Characterisation of markers associated with systemic inflammation in children with Chronic Kidney Disease

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Declaration

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

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Judith Nairn

Signature _____ Date _____

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Abstract

Chronic Kidney Disease (CKD) is a progressive condition that in the majority of cases leads to End Stage Renal Failure (ESRD) and the need for dialysis, with the only cure being renal transplant. CKD affects both adults and children; however the underlying causes of the disease are different. CKD in adults is most commonly secondary to diabetes and/or hypertension while CKD in children is usually caused by congenital structural abnormalities that result directly in renal dysfunction.

There have been numerous reports of inflammatory and immunological disturbances in adult CKD that involve both the cellular and humoral immune systems. Consequences of these include an increased rate of cardiovascular disease (CVD), decreased response to vaccinations, as well as increased rates of infection, anaemia and malnutrition. Children with CKD display many of the clinical complications seen in adult kidney disease that are associated with inflammatory and immunological changes.

In adults however, many of the primary conditions associated with CKD are inherently pro-inflammatory; therefore it is not clear whether the inflammatory changes observed in adults with CKD are due to pre-existing inflammatory conditions, renal disease *per se* or a combination of both.

The majority of CKD in children is caused by conditions that are not inflammatory in nature. This presents a unique opportunity to study the inflammatory consequences of CKD alone, without the added complication of underlying inflammatory disorders.

Despite this, there has been little investigation of the inflammatory and immunological status of children with CKD. Some very recent studies have

shown that children with CKD have an increased systemic inflammatory state[1-3], however the nature of these immunological and inflammatory changes remains poorly defined. Identification of the specific inflammatory processes that occur in CKD may provide new treatment targets and the opportunity to develop urgently needed new therapies.

The purpose of this thesis is to investigate the presence of immunological changes associated with inflammation in children with CKD. This is the first study to include children with very mild disease, and the significant changes that are present in the early stages of the disease are of particular note. I have shown that CKD in children is an intrinsically inflammatory condition, with increased accumulation of markers of oxidative stress and production of pro-inflammatory cytokines. The inflammatory markers identified in this study may be applied as a foundation for more sensitive diagnostic markers of disease progression as well as provide a basis for novel treatment strategies in this group of patients.

Early identification of increased inflammation is a prerequisite for the application of preventive strategies. In addition, a better understanding of the level and mechanisms of systemic inflammation in children with CKD may enable a more accurate assessment of their risk of other inflammatory conditions such as CVD, anaemia, muscle wasting, and malnutrition. Future research that specifically focuses on the reasons and mechanisms for different rates of disease progression may emerge as a result of this study.

Importantly, the findings of this study may have implications in the long term treatment of disease and may allow identification of new treatment strategies to achieve better patient outcomes.

The outcomes of the study are:

- Better definition of inflammatory profiles in paediatric CKD and correlation with disease severity and progression, which should contribute to improved management strategies.
- Identification of new treatment targets to reduce the damage caused by chronic systemic inflammation.
- Mechanistic understanding of the relationship of the inflammatory profile in regard to source leucocytes or other contributing cell types.

Abbreviations

µg	microgram
µL	microlitre
µmol/L	micromoles per litre
ADPCKD	Autosomal Dominat Polycystic Kidney Disease
AGE	Advanced Glycation End Products
AOPP	Advanced Oxidation Protein Products
BA	Brefeldin A
CBA	Cytometric bead array
CBP	Complete Blood Picture
CCR	Chemokine Receptor
CD	Cluster Differentiation
CKD	Chronic Kidney Disease
CRP	C-reactive protein
CVD	Cardiovascular Disease
DN	Double Negative
ELISA	Enzyme Linked Immuno-Sorbent Assay
ESR	Erythrocyte Sedimetation Rate
ESRF	End Stage Renal Failure
FACS	Fluorescence Activated Cell Sorter
FACSperm	FACS permeabilising solution
FITC	Fluorescein isothiocyanate
FL	Fluorescence Channel
FSC	Forward Scatter
g	gravitational force

GFR	Glomerular Filtration Rate
HLDA	Human Leucocyte Differentiation Antigen
HMG-CoA	Hydroxymethylglutaryl-coenzyme A
hr	hours
HUS	Haemolytic Uremic Syndrome
I	Ionomycin
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
IL-12R	IL-12 receptor
LPS	*E. coli* Lipopolysaccharide
Mab	Monoclonal Antibody
MCP	monocyte chemotactic protein
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibilty Complex
MIA	malnutrition, inflammation and atherosclerosis
min	Minutes
mL	millilitre
mRNA	messenger RNA
ng	nanogram
NIH	National Institute of Health
NK	Natural Killer
o/n	overnight
PBMC	Peripheral Blood Mononuclear Cells

PC-5	PE-CY5
PE	Phycoerythrin
PHA	phytohemagglutinin
PKD	Polycytic kidney disease
PMA	phorbol 12-myristate 13-acetate
PMT	Photomultiplier tube
PTH	Parathyroid hormone
PTS	phosphatidylserine
PUJ	Pelviureteric junction
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
RT	Room temperature
sE-selectin	Soluble E-selectin
SLE	systemic lupus erythematosis
SSC	Side Scattter
Tc	Cytotoxic T cells
Th	T Helper cells
TNF	Tumour Necrosis Factor
uL	microlitre
VCAM	vascular cell adhesion molecule
WBC	White Blood cells

Chapter One

Introduction, Hypothesis and Aims

1.1 Synopsis

Chronic Kidney Disease (CKD) is a condition that in many cases leads to End Stage Renal Failure (ESRF) and the need for dialysis, with the only cure being renal transplant. Although CKD affects both adults and children, the underlying causes of the disease are different. CKD in adults is most commonly secondary to diabetes and/or hypertension while CKD in children is usually caused by congenital structural abnormalities that result directly in renal dysfunction such as renal dysplasia or reflux nephropathy.

Immune disturbances in adults with CKD involve both the cellular and humoral immune systems [4]. Consequences of these alterations include an increased rate of cardiovascular disease (CVD), decreased response to vaccinations, and increases in infections, anaemia and malnutrition [5-7]. A persistent low grade inflammatory response leads to changes in acute phase markers such as C-reactive protein (CRP) and pro-inflammatory cytokines such as Interleukin (IL)-12 and Tumour Necrosis Factor (TNF) -α [8-15].

Children with CKD display many of the clinical complications seen in adult kidney disease that may be associated with inflammatory and immunological changes. These include changes in lipid metabolism, increased protein catabolism and induction of acute phase proteins [16, 17]. In addition, the rate of CVD in children with renal disease has been reported as 1000 times the rate of an age matched population without renal disease [18].

Despite the clinical importance of CKD, there has been little investigation of the inflammatory and immune status of children with impaired renal function.

Some very recent studies have shown that children with CKD have an increased systemic inflammatory state [1-3], however the nature of these immunological and inflammatory changes remains poorly defined.

In adults, many of the primary conditions associated with CKD are inherently pro-inflammatory; therefore it is not clear whether the inflammatory changes observed in adults with CKD are due to pre-existing inflammatory conditions, renal disease *per se* or a combination of both. In contrast, the majority of CKD in children is caused by conditions that are not inflammatory in nature. The major causes of CKD in children are renal dysplasia, reflux nephropathy, hereditary diseases such as polycystic kidney disease (PKD) and Alport's syndrome, with a smaller proportion caused by systemic diseases such as diabetes and systemic lupus erythematosis (SLE)[19]. This presents a unique opportunity to study the inflammatory consequences of CKD alone, without the added complication of underlying inflammatory disorders.

Such an investigation has the potential to identify new and novel targets for treatment strategies. The importance of this work was identified in a recent NIH review of research priorities for paediatric nephrology that specifically suggested that characterisation of systemic inflammation is essential for the development of new treatment strategies for CKD [20].

This thesis describes the investigation of the presence of immunological changes associated with inflammation in children with non-inflammatory causes of CKD. This is the first study to include children with very mild disease, and the significant changes that are present in the early stages of the disease are of particular note. Experimental evidence in this thesis

shows that CKD in children is an intrinsically inflammatory condition, with increased accumulation of markers of oxidative stress and production of pro-inflammatory cytokines.

The inflammatory markers identified in this study may be applied as a foundation for more sensitive diagnostic markers of disease progression in this group of patients. Early identification of increased inflammation is a prerequisite for the application of preventive strategies. In addition, a better understanding of the level and mechanisms of systemic inflammation in children with CKD may enable a more accurate assessment of their risk of other inflammatory conditions such as CVD, anaemia, muscle wasting, and malnutrition. Future research that specifically focuses on the reasons and mechanisms for different rates of disease progression may emerge as a result of this study.

Importantly, the findings of this study may have implications in the long term treatment of CKD and may allow identification of new treatment targets and strategies to achieve better patient outcomes.

1.2 Expected Outcomes

The outcomes of the study are expected to be:

- Improved definition of the inflammatory profiles in paediatric CKD and correlation with disease severity and progression, which may contribute to improved management strategies.
- Identification of new treatment targets to reduce the damage caused by chronic systemic inflammation.

- Mechanistic understanding of the relationship of the inflammatory profile of leucocytes or other contributing cell types.

1.3 Definition of CKD

Until recently, CKD severity and staging has not been well defined in children. There has been a lack of agreement about methods to evaluate kidney function in children and adolescents, and there has been no simple classification of the stages of CKD.

Glomerular filtration rate (GFR) is the volume of fluid filtered from the renal glomerular capillaries into Bowman's capsule per unit time and is used as a measure of renal function. GFR declines as renal impairment increases. Recently the National Kidney Foundation developed guidelines for classification and staging of CKD based on GFR which are outlined in Table 1 [21]. It is important to note that patients with near normal GFR may have abnormalities in the composition of blood or urine which are indicative of kidney damage [21], hence the inclusion of these patients in the classification of CKD.

NOTE: This table is included on page 6 of the print copy of the thesis held in the University of Adelaide Library.

Table 1: Classification of CKD. *The actions listed in the more severe stages of CKD also include actions from less severe stages. Taken from [21]. Used with permission.

1.4 Causes of CKD in children and adults

The cause of CKD in children follows a different pattern to adults. In the adult population, obesity, diabetes, hypertension or hyperlipidaemia confer an increased risk of CKD and the majority of CKD in adults is concomitant with one or more of these conditions [22].

In contrast, in the Australian population from birth to age 4 years, birth defects and hereditary diseases are by far the leading causes of kidney failure. Between ages 5 and 14 years, hereditary diseases such as PKD and Alport's syndrome, continue to be the most common causes, followed by glomerular diseases. In the 15-to 19-year-old age group, glomerular diseases are the leading cause, and hereditary diseases become rarer [19]. Most of the causes of CKD in children are

not inherently inflammatory. Over half of all paediatric CKD is caused by obstructive uropathy and congenital aplasia/hypoplasia/dysplasia [23]. These defects are present at birth, therefore, a substantial proportion of paediatric CKD sufferers develop renal insufficiency very early in life, meaning that there is a need for long term treatment and a significant disease burden for life.

CKD incidence is increasing at a rate of 8% per year in the adult population, mainly due to the increase in conditions such as diabetes, hypertension and obesity [24]. As these conditions become more prevalent in the paediatric population it is expected that the rate of paediatric CKD will increase in a similar way to that of the adult population. It is also possible that these conditions may affect those children with a congenital or hereditary cause of CKD and contribute to a faster disease progression.

Many cases of CKD in both adults and children eventually progress to ESRF requiring dialysis and/or transplantation. Thus, there is an urgent need to develop new therapies to further slow or halt the progression of the disease to avoid or delay the requirement for dialysis and/or transplantation.

1.5 Current treatments for CKD

Current treatments for CKD centre on specific therapies based on diagnosis, evaluation and management of co-morbid conditions (such as hyperlipidaemia, hypertension, anaemia, acidosis, growth failure) and slowing the loss of renal function [21]. Treatments to preserve residual renal function in both adults and children with CKD for as long as possible are important both for quality of life and health care resource implications. The treatment of children with dialysis for ESRF presents special difficulties such

as difficult vascular access, increased infection risks and psychosocial issues. Transplantation solves the difficulties associated with dialysis, but presents a different group of health care challenges.

Australian data on renal transplant graft survival shows that about half of all paediatric patients who receive a transplant under 18 years of age will have the graft fail within 10 years [3]. This is similar to other parts of the world with an equivalent health care system. A large Dutch study found graft survival for kidneys transplanted into children under fifteen years was 59.2%, 45.3%, 35.4%, and 30.3% at 5, 10, 15, and 20 years respectively [25].

Whilst newer anti-rejection therapies are improving graft survival, patients who receive renal transplants as young children will almost inevitably require renal replacement therapy or a subsequent transplant at some stage throughout their lifetime. Children who receive a successful transplant still have major health issues. They are at higher risk of post transplant infectious complications and lymphoproliferative disorders [26], and side effects from steroids used for immunosuppression often cause impairment of growth and development. Non-compliance of adolescent transplant recipients with prescribed therapies remains a significant issue [27], resulting in a high risk of graft loss during the adolescent years. New treatment strategies that slow or halt the progression of CKD and prolong residual renal function would significantly improve outcomes and possibly reduce the need for subsequent kidney transplant in this group of patients.

A recent National Institute of Health (NIH) review of research priorities for paediatric nephrology suggested that identification and characterisation of systemic inflammation in children with CKD is essential for the development

of new treatment strategies [20]. In particular, the review highlighted the need for comprehensive studies such as the one described in this thesis that define the inflammatory and immunological changes present in children with CKD with a view to identifying new treatment targets. Of particular note is the inclusion in this study of children with very mild disease as a basis for a better understanding of the changes that are present in the early stages of CKD.

1.6 Systemic Inflammation

Systemic inflammation is characterised by changes in the immune system, including increased activation of leucocytes and associated changes in inflammatory cytokine production [28]. A persistent systemic inflammatory response causes characteristic acute phase changes. These include changes in lipid metabolism, hypoferraemia, increased protein catabolism, activation of the complement pathway, hormonal changes and induction of acute phase proteins such as CRP [16, 17].

Induction of acute phase changes is regulated by cytokines and other extracellular signalling molecules that are produced by a variety of cell types. Patterns of cytokine production and the acute phase response vary in different inflammatory conditions [28]. Under normal conditions, the acute phase response lasts a few days, but in chronic inflammatory disorders, the acute phase response is prolonged and has been shown to be associated with endothelial dysfunction, atherosclerosis, cardiovascular disease and anaemia [29, 30].

Various biological markers such as pro-inflammatory cytokines, leucocyte activation markers and CRP can be measured in blood and used to assess the presence of systemic inflammation.

Inflammatory processes are now recognized to play a central role in the pathogenesis of atherosclerosis and its complications. Plasma levels of several markers of inflammation have been found to be associated with future cardiovascular risk in a variety of clinical settings. These markers include cell adhesion molecules, cytokines, enzymes and C-reactive protein (CRP) [31].

1.7 CKD as a systemic inflammatory state

Chronic systemic inflammation is a common feature of adult CKD [32] and has been widely studied in those patients who have progressed to dialysis. Foe example, in adult dialysis patients, especially those on haemodialysis, significantly increased levels of markers of systemic inflammation such as CRP and IL-6 have been reported [8, 33]. The causes of this inflammatory state are variable but can include vascular access infection, bioincompatible dialyser membranes, backfiltration of non-sterile dialysate and pyogenic infections [34]. Optimised haemodialysis therapy can reduce levels of markers of systemic inflammation, however they do not return to normal. This suggests that other factors besides dialysis contribute to the inflammatory state [35]. This is supported by other studies that have considered levels of inflammation in pre-dialytic patients and shown increases in pro-inflammatory markers [36, 37].

Chronic systemic inflammation is of particular concern for patients with CKD , as it may lead to many of the complications associated with renal disease such as anaemia, muscle wasting, malnutrition and CVD [38]. Of particular note is that After stratification for age, race, and gender, cardiovascular disease mortality rates for dialysis dependent adults are approximately 10-20 times those of the general population [39]

In adults with CKD, systemic inflammation contributes to a syndrome known as the malnutrition, inflammation and atherosclerosis (MIA) syndrome [40].

The clinical state of uraemia has been reported as one cause of systemic inflammation in CKD due to increased oxidative stress and protein carbonylation [41, 42] resulting from a reduction in GFR causing an increase in accumulation of urea and other toxins in the blood. However, other pre-existing or underlying disease states may also contribute to systemic inflammation in adult patients with CKD [36, 40], and this makes the contribution of uraemia *per se* to the inflammatory state unclear.

In many cases, renal disease is not detected in adults until the disease is quite advanced and there has been a significant decline in renal function causing symptoms. Most studies of adults with CKD have been performed in patients with stage 4 and 5 disease, thus presence of systemic inflammation early in the disease and the contribution of uraemia induced inflammation to the progression of the disease has not been widely examined.

This study in children, which includes subjects with mild disease and excludes those with any concurrent inflammatory disorders, thus presents a unique opportunity to study the contribution of uraemia *per se* to systemic inflammation in renal disease.

1.8 Oxidative stress in CKD

Oxidative stress is the disturbance of the natural balance of oxidant production to antioxidant defence. The resulting accumulation of reactive oxygen species (ROS) results in toxic effects on cells as well as increases in the production of pro-inflammatory cytokines [43].

CKD is reported to be associated with an increase in oxidative stress due to an accumulation of reactive aldehydes because of decreased renal catabolism and increased production [44]. Patients with CKD also show a reduction in reduced thiol groups [45], which have an antioxidant function as redox buffers, and an increase in oxidised thiols such as homocysteine which may have pro-inflammatory effects. Numerous markers of oxidative stress have been evaluated in adults with CKD, and it is now well accepted that CKD in adults is accompanied by an increase in oxidative stress [44].

There has been little investigation of oxidative stress in children with CKD, particularly those that have not yet progressed to dialysis. One study by Sebekova et al [46] reported an increase in advanced glycation end products (AGE) in children with CKD dialysed or not. Another study performed in children with stage 4 and 5 CKD that was published after this study commenced found increased levels of oxidative stress accompanied by increases in TNF-α and IL-6 as well as early cardiovascular damage measured via left ventricular mass and intima media thickness [47]. This indicates a link between oxidative stress and inflammation in this group and supports my original hypothesis.

Since an increase in oxidative stress is likely to contribute to increased systemic inflammation, the investigation of levels of oxidative stress in

combination with a comprehensive evaluation of systemic inflammation has been performed in this study.

1.9 Inflammatory markers in adults with CKD

Cytokines are a group of small molecular weight proteins that are secreted by cells in response to an external stimulus. They act as inter and intra cellular signalling molecules and are physiological messengers of the inflammatory response. Most cytokines have multiple sources, targets and functions [28]. There have been many reports of increased level of circulating pro-inflammatory cytokines in adults with CKD [11, 36, 37, 48-50]. The cytokines that participate in the inflammatory response are responsible for stimulating production of acute phase proteins. These pro-inflammatory cytokines include interleukin IL -6, IL-8, IL-1β, IL-12p70, and TNF-α. Selected cell and inflammatory cytokine interactions of the immune system are presented in Figure 1.1.

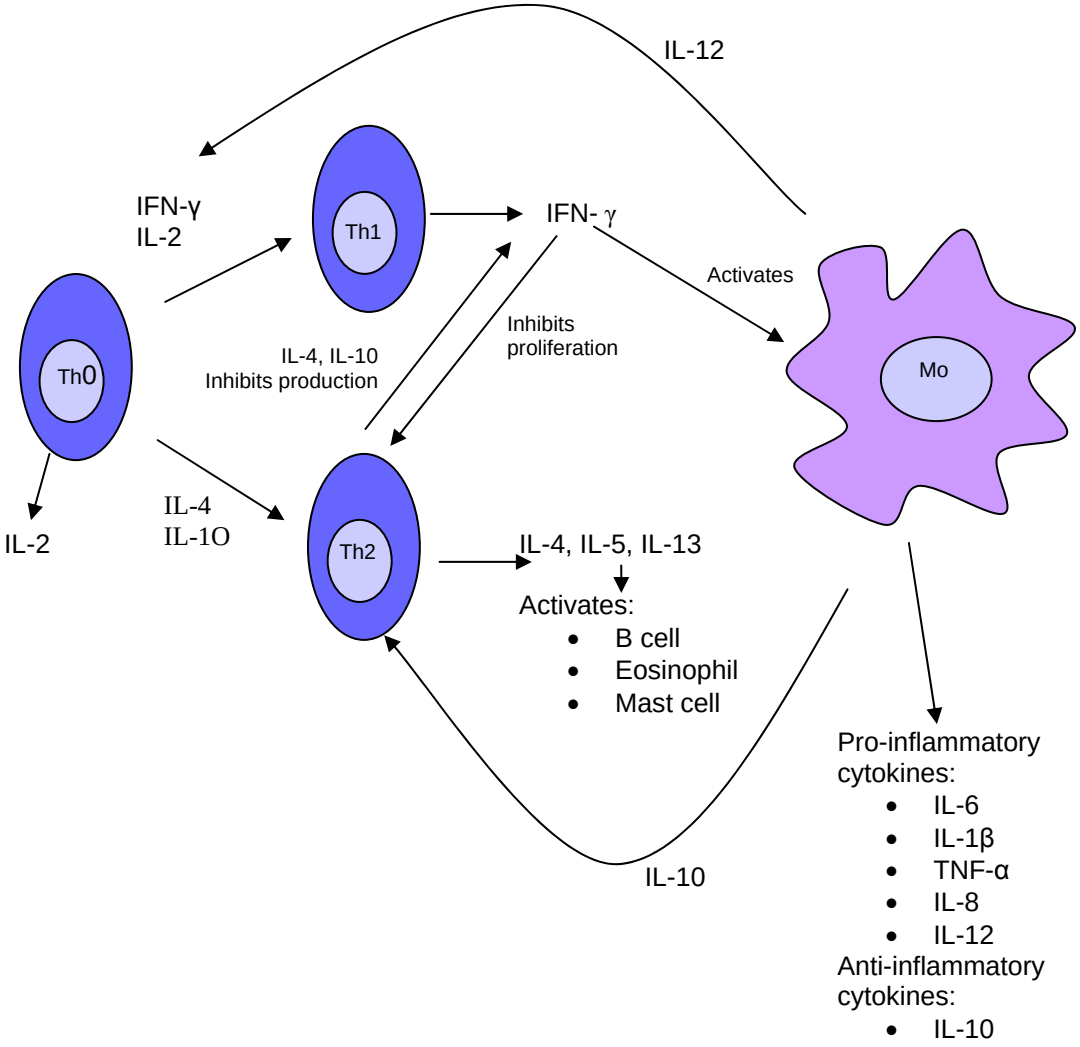


Figure 1.1: Selected cell and cytokine interactions of the immune system. Th1 cell production of IFN- γ counter regulates Th2 cell production of IL-4, IL-5 and IL-13 and vice versa. Monocyte production of IL-12 influences cells towards a Th1 phenotype, whereas monocyte IL-10 production promotes a Th2 response.

IL-6 upregulates CRP by increasing the transcription of CRP messenger RNA (mRNA). IL-1β further regulates the production of CRP by amplifying the stimulatory effect of IL-6 [51]. In the presence of IL-6, TNF-α has also been shown to up regulate the production of CRP [28]. IL-12p70 is an inflammatory cytokine produced primarily by monocytes that activates T & Natural Killer (NK) cells. IL-12p70 has been shown to be associated with other chronic inflammatory conditions such as inflammatory bowel disease and systemic lupus erythematosus [52-54]. Other pro-inflammatory cytokines contribute to the acute phase response depending on the presence or absence of other cytokines and their inhibitors.

Two reasons for increased cytokine levels in CKD patients have been reported; (i) elevated numbers of circulating monocytes, (ii) an increased level of production of cytokines per monocyte [55]. Panichi *et al* [9] measured levels of CRP and IL-6 in adults with pre-dialytic renal failure. This study compared levels of these analytes with established normal reference ranges. CRP and IL-6 results showed an inverse correlation to renal function (as measured by creatinine clearance), thus suggesting that decreased renal clearance of CRP and/or cytokines may contribute to the accumulation of acute phase reactants.

Two studies by Herbelin *et al* [50, 56] examined levels of pro-inflammatory cytokines in adult dialysis patients. The first study [50] compared plasma IL-1 and TNF-α in dialysis patients compared with healthy controls. Patients were divided into three groups; healthy controls, patients undergoing their first dialysis session (i.e. uraemic pre-dialysis), and long term dialysis patients. Specimens were collected from the dialysis groups both pre and

post dialysis so that the effect of a dialysis session on IL-1 and TNF-α could be measured.

First session dialysis patients showed significantly increased levels of TNF-α prior to their first dialysis session compared to healthy controls. Long term dialysis patients had significantly increased IL-1 and TNF-α pre dialysis. The dialysis session caused a further significant increase in the level of IL-1 compared to pre dialysis in the long-term dialysis patients only. Plasma levels of T cell derived cytokines IL-2 and Interferon (IFN) -γ were also measured, but these were undetectable in all groups. In the second study performed by this group [56], IL-6 and the relationship between IL-1 and TNF-α was examined. IL-6 was significantly increased in both non-dialysed and dialysed patients with CKD, however there was no significant difference in the levels of IL-6 pre and post dialysis in the dialysis group indicating that the dialysis procedure itself did not induce IL-6. The study showed significant correlation between IL-6 and IL-1 and between IL-6 and TNF-α. The results from both studies support the hypothesis that the increased levels of plasma cytokines in CKD are secreted by activated monocytes although this cannot be confirmed as no measurements of monocyte activation were performed in either study.

A study by Malaponte *et al* [13] investigated levels of IL-1β, IL-6 and TNF-α in plasma and cultured monocytes stimulated with LPS. Blood was collected from healthy controls, pre-dialysis renal failure and dialysis patients. Dialysis patients were divided into three groups based on the length of time they had been on dialysis (short, medium or long term). Plasma levels of pro-inflammatory cytokines were increased compared to controls in all CKD

groups, with dialysis patients showing higher levels than non-dialysis subjects. Levels of cytokines produced by cultured monocytes decreased in proportion to dialytic age. This inverse relationship to the levels of circulating cytokines suggests that monocytes are progressively activated by dialysis, which ultimately leads to monocyte dysfunction.

1.10 Systemic inflammation in children CKD

Systemic inflammation in children with CKD has not been characterised in detail. As previously discussed, adult renal disease is nearly always the cumulative result of a number of concurrent disease states that may directly increase underlying levels of inflammation. As well, in an adult population, there are other lifestyle factors such as alcohol consumption, smoking and obesity which contribute to the inflammatory process [57].

In contrast, the majority of paediatric CKD is caused by congenital abnormalities with no other underlying diseases or inflammatory states [19]. In addition, compounding lifestyle factors such as smoking and alcohol consumption are not usually present. Patterns of inflammatory response and cytokine production may vary depending on inflammatory stimulus; thus the inflammatory state observed in adults with renal disease cannot be directly translated to children with CKD because of a combination of differing stimuli and immaturity of their immune system.

Despite these differences, children with renal disease exhibit many of the complications seen in adult renal disease that are associated with inflammation, and it is possible that the inflammatory abnormalities observed in adults are replicated in children. Very little research has been done to

define inflammatory markers and immune system abnormalities in children with CKD despite the fact that these changes are likely to contribute to the progression of CKD and many of the co-morbid conditions that are observed in this group of patients.

1.11 Pro-inflammatory cytokines in children with CKD

There have been few studies examining circulating cytokine levels in children with CKD. No studies have comprehensively examined a range of plasma cytokines or investigated cytokine production on a cellular level. Sebekova *et al* [58] studied plasma levels of CRP, TNF-α and IL-6 in paediatric pre-dialysis, dialysis and transplant patients. Plasma TNF-α was significantly elevated and there were marginal increases in other pro-inflammatory markers in all CKD groups. In this study, none of the parameters measured was compared to a control group of healthy subjects.

A study performed by Zwolinska *et al* [59] examined plasma levels of IL-2, IL-6 and TNF-α in children on dialysis. This study found that there was no significant difference in IL-6 levels, but both IL-2 and TNF-α were significantly higher in dialysis patients when compared with healthy controls. The study did not consider children who had not yet progressed to dialysis. The finding of increased IL-2 levels suggests that children may show evidence of T-cell activation as this cytokine is primarily produced by T cells.

IL-10 has been classified as a Th2 or Th3 anti-inflammatory cytokine that negatively regulates the production of Th1 pro-inflammatory cytokines [60]. Increased levels of circulating IL-10 have the potential to limit the level of

Th1 pro-inflammatory cytokine levels and hence systemic inflammation. In this regard, a polymorphism in the IL-10 gene that causes variability in IL-10 production has been reported in adult CKD groups [61]. This variability in ability to produce IL-10 leads to distinct patient groups that results in alterations to uraemia and dialysis induced inflammation [10]. No studies to date have measured levels of the regulatory cytokine IL-10 in children with CKD.

Experiments performed in this thesis examine both circulating plasma cytokines and leucocyte production of cytokines at a cellular level to give a comprehensive overview of changes in pro and anti-inflammatory cytokines associated with paediatric CKD.

1.12 Leucocyte subsets in CKD

Few studies have examined leucocyte subsets in children with CKD, with conflicting results. Bouts *et al*. [1, 62] characterised leucocyte subsets in four groups of children (haemodialysis, peritoneal dialysis, non-dialysed kidney failure and healthy controls). Total monocyte counts were significantly reduced in the pre-dialysis renal failure and peritoneal dialysis groups but increased in the haemodialysis group compared with age matched controls. Total lymphocyte counts were lower in the renal failure group when compared with controls. Total T-cells, B cells, NK cells and cytotoxic (Cluster Differentiation (CD) 8^{+}) T cells were all reduced. The CD4/CD8 ratio was increased in contrast with similar studies in adults that report normal CD4/CD8 ratios [4, 63]. Taken together, the authors suggest that these changes may favour the frequent occurrence of infections

A second study by Bouts [1], reported elevated lymphocyte and monocyte levels of Complement Receptor 3 (CD11b) in children with both dialysed and non-dialysed CKD. As well as acting as a complement receptor, CD11b also functions as an adhesion molecule to mediate the adhesion of leucocytes to the endothelium and in this regard may contribute to endothelial dysfunction and early cardiovascular damage [64].

Two further studies have partially examined lymphocyte subsets in children with ESRF on dialysis with conflicting results [63, 65]. A study by Deenitchina et al [63] found an increase in total T cell ($CD3^+$) but normal $\gamma\delta$ T cell numbers as well as a decrease in B cell numbers in dialysis dependant children.

This is in contrast to a study in children by Szczepanska et al [65] that investigated T cell subsets and levels of activation in children on dialysis. Their results showed a significant increase in total T-cells ($CD3^+$) and activated T cells ($CD25^+$) as well as significant reductions in $\alpha\beta$ and $\gamma\delta$ T cell numbers.

$\gamma\delta$ T cells make up about 5% of the total circulating T cell pool [66] and have the ability to recognise antigens directly, without the need for MHC molecules. They have a specific and specialised role in the protective immunity via cytokine secretion and cytotoxic activity and influence CD8 $\alpha\beta$ T cells to differentiate into cytotoxic lymphocytes [67]. A reduction in $\alpha\beta$ and $\gamma\delta$ T cells may thus be partly responsible for the impaired T cell responsiveness which observed in children undergoing dialysis therapy. In this study I have quantified both $\alpha\beta$ and $\gamma\delta$ T cell subsets to determine whether this subset is affected by uraemia *per se*.

No prior studies have examined lymphocyte subsets in children with pre-dialytic CKD, therefore it is not known whether the changes that are observed in dialysis patients are due to the dialysis procedure or uraemia. Studies performed in this thesis examine a large range of functional leucocyte subsets to determine the effect of uraemia on leucocyte subset profiles.

1.13 Leucocyte activation in CKD

Whilst the presence of increased levels of circulating pro-inflammatory cytokines in adults with CKD is well documented, there has been limited investigation as to the source of these circulating cytokines. As discussed in 1.9, it is suggested that the most likely source of cytokines is activated monocytes and macrophages, since the immune activation in these patients appears not to be antigen specific [10].

Increased activation of antigen presenting cells, particularly monocytes, and associated increased cytokine production have been recognised as factors contributing to the inflammatory state observed in adult CKD patients [68]. To my knowledge there are no studies that have examined the activation state of *ex vivo* leucocytes from patients with non-dialysed CKD. A study by Sester *et al* [55] showed that adult dialysis patients had a significantly increased level of activated pro-inflammatory ($CD14^{+}/CD16^{+}$) versus classical non-activated ($CD14^{++}/CD16^{-}$) monocytes. In dialysis patients, pre-dialysis specimens showed 16.3% of monocytes were of the $CD14^{+}/CD16^{+}$ pro-inflammatory phenotype versus 11.2% for healthy controls.

Investigation of the activation status of leucocytes in CKD, especially non-dialysed patients is thus warranted to provide a better insight into the mechanisms of immunological changes in the disease. Such data may provide a basis for new treatment strategies.

1.14 Identification of potential therapies

Once CKD in children is confirmed as being associated with a chronic systemic inflammatory state, the effectiveness of new and novel anti-inflammatory and anti-oxidant therapies can be assessed. In this regard, several therapeutic agents have recently been found to have anti-inflammatory properties in addition to their traditional applications. Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) have traditionally been used as primary therapy to reduce cholesterol levels. Recently however, statins have been recognised as having a number of pleiotropic effects including improvement of endothelial function, anti oxidant properties and inhibition of inflammatory processes [69]. Statin therapy for non-vascular inflammatory conditions has recently been used successfully to reduce systemic inflammation in a number of conditions such as multiple sclerosis and rheumatoid arthritis [69]. Whilst there is no published literature that reducing inflammation in children with CKD will delay the progression of the disease, the anti-inflammatory effect of statins may make them an attractive candidate as a new treatment for CKD, with the possibility of reducing CVD co-morbidities in the patient group.

Anti-oxidant therapies such as vitamin E, vitamin C, N-acetyl cysteine and L-arginine are potentially useful treatments to reduce cell and tissue injury

related to oxidative stress. These have been used successfully to ameliorate oxidative effects of dialysis [70, 71]. In addition several other agents have also been recently recognised to have anti-inflammatory effects and may be possible future adjunct treatments for CKD. These include resveratrol, a naturally occurring phenolic anti-oxidant [72, 73], gabexate mesilate, a synthetic protease inhibitor used in the treatment of pancreatitis [74, 75] and curcumin, a polyphenol with antioxidant and anti-inflammatory properties [76]. It is possible that the data generated from this study will allow the selection of likely treatment agents based on the observed alterations of inflammatory markers. These could then be assessed clinically in future studies for their effectiveness in treating oxidative stress and inflammation. Well designed, randomised controlled trials will be required to assess the utility of these agents in CKD.

1.15 Summary

In summary, the inflammatory and immunological status of children with CKD is presently unclear and the literature contains conflicting information. A recent report from a National Institute of Health task force in the USA recommended that the characterisation of the state of inflammation in children with CKD should be a priority [20]. Clear characterisation of the inflammatory state of children with CKD is the first step in identifying potential therapeutic targets. Of particular importance in this study, and a major point of difference from other studies, is the inclusion of children with early stage CKD, which may identify early treatment options with the potential to maintain native renal function.

1.16 Hypotheses

- Children with CKD will show evidence of increased circulating and/or cellular inflammatory and oxidative stress markers that are not associated with acute infection.
- Increased inflammatory markers will be accompanied by changes in leucocyte subset numbers and surface molecules associated with leucocyte activation and adhesion.

1.17 Aims

To characterise inflammatory markers and immune cell subsets in children with CKD by measurement of:

- Circulating pro and anti inflammatory cytokines
- Cellular cytokine production after *in vitro* stimulation of T cells & monocytes.
- Markers of oxidative stress
- Circulating E-selectin
- Functional leucocyte subsets
- Leucocyte activation markers

Chapter 2

Research plan, study subjects and general methods

2.1 Overview

This chapter includes details of the study population and general techniques used in these investigations. Details of specific methods are provided in the relevant chapters. Twenty two CKD patients with disease grades 1-4 (i.e. not receiving dialysis therapy) were recruited for the study along with 24 age matched controls. Blood was collected from all participants. Specimens were analysed for acute phase markers of inflammation – specifically plasma CRP and erythrocyte sedimentation rate (ESR). Complete blood picture (CBP) with white cell differential and plasma creatinine were performed. Plasma was frozen at -70^{o}C for later analysis of markers of oxidative stress, pro and anti inflammatory cytokines and soluble E-selectin (sE-selectin). Surface marker antigens that determine leucocyte subsets, leucocyte function and activation and intracellular cytokine staining were all performed on peripheral blood. Full details of all leucocyte markers and cytokines as well as the cell types that were studied can be found in the relevant chapters.

2.2 Research Plan

2.2.1 Subjects

The study protocol was approved by the ethics committee of the Women's and Children's Hospital (approval no 1555 2/2007). Informed consent was obtained prior to blood collection from all children and/or parents The information sheet and consent forms used in the study can be found in appendices 1 & 2. Twenty two children with CKD defined as stage 1, 2, 3 or 4 according to the National Kidney Foundation system [21] but not on dialysis and 24 age matched controls were enrolled in the study.

Exclusion criteria for all subjects included infection, fever >38^{o}C, white cell count <4.0 or >11.0 cells/10^{9}/L, abnormal white cell differential, CRP >15mg/L and the use of immunosuppressive or anti-inflammatory drugs. Control patients were excluded if they had a GFR of <100 mL/min/1.78m^{2}. All study participants underwent a general medical examination on the day of blood collection and were reported as being completely well in the two weeks prior to blood collection.

The CKD group was recruited from CKD patients that are seen regularly by a multi-disciplinary team who manage their care. The clinical and nutritional management strategies used in this clinic have been published previously [77] and growth and developmental outcomes meet best practice expectations. The median age of the CKD group was 14 years (range 5-17) and there 12 male and 7 female subjects. The control group was a group of otherwise healthy children with no history of any chronic disease who were undergoing minor day surgery. The median age was 9 years (range 4-17) with 15 males and 9 females.

None of the patients in either group had evidence or history of an altered immune or inflammatory state. Patient demographics and causes of CKD for the CKD group are outlined in Table 2.1.

Patient ID number	Age / Gender	GFR (mL/min/1.78m^2)	Stage of CKD	Cause of CKD
1	13 / F	43	3	Reflux nephropathy
2	13 / M	57	3	HUS
3	10 / M	84	1	Reflux nephropathy
4	15 / F	23	4	Nephronopthisis
5	17 / M	69	2	Reflux nephropathy
6	17 / F	40	3	HUS
7	11 / M	58	3	Reflux nephropathy
8	13 / M	91	1	HUS
9	15 / F	16	4	Nephronopthisis
10	13 / M	65	2	Uncertain
11	16 / M	80	2	Post urethral valves / ? ADPCKD
12	14 / M	51	3	HUS

Patient ID number	Age/ Gender	GFR (mL/min/1.78m^2	Stage of CKD	Cause of CKD
13	15 / F	32	3	Cortical necrosis
14	15 / M	69	2	Reflux nephropathy
15	7 / M	15	4	HUS
16	12 / M	52	3	Reflux nephropathy (+ right nephrectomy)
17	15 / M	38	3	Reflux nephropathy
18	10 / M	20	4	HUS
19	6 / F	52	3	Renal dysplasia (Melnick – Fraser syndrome)
20	13 / M	42	3	Neurogenic bladder
21	15 / F	46	3	PUJ obstruction
22	5 / M	43	3	Cortical necrosis

Table 2.1: Patient demographics and causes of CKD. Abbreviations HUS – Haemalytic Uremic Syndrome, ADPKD – Autosomal Dominant Polycystic Kidney Disease, PUJ – Pelviureteric junction.

2.2.2 Blood Collection and analysis

All CKD subjects underwent routine blood collection as part of their clinical management. An additional 4 – 6 mL of heparinised blood was collected at the same time as routine sample collection. Control subjects had approximately 4 – 6 mL of heparinised blood collected prior to induction of anaesthesia.

Routine diagnostic haematology (CBP, ESR) and biochemistry tests (electrolytes, renal function, liver function, lipids, CRP, and parathyroid hormone (PTH)) were performed on the CKD group. CBP, ESR, Creatinine and CRP were determined for the control group. All routine diagnostic analyses were performed by the nationally accredited pathology laboratories at the Women's and Children's Hospital, Adelaide, South Australia.

Renal function was estimated from serum Creatinine values by using a modified Schwartz formula [78] to calculate GFR. The formula and constants used in calculation of GFR were as follows:

$$\frac{\text{Creat (µmol/L) x constant*}}{\text{Height (cm)}}$$

*constant:

- Pre-pubescent male 44
- Post-pubescent male 40
- Female 44

Plasma was stored at -70^{o}C for soluble cytokine assays, sE-selectin and measurement of markers of oxidative stress. Cells were processed within one hour of collection for flow cytometric determination of leucocyte subsets and leucocyte functional markers, including activation and adhesion markers and co-stimulatory molecules. Details of the specific techniques for each analysis are outlined in the relevant chapters.

Blood cultures with appropriate cell stimulation were set up to measure intracellular cytokine production via flow cytometry and cytokine secretion via enzyme linked immunosorbent assay (ELISA). The specific techniques for cell culture and cytokine analysis are outlined in the relevant chapters.

2.3 Flow Cytometry

2.3.1 Overview

Many of the techniques in this study utilise flow cytometry. A general overview of the principles of and general methods for flow cytometry are outlined below. Specific techniques are outlined in the relevant chapters.

Flow cytometry is a method for quantitation of cell populations and determination of cellular features. It allows multiparametric analysis of single cells, and has the capacity to analyse many thousands of cells per second. Cells are suspended in a stream of fluid and pass through a laser beam individually, disrupting and scattering the incident light as they pass. Several photomultiplier tubes (PMT) are used to detect the patterns of light scatter. A detector in line with the light source records narrow angle scatter (1^o – 10^o) – referred to as forward scatter (FSC) – which is correlated approximately with cell size. Several detectors are perpendicular to the light source, one detects wide angle scatter – referred to as side scatter (SSC) – and the others are fluorescent detectors. The SSC properties of a cell depend on its internal characteristics (i.e. shape and complexity of the nucleus, amount and type of cytoplasmic granules). Fluorescent detectors are used to measure light emitted from fluorochromes that can be excited by the laser in the flow cytometer. Antibodies to the antigen of interest are tagged with a

fluorochrome which is then used to stain cells for surface or intracellular antigens of interest.

Fluorescent dyes absorb the blue (488nm) light produced by the argon laser, and emit a portion of this light in different regions of the spectrum. With the use of optical filters, the emission signals are separated by the flow cytometer. Light emitted in the green region of the spectrum is measured in Fluorescence Channel (FL)-1, light emitted in the orange region in FL-2 and light emitted in the far red in FL-3. The fluorochromes used in this study were fluorescein isothiocyanate (FITC), a small organic molecule, typically conjugated to proteins via primary amines, Phycoerythrin (PE), a phycobiliprotein- based fluorochrome, derived from algae, which can be conjugated to antibodies for use in immunophenotyping, and PE-Cy5 (PC-5). PC-5 is a tandem dye consisting of an indotricarbocyanine dye coupled to PE.

Absorption and emission wavelengths of the fluorochromes used in this thesis are outlined in Table 2.2.

Flourochrome	Absorption	Emission (nm)
Fluorescein isothiocyanate (FITC),	490	530 (green)
Phycoerythrin (PE)	480	578 (orange)
PE-CY5 (PC-5)	480	660-697 (red)

Table 2.2: Absorption and emission wavelengths of fluorochromes used in this study.

All flow cytometry techniques in this study were performed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA (BD)) fitted with an air-cooled 488nm argon laser. This flow cytometer allows the detection of up to three different fluorescent parameters

2.3.2 Definition of terms used in flow cytometry

Cluster Differentiation (CD) system

The identification of monoclonal antibodies with similar patterns of reactivity with human cells has been the focus of the Human Leucocyte Differentiation Antigen (HLDA) international workshops. Each group of antibodies has been assigned a CD number. Not all antibodies in a CD group react with identical portions (epitopes) of their target antigen. An antigen recognised by a given cluster of antibodies (e.g., CD4) is referred to as a ‘CD antigen’ (e.g., CD4 antigen).

Monoclonal antibodies (Mabs)

Highly specific antibodies are obtained by fusing mouse immune B-cells from the spleen with tumour cells to produce hybridomas, each of which will then secrete a single antibody. Such antibodies can then be labelled with fluorescent dyes. The epitope (antigenic determinant) is that portion of an antigen against which the specific binding region of a monoclonal antibody reagent is directed. Epitopes may be linear sequences of as few as six amino acids sections of the antigen; each antigen typically contains multiple epitopes.

Immunophenotyping

Discrimination among subsets of leucocytes utilising fluorescence labelled Mabs that recognise membrane associated molecules.

Data display

Data may be displayed as dual or single parameter displays.

- Dual parameter displays (dot plot, histogram, contour plot) are graphic representations of data in which corrected intensities for two different parameters are measured on the same cell and plotted on an x,y grid (eg, log FITC and log PE immunofluorescence). From this display, percentages showing positive fluorescence for a particular marker could be calculated.
- A single parameter display (histogram) shows frequency distribution of measured signal intensities observed for cells within a population.

Forward Angle Light Scatter (FSC)

Measurement of light at a low radial angle relative to the incident light source. Measured values are a function of the cross-sectional area of a cell or particle and the wavelength used for measurement. It is commonly used as a measure of the relative size of a cell.

Side scatter (SSC, Ninety Degree Light Scatter)

Measurement of light at right angles to the incident light source. This measurement is related to cytoplasmic granularity, membrane irregularity and/or nuclear shape of a cell.

Gate, gating

A set of parameters used to evaluate additional parameters of a particular cell. Typically, a region of interest was defined based on one set of parameters (eg, FSC vs. 90^{0} SSC) and then other properties (eg, fluorescence) are evaluated only for cells within the defined region.

Autofluorescence

The intrinsic fluorescence of unstained cells which is generally caused by pyrimidines and flavin nucleotides. The level of autofluorescence varies depending on the cell type being analysed and/or the state of cellular activation.

Quadrant markers

Background staining occurs due to a combination of natural autofluorescence of cells and non-specific binding of Mabs to cells. This staining is defined using IgG mouse antibodies directed to *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in humans. The dual parameter display (eg, dot plot) that is obtained after staining with

IgG mouse antibodies is divided into rectangular quadrants by the use of two perpendicular boundaries (quadrant markers). These markers are then set so that background readings of less than 1% are obtained.

Resolution

The ability to discriminate between cells having different signal intensities. Such ability is a function of biological factors (heterogeneity of signal within each population, difference in signal intensities between populations) as well as instrumental factors (sensitivity).

Sensitivity

The ability to distinguish signals of interest from background ‘noise’. ‘Noise’ may be instrumental (optical or electronic signals arising when no fluorochrome or cell is present) or biological (autofluorescence, non-specific reagent binding).

Colour compensation

Electronic subtraction of a fraction of one signal from a second, typically used in correcting for overlapping fluorescence from one fluorochrome in the wavelength region to the second.

Threshold

Level of signal above which a measured value is considered to be significantly different to background ‘noise’. Threshold for instrument fluorescence sensitivity is the level of signal found for what is considered to be a non-fluorescent object. Threshold for determination of positive antibody staining is the level of signal found for cells not believed to react specifically with a given antibody reagent.

Linear amplification

A linear amplifier produces a signal output proportional to the input signal amplitude. For example, a linear amplifier could have output varying from 1 to 5 volts as the input signal varies from 0.01 to 0.05 volts.

Logarithmic amplification

A logarithmic amplifier produces a signal output proportional to the logarithm of the input signal amplitude. For example, a three decade logarithmic amplifier will have an output varying from 0 to 10 volts as the input signal varies by a factor of 1000. Logarithmic amplifiers are useful when analysing samples containing cells whose measured parameters differ by orders of magnitude

2.3.3 Set up of flow cytometer for optimal performance

Weekly alignment of the flow cytometer was carried out using AutoCOMP Software (BD), to ensure that the cytometer recorded intensity of events consistently. AutoCOMP settings were stored in a file, and retrieved every time an alignment was carried out. The software is a menu-driven program that adjusts the photo-electric components of the FACSCalibur according to known standards. Gain settings and fluorescence compensation values were adjusted to standard samples of CaliBRITE beads (plastic microspheres) (BD). There are three different types of beads supplied:

i. Unlabelled
ii. Labelled with FITC
iii. Labelled with PE.

Three FACSCalibur adjustments were performed.

i. **Gating of single events** - upper and lower gates for FSC intensity were set to minimise the effects of doublets and debris, and eliminate data which did not fall within the set light scatter limits. By acquisition and analysis of 5,000 events from unlabelled beads, the program automatically set the upper and lower gates by statistical analysis of the resulting frequency histogram.
ii. **Adjustment of PMT voltage** - variable voltage settings of PMT influence their detection sensitivity so that increasing the voltage results in greater amplification of signal and alteration in channel distribution. The PMT was automatically set so that unlabelled CaliBRITE beads had a mean within 2 channels of target value. The

FSC photodiode does not have variable voltage setting so was unaffected by this procedure.

iii. **Fluorescence compensation** - FITC and PE have some overlap of emission spectra. Despite the use of emission filters in the flow cytometer which minimise the overlap, some FITC radiation reaches the PE detector and visa-versa. AutoCOMP compensated for this by electronically subtracting the unwanted signal from the signal of interest.

***Method**:*

i. One drop of unlabelled CaliBRITE beads was added to 1 ml Isoton II (Coulter, Immunotech, USA).

ii. One drop of each of unlabelled and FITC and PE labelled CaliBRITE beads were added to 3 ml Isoton.

iii. To adjust the PMT voltage, the tubes containing the beads were put on the FACSCalibur, making sure that the fluidics Mode Selector was set to HIGH.

Fluorescence compensation and sensitivity testing was then automatically performed. As PC-5 beads could not be obtained commercially, compensation of spectral overlap between FL-2 and FL-3 was performed manually during the procedure for optimisation of stained lymphocytes outlined below.

Following the AutoCOMP procedure, PMT settings were optimised for stained lymphocytes. Lymphocytes were stained using the whole blood lysis technique described in Chapter 5. Cells were stained with CD8 FITC or CD4 PE or CD3 PC-5 individually. Each individual stain was analysed using a FSC vs SSC gate set on lymphocytes. Individual PMT settings were optimised so that negatively stained

cells fell within the 10^0 - 10^1 scale. These settings were then used for subsequent acquisition of data.

2.3.4 Controls

Various controls are necessary in flow cytometry to confirm that cell staining is specific and reproducible. The types of controls used in this study are outlined as follows:

Sub-class control

Subclass refers to variations in immunoglobulin heavy and light chains. Most Mabs used in flow-cytometry are either IgM, or various subclasses of IgG (IgG1, IgG2a, IgG2b, or IgG3). Thus, a subclass control is an immunoglobulin of the same isotype (class or subclass) as the Mab of interest but without specificity for any known human antigens. This type of control confirms the specificity of the Mab to the antigen of interest.

Autofluorescence control

A tube containing unstained cells allows the determination of the background fluorescence of the cells.

Positive control

A peripheral blood specimen from a “normal” adult, used to verify the performance of reagents and staining procedures and to test new reagent lots. Variability for new lots of reagents compared to current lots is accepted when it is no greater than the variability found for replicate samples of the current lot.

Rainbow beads

Beads (plastic microspheres) with several fluorescent intensities and excitation wavelengths. These are used to ensure that the cytometer records intensity of events consistently. Beads were purchased commercially (BD).

2.4 Statistics and Analysis of data:

Analysis of data was performed using FACSCalibur flow cytometer and Cellquest software. Statistical comparisons between the groups were made using a non-parametric Mann-Whitney U-test. The analysis was performed using SPSS software version 11.0 (SPSS Inc, Chicago, IL, USA), p values <0.05 were considered significant.

2.5 Summary

This chapter contains:

- Research plan
- Details of the Study Population
- Overview of the principles of flow cytometry
- Instructions for set up of flow cytometer
- Rationale for statistics used

Specific methods for each of the techniques are found in the appropriate chapters.

Chapter 3

Assessment of oxidative stress and circulating pro-inflammatory cytokines

3.1 Introduction

3.1.1 Overview

As outlined in Chapter 1, CKD in adults is associated with an increase in circulating pro-inflammatory cytokines and markers of oxidative stress [12, 14, 34, 36, 41, 51, 79, 80]. The majority of previous studies have been performed in adults with relatively advanced pre-dialytic CKD (stage 3 or 4), or patients that have already progressed to dialysis. The present study is unique in that it investigates inflammatory markers in children with relatively mild renal impairment (majority of subjects are stage 2 and 3) and no underlying inflammatory conditions. This gives a unique insight into the inflammatory changes that are caused by uraemia *per se.*

In this chapter I investigate levels of circulating plasma cytokines via two different cytometric bead arrays (CBA) and assess oxidative stress by measuring Advanced Oxidation Protein Products (AOPP).

3.1.2 Hypothesis

Children with CKD will have increased levels of circulating cytokines and markers of oxidative stress that may indicate an increase in systemic inflammation.

3.1.3 Specific Aims

- To determine circulating plasma levels of AOPP, a marker of oxidative stress.
- To determine circulating plasma levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ using CBA.

3.1.4 Evaluation of Oxidative Stress – Background

CKD in adults is reported to be associated with an increase in oxidative stress due to an accumulation of reactive aldehydes caused by decreased renal catabolism and increased production [44]. Patients with CKD show a reduction in reduced thiol groups [45], which have an antioxidant function as redox buffers, and an increase in oxidised thiols such as homocysteine which may have pro-inflammatory effects [81]. Numerous markers of oxidative stress have been evaluated in adults with CKD; however there has been little investigation of oxidative stress in children with CKD. Based on the likely link between oxidative stress and inflammation, this study therefore evaluated the level of a marker of oxidative stress.

3.1.5 Choice of AOPP to evaluate oxidative stress

Two commonly used plasma markers for oxidative stress are advanced glycation end products (AGE) and AOPP. In uraemia, AGE are formed by the non-enzymatic glycation of proteins caused by increases in free radicals and reactive carbonyl species [82]. AOPP are formed during oxidative stress by the action of chlorinated oxidants and cause induction of pro-inflammatory cytokines [83]. This is represented diagrammatically in Figure 3.1

Both compounds are produced when there is an increase in oxidative stress and AOPP and AGE generally correlate well [84].

Many studies of renal disease have used AOPP as a marker of oxidative stress [42, 79, 83, 85, 86]. Other studies have shown that increases in AOPP are associated with accelerated atherosclerosis in uraemic patients

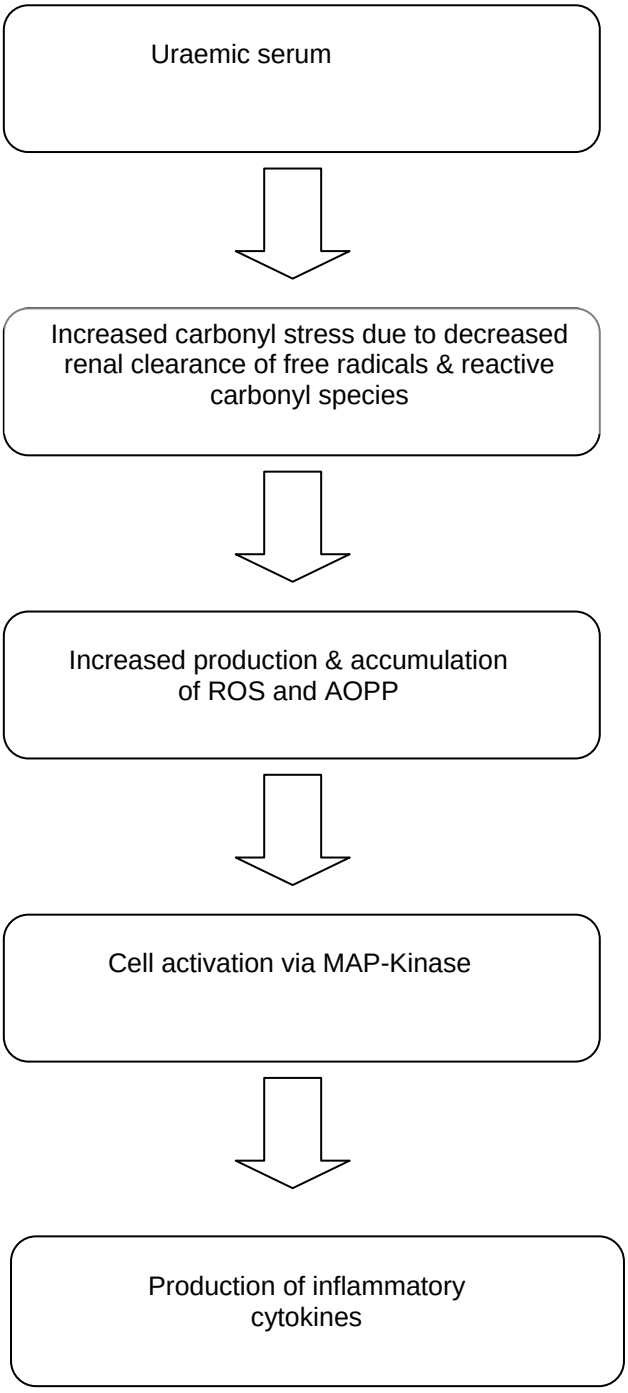


Figure 3.1: Effect of uraemic serum and accumulation of ROS on inflammatory cytokine production. Abbreviations ROS – Reactive Oxygen Species, AOPP – Advanced Oxidation Protein Products, MAP – Mitogen Activated Protein.

[86]. In the present study AOPP was chosen over AGE because the assay is more widely used in uraemic patient groups, is technically simple, sensitive and reliable, as well as requiring a smaller sample volume. The latter is important due to the limited sample volume available in this paediatric study.

My aim was to determine whether the impairment of renal function in the patient group was sufficient to cause an accumulation of reactive oxygen species and carbonyl compounds, resulting in an increase in AOPP.

3.1.6 Evaluation of pro-inflammatory cytokines in CKD

Oxidative stress may lead to systemic inflammation associated with an increased production of pro-inflammatory cytokines. As outlined in Chapter 1, many previous studies have shown increased levels of various circulating cytokines in CKD [10, 12, 14, 37, 48, 53, 58, 59, 87], however very few of these studies examined cytokines in children with CKD and none to date comprehensively examined a range of circulating plasma cytokines.

Patterns of inflammatory response and cytokine production vary depending on inflammatory stimulus. The inflammatory state noted in adults with renal disease cannot be directly translated to children with CKD because of differing inflammatory stimuli caused by coexisting and possibly inflammatory disease states in adults. Thus, this study presented a unique opportunity to examine inflammatory changes that are caused by uraemia *per se.*

To study this, a range of plasma pro-inflammatory and Th1/Th2 cytokines were investigated in children with CKD.

3.1.7 Choice of the cytometric bead array for measurement of cytokines

The cytometric bead array is a bead based ELISA technique that utilises flow cytometry to allow the simultaneous measurement of six cytokines from only 50 µL of sample. The assay uses beads with different fluorescent intensities with appropriate anti-cytokine antibodies bound to them. The major advantage of the bead array for this paediatric study is the small sample volume that is required. Without this technology, the measurement of such a large range of plasma cytokines in this paediatric population would not have been possible due to the large amount of sample required for traditional ELISA analyses. A further advantage of this technology is the significant time saving that comes from measuring all the cytokines simultaneously.

Details of the cytokines measured, their function and cellular origin are outlined in Table 3.1.

Cytokine	Major cell source	Function	Array
IL-1β	Activated monocyte Macrophage	Activates T/B cells. Induces IL-2 production.	Inflammatory
IL-6	Monocyte Macrophage	Major mediator of acute phase response. Stimulates B cell antibody production.	Inflammatory
IL-8	Monocyte	Activates Neutrophils. Chemotactic for migratory immune cells	Inflammatory
IL-12p70	Monocyte	T cell activation/proliferation. Differentiation of T cells to Th1 phenotype. Induces production of IFN-γ by T cells	Inflammatory
IL-10	Monocyte	Inhibits synthesis of pro-inflammatory Th1 cytokines	Inflammatory Th1/Th2
TNF-α	Monocyte Macrophage T cells	Cytotoxic. Promotes thrombotic processes, decreases thrombomodulin	Inflammatory Th1/Th2
IFN-γ	Th1 cells NK cells	Promoted cell mediated immunity required for antiviral and antimicrobial defence, immunoregulatory, inhibits Th2 cell activity.	Th1/Th2
IL-2	T cells	T,B and NK cell activation. Stimulates production of IFN-γ, IL-1, TNF-α, & TGF-β	Th1/Th2
IL-4	Th2 cells	Differentiation of T cells to Th2 phenotype. Enhances expression of MHC molecules on B-cells. Promotes humoral response	Th1/Th2
IL-5	T cells	Stimulates eosinophil growth and differentiation	Th1/Th2

Table 3.1: Cytokine measured in each array and cell sources and function of each cytokine. (Abbreviations IL – Interleukin, TNF – Tumour necrosis factor, IFN – Interferon TGF – Transforming growth factor, Th – T helper, MHC – Major histocompatibility complex) Compiled from Ibelgaufts et al 2006 and Jason et al 2001 [88, 89]

3.2 Material and Methods

3.2.1 Subjects

Twenty two children with CKD and 24 age matched control subjects were recruited. None of the subjects had any history of other underlying immunological abnormalities and all had been completely well in the 2 weeks prior to the study. Full details of the study populations, as well as exclusion criteria are outlined in detail in Chapter 2.

3.2.2 Samples

Heparinised blood was collected from subjects from the CKD group during routine clinic visits and from the control group prior to the induction of anaesthesia. Samples were centrifuged at 3000 rpm for 10 min within 1 hr of collection. Plasma for all assays was frozen at –70^{o}C immediately and stored for later analysis.

3.2.3 Measurement of AOPP

Measurement of AOPP was based on a spectrophotometric detection method described by Witko-Sarset et al [83]. Briefly, 200 µL of chloramine-T standard or plasma (diluted 1/5) was added to a 96 well microtitre plate. Twenty µL of glacial acetic acid was added to all wells, followed by addition of 10µL of Potassium Iodide to standard wells only. Plates were read at 340nm on a spectrophotometer as soon as possible and results expressed in chloramine units (µmol/L).

Reagent recipes and a detailed method can be found in Appendix 3.

3.2.4 Measurement of pro-inflammatory cytokines

Circulating plasma cytokines were measured using a bead based ELISA technique Cytometric Bead Array (CBA) manufactured by BD. An inflammatory CBA and a Th1/Th2 cytokine CBA were used according to the manufacturer's instructions to measure the cytokines of interest that were previously outlined in Table 3.1.

Briefly, individual capture beads for each cytokine were mixed to form the capture bead suspension. This was then centrifuged and the capture beads resuspended in serum enhancement buffer. Fifty µL of mixed capture bead suspension was added to 50 µL of the supplied detection reagent. This was followed by the addition of 50 µL of plasma or standard as appropriate. After 3 hr incubation at room temperature protected from light, samples were washed with the supplied wash buffer. They were then analysed immediately on a FACScalibur flow cytometer (BD) that had been calibrated with the cytometer set up beads supplied with the CBA kit.

3.3 Results

3.3.1 AOPP

There was a statistically significant increase in AOPP in the CKD group compared to control (CKD median 15.4 chloramine units µmol/L, range 8.7 – 81.8 chloramine units µmol/L, control median 12.3 chloramine units µmol/L, range 6.9 - 35 chloramine units µmol/L, p=0.044 Mann-Whitney). Pearson's correlation showed a statistically significant negative correlation between GFR and AOPP (Figure 3.2).

Results from the control group were used to calculate a 95% confidence interval (12.3 – 29.3 chloramine units µmol/L). There were three CKD patients with results that exceeded the control 95% confidence interval.

3.3.2 Cytokines

There were no statistically significant changes in any plasma cytokines measured between patient and control groups using the CBA bead array. Cytokine levels from the control group were used to calculate a 95% confidence interval.

All patients in both groups had levels of IL-2, IL-4 and IL-5 that were below the detection level of the CBA kit (5pg/mL). Eleven (50%) of the CKD patients showed an increase above the 95% confidence interval for the control group in at least one of the cytokines measured (Table 3.2). All of the subjects with increased AOPP also showed increases in at least one cytokine. The two subjects with the greatest increase in cytokines and AOPP had a severe reduction in GFR (CKD stage 4), whilst the third patient

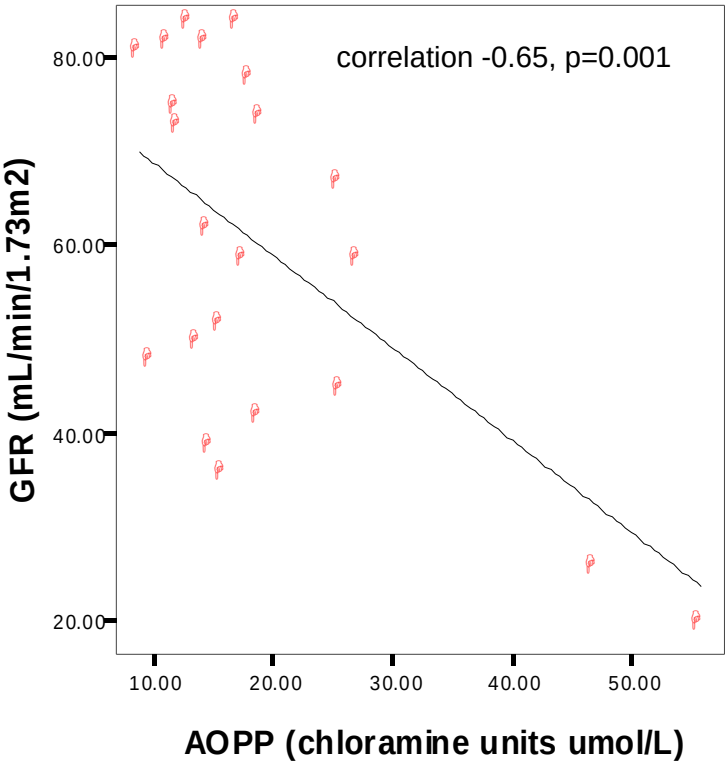


Figure 3.2: Correlation between GFR and AOPP. There was a statistically significant inverse correlation between GFR and AOPP (Pearson's correlation).

had a moderate reduction in GFR (CKD stage 3) associated with a modest increase in both the number and magnitude of cytokines (Table 3.2).

	GFR (Patient ID – table 2.1)	IL-12	Il-1b	TNF-a	IL-10	IL-6	IL-8	IFN-g	↑ AOPP
Cytokine 95% confidence interval		<10	<10	<5	<5	<5	<30	<12	
	43 (1)	7.8	**39**	<0	**10**	<0	13.3	10.3	
	57 (2)	**16**	0	0	0	0	5.9	3.5	
	84 (3)	**216**	**1001**	**350**	**125**	**40.2**	29.2	**18**	
	16 (9)	**174**	**891**	0	0	**45.3**	**39**	9.5	
	51 (12)	**382**	**1369**	**6**	0	**96.2**	28.1	7.7	
	32 (13)	**3.8**	**715**	0	0	0	4.6	5.7	
	15 (15)	**19**	**194**	0	**8**	0	6.1	5.5	*
	20 (18)	**65**	**419**	**12**	**95**	**4.2**	**34**	**16**	*
	52 (19)	**37**	**438**	0	0	0	21.9	6.2	
	46 (21)	**33**	<0	<0	1.4	<0	17.2	11.5	
	43 (22)	<0	<0	<0	2.9	1.4	**67**	10.5	*

Table 3.2: Cytokine profiles of patients showing increased cytokine levels compared to control values. Increases above the 95% confidence interval of the control group are shown in bold. Complete demographics and the cause of CKD for each patient with increased cytokines can be identified in Table 2.1 using each patient's ID number.

3.4 Discussion

Previous studies have measured single cytokines in the plasma of children with CKD; however this is the first comprehensive study of multiple plasma cytokines in this group of patients, combined with an evaluation of a marker oxidative stress.

Three patients showed an increase in AOPP, all of whom had significantly reduced GFR (2 Stage 4, 1 stage 3). A weak but significant correlation between AOPP levels and GFR was observed.

One previous study in children by Sebekova et al [46] reported an increase in oxidative stress measured via AGE in children with CKD both dialysed and non-dialysed, however the levels of uraemia in non-dialysed subjects was much higher than the subjects in this study. A very recent study in a group of children with severe (stage 5) CKD, many of whom were not undergoing dialysis therapy, examined markers of oxidative stress and a limited range of inflammatory markers [47]. This study found significantly increased levels of serum malondialdehyde (MDA), a marker of oxidative stress and significantly increased IL-6, TNF-alpha and CRP.

The children in our clinical setting are managed by a multi – disciplinary team including a nephrologist and a dietician. The clinical and nutritional management strategies used in this clinic have been published previously [77] and growth and developmental outcomes meet best practice expectations. Patients have a reduced rate of deterioration in renal function and earlier intervention with renal replacement therapy (dialysis) when compared with care available in other parts of the world. Dialysis is commenced in our unit when children reach stage 4 CKD, hence I was

unable to assess oxidative stress in uraemia in stage 5 CKD, nevertheless, the findings reveal that children with relatively mild renal disease show evidence of inflammatory changes, and that these are present earlier in the disease process than previously thought.

Eleven (50%) patients in the CKD group showed an increase in at least one of the pro-inflammatory cytokines measured. There were increases in plasma IL-12 and/or IL-1β in ten of the patients with CKD (45%), Table 3.2. One patient showed an isolated increase in IL-8. We did not observe any correlation between increases in cytokines and disease severity, however this may be due to a lack of power in the current study as a result of the relatively small number of patients. All patients with increased AOPP also showed an increase in at least one cytokine.

Interestingly, the cytokines that were elevated in the present study are produced primarily by monocytes, suggesting a possible role for monocyte activation. To examine this, *ex vivo* markers of monocyte activation were determined in the CKD and control patient cohorts (Chapter 6).

IL-12 is produced by monocytes and is a major inducer of Th1 cells [90]. IL-1β has been implicated in the development of atherosclerosis via interactions with vascular cell adhesion molecule (VCAM)-1 and monocyte chemotactic protein (MCP)-1 [91].

IL-10 is a pleiotropic cytokine produced primarily by monocytes that has potent inhibitory effects on monocyte/macrophage function including the inhibition of macrophage inflammatory protein (MIP) - 1α [92]. IL-10 is a major counter regulator of Th1 pro inflammatory cvtokines. [93]

The findings of increased IL-12 and IL-1β in three CKD patients and an increase in IL-10 in all of these patients indicates a possible counter regulatory response of anti-inflammatory IL-10. Results of increased IL-1βas well as increased IL-10 in one patient further supports this notion.

Plasma half lives of pro-inflammatory cytokines in subjects with normal renal function are very short due to rapid endogenous clearance. In this regard, the half life of IL-12 has been reported as approximately 5 hr [94] and the half life of IL-1β has been reported to be as low as 10 min [95]. Thus, although only about ½ of the CKD group showed an increase in circulating cytokines, this may be due to the presence of inhibitors, or the transient nature of cytokine presence in the circulation. Therefore, the ability of monocytes and T cells from CKD patients to produce cytokines in response to stimulus is investigated (Chapter 4). The hypothesis that the CKD group will show an increase in intracellular cytokine production by monocytes and possibly T cells in response to stimulus will be investigated.

Although there were no significant correlations between the level of oxidative stress and pro-inflammatory cytokines (data not shown), three CKD patients with high levels of one or more of IL-12, IL-1β IL-8 and TNF-α also exhibited high levels of oxidative stress. These findings support a link between pro-inflammatory cytokines and oxidative stress as previously reported by Ece at al [47].

This is the first study to identify that children with mild (stage 2) renal failure show evidence of increased oxidative stress and changes in inflammatory cytokines. This patient group may possibly benefit from early intervention with anti-oxidant therapy such as supplementation with anti-oxidant vitamins

such as Vitamin E, or by treatment with drugs with anti-oxidant properties such as Resverotrol or Melatonin. Drugs such as statins that have the ability to reduce circulating pro inflammatory cytokines may also reduce future morbidity in this patient group.

Chapter 4

Cellular cytokine production

Introduction

4.1.1 Overview

In the previous chapter I showed that some children with CKD have increased plasma pro-inflammatory cytokine levels. Limited studies in adults have suggested that the most likely source of these cytokines is activated monocytes and macrophages, since the immune activation in these patients appears not to be antigen specific [10]. Monocytes are a source of both pro and anti inflammatory cytokines, and alterations to peripheral blood cytokines often reflect a systemic inflammatory process. Whilst tissue-based cells are also capable of producing cytokines, such production usually produces a more localised response. It was therefore of interest to ascertain the cellular source of the pro-inflammatory cytokines in children with CKD.

Intracellular cytokine measurement by flow cytometry is a powerful tool which allows the characterisation of cytokine producing cells in a heterogeneous population by providing a direct measure of cytokines at the cellular level, thus allowing the determination of the effect of uraemia on specific functional leucocyte subsets. This method also overcomes the limitations of transient expression circulating cytokines. Further, the measurement of cytokine levels in the supernatant of an equivalent culture without a Golgi block allows the determination of the total amount of cytokine produced by the cells in culture. I therefore utilised both these techniques to assess the ability of monocytes and T cells of children with CKD and a control group to produce cytokines of interest.

4.1.2 Hypothesis

Stimulated monocytes and T cells from children with CKD will produce increased levels of pro-inflammatory cytokines.

4.1.3 Specific Aims

- To apply flow cytometry to determine the percentage of stimulated CD4 and CD8 T cells producing IL-4, IFN-γ, IL-2 and TNF-α, and the percentage of monocytes producing IL-12, IL-10, TNF-α, IL-6, IL-8 IL-1α and IL-1β.
- To apply CBA to measure total production of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ in supernatants of stimulated whole blood cultures.

4.1.4 Cytokines of interest

Monocytes are a source of many cytokines including the pro-inflammatory cytokines IL-12, IL-1β, IL-1α, IL-6, IL-8, TNF-α and the anti-inflammatory cytokine IL-10. Alterations to these cytokines in peripheral blood may reflect systemic inflammatory status. Further, cytokines produced by subsets of T cells regulate monocyte immunological activity. For example, Th1 type cytokines such as IFN-γ induce monocytes to express pro-inflammatory cytokines such as IL-12 and TNF-α and therefore may have particular relevance in inflammatory disorders such as CKD, whereas Th2 type cytokines such as IL-4 promote humoral antibody responses rather than cellular immunity and result in disorders such as atopy and asthma [96]. In

this study I measured a range of cytokines produced by both T cells and monocytes; these cytokines and their main actions are outlined in table 4.1.

NOTE: This table is included on page 61 of the print copy of the thesis held in the University of Adelaide Library.

Table 4.1: Cell source of cytokines measured in this chapter and their actions. Adapted from [88].

4.2 Methods

4.2.1 Subjects

Twenty two children with CKD and 24 age matched control subjects were recruited. None of the subjects had any history of immunological abnormalities and all had been completely well in the 2 weeks prior to the study. Full details of the study populations, as well as exclusion criteria are outlined in detail in Chapter 2.

4.2.2 Samples

Heparinised blood was collected from the CKD group during routine clinic visits and from the control group prior to the induction of anaesthesia. Samples were processed within one hr of collection.

4.2.3 Cell cultures for intracellular cytokine measurements

Five hundred μL of blood was added to an equal volume of RPMI medium in 10mL sterile conical polystyrene tubes (Johns Professional Products, Sydney, Australia). For measurement of T cell cytokines, 25 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, Sydney, Australia) and 1μg/mL ionomycin (I) (Sigma) was added. Monocytes were stimulated with 1 μg/mL of *E. coli* lipopolysaccharide (LPS)(Sigma) added. Ten μg/mL of brefeldin A (BA)(Sigma) was added to all cultures as a “Golgi block” to prevent the release of cytokines from cells. Tubes were incubated o/n (18-20hr) at 37^0C in humidified air with 5%CO_2.

4.2.4 Cell cultures for measurement of released cytokines (CBA)

Five hundred μL of blood was added to an equal volume of RPMI medium in 10mL sterile conical polystyrene tubes (Johns Professional Products). In cultures for T cell cytokines, 10μg/mL phytohemagglutinin (PHA) (Sigma) was added. For monocyte stimulation 10 μg/mL of LPS was added instead of PHA. Tubes were incubated o/n at 37^0C in humidified air with 5%CO_2. Due to limitations in sample volume, I was only able to set up the cytokine release culture in 14 CKD subjects and 21 controls.

Detailed methods and reagent recipes can be found in Appendix 4.

4.2.5 Intracellular Cytokine measurement

Following incubation, 100μL of 20mM EDTA in PBS was added to each of the cultures with BA. Tubes were vortexed vigorously to remove adherent cells. After 10 min, tubes were vortexed again and aliquots (200μL for T cells and 300 μL for monocytes) were placed in 5mL FACS tubes (Evergreen Scientific, Sydney, Australia). Red cells were lysed by the addition of 2mL FACSlyse solution (BD). After 10 min, tubes were centrifuged at 500xg for 1 min and the supernatant discarded. Cells were permeabilised by the addition of 0.5mL of FACS permeabilising solution (FACSperm)(BD) to each tube. Tubes were vortexed and incubated a further 10 min at room temperature (RT). Two mL of 0.5% BSA (Sigma) in Isoton II (Beckman Coulter) (wash buffer) was added and tubes centrifuged and supernatant discarded. Fc receptors were blocked by adding 20μL of 60μg/mL human immunoglobulin (Commonwealth Serum Laboratories

(CSL), Parkville, Vic, Australia) for 10 min at RT. Two and a half μL of appropriately titrated monoclonal antibodies (Mabs) to the cytokines of interest or isotype controls were added as appropriate. Details of the panel of Mabs and suppliers are supplied in Table 4.2. Tubes were incubated for 15 min in the dark at RT, washed with wash buffer, centrifuged and the supernatant discarded. Cells were resuspended in 50μL of Isoton II and data acquisition was performed immediately using a FACSCalibur flow cytometer (BD). This procedure is represented in Figure 4.1.

Data analysis was performed using CellQuest software (BD). T cells and monocytes were isolated by gating the CD3 vs SSC and CD14 vs SSC populations respectively (Figure 4.2 a&b). T cells were further defined as CD8+ or CD8- (CD4+) T cells by the presence or absence of CD8 respectively. Quadrant markers were set using isotype matched negative antibodies. The negative control was set on a 2% false positive value (Figure 4.3).

FITC	**PE**	**PC5**
IL-8	IL-10	CD14
TNF-α	IL-6	CD14
IL1-α	IL-12	CD14
	IL1-β	CD14
CD8	IFN-γ	CD3
CD8	IL-4	CD3
CD8	IL-2	CD3
CD8	TNF-α	CD3

Table 4.2: Panel of Mabs used to detect intracellular cytokines. CD3 and CD14 supplied by Beckman Coulter, all anti-cytokine antibodies were supplied by BD.

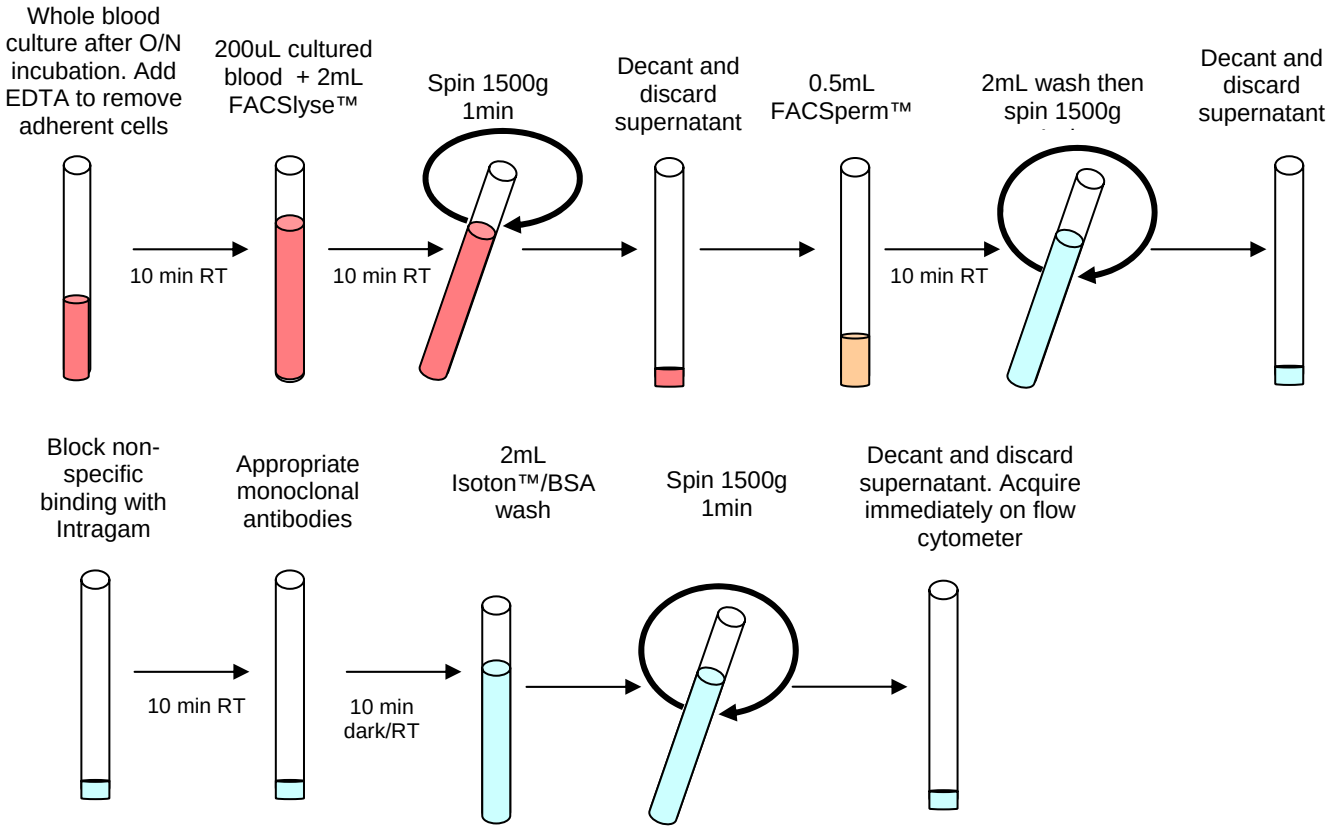


Figure 4.1: Preparation of whole blood cultures for cytokine expression. After o/n culture with appropriate stimuli 100 µL of 20mM EDTA was added to cell cultures to remove adherent cells. Two hundred µL of culture was transferred to flow cytometry tubes. Red blood cells were lysed with FACSlyse and then spun to pellet white blood cells. Supernatant was discarded, cells were treated with FACSperm to permeabilise cell membranes. After washing, Fc receptors were blocked to with Intragam to prevent non-specific binding and then the appropriate Mabs were added. Cells were then washed with wash buffer and acquired immediately on a FACSCalibur flow cytometer.

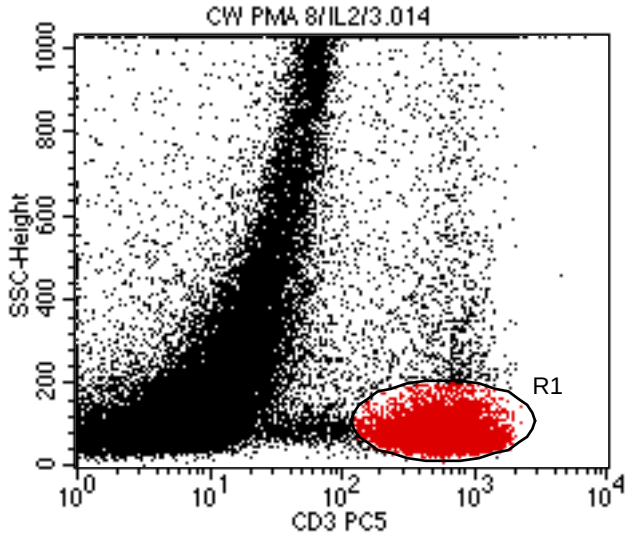


Figure 4.2a: Representative Flow cytometer dot plot showing gating of T cells by positive CD3 staining vs SSC

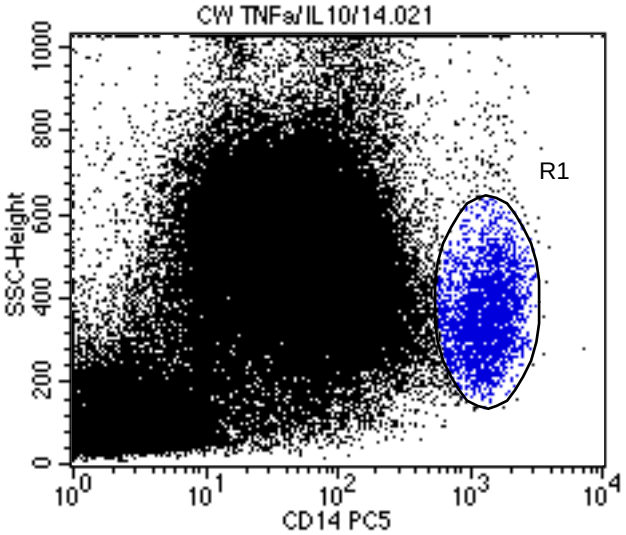
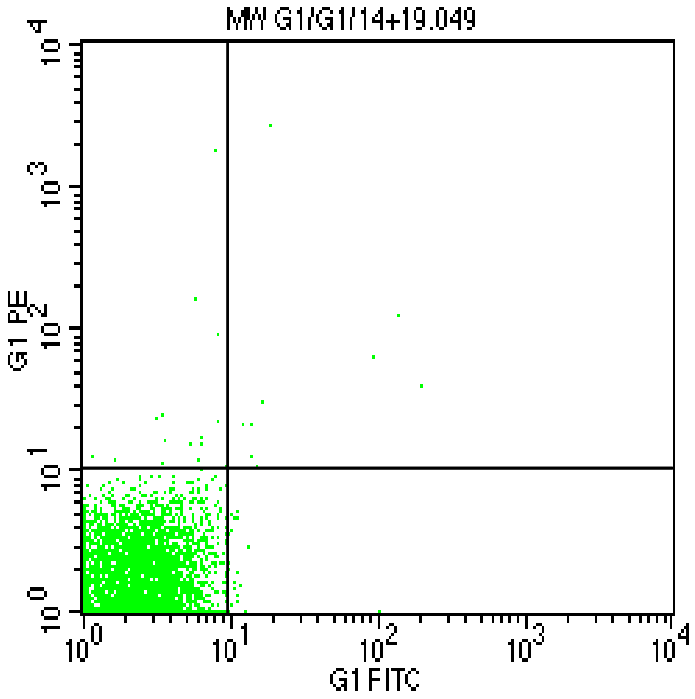


Figure 4.2b: Representative Flow cytometer dot plot showing gating of monocytes by postive CD14 staining vs SSC



Quadrant Statistics

File: MW G1/G1/14+19.049 Log Data Units:
Sample ID: Patient ID:
Tube: Panel:
Acquisition Date: 03-Mar-05 Gate: G3=R1+R
Gated Events: 5402 Total Events: 10
X Parameter: FL1-H G1 FITC (Log) Y Parameter: Fl
Quad Location: 10, 10

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	15	0.28	0.01	5.23	147.88
UR	9	0.17	0.01	58.30	331.89
LL	5359	99.20	5.36	2.75	1.83
LR	19	0.35	0.02	15.51	2.57

Figure 4.3: Flow cytometric dotplot of Isotype control showing quadrant markers set on a ≤ 2.0% false positive value for staining with FITC (x axis) and PE (y axis).

4.2.6 Measurement of cytokine levels in culture supernatant

After incubation o/n, cultures without BA were centrifuged at 3000xg for 5 min and the supernatant stored at -70^{o}C for subsequent cytokine analysis. IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ, IL-8, IL-1β, IL-6 and IL-12p70 released into cell culture supernatants were measured using the CBA employed in Chapter 3.

4.3 Results

4.3.1 Monocyte Intracellular cytokines

Blood monocytes from CKD patients showed a significantly greater ability to produce the pro-inflammatory cytokines IL-12 and IL-1β. There were no changes in the percentage of cells producing IL-8, IL-6, IL-10, IL-1α or TNF-α. Details of results are shown in Table 4.3

Cytokine	Median CKD	Range CKD	Median Control	Range Control	p value
IL-1α	85	73 – 98	90	54 – 99	0.241
IL-1β	**74**	**59 – 85**	**52**	**33 – 86**	**0.009**
IL-6	57	26 – 87	55	10 – 87	0.384
IL-8	84	58 – 97	91	35 – 98	0.886
IL-10	3.2	1.4 – 10.7	3.3	0.7 – 10.6	0.843
IL-12	**39**	**10 – 62**	**24**	**4 – 53**	**0.001**
TNF-α	83	42 – 97	84	18 – 97	0.747

Table 4.3: Percentage of monocytes producing cytokines. Significant differences are presented in bold. ($p<0.05$ Mann Whitney U test).

Representative dot plots of IL-1β and IL-12 results are shown in Figures 4.4 & 4.5.

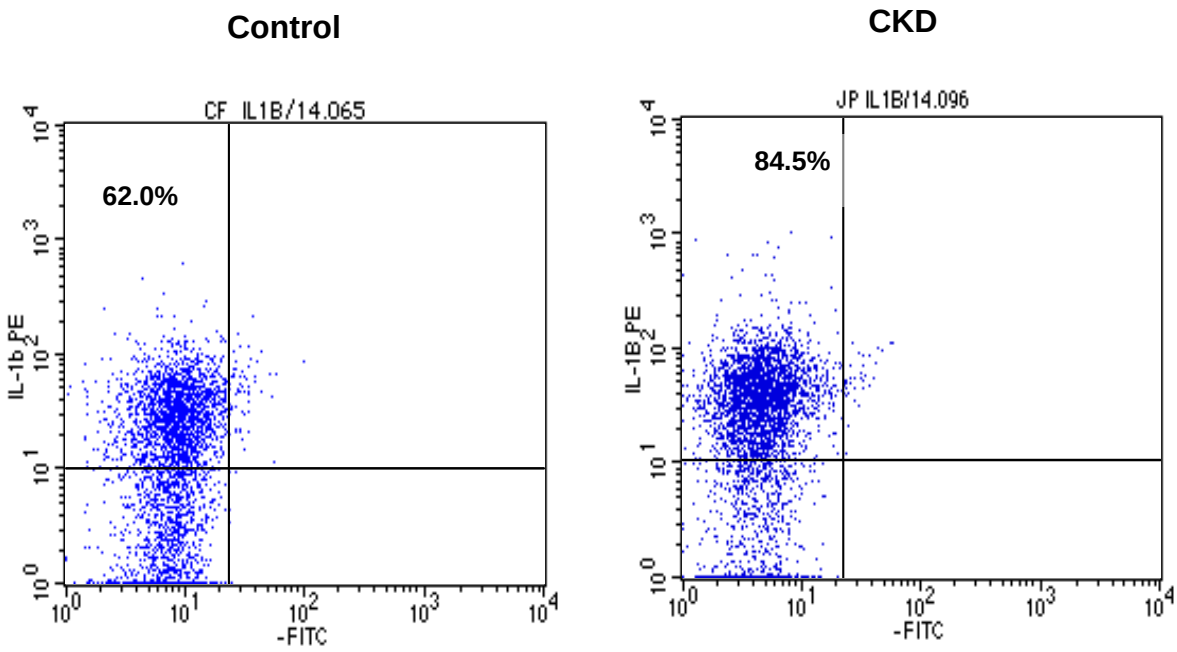


Figure 4.4: Flow cytometric dot plot showing significantly increased IL-1β production by monocytes in a patient from the CKD group (84.5%) compared with a control (62.0%).

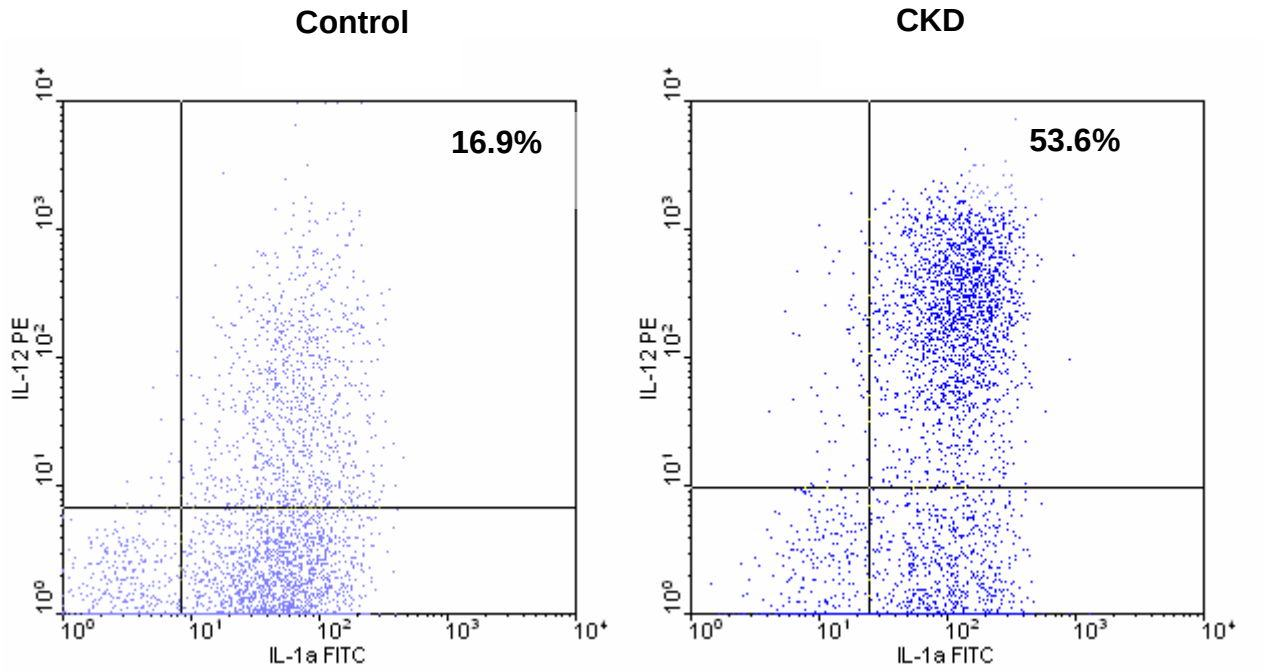


Figure 4.5: Flow cytometric dot plot showing significantly increased IL-12 (but not IL-1α) production by monocytes in a patient from the CKD group (53.6%) compared with a control (16.9%).

4.3.2 T cell Intracellular cytokines

There was a significant increase in the percentage of CD4, CD8 and CD3+all types of T cells producing IL-4 in CKD versus control subjectts. The CKD group also showed a significant increase in the percentage of CD3 cells producing IL-2 and a significant decrease in the percentage of CD8- (CD4) T cells producing IFN- γ compared to the control group. Details are presented in Table 4.4. Dot plots illustrating typical IL-2 and IL-4 and IFN-γ production in CKD and control subjects are shown in Figures 4.6, 4.7 and 4.8.

Cytokine		**Median CKD**	**Range CKD**	**Median Control**	**Range Control**	**p value**
IL-2	CD4	44.9	13.2 – 62.0	36.7	21.7 – 67.3	0.09
	CD8	13.0	4.0 – 31.5	11.2	2.6 – 43.6	0.14
	CD3	**33.2**	**9.1 – 50.8**	**28.6**	**16.3 – 54.0**	**0.04**
IL-4	**CD4**	**3.4**	**1.0 – 14.4**	**2.5**	**0.9 – 6.5**	**0.04**
	CD8	**2.4**	**0.8 – 7.2**	**1.2**	**0.1 – 3.3**	**0.004**
	CD3	**3.2**	**0.9 – 11.1**	**2.1**	**0.9 – 4.7**	**0.02**
IFN-γ	**CD4**	**24.8**	**12.0 – 35.3**	**27.3**	**14.8 – 43.4**	**0.04**
	CD8	39.6	16.4 – 56.7	42.1	42.5 – 64.7	0.33
	CD3	31.0	15.4 – 41.5	32.4	17.6 – 49.1	0.21
TNF-α	CD4	45.9	24.5 – 76.5	48.2	25.2 – 83.1	0.39
	CD8	34.3	13.1 – 73.4	34.9	18.9 – 70.9	0.91
	CD3	40.7	21.5 – 58.4	42.1	26.8 – 78.2	0.39

Table 4.4: Percentage of CD4, CD8 and CD3 T cells producing cytokines. Significant differences are presented in bold. ($p<0.05$ Mann Whitney U test).

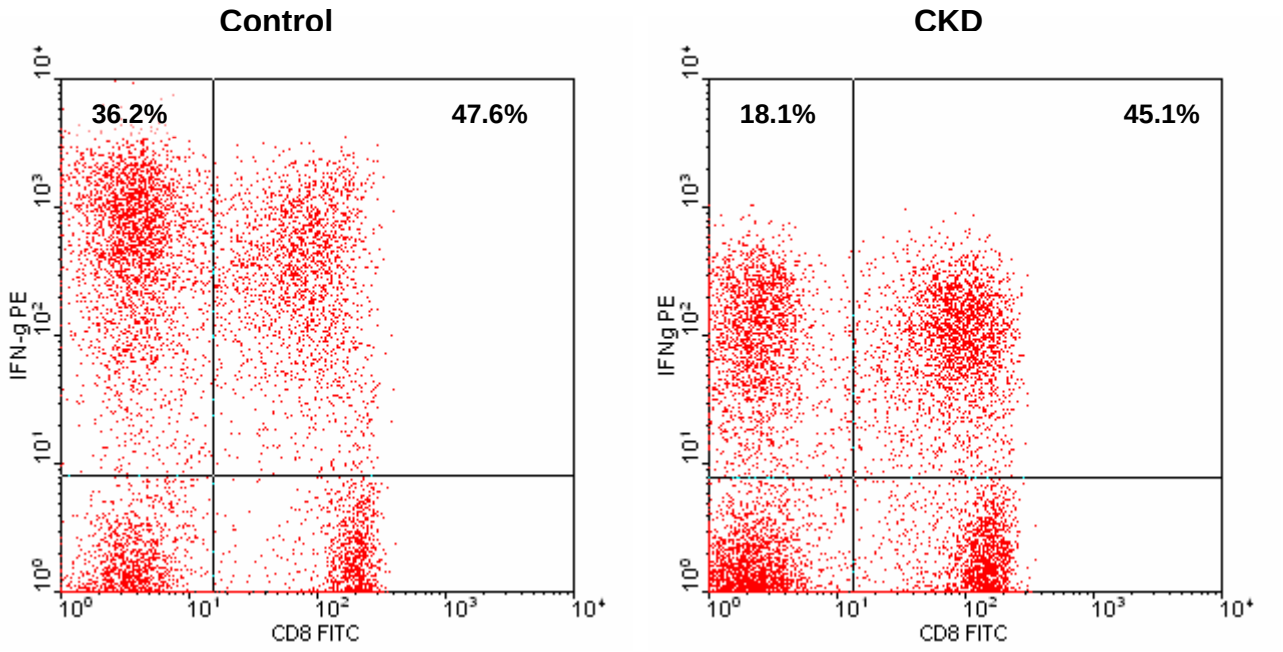


Figure 4.6: Flow cytometric dot plot showing significantly decreased IFN-γ production by CD8- (CD4) T cells in a patient from the CKD group (18.1%) compared with a control patient (36.2%).

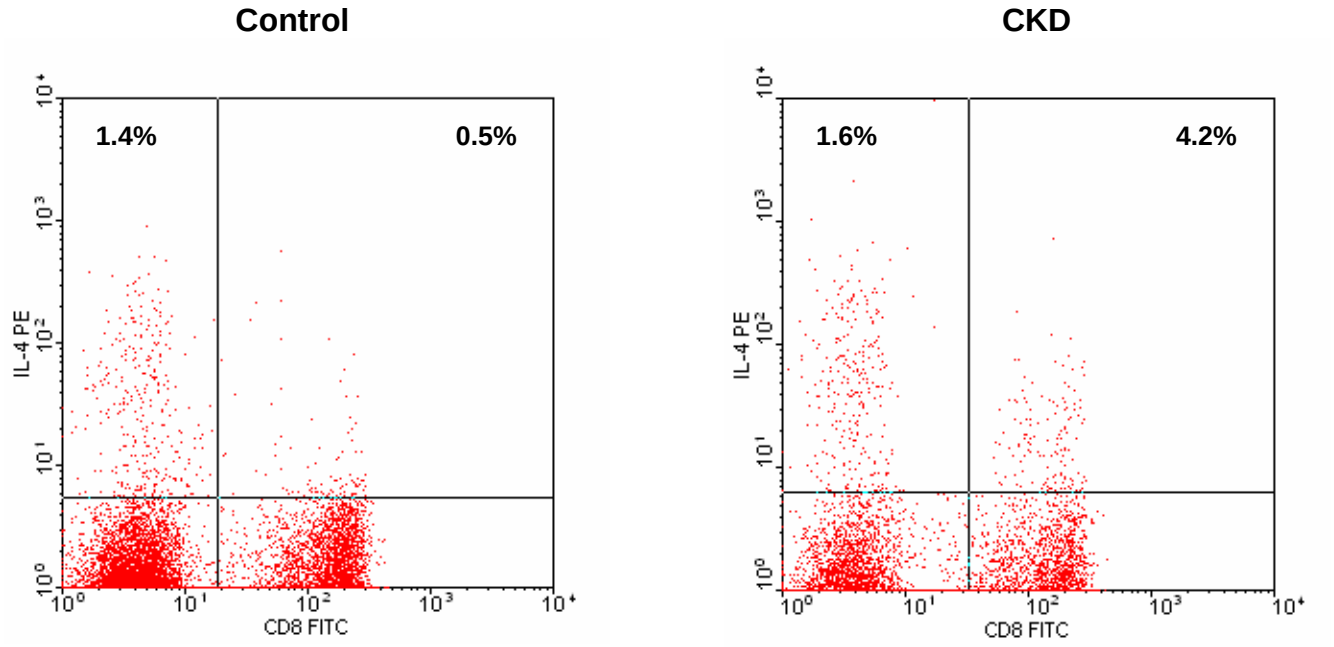


Figure 4.7: Flow cytometric dot plot showing significantly increased IL-4 production by T cells in a patient from the CKD group (5.8%) compared with a control patient (1.9%)

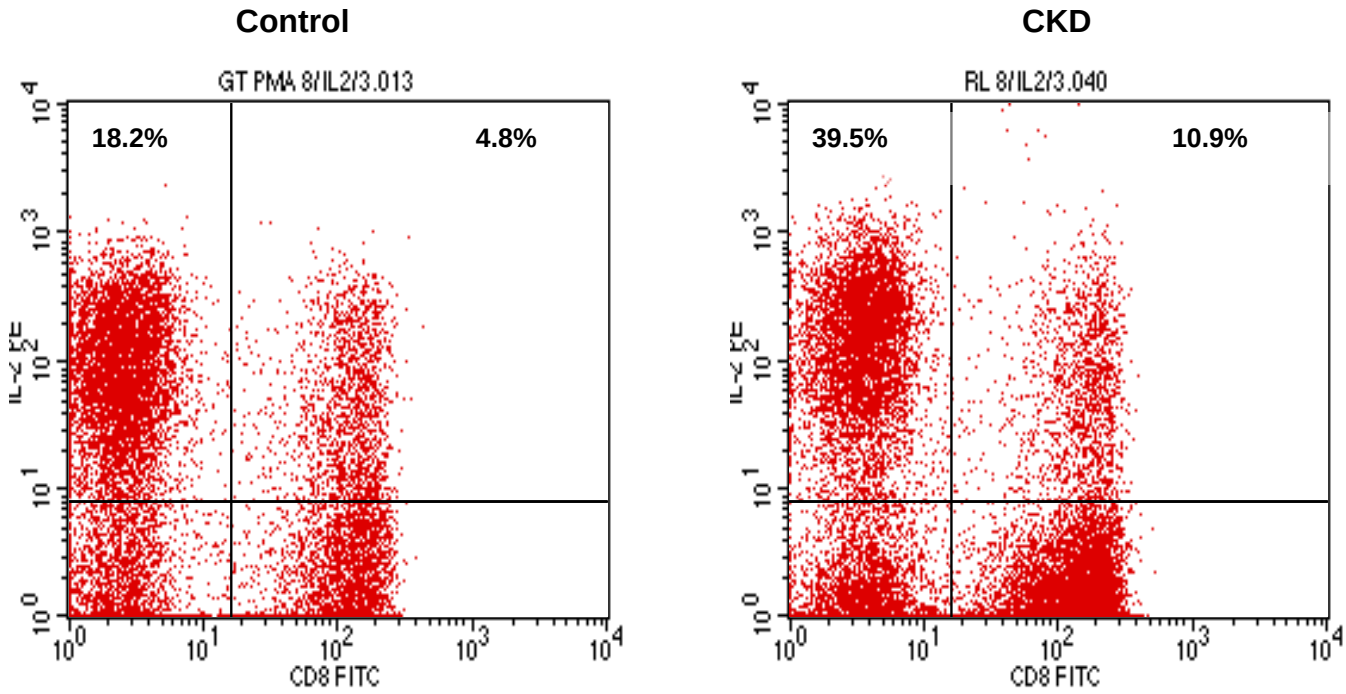


Figure 4.8 Flow cytometric dot plot showing significantly increased IL-2 production by T cells in a patient from the CKD group (50.4%) compared with control patient (23.0%)

4.3.3 Supernatant cytokines

Consistent with the intracellular cytokine production, the CKD group showed a significant increase versus control in secreted IL-12 and IL-1β in the supernatant from cultures stimulated with LPS. These are illustrated in Figures 4.9 and 4.10.

There were no significant differences between CKD and control in any of the cytokines measured in supernatants from cultures stimulated with PHA.

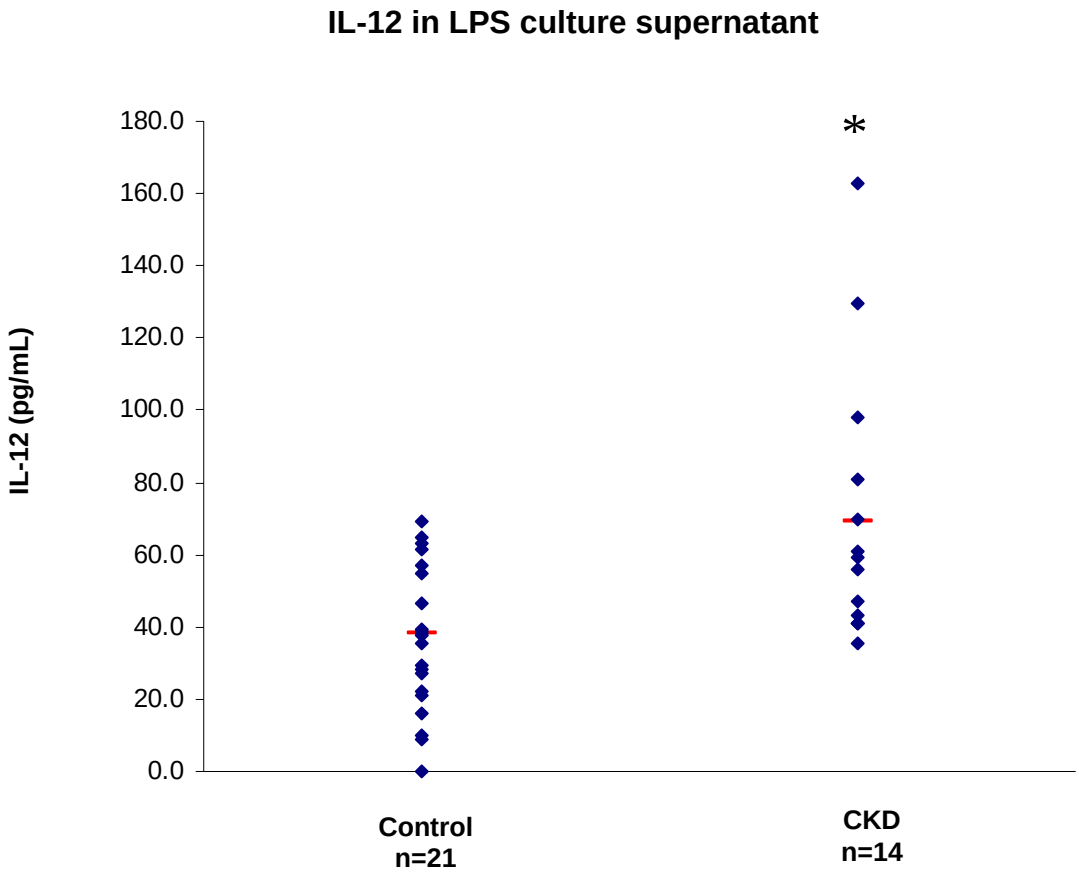


Figure 4.9: Scatter plot showing significantly increased IL-12 release into the supernatant of whole blood cultures stimulated with LPS in CKD group versus control (* p = 0.003 Mann Whitney). Red bar represents mean.

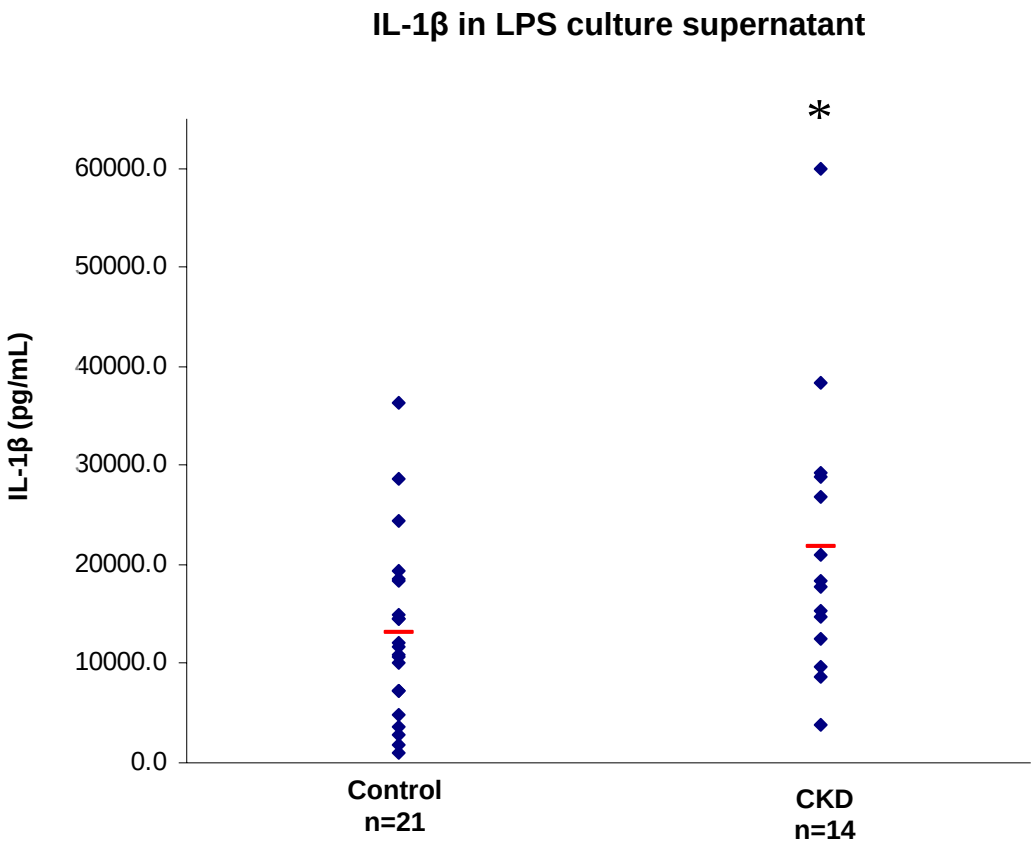


Figure 4.10: Scatter plot showing significantly increased IL-1β release into the supernatant of whole blood cultures stimulated with LPS in CKD group versus control (* p = 0.03 Mann Whitney). Red bar represents mean.

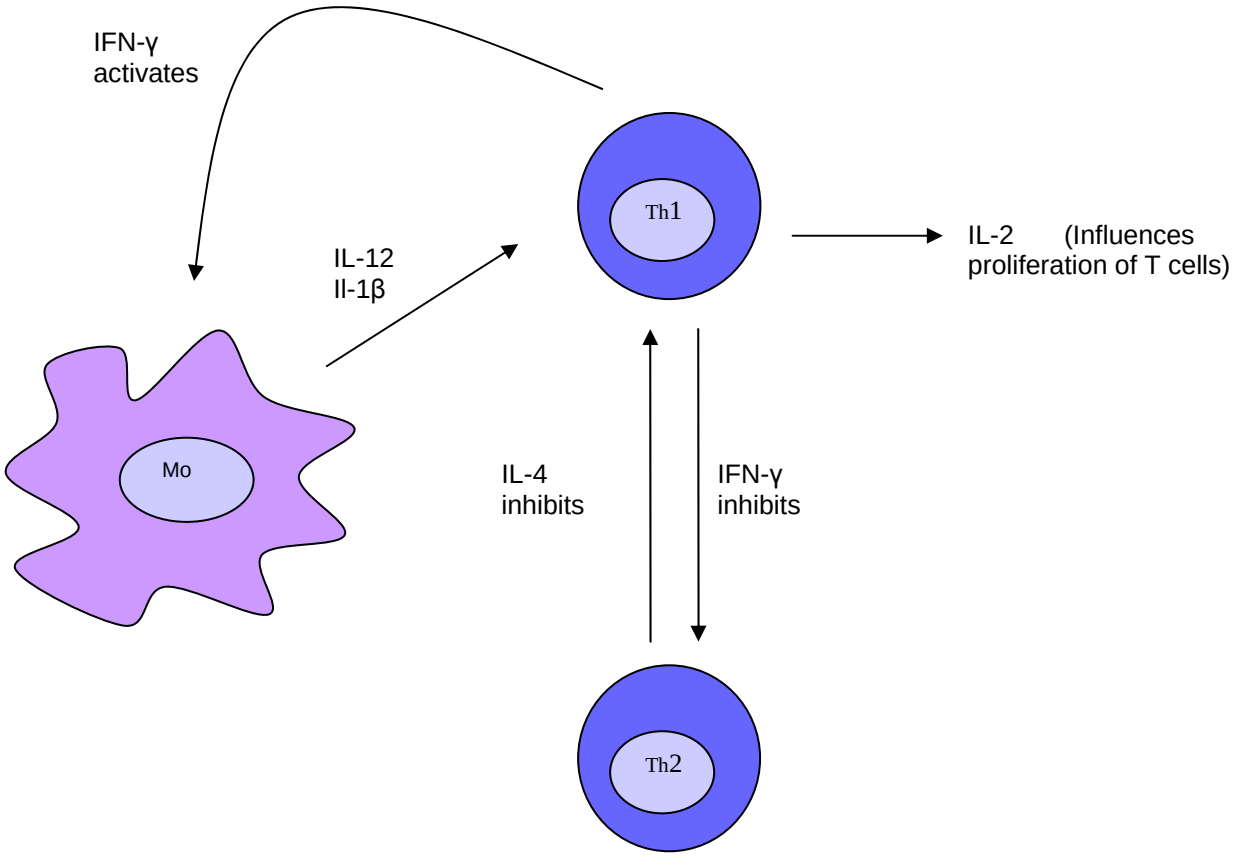
4.4 Discussion

This is the first report showing alterations of intracellular cytokine production by peripheral blood monocytes and T cells from children with CKD. In the previous chapter I showed that some children with CKD had increased circulating plasma cytokine levels that were not related to their level of residual renal function or drug treatment. Half of the CKD group showed an increase in circulating IL-12 and / or IL-1β.

Measuring circulating cytokines has a number of limitations; the presence of cytokines in the circulation is short-lived, and may be affected by receptor binding, the presence of inhibitors, or breakdown within reacting cells [97]. These effects may be responsible for the inconsistent changes in plasma cytokines that were observed. I now show that monocytes are the likely source of these increased inflammatory cytokines and that the CKD group showed an increased production of these cytokines *in vitro.*

Both these cytokines have been described as being pro-atherogenic [98] and may contribute to the increased levels of CVD that are reported in CKD.

A major role of IL-1 is to stimulate T helper cells to produce IL-2 (Figure 4.11). In this regard, my study found a small but statistically significant increase in the number of CD3 T cells producing IL-2.


IFN-γ
activates
Th1
IL-2 (Influences
proliferation of T cells)
IL-12
Il-1β
Mo
IL-4
inhibits
IFN-γ
inhibits
Th2

IL-12 is an important promoter of cell mediated immunity and a major inducer of Th1 cells that produce IFN-γ, thus I predicted that increased monocyte IL-12 production would be associated with increased IFN-γ production in the CKD group. Surprisingly, the study showed a reduction in the production of IFN-γ by CD4 T cells, indicating a possible reduction in the number of this Th1 T cell subset. Consistent with these findings, a previous report has shown that different subsets of Th1 cells produce IFN-γ and IL-2 [99]. There are several possible reasons for the reduction in the IFN-γ producing subset of CD4+ in the present study.

One reason may be due to a decrease in proliferation or an increase in apoptosis of these cells. Recent findings of increased apoptosis of the Th1 subset in non-dialysed adults with renal failure approaching dialysis [100] suggest the latter as a cause for these decreased cell numbers.

A second reason may be defective binding of IL-12 to the IL-12 receptor (IL-12R) on CD4+ T-cells or a dysfunctional signal transduction mechanism. There are several possible explanations for this. IL-12R may be down regulated on T cells or IL-12 may be unable to bind to the receptor, due to the presence of an inhibitor in uraemic plasma or an alteration in structure. The IL-12R has two sub-units (β1 and β2) that are only expressed on T cells after activation [101]. IL-12Rβ2 expression has been suggested as a determinate of Th cell differentiation towards a Th1 phenotype, thus a reduction in expression or function of this receptor may cause a bias towards a Th2 phenotype [102] with an associated decrease in the number of cells producing IFN-γ. In this regard, I have recently developed a

functional assay for IL-12Rβ1 and IL-12Rβ2 which is the subject of a future planned study.

A third reason may be increased levels of oxidative stress that have been reported to polarise T cell differentiation towards a Th2 phenotype [103].

In this regard, I also showed that children with CKD have an increased number of CD3 (both CD4 and CD8) T-cells that produce IL-4. IL-4 is an important Th2 cytokine that has been shown to negatively regulate T cell IFN-γ production [104] and may contribute to the decreased IFN-γ production by CD4 T cells noted in our patient group. Both increases and decreases in intracellular IL-4 have been shown in other forms of renal disease. Zachweija et al reported a decrease in production of IL-4 in nephrotic syndrome [105], while Masutani et al report increased IL-4 in membranous nephropathy [106]. The reasons for these conflicting results are not clear, however it is likely that these differences in immune disturbance are related to different mechanisms of disease in each of the conditions. Investigation of the reduction in the IFN-γ producing subset of CD4+ T cells will also be the focus of future studies.

I was unable to detect any significant changes in the production of IL-8, IL-10 IL-6 IL-1α or TNF-α by monocytes or TNF-α production by T cells in the present study, consistent with the plasma cytokine results in the previous chapter. This is somewhat surprising since these cytokines are known to contribute to the inflammatory process. It is possible that our staining technique may have been insufficiently sensitive to detect what may be subtle changes in the production of these cytokines, alternatively

uraemia may cause specific alterations to monocyte production of IL-12 and IL-1β and T cell IL-4 and IFN-γ.

The alterations in cytokine production observed in this study may influence the clinical immune function of this group of patients. Monocytes produce pro-inflammatory cytokines early in infection and enhance a host's innate resistance at the same time as shaping the ultimate antigen-specific immune response [101]. IL-12 is a major inducer of Th1 cells which are essential for immunity to intracellular pathogens [107]. Lack of effect of IL-12 on T-cells and the subsequent decrease in IFN-γ may have a negative effect on the development of T cell mediated immunity in children with CKD.

Chapter 5

Determination of leucocyte subsets

5.2 Introduction

5.1.1 Overview

In the previous chapters I describe changes in cytokine production by blood T cells and monocytes from children with CKD. Alterations in cytokine production can result from changes in the relative proportions of certain lymphocyte subsets including T cells and their subsets, B and NK cells. Alterations in cytokine production can also be caused by increased activation of leucocytes and this is examined in Chapter 6.

In the present chapter I define previously unidentified changes in leucocyte subsets in children with CKD which may contribute to the changes in cytokine production in these patients.

Significant changes in the numbers of T, B and NK cells and alterations in T cell subsets have been demonstrated in other groups of CKD patients; ie both dialysed and non-dialysed adults and children on dialysis (outlined in detail in Chapter 1). In this regard, lymphopaenia is a common finding in many types of renal disease [63]. I therefore investigated T, B and NK cells in children with CKD because there have been numerous reports that disturbances in the ratios and numbers of these subsets contribute to lymphopaenia in CKD [108-110]. As discussed in Chapter 1, alterations in T cell subsets, particularly the ratio of CD4:CD8 T cells, memory cells and αβ / γδ T cells have been reported [65, 111, 112]. To date, these lymphocyte subsets have not been elucidated in pre-dialytic CKD in either adults or children and the contribution of uraemia versus dialysis is unclear.

I therefore hypothesised that the children in the present study, many of whom have relatively mild disease would show similar changes in leucocyte subsets to the cohorts of patients in other reports with more severe disease.

To test this hypothesis I applied flow cytometry to extensively examine the expression of relevant cell surface antigens that define functional leucocyte subsets in peripheral blood of children with CKD. The majority of children in this study have CKD that was classified as stage three or less (i.e. they had not yet progressed to significant renal impairment). This provided an excellent opportunity to characterize the changes in functional leucocyte subsets that are due to CKD *per se* as opposed to those changes caused by dialysis and/or other co-morbid conditions.

Leucocyte subsets were defined using flow cytometry to determine the phenotype of various leucocyte and lymphocyte sub-populations. Table 5.1 outlines the CD markers that were used to determine the *ex vivo* phenotype of the leucocyte sub-populations.

Leucocyte type	**Surface Antigen**	**Specificity**
Lymphocyte	CD3	T cells
	CD19	B cells
	CD16+/CD3-	NK cells
T cell subsets	CD3/CD4	T Helper/inducer
	CD3/CD8	T suppressor/cytotoxic
	CD3/αβ	T cell receptor heterodimer
	CD3/γδ	T cell receptor – direct antigen recognition
	CD3/CD45RO^{+}	Memory cell
	CD3/CD45RO+/CD62L^{-}	Th1 cell
	CD3/CD45RO+/CD62L+	Th2 cell

Table 5.1: CD markers measured on the surface of resting cells.

5.1.2 Hypothesis

Children with CKD will show

- Alterations in lymphocyte numbers and specific lymphocyte subsets
- Changes in functional T cell subsets and phenotypes

5.1.3 Specific Aims

To quantify the following lymphocyte subsets:

- T (CD3), B (CD19) and NK (CD16) lymphocytes
- T Helper/Suppressor (CD4), Cytotoxic/Precursor (CD8) T cells
- αβ and γδ T cell numbers
- Memory T cells as defined by CD45RO/CD3
- Th1/Th2 T cells as defined by CD62L/CD45RO/CD3

5.2 Materials and methods

5.2.1 Subjects

Twenty two children with CKD and 24 age matched control subjects were recruited. None of the subjects had any history of other immunological abnormalities and all had been completely well in the 2 weeks prior to the study. Full details of the study populations, as well as exclusion criteria are outlined in detail in Chapter 2.

5.2.2 Blood Collection and sample preparation

Venous blood was collected from study subjects into a vacuette tube containing Lithium heparin as anti-coagulant (Greiner Bio-science, Kremsmunster, Austria).

Total white cell count and differential was performed on an automated cell counter (Cell-Dyn 4000, Abbott Diagnostics, Abbott Park, IL, USA). Samples were centrifuged at 3000xg for 10 min. Plasma was removed from the heparinised sample and frozen at –70^{o}C for circulating cytokine analysis. Cells were washed once in wash buffer and then resuspended to the original whole blood volume in the same buffer.

5.2.3 Flow cytometry surface marker determination

Samples were processed for flow cytometry analysis within 1 hour of collection. Samples were analysed using a panel of fluorescently conjugated Mabs to characterise leucocyte types as described in Table 5.2. Mabs were purchased from BD, Dako (Glostrup, Denmark), Immunotech, or American Diagnostic (Greenwich, CT, USA).

FITC	**PE**	**PC5**
CD3	CD16	CD19
CD8	CD4	CD3
CD62L	CD45RO	CD3
TCR αβ		CD3
TCR γδ		CD3

Table 5.2: Panel of Mabs used to determine lymphocyte subsets.

Fifty μL of resuspended blood cells were incubated with the appropriate pre-titrated fluorochrome conjugated Mab for 15 min at room temperature in the dark. Red cells were lysed by the addition of 2 mL of Facslyse (BD) and samples incubated a further 10 min at room temperature in the dark. Samples were centrifuged at 1500xg for 1 min and the supernatant discarded. White cells were washed with 2 mL wash buffer and resuspended in 50μL of wash buffer. Data acquisition was performed immediately using a FACSCalibur flow cytometer (BD). A diagrammatic representation of this method is presented in Figure 5.1.

Data analysis was performed using CellQuest software (BD). Quadrant markers were set using isotype matched negative antibodies. The negative control was set on a 2% false positive value. Statistical Analysis was performed using SPSS software. Differences between CKD and control subjects were determined using a non-parametric Mann Whitney U-test, p values <0.05 were considered significant.

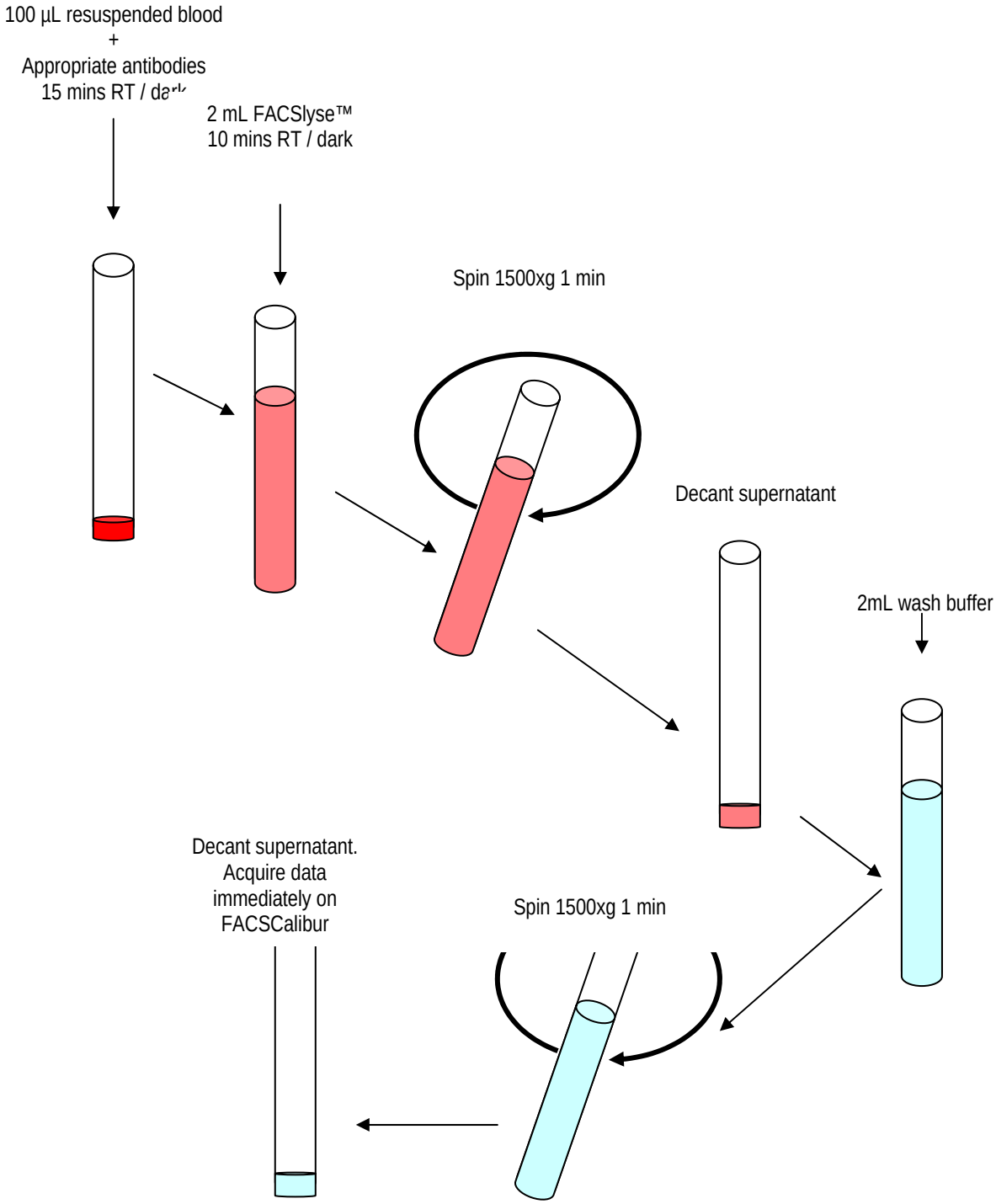


Figure 5.1: Preparation of whole blood for surface marker expression. Whole blood was collected into tubes containing Lithium Heparin as anticoagulant. Cells were washed once in wash buffer and resuspended to the original whole blood volume in the same buffer. One hundred µL of resuspended cells were transferred to flow cytometry tubes, already containing appropriate anti-human monoclonal antibodies conjugated to fluorescent tags. After staining at RT, RBC were lysed with FACSlyse and then spun to pellet WBC. Supernatant was discarded, cells washed with wash buffer and then acquired immediately on a FACSCalibur flow cytometer.

5.3 Results

5.3.1 Complete Blood Picture (CBP)

There were no significant changes in the absolute parameters of the CBP. There was an increase in the percentage of eosinophils in the CKD group versus control; however results for both groups were within the stated reference ranges. Details of the CBP results are outlined in Table 5.3

Cell type	Median CKD	Range CKD	Median control	Range control	p value
Total WBC x10^9/L	7.0	4.4 – 9.9	7.4	4.9 – 10.4	0.640
Neutrophil x10^9/L	3.7	2.2 – 5.3	3.8	1.6 – 6.7	0.975
Neutrophil (% of total WBC)	50.6	41.2 – 60.4	47.6	30.4 – 69.2	0.257
Lymphocyte x10^9/L	2.4	1.6 – 4.2	2.5	1.9 – 4.9	0.244
Lymphocyte (% of total WBC)	35.7	25.7 – 47.3	39.3	21.1 – 40.5	0.105
Monocyte x10^9/L	0.5	0.3 – 1.1	0.6	0.2 – 1.0	0.424
Monocyte (% of total WBC)	7.5	5.3 – 12.3	8.1	2.4 – 11.5	0.371
Eosinophil x10^9/L	0.30	0.17– 0.67	0.25	0.04 – 0.82	0.066
Eosinophil (% of total WBC)	4.0	2.0 – 8.5	2.8	0.5 – 10.1	0.012
Basophil x10^9/L	0.04	0.01 – 0.17	0.05	0.01 – 0.18	0.942
Basophil (% of total WBC)	0.7	0.2 – 2.2	0.7	0.1 – 1.9	0.771

Table 5.3: Details of CBP results for CKD and control groups. p value from non parametric Mann Whitney U-test.

5.3.2 Lymphocyte Subsets

Lymphocyte and T cell subsets were determined using three colour flow cytometry. Lymphocytes were gated on FSC and SSC as shown in Figure 5.2a. Lymphocyte purity was determined by positive staining for CD45 and negative staining CD14. The FSC / SSC gate for lymphocytes was adjusted so that >98% of cells were CD45+/CD14- (Figure 5.2b).

5.3.3 T, B and NK cells

T, B and NK cells were determined by the percentage of CD3+ (T cells), CD19+ (B cells) and CD16+/CD3- (NK cells) that were present in the lymphocyte gate (Figure 5.3). The lymphocyte subsets were also backgated to determine their distribution. Absolute numbers of each lymphocyte subset were then determined by calculating the absolute number of each cell type from the percentage of each lymphocyte subset in the total lymphocyte count.

There was a significant increase in T cells (CD3+) and decrease in B (CD19+) and NK (CD16+/CD3-) cells when considered as a percentage of the total lymphocyte population (Figure 5.4). Absolute counts showed a decrease in B and NK cells but no change in T cells (Figure 5.5).

5.3.5 T cell subsets

The total T cell population was determined by gating CD3 positive cells vs SSC (Figure 5.6). This subset was then back-gated on the lymphocyte FSC vs SSC gate to ensure that all CD3 cells fell within the lymphocyte gate. This population of cells was then used to determine T cell subsets.

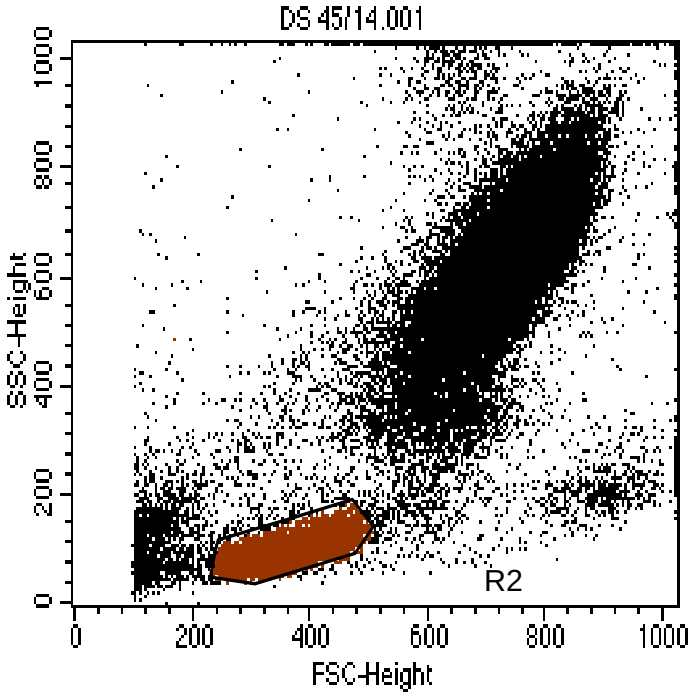
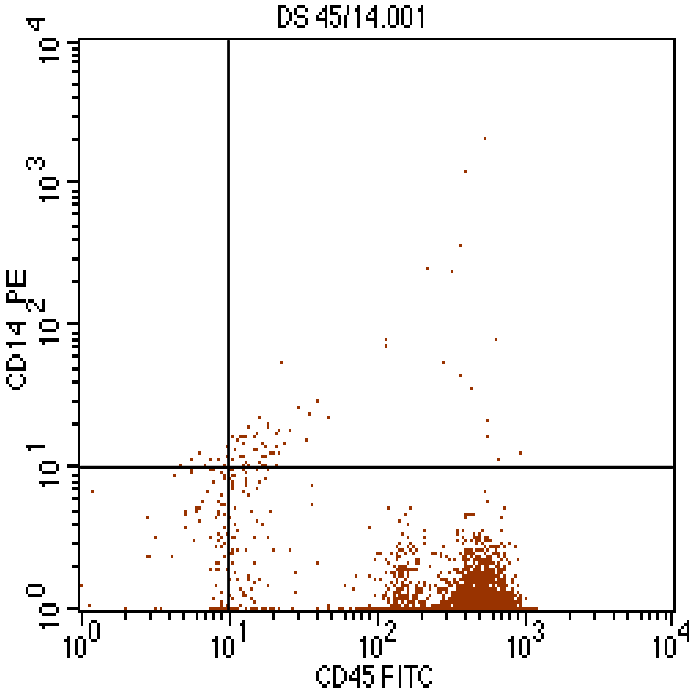


Figure 5.2a: Representative flow cytometer dot plot showing gating of lymphocytes by FSC vs SSC



Quadrant Statistics

File: DS 45/14.001 Log Data U
Sample ID: Patient ID:
Tube: Panel:
Acquisition Date: 08-Feb-05 Gate: G2=L
Gated Events: 28695 Total Event
X Parameter: FL1-H CD45 FITC (Log) Y Paramete
Quad Location: 10, 10

Quad	Events	% Gated	% Total	X Mean	Y Mean
UL	10	0.03	0.01	7.53	11.19
UR	62	0.22	0.06	119.52	85.95
LL	82	0.29	0.08	7.46	3.56
LR	28541	99.46	27.76	557.24	1.03

Figure 5.2b: Representative flow cytometer dot plot of lymphocyte region showing 99.46% lymphocyte purity (i.e. CD45+/CD14-)

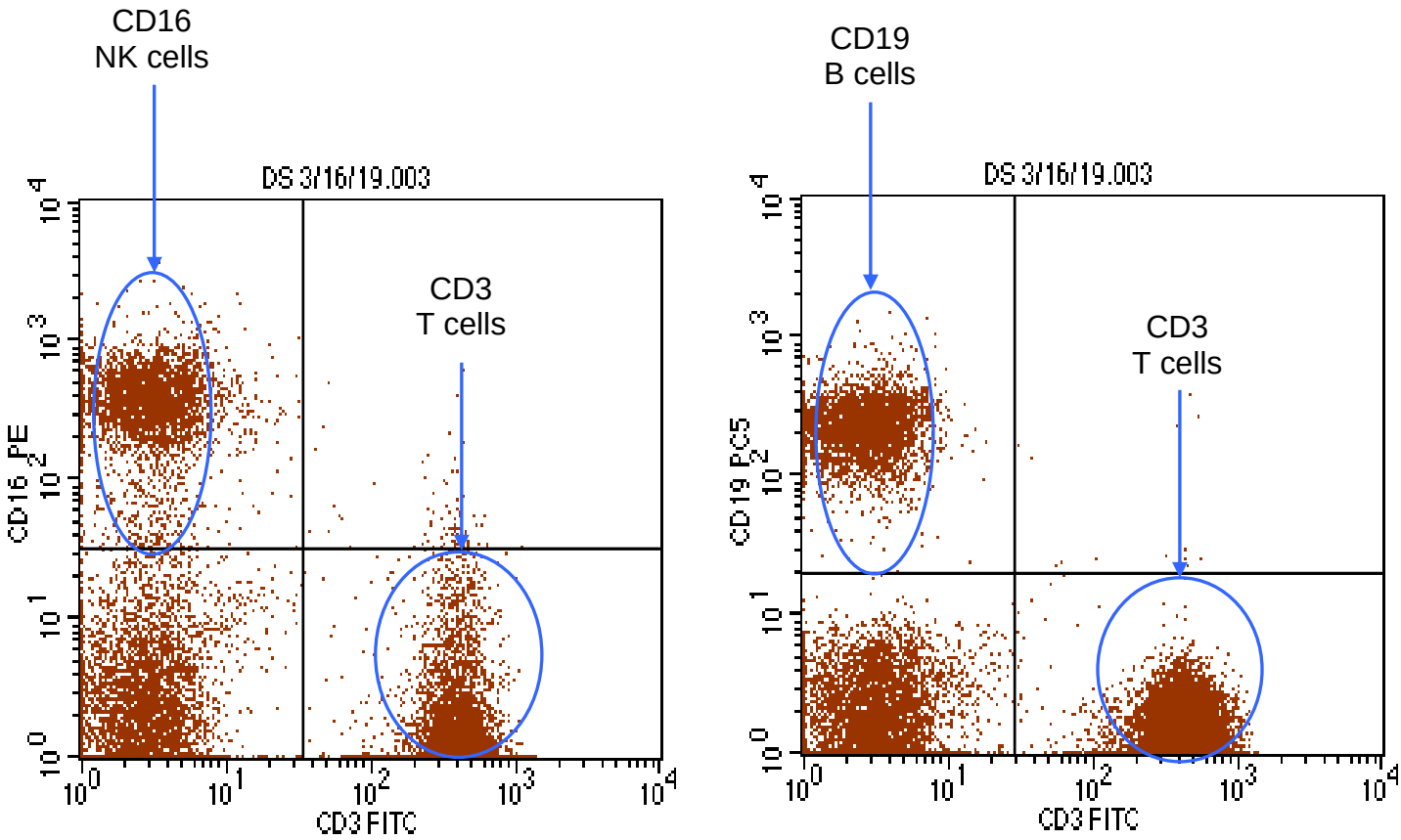


Figure 5.3: Representative flow cytometer dot plot of the determination T, B and NK cell populations. Lymphocytes were gated according to figure 5.2 and the percentages of each cell population determined.

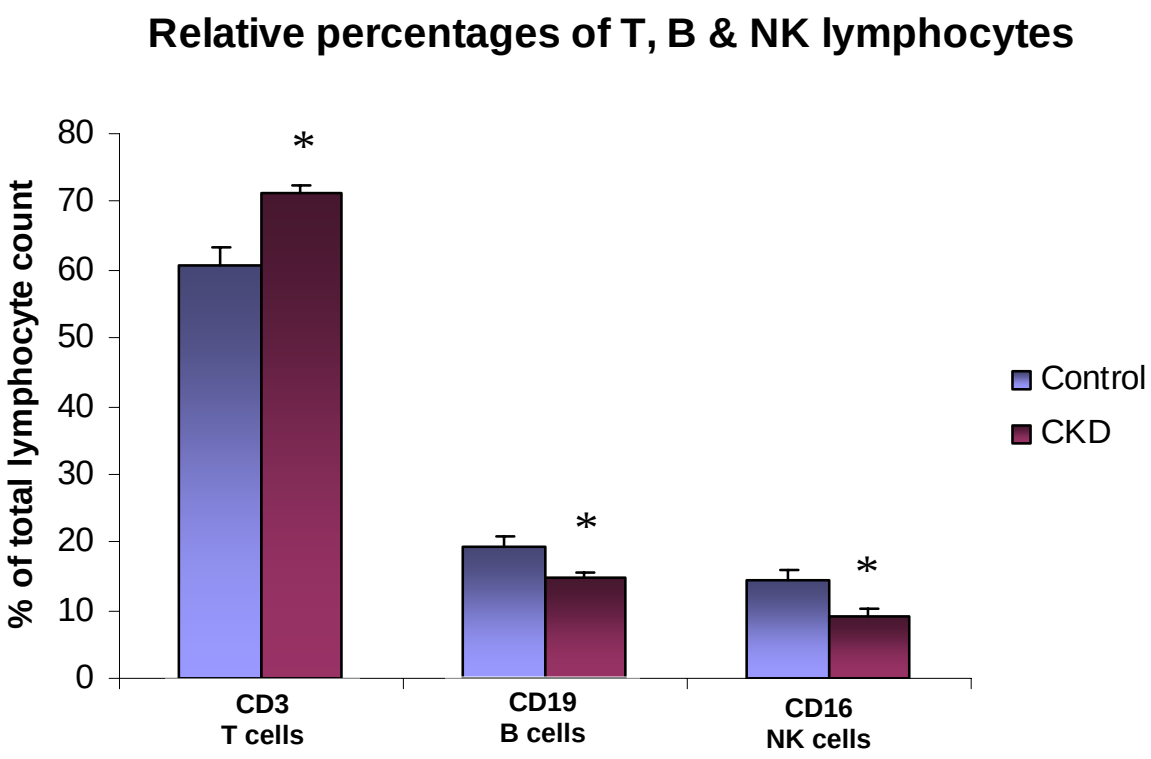


Figure 5.4: Lymphocytes were gated as described in Figure 5.2, and percentages of T (CD3), B (CD19) and NK (CD16) cells were determined as shown in Figure 5.3. CKD patients showed a greater percentage of T lymphocytes ($p<0.001$) and a lower percentage of B and NK cells compared to controls ($p=0.004$ and $p=0.003$ respectively). Mann-Whitney U test, Error bars represent SEM.

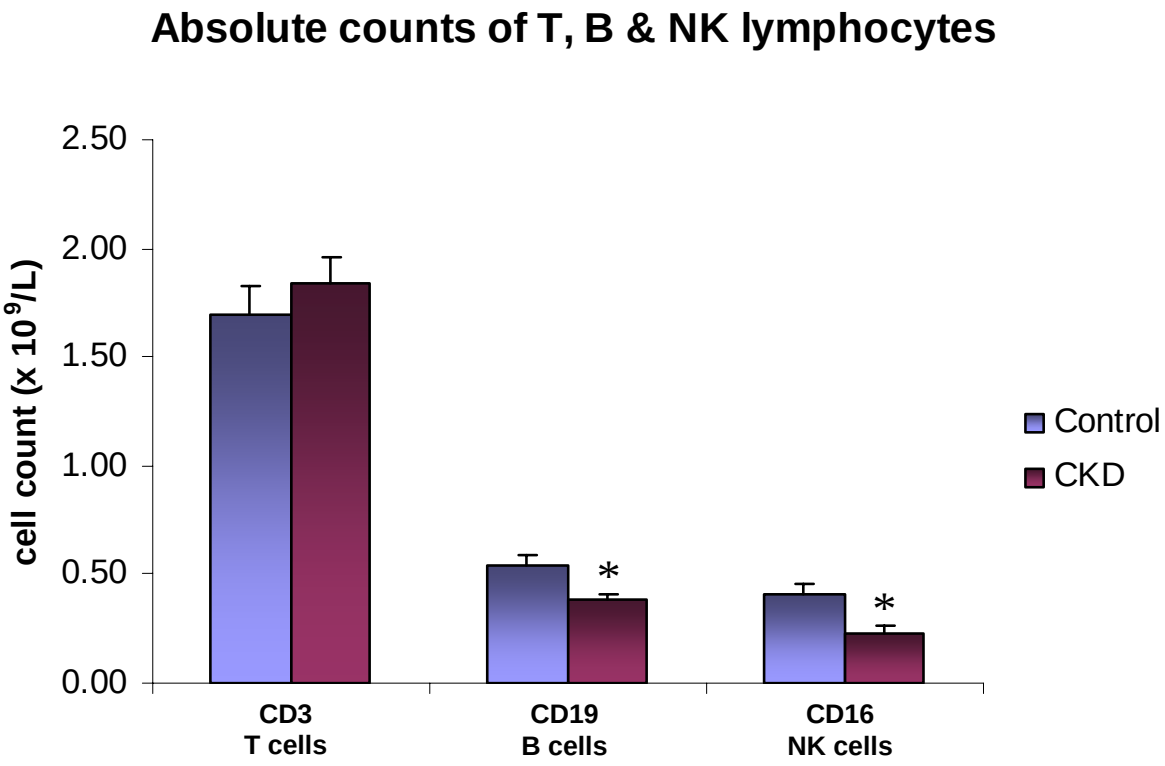


Figure 5.5: Lymphocytes were gated as described in Figure 5.2, and percentages of T (CD3), B (CD19) and NK (CD16) cells were determined as shown in Figure 5.3. Absolute numbers of T (CD3), B (CD19) and NK (CD16) cells were calculated from the total lymphocyte count. There was no difference in absolute numbers of T cells, but absolute counts of B and NK cells were significantly lower compared to controls (p=0.001) for both cell types. Mann-Whitney U test, error bars represent SEM.

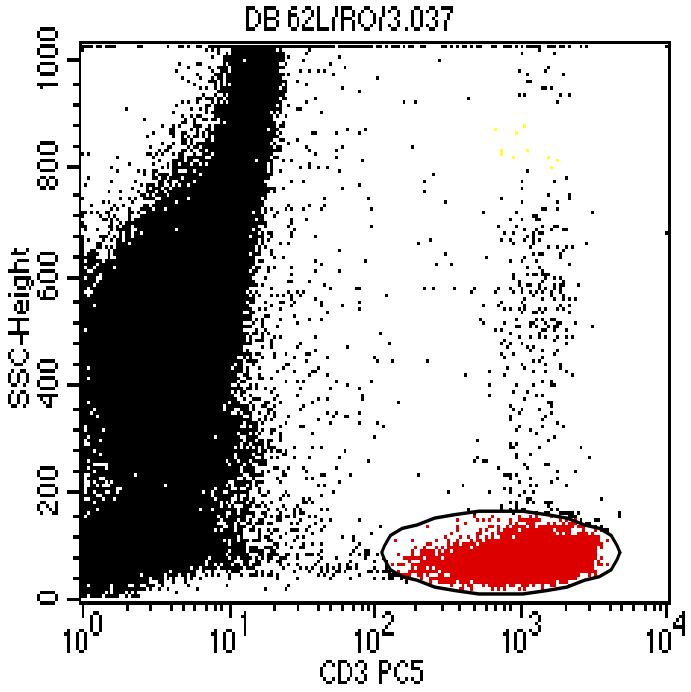


Figure 5.6: Representative flow cytometer dot plot of CD3 gating showing CD3 vs SSC. This population of cells was then back gated on the lymphocyte FSC vs SSC gate to ensure that all CD3+ cells fell within the lymphocyte gate. This population of cells was then used for all subsequent T cell analysis.

5.3.5 T Helper and Cytotoxic T cells

T Helper/Suppressor and cytotoxic T cells were defined as those CD3 T cells that stained positive for CD4 and C8 respectively. There was a significant increase in the percentage of CD4 T helper cells in children with CKD vs control. CKD median 52.2% (range 33.7 – 74.3%) vs control 59.5 (range 50.3 – 72.8%) p=0.02. There was a trend towards a decrease in the percentage of CD8 cytotoxic T cells 33.6% (range 20.6 – 43.8%) vs control 36.5% (range 24.8 – 57.5%) p=0.09. There were no statistically significant differences between groups in the in the absolute numbers of CD4 or CD8 T cells.

The change in percentage of CD4 T cells was reflected in a significant increase in the CD4:CD8 ratio in the CKD group versus control. CKD median 1.75 (range 1.2 – 3.5) vs control 1.49 (range 0.61 – 2.90) p=0.03.

The percentage of double negative (double negative) (CD4-/CD8-/CD3) T cells was significantly lower in the CKD group. CKD median 6.8% (range 3.5 – 12.5%) vs control 10.2 (range 3.7 – 19.6%) p=0.007. There was no difference in absolute numbers of double negative T cells between the groups.

5.3.6 γδ and αβ T cell subsets

γδ and αβ T cells were defined as those CD3 T cells that stained positively for the γδ and αβ T cell receptors respectively. There was a significant reduction in the percentage of γδ T cells in the total CD3 T cell pool compared to control (Figure 5.7). There was also a trend towards an increase in the percentage of αβ T cells p=0.06.

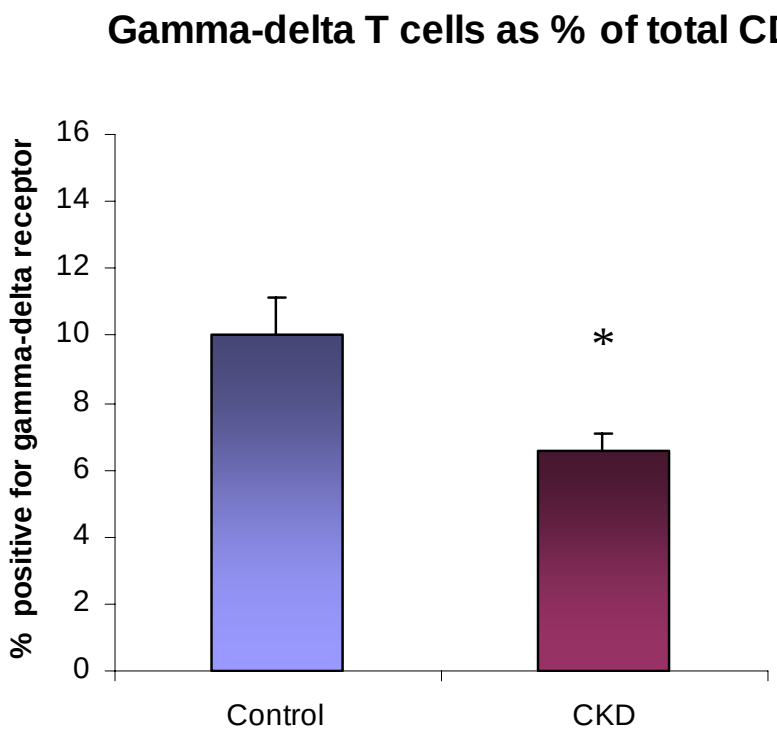


Figure 5.7: T cells were gated as described in Figure 5.6 and the percentage of cells positive for the γδ T cell receptor was measured. CKD patients showed a significant reduction in the percentage of the total T cells that were positive for the γδ T cell receptor compared to controls (p=0.04). Mann-Whitney U test, Error bars represent SEM.

5.3.7 Memory Cells

Memory T cells were defined as CD45RO positive CD3 T cells (Figure 5.8). CKD patients showed a significant reduction in the absolute number of memory T cells. CKD median 0.505 x 10^9 cells/L (range 0.10 – 0.72 x 10^9 cells/L) vs control 0.565 x 10^9 cells/L (range 0.27 – 1.01 x 10^9 cells/L) p=0.02.

5.3.8 Memory cell subsets

The Th1 subset can be defined as CD62L- and Th2 as CD62L+ memory T-cells (CD45RO+) [113]. To define Th1/Th2 memory T cells, CD62L positive and negative subsets of memory T cells were determined as presented in Figure 5.9. Absolute numbers of the CD3/45RO+/CD62L+ (Th2) memory cell subset were significantly reduced in the CKD group compared to control. CKD median 0.224 x 10^9 cells/L (range 0.028 – 0.410 x 10^9 cells/L) vs control 0.345 x 10^9 cells/L (range 0.146 – 0.921 x 10^9 cells/L) p=0.0002. There was no significant difference in the CD3/45RO+/CD62L- (Th1) memory cell subset compared to control.

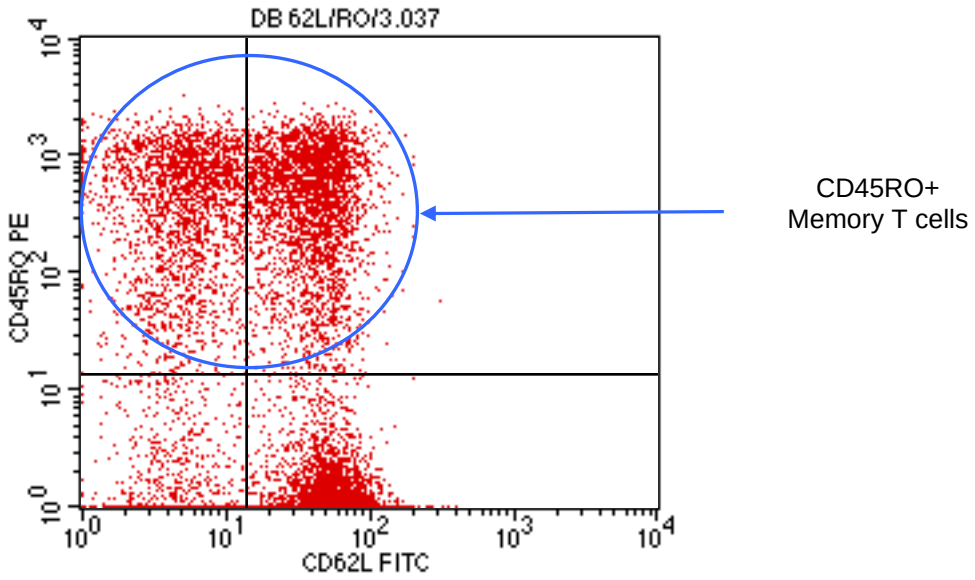


Figure 5.8: Representative flow cytometer dot plot of CD45RO+ memory T cells. T cells were gated as described in Figure 5.6 and the percentage of CD45RO+ T cells was determined.

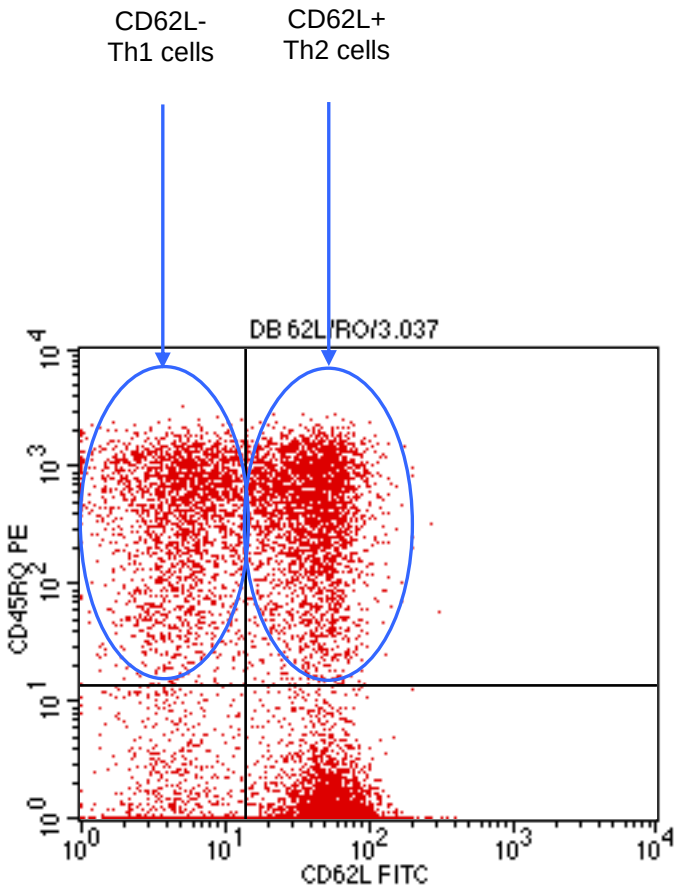


Figure 5.9: Representative flow cytometer dot plot of CD62L-/CD45RO+ Th1 cells and CD62L+/CD45RO+ Th2 cells. T cells were gated as described in Figure 5.6 and the percentages of Th1 and Th2 cells were determined.

5.4 Discussion

5.4.1 Lymphocyte subsets

These experiments define significant and previously unknown changes in immune cell subsets in children with CKD. The results show that the children in this study, most of whom have relatively mild renal disease, show substantial changes in leucocyte subsets which may contribute to the clinical manifestations of the disease.

Consistent with previous reports, there was a significant reduction in absolute numbers of B cells and NK cells, [62, 110, 114]. There was no difference either in total leucocyte or lymphocyte counts, contrary to previous reports of lymphopaenia in more severe CKD [109, 115, 116]. There was no change in total T cell numbers.

B cells are essential for effective antibody response to pathogens. A reduced number of B cells, as found in the present study may result in poor antibody response to foreign antigens, including a poor response to vaccination. This may partly explain the sub-optimal responses to vaccination that has been observed by others in a similar patient group [117].

It has been suggested that decreases in B cells in CKD may be due to increased apoptosis via dysregulation of Bcl-2 [110]. Apoptosis of lymphocyte subsets is therefore examined in Chapter 7.

NK cells are essential for an effective response to viral infections. My finding of a reduction in NK cells in the CKD group with mild disease thus suggests that these children may have an impaired response to viral infections.

Taken together, these findings may predispose this group of patients to increased bacterial and viral infections. Anecdotally there are many reports of increased infection rates in CKD, however at present there are no studies to support this and the hypothesis remains speculative. Interestingly, a very recent study by Cazzavillan et al reports an increase in sub-clinical infections in dialysed CKD patients using molecular methods to detect bacterial DNA [118]. Sub-clinical infections have also been proposed as a cause of atherosclerosis and CVD [119] and hence this may be a contributing factor to the increased rate of CVD seen in our patient cohort.

The higher percentage of T cells that was observed can be accounted for by the reduced numbers of B and NK cells causing a proportionally greater percentage of T cells in the overall lymphocyte pool.

5.4.2 T Helper and Cytotoxic T cells

My study has shown an increased CD4:CD8 ratio that was caused by an increase in the percentage of CD4 T helper cells and a trend towards a decreased percentage of CD8 cytotoxic T cells. The altered ratio appears to been augmented by a decrease in the percentage of CD4-/CD8-/CD3+ (double negative) T cells which caused a corresponding increase in the proportion but not the absolute number of CD4 T cells.

Double negative T cells have been reported as having an important regulatory role in various immune responses including immune tolerance and down-regulation of immune responses [120]. There have been few studies examining double negative T cells and their role in disease pathology, however this subset of cells play a regulatory role [121], and may

thus contribute to any disturbance in immune responses in CKD. It is likely that our finding of a reduction in double negative T cells is due to our observed decrease in the γδ T cell population.

5.4.3 αβ and γδ T cell subsets

I have shown a significant decrease in the number of γδ T cells in the CKD group versus control. γδ T cells are generally double negative and usually make up about 5-10% of the total circulating T cell pool [66]. γδ T cells share numerous features and functions with αβ T cells but also have a number of properties that distinguish them from αβ T cells. Probably the most important of these is the ability of T cells expressing the γδ T cell receptor to recognise antigen via a different mechanism from T cells that have the αβ T cell receptor. γδ T cells are able to recognise antigen independently of MHC molecules and are able to process and present soluble antigen directly [122, 123]. γδ T cells have a specific and specialised role in protective immunity via cytokine secretion and cytotoxic activity [67]. They influence CD8 αβ T cells to differentiate into cytotoxic lymphocytes [67]. Others have reported that dialysed children show a reduction in the number of γδ T cells present in the circulation [65]. This reduction may be partly responsible for the impaired T cell responsiveness which is seen in CKD. This study is the first to show that the γδ T cell subset appears to be reduced by uremia *per se.* A decrease in γδ T cells may result in an increase in susceptibility to various microbial infections in our patient group and warrants further investigation, particularly given the recent report of increased subclinical infections in dialysis patients [118].

5.4.4 Reduction in memory T cells

CKD patients showed a significant decrease in absolute CD3+/CD45RO+ memory T cell counts compared to controls. Although other studies have reported decreased total CD3 T cell counts [62, 65, 124] in adults and children with CKD, this study is the first to report a decrease in memory T cells in children with non-dialysed CKD. We observed no differences in any parameters between patients with different causes of CKD, suggesting that the presence of uraemia itself is most likely the major cause of these immunological changes. The decrease in memory T cells may be due to either increased apoptosis and/or decreased production of these cell types. A previous study by Matsumoto et al [125] has suggested that T cells in adults with end-stage renal disease may undergo increased apoptosis. If this is also the case in children then this may account for the decreases in lymphocyte subsets seen in this study. It is also possible that alterations in T cell trafficking may result in sequestration of these cells within tissues and a reduction in the number of circulating cells.

5.4.5 Th1/Th2 memory cell subtypes

The increase in T cell production of IL-4 and the decrease in IFN-γ described in the previous chapter suggest an alteration of Th1/Th2 balance towards a Th2 phenotype in children with CKD. The Th1 subset can be defined as CD45RO+ memory T-cells that are negative for CD62L [113]. In light of my findings from the previous chapter, I hypothesised that CKD patients would show an increased number of T cells expressing CD62L, indicating a Th2 bias which would account for the increase in IL-4 and

decrease in IFN-γ seen in the T cells from the CKD group. Paradoxically, there was a loss of CD62L from the CD45RO/CD3 memory T cells which may indicate a skewing of memory T cells towards a Th1 phenotype. This is a finding consistent with previous reports in adult haemodialysis patients [126], but unexpected in this study given the pattern of intracellular T cell cytokine production seen in the previous chapter.

Expression of L-selectin on lymphocytes is required for effective homing to peripheral lymph nodes and Peyer's patches [127]. Loss of CD62L from the memory lymphocytes may inhibit the homing and adhesion of lymphocytes to lymph nodes. Thus, even if lymphocytes are unable to extravasate due to a loss of CD62L, the circulating number may still be reduced. A previous study by Matsumoto et al [125] has suggested that T cells in adults with end-stage renal disease may undergo increased apoptosis. If this is also the case in children then this may account for the decreases in lymphocyte subsets seen in this study. Apoptosis of leucocyte subsets is examined in Chapter 7.

CD62L is rapidly lost upon activation of lymphocytes as a result of proteolytic cleavage. Activation of leucocytes can also be a cause of increased cytokine production which has already been examined in Chapter 4, Therefore I have examined leucocyte activation in Chapter 6.

My finding of a decreased number of memory T cells and significantly reduced CD62L expression may explain the decreased response to vaccination in children with CKD and could predispose these children to increased infection rates [6, 62].

5.4.6 Summary

Taken together, these immunological changes, including the decreased numbers of B and NK cells may contribute to some of the clinical symptoms associated with CKD. Children with CKD have variable and sometimes poor responses to common vaccines [117]. This is a particular problem with Hepatitis B vaccine and constitutes a significant issue for “at risk” populations [128]. In addition, a high infection rate is seen in individuals with advanced renal failure, the cause of which is likely to be multifactorial (increased infectious load, poor nutritional state, adverse mechanical factors). It is conceivable that the changes described herein create additional susceptibility that is not readily identified in these circumstances.

The findings provide a more accurate understanding of the immunological changes that may contribute to the clinical manifestations of CKD.

Chapter six

Leucocyte activation markers

6.1 Introduction

In the previous chapters I have shown that children with CKD display changes in both circulating cytokine levels and leucocyte cytokine production. Alterations in cytokine production can result from leucocyte activation, which is known to occur as a result of many systemic inflammatory conditions such as rheumatoid arthritis, asthma and atherosclerosis [129]. Thus, I hypothesised that children with CKD would show increased numbers of activated leucocytes *ex vivo*. Cell adhesion molecules have an integral role in various inflammatory processes, especially CVD, with the accumulation of leucocytes on the endothelial wall being one of the first stages of atherotic plaque formation.

Since this group of children have a rate of CVD 1000 times greater than the equivalent group of children without CKD [18], I further hypothesised that children with CKD would display changes in the expression of adhesion molecules that are associated with the attachment of leucocytes to the endothelium.

To test this hypothesis I applied flow cytometry to examine the expression of relevant cell surface antigens that define leucocyte activation and adhesion status in children with CKD and a control group. I also measured E- selectin in plasma a soluble adhesion molecule known to be shed from the endothelium.

In this study I investigated the two major classes of adhesion molecules, selectins and integrins. The selectins consist of three different molecules – L-selectin E-selectin and P-selectin – with 60% homology in their structure. These are expressed on leucocytes, endothelial cells and platelets

respectively. L-selectin is shed during the initial interactions (tethering/rolling) of leucocytes with activated endothelium [130]. E-selectin is expressed on activated endothelial cells and is a mediator of the attachment of leucocytes to the endothelium [130]. Expression is transient, peaking about 6 hr after activation by inflammatory cytokines such as IL-1β and then declining, with the shedding of soluble E-selectin (sE-selectin) [131]. Thus, the increase in IL-1β observed in this study has the potential to result in an increase in sE-selectin caused by activation of the endothelium.

Integrins mediate the firm adhesion of leucocytes – especially neutrophils and monocytes – to the activated endothelium. The CD11/CD18 family are principal integrins and comprise CD11a, CD11b, and CD11c [132, 133]. The interaction between selectins and integrins is illustrated in Figure 6.1.

I therefore measured expression of L-selectin leucocytes and plasma sE-selectin as well as expression of integrins on neutrophils and monocytes. Further, a range of surface antigens to define the *ex vivo* activation status of leucocyte sub-populations were measured and are outlined in Table 6.1.

Figure 6.1: Rolling, tethering and adhesion of a neutrophil to the endothelium showing the loss of L-selectin and the interaction between an integrin on the neutrophil surface and E-selectin on the endothelium.

Image adapted from

Leucocyte type	Surface Antigen	Specificity
Lymphocytes	$CD62L^-$	Activated lymphocyte / L-selectin
T cells	CD3	T cell (T cell receptor)
	CD3/CD69	Very early activated T cell
	$CD3/CD62L^-$	Very early activated T cell / L-selectin
	CD3/CD25	Early activated T cell
Monocytes	CD14	Monocyte (LPS receptor)
	CD14/CD69	Very early activated monocyte
	$CD14/CD62L^-$	Very early activated monocyte / L-selectin
	CD14/CD25	Early activated monocyte
	CD14/HLA-DR++	Late activated monocyte
	CD14/CD11b++	Activated monocyte / integrin
Neutrophil	$CD62L^-$	Activated Neutrophil / L-selectin
	CD11a++	Activated Neutrophil / β2-integrin
	CD11b++	Activated Neutrophil / β2-integrin
	CD11c++	Activated Neutrophil / β2-integrin

Table 6.1: Activation and adhesion molecules measured on the surface of resting cells. Compiled from [134].

6.1.2 Hypothesis

Children with CKD will show:

- Decreased expression of L-selectin on leucocytes.
- Increased plasma sE-selectin shed from the endothelium.
- Increased expression of markers of ex vivo activation of leucocyte subsets.

6.1.3 Specific Aims

- To quantify the ex vivo activation and adhesion status of various blood leucocyte subsets by measurement of the surface expression of selected activation and adhesion markers by flow cytometry.
- To quantify plasma sE-selectin shed from the endothelium using an ELISA.

6.2 Materials and methods

6.2.1 Subjects

The same study population of 22 children with CKD and 24 age matched control subjects as used in previous chapters were used for this part of the study. None of the subjects had any history of other immunological abnormalities and all had been completely well in the 2 weeks prior to the study. Full details of the study populations, as well as exclusion criteria are outlined in detail in Chapter 2.

6.2.2 Blood Collection and sample preparation

Venous blood was collected from study subjects into a vacuette tube containing Lithium heparin as anti-coagulant.

Total white cell count and differential was performed on an automated cell counter as described in Chapter 5. Samples were centrifuged at 3000xg for 10 min. Plasma was removed and frozen at -70^{o}C for subsequent analysis. Cells were washed once in wash buffer and then resuspended to the original whole blood volume in the same buffer. Processing for flow cytometrical analysis was completed within 1 hr.

6.2.3 Flow cytometry leucocyte surface marker determination

Leucocyte surface markers were determined using the same technique outlined in Chapter 5 (Figure 5.1). Briefly, 50 µL of prepared sample was added to appropriately titrated monoclonal antibodies. After 10 min incubation at RT in the dark, red cells were lysed by the addition of Facslyse for 10 min and then cells were washed again before immediate acquisition on a FACSCalibur flow cytometer.

Data analysis was performed using CellQuest software (BD). Differences between the CKD and control groups were determined using a non-parametric Mann Whitney U test and SPSS statistical software, p values <0.05 were considered significant.

6.2.4 Measurement of sE-selectin

Soluble E-selectin was measured using a commercially available quantitative sandwich enzyme immunoassay (R&D Systems MN, USA) according to the manufacturers instructions. Briefly, a 96 well micro plate coated with an antibody specific for sE-selectin was supplied. Standards, plasma samples, controls and a conjugate were pipetted into the wells of the micro plate. Any sE-selectin present was sandwiched by the immobilised antibody and a second enzyme linked monoclonal antibody specific for sE-selectin. Excess reagents were removed via washing and a substrate solution was added. The colour that developed in proportion to the amount of sE-selectin bound to the micro plate was measured using a micro plate reader (Perkin Elmer MA, USA) set at a wavelength of 450nm with correction at 620nm. A standard curve was created using Multicalc software

(Wallac, UK) to generate a four parameter logistic curve-fit. Concentrations of sE-selectin for study samples were then determined by reading from the standard curve.

6.3 Results

6.3.1 Lymphocyte activation and adhesion markers

Lymphocytes were identified as described in Chapter 5 (Figure 5.2).

There was a significant reduction in both the percentage and absolute number of lymphocytes expressing CD62L in the CKD group versus control. This is illustrated in Figures 6.2 and 6.3.

6.3.2 T cell activation and adhesion markers

T cells were gated using CD3 expression vs. SSC as described in Chapter 5 (Figure 5.6). There we no significant differences between the percentages of cells expressing any of the activation and adhesion markers that were measured. Results are detailed in Table 6.2.

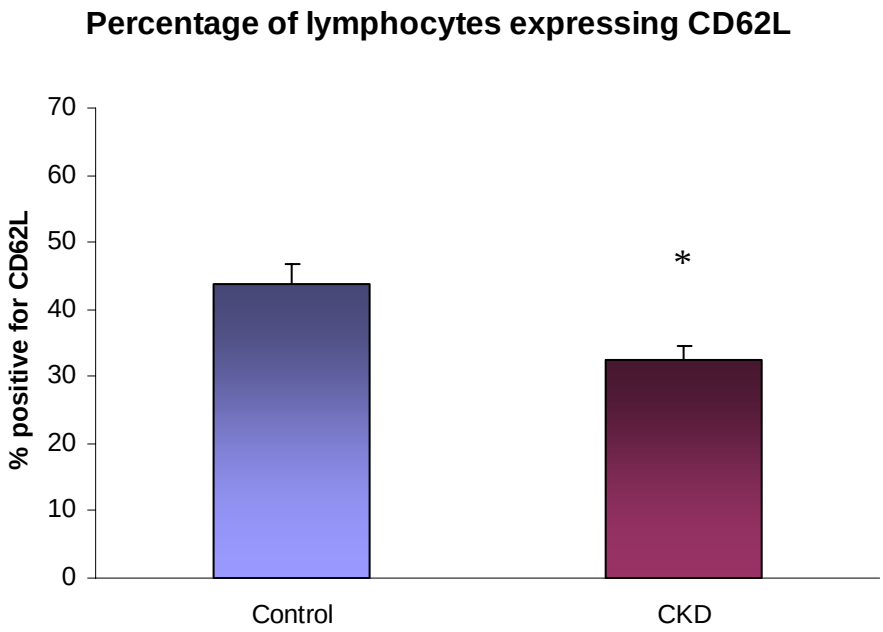


Figure 6.2: The CKD group showed a decrease in the percentage of lymphocytes expressing CD62L compared with controls. Data expressed as mean ± SEM,* p<0.001 Mann-Whitney.

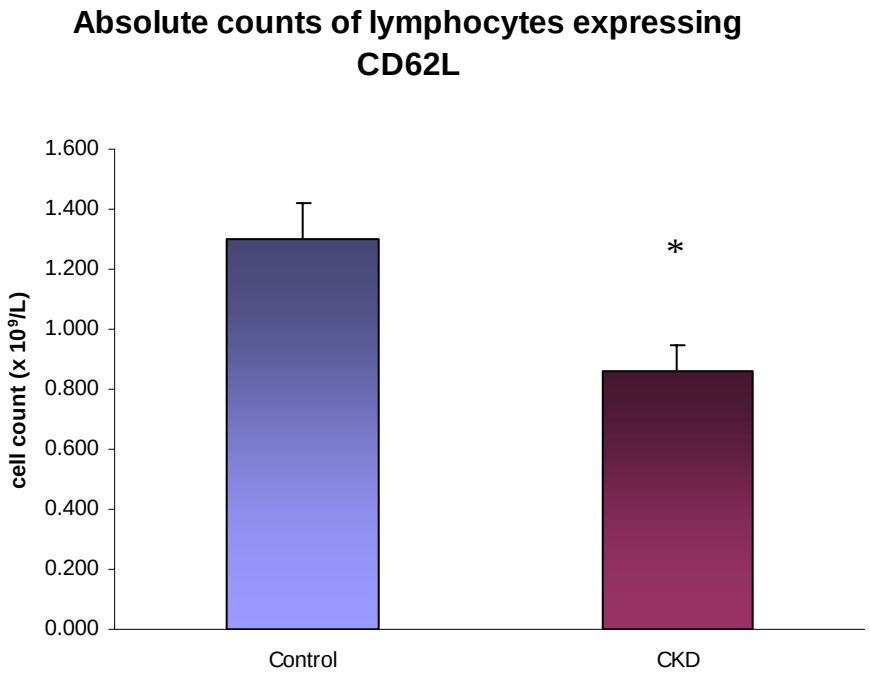


Figure 6.3: The CKD group showed a decrease in the absolute number of lymphocytes expressing CD62L compared with controls. Data expressed as mean ± SEM,* p<0.001 Mann-Whitney.

Marker (%)	Median CKD	Range CKD	Median Control	Range Control	P value
CD69	8.2	2.9 – 15.2	6.9	1.3 – 21.9	0.65
CD62L	22.4	14.1 – 37.2	24.0	17.7 – 25.8	0.87
CD25	10.6	1.2 – 24.3	11.0	3.6 – 23.7	0.42

Table 6.2: The percentage of CD3 T cells expressing activation and adhesion molecules. There were no significant differences between the percentages of cells expressing any of the markers that were measured.

6.3.3 Monocyte activation and adhesion markers

The monocyte population was determined by gating CD14 positive events vs. SSC (Figure 6.4). Expression of activation and adhesion markers was determined by either the percentage of total monocytes of cells that stained positive or the MFI of the positive population

There were no changes in any of the activation markers that were examined when the groups were considered as a whole. Results are outlined in Table 6.3.

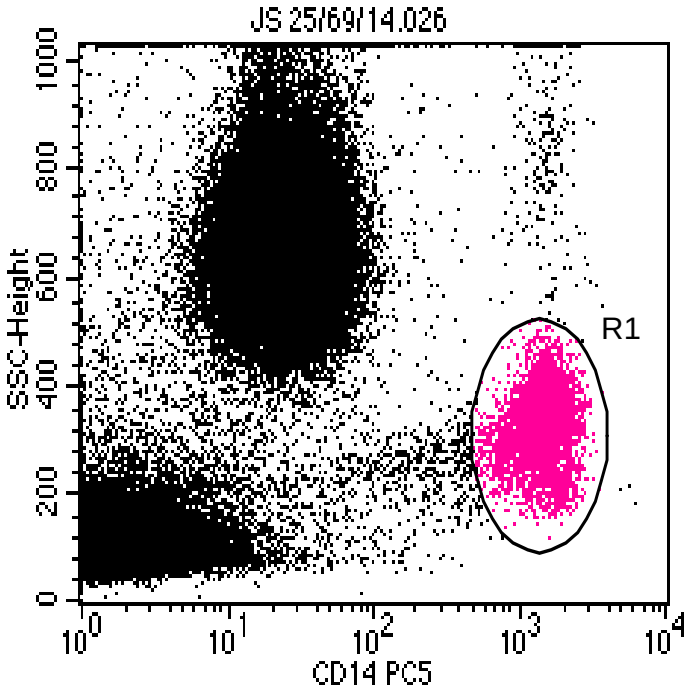


Figure 6.4: Representative flow cytometer dot plot of CD14 gating showing CD14 vs SSC. This population of cell was then used for all subsequent monocyte analysis.

Marker	Median CKD	Range CKD	Median Control	Range Control	P value
CD69 (%)	9.2	1.0 – 70.3	11.4	2.1 – 46.3	0.99
CD62L (%)	95.9	29.6 – 99.8	96.5	60.5 – 99.5	0.91
CD25 (%)	1.9	0.9 – 8.3	1.9	0.9 – 10.8	0.84
CD11b (MFI)	1955	867 – 3432	1911	390 – 2944	0.62
HLA-DR (MFI)	757	186 – 2654	545	395 – 1305	0.33

Table 6.3: The percentage or MFI of monocytes expressing activation and adhesion molecules. There were no significant differences between the percentages or MFI of cells expressing any of the markers that were measured.

Investigation of a cohort of eleven CKD patients who showed increases in circulating cytokines in (Chapter 3) revealed a significant increase in the MFI of HLA-DR compared with the control group (median 1539 (range 526 – 2654) versus control 545 (range 395 – 1305) p = 0.002) but no change in the expression of the other monocyte surface markers .

6.3.4 Neutrophil activation and adhesion markers

Neutrophils were identified by gating on FSC and SSC as shown in Figure 6.5.

There was a significant increase in the MFI of CD11b in the CKD group versus control (Figure 6.6). There was no change in the expression of other integrins (CD11a, CD11c) or L-selectin (CD62L). These results are outlined in Table 6.4. There were no significant changes in expression of neutrophil adhesion molecules in the cohort of eleven CKD patients who showed increases in circulating cytokines in Chapter 3.

Marker	**Median CKD**	**Range CKD**	**Median Control**	**Range Control**	**P value**
CD62L (%)	96.8	23.4 – 99.9	96.7	21.6 – 99.7	0.46
CD11a (MFI)	82	11 – 627	91	24 – 597	0.52
CD11b (MFI)	**3250**	**1643 – 4443**	**2231**	**1095 – 2397**	**0.005**
CD11c (MFI)	35	19 – 155	32	20 – 60	0.39

Table 6.4: The percentage and MFI of neutrophil expression of activation and adhesion molecules. The CKD group showed significant up regulation of CD11b compared with control.

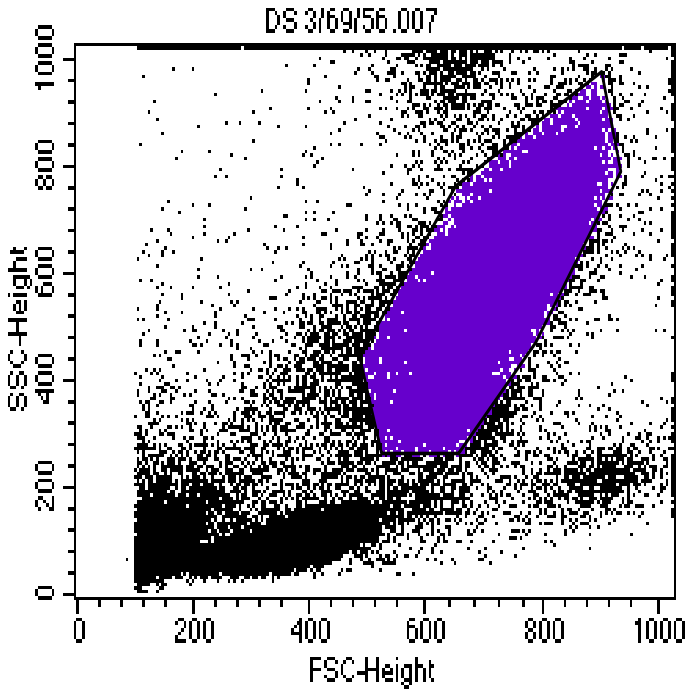


Figure 6.5: Representative flow cytometer dot plot of neutrophil gating showing FSC vs SSC. This population of cell was then used for all subsequent neutrophil analysis.

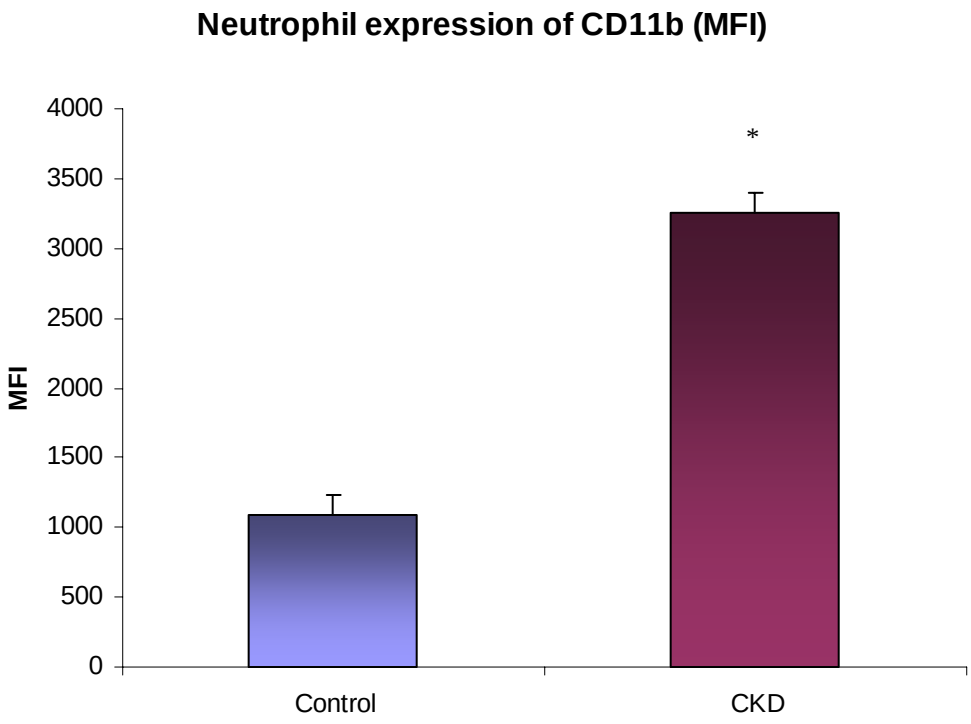


Figure 6.6: The CKD group showed an increase in the MFI of neutrophil expressing CD11b compared to controls. Data expressed as mean ± SEM, *p<0.001 Mann-Whitney.

6.3.5 Soluble E-selectin

There was no difference between the CKD group as a whole or the cohort of eleven CKD patients who showed increases in circulating cytokines (Chapter 3) in plasma sE-selectin, CKD median 46.0 ng/mL (range 19.4 – 70.2), increased cytokine cohort median 48.3 ng/mL (range 19.4 – 68.3) versus control 51.1 ng/mL (range 20.2 – 107.0).

6.4 Discussion

The uraemic milieu contains many elements that are toxic to the body including small water soluble molecules, protein bound molecules and larger molecules such as Cystatin C and β-2 microglobulin [135]. Many of these may potentially activate leucocytes either alone or in combination. I hypothesised that leucocytes from children with CKD would show signs of increased activation because of their exposure to the inflammatory elements in the uraemic environment. Surprisingly, in contrast with studies in adults [136-138], there were few signs of increased activation of any leucocyte type in the current study when the CKD group was considered as a whole. There were however, some significant changes in the expression of surface markers involved in cell trafficking and adhesion which may have clinical implications in this group.

The total lymphocyte pool showed significant down-regulation or loss of L-selectin. L-selectin is rapidly lost upon activation of lymphocytes as a result of proteolytic cleavage. Binding of L-selectin expressed on lymphocytes to carbohydrate ligand(s) on lymph node high endothelial

venules is thought to initiate lymphocyte extravasation from blood to lymph nodes and Peyer's patches during recirculation and localization to sites of antigen exposure [139], This process may be inhibited by the reduced L-selectin expression on lymphocytes [139]. Migration of naive T cells through lymph nodes increases the likelihood of these T cells encountering a primary antigen [140], consequently significantly reduced L-selectin expression and lack of subsequent exposure to antigens in lymph nodes and Peyer's patches may explain the decreased response to vaccination that is seen in children with CKD and may predispose these children to increased infection rates [6, 62]. A recent study by Cazzavillan *et al* used a molecular method to screen for sub clinical infection by detection of bacterial DNA. Their study of 81 adult CKD patients undergoing haemodialysis found increased bacterial DNA that correlated with levels of IL-6, CRP and AOPP in a population of adults with ESRD. A future study of my patient group aimed at detecting sub clinical infection by comparing levels of bacterial DNA with those of a control group would be of value.

Interestingly, the cohort of CKD patients with increased plasma cytokines also had a significantly increased monocyte HLA-DR expression. This indicates that these patients may have increased monocyte activation *in vivo* that may lead to the increased cytokine production. My *ex vivo* experiments in Chapter 4 showed that monocytes from the CKD group as a whole were able to make more cytokines after stimulation with LPS, possibly a result of monocyte priming by the uraemic environment. Future planned experiments to measure cytokine production in monocytes from non-uraemic patients that have been exposed to uraemic toxins will test the

hypothesis that the uraemic milieu is able to prime monocytes to produce larger amounts of cytokines in response to stimulus in vitro.

Alternatively, the cohort of CKD patients that had evidence of increased monocyte activation may be more sensitive to some element in uraemic plasma than other CKD patients. The reason for this are unclear, however several studies have identified numerous functional polymorphisms that may affect production of inflammatory cytokines [141, 142] and further studies to determine whether there is a polymorphism or other factor in this group that predisposes them to more easily activate monocytes and produce cytokines would be worthwhile.

The CKD group showed significant up-regulation of the adhesion integrin CD11b on neutrophils. This is consistent with findings in adult CKD patients of increased CD11b as well as decreased L-selectin [64]. A recent report from Bouts *et al* [1] also reported increased CD11b in children with CKD in the context of it's function as complement receptor 3.

Beta-2 integrins such as CD11b control recruitment and adhesion of leucocytes to the vascular endothelium [143]. Increased cell adhesion to the endothelium promotes the formation of atherosclerotic plaques and increased incidence of CVD [29]. In this regard, the increase in CD11b that I observed in my study may be a factor in the increased incidence of CVD in children with CKD.

Interestingly, there was no increase in the shedding of E-selectin in this patient population. Consistent with my results, a recent study by Benitez *et al* [144] found no differences in sE-selectin levels in patients with primary hypertriglyceridaemia compared with a control group. The study did

however find differences in the levels of intercellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM). Due to sample volume limitations, I was unable to measure a range of soluble adhesion molecules, however, given the changes in L-selectin and CD11b, further study of other soluble adhesion molecules in children with CKD would also be worthwhile.

Recent studies have reported increased endothelial dysfunction and CVD in both adults and young people with CKD [22, 145]. These increases in CVD do not correlate with traditional risk factors such as increased lipids and hypertension [146], suggesting that there are additional contributing factors. Increased β-2 integrin dependent leucocyte-membrane interactions may contribute to the increased rate of CVD observed in children with CRF. This notion is supported by a study of patients with stable CVD and hypercholesterolaemia that showed increased expression of CD11b on monocytes, when compared to control patients without CVD [147]. There have been recent reports that CD11b expression can be reduced by drugs such as statins and aspirin [147, 148]. Therefore, therapeutic targeting of CD11b with these drugs cohort has the potential to reduce the incidence of CVD in children with CKD.

Summary

These results indicate that children with CKD do not appear to have a generalised increase in activation of WBC, rather, there appears to be a number of changes in leucocyte phenotype that may result in altered adhesion of leucocytes to the endothelium and trafficking through the lymphatic system.

This may have clinical effects by a reduction in trafficking of lymphocytes between lymph nodes and the circulation and therefore reduced opportunity for lymphocyte exposure to antigen causing a decreased response to vaccination and an increased predisposition to infection.

Increased adhesion of neutrophils to the endothelium, mediated by CD11b, may allow neutrophils to congregate on the endothelial wall and form a base for atherotic plaques, therefore contributing to the increased rate of CVD seen in this cohort. Further studies are required to confirm these results and to elucidate whether the changes in leucocyte expression of adhesion molecules are accompanied by changes indicative of endothelial dysfunction. This will provide a treatment target for anti-inflammatory drugs such as statins.

Chapter seven

Apoptosis of lymphocyte subsets

7.1 Introduction

In Chapter 5 I reported decreases in the numbers of B, NK and memory T cells in children with CKD compared with healthy controls. Numerous reports suggest that some lymphocyte subsets, in particular Th1 and memory T cells in patients with CKD, undergo selective increased apoptosis (programmed cell death)[100, 110, 112, 125]. Although the children in the present study had more mild renal failure than the patients in any of the prior studies, I aimed to determine whether this group would also show increased apoptosis of selected lymphocyte subsets consistent with the previously reported findings. Such increases in apoptosis may explain the differences in lymphocyte subset numbers described in Chapter 5.

Apoptosis is a highly regulated pathway that is important in normal developmental processes as well as disease. Cells undergoing apoptosis are identifiable by a number of cell membrane changes, including expression of phosphatidylserine (PTS) on the membrane surface. Annexin-V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein with a high affinity for PTS. Annexin-V may be conjugated to fluorochromes such as FITC whilst retaining its high affinity for PTS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis [149].

Annexin-V staining is ideally performed on isolated cells or cell lines because of possible damage to white cell membranes that may occur during the red cell lysis process [150]. Because of the small sample volume available in this study (0.1mL), pre-treatment of samples to isolate the cells of interest was not possible. Thus, I performed apoptosis studies using an

Annexin-V staining technique for whole blood which was based on a published method that had previously been used successfully in our laboratory [151].

7.2 Hypothesis

- Children with CKD will show an increased level of apoptosis of lymphocyte subsets that may account for the differences in subset numbers described in Chapter 5.

7.3 Aims

- To use a whole blood flow cytometric assay based on Annexin-V binding to PTS to detect apoptotic lymphocyte subsets.
- To enumerate the numbers of apoptotic T, memory T, B and NK cells using this assay.

7.4 Materials and Methods

7.4.1 Sample collection

Samples for the initial experiments were collected from 10 CKD (patient ID's 1, 3, 5, 7-10 and 12-15 from Table 2.1) and 10 control patients in the same manner as outlined in Chapter 2. Samples for the subsequent RBC lysis studies were collected from 5 normal adult donors recruited from laboratory staff who gave informed consent.

7.4.2 CKD vs Control Annexin-V staining

Lymphocyte subsets were determined as in previous chapters using the surface marker staining technique outlined in Chapter 5 (Figure 5.1) and then subsequently stained for Annexin-V binding. Briefly, 50 μL of resuspended blood cells were incubated with the appropriate pre-titrated PE or PC-5 conjugated Mabs (Table 7.1) for 10 min at RT in the dark, RBC were lysed by incubation with FACSlyse for 10 min. Samples were centrifuged at 1500xg for 1 min and the supernatant discarded. WBC were washed with 2 mL Annexin binding buffer (Appendix 5) and resuspended in 50μL of Annexin binding buffer. Five μL of appropriately titrated FITC conjugated Annexin-V was added. Data acquisition was performed immediately using a FACSCalibur flow cytometer (BD). Data analysis was performed using CellQuest software (BD). Quadrant markers were set using isotype matched negative antibodies. Annexin-V FITC positive and negative staining was identified visually (as in Figure 7.2).

FITC	PE	PC5	Cell type
Annexin-V	CD3	CD56	NK cells (CD3-/CD56+)
Annexin-V		CD19	B cells
Annexin-V	CD45RO	CD3	T cells, Memory T cells

Table 7.1: Mabs used to define lymphocyte subsets.

7.3.3 Subsequent staining to test RBC lysis techniques

Surface marker staining was performed as above until the RBC lysis step. RBC were then lysed using one of the techniques in outlined in Table 7.2. After the RBC lysis step was complete, samples were washed with Annexin binding buffer and stained for Annexin-V as above.

Lysing agent	**Time (min)**
FACSlyse	3
FACSlyse	5
FACSlyse	10
Ammonium chloride (8.3g/L in 0.01M Tris buffer)	10
Ammonium chloride	20
Deionised water (hypotonic)	1.5

Table 7.2: Lysing agents and times used to remove RBC from samples prior to Annexin-V staining

7.4 Results

7.4.1 Annexin-V staining following RBC lysis with FACSlyse

The RBC lysis step was performed using FACSlyse and the standard time recommended RBC by the manufacturer (10min). This had been used successfully in our laboratory on previous occasions [151]. It was noted that there were very large differences between patients in the numbers of Annexin-V positive cells in both the control and CKD groups (Table 7.3).

Cell Type	**% Cells Annexin-v positive (min – max) CKD**	**% Cells Annexin-v positive (min – max) Control**
T cell (CD3)	0.6 – 56.7	1.2 – 70.3
Memory T cell (CD3/CD45RO)	0.2 – 60.4	0.1 – 56.5
B cell (CD19)	1.0 – 47.2	1.6 – 68.2
NK cell (CD56)	0.9 – 50.9	1.4 – 38.6

Table 7.3: The range of Annexin positive staining cells for each lymphocyte subset using the standard 10 min FACSlyse RBC lysis step.

It was further noted that some patient samples lysed much more readily than others. In some samples, cells would appear lysed (i.e. specimen looked clear) after only 2-3 min while others still appeared to have unlysed RBC (i.e. specimen looked cloudy) after 7-8 min. All samples were completely lysed after the recommended 10 min incubation with FACSlyse. It was noted that the samples that lysed more quickly generally had higher levels of Annexin-V staining than those that took longer to lyse. Upon further

investigation we observed that the time of exposure to FACSlyse was directly correlated to the number of Annexin-V positive cells. This is illustrated in Figure 7.1. When samples that lysed readily were treated for a shorter time with FACSlyse, the Annexin staining was less (Figure 7.2). When those samples that took longer to lyse were treated for a shorter time with FACSlyse, there were too many intact RBC present to be able to distinguish the lymphocyte population. Thus I was unable to determine a standard time for treatment of samples with FACSlyse.

7.4.2 Annexin-V staining following RBC lysis with ammonium chloride

Ammonium chloride is a gentle lysing reagent that generally produces excellent light scatter characteristics to identify leucocytes. I hypothesised that the damage caused to cell membranes by FACSlyse would be ameliorated by the gentle action of ammonium chloride. Although the Annexin-V staining results with ammonium chloride were generally lower, similar variations in sample propensity for lysis and Annexin-V staining were observed using the ammonium chloride lysis method (Figure 7.3).

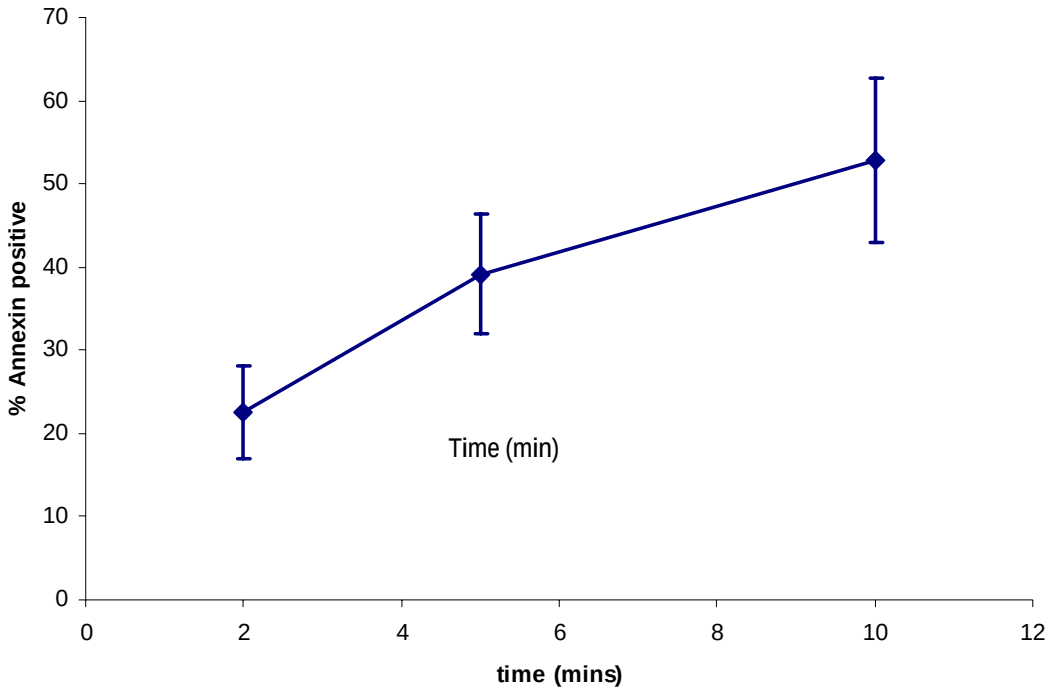


Figure 7.1: The relationship between the time of exposure to FACSlyse and the number of cells staining positive for Annexin-V in a typical sample. Mean ± SD of 3 replicates.

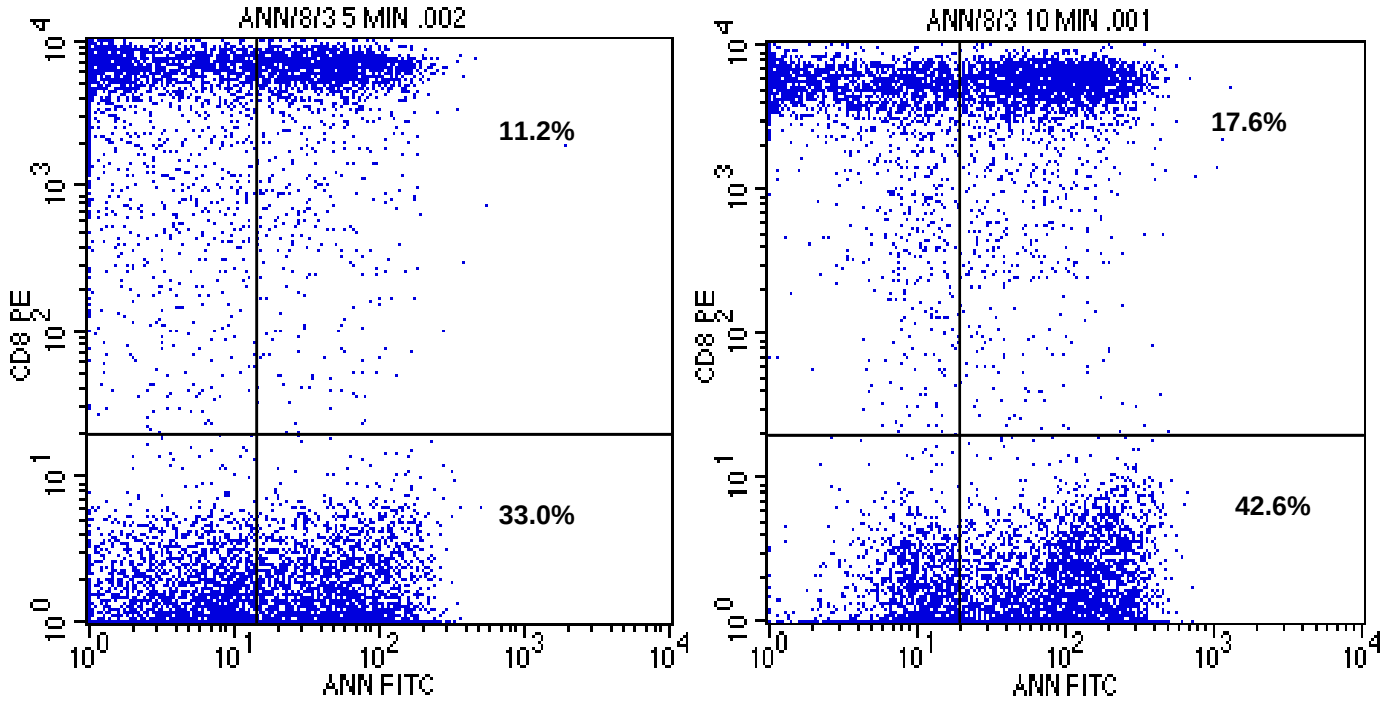


Figure 7.2: Typical dot plot of demonstrating the rise in Annexin-V in the same patient sample with increased exposure time to FACSlyse. Plots show total Annexin-V staining on CD3 positive T cells at 5 min (44.2%) vs 10 min (60.2%).

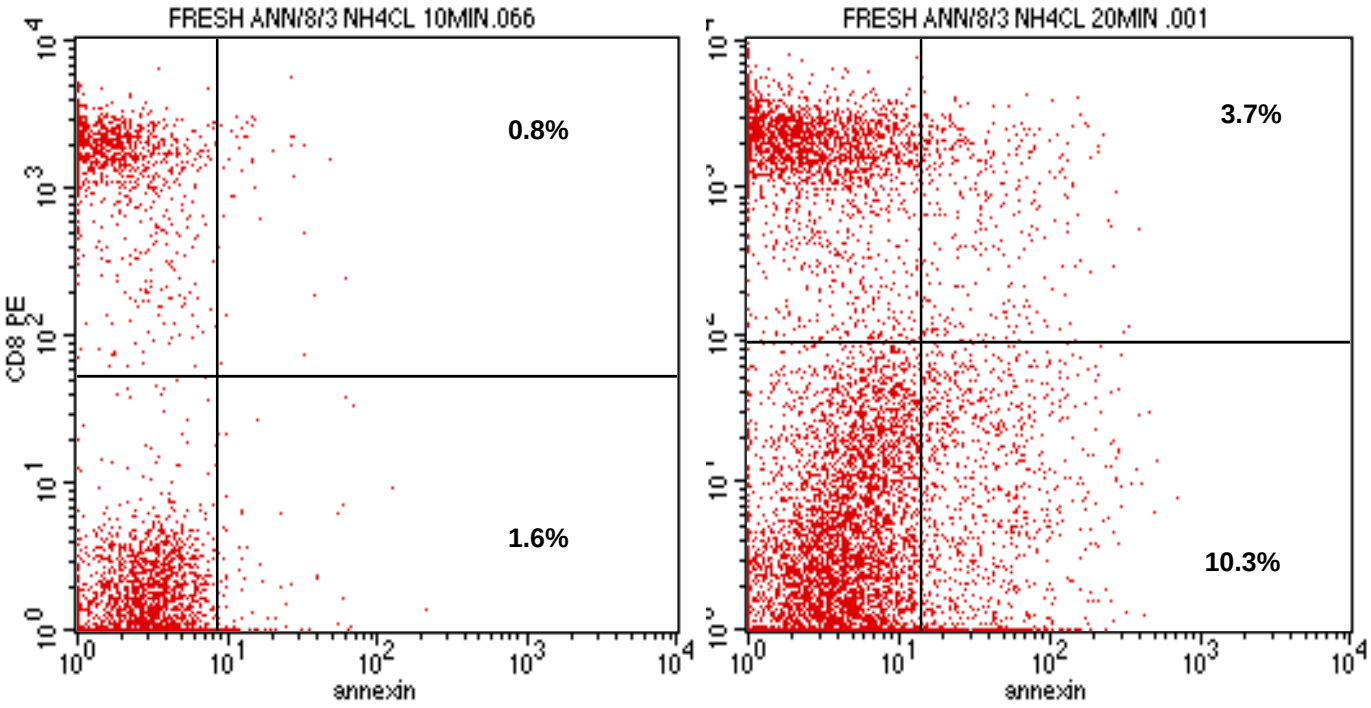


Figure 7.3: Typical dot plot demonstrating a generally lower level of Annexin-V staining in the same patient sample as Figure 7.2 using Ammonium Chloride as lysing agent and showing increased Annexin V staining as exposure time to lysing agent is increased. Plots show total Annexin-V staining on CD3 positive T cells at 5 min (2.4%) vs 10 min (14.0%).

7.4.3 Annexin-V staining following RBC lysis with deionised H_2O

Hypotonic lysis of RBC using deionised H_2O was unsuccessful in my hands. It appeared that both RBC and WBC were being lysed by this treatment, so that the WBC numbers in samples lysed with deionised water were very low. There was also a large amount of variability between specimens as to how many RBC remained intact after the treatment. Numerous time points were tried, however I was unsuccessful in finding a time point that would consistently lyse the RBC whilst leaving the WBC intact.

7.5 Discussion

Due to technical difficulties with the lysis of red blood cells I was unable assess the levels of lymphocyte subset apoptosis in this patient group. We were unable to determine from the manufacturer whether the formulation of FACSlyse had been changed since our previously successful experiments, however this may explain why this method was unsuccessful in my hands.

The number of Annexin-V positive cells increased as exposure to the lysing agent was increased. This is most likely due to damage of the white cell membrane during the lysis process thereby exposing PTS and giving a false positive stain. I was unable to standardise the time required for the cell lysis step to obtain reproducible results.

Whilst there are numerous alternative techniques that can be used to determine the number of apoptotic cells in a sample, all of these require pre-treatment of the sample to remove red blood cells, either via a density gradient or cell lysis. None of these alternatives was a viable option in this study due to the limited sample volume available (0.1 mL).

A future study to explore the mechanism behind the reduction of the lymphocyte subsets in this group is planned. This study will examine both apoptosis and proliferation of these cell types since total cell numbers depend on the balance between cell proliferation and cell loss.

In contrast to the current study, where the priority for specimen use was to determine inflammatory phenotype and cytokine production, blood specimens will be collected specifically for the apoptosis / proliferation study, hence there will be sufficient specimen to isolate peripheral blood mononuclear cells (PBMC) using a density gradient and then measure both apoptosis and proliferation.

It has been reported that some cell isolation techniques can be toxic to cells [151] and that there may be a selective loss of some lymphocyte subsets during PBMC isolation [152]. To explore whether the changes in lymphocyte subsets in CKD are caused by selective apoptosis of any particular subset, PBMC will be phenotyped for lymphocyte subsets to confirm our whole blood results and then levels of lymphocyte apoptosis and proliferation will be studied.

I have performed some initial experiments on normal donors using PBMC isolated via a Ficoll density gradient, followed by Annexin-V staining. These have produced consistent and reproducible results for both phenotype and Annexin-V staining for all lymphocyte subsets. My future planned study will help to elucidate the mechanism behind the changes in lymphocyte subsets that I have observed in the current study

Chapter 8

Discussion and Future Directions

8.1 Introduction

CKD is a dynamic disease governed by multiple factors that affect its progression and prognosis. This is the first study that comprehensively defines plasma levels and leucocyte production of pro-inflammatory cytokines and leucocyte immunophenotypes in children with pre-dialytic CKD. The study provides an improved understanding of some of the mechanisms which may contribute to the clinical manifestations of the disease and the potential for future development of co-morbid conditions as well as providing a basis for the formulation of potential new treatment strategies.

Although it is now well accepted that adult CKD is associated with systemic inflammation and oxidative stress [34, 38, 44, 153-157], there are difficulties in defining the inflammatory changes that are due to renal disease *per se* because adults have a high prevalence of co-morbid conditions that are inherently inflammatory. These include hypertension, diabetes and CVD, all of which are likely to play a role in the development of systemic inflammation in adults with CKD. It is likely that the increase in inflammation in adults with CKD is caused by a combination of uraemia and other co-morbid conditions.

This study in children presented a unique opportunity to determine inflammatory alterations caused by CKD *per se*, since the co-morbid conditions often associated with CKD in an adult population are largely absent in children. This has allowed the assessment of the inflammatory state of this group without any confounding factors caused by other co-morbid conditions.

As a result I have identified numerous markers that confirm the presence of pro-inflammatory mediators in children with CKD, independent of the stage of the disease.

8.2 CKD, Inflammation and CVD

Adults with CKD have rates of CVD higher than the general population, even after correction for other risk factors. There is some evidence that the chronic inflammatory state induced by CKD in adults contributes to this [158]. Children with CKD also have excessive CVD mortality [18, 47]. This is the first study to identify evidence of chronic systemic inflammation in children with CKD. The majority of children and young people with CKD survive well into adult life. Accordingly, they will be exposed to the pro-inflammatory mediators that are risk factors for CVD for longer periods than their adult counterparts. Thus, the inflammatory mediators that I have identified in children with CKD may represent a modifiable risk factor for CVD and provide a sound basis for treatment strategies to lessen the risk of CVD in this group of patients.

As such, a more aggressive management strategy using treatments that are of proven benefit in other settings is likely to be beneficial. Early normalisation of hyperlipidaemia, and reduction of inflammation through treatments such as Hydroxymethylglutaryl-coenzyme A (HMG CoA) reductase inhibitors (statins), aspirin, B-vitamins, and antioxidant vitamins may help to prevent the occurrence of the co-morbid conditions that are commonly seen in the adult CKD population [159].

8.3 Circulating Cytokines and AOPP

To my knowledge, this is the first study to have comprehensively measured a wide range of circulating pro and anti inflammatory cytokines in the plasma of patients with CKD. This comprehensive overview of the circulating cytokine profile, as well as an evaluation of oxidative stress in patients with CKD supports my initial hypothesis that children with CKD would show increased pro-inflammatory and oxidative stress markers indicative of an increased systemic inflammatory response.

Fifty percent of patients in the CKD group showed an increase in at least one of the circulating pro-inflammatory cytokines (Chapter 3), supporting my hypothesis of an increase in systemic inflammation in this group. I was unable to demonstrate any relationship between increased circulating cytokines and the level of renal function or to drug treatments. This may be due to a lack of statistical power due to the relatively small numbers in the current study. Expansion of this study by measurement of circulating cytokines in a larger patient cohort is thus warranted. Further, a longitudinal study to investigate levels of circulating cytokines over the course of the disease would provide further insight into the relationship (if any) between increased circulating plasma cytokines and the stage of CKD.

Interestingly there were increases in plasma IL-12 and/or IL-1β in 45% of the CKD group. These cytokines are produced primarily by monocytes, suggesting a role for monocyte activation in CKD. IL-12 production by monocytes results in the induction of Th1 cells. IL-1β has been implicated in the development of atherosclerosis via interactions with vascular cell adhesion molecule (VCAM)-1 and monocyte chemotactic protein (MCP)-1

[91]. It is therefore likely that the increases in these pro-inflammatory cytokines are a contributing factor to the increased CVD observed in this group.

Four patients showed an increase in the anti inflammatory cytokine IL-10, and three of these patients also had increases in both IL-12 and IL-1β. IL-10 provides negative regulation of the Th1 response, indicating a possible compensatory response to the increased presence of inflammatory cytokines in these patients.

Three patients, all with signifiacantly reduced GFR showed an increase in oxidative stress measured via AOPP. A weak but statistically significant correlation between increased AOPP and decreased GFR was identified and would be further strengthened by study of a larger patient cohort. These investigations will be the target of a future planned study.

50% of the CKD group showed an increase in at least one circulating pro-inflammatory mediator. This may be an underestimation of the actual number of patients experiencing high levels of these cytokines due to the transient nature of cytokines in the circulation and the presence of inhibitors [89]. It further highlights the complexity of the regulatory mechanisms that control and influence cytokine production. To overcome variations in circulating cytokine levels, I investigated the ability of monocytes and T cells from CKD patients to produce cytokines in response to stimulus. Hence the next part of my study detailed cytokine production by peripheral blood monocytes and T cells from children with CKD.

8.4 Cellular Cytokine Production

I hypothesised that the CKD group would show an increase in the production of cytokines by monocytes and possibly by T cells in response to stimulus compared with age matched healthy controls. This is the first report detailing cytokine production by peripheral blood monocytes and T cells from children with CKD. In Chapter 4 I have shown increases in monocyte production of IL-12 and IL-1β indicating that monocytes are the likely source of the increased circulating IL-12 and IL-1β that were observed in some CKD children in Chapter 3. Both of these cytokines have been described as being pro-atherogenic [98] and may contribute to the increased rates of CVD that are seen in this patient cohort.

IL-12 is also an important promoter of cell mediated immunity and a major inducer of Th1 cells that produce IFN-γ, thus I had predicted that increased monocyte IL-12 production would be associated with increased T cell IFN-γ production in our patient group. Surprisingly, I found a reduction in the production of IFN-γ by CD4 T cells from children with CKD, the mechanism of which is presently unclear and will be the subject of future planned studies. One possibility is a reduction in the IFN-γ producing subset of CD4+ cells due to a decrease in proliferation or an increase in apoptosis of this T cell subset. An alternative mechanism may be a decrease in IL-12 binding to the IL-12 receptor (IL-12R) on CD4+ T-cells or dysfunctional signal transduction mechanism following cytokine binding to IL-12R.

Interestingly, children with CKD had increased numbers of CD3+ (both CD4+ and CD8+) T-cells that produce IL-4. IL-4 is an important Th2 cytokine that has been shown to negatively regulate T cell IFN-γ production [104]

which may explain the decreased IFN-γ production by CD4 T cells noted in our patient group. Since increased oxidative stress reportedly polarises T cells towards a Th2 phenotype [103], this may further explain the decrease in IFN-γ and increase in IL-4.

The alterations in cytokine production observed in this study may influence the clinical immune function of this group of patients. Monocytes produce pro-inflammatory cytokines early in infection and enhance a host's innate resistance at the same time as shaping the ultimate antigen-specific immune response [101]. IL-12 is a major inducer of Th1 cells which are essential for immunity to intracellular pathogens [107]. Lack of effect of IL-12 on T-cells and the subsequent decrease in IFN-γ may have a negative effect on the development of T cell mediated immunity.

IFN-γ plays an important role in the initiation of macrophage activation. The decreased production of IFN-γ noted in this study may suggest a different mechanism for monocyte activation, possibly due to direct monocyte stimulation by factors in uraemic plasma such as AOPP [138].

In summary, the pattern of cytokine production may suggest that elements in the uraemic milieu have the ability to activate or prime monocytes for increased pro-inflammatory cytokine production. Paradoxically, T cell cytokine production appears to be polarised towards a Th2 phenotype, which was unexpected given the pattern of monocyte cytokine production. Future studies to fully define the Th1/Th2 phenotype using a range of surface markers and chemokine receptors (CCR) such as CCR4 (Th2), CCR5 and CXCR3 (Th1) would be worthwhile and may provide further insight into T cell function in this group.

8.5 Lymphocyte subsets

The experiments in Chapter 5 define significant and previously unknown lymphocyte subset changes in children with CKD. The results show that children in this study, the majority of whom have relatively mild renal disease, show substantial changes in leucocyte subsets which may affect immune function and contribute to the clinical manifestations of the disease.

There were no differences in total leucocyte or lymphocyte counts, contrary to many reports of lymphopaenia in CKD [109, 115, 116], possibly because the children in our study had earlier stage CKD than those in the other studies. This suggests that the immunophenotypic changes noted in mild renal disease are less obvious than those present later in the disease. There was no change in T cell numbers and a significant reduction in absolute numbers of B cells and NK cells, consistent with previous reports [62, 110, 114].

It has been suggested that decreases in B cells in CKD may be due to increased apoptosis via dysregulation of Bcl-2 [110]. Due to the methodological and sample limitations discussed in Chapter 7, examination of apoptosis and proliferation of lymphocyte subsets was not determined in the present study; however a future study to comprehensively examine apoptosis and proliferation of lymphocyte subsets would be worthwhile and could define a mechanism for the alterations observed in lymphocyte subsets.

B cells are essential for effective antibody response to pathogens. Reduced B cell numbers may result in poor antibody response to foreign antigens, including a poor response to vaccination. This may partly explain the sub-

optimal response to vaccination that has been observed by others in a similar patient group [117].

NK cells are essential for an effective response to viral infections. A reduction in NK cells, as noted in the present study, may result in an impaired response to viral infections in this group of patients, although this has not been clearly established [160, 161].

The increased percentage of T cells in the total lymphocyte pool is probably due to the reduced numbers of B and NK cells causing a proportionally greater percentage of T cells overall.

In summary, my data suggests an impaired immune response in children with CKD due to decreased absolute numbers of B and NK cells.

8.6 Lymphocyte phenotype

Extensive lymphocyte immunophenotyping of children with CKD was performed (Chapter 6). The major finding was a significant down-regulation or loss of L-selectin (CD62L) on the total lymphocyte population. L-selectin is rapidly lost upon activation of lymphocytes as a result of proteolytic cleavage, however there was no other evidence of lymphocyte activation as determined by up regulation of lymphocyte CD25 or CD69 expression.

Expression of L-selectin on lymphocytes is required for effective homing to peripheral lymph nodes and Peyer's patches [127]. Binding of L-selectin initiates lymphocyte extravasation from blood to lymph during recirculation and localization to sites of antigen exposure [139]. As such, a decrease in L-selectin on lymphocytes may inhibit the homing and adhesion of lymphocytes to lymph nodes and Peyer's patches [139]. Migration of naive T

cells through lymph nodes increases the likelihood of these T cells encountering a primary antigen [140]. Children with CKD have variable and sometimes poor responses to common vaccines [117], which may be partially explained by the loss of L-selectin from lymphocytes and lack of subsequent exposure to antigens in lymph nodes and Peyer's patches. This is a particular problem with Hepatitis B vaccine in the CKD population and constitutes a significant issue for this "at risk" population [128].

Further, this may predispose children with CKD to a higher rate of minor infections than healthy controls, although there are presently there are no published clinical studies to support this. This is likely due to the difficulty in detecting all minor infections (coughs, colds etc) in both a control and CKD group. Medical attention is often not sought for these conditions, and some infections may even be sub-clinical. In this regard, a recent study by Cazzavillan *et al* provides some evidence for an increased susceptibility to infection [118]. Their molecular method to screen for sub clinical infection found increased bacterial levels of DNA that correlated with levels of the inflammatory markers IL-6, hs-CRP and AOPP in a population of adults with ESRD. In addition, high infection rates are seen in individuals with advanced renal failure [162]. The cause of this is likely to be multifactorial (increased infectious load, poor nutritional state, adverse mechanical factors).

It is conceivable that the change in lymphocyte numbers and phenotype described in these studies creates an additional susceptibility to infection that is not easily defined.

8.7 T Helper and Cytotoxic T cells

The results described herein show changes in T cells subsets. An increased CD4:CD8 ratio may be due to numerous factors. There was an increase in the percentage of CD4 T helper cells and a trend towards a decreased percentage of CD8 cytotoxic T cells. There was also a significant decrease in the percentage of double negative T cells (i.e. CD4-/CD8-/CD3+). This resulted in a corresponding increase in the proportion but not the absolute number of CD4 T cells.

Double negative T cells have been reported as having an important regulatory role in various immune responses including immune tolerance and down-regulation of immune responses [121]. It is likely that the finding of reduced double negative T cells is due to the significant decrease observed in the γδ T cell population which is also generally negative for both CD4 and CD8 [123].

γδ T cells are able to recognise antigen independently of MHC molecules and process and present soluble antigen [122, 123]. They play a specialised role in protective immunity via cytokine secretion and cytotoxic activity. A reduction in γδ T cells has previously been reported in dialysed end stage renal failure [65, 111], however, from this study it was not possible to distinguish whether this change was caused by the dialysis procedure or uraemia *per se*. The current study is the first to show a reduction in the γδ T cell subset in non-dialysed CKD and shows that this reduction may be due to uraemia *per se.* A decrease in γδ T cells may reduce the ability of the immune system to process antigens directly [121] thereby resulting in an

increase in susceptibility to various microbial infections in our patient group. Further study of this relationship is therefore warranted.

8.8 Reduction in memory T cells

This study is the first to show that non-dialysed CKD patients show a significant decrease in absolute CD3+/CD45RO+ memory T-cell numbers compared to age matched controls. As discussed in Chapter 5, the underlying cause of CKD does not appear to be a contributing factor to the changes observed in the study, suggesting that uraemia itself is the major cause of these immunological changes. The decrease in memory T cells may be due to increased apoptosis and/or decreased production of these cell types. A previous study by Matsumoto et al [125] has suggested that T cells in adults with end-stage renal disease may undergo increased apoptosis. If this is also the case in children then this may account for the decreases in memory T cells noted in this study. Due to the limitations of the current study, I was unable to assess memory T cell apoptosis and this will be the subject of future studies.

8.9 Th1/Th2 memory cell subtypes

The increase in T cell production of IL-4 and the decrease in IFN-γ described in Chapter 4 suggests an alteration of Th1/Th2 balance towards a Th2 phenotype. Th1 cells have been described as those memory cells that are negative for CD62L [113], so the lymphocyte phenotype results in this study point to a possible bias towards a Th1 phenotype (which is in contrast to the pattern of cytokine production). However, CD62L is not a definitive marker for Th1/Th2 cells. Ideally a combination of markers including

chemokine receptors such as CCR4 (Th2), CCR5 and CXCR3 (Th1) could also be used to define the Th1/Th2 ratio. Future studies to clearly define the Th1/Th2 status would provide useful insights into T cell regulation and function.

Increased apoptosis and/or decreased production of these cell types could also cause the alterations in memory cell numbers that were observed and may account for the decreases in lymphocyte subsets observed in this study. Technical difficulties precluded the assessment of apoptosis and proliferation of leucocyte subsets in this study, however further studies are planned to investigate this. This will help to define the mechanism responsible for the alterations in cell numbers that have been observed in this study.

8.10 Leucocyte activation

I hypothesised that leucocytes from children with CKD would show signs of activation because of their exposure to the uraemic environment. Surprisingly, in contrast with studies in adults [136-138], there were few signs of a generalised increase in activation of any cell type in the current study.

There was a cohort of CKD patients that showed increased circulating inflammatory cytokines. In Chapter 6 I showed that this cohort had increased expression of HLA-DR on monocytes indicative of increased monocyte activation.

Monocytes from the CKD group produced more cytokines than the control group following stimulation with LPS. This may suggest that a uraemic

environment may prime monocytes to more easily become activated in response to stimulus through the LPS receptor. Future studies are needed to investigate the effect of uraemic plasma exposure on the cytokine production of leucocytes from children without CKD.

The CKD group showed significant up-regulation of the adhesion integrin CD11b on neutrophils. Beta-2 integrins such as CD11b control recruitment and adhesion of leucocytes to the vascular endothelium [143]. Increased cell adhesion to the endothelium promotes the formation of atherosclerotic plaques [29]. Interestingly, recent studies have reported increased endothelial dysfunction and CVD in both adults and young people with CKD [22, 145]. The increases in CVD do not correlate with traditional risk factors such as increased lipids and hypertension [146], suggesting that there are additional contributing factors. Increased β-2 integrin dependent leucocyte-membrane interactions may contribute to the increased rate of CVD observed in children with CKD. There have been recent reports that CD11b expression can be reduced by drugs such as statins and aspirin [147, 148]. Therapeutic targeting of CD11b with these drugs in this patient cohort may have the potential to reduce the incidence of CVD in this group of patients.

8.11 Summary

The expression of cell surface molecules plays an important role in the regulation of inflammatory processes. The induction of inflammatory cytokines may contribute to and sustain an increase in systemic inflammation that may have detrimental clinical effects. It is important to be aware of the contribution that systemic inflammation caused by CKD may

make to the increased risk of development of various other co-morbid conditions such as CVD in children with CKD. The findings of this study improve our understanding of the immunological changes that may contribute to the clinical manifestations of CKD.

Taken together, these immunological changes, such as the loss of L-selectin from lymphocytes and the decreased numbers of B and NK cells may contribute to some of the clinical symptoms associated with CKD. These include a variable and often poor response to vaccination [6].

These results indicate that children with CKD exhibit alterations in leucocyte phenotype that may result in altered adhesion and trafficking of leucocytes and an increase in several pro-inflammatory mediators. These changes may contribute both to disease progression and the development of other co-morbid conditions such as CVD that further complicate the management of children and young adults with CKD.

The information obtained in this study provides the basis for the identification of treatment targets. Recently, statin therapy for non-vascular inflammatory conditions has been used successfully to reduce systemic inflammation in a number of conditions such as multiple sclerosis and rheumatoid arthritis [69]. Currently available treatments such as statins or anti-oxidants or the development of new treatments based on inhibiting the pro-inflammatory mediators identified in these studies may be of benefit to the long term outcomes of this patient group.

This extensive investigation has provided novel insights into the immune cellular processes and responses in CKD. Mild CKD is associated with significant inflammatory and immunological changes that are likely to affect

immune function. These changes may initially appear to have few clinical effects but it is probable that they predispose this group to a range of co-morbid conditions as well as altered immune function that may contribute to a future significant disease burden.

These findings are particularly relevant in relation to the management of young patients with CKD and the development of new treatment strategies to prolong renal function and prevent co-morbid complications and thus improve quality of life.

Appendices

Appendix 1 Information sheets for study subjects

A1.1 Information sheet for CKD subjects

Women's and Children's Hospital
Information sheet for research subjects

PROJECT TITLE: **Characterisation of systemic inflammation in children with chronic kidney disease.**

Why have I been asked to take part in the study?

You have been diagnosed with chronic kidney and are being managed by the multi-disciplinary team at the women's and Children's hospital.

OR

You have no evidence of kidney abnormalities and are being asked to be part of a normal control group.

This will allow us to compare differences in inflammatory markers between children with CKD and children who have normal kidneys.

Who is doing the research?
This research is being undertaken by the Renal Unit, Women's and Children's Hospital, North Adelaide.

What is the aim of the project?
A pilot study in our laboratory has shown that children with kidney disease showed some evidence of increased inflammation. This inflammation may contribute to the progression of kidney disease. It has also been shown to be associated with some of the clinical complications which can be associated with kidney disease.
The aim of this study is to determine whether children with chronic kidney disease all show similar and constant patterns of inflammation.
To do this we will measure markers of inflammation in your blood. This information may assist in understanding of how this inflammation happens. This may help decide the future direction of research aimed at preventing this inflammation occurring. The results of this research will not influence medical management in the foreseeable future, but can be discussed with you if you wish.

What will happen if I agree to participate in the study?
At the time you come for routine blood tests, an extra 5-10mL (1-2 teaspoonsful) of blood will be collected. **There will be no extra needles or procedures required**. This blood will be analysed by scientific staff in the laboratory to measure the levels of cytokines and to determine if there is any increase in the activation levels of white blood cells.
We will also look at your medical record so that we can classify the cause of your kidney disease.

What is the duration of the study?
We hope to complete the study within 24 months. Confidentiality with respect to results will be maintained. Any data for publication will not contain any information that identifies you or your child. You will not be paid for the study, and may withdraw at any time with no influence on your future treatment or care.

This study has been scrutinised and approved by the Women's and Children's Hospital Research Ethics Committee. If you have any questions or concerns relating to the ethics approval process you may contact Ms Brenda Penny, Research Secretariat, ph 8161 6521. If you have any other queries about the study, please feel free to contact the following staff in the Renal Unit:

• Ms Judi Nairn	Medical Scientist, Renal Unit	Ph 8161 7303
• Dr Paul Henning	Medical Unit Head, Renal Unit	Ph 8161 7303
• Dr Sam Crafter	Fellow, Renal Unit	Ph 8161 7303

A1.2 Information sheet for Control subjects

Women's and Children's Hospital
Information sheet for research subjects

PROJECT TITLE: **Characterisation of systemic inflammation in children with chronic kidney disease.**

Why have I been asked to take part in the study?

You have chronic kidney disease or you have received a renal transplant.

OR

You have no evidence of kidney abnormalities and are being asked to be part of a normal control group.

This will allow us to compare differences in inflammatory markers between renal patients and people who have normal kidneys.

Who is doing the research?
This research is being undertaken by the Renal Unit, Women's and Children's Hospital, North Adelaide.

What is the aim of the project?
A pilot study in our laboratory has shown that children with kidney disease showed some evidence of increased inflammation. This inflammation may contribute to the progression of kidney disease. It has also been shown to be associated with some of the clinical complications which can be associated with kidney disease.
The aim of this study is to determine whether children with chronic kidney disease all show similar and constant patterns of inflammation.
To do this we will measure markers of inflammation in your blood. This information may assist in understanding of how this inflammation happens. This may help decide the future direction of research aimed at preventing this inflammation occurring. The results of this research will not influence medical management in the foreseeable future, but can be discussed with you if you wish.

What will happen if I agree to participate in the study?
When the anaesthetist inserts the intravenous line for your anaesthetic, they will take 5-10mL (1-2 teaspoonsful) of blood from the line. **There will be no extra needles or procedures required**. This blood will be analysed by scientific staff in the laboratory to measure the levels of cytokines and to determine if there is any increase in the activation levels of white blood cells. Renal patients will be required to have three extra blood samples collected when you have your routine blood test on three separate visits, to see whether any inflammation we detect changes over time.
Control patients will only have one blood sample collected, during the anaesthetic. If you are part of the chronic kidney disease group, we will look at your medical record so that we can classify the cause of your kidney disease.

What is the duration of the study?
We hope to complete the study within 12 months. Confidentiality with respect to results will be maintained. Any data for publication will not contain any information that identifies you or your child. You will not be paid for the study, and may withdraw at any time with no influence on your future treatment or care.

This study has been scrutinised and approved by the Women's and Children's Hospital Research Ethics Committee. If you have any questions or concerns relating to the ethics approval process you may contact Ms Brenda Penny, Research Secretariat, ph 8161 6521. If you have any other queries about the study, please feel free to contact the following staff in the Renal Unit:

- Ms Judi Nairn — Medical Scientist, Renal Unit — Ph 8161 7303
- Dr Paul Henning — Medical Unit Head, Renal Unit — Ph 8161 7303

Appendix 2 Consent form for study subjects

WOMEN'S & CHILDREN'S HOSPITAL RESEARCH ETHICS COMMITTEE

CONSENT FORM

I

_____
_

hereby consent to my/my child's involvement in the research project entitled:**

Clinical implications of systemic inflammation in children with Chronic Kidney Disease

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it, and agree for myself (**my child) to take part.

2. I understand that I (**my child) may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that while information gained in the study may be published, I (**my child) will not be identified and information will be confidential.

5. I understand that if I am part of the Chronic Kidney Disease group that the researchers will access my (**my child's) medical record in order to classify my (**my child's) renal disease.

6. I understand that I (**my child) can withdraw from the study at any stage and that this will not affect medical care or any other aspects of my (**my child's) relationship with this hospital.

7. I understand that there will be no payment to me (**my child) for taking part in this study.

8. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

9. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

10. a) I consent to a specimen of blood being taken from me (**my child) and being used in the above project.

b) I do / do not consent to the blood samples being used in any other research project, provided the project has the approval of the Women's & Children's Hospital Research Ethics Committee.

Signed: .....

Relationship to Patient: .....

Full name of patient: .....

Dated:.....

I certify that I have explained the study to the parent (**patient)(**and/or child) and consider that he/she understands what is involved.

Signed: ..... Title: .....

Dated: .....

** ***Please delete either the phrase, or the brackets, as appropriate.***

Appendix 3 Quantification of Advanced Oxidation Protein Products

A3.1 Reagents

1. Phosphate Buffered Saline (PBS) – pH7.4

20 x stock

32g $Na_2HPO_4.2G_2O$

6g $NaH_2PO_4.2H_2O$

164 NaCl

Make up to 900mL in deionised water (DIW)

Working Solution

Dilute stock 1/20 ie 5mL stock + 95 mL DIW

2. Chloramine T standards (MW 281.7)

   10mM stock

   - 0.2817g made up to 100mL with DIW

   Dilute to working standards:

   - Dilute 10mM stock 1/100 and then do 4 serial dilutions to give standard concentrations of 100µM, 50µM, 25µM, 12.5µM, 6.25µM

3. 1.16M Potassium Iodide (MW 166)

   1.9256g made up to 10mL with deionised water

4. Glacial Acetic Acid

A3.2 Equipment required

- 96 well microtitre plate
- Automatic pipettes capable of delivering volumes ranging from 10 – 200ul
- Plate reader capable of measuring at 340nm

A3.3 Method

Standards

1. Add 200µL blank (PBS) or standard to a 96 well plate in duplicate
2. Add 20µL acetic acid
3. Add 10µL KI
4. Read ASAP at 340nm

Samples

1. Dilute plasma 1/5 (100µl+400µl) in 20mM phosphate buffer (pH 7.4)
2. Add 200µL of diluted sample to a 96 well plate in duplicate
3. Add 20µL acetic acid
4. Read ASAP at 340nm

Appendix 4 Set up of whole blood cultures for cytokine production

A4.1 Reagents

1 Phorbol 12-Myristate (PMA)

Supplier:Sigma (product number P-8139)

Stock solution:

- Reconstitute 1 mg vial with 20 ml DMSO (sterile) (i.e. 50ng/mL)
- Store 20 µL aliquats at -70^0C
- Stable 1 year

Working solution:

- Add 980 µL RPMI (no FCS) to stored 20 µL aliquat and mix. (i.e. 1 ng/mL)
- Use 25 µL / 1 mL cell suspension. (ie 25 ng/mL)
- Discard any remaining solution after use

2 Ionomycin (I)

Supplier:Sigma (product number I-0634)

Stock solution:

- Reconstitute 1 mg vial with 2 mL 98% ethanol / 2%& methanol (sterile) (i.e. 0.5 µg/µL)
- Store 20 µL aliquats at -70^0C
- Stable 1 year

Working solution

- Add 180 µL RPMI (no FCS) to stored 20 µL aliquat and mix (i.e. 50ng/µL).

- Use 20 µL / 1 mL cell suspension (i.e.1 µg/mL)
- Discard any remaining solution after use

3 *E. coli* Lipopolysaccharide (LPS)

Supplier:Sigma (product number L-2654)

Working solution:

- Reconstitute 1 mg vial with 10mL RPMI (no FCS, sterile) (ie 0.1µg/µL)
- Store 50 µL aliquats at -20^{0}C
- Stable 6 months
- Use 10 µL / 1 mL cell suspension. (i.e. 1 µg/mL)
- Discard any remaining solution after use

4 PHA

Supplier:Sigma (product number L-)

Working solution:

- Reconstitute 5 mg vial with 5 mL RPMI (no FCS, sterile) (ie 1 µg/µL)
- Store 50 µL aliquots at -20^{0}C
- Stable 6 months
- Use 10 µL / 1 mL cell suspension. (i.e. 10 µg/mL)
- Discard any remaining solution after use

5 Brefeldin A

Supplier:Sigma (product number I-0634)

Stock solution:

- Reconstitute 5 mg vial with 1 mL DMSO (sterile) (i.e. 5µg/µL)
- Store 20 µL aliquots at -70^0C
- Stable 1 year

Working solution

- Add 180 µL RPMI (no FCS) to stored 20 µL aliquot and mix (i.e. 5µg/µL).
- Use 20 µL / 1 mL cell suspension (i.e. 10 µg/mL)
- Discard any remaining solution after use

6 Cell culture Media

RPMI media (Ordered from Hanson Centre, Adelaide, S. Aust)

1 vial Streptomycin / Penicillin (Gibco cat # 15075-031)

Mix together, store at 4^0C

Keep sterile

Stable 6 months

A4.2 Method

1. Remove stainless steel cover from laminar flow cabinet
2. Turn on laminar flow cabinet by switching both switches to the opposite position
3. All equipment and reagents that are to be used in the cabinet must be sterilised by spraying with 70% alcohol.
4. Wear gloves and spray with 70% alcohol before putting hands in cabinet
5. Aliquat specimen for cell stimulations as follows

T-cell stimulations

- 0.5mL blood + 0.5mL RPMI +20uL PHA (ie 10ug)
- 0.5mL blood + 0.5mL RPMI +25uLPMA + 20uLIonomycin
- 0.5mL blood + 0.5mL RPMI +25uLPMA + 20uLI + 20uLBA

Monocyte stimulation

- 1.0mL blood + 1.0mL RPMI +20uL LPS
- 1.0mL blood + 1.0mL RPMI +20uL LPS (1ug) +40uL BA

6. Incubate o/n at 37^0C with 5% CO_2

Appendix 5 Annexin Binding Buffer

A5.1 Reagents

10mM Hepes (MW 283.3)

150mM NaCl (MW 58.44)

5mM KCl (MW 74.56)

1.8mM $CaCl_2$ (MW 147.02)

1mM $MgCl_2$ (Mw 203.31)

A5.2 Method

For 1 litre:

2.383g Hepes

8.766g NaCl

0.3728g KCl

0.2646g $CaCl_2$

0.2033g $MgCl_2$

pH to 7.4 with NaOH

References

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