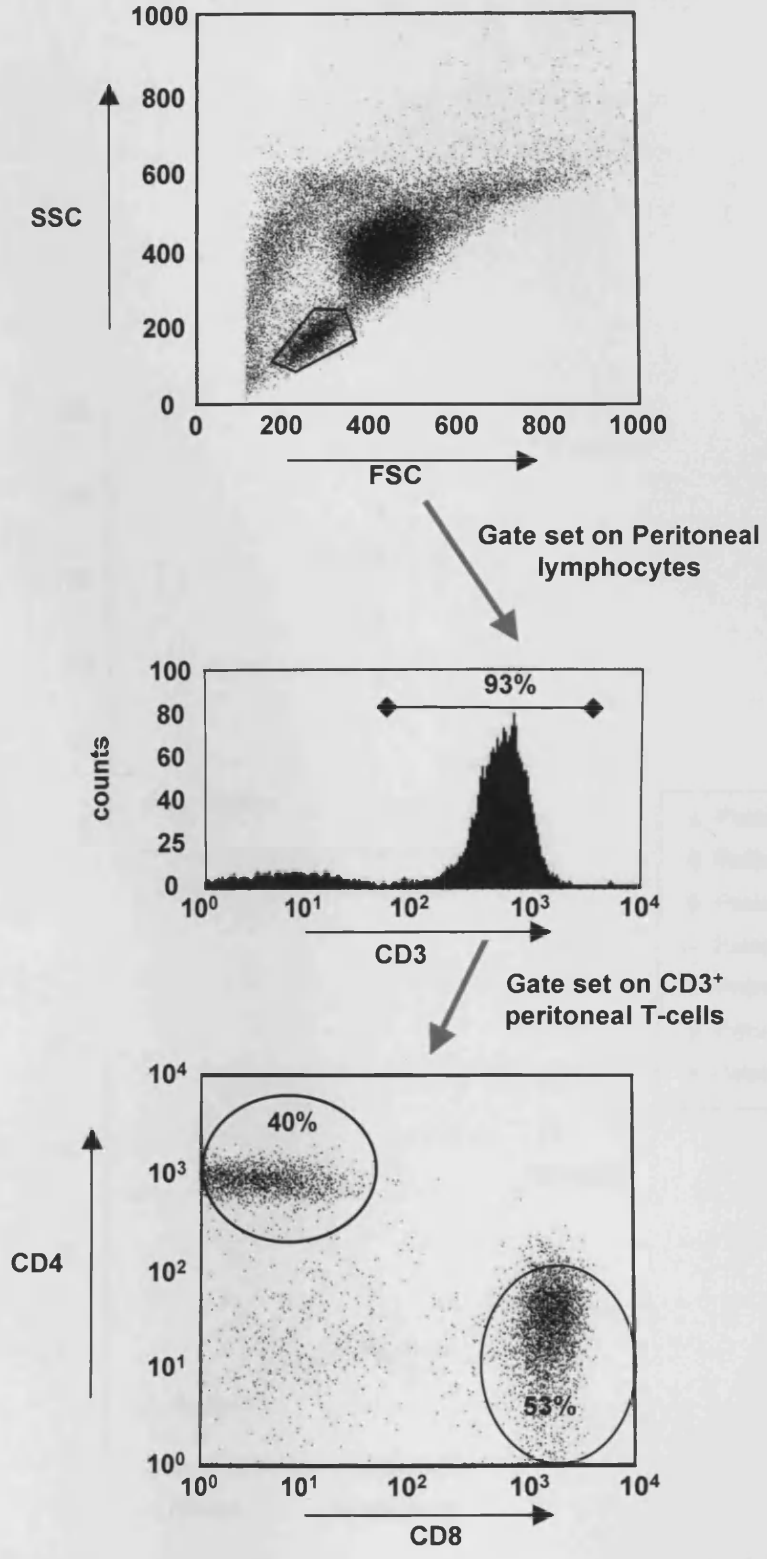


Figure 3.1 Basic phenotype of peritoneal T-cells. Peritoneal leukocytes were isolated from overnight PD effluent. Cells were labeled with fluorescently labeled monoclonal antibody directed against CD3, CD4 and CD8. A gate was set on lymphocytes according to their characteristic FSC:SSC profile. The percentage of $CD3^+$ cells within the lymphocyte gate is shown. A further gate was set on $CD3^+$ cells and the percentage of $CD4^+/CD8^+$ cells within this gate was calculated. Representative example shown from 20 separate patients (see appendix for patient demographics).

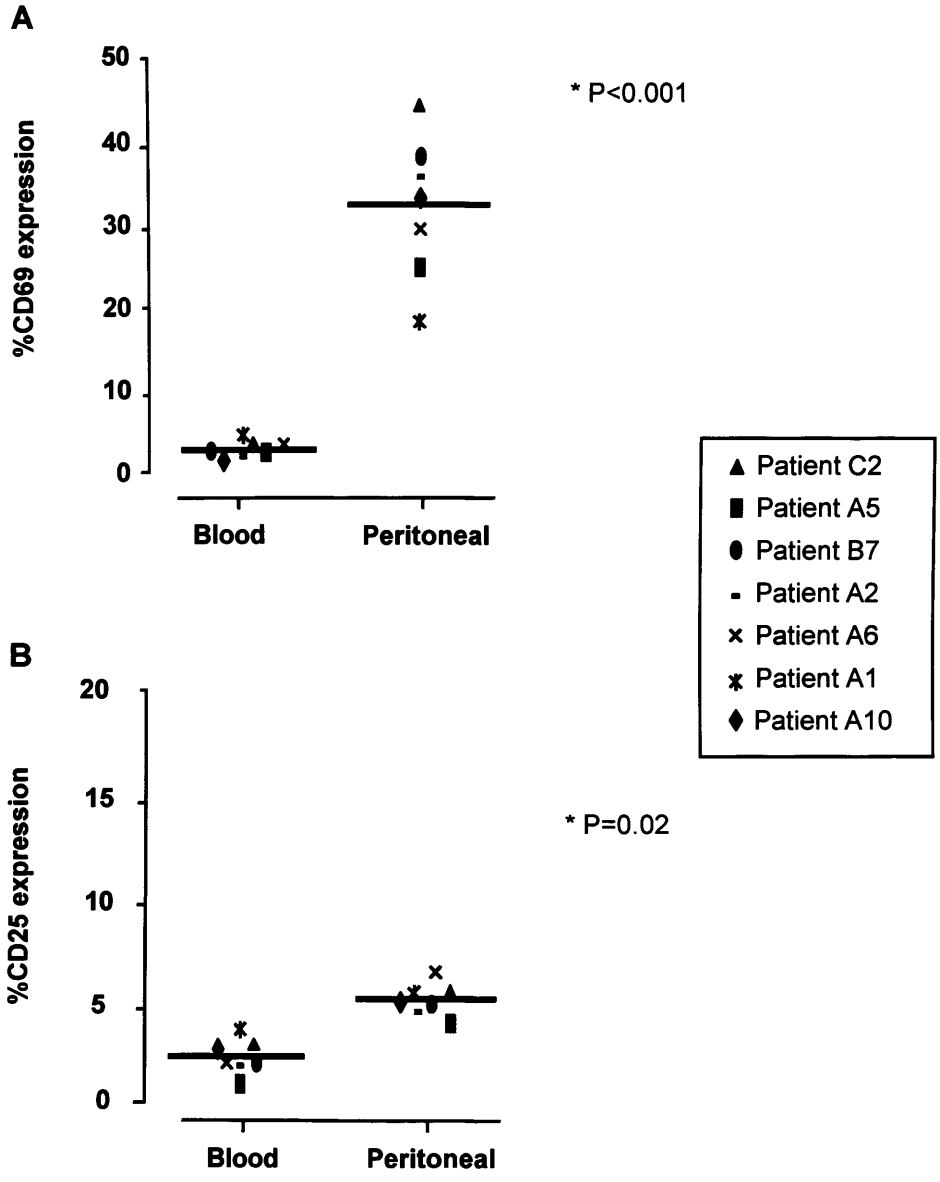


Figure 3.2. Expression of cell surface activation markers on peritoneal and peripheral blood T-cells. Paired samples of peripheral blood and peritoneal leukocytes were collected from PD patients. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3, CD69 and CD25. A combined gate was set T-cells based on their FSC:SSC profiles and their expression of CD3. Results show the percentage of $CD3^+$ cells expressing CD69(A) or CD25(B). Horizontal lines represent the mean. Differences between groups were tested using the Wilcoxan signed ranks test (see appendix for patient demographics).

this apparent local T-cell "activation" in the dialysed peritoneum have not been identified. We speculated that it was related either to exposure to PD solutions or that the process of transmigration into the peritoneal cavity results in peritoneal T-cell activation.

To assess the impact of PD fluid on T-cell activation status, peripheral blood T-cells were cultured overnight in the presence of either Dianeal® (1.36% anhydrous glucose) or Extraneal® (7.5% icodextrin). As shown in Figure 3.3A, this did not, however, result in any significant up-regulation of activation markers.

To determine whether changes in T-cell phenotype were induced by the transmigration process, we utilised a validated transmigration chamber system. A mesothelial monolayer was formed on the underside of a trans-well insert [83] (Figure 3.3B). As shown in Figure 3.3C, the transmigration of PBMC across this cytokine stimulated mesothelial monolayer did not result in any significant up-regulation of CD69 expression on CD3 positive cell population.

3.2.3 Polyclonal stimulation of peritoneal T-cell

Given that peritoneal T-cells already display an activated phenotype, some authors have suggested that these cells may be "anergic" and therefore resistant to further stimulation [90]. To ascertain whether peritoneal T-cells have the capacity to respond to stimulation, isolated $CD3^+$ peritoneal T-cells from stable PD patients were cultured overnight in the presence of optimal concentrations of Phytohemagglutinin (PHA), a polyclonal T-cell activator. As demonstrated in Figure 3.4, stimulation of peritoneal T-cells with PHA results in increased expression of both CD69 and CD25 suggesting that these T-cells can indeed be further activated (*in-vitro*).

3.2.4 TH1 vs. TH2 polarisation

TH1/TH2 polarisation of T-cells is evidenced by the production of IFN-γ and IL-4 respectively. In an attempt to define the TH1/TH2 profile of resident

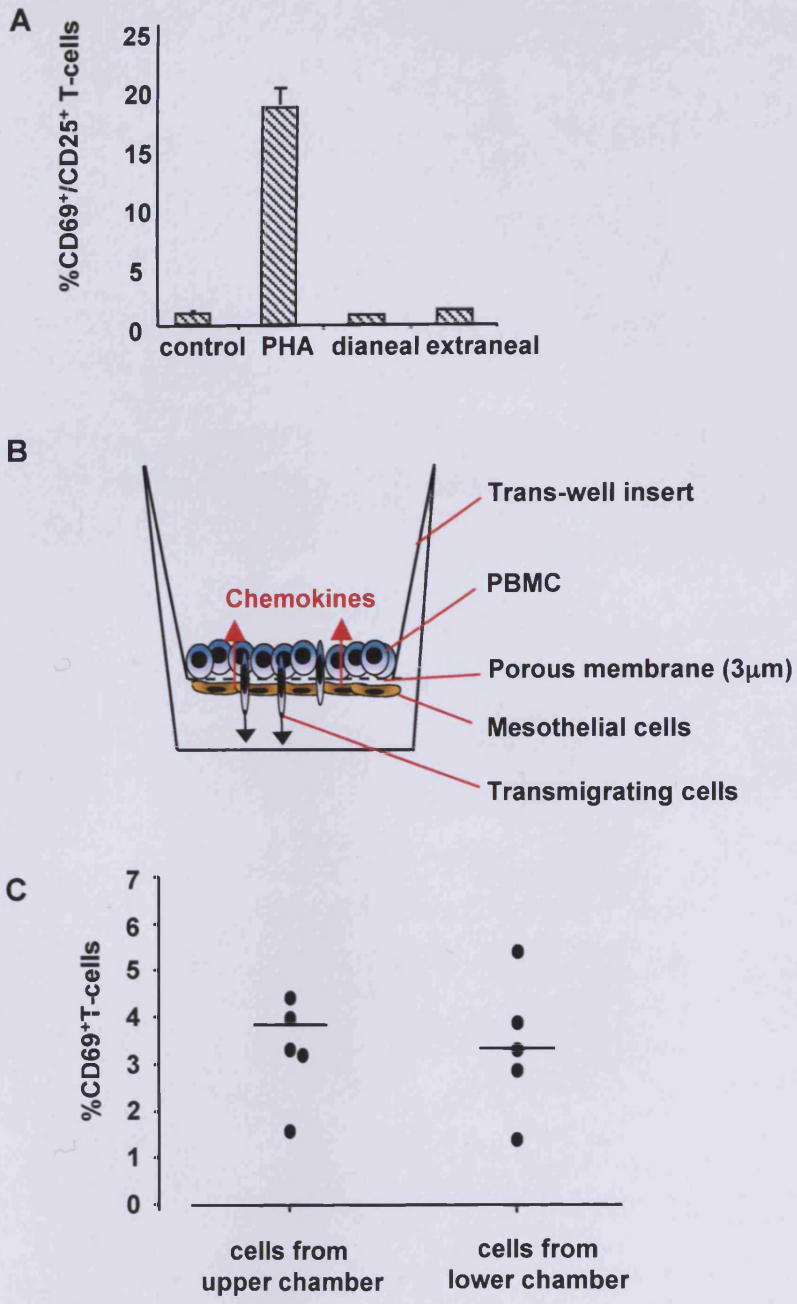


Figure 3.3. Exploring possible mechanisms of T-cell activation (A) PBMC were isolated from the blood of healthy volunteers (n=5) by ficoll density gradient centrifugation. PBMC were cultured overnight in the presence of culture medium alone, PHA, extraneal (diluted 1:2) or dianeal (diluted 1:2). T-cell activation (defined as increased expression of CD69 and CD25) was assessed by flow cytometry at 24, 48 and 72h (data at 48h shown as a representative example). (B) A mesothelial monolayer was formed on the underside of a tran-swell insert. Mesothelial cells were stimulated for 24h with IL-1 (50pg/ml), then washed. PBMC (isolated from the blood of 5 healthy volunteers by ficoll density gradient centrifugation) were placed in the upper chamber of the trans-well. (C) Samples were taken from the upper and lower chambers at 24hrs. T-cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3 and CD69. A combined gate was set T-cells based on CD3 expression and their FSC:SSC profiles. Results show the percentage of $CD3^+$ cells expressing CD69 (horizontal line represents mean).

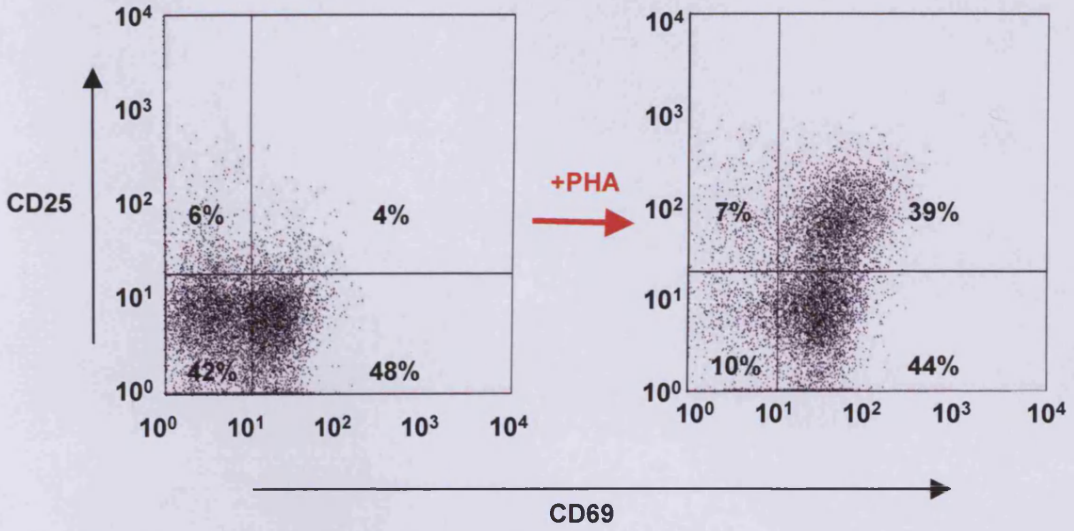


Figure 3.4 Activation of peritoneal T-cells with Phytohemagglutinin(PHA). Peritoneal leukocytes were collected on ice from a timed overnight exchange. Leukocytes were stimulated with PHA (10µg/ml) for 12h. T-cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3, CD69 and CD25. A combined gate was set T-cells based on CD3 expression and their FSC:SSC profiles. The percentage of T-cells expressing CD25, CD69 or both was calculated. Representative example shown from 5 separate donors (see appendix for patient demographics).

peritoneal T-cells, we utilised intracellular flow cytometry, to assess cytokine production at a single cell level. As shown in Figure 3.5, the majority of isolated $CD3^+$ peritoneal T-cells were polarised towards a TH1 type response characterised by the capacity to produce IFN-γ with very few peritoneal T-cells producing IL-4. When PD effluent obtained during peritonitis was analysed using Luminex array technology, increased levels of IFN-γ were detected but only very low levels of IL-4, suggesting that the *in vivo* response during infection is also apparently skewed toward a TH1 phenotype.

3.2.5 Proliferation of peripheral blood and peritoneal T-cells isolated from stable PD patients

In order to reproducibly and accurately quantify proliferation, T-cells from the peripheral blood and the peritoneum of stable PD patients were isolated by negative selection using magnetic beads (see Materials and Methods, section 2.6.1). Isolated T-cells were then stimulated with the T-cell specific mitogen OKT3 (anti-CD3). When isolated peripheral blood T-cells from stable PD patients (no infection in the past 3 months) were stimulated with OKT3 (0-100ng/ml) there was a dose dependant increase in proliferation (Figure 3.6A). Peritoneal T-cells isolated from the same patients had negligible proliferation in response to OKT3 (Figure 3.6A). To confirm this lack of response, peritoneal T-cell proliferation experiments were also performed with a range of alternative mitogenic stimuli including OKT3 coated plates, Concanavalin A and a combination of anti-CD3 and anti-CD28. None of these stimuli were able to induce the proliferation of peritoneal T-cells (data not shown).

To ascertain whether the non-proliferating cells were being activated, isolated peritoneal T-cells from these same donors were examined for their expression of CD69. OKT3 treatment resulted in a dose dependant increase in CD69 expression in all patients examined suggesting that these cells are indeed capable of being activated but are not able to proliferate in response to mitogenic stimulation (Figure 3.6B).

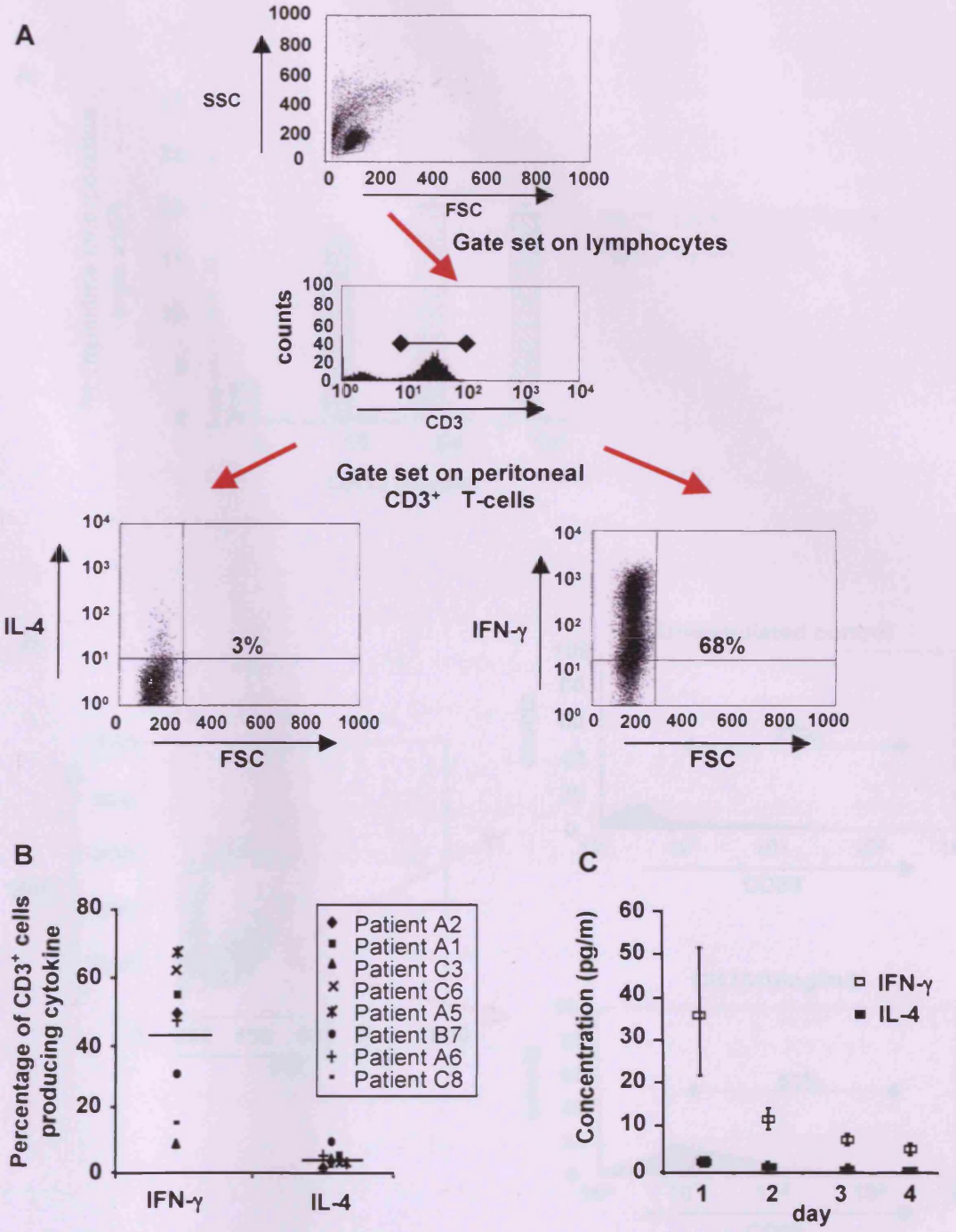


Figure 3.5 TH1 vs. TH2 polarisation of peritoneal T-cells. Peritoneal leukocytes ($5x10^5$/well) were stimulated with PMA (500ng/ml)/ Ionomycin (50ng/ml) for 4 hours in the presence of monensin (2μM). Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3. Cells were subsequently fixed, permeabilized then stained with fluorescently labeled anti-IFN-γ or anti-IL-4. A combined gate was set on peritoneal T-cells based on their FSC:SSC profiles and their CD3 expression. The percentage of IFN-γ$^+$ or IL-4$^+$ T-cells was calculated. (A) Representative FACS plot. (B) Intracellular Flow cytometry data from 8 separate patients (results shown as % of CD3$^+$ T-cells expressing either IFN-γ or IL-4; the horizontal line represents the mean values) .(C) Serial samples of peritoneal effluent was collected from patients suffering from acute peritonitis. IL-4 and IFN-γ levels were analysed with a multiplex bead-based cytokine assay. (Results show mean ±SE of 10 separate patients; see appendix for patient demographics).

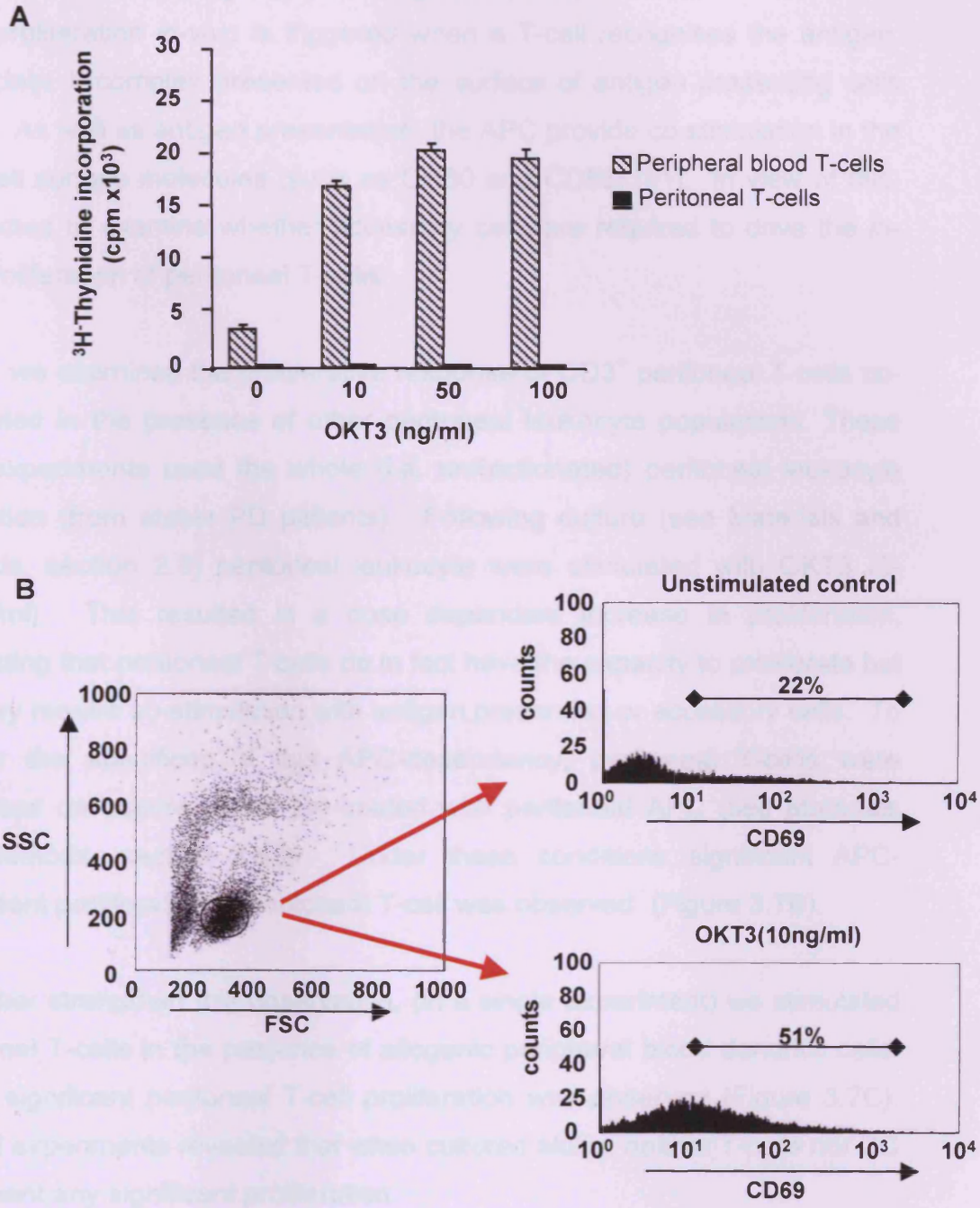


Figure 3.6 OKT3 stimulation of peritoneal and peripheral blood T-cells. (A) Peripheral blood and peritoneal T-cells were isolated from peritoneal dialysis patients by magnetic column negative separation. Isolated T-cells were stimulated with increasing concentrations of OKT3 (0-100ng/ml) in the presence of IL-2 (25U/ml). Proliferation (^{3}H-Thymidine incorporation) was assessed at 96h. Proliferation results show mean ± SE of duplicate cultures (representative example shown from 6 separate donors). (B) Magnetic column isolated peritoneal T-cells were stimulated for 4 hours with OKT3 (10ng/ml). Cells were stained with fluorescently labeled monoclonal antibody directed against CD69. A gate was set on T-cells based on their FSC/SSC profile. Results show the percentage of T-cells expressing CD69 (see appendix for patient demographics).

3.2.6 The role of antigen presenting cells (APC) in T-cell activation

T-cell proliferation *in-vivo* is triggered when a T-cell recognises the antigen: MHC class II complex presented on the surface of antigen presenting cells (APC). As well as antigen presentation, the APC provide co-stimulation in the form cell surface molecules (such as CD80 and CD86) [91]. In view of this, we elected to examine whether accessory cells are required to drive the *in-vitro* proliferation of peritoneal T-cells.

Initially we examined the proliferative response of $CD3^+$ peritoneal T-cells co-stimulated in the presence of other peritoneal leukocyte populations. These initial experiments used the whole (i.e. unfractionated) peritoneal leukocyte population (from stable PD patients). Following culture (see Materials and Methods, section 2.8) peritoneal leukocyte were stimulated with OKT3 (0-100ng/ml). This resulted in a dose dependant increase in proliferation, suggesting that peritoneal T-cells do in fact have the capacity to proliferate but that they require co-stimulation with antigen presenting or accessory cells. To confirm the specificity of this APC-dependency, peritoneal T-cells were stimulated on culture plates pre-coated with peritoneal APC (see Materials and Methods, section 2.8.2). Under these conditions significant APC-dependent proliferation of peritoneal T-cell was observed (Figure 3.7B).

To further strengthen this observation, (in a single experiment) we stimulated peritoneal T-cells in the presence of allogenic peripheral blood dendritic cells. Again, significant peritoneal T-cell proliferation was observed (Figure 3.7C). Control experiments revealed that when cultured alone; neither T-cells nor DC underwent any significant proliferation.

Subsequent experiments utilising CFSE staining suggested that it was indeed the peritoneal T-cell population that was specifically proliferating in these experiments (Figure 3.7D). It is apparent from Figure 3.7D that the CFSE staining pattern of proliferating peritoneal T-cells is atypical in that there is a general shift of the whole population to the left in contrast to the well defined peaks typically seen with proliferating PBMC. The reasons for this are not clear, since the methodology has previously been validated in PBMC

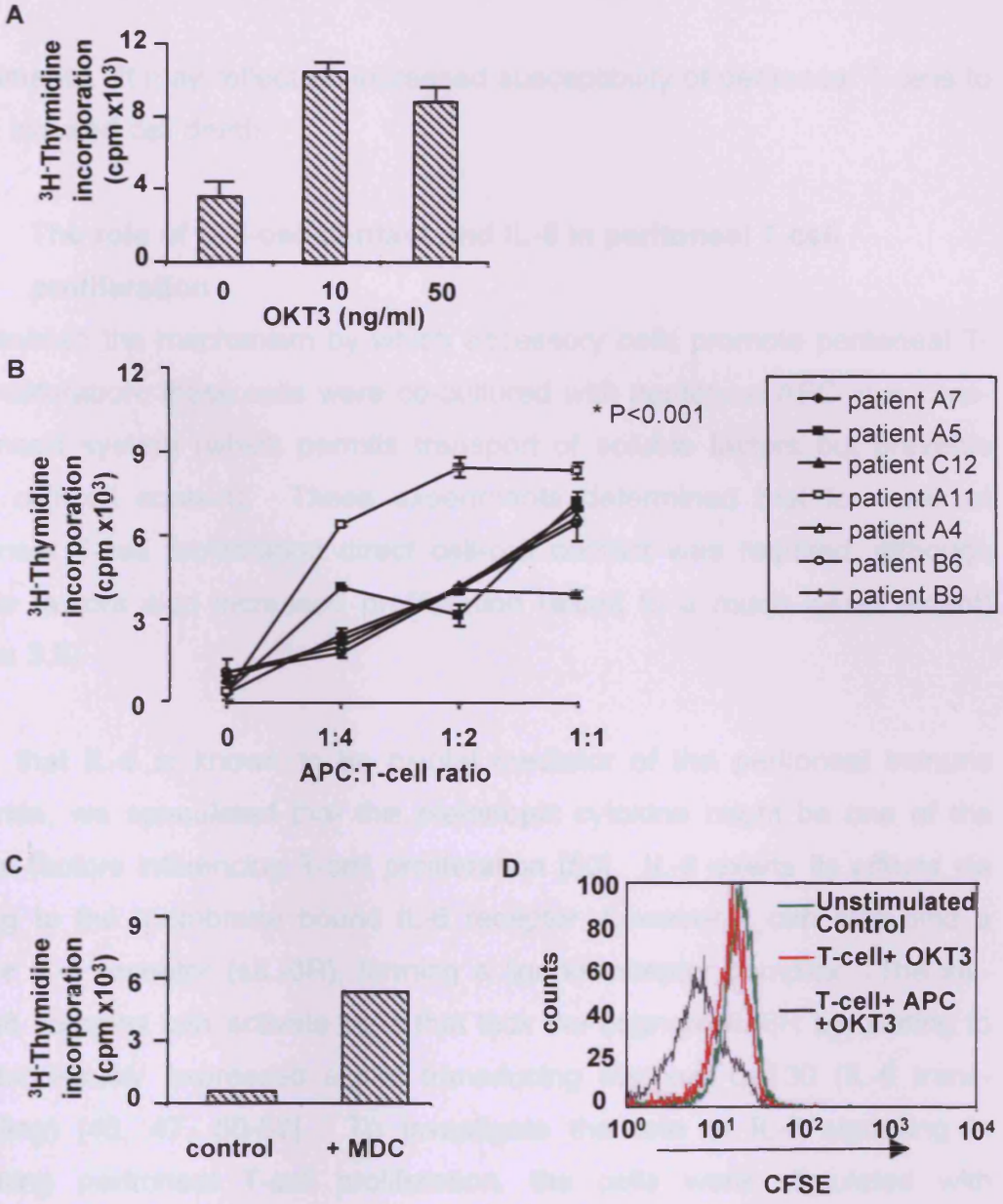


Figure 3.7 The role of antigen presenting cells (APC) in peritoneal T-cell proliferation. (A) Peritoneal leukocytes were isolated from overnight PD effluent. The unfractionated leukocytes were stimulated with increasing concentrations of OKT3 (0-50ng/ml). Proliferation (^{3}H-Thymidine incorporation) was assessed at 96h (results show mean ± SE of 3 separate experiments). (B) APC coated plates were prepared by adhering peritoneal leukocytes to 96-well plates for 1 hour then removing non-adherent leukocytes. Isolated peritoneal T-cells were stimulated with OKT3 in the presence of increasing numbers of APC. Proliferation (^{3}H-Thymidine incorporation) was assessed at 96h (proliferation results show mean ±SE of duplicate cultures). Means were analysed using repeated measures analysis of variance. (C) Blood derived BDCA-1+ myeloid dendritic cells (MDC) were isolated from peripheral blood by 2-stage magnetic separation. Peritoneal T-cells were isolated from overnight PD effluent by magnetic column negative separation. Peritoneal T-cells were stimulated with OKT3 in the presence of allogenic peripheral blood MDC(T:cell to MDC ratio 5:1). Proliferation (^{3}H-Thymidine incorporation) was assessed at 96h. (D) Peritoneal T-cells were labeled with CFSE then stimulated with OKT3(10ng/ml) in the presence or absence of allogenic APC. CFSE fluorescence within the gated T-cell population was analysed by flow cytometry at day 7 (see appendix for patient demographics).

experiments. It may reflect an increased susceptibility of peritoneal T-cells to CFSE induced cell death. .

3.2.7 The role of cell-cell contact and IL-6 in peritoneal T-cell proliferation

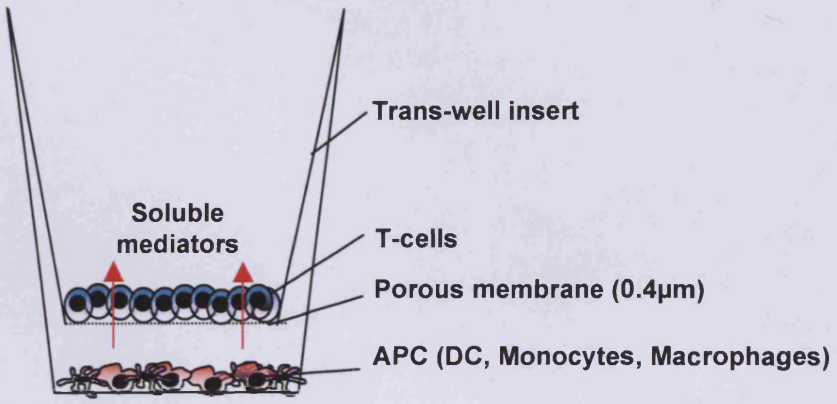
To establish the mechanism by which accessory cells promote peritoneal T-cell proliferation, these cells were co-cultured with peritoneal APC in a trans-well insert system (which permits transport of soluble factors but prevents direct cell-cell contact). These experiments determined that for maximal peritoneal T-cell proliferation direct cell-cell contact was required, although soluble factors also increased proliferation (albeit to a much lesser extent) (Figure 3.8).

Given that IL-6 is known to be pivotal mediator of the peritoneal immune response, we speculated that this pleiotropic cytokine might be one of the soluble factors influencing T-cell proliferation [50]. IL-6 exerts its effects via binding to the membrane bound IL-6 receptor, however it can also bind a soluble IL-6 receptor (sIL-6R), forming a ligand-receptor complex. The sIL-6R/IL-6 complex can activate cells that lack the cognate IL-6R by binding to the ubiquitously expressed signal transducing element gp130 (IL-6 trans-signalling) [46, 47, 50-52]. To investigate the role of IL-6 signalling in regulating peritoneal T-cell proliferation, the cells were stimulated with increasing concentrations of IL-6 or increasing concentrations of soluble gp130 (which blocks IL-6 trans-signalling). In these limited number of experiments, IL-6 did not appear to have a significant impact on peritoneal T-cell proliferation (Figure 3.9A,B).

3.2.8 T-cell proliferation during peritonitis

We next speculated that effector T-cells recruited into the peritoneum during peritonitis might behave differently to resident peritoneal T-cells present during the steady (non-infected) state. To address this question peritoneal T-cells were isolated from patients during acute peritonitis episodes and stimulated in the presence or absence of peritoneal APC. In contrast to peritoneal T-cells isolated from stable PD patients, peritoneal T-cells isolated

A



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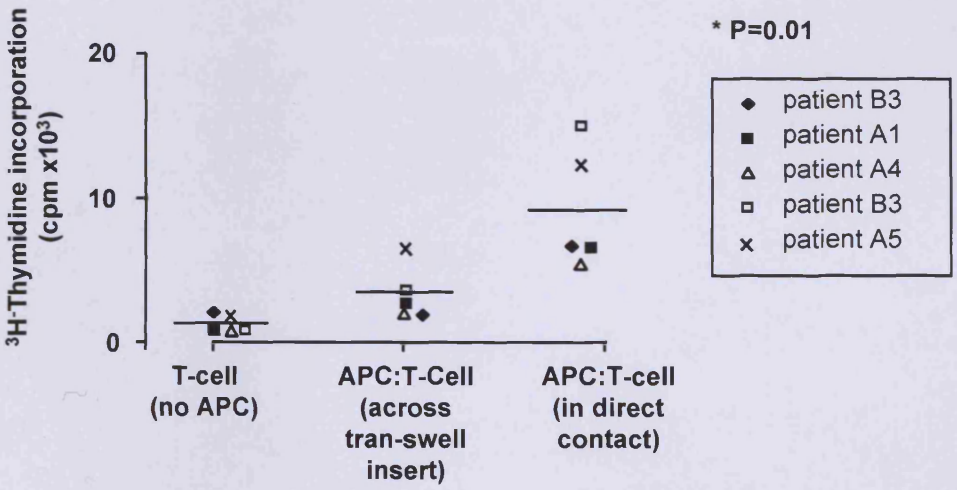

Figure 3.8. The impact of cell-cell contact on peritoneal T-cell proliferation. Peritoneal T-cells were isolated from overnight PD effluent by magnetic column negative separation. APC coated plates were prepared by adhering allogenic peritoneal leukocytes to plates for 1 hour then removing non-adherent leukocytes. (A) Trans-well inserts were used to prevent direct cell-cell contact between APC and T-cells. (B) Peritoneal T-cells were cultured (in duplicate) in medium alone, in direct contact with APC or with APC on the opposite side of the tran-swell insert membrane. Mean proliferation (^{3}H-Thymidine incorporation) was assessed at 96h. Means were analysed using repeated measures analysis of variance.

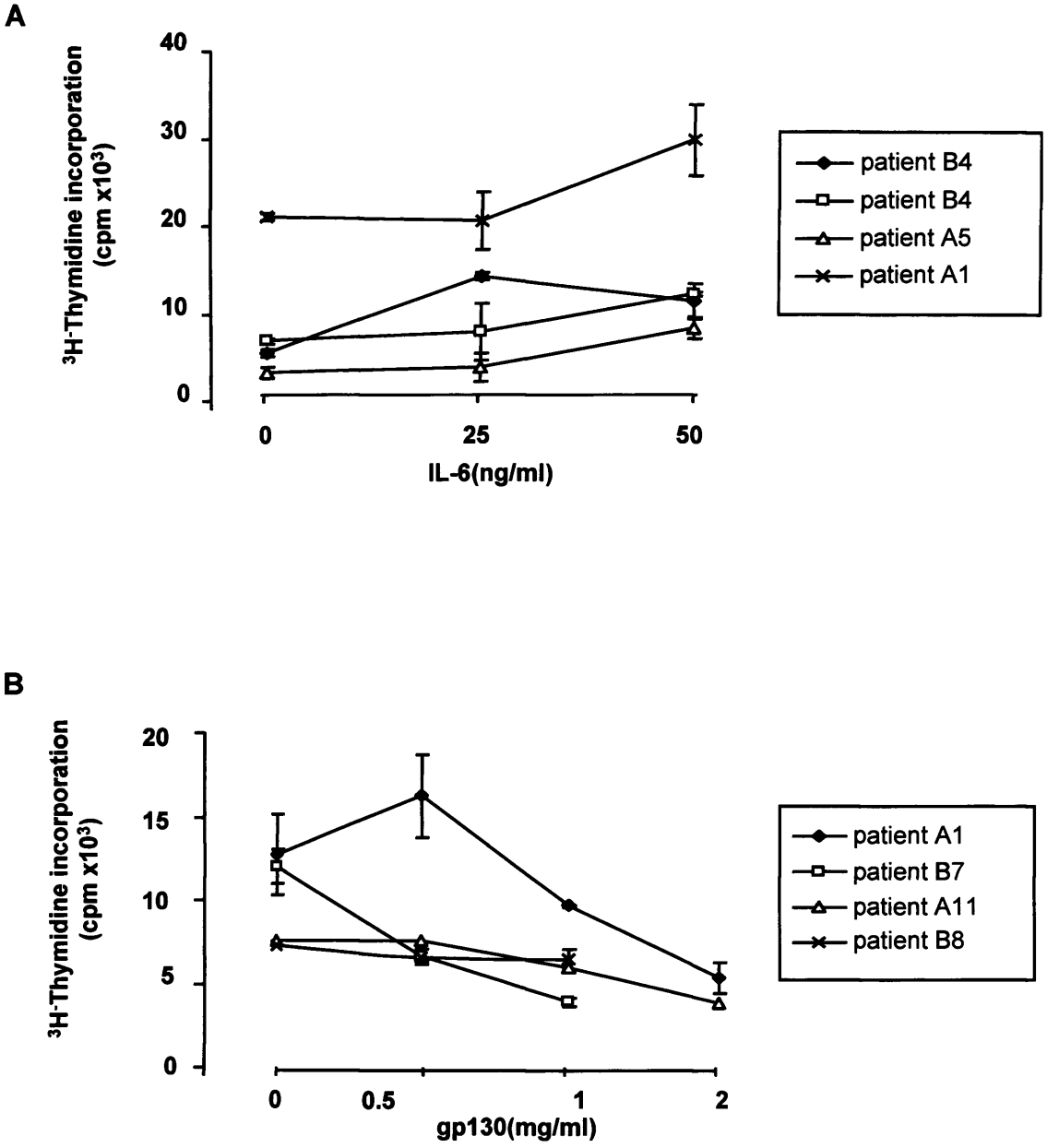

Figure 3.9 The impact of IL-6 signalling on peritoneal T-cell proliferation. Peritoneal T-cells were isolated from overnight PD effluent by magnetic column negative separation. APC coated plates were prepared by adhering allogenic peritoneal leukocytes to plates for 1 hour then removing non-adherent leukocytes. (A) Peritoneal T-cells were cultured in the presence of APC and increasing concentrations of IL-6 or (B) increasing concentrations of soluble gp130 (sgp130). Proliferation (^{3}H-Thymidine incorporation) was assessed at 96h. Results show mean ±SE of duplicate cultures (see appendix for patient demographic).

during acute peritonitis are able to proliferate in the absence of APC and, in fact allogenic peritoneal APC down-regulate the proliferation of these cells (Figure 3.10A).

3.2.9 Apoptosis of recruited T-cells

To establish the mechanism whereby APC down regulate recruited T-cell proliferation we analysed the expression of the early apoptosis marker Annexin V. As shown in Figure 3.10B, stimulation of recruited peritoneal T-cells in the presence of APC resulted in increased expression of Annexin V, suggesting that these cells were undergoing apoptosis.

3.3 Discussion

To date, the phenotype, function and regulation of human peripheral tissue T-cells have remained poorly defined. The lack of readily available samples (especially during the "non-inflamed" state) means that we are heavily reliant on data extrapolated from peripheral blood cells or from animal models. Though the basic surface phenotype of peritoneal T-cells has been previously documented, a more detailed functional analysis of this leukocyte population remains to be defined [29-33]. In view of the potential importance of T-cells in regulating peritoneal immune function and host defence, we undertook a more detailed functional analysis of the resident peritoneal T-cell population focussing in particular on their activation status, cytokine profile and proliferative capacity.

In agreement with previous studies we have shown that, as compared to peripheral blood, the peritoneal T-cells from patients on PD express significantly increased levels of CD69 (a very early marker of T-cell activation [92]. This suggests that peritoneal T-cells may be more "activated" than peripheral blood T-cells. In trying to delineate the mechanisms that lead to this increased CD69 expression, we performed a series of experiments attempting to mimic some of the factors that contribute to the unique intra-

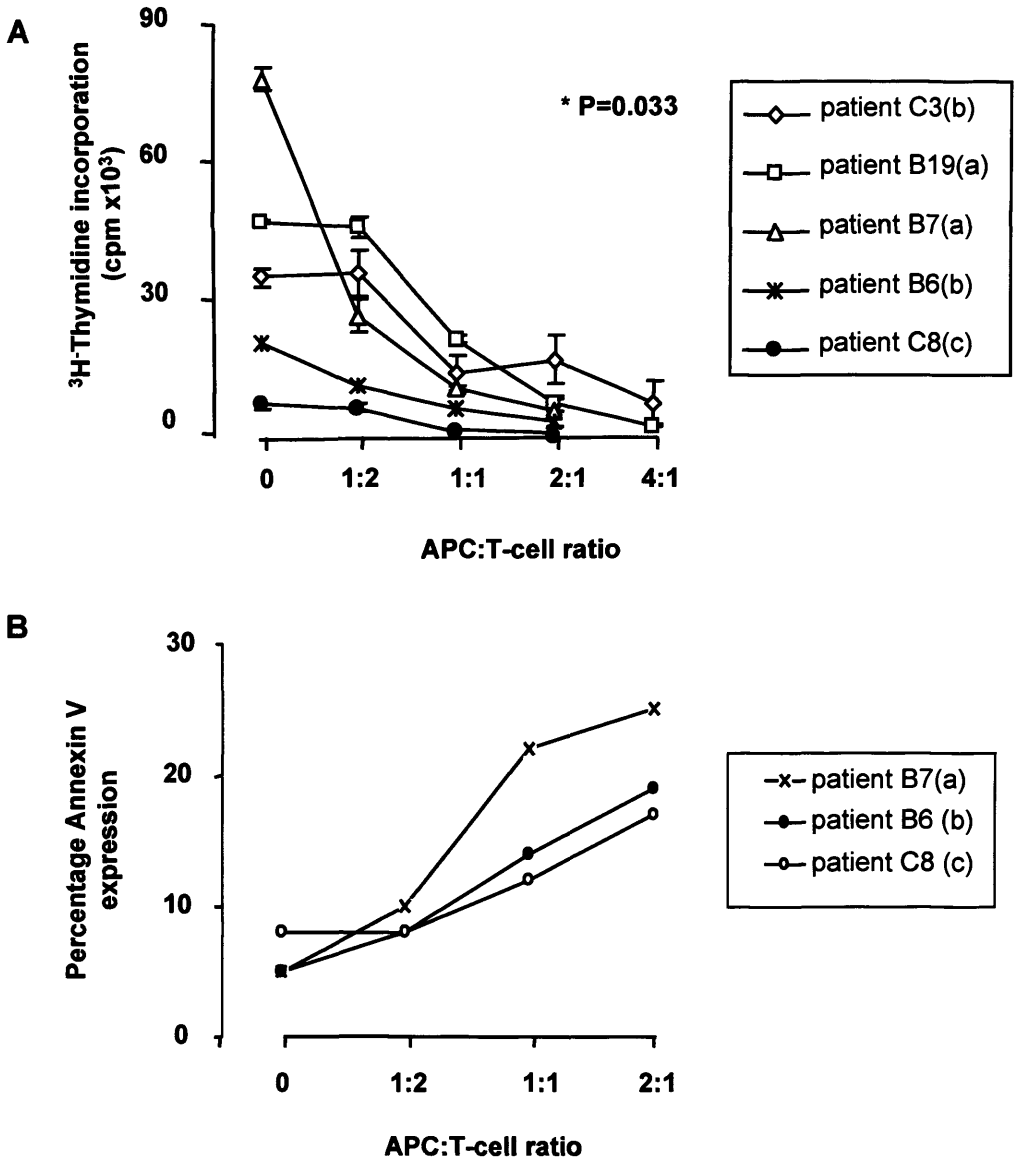

Figure 3.10 Proliferation of peritoneal T-cells collected during bacterial peritonitis. (A) Timed overnight PD effluent was collected from patients suffering from acute bacterial peritonitis (day 4). Peritoneal T-cells were isolated by magnetic column negative separation. APC coated plates were prepared by adhering allogenic peritoneal leukocytes to plates for 1 hour then removing non-adherent leukocytes. Cells were stimulated with OKT3(10ng/ml). Proliferation (^{3}H-Thymidine incorporation) was assessed at 96h (proliferation results show mean ±SE of duplicate cultures). Means were analysed using repeated measures analysis of variance. (B) Overnight PD effluent was collected from patients suffering from acute bacterial peritonitis (day 4). Peritoneal T-cells were isolated by magnetic column negative separation. APC coated plates were prepared by adhering allogenic peritoneal leukocytes to plates for 1 hour then removing non-adherent leukocytes. Cells were stimulated with OKT3. Following 48h of stimulation, cells were stained with fluorescently labeled monoclonal antibody directed against CD3 and Annexin V. A combined gate was set on T-cells based on their CD3 and FSC:SSC profiles. Results show the percentage of CD3+ T-cells expressing Annexin V (see appendix for demographics, peritonitis episode and microbiology data).

peritoneal environment. Neither *in vitro* exposure of PBMC to unused PD solutions, nor transmigration across a mesothelial monolayer resulted in significant up-regulation of CD69 on peripheral blood T-cells. Clearly these *in vitro* experiments are limited in that they do not mimic the sophisticated cytokine/cellular milieu of the human peritoneal cavity. They are also limited by the absence of an adequate positive control showing that the transmigrated cells are capable of up-regulating CD69 expression. In spite of this, the findings suggest that other mechanisms may be responsible for the increased "activation" of these cells.

Interestingly, the number of $CD69^+$ T-cells was much higher than the number of $CD25^+$ cells, and only a few cells (<10%) were CD25/CD69 double positive. This was unexpected, since both CD25 and CD69 are markers of T-cell activation and *in vitro* stimulation of peritoneal T-cells with mitogens leads to increased expression of both molecules [93]. There are two possible explanations for this. The first explanation lies in the differing temporal expression profiles of these markers. CD25 is a "late" activation marker and requires a more sustained period of TCR stimulation than does CD69 [93]. The putative *in vivo* stimulus leading to CD69 up-regulation may be transient and incomplete and hence insufficient to increase CD25 expression (moreover, many of the $CD25^+$ cells may be T-regs rather than activated T-cells – see Chapter 4, section 4.2.6). Accordingly, the $CD69^+$ cells are in effect "partially activated" and may be resistant to further stimulation. Analogous to this, peripheral blood T-cells from Lupus Nephritis patients show an increased expression of CD69, but are hypo-responsive to further stimulation. Additionally, human synovial fluid T-cells display hypo-responsiveness that parallels their CD69 expression [89, 94-96].

The alternative explanation is that CD69 expression may not only signify T-cell activation; it may delineate a distinct T-cell subset with unique effector functions. The evidence supporting this comes from a series of experiments analysing the pathogenesis of collagen-induced arthritis (CIA). Rather unexpectedly, it was shown that CD69 deficient mice developed an exacerbated form of disease associated with a hyper-immune response and

decreased levels of TGFβ. Together with similar observations in the NK-sensitive tumor model these findings have revealed a novel regulatory/ suppressive role for CD69 signalling, which may be mediated via secretion of anti-inflammatory mediators. These findings hint at the intriguing possibility that some of the $CD69^+$ peritoneal T-cells may perform a regulatory function, dampening down local immune responses [89, 94-96].

To further examine the reactivity pattern of the peritoneal T-cells, we investigated their TH1/TH2 cytokine profile. According to the TH1vs.TH2 paradigm, effector T-cells are defined according to their cytokine profiles. TH1 cells produce IFN-γ and drive cell-mediated immune responses whereas TH2 cells produce IL-4 and are associated with B-cell help and humoral responses [56, 58, 59]. Whilst previous studies have suggested that IFN-γ levels are elevated during acute peritonitis, a detailed analysis of the TH1/TH2 axis in resident peritoneal T-cells has been lacking [54, 55, 97].

From our experiments we can conclude that *in vitro* stimulated peritoneal T-cells mount a pre-dominantly TH1-skewed immune response. TH1 responses also appear to dominate the *in vivo* situation as evidenced by the increased levels of IFN-γ (but very little IL-4) detected in PD effluent during both peritonitis and the steady state.

The peritoneal TH1 bias is probably due to a combination of preferential recruitment of TH1 cells and DC driven polarisation of local undifferentiated cells. Many murine studies have shown that TH1 and TH2 cells display distinct migration patterns, with TH1 cells entering inflamed peripheral tissues, whereas TH2 cells enter lymph node and spleen (the inflamed asthmatic lung is an exception here as it contains an abundance of TH2 cells) [98]. Evidence for the role of DC in peritoneal T-cell polarisation comes from *in vitro* work showing that DC skew stimulated PBMC towards a TH1 reactivity profile [26]. Further evidence comes from the observation that IL-12 levels are greatly increased during the early stages of peritonitis. The formation of a TH1 response is highly dependant on IL-12, suggesting that this DC derived

cytokine is skewing the local peritoneal immune response towards a TH1 bias [97, 99].

The TH1 response is pivotal for both bacterial clearance and immuno-resolution. In terms of pathogen clearance, IFN-γ serves to activate peritoneal macrophages and promote bactericidal activity. In terms of resolution, IFN-γ (in combination with IL-6) has been shown to abrogate neutrophil recruitment and promote neutrophil apoptosis thus limiting host damage [46, 52]. It should be noted that we did not attempt to deliberately skew the peritoneal T-cells to a TH2 phenotype in these experiments (e.g. by culturing in an IL-4 rich environment). Consequently it remains feasible that under appropriate conditions (e.g. within omental milky spots or peritoneal lymph nodes) peritoneal T-cells may adopt a TH2 phenotype allowing them to stimulate/support local B-cells

In addition to cytokine production, immuno-competent T-cells must retain proliferative capacity so that they can generate a secondary wave of antigen specific effector cells. Some authors have suggested that resident peritoneal T-cells exist in a state of anergy and do not proliferate in response to stimulation [100]. Our initial work seemed to confirm this, since unlike matched peripheral blood T-cells, purified peritoneal T-cell did not appear to proliferate in response to OKT3. We subsequently refined these experiments and were able to demonstrate that peritoneal T-cells can in fact proliferate when cultured in the presence of allogenic peritoneal APC.

Why is peritoneal T-cell proliferation so highly dependent on APC? A more refined analysis of the peritoneal T-cell: APC interaction suggests that this accessory cell requirement is mainly dependent on direct cell-cell contact. One possible explanation for this relates to the fact that OKT3 is a monoclonal antibody. The APC contain receptors capable of binding the Fc portion of the antibody, thus creating a matrix of cross-linked TCRs and activating the T-cells (Figure 3.11).

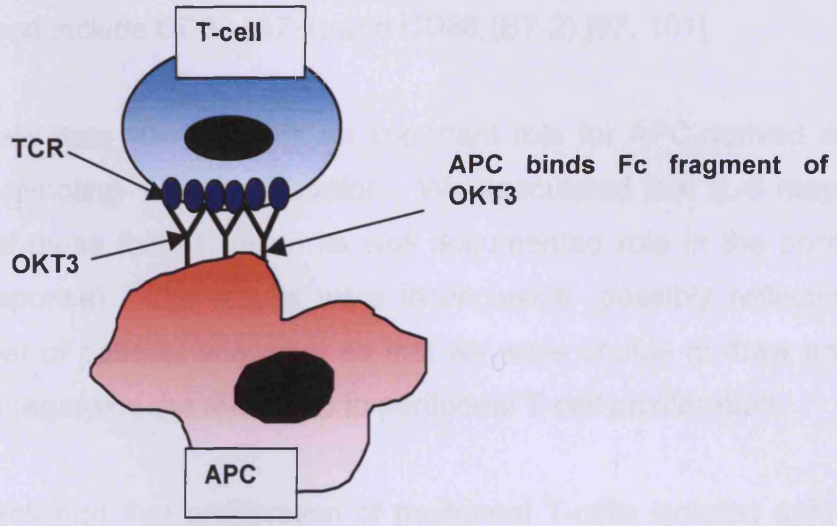

Figure 3.11 TCR cross linking – a possible explanation for APC dependence. APC bind the Fc portion of the OKT3 molecule. Subsequent clustering of the TCR results in T-cell activation

If this explanation were correct then plate bound OKT3 should mimic this effect by cross-linking TCR. The poor proliferation observed with plate bound OKT3 and plate bound CD3 suggests that APC are not acting solely via engagement of Fc binding. We speculate that other factors such as contact with APC derived co-stimulatory molecules are necessary to drive peritoneal T-cell proliferation. These co-stimulatory molecules have been well described elsewhere and include CD80 (B7-1) and CD86 (B7-2) [97, 101].

The trans-well data also highlight an important role for APC-derived soluble factors in promoting T-cell proliferation. We speculated that IL-6 may have been one of these factors (given its well documented role in the peritoneal immune response). Our results were inconclusive (possibly reflecting the small number of patients analysed) so that we were unable to draw any firm conclusions regarding the role of Il-6 in peritoneal T-cell proliferation.

Having established that proliferation of peritoneal T-cells isolated during the steady (non-infected) state are dependant on APC co-stimulation, we next extended our study to examine the proliferation of peritoneal T-cells isolated during acute peritonitis. These T-cells are a short lived population, recruited in large numbers from the blood on days 4-6 of infection [C Parker, unpublished data] then rapidly cleared from the peritoneum. We discovered that (similar to peripheral blood T-cells) these cells were not dependant on APC for *in-vitro* proliferation and in fact the peritoneal APC appeared to have a suppressive effect on the proliferation of these cells, promoting activation induced cell death (AICD).

These findings hint at a dual role for peritoneal APC in regulating T-cell proliferation. During the steady state, slow turnover of resident peritoneal T-cells is likely to occur only in peritoneal lymph nodes where T-cells come into contact with peritoneal DC. This dependency on DC co-stimulation minimises non-specific T-cell proliferation in response to innocuous peritoneal or environmental antigens. During acute peritonitis vast numbers of effector T-cells are recruited from the blood into the peritoneal cavity. Following pathogen clearance, it is imperative that the majority of these (now redundant)

effector cells are removed, DC appear to expedite this process by promoting apoptosis in this population.

3.4 Conclusion

In conclusion, we have identified significant differences between peripheral blood and peritoneal T-cells with regards to their activation status and proliferative capacity. Peritoneal T-cells express increased levels of CD69, (which may serve a regulatory function) and respond to stimulation with a TH1 mediated response. They appear to have a higher activation threshold than peripheral blood cells and their proliferation is tightly regulated by peritoneal DC. This suggests that intricate control mechanisms are in place to streamline the peritoneal immune response, contributing to replete host defence without causing host damage.

Chapter 4

Characterisation of Specialised Peritoneal T-cell Sub-Populations

4.1 Introduction

Amongst the most exciting recent developments in the field of immunology has been the identification of specialised effector subsets within the T-cell repertoire. These include memory subsets, regulatory T-cells, IL-17 producing T-cells and Vγ9δ2 cells. Their characterisation has provided new insights into the complexity of the human innate and adaptive immune responses and how T-cells contribute to immune-surveillance [59, 61, 62, 66, 67, 75-79].

The current definition of memory T cells recognises central memory cells (T_{CM}), effector memory cells (T_{EM}) and terminally differentiated effector memory cells (TEMRA). These memory subsets differ in both phenotype and function. T_{CM} (CD45R0/CCR7^{+}/CD62L^{Hi}) express lymph node homing receptors and localize to lymphatic tissue where they are thought to respond to TCR stimulation by proliferation. T_{EM} (CD45R0/CCR7^{-}/CD62L^{Lo}) lack these lymph homing receptors and localize to peripheral tissues, providing an immediate response to infection [59, 79, 80]. These subsets have not, as yet been characterised in the peritoneal cavity.

Regulatory T-cells (T-regs) are well known to maintain peripheral tolerance and prevent autoimmunity. A growing body of evidence also suggests that T-regs are pivotal in regulating the host's response to infection [68-71]. Closely related to T-regs are the IL-17 producing CD4^{+} T cells (TH17 cells). There is emerging evidence that these cells are instrumental in the pathogenesis of numerous human diseases including psoriasis, colitis and multiple sclerosis (MS) [61]. Their role in the host response to bacterial infection is less well defined.

Another recently identified T-cell subset that have attracted much attention are the Vγ9δ2 T-cells. Whilst most peripheral blood T-cells express the TCRαβ on their surface, a minority (2-20%) express the TCRγδ. The most abundant γδ T-cells are the subset expressing the Vγ9δ2 TCR. These cells

are unique in that they recognise low molecular weight non-peptide compounds in an MHC independent manner. The most potent stimulant of Vγ9δ2 T-cells is E-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), a metabolite produced by many infective agents including most species of Gram-negative bacteria. There is growing evidence that these cells may govern the early phase of the innate immune response to bacterial infection, although no data exists on their role in the peritoneal cavity [75-78].

The interest generated by these findings is reflected in the developing literature describing the phenotype and function of these T-cell sub-populations. It should however be noted that the vast majority of these studies describe T-cells obtained from murine models of inflammation and disease. Concerns have thus been raised that the findings of such studies may have limited applicability to human disease. Since the mice are kept in germ-free or essentially pathogen-free facilities it is well recognised that they have much lower levels of endogenous T-cell activation than the human system [70]. Clearly the simple answer is to perform all research on human samples, however this is not always feasible since access to human (especially peripheral tissue) cells is hampered by the need for invasive access. The unique nature of PD therapy has given us a rare opportunity to analyse specialised T-cell subsets within the context of human peripheral tissue inflammation.

The specific aims of this chapter are:

a) Characterise the memory phenotype of the peritoneal T-cell population.

b) Identify and characterise peritoneal T-cell sub-populations (T-regs / TH17 cells and Vγ9δ2 T-cells).

c) Examine the role of Vγ9δ2 T-cells in the peritoneal immune response to infection.

4.2 Results

4.2.1 Peritoneal T-cell memory subsets

To determine the "memory phenotype" of peritoneal T-cells, we analysed the surface expression of CD45R0 and CCR7 in paired samples of cells isolated from peripheral blood and PD effluent (Figure 4.1). When compared to peripheral blood, the peritoneum is depleted of naïve cells and enriched in T_{EM} cells (Figure 4.2). We further extended our phenotypic analysis to include the expression of the lymph node homing signal CD62L. We observed that CD62L expression is lower on peritoneal T-cells than peripheral blood T-cells. We also found that within the memory subsets, CD62L expression decreases progressively from naïve through T_{CM} to T_{EM} (Figure 4.3) [59, 80].

4.2.2 CD127 expression pattern of memory subsets

In an attempt to distinguish long-lived effector memory cells from recently activated effector cells (both of which express CD45R0) we examined CD127 expression on peritoneal T-cells. IL-7 signalling via CD127 (the IL-7R) is thought to confer anti-apoptotic properties to T-cells. We observed that (as compared to naïve cells) a sub-group of peritoneal memory cells express very high levels of CD127 (Figure 4.4).

4.2.3 Peritoneal T-cells mount a TH1 response to standard recall antigens

Murine data suggests that immediate effector cytokine production is a function of the T_{EM} subset. To determine whether this was also true of peritoneal T-cells we stimulated them with PMA(500ng/ml) and Ionomycin (50ng/ml) then analysed IFN-γ production. As predicted, the predominant early TH1 response to stimulation came from the T_{EM} subset (Figure 4.5).

Next, we used an ELISpot assay to investigate the antigen specificity of this response. Paired samples of peripheral blood and peritoneal T-cells were exposed to the standardized recall antigens PPD (purified protein derivative of *Mycobacterium tuberculosis*), HA (Haemagglutinin antigen derived from

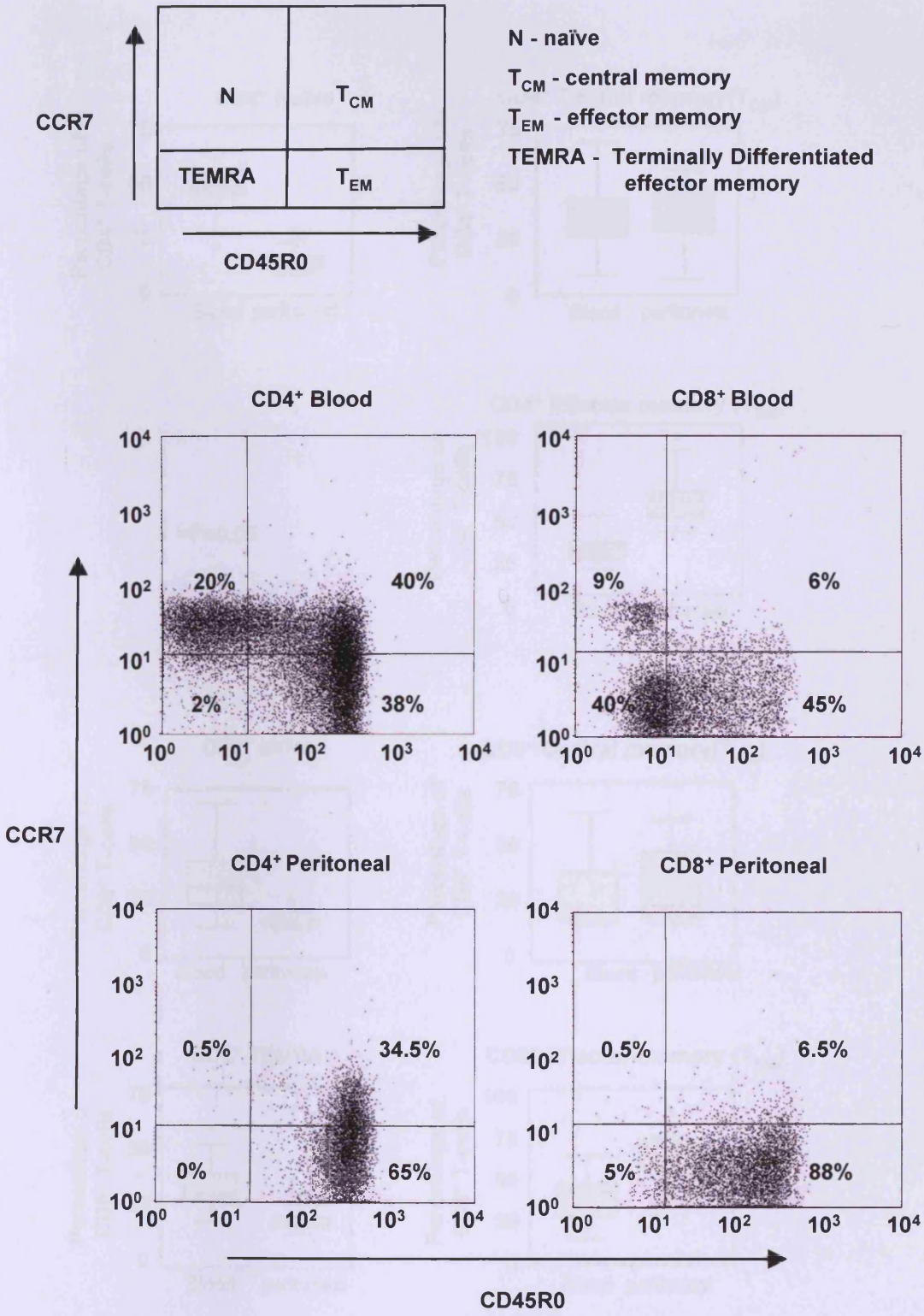

Figure 4.1 A comparison of peritoneal and peripheral blood T-cell memory subsets. Paired samples of PBMC and peritoneal leukocytes were collected from PD patients. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD4/CD8/CCR7 and CD45R0. A combined gate was set on peritoneal T-cells based on CD4 or CD8 expression and their FSC:SSC profiles. Results show representative example from 20 patients (see appendix for patient demographics).

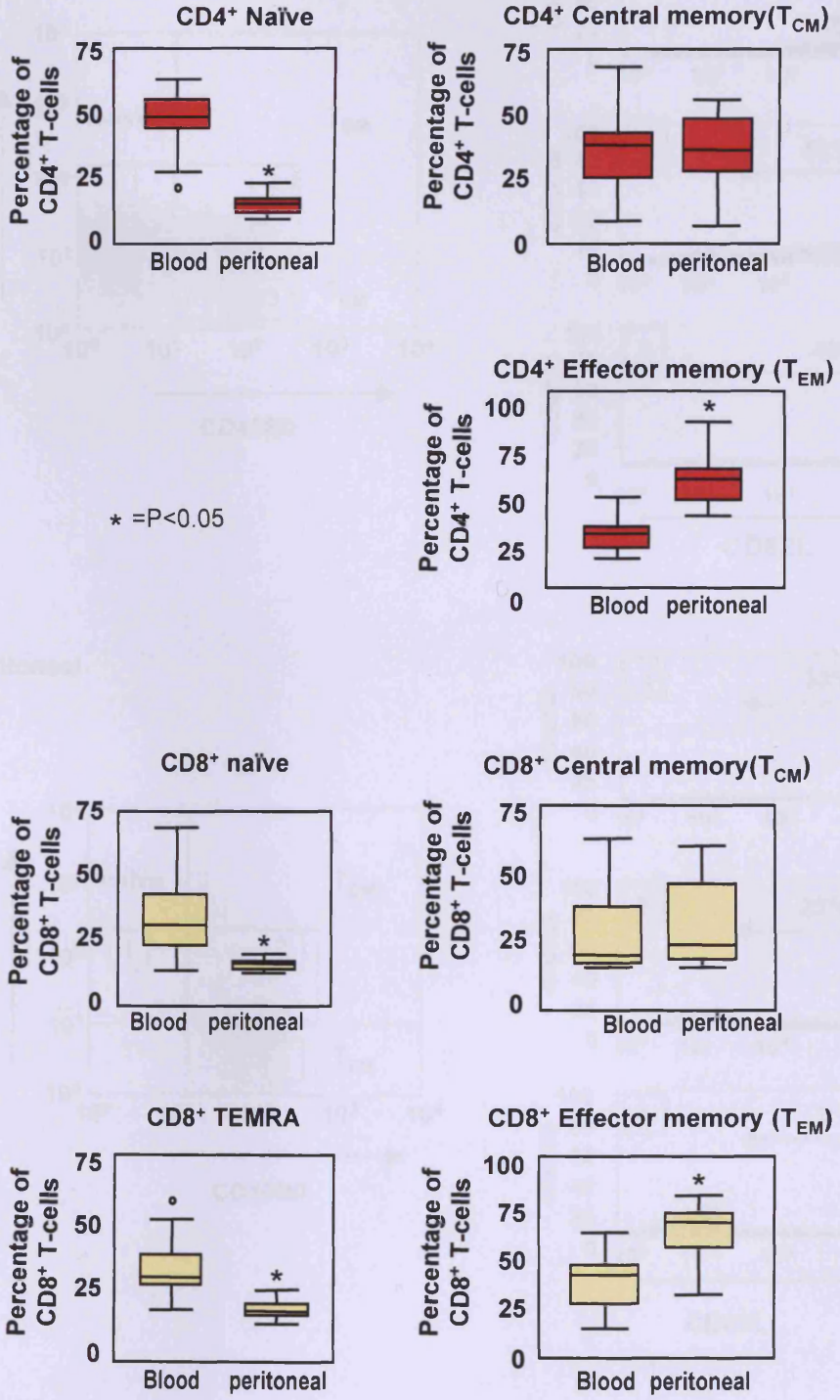

Figure 4.2 A comparison of peritoneal and peripheral blood T-cell memory subsets. Paired samples of PBMC and peritoneal leukocytes were collected from PD patients. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD4/CD8/CCR7 and CD45R0. A combined gate was set on peritoneal T-cells based on CD4 or CD8 expression and their FSC:SSC profiles. Summary of data obtained from paired blood and peritoneal T-cells obtained from 20 patients. Differences between groups were tested using the Wilcoxan signed ranks test (see appendix for patient demographics).

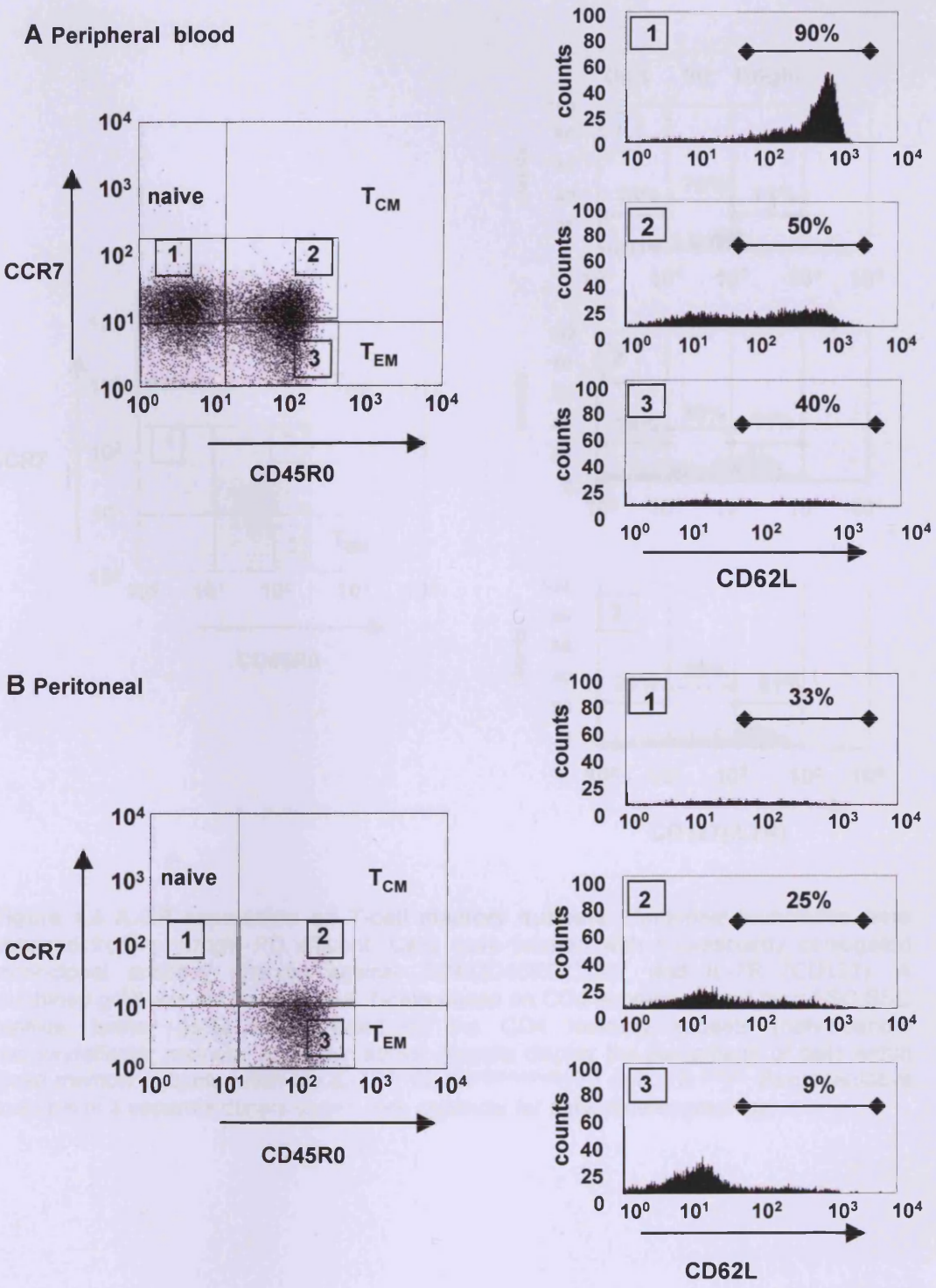

Figure 4.3 CD62L expression on (A) peripheral blood and (B) peritoneal T-cell memory subsets. Paired samples of PBMC and peritoneal leukocytes were collected from PD patients. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD4/CD62L/CCR7 and CD45R0. A combined gate was set on T-cells based on their CD4 expression and their FSC:SSC profiles, further gates were plotted on the CD4 memory subsets (naïve/central memory/effector memory) as shown above (see appendix for patient demographics).

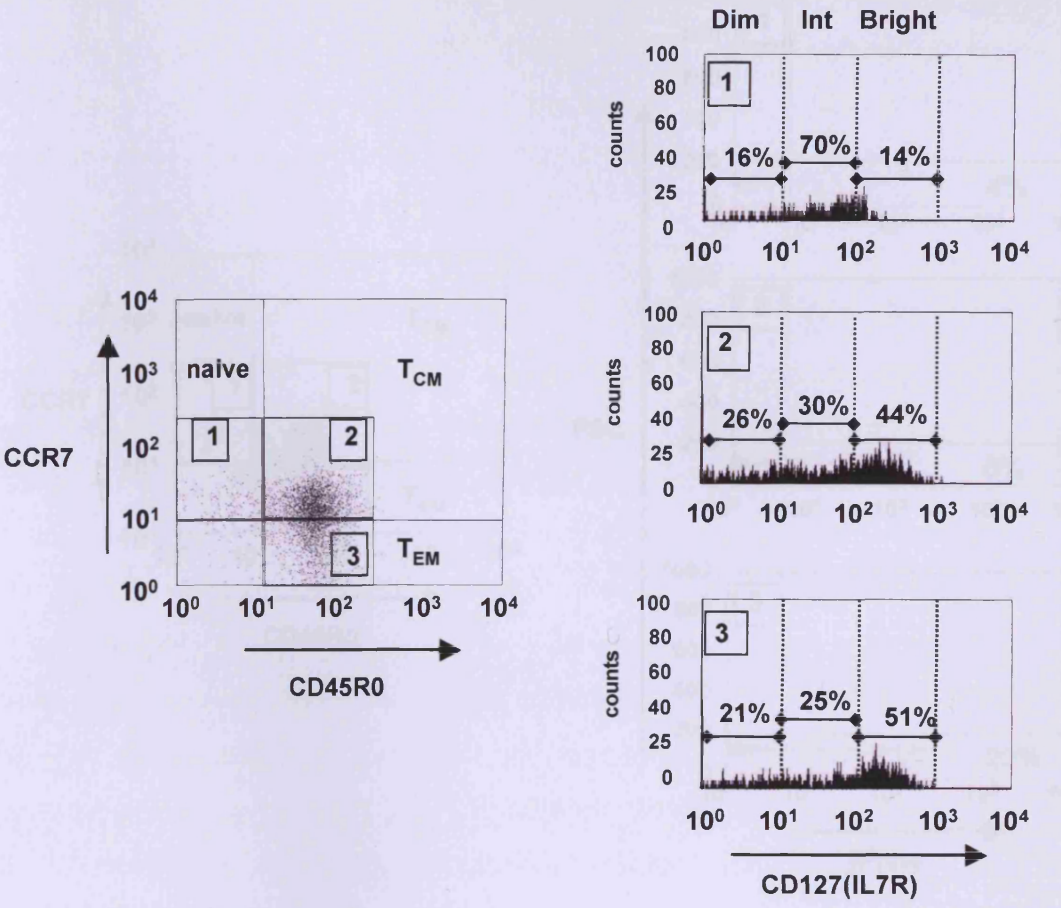

Figure 4.4 IL-7R expression on T-cell memory subsets. Peritoneal leukocytes were obtained from overnight PD effluent. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD4/CD45R0/CCR7 and IL-7R (CD127). A combined gate was set on peritoneal T-cells based on CD4 expression and their FSC:SSC profiles, further gates were plotted on the CD4 memory subsets (naïve/central memory/effector memory) as shown above. Results display the percentage of cells within these memory subsets which are IL-7R^{dim}/IL-7R$^{Intermediate(INT)}$ or IL-7R ^{Bright}. Representative example of 3 separate donors shown (see appendix for patient demographics).

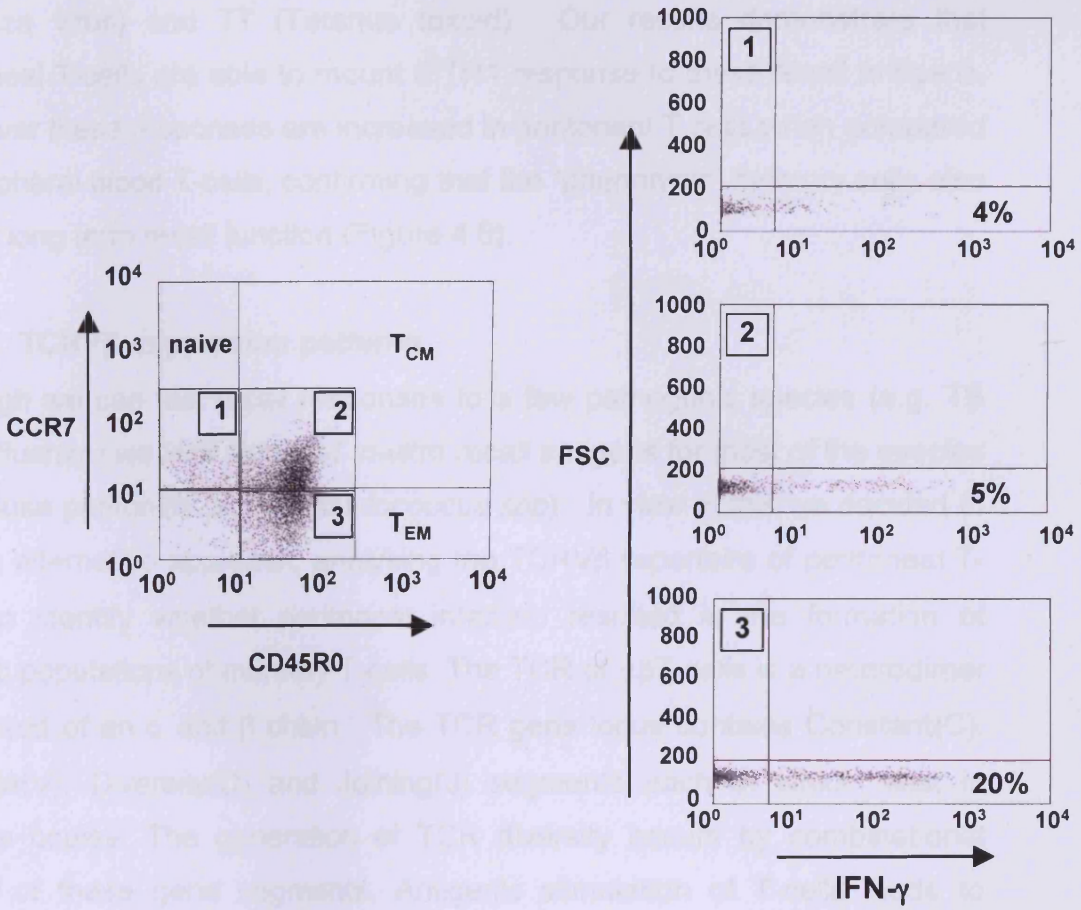

Figure 4.5 Peritoneal memory subset TH1 response. Peritoneal leukocytes ($2x10^5$/well) were stimulated with PMA (500ng/ml)/Ionomycin (50ng/ml) for 2 hours. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD4/CD45R0/CCR7. Cells were subsequently fixed and permeabilized then stained with anti-IFN-γ. A combined gate was set on peritoneal T-cells based on CD4 expression and their FSC:SSC profiles, further gates were plotted on the CD4 memory subsets (naïve/central memory/effector memory). Results show the percentage of IFN-γ^+ cells within these memory subsets. Results are a representative example of experiments performed on 4 separate donors (see appendix for patient demographics).

Influenza virus) and TT (Tetanus toxoid). Our results demonstrate that peritoneal T-cells are able to mount a TH1 response to these recall antigens, moreover these responses are increased in peritoneal T-cells when compared to peripheral blood T-cells, confirming that the "phenotypic" memory cells also exhibit long term recall function (Figure 4.6).

4.2.4 TCRVβ expression patterns

Although we can test recall responses to a few pathogenic species (e.g. TB and Influenza) we lack standard *in-vitro* recall antigens for most of the species that cause peritonitis (e.g. *Staphylococcus spp*). In view of this we decided to use an alternative approach, analysing the TCRVβ repertoire of peritoneal T-cells to identify whether peritoneal infection resulted in the formation of specific populations of memory T-cells. The TCR of αβT-cells is a heterodimer composed of an α and β chain. The TCR gene locus contains Constant(C), Variable(V), Diversity(D) and Joining(J) segments each of which exist in multiple copies. The generation of TCR diversity occurs by combinational joining of these gene segments. Antigenic stimulation of T-cells leads to oligoclonal expansion of cells expressing common VDJ segments, whereas superantigen stimulation leads to polyclonal expansion of cells expressing a particular TCRVβ family [102, 103]. We speculated that if peritonitis resulted in clonal expansion of peritoneal memory cells, these cells would be detectable as a local clonally expanded population, all sharing the same TCRVβ repertoire.

TCRVβ expression was examined by flow cytometry in paired samples of peripheral blood and peritoneal T-cells. The blood/peritoneal TCR repertoire was similar in patients with no previous peritonitis or previous *Staphylococcus epidermidis* peritonitis. In contrast, patients who had a previous history of *Staphylococcus aureus* peritonitis displayed polyclonal expansion of peritoneal T-cells (Figure 4.7).

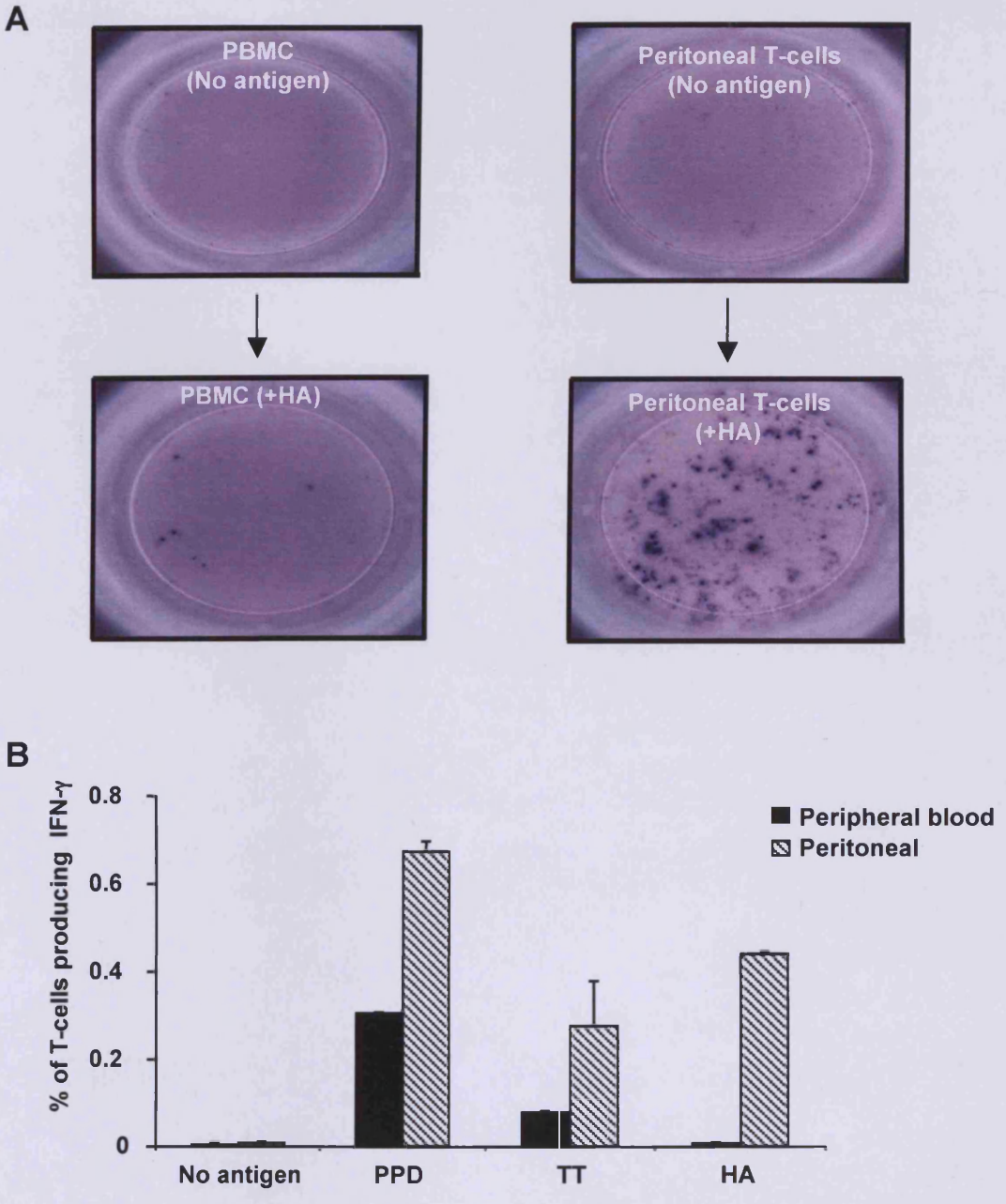

Figure 4.6 Peritoneal T-cell recall responses. Paired samples of PBMC and peritoneal leukocytes were collected from PD patients. ELISpot plates were coated with 50 µL anti-IFN-γ antibody in sterile PBS (10µg/ml) and incubated at 4°C for 180mins. Peritoneal leukocytes and PBMC were plated out at $2x10^5$ cells per well. Cells were incubated at 37°C for 16h in the presence of purified protein derivative (PPD) (10 µg/ml), Tetanus Toxoid (TT) (5 µg/ml) or Haemaglutinin (HA) (5µg/ml). The ELISpot assay was developed according to the manufacturer's instructions. (A) Representative example of Haemaglutinin recall response in peripheral blood and peritoneal T-cells. (B) Summary of recall response data from an individual patient. Results show mean ±SE of triplicate observations. Representative example of 4 donors is shown (see appendix for patient demographics).

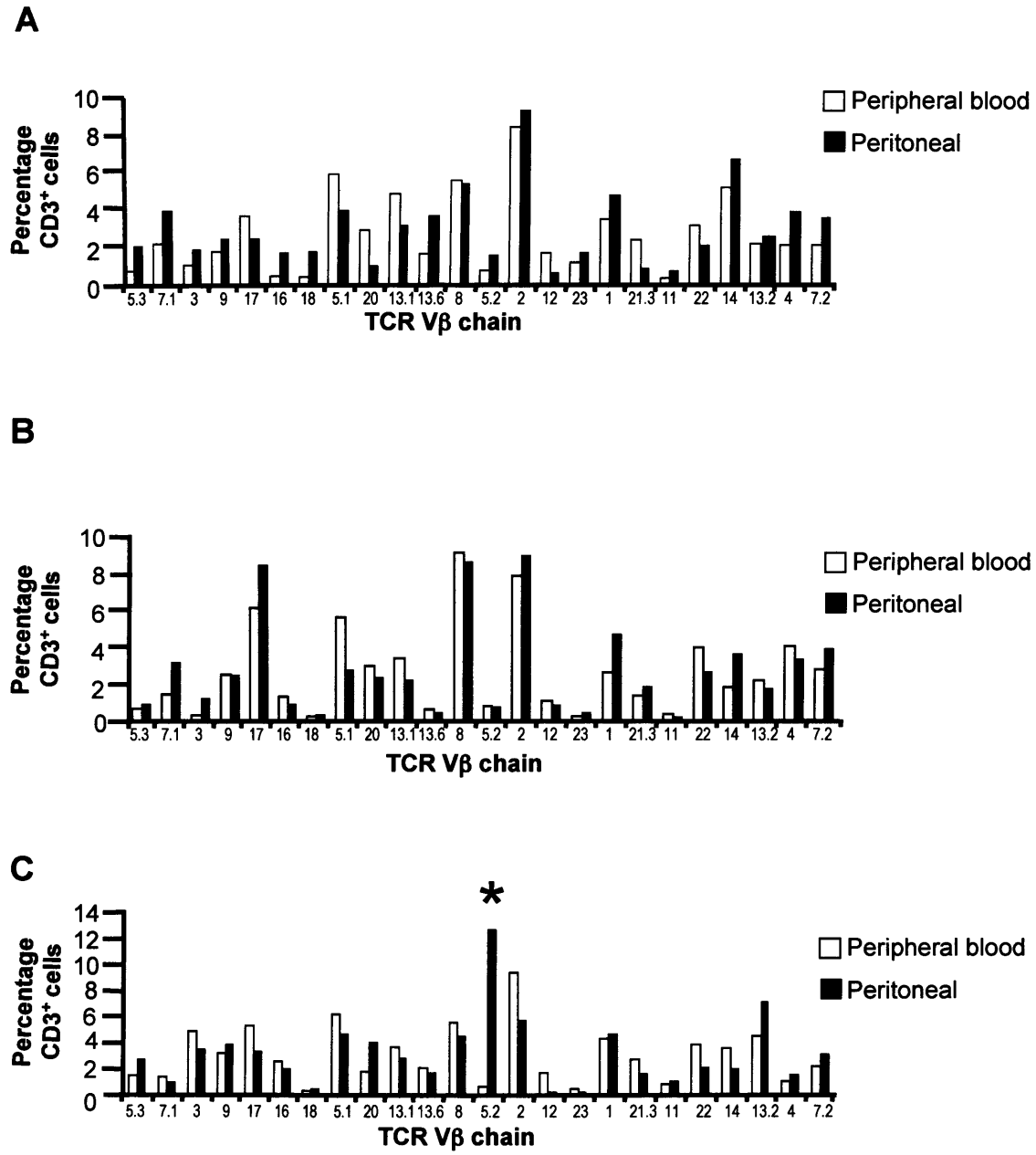

Figure 4.7 TCRVβ repertoire of peripheral blood and peritoneal T-cells. Paired samples of PBMC and peritoneal leukocytes were collected from PD patients. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3 and the various TCRVβ families. A combined gate was set on peritoneal T-cells based on CD3 expression and their FSC:SSC profiles. (A) Representative TCRVβ repertoire from never infected patients (n=5). (B) Representative TCRVβ repertoire from a patient with previous *Staphylococcus epidermidis* peritonitis (n=10). (C) Representative TCRVβ repertoire from a patient with previous *Staphylococcus aureus* peritonitis (n=3). Results show the percentage of $CD3^+$ T-cells expressing the TCRVβ family (see appendix for patient demographics).

4.2.5 Characterisation of peritoneal regulatory T-cells (T-regs)

To determine whether the peritoneal cavity of stable PD patients contains a population of T-regs, we analysed the peritoneal effluent for the presence of $CD3^+/CD4^+/CD25^+/Foxp3^+$ T-cells. As shown by the representative scatter plots (Figure 4.8A) the peritoneal cavity contains a distinct population of cells bearing this characteristic T-reg-like surface phenotype.

We subsequently compared the frequency of T-regs in paired samples of peripheral blood and peritoneal leukocytes isolated from a group of stable (i.e. infection free for at least 3 months prior to collection) PD patients. The frequency of T-regs within the peritoneal leukocyte pool was significantly higher ($p=0.01$) than the frequency of T-regs in the peripheral blood of the same individuals, suggesting that the peritoneal cavity is enriched in T-regs (Figure.4.8B).

4.2.6 Peritoneal T-regs are functional and retain their suppressive function

Although generally accepted that Foxp3 expression distinguishes T-regs from effector T-cells, there remains some controversy regarding the exact T-reg phenotype with some authors reporting transient up-regulation of Foxp3 expression in effector (non-suppressive) T-cells [66]. In view of this we felt it was necessary to demonstrate that peritoneal T-regs did indeed have suppressive function. To investigate this further, peritoneal T-regs were isolated by FACS (see Materials and Methods section 2.9.2) and added to stimulated autologous responder T-cells. Although the sorted cell populations were not irradiated (and thus may themselves proliferate), our results demonstrate that peritoneal T-regs display potent suppressive function. (Figure 4.9).

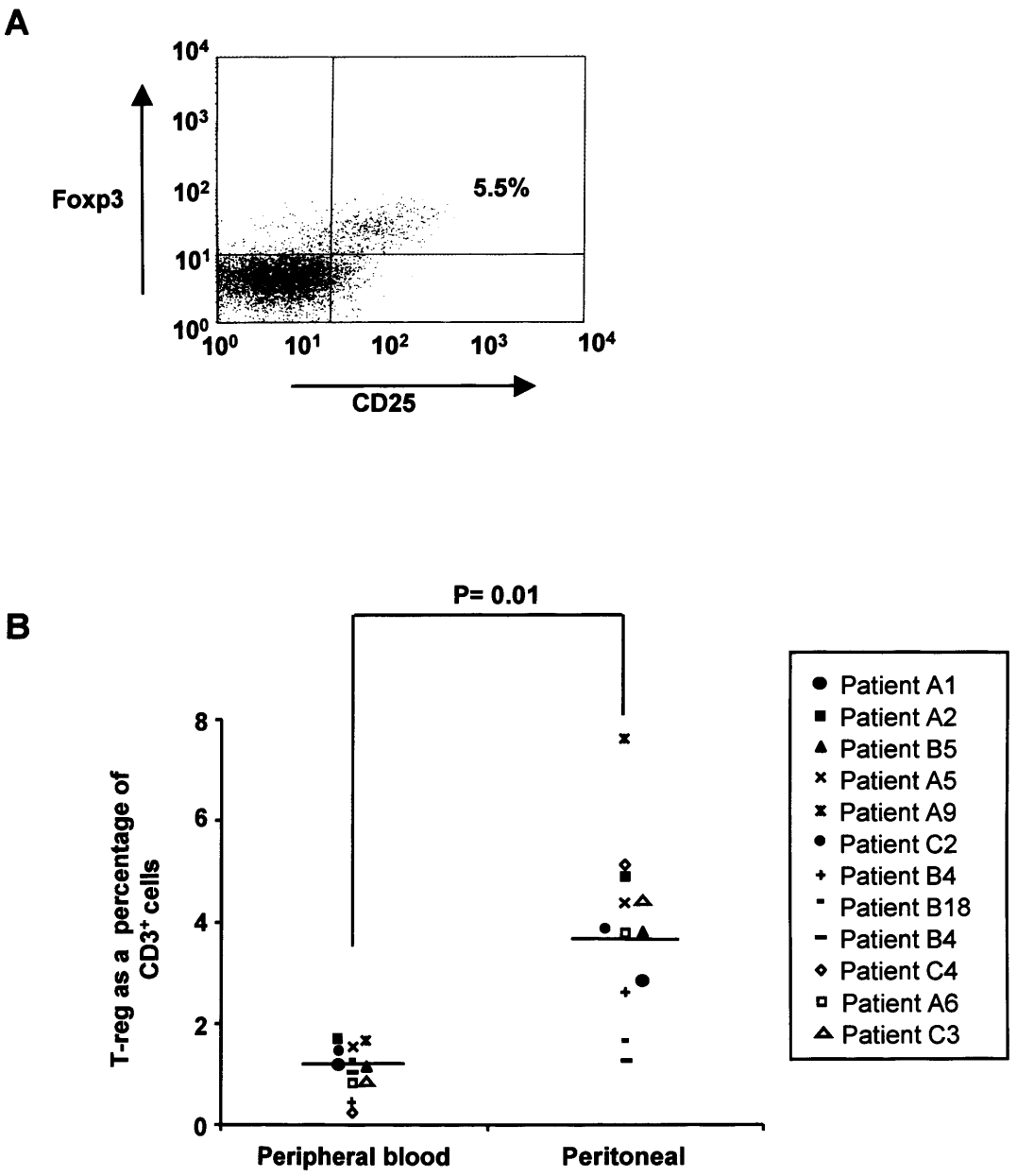

Figure 4.8 Identification of Peritoneal T-regs (A) Paired samples of peripheral blood and peritoneal T-cells were isolated from PD patients. T-cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3/CD4 and CD25. Cells were subsequently fixed and permeabilized then stained with anti-Foxp3. A gate was set on peritoneal T-cells based on their FSC/SSC profile and CD3/CD4 expression pattern. CD25 and Foxp3 expression was examined within this gated population. (A) Representative example of peripheral blood and peritoneal T-regs (results display percentage of $CD4^+/CD25^+/Foxp3^+$ T-regs within the $CD3^+$ T-cell pool). (B) Summary of data from paired blood and peritoneal T-cells obtained from 12 patients (horizontal lines represent the mean). Differences between groups were tested using the Wilcoxan signed ranks test.

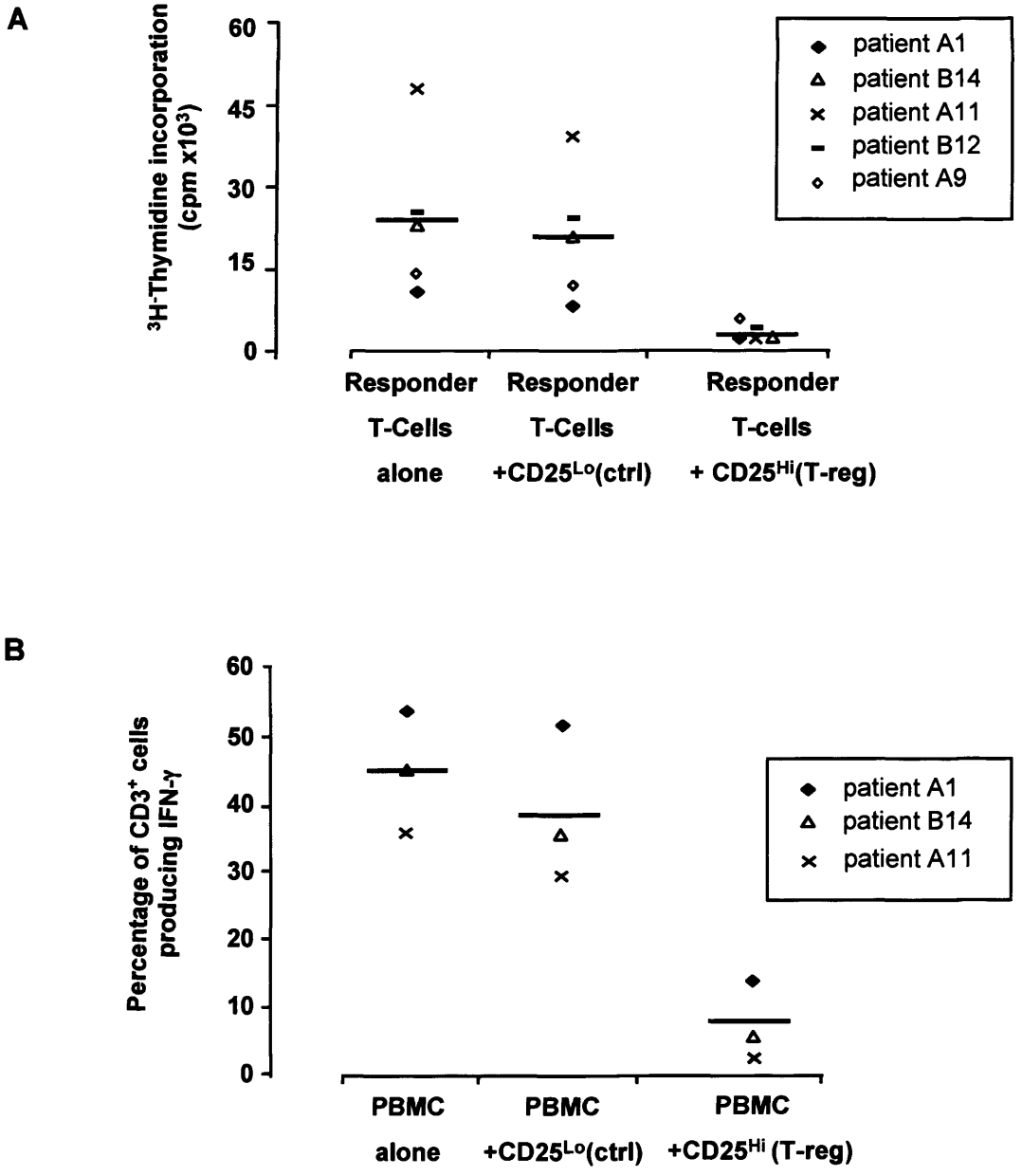

Figure 4.9 Peritoneal T-reg suppressive function. Paired samples of PBMC and peritoneal leukocytes were isolated from PD patients. Peritoneal leukocytes were labeled with fluorescently conjugated monoclonal antibody directed against CD3/CD4 and CD25. Pure populations (>98%) of $CD4^+/CD25^{Hi}$ (T-regs) and $CD4^+/CD25^{Lo}$ (control) peritoneal T-cells were isolated by flow cytometry and cell sorting (FACS). (A)The sorted cells were added to OKT3 stimulated allogenic peripheral blood T-cells (responder population) at a T-cell:T-reg ratio of 5:1. T-cell proliferation (^{3}H-Thymidine incorporation) was assessed at 96h. Proliferation results shown as mean of duplicate culture wells (horizontal lines represent mean of 5 patients) (B) The sorted peritoneal cells were added to allogenic PBMC cultures (PBMC:T-cell ratio 5:1). Cells were subsequently stimulated with PMA (500ng/ml)/Ionomycin (50ng/ml). Following 4h of stimulation, PBMC were labeled with fluorescently conjugated monoclonal antibody directed against CD3. Cells were fixed, permeabilized then stained with anti-IFN-γ. A combined gate was set on T-cells based on CD3 expression and their FSC:SSC profiles. Results show the percentage of IFN-γ^+ T-cells (horizontal lines represent mean).

4.2.7 Mechanism of T-reg suppression

To determine whether peritoneal T-reg suppression was dependent on cell-contact, purified peritoneal T-regs (from stable PD patients) were co-cultured with stimulated autologous peripheral blood T-cells in a trans-well culture system (Figure 4.10A). In these experiments direct cell-cell contact was required for maximal T-reg suppression. When the T-reg and responder PBMC were separated by the insert, cell proliferation was still observed albeit at a lower level suggesting that T-reg derived soluble factors appear also have significant suppressive function (Figure 4.10B). Since both IL-10 and TGFβ have both been previously identified as potent T-reg derived anti-proliferative and anti-inflammatory cytokines [104], we used blocking antibodies directed against both IL-10 and TGFβ in these trans-well experiments. As shown in Figure 4.10C blocking IL-10 and TGFβ only partially abrogated T-reg suppressive function.

4.2.8 Peritoneal IL-17 producing T-cells (TH17)

To identify whether TH17 cells are present or can be derived from T-cells in the dialysed peritoneal cavity, isolated peritoneal T-cells were collected from both stable patients and from patients suffering bacterial peritonitis. Cells were stimulated *in-vitro* with PMA (500ng/ml) and Ionomycin (50ng/ml) and IL-17 expression was analysed by flow cytometry. We only detected TH17 cells in 2 of the 15 patients analysed (Figure 4.11A). One of these patients had severe, non-resolving *Staphylococcus epidermidis* peritonitis, while the other had Gram-negative peritonitis (It should also be noted that in these 2 patients TH17 cells were only detectable after further *in-vitro* stimulation i.e. not immediately *ex-vivo).*

In a separate series of patients, analysis of PD effluent by LUMINEX multiplex ELISA showed detectable levels of IL-17 early (days 1 and 2) in the course of peritonitis, suggesting that small populations of peritoneal IL-17 producing cells (although not always detected by FACS analysis) are present during infection (Figure 4.11B).

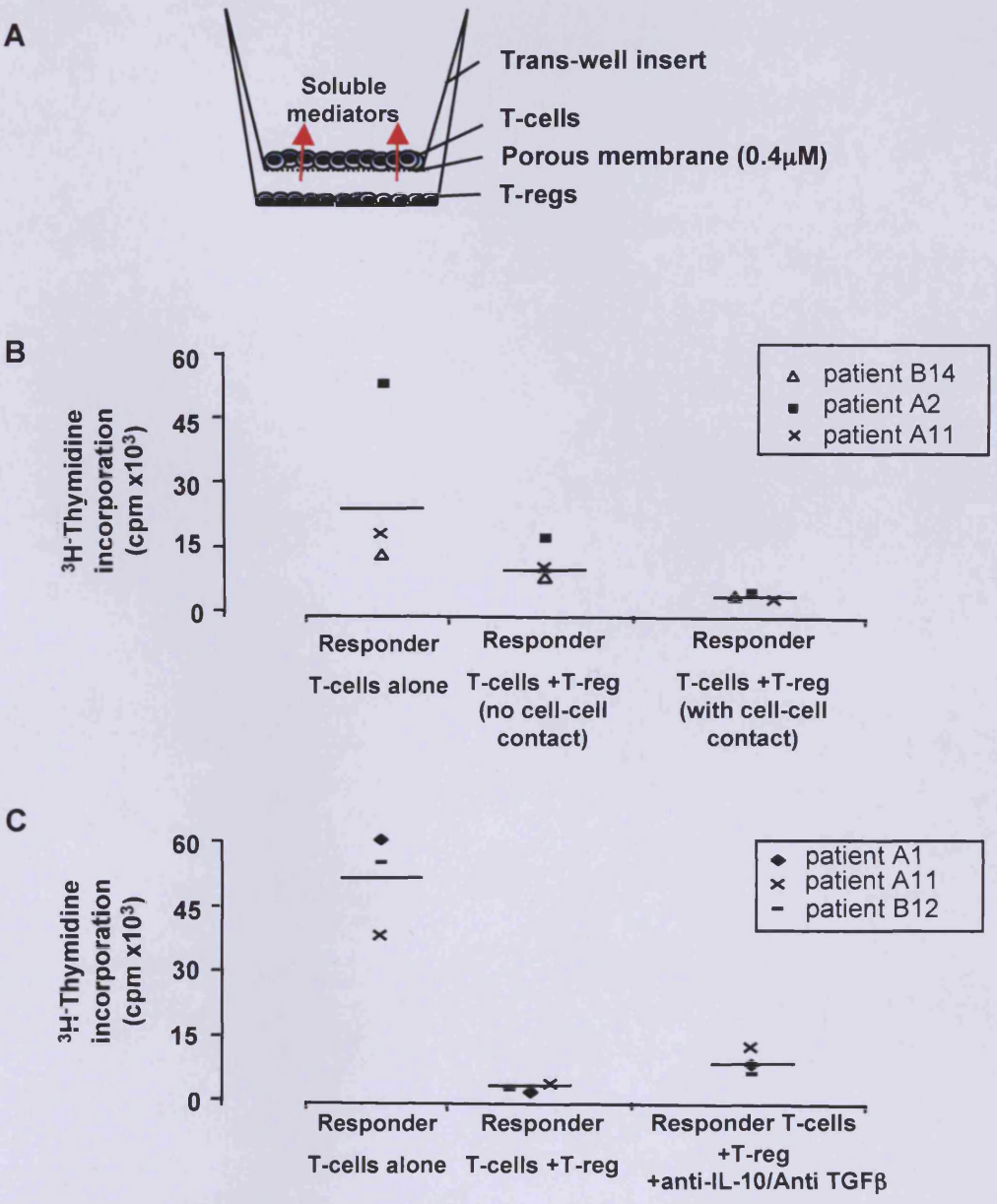

Figure 4.10 Mechanism of peritoneal T-reg suppression. Paired samples of PBMC and peritoneal leukocytes were isolated from PD patients. Peripheral blood T-cells were isolated by magnetic column negative selection. Peritoneal leukocytes were labeled with fluorescently conjugated monoclonal antibody directed against CD3/CD4 and CD25. Pure populations (>98% purity) of $CD4^{+}/CD25^{Hi}$ peritoneal T-regs were isolated by flow cytometry and cell sorting (FACS). (A) Trans-well inserts were used to prevent direct cell-cell contact. (B) Peripheral blood T-cells were stimulated with OKT3(10ng/ml) and IL-2 (25U/ml), either in direct contact with peritoneal T-regs (T-cell:T-reg ratio, 5:1) or with peritoneal T-regs on the opposite side of a trans-well insert membrane. Proliferation(^{3}H-Thymidine incorporation) was assessed at 96h. Results show mean of duplicate cultures (horizontal lines represent mean of 3 patients). (C) Peritoneal T-regs($CD3^{+}/CD4^{+}/CD25^{Hi}$) isolated by FACS were added to cultures of allogenic peripheral blood T-cells (T-cell:T-reg ratio 5:1). Cells were stimulated with OKT3(10ng/ml) and IL-2(25U/ml), in the presence or absence of anti-IL-10/anti-TGFβ(both 10μg/ml). Proliferation(^{3}H Thymidine incorporation) was assessed at 96h. Results show mean of duplicate cultures (horizontal lines represent mean of 3 separate patients; see appendix for patient demographics)

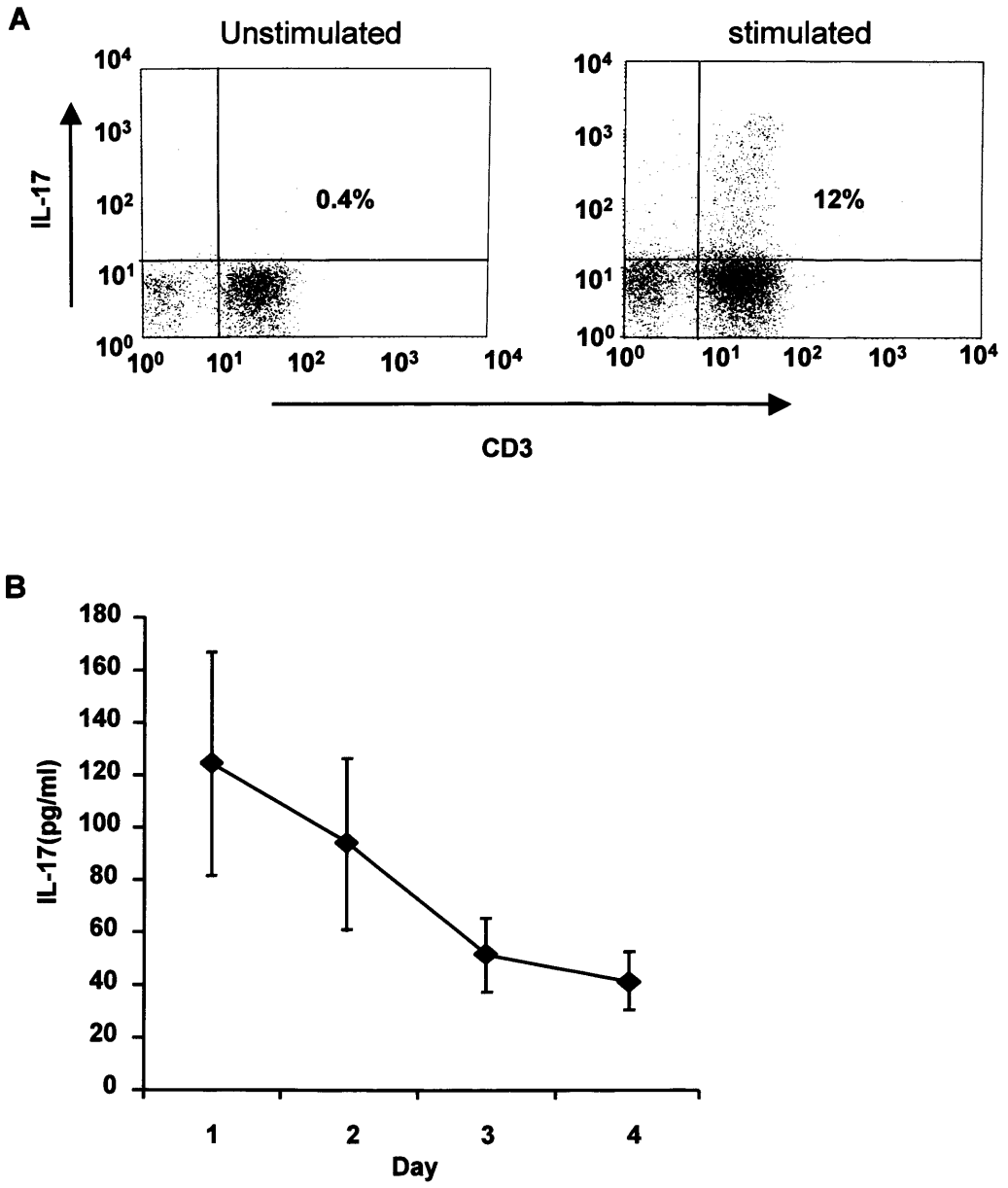

Figure 4.11 Identification of peritoneal TH17 cells. (A) Peritoneal leukocytes were collected during Gram -ve peritonitis (day 5). Cells were stimulated with PMA (500ng/ml)/ Ionomycin (50ng/ml) for 4 hours in the presence of monensin (2μM). Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3. Cells were subsequently fixed and permeabilized then stained with anti-IL17. A gate was set on peritoneal lymphocytes based on their FSC:SSC profiles, the percentage of IL-17$^+$ cells (within the CD3$^+$ population) was calculated. (B) Serial samples of peritoneal effluent was collected from patients suffering from acute peritonitis. IL-17 levels were analysed with a multiplex bead-based cytokine assay. Results show mean ±SE of n=12 separate patients (see appendix for patient demographics).

4.2.9 Peritoneal Vγ9δ2 T-cells

We next examined whether the peritoneal cavity contains a population of Vγ9δ2 cells as part of its innate immune defence armoury. As demonstrated in Figure 4.12, a population of Vγ9δ2 cells was detected by FACS analysis. These peritoneal Vγ9δ2 cells expressed an inflammatory phenotype as characterised by increased expression of CCR5 and decreased expression of CCR7 (Figure 4.12D). Stimulation of these cells with the Vγ9δ2 cells agonist HMB-PP resulted in selective activation (CD25 up-regulation) and TH1 cytokine production (Figure 4.12B and C). Furthermore, in a single experiment, exposure of PBMC to cell free peritoneal effluent freshly collected from a patient suffering Gram-negative peritonitis resulted in selective activation of Vγ9δ2 cells. These observations suggest that this effluent may contain HMB-PP or an equivalent activating principal (Figure 4.13). It should be noted that repeating this experiment with stored samples of Gram-negative PD effluent failed to activate Vγ9δ2 cells. This may reflect the documented instability of HMB-PP in biological fluids.

4.2.10 Vγ9δ2 Cell recruitment profile

Previous reports suggest that Vγ9δ2 cells regulate the early phase of the inflammation [75]. To determine their role in the peritoneal immune response, we utilised flow cytometry to follow their recruitment profile. Although limited, these data suggest that Vγ9δ2 cells appeared to be selectively recruited from the blood during the first few days of Gram-negative (but not Gram-positive) infection (Figure 4.14).

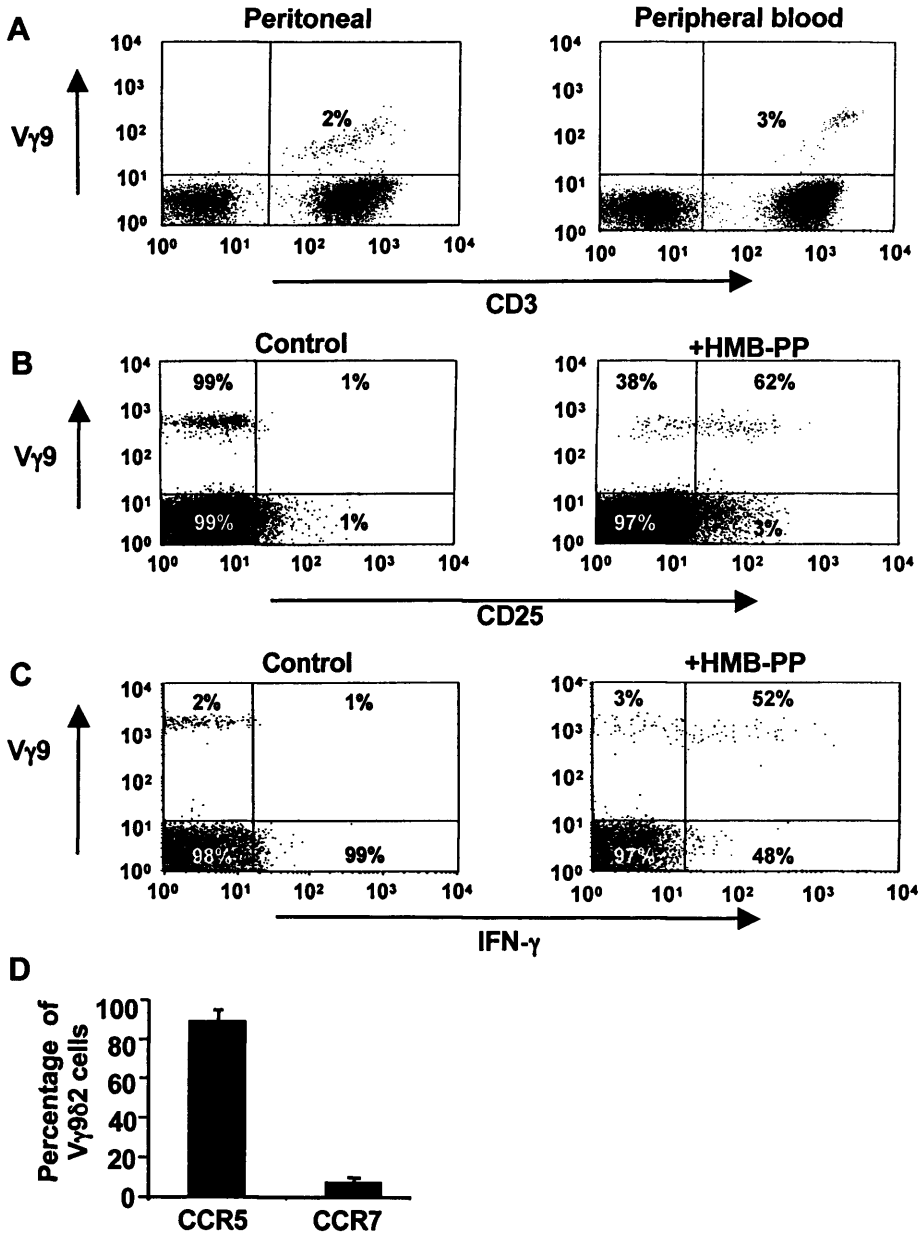

Figure 4.12 Characterisation of peritoneal Vγ9δ2 cells. (A) Paired samples of PBMC and peritoneal leukocytes were isolated from PD patients. Leukocytes were labeled with fluorescently conjugated monoclonal antibody directed against CD3 and Vγ9. Results shown as percentage of $CD3^+$ cells (representative example shown of 5 separate donors; see appendix for patient demographics.) (B) Peritoneal leukocytes were stimulated with HMB-PP(100pmol/ml) for 12 hours. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3/Vγ9/CD25. To determine the percentage of activated cells, a gate was set on either the αβ or γδ subset and the percentage of positively staining cells within this gate was calculated (representative example shown of 3 separate donors; see appendix for patient demographics). (C) Peritoneal leukocytes were stimulated for 12 hrs with HMB-PP(100pmol/ml). Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3/ Vγ9. Cells were subsequently fixed and permeabilized then stained with anti-IFN-γ. To determine the percentage of activated cells, a gate was set on either the αβ or γδ subset and the percentage of positively staining cells within this gate was calculated (representative example shown of 3 separate donors; see appendix for patient demographics). (D) Peritoneal leukocytes were labeled with fluorescently conjugated monoclonal antibody directed against CD3/Vγ9/CCR5 and CCR7. Results shown as percentage of Vγ9δ2 cells expressing CCR5 or CCR7 (mean and SE of 8 separate patients; see appendix for patient demographics).

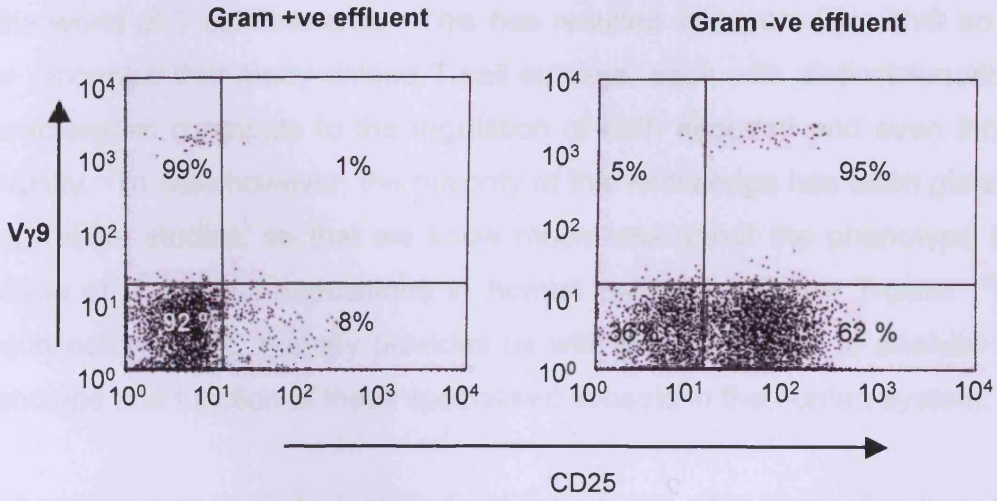

Figure 4.13 Activation of Vγ9δ2 cells by infected PD effluent. PD effluent was collected on ice during day1 of Gram + and Gram-ve peritonitis. PBMC were cultured overnight in the filtered effluent. T-cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3/Vγ9 and CD25. To determine the percentage of activated cells, a gate was set on either the αβ or γδ subset and the percentage of positively staining cells within this gate was calculated (see appendix for patient demographics).

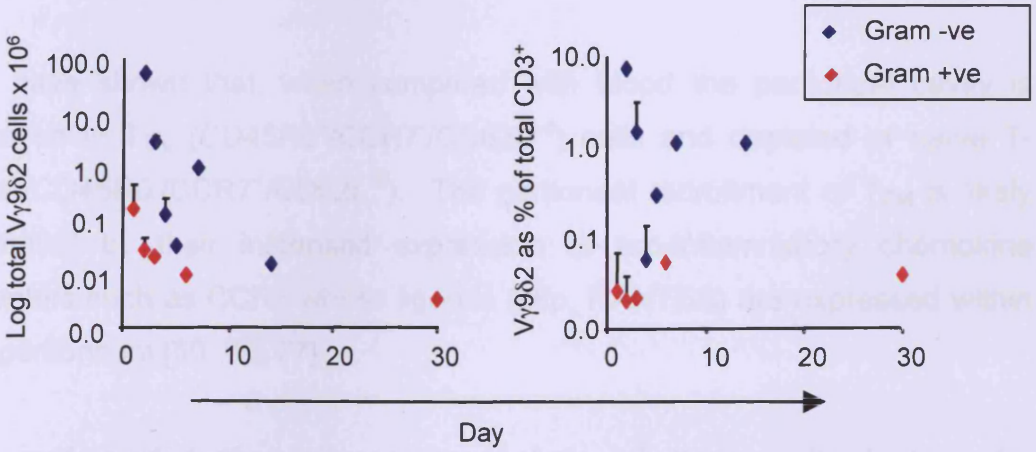

Figure 4.14 Temporal profile of Vγ9δ2 cell recruitment during peritonitis. Peritoneal leukocytes were collected during episodes of peritonitis. Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3/Vγ9 (see appendix for patient demographics).

4.3 Discussion

The past few years have witnessed numerous groundbreaking developments in the world of T-cell research. This has resulted in a paradigm shift so we now recognise that many unique T-cell subsets, each with distinct functional characteristics contribute to the regulation of both acquired and even innate immunity. To date however, the majority of this knowledge has been gleaned from murine studies, so that we know much less about the phenotype and function of these sub-populations in human peripheral tissue T-cells. The unique nature of PD therapy provides us with an opportunity to analyse the phenotype and function of these specialised subsets in the human system.

We began our analysis by looking for the presence of memory T-cells within the peritoneal cavity. The ability to generate long-lived antigen specific memory is the hallmark of the adaptive immune system. Upon re-exposure to the primary antigen, memory cells mount an immediate recall response, thus enhancing pathogen clearance. Though previous authors have suggested that peritoneal T-cells express CD45R0 (a marker of memory), these studies pre-date the current model of T-cell memory which recognises naïve/central memory (T_{CM}) / effector memory (T_{EM}) or TEMRA (terminally differentiated effector cells) subsets [59, 80].

We have shown that, when compared with blood the peritoneal cavity is enriched in T_{Em} ($CD45R0^{+}/CCR7^{-}/CD62L^{Lo}$) cells and depleted of naïve T-cells ($CD45R0^{-}/CCR7^{+}/CD62L^{Hi}$). The peritoneal recruitment of T_{EM} is likely facilitated by their increased expression of pro-inflammatory chemokine receptors such as CCR5 whose ligands (Mip, RANTES) are expressed within the peritoneum [50, 75, 77].

We performed further phenotypic analysis of these cells looking for expression of survival signals. Long-term survival is a hallmark of memory cells; it is the defining feature that distinguishes them from short-lived effector cells. This survival is antigen independent and relies on signals provided by

cytokines, in particular IL-7 and IL-15. Whereas IL-15 maintains homeostatic proliferation, IL-7 is thought to act as a survival signal, up-regulating expression of anti-apoptotic molecules [105]. Our phenotypic analysis of the peritoneal T-cells identified that CD127 (the IL-7 receptor) expression was higher on peritoneal T-cells; in particular a subset of CD45R0 cells expressed very high levels of CD127. It is likely therefore that the $CD45R0^{+}/CCR7^{-}/CD62L^{Lo}/CD127^{Hi}$ cells are the true long-lived memory cells, whereas the $CD45R0^{+}/CCR7^{-}/CD62L^{Lo}/CD127^{dim}$ group may represent short-lived effector cells. [76]. In this context it is interesting to note that previous studies have documented that stimulated HPMC produce IL-15, which suggests that the peritoneum may form a selective niche rich in survival signals that promote memory T-cell longevity [23]. The finite availability of such survival signals within the peritoneum may serve to restrict the expansion of memory cells, thus maintaining homeostasis [97, 106].

Having established the baseline phenotype of the peritoneal memory subsets we next examined their functional characteristics. In agreement with current opinion, immediate/early TH1 cytokine production was a function of the T_{EM} subset. Using a more refined, antigen specific stimulation, we were able to show that the peritoneal T_{EM} mount a TH1 recall response to standard antigens (TB, Tetanus Toxoid and Infeuenza). Of note, when compared to blood the peritoneum was enriched in cells capable of mounting a recall response. This is interesting for two reasons. Firstly it confirms that the peritoneal "memory phenotype" cells do indeed display long-term antigen-specific memory. Secondly, it gives us new insight into the trafficking mechanisms of memory cells, suggesting that cells formed at the site of vaccination migrate selectively into peripheral tissues such as the peritoneum. This may have evolved as a defence mechanisms ensuring that an immediate response is available in the periphery.

Since we lacked an *in-vitro* recall antigen to the common causes of peritonitis, we adopted an alternative strategy to determine whether peritonitis leads to local expansion of memory cells. We analysed the TCRVβ expression repertoire of peritoneal and peripheral blood T-cells isolated from PD patients.

Peritoneal T-cells from infection free or from patients with a previous history of *Staphylococcus epidermidis* peritonitis displayed similar TCRVβ expression profiles to peripheral blood T-cells. In contrast, peritoneal T-cells isolated from patients with previous *Staphylococcus aureus* peritonitis displayed some skewing of TCRVβ usage suggesting that this *pathogen* (which, unlike *S. epidermidis* produces superantigens) may promote the expansion of local long-lived memory clones. The absence of *S. epidermidis* specific memory cells, may explain in part why recurrent infection (with the same organism or the continuous presence of Coagulase negative *Staphylococcal spp.* in the bio-film lining the PD catheter) does not result in host immunity to that organism [102, 107]. Similarly the oligoclonal expansion of peritoneal T-cells triggered by *S. aureus* infection, may be responsible for the increased immune activation seen in peritonitis with this organism [54, 108].

From our analysis of peritoneal memory cell phenotype and function we can begin to predict their behaviour in the context of the peritoneal immune response. Since they lack the lymph node homing signals (CCR7 and CD62L), the peritoneal T_{EM} subset is likely to remain within the peritoneal cavity where they contribute to defence mechanisms against invading organisms. They are, in effect primed cells, responding rapidly to infection by mounting a TH1 polarised cytokine response. In contrast, T_{CM} express both CD62L and CCR7 and are likely to migrate to the peritoneal lymph tissue where they would be ideally placed to encounter antigen loaded DC (also $CCR7^{+}$). In this way T_{CM} could "screen" vast numbers of APCs for their cognate antigen. Upon re-activation T_{CM} are likely to proliferate, thus generating a wave of antigen specific effectors.

Having focused on the effector memory cell population, we next investigated the regulatory/suppressive components of the peritoneal immune response. We have now identified for the first time a T-cell subset fulfilling the phenotypic definition of T-regs existing within the peritoneal cavity of PD patients. Moreover we have shown that when compared to the peripheral circulation, T-regs are enriched in the peritoneal cavity of PD patients. The T-

reg enrichment may either represent expansion of a local population or selective recruitment of T-regs from the circulation.

We were able to demonstrate that peritoneal T-regs display potent suppressive activity and are capable of abrogating both the proliferation and TH1 (IFN-γ production) effector function of autologus peripheral blood T-cells. The mechanisms that underpin T-reg mediated suppression are complex and poorly understood. Recent *in vitro* work has suggested that suppression is cell-contact dependant, mediated via disrupting the DC: effector T-cell interaction. This contrasts somewhat with *in vivo* observations, which suggest that T-reg suppression is mediated via production of anti-inflammatory mediators, most notably IL-10 and TGFβ [74, 104]. Our own *in vitro* observations of peritoneal T-regs suggest that suppression is dependent on direct cell-cell contact with a limited role for IL-10 and TGFβ .

When considering the behaviour of peritoneal T-regs *in-vivo* we must consider that within the peritoneal cavity there is ample space for effector cells to avoid direct cell-cell contact. In view of this, we speculate that cell-cell contact mechanisms occur predominantly within the draining lymph nodes (where DC, effectors and T-regs are in close proximity) whilst secretion of IL-10 and TGFβ may maintain suppression within the cavity itself.

What role do these suppressive mechanisms play in the complex multi-faceted peritoneal immune system? The nature of PD therapy means that this system is constantly exposed to foreign antigens and inflammatory stimuli. Under such circumstances peritoneal T-regs serve to maintain homeostasis by suppressing effector cell function. During acute peritonitis, this equilibrium shifts towards a pro-inflammatory phenotype, so that the invading pathogen can be cleared. T-regs are likely to play a pivotal role in regulating the magnitude of this inflammatory response, minimising host damage, promoting immuno-resolution and preventing chronic inflammation.

Closely related to T-regs are the pro-inflammatory IL-17 producing subset (TH17). Interestingly, these two subsets with apparent divergent function are thought to derive from the same pre-cursor cell. During the steady state, the local cytokine milieu favours the formation of T-regs whereas during inflammation, the balance is shifted in favour of TH17 development [62]. Though numerous recent publications highlight the importance of TH17 in autoimmune disease, their anti-microbial role is poorly understood.

There is indirect evidence that TH17 cells may play a role in the peritoneal inflammatory response since HPMC express the IL-17 receptor and respond to IL-17 stimulation by producing C-X-C chemokines. Work from the same group has shown that intra-peritoneal injection of mice with IL-17 results in massive neutrophil accumulation [109-111]. We expanded on these observations by showing that elevated IL-17 can be detected in the PD effluent of patients suffering from peritonitis. We have also shown that TH17 cells can be detected in the PD effluent of patients who have suffered prolonged or more severe episodes of inflammation. Collectively, these findings suggest that TH17 cells may be important during the early, "pro-inflammatory" phase of the peritoneal immune response, and that their expansion may reflect underlying dysregulated inflammation which may in turn lead to a worse outcome.

The final specialised sub-set that we investigated were the Vγ9δ2 T-cells. These T-cells differ fundamentally from αβ T-cells with respect to the type of antigen recognised, mode of antigen recognition and chemokine receptor profile. Vγ9δ2 T-cell research is hampered by both the lack of appropriate animal models (Vγ9δ2 T-cells do not exist in rodents), and the lack of readily accessible human samples [75-78]. Analysis of PD effluent during both the steady state and during peritonitis has given us fresh insight into the activation and recruitment patterns of these cells.

Our data shows that Vγ9δ2 T-cells reside (in small numbers) within the non-infected peritoneal cavity. These cells retain functional activity and can be

selectively activated *in vitro* by HMB-PP. The number of intra-peritoneal Vγ9δ2 T-cells increased dramatically during the early phase (day 1-2) of Gram-negative peritonitis suggesting that they are selectively recruited into the inflamed peritoneum. This "early recruitment" of Vγ9δ2 T-cells precedes the bulk of αβ T-cell recruitment which occurs at day 5-6, suggesting that Vγ9δ2 T-cells (along with neutrophils and monocytes) may form part of the early immune response. Their recruitment is likely to be facilitated by the "pro-inflammatory" phenotype ($CCR5^+/CCR7^-$) which was displayed by the majority of circulating Vγ9δ2 T-cells, a phenotype which is shared by other "innate" system cells [75, 77].

Many of these cells display an "activated" phenotype, and though we did not measure HMB-PP levels directly, we have shown that Gram-negative effluent (but not Gram-positive effluent) selectively activates Vγ9δ2 T-cells. Though historically, Gram-positive organisms have been the most common pathogens associated with PD-related inflammation, the contribution of Gram-negative organisms such as *Pseudomonas spp.* has increased considerably over the past years. Gram-negative bacteria are well known to cause a clinically more severe infection, with higher rates of non-resolution, catheter removal, and even patient death [112, 113]. This hints to a correlation of morbidity and mortality with the potential of the causative agents to stimulate Vγ9δ2-T-cells. More recent data collected by our own group (analysing over 400 episodes of peritonitis) has confirmed this association (Figure 4.15).

Once recruited into the peritoneum, we speculate that Vγ9δ2 T-cells respond to microbial products (such as HMB-PP) in an MHC independent manner. They secrete further pro-inflammatory cytokines, amplifying the immune response before migrating to local lymph nodes as professional APCs. Within lymph nodes, Vγ9δ2 T-cells cells have the capacity to activate αβT-cells thus initiating the adaptive immune response [77]. Clearly more work is needed to confirm this hypothesis, however it is becoming clear that Vγ9δ2 T-cells (similar to peritoneal DC) are likely to form a cardinal bridge between the innate and adaptive arms of the immune response [75].

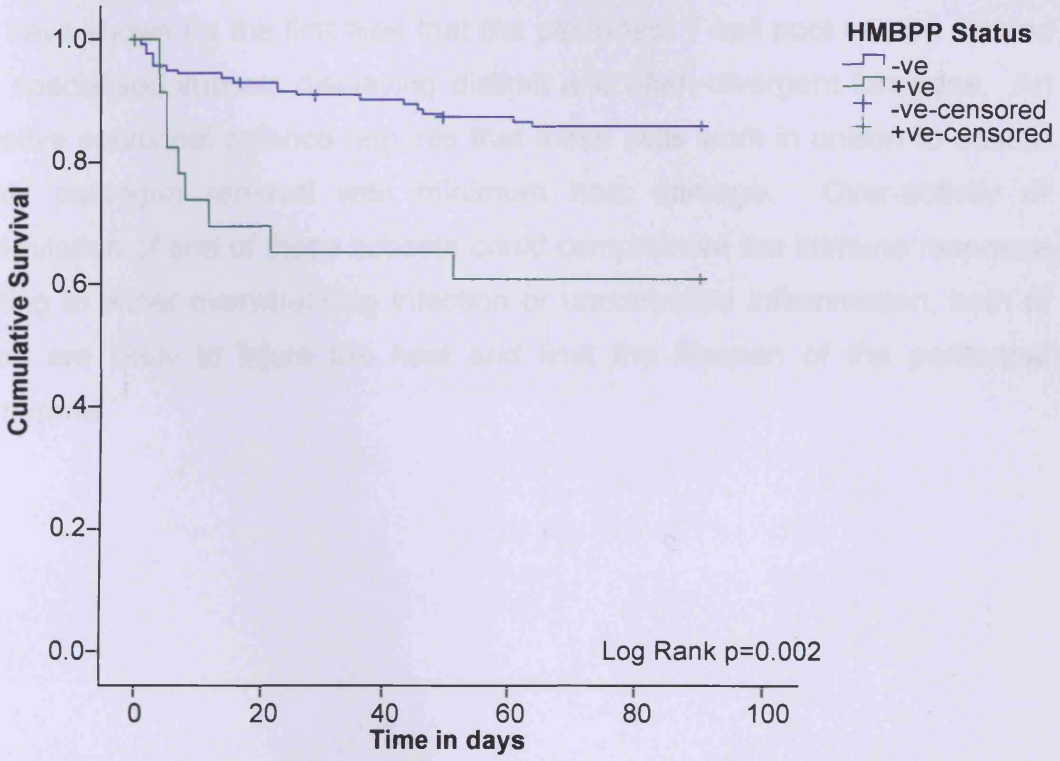

Figure 4.15 PD technique survival according to HMB-PP status of infecting organism. Data analysis based on first episode of peritonitis in 413 separate patients (Chess et al, unpublished data).

4.4 Conclusion

We have shown for the first time that the peritoneal T-cell pool can be divided into specialised subsets displaying distinct and often divergent functions. An effective peritoneal defence requires that these cells work in unison to ensure timely pathogen removal with minimum host damage. Over-activity or deregulation of one of these subsets could compromise the immune response leading to either overwhelming infection or uncontrolled inflammation, both of which are likely to injure the host and limit the lifespan of the peritoneal membrane.

Chapter 5

Peritoneal T-cell Phenotype and Function: the Impact of Recurrent Peritonitis

5.1 Introduction

Recurrent peritonitis is the Achilles heel of PD therapy and remains the main cause of treatment failure [114]. Peritoneal biopsy data shows that there is a relationship between peritonitis and membrane fibrosis, with those individuals who have suffered recurrent peritonitis likely having the greatest degree of sub-mesothelial expansion [8, 10]. This is further supported by autopsy studies demonstrating that peritonitis (but not duration of dialysis) is linked to membrane thickening [16].

We are now beginning to unravel the basic mechanisms that connect repeated infection with membrane fibrosis. It appears that recurrent infection and dialysis duration significantly alters the phenotype and function of the resident peritoneal cells, generating a chronic inflammatory milieu within the peritoneal cavity. Studies of human peritoneal macrophages have shown that recurrent infection appears to impair killing capacity and cytokine production [115]. This was believed by some authors to potentially contribute to a "burnout" phenomenon that rendered the peritoneal cavity compromised in terms of host defence. Studies by our own group have also show that recurrent episodes of peritonitis are associated with dysregulated HPMC signalling and neutrophil retention [C. Parker, unpublished]. Similar retention of inflammatory cells has been observed in the small intestine of patients suffering from Crohns disease and in the joints of patients suffering from Rheumatoid arthritis. Such aberrant leukocyte trafficking is the hallmark of chronic inflammation and is thought to be responsible for local tissue damage and fibrogenic processes [46, 47].

Since most host defence studies have concentrated on the peritoneal macrophage, we know very little about the impact of recurrent infection on the peritoneal T-cell. Data from the murine model of peritonitis suggests that infection leads to T-cell dysregulation, which in turn is a pivotal trigger for chronic inflammation [Topley, Jones unpublished data].

The aims of this chapter are:

a) Characterise the impact of infection on resident peritoneal T-cell phenotype.

b) Characterise the impact of recurrent infection on resident peritoneal T-cell function.

c) Explore possible mechanisms that link recurrent infection with altered phenotype and function.

5.2 Results

5.2.1 Impact of recurrent infection on peritoneal T-cell effector function

To analyse the impact of infection on peritoneal T-cell proliferation, we first stratified patients according to their previous infection history (no episodes, 1-2 episodes, >2 episodes). Peritoneal T-cells were isolated from these patients during infection-free periods (defined as a period of at least 3 months since their last episode of peritonitis) and stimulated *ex vivo*. As shown in Figure 5.1 multiple previous episodes of peritonitis are associated with significantly impaired *ex vivo* T-cell proliferation and IFN-γ producing capacity. A stepwise multivariate analysis revealed that only the number of previous infections (and not time on PD) was significantly correlated with peritoneal T-cell effector function ($p=0.028$ for T-cell proliferation, $p=0.001$ for IFN-γ).

We performed further experiments to determine whether the *ex vivo* T-cell changes observed in peritonitis patients are mirrored *in vivo*. To assess *in vivo* proliferation, peritoneal T-cells freshly isolated from the peritoneal cavity of infection free and previous peritonitis patients were stained immediately with antibody directed against the proliferating nuclear cell antigen Ki67. This nuclear protein is expressed by proliferating cells in all phases of the active

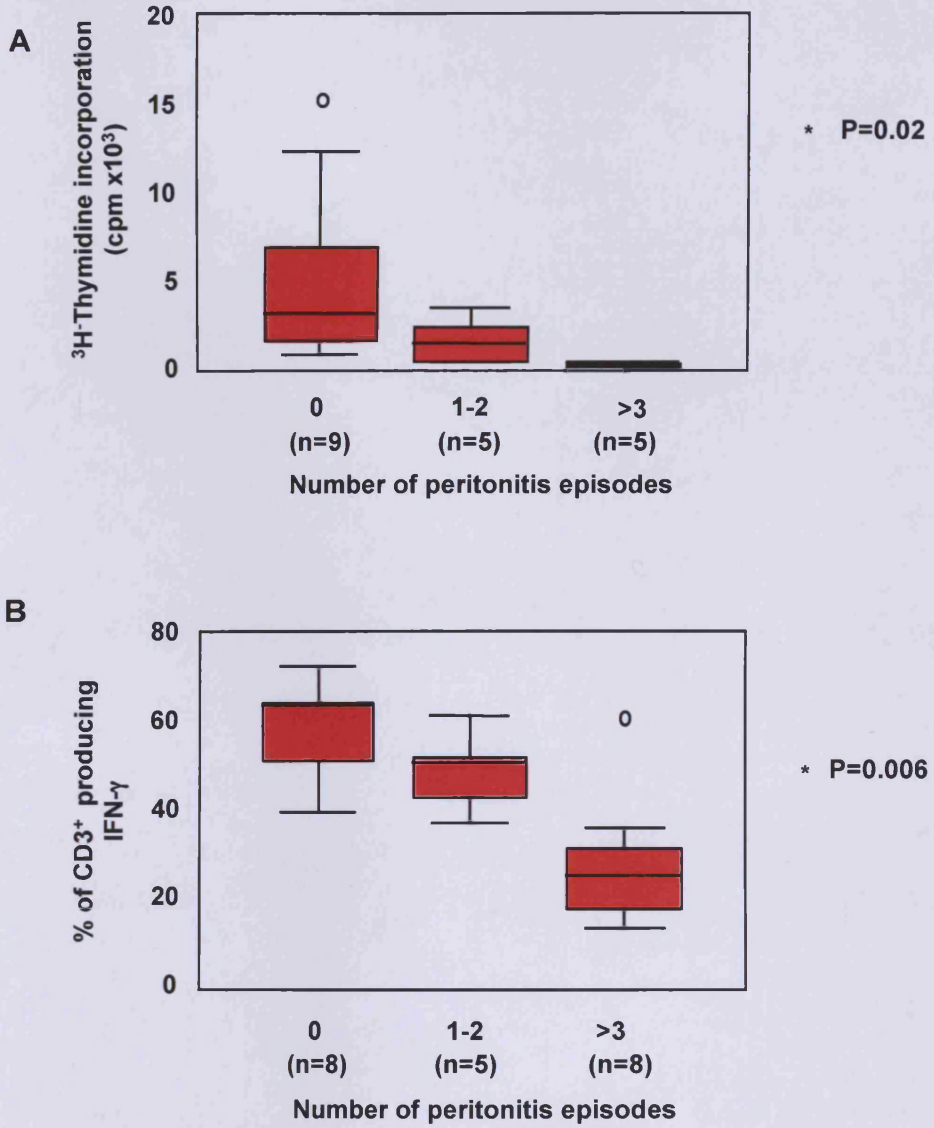

Figure 5.1. Impact of recurrent peritonitis on peritoneal T-cell effector function. Peritoneal leukocytes were collected from stable (no infection in the past 3months) patients. (A) Peritoneal T-cells (isolated by magnetic column negative selection) were stimulated with OKT3 in the presence of allogenic peritoneal APC. Proliferation (^{3}H-Thymidine incorporation) was assessed at 96h. For data analysis patients were stratified into subsets according to their previous history of peritonitis (data presented as box and whisker plot for each subset). (B) Peritoneal leukocytes ($2x10^5$/well) were stimulated with PMA (500ng/ml)/Ionomycin (50ng/ml) for 4 hours in the presence of monensin (2μM). Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3. Cells were subsequently fixed and permeabilized then stained with anti-IFN-γ. A combined gate was set on peritoneal T-cells based on CD3 expression and their FSC:SSC profiles, the percentage of CD3$^+$/IFN-γ$^+$ cells was calculated. For data analysis patients were stratified into subsets according to their previous history of peritonitis. The data for each subset is presented as box and whisker plots. Overall p values were calculated using the Kruksal-Wallis test (see appendix for patient demographics).

cell cycle but is absent in resting cells and hence serves as a surrogate marker of cell proliferation [116]. In the limited number of experiments of this type that we performed there was a significantly lower Ki67 staining in peritoneal T-cells obtained from recurrently infected patients suggesting that the observed *ex vivo* proliferative defects are manifest in the *in vivo* setting (Figure 5.2A).

To assess cytokine production *in-vivo*, we also measured PD effluent cytokine levels in these same groups of patients. As shown in Figure 5.2B, IFN-γ was present in the peritoneal cavity during steady state PD in patients without a history of infection, but was below the detection limit in patient samples from those with a history of peritonitis.

5.2.2 Impact of recurrent infection on TH1/TH2/TH17 axis

To determine whether recurrent infection caused a shift in polarisation from TH1 to TH2 or TH17, peritoneal T-cells from recurrently infected patients were stimulated with PMA (500ng/ml) and Ionomycin (50ng/ml). As shown in Figure 5.3, the decreased TH1 response was not accompanied by an increased TH2/TH17 effector response.

5.2.3 Impact of recurrent infection on peripheral blood T-cell effector functions

During the collection of peritoneal effluent samples, we also obtained paired samples of peripheral blood T-cells from a subset of these patients. When compared to peritoneal T-cells, peripheral blood T-cells obtained from recurrently infected patients retained their effector function (as evidenced by no differences in IFN–γ production between infected and never infected patients) (Figure 5.4).

5.2.4 Impact of recurrent infection on peritoneal T-cell phenotype

To analyze whether the observed alterations in T-cell function were due to changes in their global phenotype, we performed flow cytometric analysis on peritoneal T-cells obtained from the same patient cohort. We did not find any

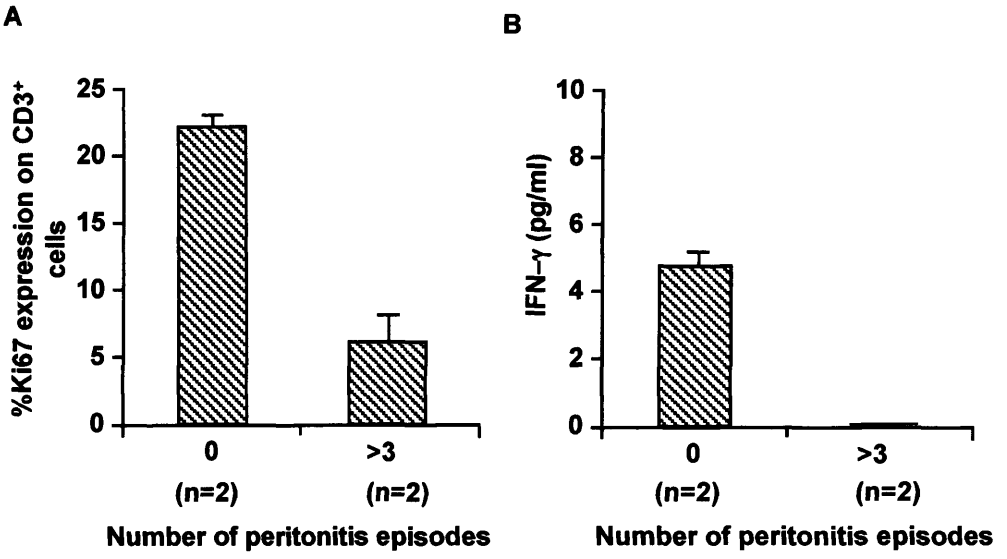

Figure 5.2 *In-vivo* effector function of peritoneal T-cells. Peritoneal leukocytes were collected from stable (no infection in the past 3 months) patients. (A) Peritoneal leukocytes were stained with monoclonal antibody directed against CD3. Cells were subsequently fixed, permeabilised and stained with monoclonal antibody directed against Ki-67. Results show the percentage of $CD3^+$ cells expressing ki67. (B) Peritoneal effluent was collected from patients during the steady (non-infected) state. IFN-γ levels were analysed with a multiplex bead-based cytokine assay. For data analysis patients were stratified into subsets according to their previous history of peritonitis (mean ±SE for each subset shown; see appendix for patient demographics).

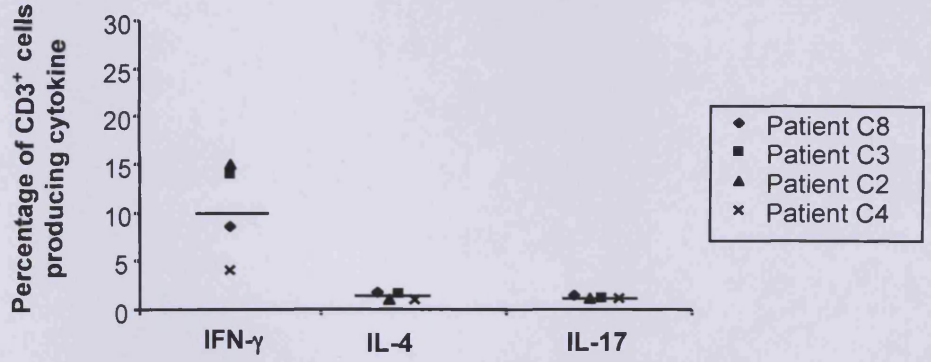

Figure 5.3. TH1/TH2/TH17 axis of peritoneal T-cells isolated from recurrently infected patients. Peritoneal leukocytes were isolated from a subset (n=4) of the recurrent peritonitis cohort. Peritoneal leukocytes ($2x10^5$/well) were stimulated with PMA (500ng/ml)/Ionomycin (50ng/ml) for 4 hours in the presence of monensin (2μM). Cells were labeled with fluorescently conjugated monoclonal antibody directed against CD3. Cells were subsequently fixed and permeabilized then stained with anti-IFN-γ/anti-IL-4/anti-IL-17. A combined gate was set on peritoneal T-cells based on CD3 expression and their FSC:SSC profiles, the percentage of cytokine producing T-cells was calculated (horizontal lines represent the mean; see appendix for patient demographics).

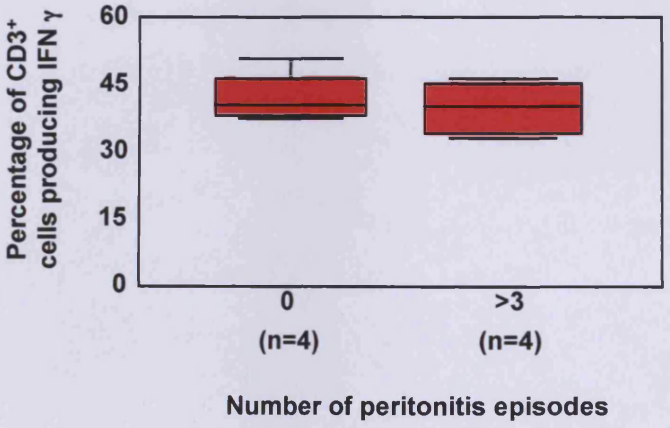

Figure 5.4 Impact of recurrent peritonitis on peripheral blood T-cell effector function. Peripheral blood was collected from a subset of patients (n=8) and PBMC were isolated by Ficoll density gradient centrifugation. PBMC ($2x10^5$/well) were stimulated with PMA (500ng/ml)/Ionomycin (50ng/ml) for 4h in the presence of monensin (2μM). Cells were labelled with fluorescently conjugated monoclonal antibody directed against CD3. Cells were subsequently fixed and permeabilized then stained with anti-IFN-γ. A combined gate was set on T-cells based on CD3 expression and their FSC:SSC profiles. The percentage of IFN-γ^+ cells was calculated. For data analysis patients were stratified into subsets according to their previous history of peritonitis. The data for each subset is presented as box and whisker plots.

significant difference in the proportion of T_{EM} nor T_{CM} in these patients (Figure 5.5). We did however find a trend towards increased T-regs within the peritoneal cavity of patients who had suffered recurrent peritonitis, though this did not reach statistical significance (Figure 5.6).

5.2.5 T-cell telomere length

To determine whether the observed changes in peritoneal T-cell function are related to cellular senescence, highly purified paired populations of peripheral blood and peritoneal $CD3^+$ T-cells were obtained by FACS. T-cell telomere length was assessed by Single Telomere Length Analysis (STELA) [88]. Peritoneal T-cell telomeres were considerably shorter than matched peripheral blood T-cells, moreover PD patients had shortened peripheral blood T-cell telomeres as compared to healthy age-matched controls. Recurrent peritonitis did not have a significant effect on peritoneal T-cell telomere length (Figure 5.7).

5.3 Discussion

Recurrent peritonitis is known to have a significant detrimental impact on technique and patient survival [9, 112, 113]. To date the impact of infection on the human peritoneal immune system is poorly described. Though some evidence suggests that macrophage function is altered [21], the effect on peritoneal T-cell function has been largely ignored. There is emerging evidence from the mouse model of peritonitis to suggest that peritonitis has a significant effect on T-cell function, with T-cells adopting a primed "active" phenotype following repeated episodes of inflammation [Topley, Jones unpublished data]. In view of these findings, we assessed the impact of recurrent peritonitis on human peritoneal T-cell phenotype and function.

Our results suggest that repeated (>2 episodes) peritonitis has a significant impact on T-cell effector capacity based on both *in vitro* stimulation protocols or more direct assessment of function in freshly isolated peritoneal T-cells.

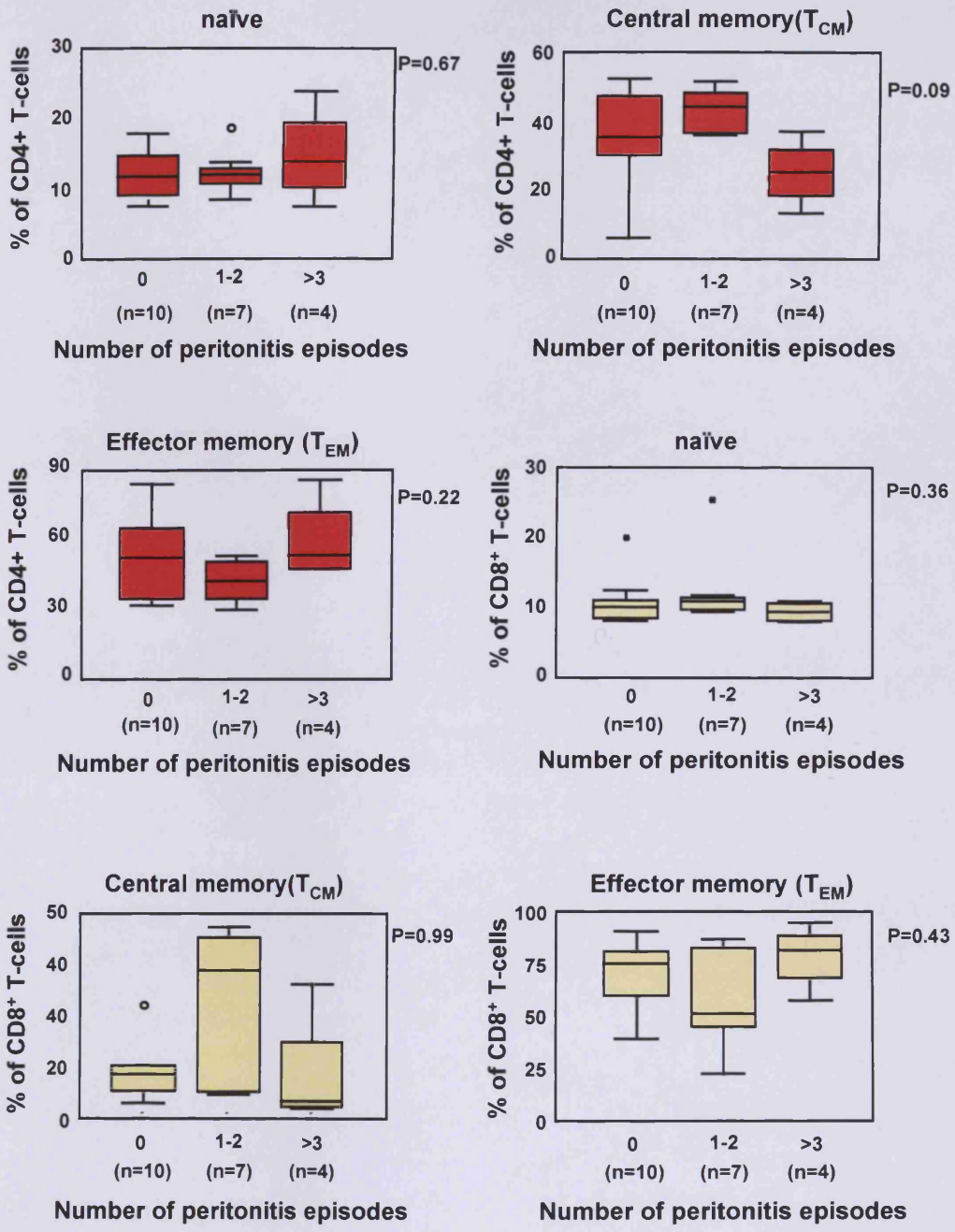

Figure 5.5 Impact of recurrent peritonitis on T-cell memory phenotype. Paired samples of PBMC and peritoneal leukocytes were collected from PD patients (n=21) and cells were labelled with fluorescently conjugated monoclonal antibody directed against CD4/CD8/CCR7 and CD45R0. A combined gate was set on peritoneal T-cells based on CD4 or CD8 expression and their FSC:SSC profiles. For data analysis patients were stratified into subsets according to their previous history of peritonitis. The data for each subset is presented as box and whisker plots. Overall p values were calculated using the *Mann-whitney test* (see appendix for patient demographics).

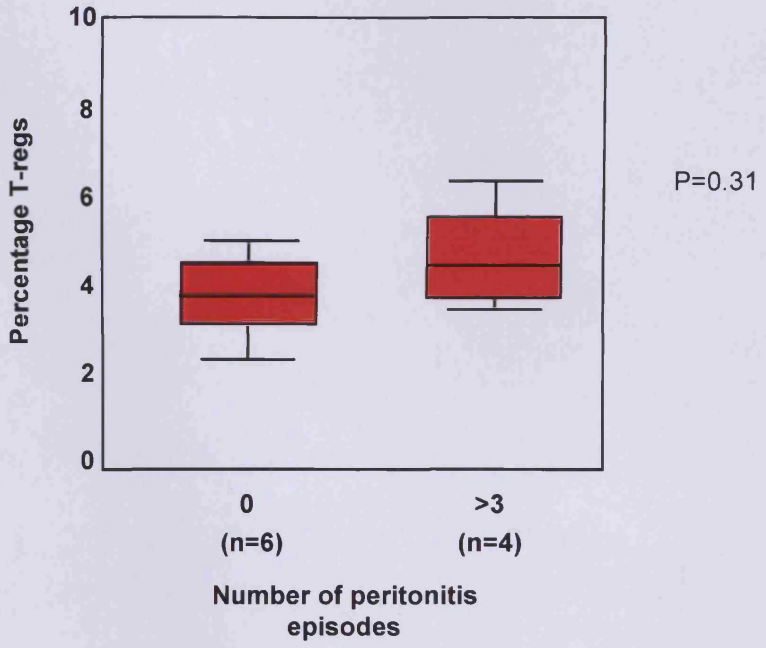

Figure 5.6 Impact of recurrent peritonitis on peritoneal T-regs. Peritoneal leukocytes were labelled with fluorescently conjugated monoclonal antibody directed against CD3/CD4 and CD25. Cells were subsequently fixed and permeabilized then stained with anti-Foxp3. A gate was set on peritoneal T-cells based on their FSC/SSC profile and CD3/CD4 expression pattern. CD25 and Foxp3 expression was examined within this gated population and the percentage of $CD4^+/CD25^+/Foxp3^+$ T-regs calculated. For data analysis patients were stratified into subsets according to their previous history of peritonitis. The data for each subset is presented as box and whisker plots. Statistical analysis was performed using the *Mann-Whitney* test (see appendix for patient demographics).

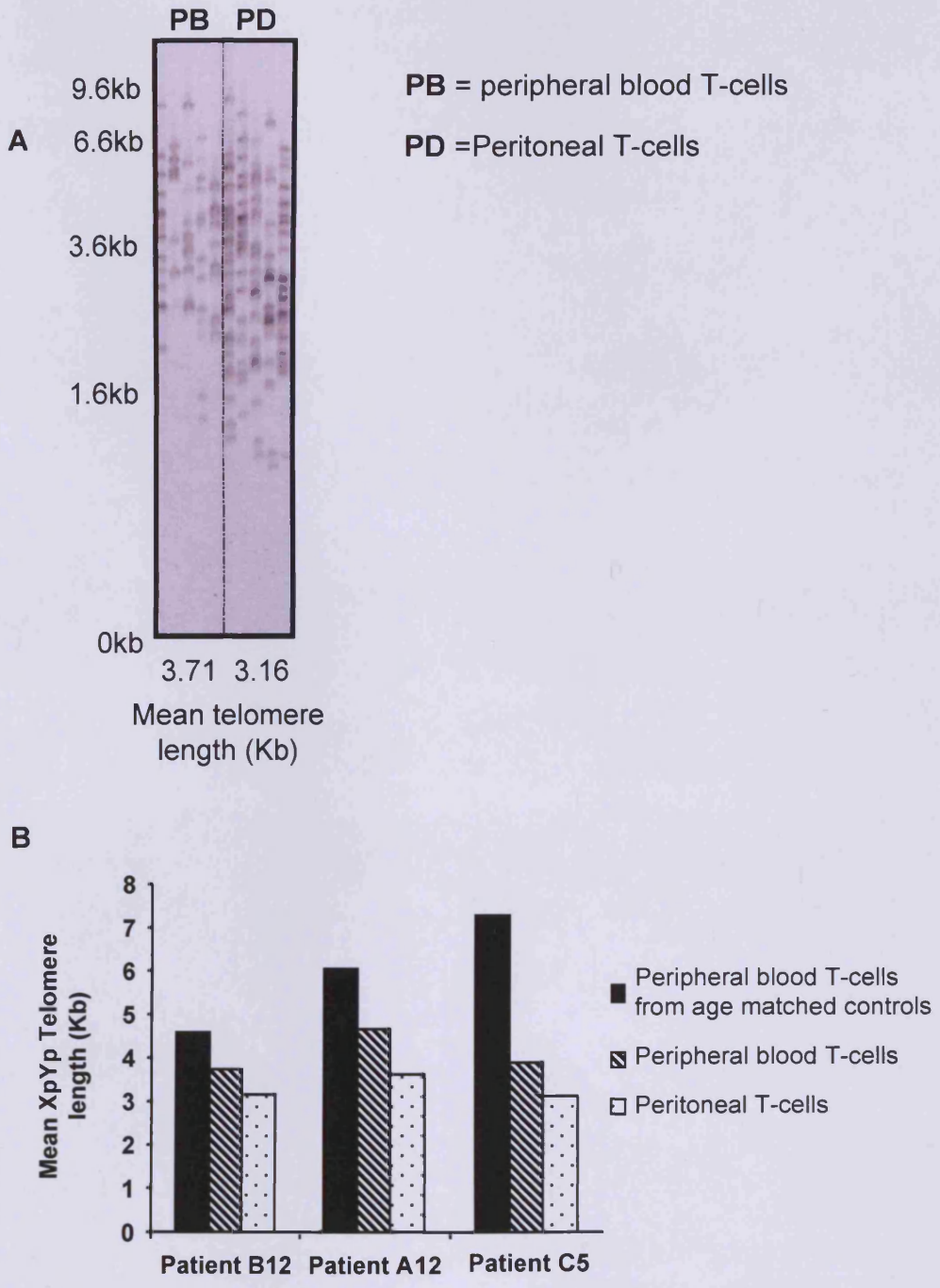

Figure 5.7 Single telomere length analysis (STELA) of peripheral blood and peritoneal T-cells. Samples of peritoneal and peripheral blood leukocytes were obtained from PD patients and healthy age-matched volunteers. Highly purified (>98%) T-cell populations were isolated by flow cytometry and cell sorting (FACS). T-cell telomere lengths were measured by single telomere length analysis (STELA). (A) Representative example of STELA data from patient C5. (B) Mean XpYp telomere lengths (see appendix for patient demographics).

We have shown that peritoneal T-cells isolated from the recurrently infected cohort had markedly decreased *in vitro* proliferation and TH1 cytokine production. In addition, analysis of Ki67 expression and effluent cytokine levels indicates that these cells are indeed potentially hypo-responsive *in vivo*.

In trying to link peritoneal T-cell function to previous infection, we must consider that there are a large number of confounding variables that might impact on the analysis. In general the longer you have been on PD therapy (vintage), the more likely it is that you will suffer from an episode of infection. In spite of this, a stepwise multivariate analysis revealed that only the number of previous infections (and not time on PD) was significantly correlated with peritoneal T-cell effector function.

Another possible explanation for T-cell dysfunction is that recurrently infected patients are a "sicker" population with a higher burden of co-morbid disease and increased systemic inflammation (e.g. elevated plasma CRP) that reflects their poor outcome on dialysis [4]. By this reasoning, recurrently infected patients would also be "systemically unwell" and hence expected to be "systemically immuno-deficient". This is not however what we observed upon analysing peripheral blood T-cells from this patient group. Peripheral blood T-cells retained their effector function, implying a local specificity of this immuno-deficient effect on T-cells localised to the peritoneal cavity. This data is perhaps not surprising given the evidence presented in this thesis for the phenotypic and functional specificity of peritoneal cavity T-cell populations.

In trying to elucidate the mechanism driving local T-cell hypo-responsiveness, we speculated that this effect of peritonitis might be related to global changes in T-cell phenotype. Within the memory subsets, the effector memory (T_{EM}) sub-sets are "less proliferative" in *vitro* than naïve or central memory (T_{CM}) cells [58]. We were not, however able to show that peritonitis resulted in a shift towards this T_{EM} phenotype. Our phenotypic analysis does hint that recurrent infection (>2) leads to intra-peritoneal accumulation of T-regs. Though our experiments did not asses the functional capacity of these T-regs, it is tempting to speculate that recurrent infection increases their inhibitory

function. Previous work in our laboratory has shown that sIL-6R levels and T-cell IL-6R expression are significantly decreased in patients with a past history of peritonitis (C Parker, MD thesis). As previously described, IL-6 signalling may be critical to T-cell effector function by allowing cells to overcome T-reg suppression. Consequently, defective IL-6 signalling in the recurrently infected cohort may contribute to T-cell anergy by preventing the "T-reg release" phenomenon [117]. This facet of T-reg function is currently under investigation.

It has been previously suggested in studies of PMØ that the continuous drainage of cell from the peritoneal cavity of PD patients contributes to significant alterations in phenotype and function. This was believed by some authors to potentially contribute to a "burnout" phenomenon that rendered the peritoneal cavity compromised in terms of host defence [21]. In the context of these studies we also hypothesised that recurrent peritonitis may be driving peritoneal T-cells towards cellular senescence so that they are no longer responsive to re-stimulation. To further assess this we collaborated with Dr. Duncan Baird (Department of Pathology, School of Medicine, Cardiff University) to measure peritoneal T-cell telomere lengths.

In the past, analysis of telomere lengths in peritoneal T-cells would have been extremely difficult. The most widely used approach, terminal restriction fragment (TRF) analysis suffers from a low overall sensitivity and requires large cell numbers, making it unsuitable for analysis of the small populations such as the peritoneal T-cells [118]. Another widely applied method quantitative fluorescence *in situ* hybridization (Q-FISH) requires metaphase chromosomes which restricts analysis to cells that are still proliferating [119]. Again this may be unsuitable for poorly proliferating peritoneal T-cells. More recently, a technique known as single telomere length analysis (STELA) has emerged which overcomes the problems highlighted above. STELA is a robust, PCR-based approach that accurately measures the full spectrum of telomere lengths from individual chromosomes [87, 88]. Using STELA we were able to compare the telomere lengths between purified peripheral blood and peritoneal T-cell populations.

We observed that peritoneal T-cells from both infection free and recurrently infected patients had shortened telomeres compared to peripheral blood, with some telomere lengths approaching the senescent range (1-2Kb). This significant difference between blood and peritoneal cells can be explained by two mechanisms. Either the blood and peritoneal pools are mutually exclusive, undergoing cell division at separate rates, or the peritoneum may selectively recruit cells which have already undergone numerous cell divisions i.e. antigen experienced cells (e.g. memory cells). In truth, both of these mechanisms probably occur to some extent. Clearly there is communication and transmigration between the two compartments (as proven by our memory cell recall response data), however the T-cells with very short telomere lengths do not appear to exist in the peripheral blood. These may represent a true "resident" T-cell population that have undergone multiple cell divisions locally within the PD cavity. In order to survive within the peritoneal cavity, these cells must avoid being "flushed out" with each PD exchange; this is likely to be achieved by migrating to local peritoneal lymphatic tissue such as the milky spots. Within these APC enriched zones the T-cells are likely to receive TCR stimulation resulting in localized peritoneal T-cell proliferation. Following proliferation these cells are released into the peritoneal cavity and may represent the actively proliferating cells detected with Ki67 staining.(see figure 5.8).

The shortened telomere lengths may explain in part the generally lower responsiveness between peripheral and peritoneal cells as some of the latter may have reached cellular senescence.

We also observed that, when compared with healthy age-matched controls, our PD patients had considerably shortened peripheral blood T-cell telomere length. This telomere shortening may be secondary to the increased inflammation and immune stresses that these patients have faced during the development of end stage renal failure. Similar telomere shortening has been observed in lymphocytes obtained from pre-dialysis and HD patients, suggesting that renal failure *per se* may play some role in the process [120].

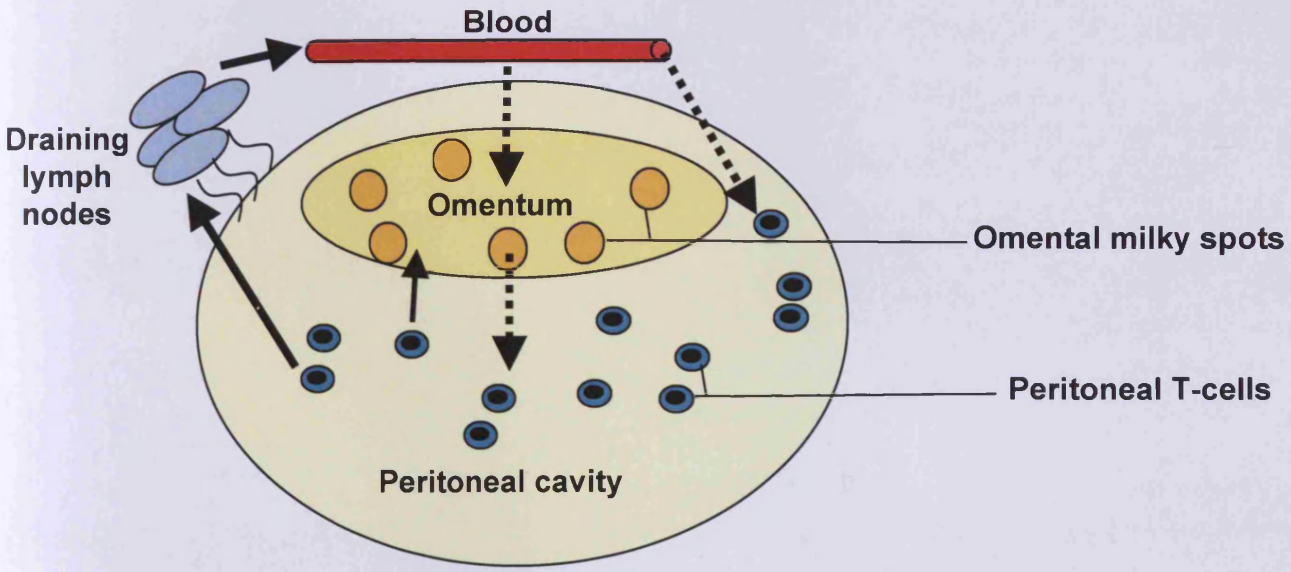

Figure 5.8 Peritoneal T-cell recruitment and re-circulation. T-cells are recruited from the peripheral blood into the peritoneal cavity. T-cells re-circulate through peritoneal lymph nodes and omental milky spots.

This may have prognostic implications for PD patients, since previous population based studies have shown that in a "normal", healthy cohort, shortened peripheral blood telomere lengths are associated with increased mortality. Of note, individuals with shorter peripheral blood telomeres have a three-fold increase in cardiovascular mortality and an eight fold higher mortality from infectious diseases [121].

In this limited analysis there were however, no significant difference in telomere lengths between the patient groups and thus we cannot conclude that cellular senescence is the major reason for the decreased peritoneal T-cell function seen following recurrent peritonitis. These observations clearly need to be followed up in a larger cohort and its relationship to other parameters such as time on PD, PD regimen, biocompatibility of fluid established.

Though the exact cause of peritoneal T-cell anergy (defined for these purposes as a state of unresponsiveness to further stimulation) remains poorly defined and is likely multi-factorial and variable between individuals, we can predict that it will have profound effects on the host immune system. Firstly such patients are "intra-peritoneally" immuno-suppressed which puts them at risk of subsequent infection. We have in effect a downward spiral whereby infection leads to T-cell anergy, which in turn leads to further infection. Secondly, the impaired TH1 response is likely to affect immuno-resolution. Previous work in our laboratory has shown that recurrent peritonitis is associated on-going production of IL-8 and continued neutrophil recruitment during the steady state [47, 52]. We also know that IFN-γ (together with IL-6) is a pivotal trigger that switches off IL-8 production. Impaired IFN-γ production by resident T-cells may thus contribute to continued IL-8 production and on-going neutrophil recruitment [47, 52]. We now have some murine data to support this; IFN-γ deficient mice clear peritoneal *Staphylococcal infection* much more slowly than wild type mice, which is paralleled by impaired neutrophil trafficking [Topley, Jones, unpublished data].

5.4 Conclusion

These data suggest that recurrent infection has a negative impact on peritoneal T cell effector function. Peritoneal T cells isolated from patients who have suffered multiple episodes of peritonitis display impaired proliferative capacity and TH1 effector function. The impaired TH1 function is not the result of a switch in T-cell polarisation as the cells also display impaired IL-4 and IL-17 production. Although peritoneal T-cells have shortened telomere lengths, this is not apparently related to recurrent peritonitis but may nevertheless confer a more general susceptibility to infection. Regardless of the mechanism, the impaired T-cells responses are likely to result in both increased risk of infection and impaired immuno-resolution, both of which will likely contribute to membrane damage and treatment failure.

Chapter 6
General Discussion

Since its introduction more than 30 years ago, PD has been beset by infectious complications. Despite improvements in connection techniques (particularly the introduction of disconnect Y-set systems) and patient education, peritonitis rates remain unacceptably high and still contribute significantly to treatment failure. More worryingly over the past decade there has been a shift in the spectrum of organisms causing infection with an increased incidence of Gram-negative peritonitis which usually has a worse clinical course and is associated with higher mortality and technique failure [112, 113] (Table 6.1).

Species	% Non-resolved infections	% Catheter removals	% Patient deaths
Gram-positive	12.7	4.8	1.4
Staphylococcus, Streptococcus	12.7	4.7	1.4
Gram-negative	36.4	16.6	3.7
E. coli, Enterobacter	25.0	5.8	2.9
Pseudomonas, Serratia, Klebsiella	62.5	37.0	7.4

Table 6.1 Morbidity and mortality in bacterial peritonitis [112, 113].

It is apparent that for PD to thrive as a treatment modality, we must develop a better grasp of the basic disease processes that lead to structural and functional changes in the peritoneal membrane. In particular we need to understand the fundamental processes that link repeated inflammation with tissue fibrosis and treatment failure.

To date, most studies of the peritoneal immune system have focussed on the early stages of peritonitis, the biology and phenotype of the peritoneal

macrophage and the role of the peritoneal mesothelial cells in regulating peritoneal immunity and responses to infection [33-41]. Although these analyses have provided significant insight into our understanding of the peritoneal immune responses, to date the contribution of the peritoneal T-cells to the processes that regulate inflammation and resolution have received much less attention particularly in studies of PD.

Recent years have seen an exponential increase in the number of publications based on T-cell research. This has largely been fuelled by the interest in the newly identified subsets such as T-regs, TH17 and memory cell phenotypes. Most of this research centres on animal models of disease so that there continues to be a paucity of human data, especially data derived from human peripheral tissue T-cells. The peritoneal cavity of PD patients, with its continuous access (via the indwelling catheter) presents a unique opportunity to study human T-cell phenotype, activation status and contribution to local immunity during both stable dialysis and during episodic infection. The aim in undertaking this research was to develop a better understanding of the basic phenotype and reactivity pattern of peritoneal T-cells so that the impact of recurrent peritonitis on the behaviour of these cells could be assessed in the context of global peritoneal immunity.

Previous studies of peritoneal T-cells have been somewhat contradictory with some suggesting that the cells are primed and activated while others suggest that they are anergic and unlikely to respond to stimulation [8, 45-48, 70, 104, 114, 121, 136]. In view of this we commenced our studies by analysing the activation status and reactivity profile of resident peritoneal T-cells. Our studies revealed that although peritoneal T-cells expressed markedly increased levels of CD69, this was not matched by a corresponding increase in CD25. Indeed, the small increase in peritoneal CD25 levels is likely due to the expression of this molecule by T-regs rather than by activated T-cells. From this we can conclude that $CD69^{+}$ peritoneal T-cells are not completely activated, a factor that suggests that either they are in a state of partial activation or they represent the peritoneal equivalent of the recently described

$CD69^+$ "adaptive regulatory T-cells" which are characteristic of chronic inflammatory diseases such as lupus or RA [96].

We next investigated the reactivity of these cells, concentrating first on their cytokine expression profile. We have shown that during both the steady state and during peritonitis, the peritoneal T-cells are skewed toward a TH1 polarity. The TH1 response generated serves a dual purpose, not only does it enhance macrophage bacterial clearance, it also promotes immuno-resolution by promoting neutrophil apoptosis through IL-6/sIL-6R dependent mechanisms [47, 52]. This localised TH1 bias is likely to be driven by peritoneal DC derived signals such as IL-12 [26, 27, 55].

The influence of DC on T-cell reactivity was further emphasised by our analysis of peritoneal T-cell proliferation. We were able to demonstrate that the proliferation of peritoneal T-cells cells (*in vitro*) requires co-stimulation from APC [26, 27, 55]. This co-stimulation occurs via both cell-cell contact dependant mechanisms and also via the production of soluble mediators which. This intimate dependence on APC was in contrast to peripheral blood T-cells that appear much less reliant on APC for their proliferative responses *in vitro*. Thus we conclude that peritoneal T-cells have a much higher activation/proliferation threshold than peripheral blood T-cells. This may serve as a control mechanism to limit activation of these cells in the peritoneal cavity.

Having focussed on peritoneal T-cells obtained during infection-free PD, we next examined the proliferative capacity of peritoneal T-cells isolated during peritonitis. The complex mechanisms that govern effector T-cell clearance during acute inflammation are poorly defined, however it is accepted that effector cells have a propensity to survive and that elimination of these cells is an instructional rather than a default process [97, 106]. Our analysis of peritoneal T-cell:APC interaction during peritonitis has provided us with further insight into the regulation of this process. We discovered that peritoneal APC inhibit proliferation and induce apoptosis in the recruited T-cell population. This mechanism may serve to promote immuno-resolution, minimising host

damage by preventing the accumulation of highly activated but now redundant effector cells.

Having established their reactivity profile, we next investigated whether the peritoneal T-cell pool has a memory component. Antigen specific memory defines the adaptive immune response, protecting the host during re-exposure to previously encountered foreign antigen. Our results have clearly demonstrated that the peritoneal cavity is enriched in cells displaying an effector memory phenotype. This observation is in agreement with the current paradigm of T-cell memory and is likely explained by the pro-inflammatory chemokine receptor profile expressed by this subset of cells [79]. Our functional analysis showed that these memory cells mount a potent TH1 skewed recall response to Tuberculosis, Influenza antigen and Tetanus Toxoid. This implies that circulating memory cells are selectively recruited into the peritoneal cavity from their sites of formation, forming a first line of defence at peripheral sites. In addition, our analysis of the TCRVβ repertoire suggests that *Staphylococcus aureus* peritonitis may result in the local polyclonal expansion of peritoneal cells, so that at least some memory may be formed locally.

Further insight into the dynamics of T-cell trafficking was obtained from our analysis of T-cell telomere lengths. Though the term "resident peritoneal cell" is often used to describe cells obtained from PD effluent, this term is misleading since a T-cell recruited from the blood into the cavity then drained off minutes later would still be classed as a resident peritoneal cell. It could be argued that resident peritoneal T-cells do not in fact exist, and that cells obtained in PD effluent are continually trafficking between blood, PD fluid and peritoneal lymphatics. Until now it has been very difficult to disprove this argument since cell labelling studies may be logistically difficult to perform in humans. Our telomere data allows us to argue that there may in fact be a truly resident peritoneal T-cell population that resides and divides locally within the peritoneum. The evidence supporting this comes from the finding that T-cells with very short telomere lengths were found only in the peritoneum but not in the peripheral circulation. Had these cells been

recruited from the blood then we would have seen a corresponding telomere band in the peripheral blood, but this is not in fact the case. These cells are likely to have entered the peritoneum from the blood, and undergone further cell division locally. They are "truly resident" cells, which may avoid being drained off by adhering to the peritoneal membrane or trafficking to local lymph tissue. Thus we conclude that "resident peritoneal T-cells" are likely to consist of a mixture of truly resident cells that remain within the local environment and cells that are constantly re-circulating between blood, peritoneum and draining lymph nodes. It is tempting to speculate that the local cells (with shorter telomere lengths) represent the long-lived effector memory pool whereas the re-circulating cells are central memory or naïve cells.

From our studies it is becoming clear that the peritoneal T-cell pool contains a sophisticated functional effector arm capable of initiating/propagating a potent immune response. This response must be tightly controlled, since an overactive response will likely result in host damage. The human body has developed numerous strategies that serve to regulate this process and minimise self-harm. Chief amongst these regulatory mechanisms are the specialised T-cell subset known as T-regs [72-74]. Though most T-reg studies have focused on the role that they play in autoimmune diseases, there is now an abundance of data to suggest that they also regulate host responses to infection. This is exemplified by the analysis of murine *Pneumocystis carinii* infection. Infection of RAG2 mice (which are deficient in T and B cells) normally results in very little tissue damage. Concomitant transfer of effector T-cells into this population results in a florid pneumonitis rapidly followed by death. Transfer of T-regs can prevent this pneumonitis by dampening down the immune response and restoring homeostasis [122, 123].

We have shown for the first time that the human peritoneal cavity is specifically enriched in $Foxp3^+$ T-regs that display potent suppressive function mediated via cell-cell contact and (to a lesser extent) via production of soluble mediators (IL-10 and TGFβ). We speculate that during the non-infected state, peritoneal T-regs maintain the integrity of the host tissue by minimising non-

specific immune responses to innocuous antigens or host flora. This suppression is likely to occur via production of IL-10 and TGFβ within the cavity itself (where direct cell-cell contact is less likely), while cell-cell contact suppression may occur within the draining nodes where T-regs, effector leukocytes and DC are in close proximity. In this way T-regs are critical in maintaining homeostasis. This contrasts somewhat with the situation that occurs during peritonitis, where T-reg activity may be seen as counterproductive, preventing the host from mounting an adequate immune response, in effect protecting the pathogen. To prevent this, DC derived cytokines (in particular IL-6) allow effector T-cells to overcome T-reg suppression and amplify the inflammatory response [117].

Given the likely importance of peritoneal T-regs as "*peace keepers*" within the pro-inflammatory milieu of the peritoneal cavity, it is evident that any manipulation in the function of these cells may have consequences for the host. Strategies aimed at enhancing T-reg number or activities have shown promise in reducing chronic inflammation in animal models [124]. Conversely, strategies that deplete the T-reg population are likely to exacerbate any underlying inflammation. This would be a clear benefit in oncology where T-reg suppression may be protecting the tumor from the host immune defences [124]. T-reg depletion may also inadvertently occur with commonly used immunosuppressive regimens. Since T-regs are highly dependant on IL-2 for their function and survival, treatments that target effector T-cells via this pathway (e.g. anti-CD25 and calcineurinin inhibitors) may inadvertently disable T-regs [125, 126]. Here we can speculate as to the pathogenesis of post transplant encapsulating peritoneal fibrosis. This paradoxical increase in peritoneal inflammation and fibrosis in spite of immunosuppression may reflect selective peritoneal T-reg depletion, which unmasks a "primed" pro-inflammatory peritoneal response.

Closely linked to T-regs, but displaying divergent function are the highly pro-inflammatory TH17 sub-set. The role of TH17 cells in regulating the host response to microbial infection remains poorly defined. This is surprising

given that most authors agree that the TH17 lineage evolved to manage infections not dealt with adequately by the TH1/TH2 responses [62-65].

TH17 development is driven by IL-6 and TGFβ. Since both these cytokines have been identified in the inflamed peritoneal cavity we hypothesised that TH17 cells would also be present in the inflamed peritoneum [127, 128]. Of the fifteen patient effluent samples analysed using flow cytometry we were only able to detect a convincing population of TH17 cells in two, both of whom had suffered prolonged or severe peritoneal inflammation. That we were not able to detect these cells in the majority of peritonitis patients suggests that they are subject to tight regulation presumably aimed at limiting both their number, activity and their potential for host damage. We speculate that prolonged or severe peritoneal inflammation (particularly with *S aureus* or Gram-negative organisms) leads to a breakdown in this equilibrium leading to the persistence of TH17 cells and on-going inflammation

Although we had difficulty isolating these cells *ex vivo*, the elevated IL-17 levels detected during peritonitis would suggest that these cells might be present (albeit in very low numbers) at an early phase of peritonitis. During this early phase they may promote neutrophil recruitment, as evidenced by previous studies demonstrating that IL-17 induces CXC chemokine production by HPMC [109, 111].

The final peritoneal T-cell subset that we investigated was the Vγ9δ2 subset (the so called gamma/delta T-Cells). These are a unique T-cell population similar in many respects to activated DC [75-78]. Previous studies have suggested that, unlike conventional αβ T-cells, Vγ9δ2 T-cell participate in the early "innate" immune responses. In accordance with this, we have shown that Vγ9δ2 T-cells are recruited early during the course of peritonitis, peaking at day1-2 (as compared to αβ T-cells that peak between days 4-6 depending on infection type). We were also able to show that these cells are selectively activated by HMB-PP, a highly conserved metabolic by-product produced by most Gram-negative species (but not *Staphylococcal spp.*). This observation

might explain why effluent from Gram-negative patients is able to activate Vγ9δ2 T-cells.

Since Vγ9δ2 T-cells have potent APC function we predict that similar to DC, they form a pivotal link between the innate and adaptive arms of the peritoneal immune response. Elucidation of the role of these cells in Gram-negative infection is of very significant importance given the high morbidity and mortality associated with these types of infections in PD patient [112, 113].

Data presented in this thesis has shown that the peritoneal T-cell population is a heterogeneous mixture of specialised subsets, with unique but interacting effector functions. These cells must exist in a controlled equilibrium in order to both maintain homeostasis and mount an effective immune defence. A paradigm of peritoneal inflammation incorporating our data on the phenotype and reactivity patterns of peritoneal T-cells is outlined in Figure 6.1-6.4.

We concluded our studies by assessing what impact recurrent infection had on the peritoneal T-cell population. Peritoneal T-cells isolated in the steady state from patients who had suffered >2 episodes of peritonitis showed markedly impaired *ex vivo* proliferation and effector function and appeared to have reached a state of adaptive tolerance (*in vivo* anergy). We speculate that increased T-reg numbers and function (possibly enhanced by the loss of IL-6 signalling) contribute to this anergic state, other plausible explanations such as impaired DC signalling remain unexplored and may also play a significant role.

Whatever the exact mechanism, this peritoneal T-cell anergy is likely to contribute to a state of immuno-suppression thus rendering the patients likely

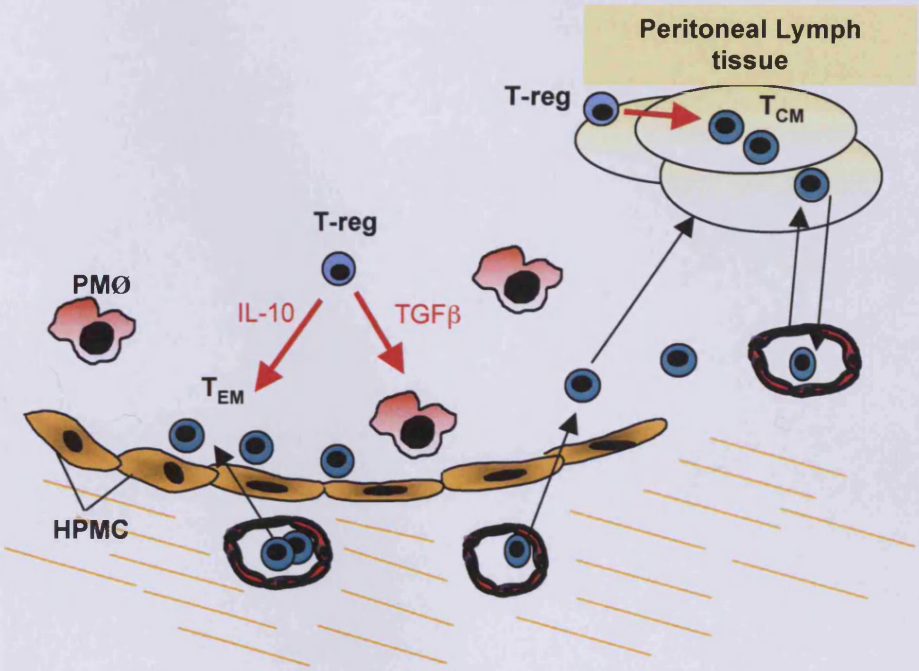

Figure 6.1 Peritoneal immune system homeostasis. Effector memory T-cells reside primarily within the peritoneal cavity, maintained by local anti-apoptotic and proliferative signals (IL-7 and IL-15). Central memory cells re-circulate through the peritoneal lymphatic system "patrolling" for antigen loaded DC. T-regs maintain homeostasis via production of suppressive cytokines within the peritoneal cavity and via contact mediated suppression within the lymph nodes.

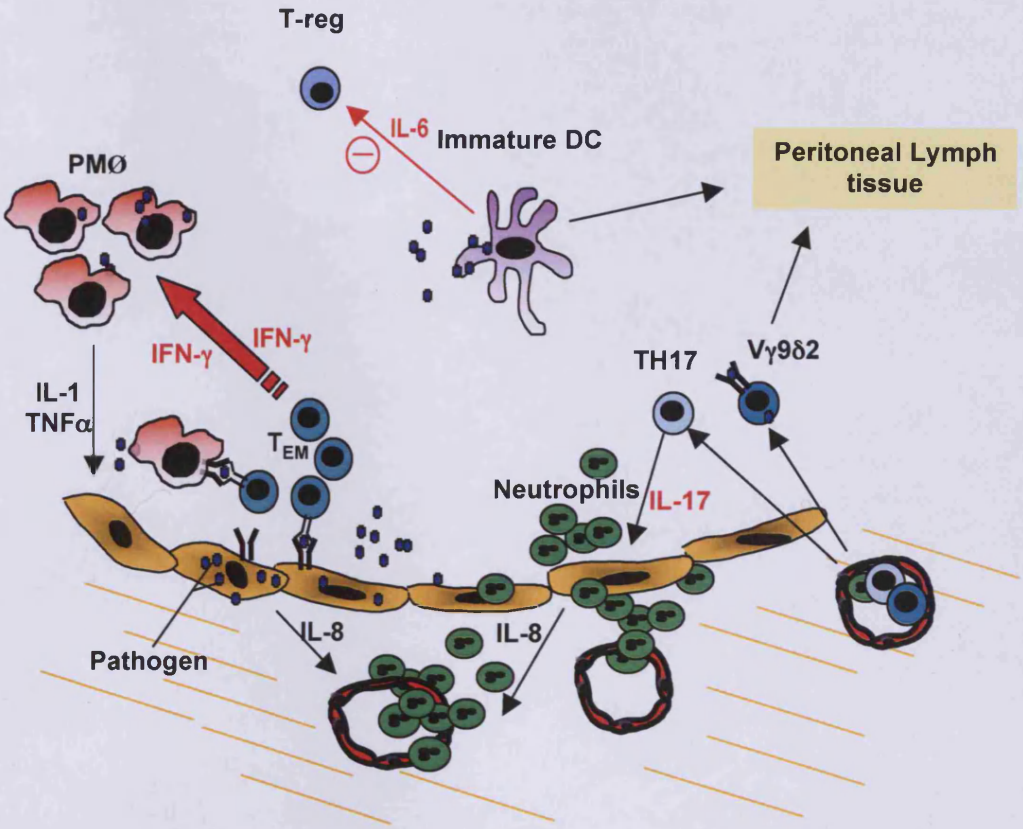

Figure 6.2 Early phase of peritonitis. Invading pathogens are ingested by phagocytic cells, including peritoneal macrophages (PMØ) and HPMC. Pathogen derived peptides are displayed on MHC class II molecules to the "resident" effector memory population. Antigen recognition is followed by an immediate TH1 polarised cytokine response. The IFN-γ thus generated stimulates macrophage bacterial killing. Concurrently HPMC derived cytokines lead to recruitment of "early phase" cells including neutrophils, Vγ9δ2 T-cells and TH17 cells. Interleukin17, IL-1 and TNFα promote further neutrophil recruitment by inducing HPMC production of CXC-chemokines. Immature peritoneal DC and Vγ9δ2 T-cells are activated and migrate to local lymph nodes as "professional" APC. DC and HPMC derived IL-6 serves to inhibit T-reg suppression allowing inflammation to proceed.

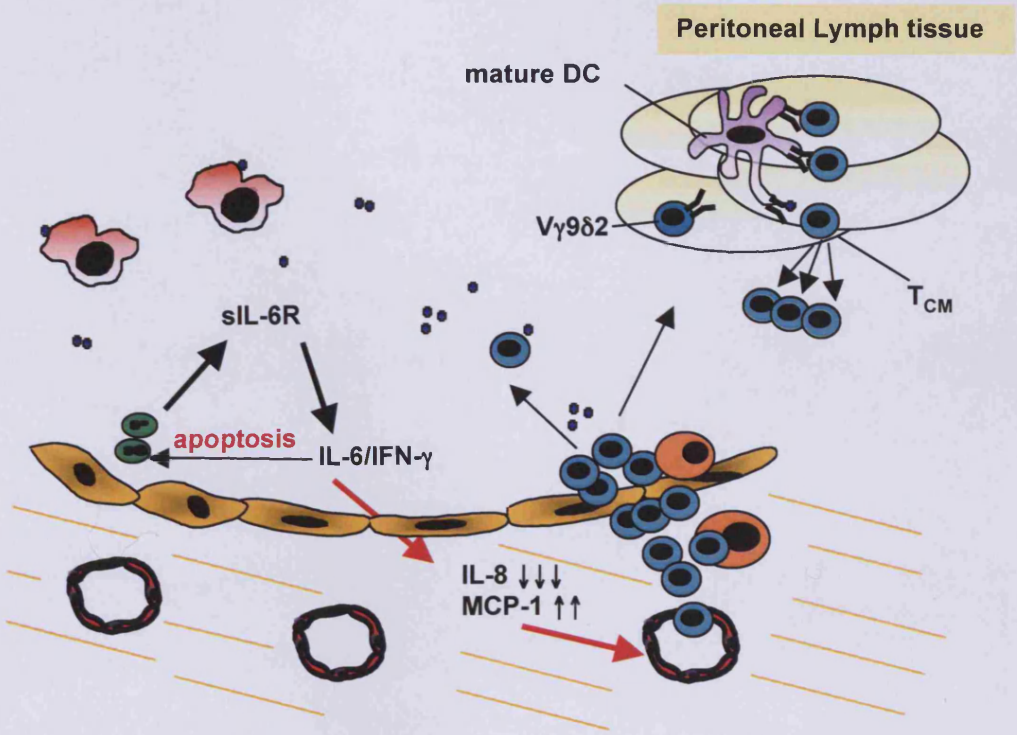

Figure 6.3 Late phase of peritonitis. IL-6 trans-signalling orchestrates a switch from neutrophil to mononuclear cell recruitment. Recruited T-cells are activated by peritoneal APC including macrophages, DC and HPMC. Activated APC (DC and Vγ9δ 2) present antigen to central memory T-cells within the peritoneal lymph nodes, thus generating a wave of antigen specific effectors.

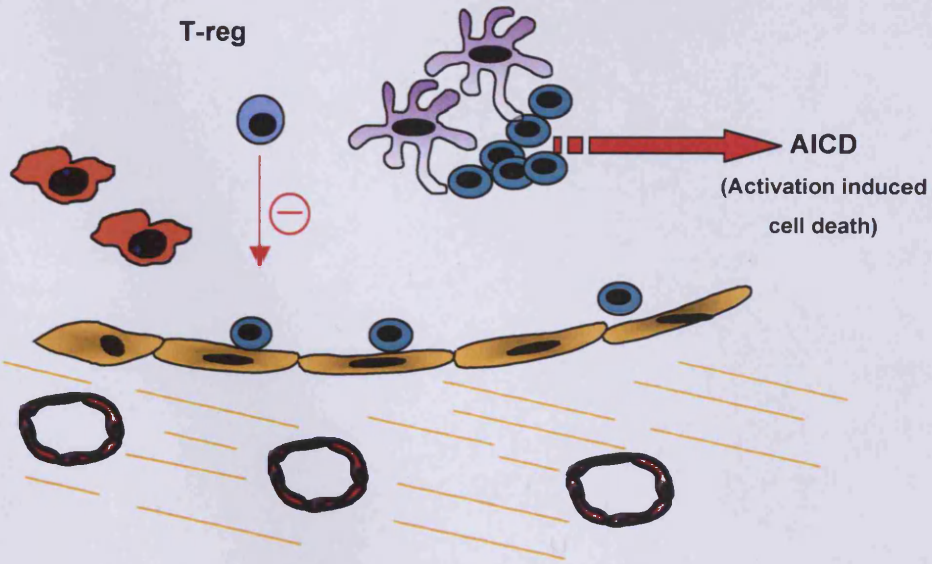

Figure 6.4 Immuno-resolution. Following prolonged APC stimulation, recruited T-cells undergo activation induced cell death. Cells that survive this process remain in the peritoneum as long-lived memory cells. As inflammation subsides, IL-6 levels falls, thus unmasking peritoneal T-reg suppression and restoring homeostasis.

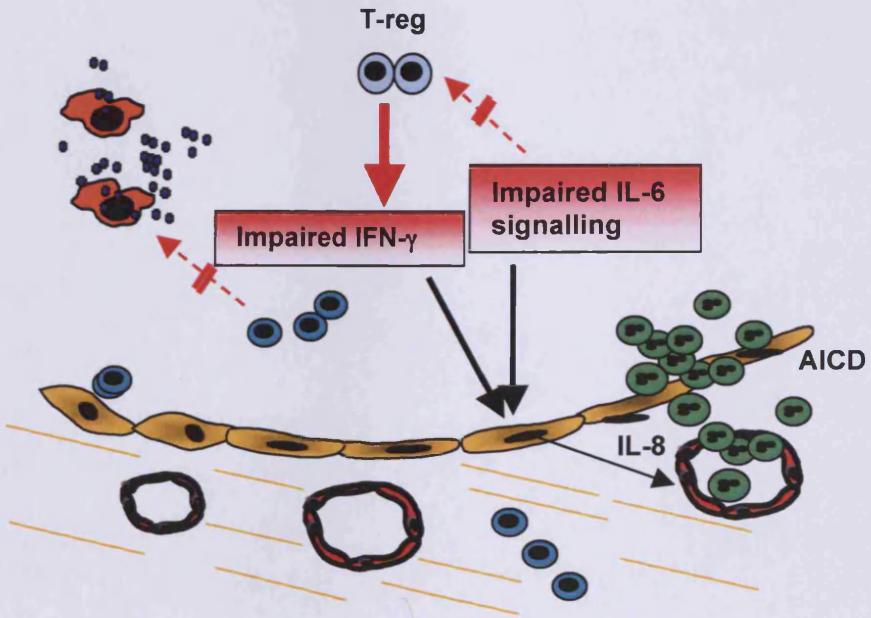

Figure 6.5 Chronic inflammation. Recurrent peritonitis results in peritoneal T-cell anergy and a defective TH1 response. Together with impaired IL-6 signalling, this leads to on-going neutrophil recruitment and impaired cellular immunity. This generates a chronic inflammatory milieu within the peritoneal cavity that contributes to membrane fibrosis and treatment failure.

more susceptible to further episodes of peritonitis. It is also likely to have a negative impact on immuno-resolution and contribute to neutrophil retention and on-going inflammation (Figure 6.5). This speculation is now confirmed by more recent work in our laboratory showing that IFN-γ deficient mice display significantly impaired bacterial clearance and aberrant neutrophil trafficking.

From our studies we can conclude that T-cell dysregulation may be a pivotal event in the early stages of chronic inflammation, less clear however is the mechanism that links dysregulated inflammation to tissue fibrosis. This thesis has not directly addressed this issue, however here is an abundance of animal model and *in-vitro* data to suggest that inflammation can trigger fibrosis, indeed our own group have demonstrated that recurrent inflammation is associated with membrane fibrosis in the murine model of peritonitis.

The role of T-cells in the genesis of fibrosis remains poorly defined however, with conflicting reports of pro or anti-fibrotic effects depending on the local environment and cytokine milieu [129]. This is exemplified by murine studies of pulmonary fibrosis. Athymic (nude) mice (with a total lack of T-cells) continue to develop pulmonary fibrosis in response to bleomycin, suggesting that T-cells play no role in the process [130]. In contrast mice deficient in CD28 signalling (a central T-cell co-stimulant) appear protected against bleomycin-induced fibrosis. Transfer of $CD28^+$ T-cells into these mice restores a normal "fibrotic" response suggesting causality [131]. A further study has shown that exposure of SCID mice (that lack functional T-cells) to asbestos results in more fibrosis (than wild type), suggesting that in this case T-cells are protective [132]. These conflicting results may reflect the "immune-adaptation" that may occur in genetically modified mice, it may also reflect the complex, often divergent functions of T-cells *in-vivo.* In the context of our studies, it is interesting to note that T-cell derived IFN-γ has been identified as an extremely potent anti-fibrotic factor, capable of inhibiting fibroblast proliferation and collagen production [133, 134]. This hints that loss of TH1 effector function (as seen following recurrent peritonitis) may contribute directly to peritoneal membrane fibrosis.

We must now consider the therapeutic options that will allow us to reverse or at least limit the detrimental impact of recurrent infection on peritoneal immune homeostasis. Over the last century the approach to combating infectious disease has focussed almost entirely on killing the invading pathogen with antibiotic therapy. The development of multiple-drug-resistant bacteria and the recognition that recurrent infection is linked to chronic inflammation highlights the need for a novel strategy to deal with microbial infections. Such an "immuno-modulatory" strategy may focus on enhancing or suppressing the immune response, aiming to restore "normality". With regard to the peritoneal immune system, such strategies may include augmenting IFN-γ (or IL-6) signalling or manipulating T-reg activity to alter the magnitude of the inflammatory response (Figure 6.6)[135]. Alternative approaches include taking advantage of the adaptive immune response and priming the peritoneal memory T-cells with vaccines targeted against the most common infective agents. Though previous attempts at vaccination have been unsuccessful [81, 136], these trials pre-dated our current understanding of T-cell biology. As we have shown, we are now able to assess the efficacy of a potential vaccine in generating functional peritoneal memory cells. Moreover, we may soon be able to modulate T-cell signalling pathways and B-cell help thus enhancing antibody responses.

The biggest challenge in designing novel immuno-modulators is the complexity of the human immune system itself. This highly refined system does not lend itself well to a "shotgun approach", highlighting the need for a better understanding of the fundamental processes involved in tissue inflammation. We hope that further studies such as this may lead to the development of novel immunotherapeutic agents that prevent or reverse the processes that drive dysregulated inflammation.

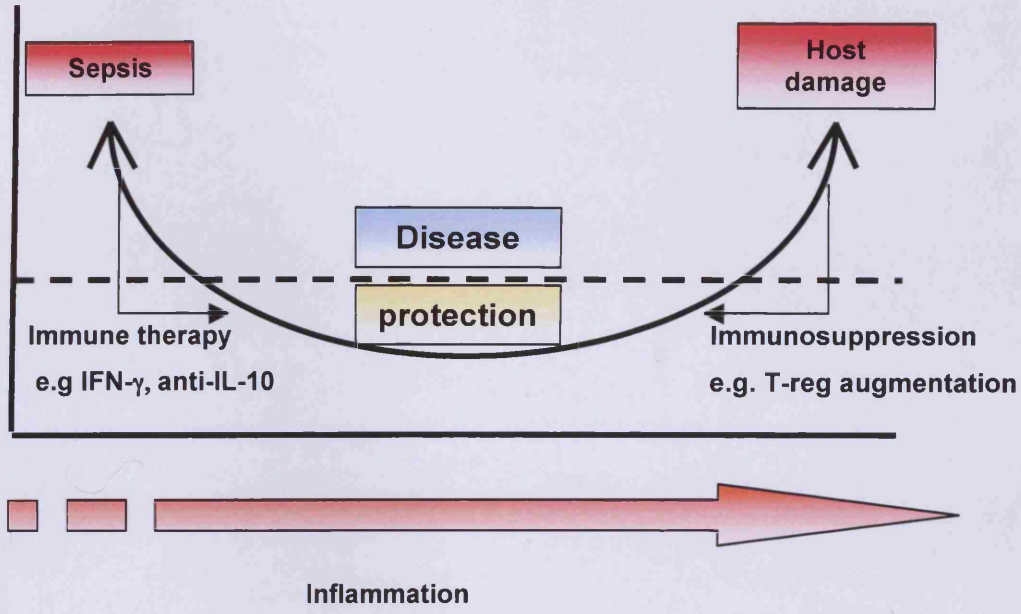

Figure 6.6 Strategies for modifying the acute inflammatory response [135]

6.2 Future work

The aim of our research was to undertake an extensive, contemporary analysis of the peritoneal T-cell repertoire rather than to focus specifically on a particular T-cell subset. Clearly many of the studies are limited by both their cross sectional nature and by the small number of patients involved. In spite of this, our findings have provided us with novel insights into the behaviour of human peripheral tissue T-cells, allowing us to focus future research on those aspects of peritoneal T-cell biology that appear important or linked to disease. Current or future avenues of peritoneal T-cell research include: -

- **The role of peritoneal Vγ9δ2 T-cells in bacterial peritonitis**: Based on data presented in this thesis we (in collaboration with Dr Matthias Eberl and Professor Bernhard Moser, Cardiff university department of medical Biochemistry) have been awarded a Welsh Office of Research and Development grant (£170,000) to study the role of Vγ9δ2 T-cells in PD peritonitis. Though the study is primarily a mechanistic analysis of peritoneal Vγ9δ2 T-cells, it will also provide insight into the trafficking of TH17, T-regs and memory T-cells during acute inflammation.
- **The impact of recurrent infection on peritoneal T-reg function.** Though our studies assessed the impact of recurrent infection on effector T-cell function, we did not investigate the impact of infection on T-reg function. We hypothesise that recurrent infection leads to increased T-reg activity, which may have a suppressive effect on local effector cells. This is the focus of current research.
- **Characterise the function of $CD69^+$ peritoneal T-cells.** We have now begun to characterise the function of FACS sorted $CD69^+$ peritoneal T-cells. Preliminary data suggests that these cells suppress peripheral blood T-cell proliferation, suggesting that they may indeed perform a regulatory role within the peritoneum. Further research is warranted to confirm and expand on these findings.

- **Explore potential vaccination strategies.** Our data shows that the peritoneal cavity is enriched in memory T-cells. Future research should focus on whether these cells can be primed to target common invading pathogens.
- **T-cell telomere length studies.** PD patients appear to have shortened telomere lengths, which may be associated with increased morbidity and mortality. This could be evaluated further in a larger prospective study. The impact of more biocompatible fluid on peritoneal T-cell telomere shortening could also be assessed.
- **Investigate the role of T-cells in peritoneal fibroblast activation/myofibroblast differentiation.** Future research may focus on the mechanisms by which T-cell activation may initiate or propagate tissue fibrosis.
- **Characterise the phenotype and function of peritoneal T-cells obtained from "healthy" individuals** e.g. women undergoing laparoscopic sterilisation.

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Appendices

Appendix I: Table1 Patient demographics

Patient	Age	Peritonitis episodes	DM (Y/N)	KT/V renal	KT/V PD	KT/V Total	Alb	Wt (Kg)	UF	Glucose exposure (g/day)	Transport Status *	PD Vintage (months)	Extraneal (Y/N)	Aetiology Of ESRF **	Outcome at study end
A1	66	0	N	1.23	1.08	2.31	33	55	1.26	108	H	4	N	Hypertensive nepropathy	CAPD
A2	58	0	Y	1.15	1.15	2.3	39	81	0.66	108	LA	4	N	Hypertensive nepropathy	CAPD
A3	66	0	Y	0	2.11	2.11	36	62	1.75	99.8	HA	17	Y	Systemic sclerosis	CAPD
A4	66	0	Y	0.13	2.03	2.17	35	60	2.22	127	LA	37	N	Diabetic nephropathy	CAPD
A5	42	0	N	0	1.72	2.06	35	77	1.55	154.2	HA	10	Y	Unknown	CAPD
A6	59	0	N	0.35	2.39	2.81	41	53	1.73	127	HA	86	N	Chonic pyelonepritis	CAPD
A7	75	0	N	0.4	1.78	2.18	34	72	1.75	81.6	HA	40	Y	Reflux	Transplant
A8	66	0	N	0.5	1.14	1.65	32	74	0.08	108	HA	13	N	Unknown	CAPD
A9	46	0	Y	0.41	1.6	2.01	32	62	2.71	99.8	HA	12	Y	Unknown	CAPD
A10	36	0	N	0.54	1.05	1.59	38	97	2.43	81.6	HA	12	Y	Unknown	CAPD
A11	57	0	Y	.0	1.59	1.59	37	59	1.81	108	LA	24	N	Interstitial nephritis	R.I.P
A12	59	0	N	0.6	1.62	2.22	39	80	1.0	108	HA	3	N	Focal segmental GN	CAPD
B1	71	1	N	1.1	1.31	2.4	39	88	2.2	81.6	HA	22	Y	Unknown	CAPD
B2	65	1	N	2.01	0.81	2.76	36	63	0.83	81.6	LA	11	N	Reflux	CAPD
B3	62	1	N	3.44	1.34	4.78	37	81	2.35	154	HA	9	Y	Renovascular disease	CAPD
B4	64	2	N	0	1.64	1.64	38	62	1.00	127	H	48	N	Unknown	HD
B5	56	1	N	0.56	1.32	1.88	31	71	1.71	108	LA	4	N	IgA GN	CAPD
B6	79	2	N	0.46	2.07	2.53	35	78	1.48	81.6	HA	15	Y	Diabetic nephropathy	CAPD
B7	70	1	Y	0.98	1.72	2.7	35	86	3.05	99.8	HA	16	Y	Diabetic nephropathy	R.I.P
B8	60	1	Y	1.25	1.47	2.72	35	73	3.19	81.6	HA	22	N	APCKD	CAPD
B9	68	2	N	0.09	1.43	1.52	28	56	0.32	81.6	H	132	Y	Analgaesic nephropathy	R.I.P
B10	66	1	N	2.21	1.61	3.81	38	62	1.9	108	HA	6	N	Unknown	CAPD
B11	62	1	N	0	3.03	3.03	42	36	2.10	81.6	H	72	N	Chonic pyelonepritis	CAPD
B12	61	1	Y	1.45	0.73	2.18	37	103	1.99	108	N/A	5	Y	Nephrectomy, Ca kidney	CAPD
B13	70	1	N	1.26	1	2.16	32	83	0.85	127	HA	48	N	Membranous GN	CAPD
B14	41	1	Y	1.6	0.93	2.53	40	103	1.83	81.6	HA	6	Y	Unknown	CAPD
B15	35	1	Y	0	2.87	2.87	34	66	1.10	113.6	HA	24	N	Unknown	CAPD
B16	74	1	Y	1.4	1.06	2.46	42	74	0.6	113.6	H	18	N	Unknown	CAPD
B17	40	1	N	0	1.72	1.72	32	65	0.61	81.6	HA	72	N	Mesangio capillary GN	CAPD
B18	42	1	N	0.61	1.6	2.21	36	80	1.5	81.6	H	38	Y	Hypertensive nepropathy	CAPD
B19	78	2	N	0.8	1.03	1.83	34	71	1.3	108	LA	36	N	CLL	R.I.P
B20	75	1	N	1.0	1.83	2.83	21	51	0.5	126.4	HA	18	N	Diabetic nephropathy	CAPD
B21	66	1	Y	0	2.11	2.11	36	62	1.75	99.8	HA	17	Y	Systemic sclerosis	CAPD
B22	65	1	N	0	2.11	2.11	36	62	1.75	99.8	HA	17	N	Hypertensive nepropathy	CAPD
B23	40	1	N	0.52	1.6	2.12	39	68	0.45	127	HA	36	N	GN	CAPD
B24	39	1	M	0	2.43	2.43	39	77	1.0	81.6	HA	29	Y	Unknown	CAPD
C1	66	3	N	0.13	1.78	1.91	33	61	0.46	113.6	HA	70	Y	IgA GN	HD
C2	74	5	N	0	2.12	2.12	32	56	1.1	127	HA	72	N	Hypertensive nepropathy	CAPD
C3	71	5	N	0.86	1.25	2.11	27	82	1.3	81.6	H	24	Y	Diabetic nephropathy	R.I.P

Patient	Age	Peritonitis episodes	DM (Y/N)	KT/V renal	KT/V PD	KT/V Total	Alb	Wt (Kg)	UF	Glucose exposure (g/day)	Transport Status *	PD Vintage (months)	Extraneal (Y/N)	Aetiology Of ESRF **	Outcome at study end
C4	57	5	N	0	1.89	1.89	36	63	0.9	93	H	89	Y	Obstructive uropathy	R.I.P (EPS)
C5	64	6	N	0.99	1.12	2.11	38	72	0.38	163.4	HA	60	N	Renovvascular disease	R.I.P
C6	50	5	N	0.5	2.13	2.63	37	78	1.7	0.36	LA	8	N	IgA GN	Transplant
C7	79	5	N	0.58	1.36	1.92	30	68	0.41	36	HA	36	Y	Unknown	R.I.P
C8	75	4	N	0.3	1.64	1.99	36	79	1.07	127	HA	60	Y	IgA GN	CAPD
C9	67	4	Y	1	0.85	1.85	32	55	0.7	127	H	47	Y	Diabetic nephropathy	CAPD
C10	69	6	N	0	1.52	1.52	28	56	0.32	81.6	H	143	Y	Interstitial nephritis	R.I.P
C11	43	1	Y	0	2.37	2.37	33	70	2.25	99.4	H	120	Y	Chronic peylonephrtits	CAPD
C12	79	3	Y	0.5	1.75	2.25	21	63	1.9	81.6	HA	24	Y	Diabetic nephropathy	R.I.P
C13	38	3	Y	0	0	2.31	30	70	0.4	113.6	H	117	Y	unknown	R.I.P (EPS)

Group A - 0 peritonitis episodes
Group B - 1-2 peritonitis episodes
Group C - >2 peritonitis episodes

*H-High
HA-high average
LA-Low average
L-Low

**GN- Glomerulonephritis
APKD- Adult polycystic kidney disease
EPS- Encapsulating peritoneal sclerosis

Appendix I: Table 2 Peritonitis data for group B patients

B1	a) *Coag. neg Staph.* May 05
B2	a) *Coag. neg Staph.* Feb 07 b) *Staph. Aureus* Feb 07
B3	a) *Coag. neg Staph.* Aug05
B4	a) *Staph. Aureus* May 06 b) *Staph. Aureus* Nov 07
B5	a)*Coryneforms* Jan 05
B6	a) *Coag. neg Staph.* Mar 06 b) *Coag. neg Staph.* Apr 06
B7	a) *E.Coli* Aug 06
B8	a) *Staph. Aureus* Sep 06
B9	a) *Coag. neg Staph.* Apr 03 b) *Coag. neg Staph.* May 05
B10	a) *Coag. neg Staph.* Aug 05
B11	a) *E.Coli* Feb 08
B12	a) *Bacteroides* Aug 07
B13	a) *E.Coli* Jun 07
B14	a) α *Haem. Strep* May 07
B15	a) *Coag. neg Staph.* Aug 07
B16	a) *Coag. neg Staph.* Oct 07
B17	a) *Coag. neg Staph.* May 03 b) *Coag. neg Staph.* Oct 07
B18	a) *Staph. Aureus* Apr 06
B19	a) α *Haem. Strep* Jun 05 b) *E.Coli* Aug 05
B20	a) *Coag. neg Staph.* Feb 08
B21	a) *E.Coli* Oct 06
B22	a) *E.Coli* Mar 08
B23	a) *Coag. neg Staph.* Oct 08
B24	a) *Coag. neg Staph.* Mar 08

Appendix I: Table 3 Peritonitis data for group C patients

C1	a) *Coag. neg Staph.* Feb 06 b) *Coag. neg Staph.* Apr 06 c) *Coag. neg Staph.* Jun06
C2	a) *Coag. neg Staph.* Jan 03 b) *Coag. neg Staph.* Aug 03 c) *Coag. neg Staph.* Feb 06 d) *Coag. neg Staph.* Mar06 e) *Coag. neg Staph.* May 06
C3	a) *Coag. neg Staph.* Jan 06 b) *Coag. neg Staph.* Mar 06 c) *Coag. neg Staph.* Nov 06 d) *Staph. Aureus* Dec 06 e) *Coag. neg Staph* Mar 07
C4	a) *Coag. neg Staph.* Jan 00 b) *Coag. neg Staph.* Apr 04 c) *Coag. neg Staph.* Nov 05 d) *Coag. neg Staph.* Feb 06 e) *Neisseria* Mar07
C5	a) *Coag. neg Staph.* Feb 02 b) *Coag. neg Staph.* Nov 03 c) *Coag. neg Staph.* Dec 03 d) *Coag. neg Staph.* Jan *04* e) *Coag. neg Staph.* Feb *04* f) *α haem. Strep* Feb 05
C6	a) *Corynebacterium* Aug 06 b) *Corynebacterium* Nov 06 c) *Corynebacterium* Jan 07 d) *Corynebacterium* Aug 07 e) *Corynebacterium* Sep 07
C7	a) *Coag. neg Staph.* Aug *04* b) *Coag. neg Staph.* Dec*04* c) *Coag. neg Staph.* Jan*04* d) *Coag. neg Staph.* Apr *04* e) *Coag. neg Staph.* Aug *04*
C8	a) *Coag. neg Staph.* Jan *00* b) *Coag. neg Staph.* Apr *04* c) *Coag. neg Staph.* Nov 05 d) *Coag. neg Staph.* Feb06
C9	a) *Coag. neg Staph.* Aug 05 b) *Coag. neg Staph.* Nov 06 c) Enterococcus Feb 07 d) *α Haem. Strep* Aug 07
C10	a) *Coag. neg Staph.* Apr 03 b) *Coag. neg Staph.* May 05 c) *Coag. neg Staph.* Jan 07 d) *Coag. neg Staph.* Feb 07 e) *Coag. neg Staph.* Apr 07 f) *Coryneforms* Oct 07
C11	a) *St Aureus* Mar 03 b) *Coag. neg Staph.* May 03 c) *Coag. neg Staph.* Jan 04 d) *Coag. neg Staph.* Jan 08 e) *Coag. neg Staph.* Jan 08
C12	a) *Coag. neg Staph.* May 04 b) *Coag. neg Staph.* Nov 04 c) *Coag. neg Staph.* May 05
C13	a) *Staph. Aureus.* Nov 98 b) *Coag. neg Staph.* Oct *04* c) *Coag. neg Staph.* Jan 07

Appendix II: Table 1 Patients used in each figure

Chapter 2

Figure 2.1 Representative example from A8

Figure 2.2 Representative example from B12

Figure 2.3 Representative example from B14

Figure 2.4 Representative example from B14

Figure 2.5 (A) Representative example from B3
(B) Representative example from A5

Chapter 3

Figure 3.1 (A) Representative example from A9 of data obtained from ..

Figure 3.2 See figure for patient data

Figure 3.3 Data derived from 5 healthy controls

Figure 3.4 Representative example shown from B14 of data derived from B14, B12, A4, A9 and A5.

Figure 3.5 (A) Data derived from A2, A6, A4, A5, B6, C3, B8, A11
(B) Data derived from B14, B15, B16, B17, B21, B22, B23, B24, C10 and C9.

Figure 3.6 (A) Data derived from A7, A8, A5, B10 and A11.
(B) Representative example shown from A7 of data derived from A7, A8 and A5.

Figure 3.7 (A) Data derived from C12, A7 and A5.
(B) See figure for patient data.
(C) Data derived from A7.
(D) Representative example shown from B14 of data derived from B14 and A9.

Figure 3.8 See figure for patient data

Figure 3.9 See figure for patient data

Chapter 4

Figure 4.1 (A) Representative example from A7 (B) Data derived from A1, B1, A3, B12, A4, A5, B6, C1, C2, A7, C3, B8, A8, A10, A11,A6, A7, C3, B21 and C4.

Figure 4.2 Representative example from B11 of data derived from B11, C3, A7 and B7.

Figure 4.3 Representative example from A6 of data derived from A6, B3, A7 and B6.

Figure 4.4 Data derived from

Figure 4.5 Representative example shown from A5 of data derived from A5, B14, A1, A12 and A11.

Figure 4.6 (A) Data derived from A2, A6, A4, A5, B6, C3, B8 and A11.
(B) Data derived from B14, B15, B16, B17, B21, B22, B23, B24, C10, and C9.

Figure 4.6 Data derived from A12, A9 and B12.

Figure 4.7 (A) Representative example shown from A10 of data derived from A10, A7, A11, A5 and A1.
(B) Representative example shown from C3 of data derived from C9, C3,B3, C2, C1, B10, C3, C1, B6, and C10.
(C) Representative example shown from B4 of data derived from B4, B8 and B14.

Figure 4.8 (A) Representative example shown from A1.
(B) Data derived from A1, A2, B4, B5, A5, B6, C1, C2, A6, A6, B7 and C4.

Figure 4.9 (A) See figure for patient data.
(B) Data derived from A1, A11 and B14.

Figure 4.10 (A) See figure for patient data.
(B) See figure for patient data.

Figure 4.11 (A) Representative example from B21 of data derived from B21 and C1(a).
(B) See figure for patient data.

Figure 4.12 (A) Data derived from A9
(B) Data derived from A11
(C) Data derived from B6
(D) Data derived from B12, A9, B9, B16,B14, C11, B22, B20

Figure 4.13 Gram negative data derived from, B12(day3, day4, day7, day20) B13 (day2, day3), B11(d5) and B22(d6). Gram positive data derived From C11(day1), C10(day1, day2), B20(day1), B17(day1, day2), B16(day1,day2) and B15(day2).

Figure 4.14 PBMC obtained from healthy volunteers, Gram negative effluent obtained from B13, Gram positive effluent obtained from.

Chapter 5

Figure 5.1 (A) Data derived from A1, A3, A4, A5, A6, A7, A8, A9, A11, B3, B6, B21, B18, B21, C1, C2, C3, C4 and C8.
(B) Data derived from A1, A2, A4, A6, A7, A8, A9, A11, B5, B6, B7, B8, B9, C2, C3, C4, C5, C6, C7, C8 and C13.

Figure 5.2 (A) Data derived from A1, A5, C4, C7
(B) Data derived from A1, A2, C3, C4

Figure 5.3 Data derived from C8, C3, C4, C2

Figure 5.4 Data derived from A1, A2, A4, A11, C2, C3, C4, C8,

Figure 5.5 Data derived from A1, B1, A3, B12, A4, A5, B6, C1, C2, A7, C3, B8, A8, A10, A11, A6, A7, C3, B21 and C4.

Figure 5.6 Data derived from A1, A2, B4, B5, A5, B6, C1, C2, A6, A6, B7 and C4.

Figure 5.7 (A) Representative example from A12. Data derived from A12 and
(B) Data derived from A12 and C5

APPENDIX III: Patient information sheet and consent form

PATIENT INFORMATION SHEET

1. **Study title**

Inflammation in Peritoneal Dialysis.

Markers of inflammation are the white blood cells that are located in the peritoneal cavity of a patient undergoing peritoneal dialysis. The chemicals which are responsible for organising these white blood cells can also be measured as markers of inflammation.

2. **Invitation paragraph**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

3. **What is the purpose of the study?**

Our immune system fights harmful microbes, such as disease-causing bacteria, viruses and tumours by using immune cells which attack these foreign substances. We carry numerous immune cells at various locations in our body, including our blood and many tissues (including the peritoneal cavity). This study is all about finding out more about our peritoneal immune cells. We want to understand how they reach the peritoneal cavity, how they recognize microbes and other harmful particles and how they protect us during episodes of peritonitis. Answers to these fundamental questions are extremely important since recurrent infection (peritonitis) remains the major reason for treatment failure in peritoneal dialysis.

4. **Why have I been chosen?**

You have been invited to take part because you are on peritoneal dialysis and the fluid from your exchange would normally be thrown away. We can use this fluid to investigate the immune cells in the body.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

6. What will happen to me if I take part?

If you agree to take part, we will use some of the fluid that you drain out during your PD exchange for research purposes. As you know this fluid would normally be thrown away. When the research group get your sample, it will have no details that can identify you with it, only a code number. The master copy with your identification details will stay with the PD Team and will not be disclosed to the researchers

7 What will happen to the sample I give?

The sample you give will be

- processed to isolate immune cells to be used in experiments. The surplus of cells (in case not all of the cells have been used) will be stored as frozen samples in case the experiment needs to be redone later or you agree that we can use the surplus for further research.
- The procedure of cell isolation and tissue culture is not dangerous to you (as blood donor) or to the researchers carrying out these experiments.
- Nobody else but the researchers involved in this study have access to your donated cells.
- Researchers handling your donated material will not be able to identify you, i.e. your material will be labelled with a code whose meaning is only known to the clinical personnel collecting your sample.
- If you wish, your sample can be destroyed at anytime (unless it has already been used in experiments)

8. Future research

We would like to store the sample you have given us for use in future research projects. If you agree the sample will be stored and may be used in future research into immune cells. This will be done in accordance with the Human Tissue Act which lays down requirements for the storage and use of all samples. No identifiable information will be stored with the sample and all research that will be done in the future will have the approval of a Research Ethics Committee. If you wish, you can agree for the sample to be used for the current project but not for future research. If so, you should only sign Part 1 of the consent form

9. Will any genetic tests be done?

No genetic testing will be done

10. What do I have to do?

There are no restrictions on your lifestyle by taking part in this study over and above those normally associated with being on peritoneal dialysis.

11. What are the possible disadvantages and risks of taking part?

There are no obvious disadvantages to taking part in this study. You should not be inconvenienced by taking part as the researcher will come to your house to collect the fluid at a time convenient to you. There is a small risk of bruising when having a blood sample taken, but as often as possible this will be done as part of your routine care and will not involve you in having an extra stab into a vein.

12. What are the possible benefits of taking part?

There is no benefit to you from taking part in the trial, however the information we get from this study may help us to improve treatment for patients undergoing PD.

13. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you. The Complaints Officer can be contacted on 02920.746296

14. **Will my taking part in this study be kept confidential?**

All information that is collected about you during the course of the research will be kept strictly confidential. No information about you will leave the hospital with your name and address on it so you cannot be recognised from it.

15. **What will happen if I don't want to carry on with the study?**

You can decide to withdraw from the study at any point. If you want to withdraw you can contact the research team and make that request. If the sample has been processed it will not be possible to return it to you or destroy it, but the information we have obtained can be withdrawn and destroyed. If the sample has not yet been processed it can be destroyed or disposed of as you see fit.

16. **What will happen to the results of the research study?**

The results of this study will be submitted for presentation and publication at PD meetings and in medical journals. You will not be identified in any presentation or publication that is submitted.

17. **Who is organising and funding the research?**

This study is being carried out by the Institute of Nephrology using their own research funds. No payment is being made to any of the researchers for including you in this study.

18. **Who has reviewed the study?**

This study has been reviewed by Panel D of the South East Wales Ethics Committee.

20. **Contact for Further Information**

Professor John Williams: 02920 748432
Professor Nick Topley: 02920 748432
Dr G Roberts: 02920 748446

Thank you for taking part in this study

PATIENT CONSENT FORM

Patient Identification Number for this trial:

Name of Researchers: Prof JD Williams, Prof N Topley, Dr G Roberts

CONSENT FORM

Title of Project: Inflammation in Peritoneal Dialysis

Please initial box

I confirm that I have read and understand the information sheet dated April 2008 (Version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that there will be no financial reward for taking part. I am also free to withdraw at any time without giving any reason, and without my care or legal rights being affected. ☐

3. I understand that relevant sections of data collected during the study, may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research.I give permission for these individuals to have access to these data. ☐

4. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature