IMMUNE DYSFUNCTIONS ASSOCIATED WITH HEMODIALYSIS MODALITIES

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ABSTRACT

Infection is a leading cause of death in hemodialysis patients, partly due to dysfunctional immunity. Frequent dialysis therapy improves patient outcomes and quality of life. We hypothesize that extended home hemodialysis (EHHD) also improves immune function compared to conventional in-hospital hemodialysis (CHD); therefore, we designed a prospective matching-cohort clinical study to assess serum inflammatory markers and the functional capacity of monocyte-derived dendritic cells (MDDCs) and T-lymphocytes. Serum CRP was decreased in EHHD patients suggesting that extended dialysis may decrease inflammatory solute/cytokine levels. Compared to controls, MDDCs from hemodialysis patients had similar endocytic capacity, expression of co-stimulatory molecules, and T-cell activation capacity. However, CHD was associated with the highest expression of CD83 and CD40. Activated T-cells in CHD patients also produced significantly more immunosuppressive IL-10 compared to EHHD patients and controls. Therefore, EHHD may improve immune function by decreasing inflammation, MDDC pre-activation, and synthesis of immunosuppressive cytokines.

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For my dad –
Without a doubt the greatest scientific mind
I've ever encountered in my academic career.

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LIST OF ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
ADP	advanced digital processing
AF488	AlexaFluor488
AKI	acute kidney injury
ANOVA	one-way analysis of variance
APC	antigen presenting cell
APC	allophycocyanin
AV	arterio-venous
CD	cluster of differentiation
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
CHD	conventional hemodialysis
CHHD	conventional home hemodialysis
CKD	chronic kidney disease
CO2	carbon dioxide
CRP	C-reactive protein
CTLA	cytotoxic T-lymphocyte antigen
CVC	central venous catheter
DC	dendritic cell
DTH	delayed-type hypersensitivity
eGFR	estimated glomerular filtration rate
EHHD	extended home hemodialysis
ELISA	enzyme-linked immunosorbent assays
ESRD	end-stage renal disease
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor

H_3PO_4	phosphoric acid
HBSS	Hank's balanced salt solution
HBV	hepatitis B virus
HCL	hydrochloric acid
HCV	hepatitis C virus
HD	hemodialysis
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	hemofiltration
HG	hemoglobin
HHD	home hemodialysis
HIV	human immunodeficiency virus
HLA-DR	human leukocyte antigen- DR
HRP	horseradish-peroxidase
HV	healthy volunteers
IL	interleukin
INF	interferon
KDOQI	Kidney Disease Outcomes Quality Initiative
L	liter
LPS	lipopolysaccharide
M	molar
MACS	magnetic activated cell sorting
MCP-1	monocyte chemotactic protein-1
MDDC	monocyte-derived dendritic cell
MEM	minimum essential medium
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIP-2	macrophage inflammatory protein-2
MLR	mixed leukocyte reaction
MO	monocyte
NHD	nocturnal hemodialysis
P	probability

PAMP	pathogen-associated molecular patterns
PB	Pacific Blue
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD	peritoneal dialysis
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohemagglutinin
PMA	phorbol 12-myristate 13-acetate
RANTES	regulated on activation, normal T cell expressed and secreted
RO	reverse osmosis
RPM	rotations per minute
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RT	room temperature
RTL	relative telomere length
SD	standard deviation
SDHD	short daily hemodialysis
SEB	Staphylococcal enterotoxin B
TCR	T-cell receptor
T_h	T-helper cell
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TNFRSF	tumor necrosis factor receptor superfamily
TREC	T-cell receptor excision circle
UF	ultrafiltration

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INTRODUCTION

1.1 Overview

Chronic kidney disease is a progressive illness that affects millions of people worldwide. When the kidneys are no longer able to sustain life, renal replacement therapy (such as hemodialysis, peritoneal dialysis, or a kidney transplant) is necessary for survival. However, these individuals still exhibit various metabolic abnormalities (Chmielewski et al., 2009), medical co-morbidities (Bentata et al., 2013; Kuznik et al., 2013), and immune deficiencies (van Dijk et al., 2001). The second leading cause of death in individuals with kidney failure is infection / sepsis (Levin et al., 2013; Sarnak and Jaber, 2000; van Dijk et al., 2001). Additionally, renal failure is often associated with chronic inflammation (Zaza et al., 2008)

plus suboptimal immune responses against vaccines and latent / chronic infections (Chang et al., 2012; Fonseca et al., 2013; Sengar et al., 1975). A uremic environment is thought to cause these immune dysfunctions; however, the exact mechanisms are not well understood. The impaired responses to vaccination and chronic infections indicate a predominant deficiency in adaptive immunity – particularly in antigen presenting cells and T-lymphocytes.

Hemodialysis, which provides extracorporeal removal of water and solutes, is the most common type of renal replacement therapy. Chronic inflammation and a uremic environment have been proposed to affect leukocyte function (Bauer and Jilg, 2006) so hemodialysis regimes that remove more toxins may improve immunity. Chronic hemodialysis is usually administered in hospital thrice weekly, but less commonly is performed at home on an almost daily basis. Patients that dialyze at home for longer intervals show many health improvements compared to conventional hospital dialysis (Culleton et al., 2007). They have improved cardiovascular health (Bergman et al., 2008), better blood pressure control with less need for anti-hypertensive drugs (Fagugli et al., 2006), and a generally improved quality of life (Culleton et al., 2007). But, it is currently unknown if these ameliorations are also correlated with enhanced immunity.

Therefore, we designed a prospective matching-cohort research study to compare immune responses in conventional hemodialysis versus extended home hemodialysis versus healthy controls. We hypothesize that patients on extended dialysis therapy have enhanced immune function secondary to enhanced removal of uremic solutes.

1.2 Background

1.2.1 The Kidneys and Chronic Kidney Disease

The kidneys are paired organs located in the abdominal cavity in a retroperitoneal position on either side of the spine. Each kidney contains approximately 1 million functional units called nephrons. The nephrons contain two segments: a glomerulus, composed of a capillary network and supporting cells, that functions as a filter and a tubular element that handles blood filtrate and leads to the formation of urine. The function of the kidneys is to process blood by removing constituents from it, or in some cases by adding elements to it. Through this mechanism, they excrete wastes and foreign substances in the urine and regulate extracellular fluid volume, blood osmolarity, ion balance, pH, and blood pressure (Vander, 1995). In total, the kidneys can process up to 180 liters of blood plasma per day, which translates into a clinically estimated glomerular filtration rate (eGFR) of 120-125 mL/min/1.73m^2 (Levey et al., 2003; Vander, 1995). In addition, cortical kidney cells have several endocrine functions. Notably, they secrete renin, erythropoietin, and calcitriol, which regulate blood pressure, red blood cell development, and calcium metabolism, respectively.

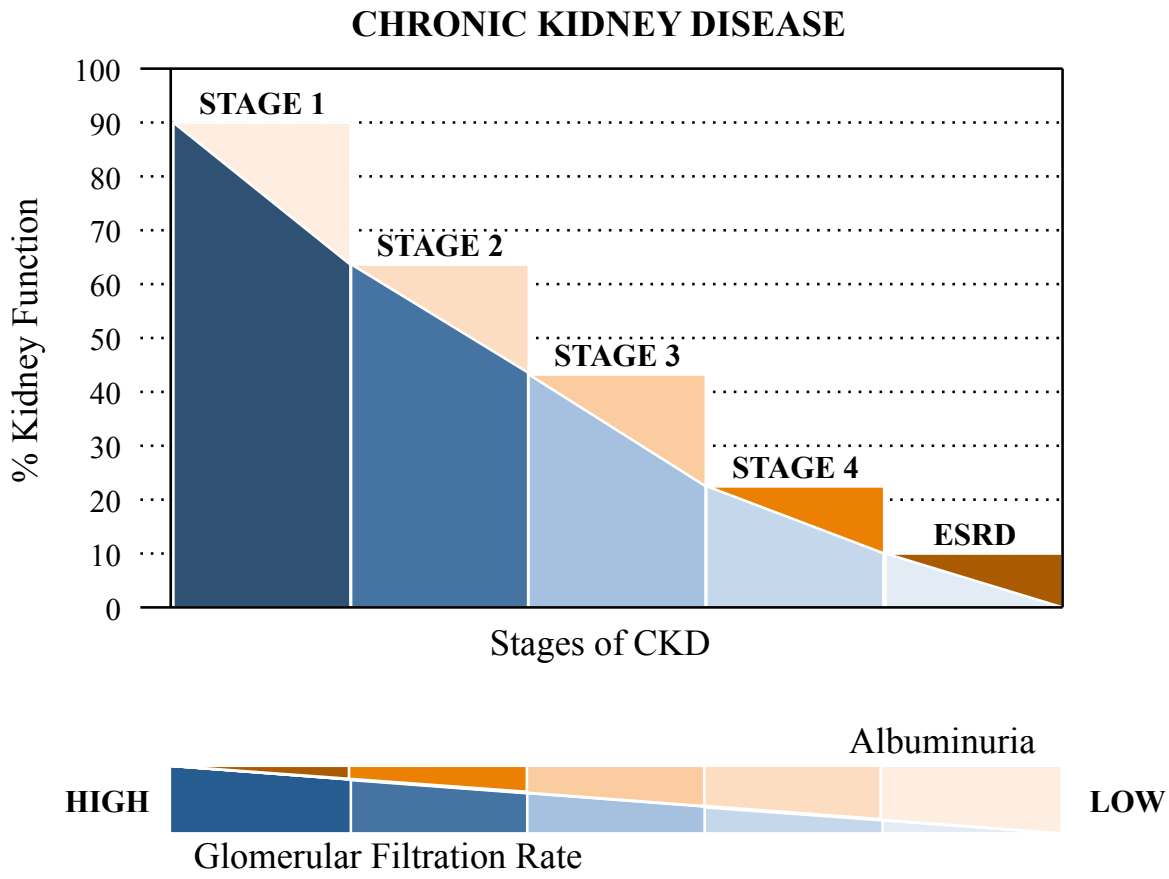
From the early stages of modern medicine, Hippocrates (460-377 BC) recognized that oddities in urine color, odor, and consistency are related to various forms of kidney disease and that modification of the urine by dietary, therapeutic, or surgical means ultimately dictates the patient's prognosis (Diamandopoulos et al., 2009). Giovanni Battista Morgagni (1682-1772), a clinical pathologist, later reported that the presence of atrophied kidneys during an autopsy is a key indication of a uremic state known as "dropsy" or edema (Antonello et al., 1999; Fogazzi, 1998). Today, kidney disease is still determined by

structural or functional abnormalities in urine sediment (albuminuria / hematuria), renal imaging, or biopsy results (Fink et al., 2012). More recently, creatinine clearance levels are used to estimate the glomerular filtration rate (eGFR). Creatinine is a metabolic waste product of muscles that is normally filtered by the glomerulus and fully excreted in urine; therefore, its accumulation in the blood indicates a decrease in GFR and overall kidney function (Vander, 1995).

Chronic kidney disease (CKD) is known as the presence of kidney damage, or the sustained loss of kidney function, for a period greater than three months (Levey et al., 2005). More specifically, CKD is defined by an eGFR less than 60 mL/min/1.73m^2 and/or the presence of protein/blood in the urine or some form of kidney damage seen by radiologic imaging (Levey et al., 2003). According to the guidelines set by the Kidney Disease Outcomes Quality Initiative (KDOQI) of the United States National Kidney Foundation, chronic kidney disease is classified into five stages based on the severity of kidney damage (albuminuria) and the decrease in eGFR (Levey et al., 2005) (Figure 1).

Approximately 2.9 million Canadians and 34 million Americans have some stage of chronic kidney disease. It is estimated that the overall prevalence of CKD is between 12.5-13.4% in Canada (Arora et al., 2013) and between 13.1-15.76% in the US (Coresh et al., 2007). In other parts of the world, the prevalence of CKD is comparable or slightly lower with 11.2% in Australia, 17.2% in India, 4.7-8.1% in Europe, and 2.5-6.8% in Asia (Arora et al., 2013; Singh et al., 2013). The leading causes of CKD in these more developed societies are hypertension, diabetes, atherosclerotic vascular disease, and certain glomerular diseases – all of which affect the nephrons and cause the kidneys to lose their filtering capacity (Levin et

Figure 1: Stages of chronic kidney disease. According to the guidelines of the Kidney Disease Outcomes Quality Initiative (KDOQI) of the United States National Kidney Foundation, chronic kidney disease is classified into five stages based on the severity of kidney damage (albuminuria) and the decrease in eGFR.


CHRONIC KIDNEY DISEASE
100
90
80
70
60
50
40
30
20
10
0
% Kidney Function
STAGE 1
STAGE 2
STAGE 3
STAGE 4
ESRD
Stages of CKD
Albuminuria
HIGH
LOW
Glomerular Filtration Rate

al., 2013). However, hypertension and diabetes are by far the most prominent and account for 52.8-64.5% and 23.4-31.6% of all CKD cases, respectively (Bentata et al., 2013; Crews et al., 2010). The prevalence of CKD is also approximately 3 times higher in adults aged over 65 versus those that are younger (Arora et al., 2013).

1.2.2 End-Stage Renal Disease

The most severe form of CKD, or stage 5, is known as end-stage renal disease (ESRD). ESRD is the complete, or almost complete, failure of the kidneys to function at a level needed for day-to-day life. In stage 5 CKD, kidney function is below 10% of normal and the eGFR is less than 15 mL/min/1.73m^2 (Hallan et al., 2006). The remaining kidney function is unable to remove water and solutes effectively, which results in the symptomatic accumulation of uremic toxins in the body. Renal replacement therapy, in the form of hemodialysis, peritoneal dialysis, or a kidney transplant, is absolutely necessary for survival.

The prevalence of ESRD is between 0.5-0.8% in the US and approximately 870,000 Americans are currently receiving treatment, along with over 350 new cases per million persons per year (Liu et al., 2006). In patients with CKD, there is a wide variation in rates of progression to ESRD, related in part to the severity of the underlying kidney pathology as well as other health co-morbidities (Li et al., 2012). In addition, numerous individuals with CKD do not reach ESRD due to competing co-morbidities and many succumb to death from cardiovascular causes rather than progress further to dialysis therapy (Tangri et al., 2011).

While dialysis is life sustaining, it is unable to replicate the normal kidney function. Even on dialysis, patients with ESRD have an unadjusted five-year mortality rate of approximately

43% (Brunner et al., 1988). These individuals have various metabolic abnormalities (such as anemia, abnormal calcium and phosphate metabolism, endocrine abnormalities, and protein energy wasting), medical co-morbidities (such as hypertension, diabetes, and cardiovascular disease), and immune deficiencies (including increased susceptibility to infections, higher rates of chronic/latent diseases, and poor immunization success). The two major causes of death for ESRD patients are cardiovascular disease and infections, which together account for approximately 70% of all deaths in this population (Kuznik et al., 2013).

1.2.3 Dialysis Therapy

Dialysis is defined as the diffusion of molecules in solution across a semipermeable membrane down an electrochemical concentration gradient. Dialysis helps restore the extracellular fluid environment by transporting solutes (e.g. urea) out of the blood into a dialysate fluid or from the dialysate (e.g. bicarbonate) into the blood (Himmelfarb and Ikizler, 2010). The diffusion rate of solutes is mainly determined by their concentration and their molecular weight. Small molecules (such as urea) diffuse rapidly and are efficiently removed by dialysis treatment. However, larger molecules (such as phosphate) or protein-bound molecules (such as p-cresol) diffuse much more slowly and are less efficiently removed during dialysis (Greene et al., 2009; Yuen et al., 2005a). In addition, dialysis is often accompanied by ultrafiltration (UF) – a process where the blood is forced across the semipermeable membrane in order to remove excess water using hydrostatic/osmotic pressure.

The principles of dialysis are based on Thomas Graham's (1805-1869) first separation of colloids and crystalloids using a semipermeable membrane and Albert Einstein's (1879-

1955) particle theory describing the motion of molecules in a solution (Gottschalk and Fellner, 1997). In 1913, John Abel dialyzed various anesthetized animals using the first dialysis machine and 10 years later the German physician, Georg Haas, performed the first dialysis treatment on humans (Gottschalk and Fellner, 1997). It wasn't until 1945 to 1962 that extracorporeal renal dialysis gained scientific acceptance. A series of improvements in dialysis machines lead to the creation of the Kolff-Brigham artificial kidney (Kelemen and Kolff, 1960). Veterans of the Korean War, and other patients suffering from acute kidney failure, were successfully treated with this apparatus and its success sparked worldwide interest, which resulted in additional advances in dialysis therapy. Notably, Fredrik Kiil significantly improved the function and efficiency of dialyzers (the Kiil dialyzer continued to be used until the 1990s) (Down et al., 1970) and Belding Scribner devised a glass and Teflon cannula, known as the Scribner Shunt, for easy and repeated access to the patients' arteries/veins during dialysis treatment (Quinton et al., 1960; Scribner et al., 1960a; Scribner et al., 1960b). In 1960, Clyde Shields became the first chronic hemodialysis patient and survived on dialysis treatment for 11 years before dying of cardiovascular complications.

Today, there are several different types, or variations, of dialysis used for the treatment of renal failure including hemodialysis (HD), peritoneal dialysis (PD), and hemofiltration (HF). Hemodialysis is the most common type and it relies on an artificial filter, called a dialyzer, to achieve the extracorporeal removal of wastes and excess water from the patient's blood (Levey et al., 2009). Peritoneal dialysis, on the other hand, uses the patient's own peritoneum (lining of the abdominal cavity) as a semipermeable membrane to move solutes and water from small capillaries into a dialysate bath placed in the patient's abdomen (Levey et al., 2009). Hemofiltration is the least common type and it is almost exclusively used for patients

with acute kidney injury (AKI) or in intensive care settings. Hemofiltration, like hemodialysis, achieves extracorporeal filtration through a dialyzer but does not require dialysate fluid because it removes water and solutes by convection rather than by diffusion (Hu et al., 2013). This thesis examines patients on hemodialysis only and from hereon the terms hemodialysis and dialysis are used interchangeably, unless otherwise specified.

1.2.4 Hemodialysis Modalities

According to the US National Kidney Foundation KDOQI guidelines, hemodialysis is usually performed in hospital or clinic with assistance from health care professionals, three times per week for 4-hour intervals (total is approximately 12 hours/week) (Levey et al., 2005). This amount of weekly hemodialysis is deemed sufficient to maintain satisfactory patient outcomes, while accommodating the large number of individuals with ESRD that require dialysis therapy. In addition, the financial cost of in-center hemodialysis as well as patient schedules, time commitments, compliance, and quality of life also contribute to the guidelines regarding minimally adequate hemodialysis (Kuznik et al., 2013).

Although less common, hemodialysis can also be administered at home by the patient. Dialysis machines and water purification systems are set up in the patient's home for more easily accessible therapy. This concept of home hemodialysis (HHD) is not new and, in fact, stems from the earliest dialysis treatments available. Before the amendment of artificial kidney dialysis to the US medicare system and the formal setup of dialysis centers, eligible patients received treatment for chronic kidney disease at home (Moorhead et al., 1970). In addition, approximately 60-years of peritoneal dialysis, often performed by the patient at home, demonstrated that ESRD patients could handle their own renal dialysis care.

For home hemodialysis, the time and frequency of treatment depend mostly on the patient's needs and personal preference. Common therapies include 1) conventional home hemodialysis (CHHD): standard thrice weekly 4-hour runs at home, 2) nocturnal hemodialysis (NHD): 6-hour sessions >5 times per week during sleep, and 3) short-daily hemodialysis (SDHD): daily 2-hour sessions (Rocco et al., 2011).

Interestingly, home hemodialysis has several benefits compared to conventional in-hospital hemodialysis (CHD), possibly due to a larger cumulative weekly dialysis dose. First, cardiovascular health is substantially improved due to better clearance of middle-sized molecules and excess fluid (Greene et al., 2009; Jun et al., 2013). Longer dialysis runs effectively remove more phosphate molecules, which are known to form harmful calcium/phosphate deposits in joints, blood vessels, and other soft tissues (Yuen et al., 2005a). In addition, daily dialysis lessens non-physiologic fluctuations in extracellular fluid volume and eases the overall stress placed on the heart muscle (Chan et al., 2009; Fagugli et al., 2006). A more constant fluid environment also reduces fatigue experienced by many patients post-dialysis. Second, home hemodialysis offers greater flexibly, convenience, and independence for some patients. The quality of life in home hemodialysis patients is remarkably improved compared to CHD (Culleton et al., 2007; Okpechi et al., 2013). And third, home dialysis is potentially more cost-effective for patients and the health care system (De Vecchi et al., 1999). There is less need for staff assistance, hospital maintenance, and transportation to-and-from in-center facilities.

However, home hemodialysis is not appropriate for all ESRD patients. Individuals on this modality are often younger and healthier compared to those on conventional dialysis (Rocco

et al., 2011). A certain degree of independence, mobility, and comfort is needed to comply with home therapy. In general, fewer than 20% of ESRD patients are placed on home hemodialysis.

1.3 Review of Literature: Immune Dysfunctions in Chronic Kidney Disease

Immune dysfunctions in end-stage renal disease are very complex and both the nature of the immune deficiency and the underlying causes are inadequately understood. Clinically, it appears that patients with ESRD are somewhat immune-compromised compared to the general population. Infections are more common in these patients and responses to some vaccines are either suboptimal or short-lived (De Vecchi et al., 1999; Levin et al., 2013; van Dijk et al., 2001). However, at a cellular/molecular level this secondary immunodeficiency is difficult to explain fully and seems to affect all branches of the immune system. On one hand, ESRD is associated with chronic low-grade inflammation and immune activation (Chung et al., 2012; Kim et al., 2011; Tsakolos et al., 1986) and, on the other, with impaired cellular maturation/function and immunosuppression (Brunet et al., 1998; Verkade et al., 2007b). The loss of function of the native kidneys, the increased age and underlying medical conditions of the patients, and the secondary complications of ESRD may all contribute significantly to changes in immune function. In addition, dialysis therapy – including dialyzer membranes, dialysate fluid, frequent access to blood vessels, drugs and other pharmacologic interventions, etc. – may also directly cause or exacerbate any immune dysfunctions in patients with ESRD (De Cal et al., 2008; Wilflingseder et al., 2008; Xue et al., 2013). Therefore, immune impairments in patients with renal failure are difficult to clearly delineate even after 50 years of active clinical and laboratory investigation. The

following sections highlight a few key findings in the literature and summarize the current state of the topic.

1.3.1 Historical Perspectives

In the 1950s, when transplantation biology was flourishing, it was observed that skin grafts and kidney transplants survived longer than expected in uremic patients (Hume et al., 1955). Although ultimately the transplants did not have the same success as today (lack of immunosuppressive drug therapy), the rejection pattern seen in experimental animals was less pronounced in patients with uremic symptoms (Dammin et al., 1957). It was suggested that uremic patients might have an immune impairment; however, initial leukocyte numbers and antibody responses were normal so an explanation was not found for the extended tissue survival (Dammin et al., 1957).

At the time, the mixed leukocyte reaction (MLR) was commonly used for tissue typing (histocompatibility) and for measuring the allogeneic response (blast formation after exposure to foreign cells). Several studies reported that MLRs were suppressed in renal failure (untreated) and dialysis (before dialysis runs) (Elves et al., 1966). Even mitogen (phytohemagglutinin) and antigen (tuberculin) failed to activate leukocytes to the same degree as healthy controls (Selroos et al., 1973). However, dialysis therapy (both HD and PD) and/or the removal of uremic plasma from cultures enhanced leukocyte reactivity in MLRs (Elves et al., 1966). In addition, delayed-type hypersensitivity (DTH) responses to intradermal injections of antigen (i.e. tuberculin) were also diminished in proportion to the severity of uremia (Huber et al., 1969). Together these studies identified early immune

impairments, particularly related to the cellular immune response in MLRs/DTH mediated by T-cells and macrophages, as well as the effect of a uremic environment on cell function.

In addition to uremia-related impairments, dialysis therapy was also known to affect immune function. Blood transfusions, cannulation, and frequent access to blood vessels increased rates of infection and bacterial colonization, as well as decreased tissue / wound healing (Bulger et al., 1965; Bunn et al., 1964; Kelemen and Kolff, 1960). Dialysate fluid was also at high risk of contamination and, even though bacteria could not cross the dialyzer membrane, endotoxin levels in patients' blood were often elevated (Bulger et al., 1965). Pneumonia, tuberculosis, and hepatitis were particularly prevalent among the dialysis population. Increased complement activation and abnormal granulocyte function (impaired phagocytosis, decreased mobility, and hyper-reactive) after exposures of blood to dialysis membranes were also associated with high prevalence of pulmonary diseases in dialysis patients (Moorhead et al., 1970; Smith et al., 1964).

1.3.2 Clinical Observations of Immune Deficiency in Uremia

Infections and sepsis contribute significantly to high mortality rates in end-stage renal disease. They are the second leading cause of death after cardiovascular disease and account for over 20% and 50% of all deaths, respectively (van Dijk et al., 2001). Hospital admissions for bacteremia/sepsis are 4 times higher in CKD patients and 10 times higher in dialysis patients compared to individuals without signs of kidney disease (Berman et al., 2004; Ishani et al., 2005; Naqvi and Collins, 2006). In order of prevalence, urinary tract infections, pneumonia, and sepsis account for the majority of these hospitalizations (Naqvi and Collins, 2006). In addition, uremic patients on dialysis are also more susceptible to chronic/latent

infections. For example, hepatitis B virus (HBV) infection in dialysis patients is associated with higher rates of chronic disease compared to immune-competent individuals (London et al., 1977). Tuberculosis is yet another example in which HD patients remain infected despite negative tuberculin skin test results (Fonseca et al., 2013; Selroos et al., 1973). Thus, renal failure and dialysis therapy are both strongly associated with a weakened immune system and an increased susceptibility to infections.

Vaccination mimics the challenge of microbial invasion and reflects the overall function of the immune system. Vaccine efficacy is measured by 1) seroconversion (development of detectable antibody titers) and 2) seroprotection (duration of sustained antibody titers). In patients with CKD, responses to vaccines are often suboptimal and/or short-lived. Diphtheria-Tetanus immunization is among the most well studied in CKD and; although patients develop a strong initial response (97% seroconversion), the antibody titers decline significantly within 6 months (62% seroprotection) (Girndt et al., 1995; Guerin et al., 1992; Kruger et al., 2001; Kruger et al., 1999). Similarly, Staphylococcus (Moustafa et al., 2012; Welch et al., 1996), Pneumococcus (Fuchshuber et al., 1996; Pourfarziani et al., 2008), and influenza (Chang et al., 2012; Labriola et al., 2011; Lertdumrongluk et al., 2012) vaccinations follow the same pattern. It is normal for antibody titers to wane over time but this decline is substantially more rapid in dialysis patients. With other vaccines, initial seroresponses are sub-optimal in CKD. Four doses of the Hepatitis B vaccine induce seroconverion in only 50-70% of patients compared to >90% in healthy individuals (Chow et al., 2010; Crosnier et al., 1981; Lin et al., 2011). Within 1-3 years dialysis patients lose their protective immunity against hepatitis B and often require additional vaccine boosters (Lin et al., 2011). Since hepatitis B vaccination is known to induce both humoral and cellular

immunity, these results indicate a general adaptive immune impairment in CKD. In addition, Grindt *et al.* recently demonstrated that a poor response to hepatitis B immunization correlates with elevated cytokine levels, in particular interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Girndt et al., 1995).

Patients with ESRD often have chronic inflammation, ranging from measurable elevations in inflammatory cytokines to inflammation-malnutrition syndromes. Hemodialysis patients have a generalized increase in cytokines and inflammatory serum biomarkers such as interleukin-6 and C-reactive protein (CRP) (Kuznik et al., 2013; Yuen et al., 2005b). Both IL-6 and CRP are strongly correlated with increased morbidity and mortality in CKD patients (Danesh et al., 2004; Honda et al., 2006). There is also increasing evidence that inflammation contributes to the development of atherosclerotic disease and increases the risk of developing cardiovascular complications, which are the leading cause of death (Kuznik et al., 2013). Chronic inflammation in CKD is believed to be a combination of reduced renal clearance of cytokines and immune activation due to infections and dialysis-related effects (artificial membranes, dialysate fluid, etc.).

1.3.3 Adaptive Immune Deficiencies

The adaptive immune response is antigen-specific and creates immunological memory after an initial exposure to pathogen. It rests on activation of two major types of lymphocytes, T-cells and B-cells, by professional antigen-presenting cells (APCs). T-cells play a central role in cell-mediated immunity, which involves activation of phagocytes, cytotoxic T-cells, and cytokines. B-cells, on the other hand, are the key cells necessary for humoral immunity, which involves production of specific antibodies with the help of T-cell activation. The

clinical manifestations of CKD suggest that the overall adaptive immune response is impaired in these patients.

1.3.3.1 Antigen Presenting Cells

Antigen presenting cells internalize and process antigen found throughout the body in order to activate T-lymphocytes who are unable to recognize free antigen. APCs activate T-lymphocytes by 1) displaying peptide fragments in MHC molecules 2) providing co-stimulation using accessory molecules and 3) producing cytokines for T-cell growth and differentiation. Several lines of evidence suggest that antigen-presenting cells are dysfunctional in patients with end-stage renal disease. For example, monocytes from ESRD patients cultured *in vitro* produce high levels of inflammatory cytokines (TNF-α, IL-1β, IL-8, and IL-6) even in the absence of activation signals (Kim et al., 2011). In addition, exposure to lipopolysaccharide (LPS) enhances cytokine production but not nearly to the same degree as in healthy monocytes (Kim et al., 2011). The magnitude of cytokine production in response to pathogen-associated molecular patterns (PAMPs) is severely diminished in CKD, which suggests a pre-activated state where APCs are unable to respond appropriately to subsequent antigen challenge. Also, monocytes from CKD patients have a decreased expression of co-stimulatory molecules like CD80 and CD86 (also called B7-1 and B7-2) (Girndt et al., 2001). These co-stimulatory molecules are critical for T-cell activation and cellular immune responses. Grindt *et al.* found that HD patients who are non-responders to the hepatitis B vaccine express fewer CD86 molecules on their monocytes (Girndt et al., 2001). Low CD86 expression also correlated with decreased peripheral blood mononuclear cell (PBMC) proliferation in response to PHA stimulation. Overall, this indicates that

monocytes in CKD patients are potentially pre-activated or unable to express the co-stimulatory molecules and cytokines necessary to activate T-cells.

Dendritic cells (DCs) are the most efficient antigen-presenting cells. There are several different subtypes of DCs (myeloid, plasmacytoid, monocyte-derived, Langerhans, and tissue-resident) with unique phenotypes and specialized cytokine profiles but they are all very potent T-cell activators. Unlike monocytes and macrophages, DCs have no intrinsic ability to eliminate pathogens and their only known function to date is T-cell activation and immune modulation. Therefore, functional impairments in these cells are likely to contribute significantly to the overall dampened adaptive immune response in CKD patients.

Like monocytes, DCs also exhibit a pre-activated state. *In vitro*, they secrete high levels of inflammatory cytokines (IL-6, TNF-α, and IL12p70), especially when cultured in uremic serum from HD patients (Choi et al., 2011). However, the magnitude of this cytokine response isn't as well studied as in monocytes. *In vivo*, renal DCs are known to contribute to inflammation and accelerated kidney damage in mouse models of experimental kidney injury (Dong et al., 2008; Macconi et al., 2009; Zhou et al., 2009). DCs were the most potent producers of inflammatory cytokines / chemokines (IL-6, TNF-α, MCP-1, MIP-2, and RANTES) and resulted in the activation and infiltration of T-cells (T_H1 an T_H17 subsets) (Dong et al., 2008).

The phagocytic capacity and phenotype of dendritic cells are also extensively studied in CKD. Dendritic cells are typically found in two states throughout the body: 1) Immature – in which they are highly phagocytic and specialize in antigen uptake / processing and 2) Mature

– in which they express high levels of co-stimulatory molecules plus major-histocompatibility complex (MHC) molecules for T-cell activation. Several studies suggest that impaired adaptive immunity in CKD is associated with DC immaturity and inability to activate effector cells (Verkade et al., 2007b). For example, DCs from dialysis patients are more phagocytic compared to healthy volunteers. In both HD patients and controls, antigen uptake decreases after exposure to LPS or cytokines but uremic DCs continue to phagocytize significantly more antigen even after maturation (Verkade et al., 2007b).

Furthermore, it is suggested that DCs from HD patients are also unable to terminally differentiate into potent T-cell activators after maturation. Expression of co-stimulatory molecules (CD80, CD86, CD83, and CD40) and MHC class II molecules are decreased on mature DC from HD patients (Lim et al., 2007; Verkade et al., 2007b). This decrease is even more evident in non-responders to the hepatitis B vaccine (Verkade et al., 2007a). In addition, Lim *et al.* showed that healthy DCs cultured in uremic serum or increasing concentrations of urea also exhibit a decreased expression of co-stimulatory molecules and HLA class II molecules (Lim et al., 2007). Thus, an enhanced phagocytic capacity and a delayed maturation phenotype suggest that DCs from dialysis patients are in an immature DC state, in which they are unable to properly activate T-cells. However, despite this prevailing theory of impaired DC terminal differentiation, other studies by Choi *et al.* and Agrawal *et al.* found that the expression of co-stimulatory molecules was high in HD patients and that the overall DC phenotype post LPS maturation was similar in HD patients and healthy subjects (Agrawal et al., 2010; Choi et al., 2011). Further studies are needed to clarify DC phenotype and function in chronic kidney disease.

In addition to functional impairments in APCs due to uremic conditions, dendritic cell counts are lower in ESRD patients before dialysis and decline further after dialysis. Plasmacytoid dendritic cells (pDCs), which produce large amounts of type I interferon (INF-γ) in response to ssRNA and CpG DNA, are the most significantly depleted (Agrawal et al., 2010). These cells recognize intracellular pathogens and play a significant role in anti-viral defense via cell-mediated immunity. This depletion of APCs following dialysis therapy is consistent with clinical indications of immune deficiency and increased susceptibility to infections in CKD patients (Berman et al., 2004).

1.3.3.2 T-Lymphocytes

T-cells represent a major component of the adaptive immune system and play a central role in cell-mediated immunity and B-cell activation. They have unique T-cell receptors (TCRs) that recognize peptide antigens in the context of MHC class I / II molecules as displayed by antigen presenting cells. Co-stimulation via receptor-ligand interactions on T-cells and APCs (such as CD28:CD86, CD40L:CD40, $CTLA_4$:CD80, and OX40:OX40L) strengthen the TCR signal; thus, resulting in clonal expansion and differentiation of effector / memory T-cells. As previously described, an imbalance in TCR:MHC interaction and/or co-stimulation by APCs can lead to sub-optimal T-cell activation and overall immune deficiency in CKD (Verkade et al., 2004; Verkade et al., 2007b). In addition, the TCR has a short membrane domain and requires additional interaction with several CD3 molecules to induce a signaling cascade. Stachowski *et al.* found that T-cells from uremic patients have fewer TCR:CD3 complexes that further blunt T-cell activation in HD patients (Stachowski et al., 1993). This low TCR:CD3 density also correlates with non-responsiveness to hepatitis B vaccination, which is common in CKD (Stachowski et al., 1994). Furthermore, polyclonal activation of T-

cells by mitogen (PHA, concavalin-A, and SEB superantigen) is also diminished in ESRD (Eleftheriadis et al., 2004; Kurz et al., 1986; Raskova et al., 1986). Mitogens bind cell surface glycoproteins, including the TCR and CD3 molecules, and thereby cause broad non-specific T-cell activation. A diminished proliferative response to mitogens suggests that intrinsic T-cell defects may also contribute to immune impairments in ESRD.

Two major classes of T-cells exist: $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells. Cytotoxic T-cells recognize antigens associated with MHC class I molecules (expressed by almost all cells in the body) and can directly destroy virally infected cells, tumor cells, and foreign tissues (i.e. transplants). Helper T-cells, on the other hand, recognize antigens associated with MHC class II molecules (expressed by professional APCs and some mucosal epithelial cells) and can induce a variety of functions from immune activation to immune suppression. In ESRD, there is substantial lymphopenia (Costa et al., 2008; Yoon et al., 2006). Depletion of naïve $CD4^+$ and $CD8^+$ T-cells is most prominent and many remaining lymphocytes express effector memory ($CD45RO^+$, $CCR7^-$) phenotypes (Betjes et al., 2011; Yoon et al., 2006). Normally, old age (>60 years) is characterized by a higher ratio of memory cells to naïve cells; however, in dialysis patients this aged T-cell phenotype is significantly augmented compared to age-matched healthy volunteers (Betjes et al., 2011; Meijers et al., 2012). These findings are confirmed by relative telomere length (RTL) and TCR excision circle (TREC) content analyses in ESRD patients (van den Dool and de Boer, 2006). Telomere shortening is proportionate to the number of cell divisions; thus, memory cells, which previously encountered antigen, have shorter chromosomal ends. On the other hand, T-cell receptor rearrangement and formation of small excision circles occur during cell development in the thymus. A decrease in TREC content signifies diminished output of

naïve T-cells from the thymus. Hence, it is evident that ESRD is associated with excessive premature immunological aging. Premature T-cell ageing has several implications for immune dysfunction in ESRD. First, aged cells are more susceptible to apoptosis, which further enhances T-cell lymphopenia (Meier et al., 2002). Second, fewer naïve T-cells are available to respond to new microbial challenges, which increases the risk of infections.

Finally, immune dysfunctions in ESRD are associated with altered $CD4^+$ helper T-cell subsets. $CD4^+$ helper T-cells differentiate into different subsets in response to their cytokine environment and are classified based on their main effector function. T_H1 cells secrete interferon-γ (INF-γ) and are the effector cells of cellular immunity whereas T_H2 cells secrete IL-4 and promote humoral immunity. T_H3 cells (also called T regulatory cells or T_{regs}) are immunosuppressive and play a fundamental role in tolerance to self-antigen. Lastly, the newly discovered T_H17 cells are involved in inflammation and anti-microbial immunity at mucosal sites. Several studies indicate that the ratios of helper T-cells are skewed in dialysis patients, although there is disagreement on this topic. For example, Sester *et al.* found that the T_H1 cell subtype is more common in hemodialysis patients (Sester et al., 2000). The T_H1 differentiation cytokine, IL-12, is elevated in chronic kidney disease and likely contributes to an increase in this subtype of $CD4^+$ helper T-cells (Lim et al., 2007). T_H1-promoting cytokines also down-regulate the T_H2 counterparts and, therefore, impairs B-cell activation and humoral immunity. In contrast, Libetta *et al.* found that helper T-cells in ESRD patients are polarized towards the T_H2 phenotype (Libetta et al., 2001). This T_H2 shift can partly explain the clinical dysfunctions in cell-mediated immunity due to increased T_H2-mediated humoral immunity. However, other studies did not find any differences between T_H1: T_H2 ratios in dialysis patients (Alvarez-Lara et al., 2004). Instead, there is growing interest in

inflammatory T_H17 subsets (Chung et al., 2012). It is currently unclear if these subsets contribute to immune dysfunctions in ESRD patients but T_H17 cells do play a role in augmenting kidney damage and perpetuating the inflammatory environment.

1.4 Thesis Outline and Experimental Statements

1.4.1 Experimental Question and Rationale

Clinical and experimental data indicate that ESRD patients have substantial adaptive immune impairments. Improving chronic inflammation and overall immunity in these patients is fundamental to reducing morbidity and mortality in this population. Home hemodialysis therapy has been shown to improve multiple complications of ESRD including blood pressure (Fagugli et al., 2006), left ventricular hypertrophy (Culleton et al., 2007), calcium-phosphate balance (Yuen et al., 2005a) and quality of life (Okpechi et al., 2013). Thus, there is a possibility that the benefits of home dialysis extend to improved immunity as well. Better clearance of uremic toxins, improved extracellular fluid balance, and a more stable physiologic environment can all contribute to improved immune function (Greene et al., 2009; Walsh et al., 2005). On the other hand, home dialysis might also exacerbate immune impairments due to increased blood exposure to artificial membranes and dialysate fluid, which may cause leukocyte activation and increase inflammation (Mares et al., 2010; Wilflingseder et al., 2008). There are no current studies, which we are aware of, that have assessed immune function, either at the clinical or molecular level, in the home dialysis population. Although, there is emerging evidence that extended hemodialysis increases vascular access complications and the risk of infections due to the high dialysis frequency (Jun et al., 2013). Therefore, we asked the following experimental question: Does extended

home hemodialysis, which we defined as a minimum of 25 hours of dialysis per week irrespective of how/when the patient chooses to dialyze, affect immune function?

1.4.2 Hypothesis

Patients receiving extended home hemodialysis (EHHD) therapy have decreased inflammation and exhibit improved immunity compared to patients on conventional in-center hemodialysis (CHD).

1.4.3 Experimental Approach

An appropriate animal model of chronic intermittent hemodialysis for the treatment of uremic symptoms is still elusive. There are extremely few adequate animal models of dialysis suitable to our experimental question. Most studies on mice, rats, rabbits, and dogs involved peritoneal dialysis or hemodialysis under limited shot-term conditions (although PD has been used to model chronic treatment) (Peng et al., 2000; Wieczorowska-Tobis et al., 1999). In some cases animals received as little as one dialysis treatment and required anesthesia during the procedure. In addition, studies examining the biocompatibility of membranes and dialysate fluid did not always use uremic animals (Zunic-Bozinovski et al., 2008). Hence, a non-interventional human trial was deemed most appropriate for this particular work.

We designed a prospective, matching-cohort, clinical research study with two groups of ESRD patients – those on extended-home hemodialysis and those on conventional hemodialysis – as well as a third group of healthy volunteers as controls. To limit possible confounding variables, all recruited patients were matched by age, gender, dialysis access

type, and medical co-morbidities. Two blood samples were obtained from all study participants in order to isolate leukocytes and measure immune competence of patients on different hemodialysis modalities (Figure 2).

A large part of this thesis is focused on the function of dendritic cells in dialysis patients. However, DCs (plasmacytoid and myeloid subsets together) constitute only between 0.5-1% of total circulating leukocytes. A larger source of DCs was thus obtained from *ex vivo* differentiation of blood monocytes into monocyte-derived dendritic cells (MDDCs) using granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). MDDCs are widely used throughout the literature and are somewhat similar to myeloid dendritic cells (Osugi et al., 2002; Sallusto and Lanzavecchia, 1994). There is also evidence suggesting MDDC occur *in vivo* during inflammatory conditions (Leon et al., 2007).

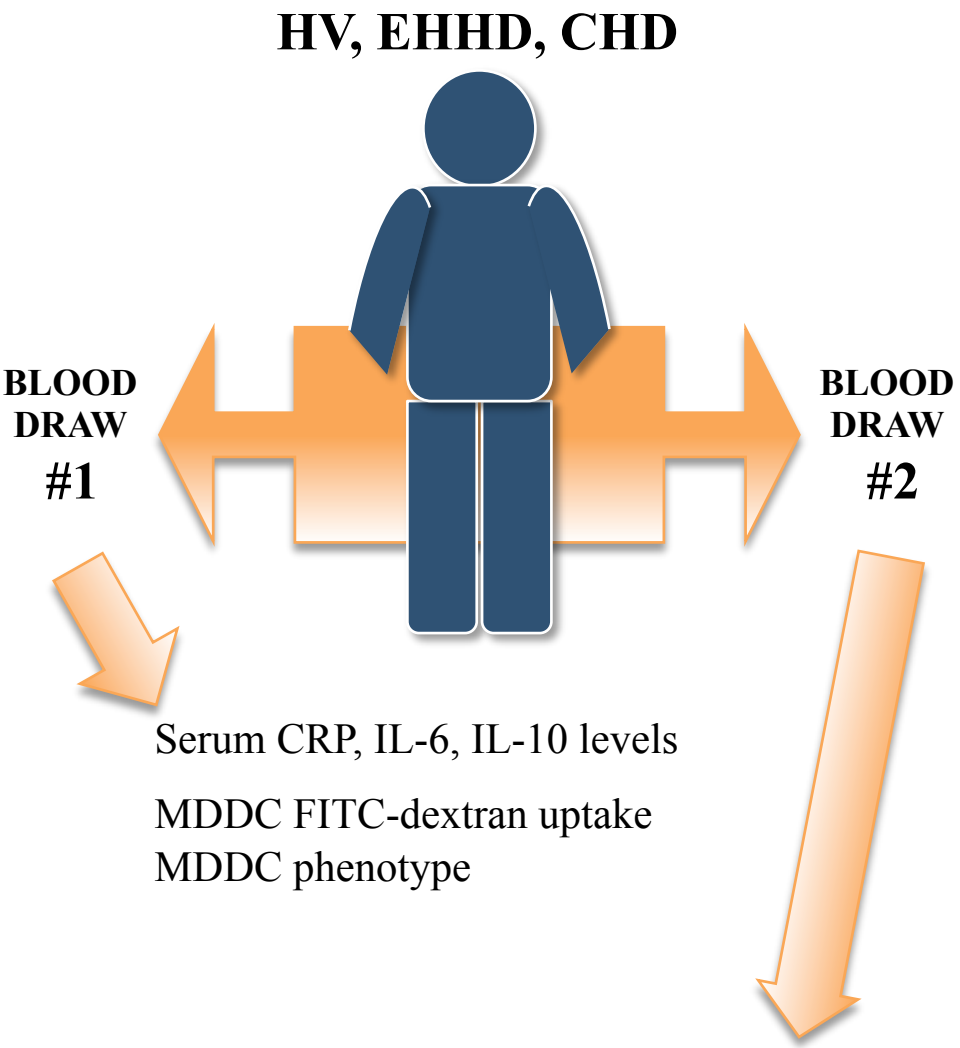
1.4.4 Specific Objectives

Three experimental objectives were used to test our hypothesis that extended home-based hemodialysis therapy improves immune function in patients with ESRD (Figure 2):

1. Measure the baseline serum markers of inflammation in ESRD patients, specifically:
    a. Interleukin-6
    b. C reactive protein
    c. Interleukin-10
2. Assess the functional capacity of dendritic cells in ESRD patients, through:
    a. Antigen (dextran) uptake
    b. Maturation via expression of cell surface markers
    c. Their ability to activate T-cells in an allogeneic mixed lymphocyte reaction

3. Test the T-lymphocyte response to stimulation in ESRD patients, via:
    a. Proliferation capacity
    b. Cytokine production and T_H-cell polarity

Figure 2: Experimental outline. All recruited healthy volunteers and hemodialysis patients were asked to donate two blood samples spaced between 1-6 months apart. For CHD patients, all blood samples were obtained pre-dialysis and mid week for accurate baseline measurements. The first blood sample was used to assess serum inflammatory markers and MDDC endocytosis plus phenotype; whereas, the second blood sample was used to assess MDDC activation capacity and T-lymphocyte proliferation.


HV, EHHD, CHD
BLOOD DRAW #1
BLOOD DRAW #2
Serum CRP, IL-6, IL-10 levels
MDDC FITC-dextran uptake
MDDC phenotype
MDDC activation capacity (allogeneic MLR)
T-cell proliferation (anti-CD3 stimulation)
T-cell cytokine (IL-10 and INF-γ) production

2

MATERIALS AND METHODS

2.1 Study Design and Participants

2.1.1 General Study Description

We designed a non-interventional, prospective matching-cohort clinical research trial with two hemodialysis patient populations and healthy volunteers as controls. Dialysis patients were grouped based on their HD modality – those receiving conventional in-center hemodialysis or those on extended-home hemodialysis. This clinical translational study was not randomized because dialysis modality is assigned according to patient preference and ability to comply with treatment. Instead, to limit possible confounding variables, CHD and EHHD patients were matched based on age/gender, medical co-morbidities, and dialysis

access type (Table 1). HVs were recruited at random and were not age/gender matched to either hemodialysis cohort since they were only used to establish a reference for optimal immune function. The research team was not blinded to the participants' research cohorts. The Ottawa Hospital Research Ethics Board approved this study and all participants provided written and verbal informed consent.

Table 1: EHHD and CHD Patient Matching Criteria

	Specific Matching Criteria
Patient demographics	Age within 5 yrs.
Dialysis characteristics	Dialysis access type (AV fistula, graft, CVC)
	Time on modality (<2 yrs., 2-5 yrs., or >5 yrs.)
Medical co-morbidities	Presence of diabetes mellitus

EHHD extended home hemodialysis, CHD conventional hemodialysis, AV arterio-venous, CVC central venous catheter.

2.1.2 Study Participants

Patients were recruited from The Ottawa Hospital Dialysis Program. Conventional hemodialysis was defined as in-center nurse-assisted hemodialysis for 4 hours thrice weekly (12 hours/week) and extended hemodialysis was defined as home-based hemodialysis performed for a minimum of 25 hours/week. For both CHD and EHHD cohorts, dialysis was achieved using Polysulfone High-Flux dialyzers (Fresenius, Waltham, MA, USA) with purified reverse osmosis (RO) water and DIASAFE®plus filters (Fresenius, Waltham, MA, USA). Patients were included in this trial if they were on their respective hemodialysis modality for a minimum of 3 months before recruitment (Table 2). Patients with signs of an active infection within 3 months and those on immunosuppressive drugs or with autoimmune

diseases, malignancies, HIV, HCV, or a failed kidney transplant within the last 2 years were excluded from this study (Table 3).

Table 2: Study Eligibility Criteria for Participants

HV	EHHD	CHD
Age >18 yrs. No medical conditions requiring care	Age > 18 yrs. On EHHD for > 3 mo. Min. 25 hours dialysis/week HG levels > 100g/L for 2 mo.	Age > 18 yrs. On CHD for > 3 mo. Session Kt/V > 1.2 for 2 mo. HG levels > 100g/L for 2 mo.

HV, healthy volunteers, EHHD extended home hemodialysis, CHD conventional hemodialysis, HG hemoglobin, Kt/V dialysis adequacy (volume filtered / total fluid volume).

Table 3: Study Exclusion Criteria for Hemodialysis Patients

Unable to provide informed consent for participation
Infection requiring antimicrobial medication within 3 mo.
Failed kidney transplant in last 2 yrs. that was not surgically removed
History of active autoimmune disease requiring immunosuppressive drugs in previous 2 yrs.
Positive for HIV and/or HCV
Concurrently receiving chemotherapy for malignancy
Life expectancy < 2yrs. as judged by their attending dialysis physician

HIV human immunodeficiency virus, HCV hepatitis C virus.

2.1.3 Experimental Design

All recruited healthy volunteers and hemodialysis patients were asked to donate two blood samples spaced between 1-6 months apart. For CHD patients, all blood samples were obtained pre-dialysis and mid week for accurate baseline measurements. The first blood sample was used to assess serum inflammatory markers and MDDC endocytosis plus

phenotype; whereas, the second blood sample was used to assess MDDC activation capacity and T-lymphocyte proliferation (Figure 2).

2.2 Blood Sample Collection and Serum Isolation

2.2.1 Blood Sample Collection

For each sample donation, approximately 40mL venous blood was collected in two heparinized syringes. On the first donation only, an additional 5mL blood was collected in a gold-top Vacutainer™ serum-separator tube (BD Biosciences, Mississauga, ON, Canada). All blood samples were transported on ice and processed within 2 hours of collection.

2.2.2 Serum Isolation

Serum-separator tubes were left at room temperature (RT) for 30 minutes. The tubes were centrifuged at 1,000 - 2,000 × *g* for 10 minutes at 5°C. The topmost layer, containing blood serum, was collected in 1.5mL Eppendorf tubes and stored at -80°C until use.

2.3 Cell Isolation and Culture

2.3.1 Peripheral Blood Mononuclear Cell Isolation

Heparinized blood was diluted with equal parts sterile 0.01M phosphate buffered saline (PBS) (Sigma-Aldrich, Oakville, ON, Canada) and then gently layered over Ficoll-Paque™ PREMIUM (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) in a 2:1 blood to Ficoll ratio. Layered blood was centrifuged at 400 × *g* for 35 minutes at 18°C with the centrifuge brake disabled. The buffy coat, containing peripheral blood mononuclear cells (PBMCs),

was gently removed from the interface between the two topmost layers. PBMCs were washed three times in PBS and centrifuged at 400 × *g* for 10 minutes. Cell counts were performed using an automated Z-series cell counter (Beckman-Coulter, Mississauga, ON, Canada) with aperture set to 5μm. Average yield was 0.5-1 × 10^6 PMBCs per 1mL undiluted blood.

2.3.2 Leukocyte Isolation

Monocytes (MO) and T-lymphocytes were sorted from PBMCs by magnetic activated cell sorting (MACS) using Human Monocyte-Isolation Kits and Pan T-cell Isolation Kits as per the manufacturer's instructions (Miltenyi Biotech, Cambridge, MA, USA). In both cases negative selection was used to enrich MO and T-cell populations. Briefly, a PBMC single cell suspension was labeled with a cocktail of biotinylated-antibodies targeted against either non-monocytes (cells expressing CD3, CD7, CD16, CD19, CD56, CD123, and CD235a) or non-T-cells (cells expressing CD14, CD16, CD19, CD36, CDCD56, CD123, and CD235a). These cells were then combined with anti-biotin antibodies conjugated to magnetic beads. They were passed through an LS column in a magnetic field (Miltenyi Biotech, Cambridge, MA, USA) to capture all cells that were not of interest and decant pure MO or T-cell suspensions. Cell counts were performed as previously mentioned. Average MO and T-cell yield was 15-25% of total PBMCs.

2.3.3 In Vitro Generation of Monocyte Derived Dendritic Cells

Freshly isolated monocytes were suspended in serum-free RPMI-1640 (Thermo Scientific Company, Toronto, ON, Canada) and left to adhere to 6-well plates (2×10^6 cells/well) for 1 hour at 37°C in a humidified 5% CO_2 incubator. Debris and non-adherent cells were

removed and remaining adherent monocytes were then cultured for 8 days in complete RPMI-1640 supplemented with 5% sterile-filtered fetal calf serum (FCS), 2mM L-glutamine, 100U/mL penicillin, 100µg/mL streptomycin, 10mM HEPES, 1mM MEM sodium pyruvate, and 100µM non-essential amino acids. During the 8-day period, monocytes were cultured in the presence of granulocyte-macrophage colony stimulating factor (1µg/mL) and interleukin-4 (1µg/mL) (PeproTech, Dollard des Ormeaux, QC, Canada) to induce differentiation of monocytes into monocyte-derived dendritic cells (MDDCs). Culture medium and cytokines were replaced at days 3 and 6. MDDCs were then matured with lipopolysaccharide (1µg/mL) for an additional 48 hours.

2.4 Monocyte-Derived Dendritic Cell Characteristics and Function

2.4.1 Immunofluorescence Staining and Flow Cytometric Analysis

Mature (LPS-treated) MDDCs were suspended in flow buffer (Hank's Balanced Salt Solution containing 0.1% NaN_3 and 1% bovine serum albumen). Cells were labeled with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, AlexaFluor488 (Alexa488)-, Pacific Blue (PB)-, or allophycocyanin (APC)-conjugated mouse monoclonal antibodies against the following human MDDC cluster of differentiation (CD) markers: CD14, CD80, CD86, CD83, CD206, CD11c, CD40, and HLA-DR (eBioscience, Inc., San Diego, CA, USA). Isotype controls were used at the same protein concentration as fluorochrome-conjugated antibodies. After 30-40 minutes incubation in the dark at 4°C, cells were washed once with PBS and re-suspended in 1% paraformaldehyde (PFA) solution. Cells were examined on a CyAn ADP analyzer (Beckman-Coulter, Mississauga, ON, Canada) and expression of CD markers was determined by both percent positive compared to isotype control and median

fluorescence intensity (MFI) using FlowJo analysis software. Before each experiment, the flow cytometer was calibrated with FlowSet™ Pro Fluorospheres (Beckman-Coulter, Mississauga, ON, Canada) to standardize the fluorescence parameters for each detector. A 5% coefficient of variation was aimed at for each fluorescent marker. A representative example of gating strategy and overall analysis is available in Appendix I.

2.4.2 Fluorescein Isothiocyanate-Labeled Dextran Internalization

Between 0.5-1×10^6 mature (LPS-treated) and immature (untreated) MDDCs were incubated with 1 μg/mL FITC-dextran for 1 hour in culture media at 37°C in a humidified 5% CO_2 incubator. Control cells were treated with equal amounts of FITC-dextran and incubated for 1 hour at 4°C. Excess FITC-dextran was removed and cells were stained with a viability dye, 7-Aminoactinomycin D (7-AAD), and incubated in the dark at 4°C for 5 minutes. Finally, cells were washed in PBS and re-suspended in 1% PFA solution. Cells were examined on a CyAn ADP analyzer and FITC-dextran uptake by live cells was measured as percent positive and MFI of internalized dextran. We calculated the percent change in dextran uptake for each individual as follows: Δ uptake = [(uptake post LPS – uptake pre LPS) / uptake pre LPS] *100. A representative example of the flow cytometric analysis is available in Appendix I.

2.5 T-Lymphocyte Proliferation

2.5.1 Carboxyfluorescein Succinimidyl Ester Cell Labeling

CFSE cell labeling was performed as previously described (Parish et al., 2009; Quah et al., 2007). Briefly, whole PBMCs or purified T-cells were suspended in sterile PBS supplemented with 5% sterile-filtered FCS. In the dark, the cells were labeled with 5mM

carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Invitrogen – Life Technologies, Burlington, ON, Canada) for 5 minutes. Afterwards, labeled cells were washed three times with 5%FCS-PBS and centrifuged at 400 × *g* for 5 minutes.

2.5.2 T-Lymphocyte Stimulation with Anti-CD3 Antibodies

CFSE-labeled PBMCs (1×10^5 cells/well) from study participants were stimulated with serial dilutions (2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, and 0.02 μg/mL) of plate-bound anti-CD3 antibodies in a 96-well flat-bottom plate for 3 days at 37°C in a humidified 5% CO_2 incubator. Non-stimulated PBMCs were used as negative control for proliferation and phorbol 12-myristate 13-acetate (PMA) (10 ηg/mL) plus ionomycin (1 μg/mL) as positive control for proliferation. Non-CFSE-labeled PBMCs were used as staining controls for flow cytometric analysis.

2.5.3 T-Lymphocyte Activation in an Allogeneic Mixed Lymphocyte Reaction

In a one-way allogeneic mixed lymphocyte reaction, LPS-matured MDDCs from study participants were tested for their ability to stimulate purified T-cells from one healthy donor. CFSE-labeled T-cells (1×10^5 cells/well), which function as the responder cell population, were stimulated with serial dilutions (1:1, 1:2.5, 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160) of mature MDDCs from a study participant in a 96-well round-bottom plate for 5 days at 37°C in a humidified 5% CO_2 incubator. Non-stimulated T-cells were used as negative control for proliferation and PMA (10 ηg/mL) plus ionomycin (1 μg/mL) stimulated T-cells as positive control for proliferation. Non-CFSE-labeled T-cells were used as staining controls for flow cytometric analysis.

2.5.4 Flow Cytometric Analysis of T-Lymphocyte Proliferation

For both the T-cell proliferation assay and the allogeneic MLR, culture plates (96-wells) were centrifuged at 400 × *g* for 5 minutes and excess supernatant was stored at -20°C until needed for cytokine measurement. For each stimulation condition, cells were stained with PE-conjugated anti-CD3 antibodies and 7-AAD for 30 minutes in the dark at 4°C. Finally, cells were washed with PBS and re-suspended in 1% PFA solution. They were analyzed on a CyAn ADP flow cytometer and the proliferation of live $CD3^+$ T-cells was measured by the degree of CFSE dilution within the cells. Cells that underwent more than one division were considered to be proliferating and were reported as percent cells over one generation plotted against the stimulation condition. In addition, the proliferation index, or the average number of divisions per proliferating cells (Roederer, 2011), was calculated by FlowJo analysis software and averaged over all the dilutions or MDDC:T-cell ratios. A representative example of the gating strategy and flow cytometric analysis is available in Appendix II.

2.6 Serum and Cell Culture Cytokine Measurements

2.6.1 Serum C-Reactive Protein and Cytokine (IL-6, IL-10) Quantification

High-sensitivity ELISA kits for CRP (Abnova, Walnut, CA, USA) and IL-6 / IL-10 (eBioscience, Inc., San Diego, CA, USA) were used for the quantitative determination of CRP and cytokines in human serum. According to the manufacturer's instructions, human serum samples were diluted accordingly and plated on pre-coated ELISA plates. Secondary biotinylated antibodies and streptavidin-horseradish peroxidase (HRP) were used to detect the colorimetric oxidative reaction of tetramethylbenzidine (TMB). For IL-6 and IL-10 only, the reaction was further amplified with biotinyl-tyramide, which increases binding sites for

streptavidin-HRP. Acid was used to stop the reaction and absorbance was measured at 450nm. The concentrations of CRP, IL-6, and IL-10 were calculated using 4-parameter standard curves.

2.6.2 Cell Culture Cytokine (IL-10 and INF-γ) Determination

Cytokine (IL-10 and INF-γ) ELISA kits (BD Biosciences, San Diego, CA, USA) were used for the quantitative determination of cytokines in T-lymphocyte proliferation assays as described previously for serum CRP / cytokine quantification. However, ELISA plates were coated with primary antibody at 4°C overnight and blocked with protein manually before addition of samples. Furthermore, no amplification reaction was necessary and substrate solution containing TMB was purchased and prepared separately (BD Biosciences, San Diego, CA, USA). Absorbance was also measured at 450nm and concentrations of IL-10 and INF-γ were calculated using 4-parameter standard curves.

2.7 Statistical Analysis

Flow cytometry data were analyzed with FlowJo (FlowJo Analysis Software version 8.8.7 for Mac, ©Tree Star, Ashland, OR, USA) and all other data were analyzed with GraphPad Prism (GraphPad Software version 5.0d for Mac, San Diego, CA, USA). All values are reported as means ± standard deviation (SD). Statistical comparisons of multiple groups were made using one-way analysis of variance (ANOVA) followed by Bonferroni post-test; whereas, statistical comparisons of two groups were made using the two-tailed unpaired student *t* test. *P* values less than 0.05 were considered significant.

3

RESULTS

3.1 Patient Characteristics

The healthy volunteers (14 female / 5 male, mean age 40.16 ± 2.65 years) enrolled in this study had no history of major illnesses and were self-assessed as healthy. All volunteers, except one, (94.7%) were of Caucasian ethnicity.

The extended home hemodialysis patients (4 female / 5 male, mean age 43.22 ± 3.50 years) were not significantly older than HV and most were Caucasian (88.9%) as well. Two patients had diabetes mellitus and two patients had a previous splenectomy. The underlying causes of ESRD were as follows: IgA nephropathy (n = 4), medullary cystic kidney disease (n = 1),

hemolytic uremic syndrome (*n* = 1), membranous nephropathy (*n* = 1), malignant hypertension (*n* = 1), and congenital renal hypoplasia (*n* = 1).

The conventional hemodialysis patients (2 females / 3 males, mean age 57.40 ± 6.79 years) did not differ in age compared to EHHD patients but were slightly older than HV ($P < 0.05$). The CHD patients were of Middle Eastern, African, or East Indian decent. The underlying causes of ESRD were as follows: diabetic nephropathy (*n* = 2), polycystic kidney disease (*n* = 1), congenital disease (*n* = 1) and unknown (*n* =1).

Compared to CHD, EHHD patients had a longer duration of renal failure (9.00 ± 4.87 vs. 5.50 ± 3.87 years) and overall hemodialysis usage (7.10 ± 4.05 vs. 3.70 ± 1.79 years) but both groups of patients were on their current dialysis modality for a similar period (4.39 ± 2.87 and 3.37 ± 1.70 years). As expected, EHHD patients also received significantly more dialysis therapy compared to CHD patients (36.56 ± 6.19 vs. 10.40 ± 3.58 hours/week, $P < 0.0001$) (Table 4).

Table 4: Characteristics of Patients and Healthy Volunteers Included in the Study

	HV (*n* = 19)	EHHD (*n* = 9)	CHD (*n* = 5)
Age (yrs.)	40.16 ± 11.55	43.22 ± 10.50	57.40 ± 15.19#
Gender, female, *n* (%)	14 (73.7%)	4 (44.4%)	2 (40.0%)
Race, Caucasian, *n* (%)	18 (94.7%)	8 (88.9%)	0 (00.0%)
Renal failure duration (yrs.)	N/A	9.00 ± 4.87	5.50 ± 3.87
Total HD duration (yrs.)	N/A	7.10 ± 4.05	3.70 ± 1.79
Current HD modality (yrs.)	N/A	4.39 ± 2.87	3.37 ± 1.70
Weekly dialysis amount (hrs.)	N/A	36.56 ± 6.19	10.40 ± 3.58*

Previous failed transplant, *n* (%)	N/A	5 (55.6%)	2 (40.0%)
Diabetes mellitus, *n* (%)	N/A	2 (22.2%)	2 (40.0%)
Visit #1: Hemoglobin (g/L)	N/C	114.8 ± 16.49	117.2 ± 4.82
White Blood Cells (10^9/L)	N/C	7.58 ± 2.03	7.16 ± 1.69
Visit #2: Hemoglobin (g/L)	N/C	119.1 ± 22.09	119.2 ± 8.67
White Blood Cells (10^9/L)	N/C	7.70 ± 3.10	7.70 ± 2.40

Data shown as mean ± standard deviation, unless otherwise indicated. HV healthy volunteers, EHHD extended home hemodialysis, CHD conventional hemodialysis, HD hemodialysis, N/A not applicable, N/C not checked.

* $P < 0.0001$, EHHD vs. CHD (unpaired t test)
# $P < 0.05$, HV vs. CHD (1way ANOVA and Bonferroni post test)

3.2 Serum Cytokines in HD Patients

3.2.1 Serum Biomarkers of Inflammation Are Elevated in HD Patients

We measured serum C-reactive protein and interleukin-6 concentrations to determine whether or not EHHD and CHD patients have similarly elevated levels of inflammatory biomarkers (Figure 3). As expected, CHD patients had significantly higher concentrations of both CRP (13.10 ± 7.65 vs. 2.47 ± 2.49 mg/L) and IL-6 (2.37 ± 1.12 vs. 0.69 ± 0.85 pg/mL) as compared to HV. EHHD patients had notably lower levels of CRP (6.24 ± 3.61 mg/L) compared to CHD patients ($P < 0.05$). This CRP amount was higher than in HVs but it was not statistically significant. In addition, levels of IL-6 (1.75 ± 1.18 pg/mL) were slightly lower than in CHD patients but significantly higher compared to HVs ($P < 0.05$).

3.2.2 Serum Anti-Inflammatory Cytokines Are Elevated in HD Patients

We also measured serum interleukin-10 concentrations to determine whether immunomodulatory cytokines are elevated in HD patients in addition to inflammatory

Figure 3: Serum IL-6 and CRP levels are elevated in HD patients compared to HVs although CRP is significantly higher in CHD patients versus EHHD patients. High-sensitivity ELISAs were used to measure levels of CRP (A) and IL-6 (B) in blood serum of HVs (*n*=18), EHHD patients (*n*=7/9), and CHD patients (*n*=5). Bars and whiskers represent mean ± standard deviation (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

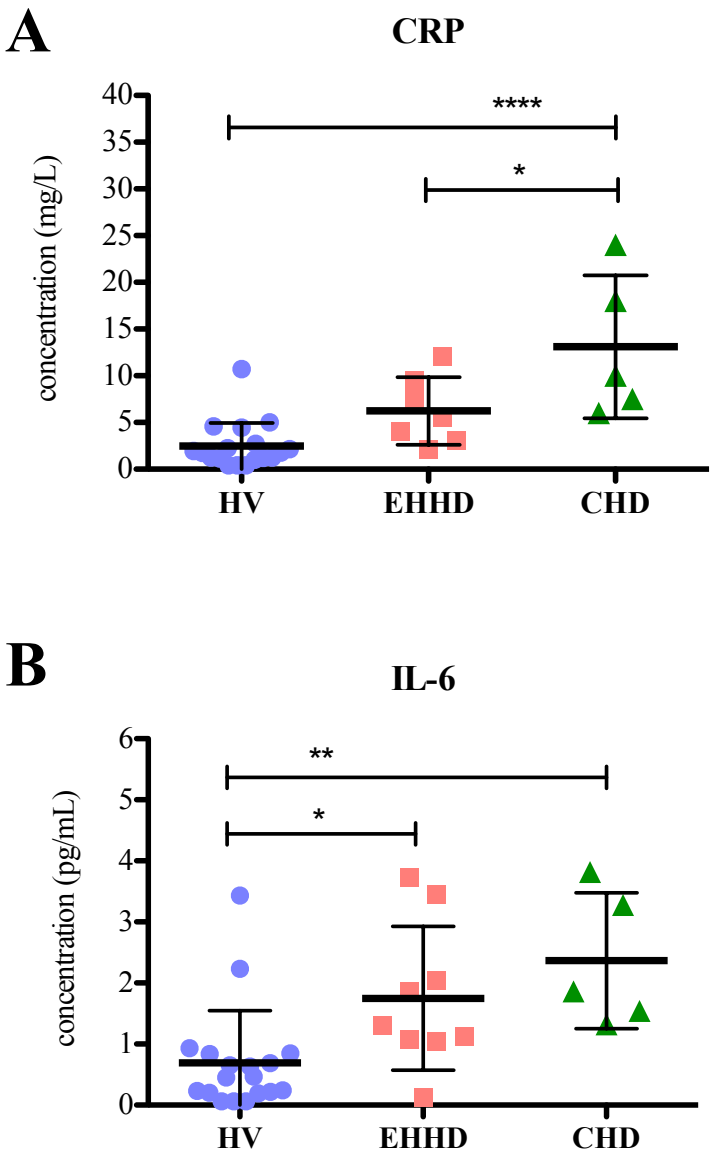
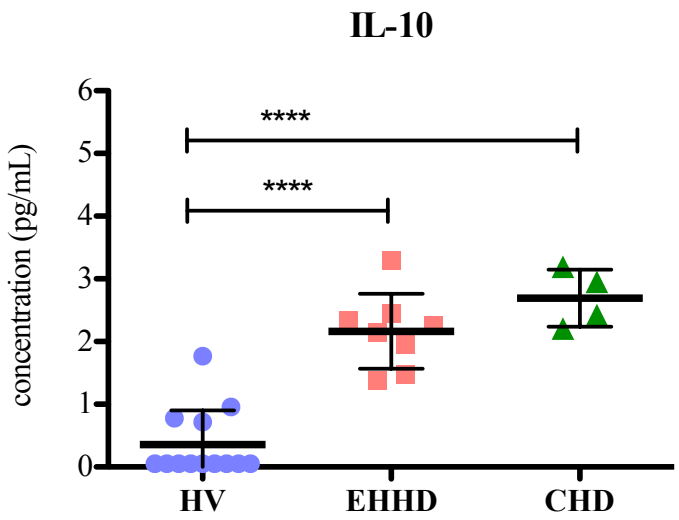

A
CRP
concentration (mg/L)
40
35
30
25
20
15
10
5
0
****
*
HV
EHHD
CHD
B
IL-6
concentration (pg/mL)
6
5
4
3
2
1
0
**
*
HV
EHHD
CHD

Figure 4: Serum IL-10 levels are elevated in HD patients compared to HVs. High-sensitivity ELISAs were used to measure levels of IL-10 in blood serum of HVs (*n*=13), EHHD (*n*=8) and CHD (*n*=4) patients. Bars and whiskers represent mean ± standard deviation (**** $P < 0.0001$).


IL-10
concentration (pg/mL)
6
5
4
3
2
1
0
****
****
HV
EHHD
CHD

biomarkers (Figure 4). CHD (2.69 ± 0.46 pg/mL) and EHHD (2.17 ± 0.60 pg/mL) patients had comparable levels of IL-10. However, levels of this anti-inflammatory cytokine were significantly lower in HVs (0.36 ± 0.54 pg/mL) with almost all values below the detection limit of our assay.

3.3 Immunophenotype of MDDCs in HD Patients

3.3.1 Hemodialysis Therapy Does Not Influence MDDC Differentiation and Viability

Monocyte yield after isolation was similar in all HVs and HD patients. After 8 days of culture with IL-4 and GM-CSF, monocytes were successfully differentiated into mature dendritic cells. MDDCs were defined as the $CD14^{low}$ and $CD11c^{+}$ cell population (Figure 5). The pan dendritic cell adhesion marker, CD11c, was more highly expressed on healthy MDDCs versus MDDCs from HD patients but this difference was not statistically significant. In addition, there was no difference in the viability of MDDCs from HVs (93.33 ± 4.48%), EHHD patients (97.50 ± 1.79%), or CHD patients (97.68 ± 1.60%) (Figure 5C).

3.3.2 MDDC Phenotypic Maturation Is Enhanced in HD Patients

LPS (1 μg/mL) was added to monocyte cultures during the last 48 hours to induce terminal differentiation of MDDCs into mature antigen presenting cells. The maturation phenotype of MDDCs was determined by flow cytometry based on the expression of T-cell co-stimulatory molecules (CD80, CD86, CD83, and CD40) and MHC class II molecules (HLA-DR), whose functions are outlined in Table 5. Mature MDDC from HVs, EHHD, and CHD patients were characterized as $CD80^{+}$, $CD86^{+}$, $CD83^{+}$, $CD40^{+}$, and $HLA\text{-}DR^{high}$. The relative expression of

Figure 5: MDDC differentiation and viability are similar in all study participants. After 8 days of culture with IL-4 and GM-CSF, MDDCs were stained with fluorochrome-conjugated antibodies against CD14 (A) and CD11c (B) as well as with the viability dye 7-AAD (C). Expression of pan dendritic cell markers and cell viability were determined by flow cytometry. For MDDC differentiation, data is shown as % positive cells (HV *n*=19, EHHD *n*=8, CHD *n*=5) and median fluorescence intensity (HV *n*=12, EHHD *n*=8, CHD *n*=3). For cell viability, data is shown as % live cells (HV *n*=8, EHHD *n*=7, CHD *n*=4). Bars and whiskers represent mean ± standard deviation.

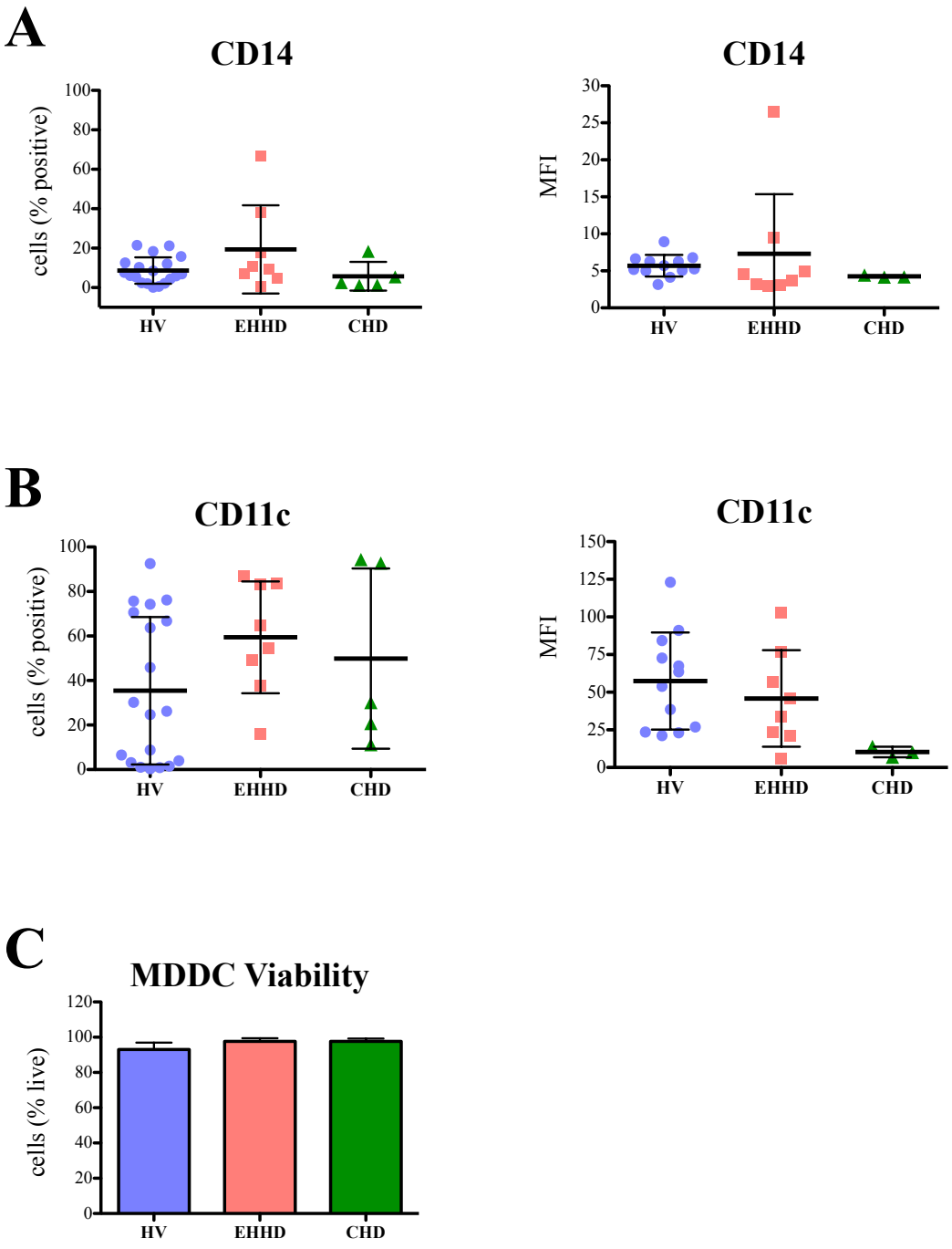
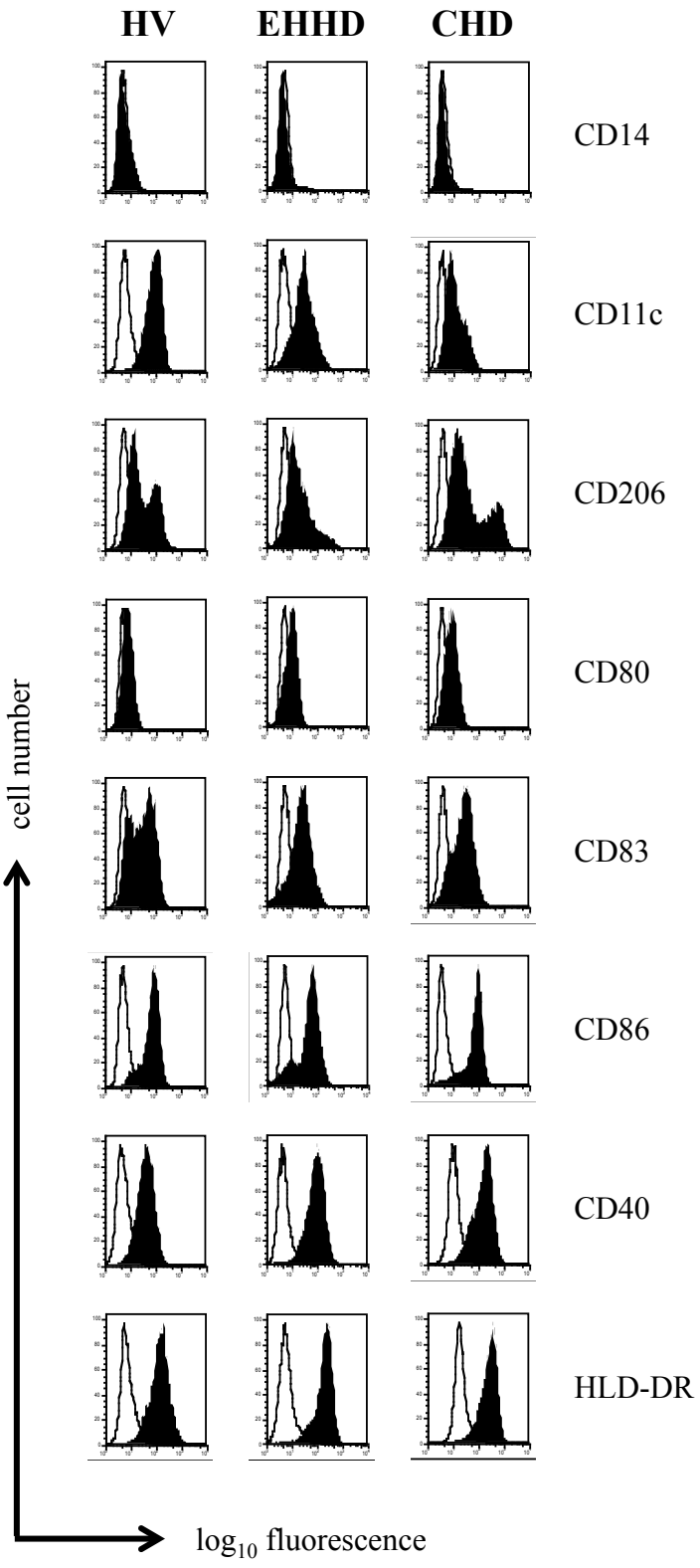

A
CD14
cells (% positive)
100
80
60
40
20
0
HV
EHHD
CHD
CD14
MFI
30
25
20
15
10
5
0
HV
EHHD
CHD
B
CD11c
cells (% positive)
100
80
60
40
20
0
HV
EHHD
CHD
CD11c
MFI
150
125
100
75
50
25
0
HV
EHHD
CHD
C
MDDC Viability
cells (% live)
120
100
80
60
40
20
0
HV
EHHD
CHD

Figure 6: Representative histogram plots of MDDC maturation phenotypes in HVs, EHHD, and CHD patients. Monocytes were cultured with IL-4 and GM-CSF for 6 days to yield MDDCs, which were then terminally differentiated with 48 hours LPS treatment. Mature MDDCs were stained with fluorochrome-conjugated antibodies against CD14, CD11c, CD206, CD80, CD83, CD86, CD40, and HLA-DR (filled histograms) and analyzed by flow cytometry. The open histograms represent background staining of the appropriate isotype controls.


HV
EHHD
CHD
CD14
CD11c
CD206
CD80
CD83
CD86
CD40
HLD-DR
cell number
log10 fluorescence

these maturation markers compared to their isotype controls is shown in representative histograms for each study cohort (Figure 6).

Table 5: Molecules expressed by terminally differentiated MDDCs for antigen presentation and T-cell activation.

Name(s)	Ligand(s)	Function
HLA-DR (MHC class II)	TCR / CD4	Antigen presentation and T-cell activation
CD80 (B7-1)	CD28 / $CTLA_4$	Co-stimulation to augment TCR signaling
CD86 (B7-2)	CD28 / $CTLA_4$	Co-stimulation to augment TCR signaling
CD83 (HB15)	CD83L	Co-stimulation to augment TCR signaling
CD40 (TNFRSF5)	CD40L	IL-12 synthesis and T-cell differentiation

MDDC monocyte-derived dendritic cell, HLA human leukocyte antigen, MHC major histo-compatibility, TNFRSF tumor necrosis factor receptor superfamily, TCR T-cell receptor, CTLA cytotoxic T-lymphocyte antigen, IL interleukin.

Compared to HVs, a significantly larger percentage of MDDCs from CHD patients expressed CD83 (50.06 ± 7.65% vs. 15.35 ± 19.74%, $P = 0.0016$) and CD40 (64.40 ± 3.67% vs. 37.36 ± 19.08%, $P = 0.025$). Similarly, CD40 was expressed on a larger percentage of MDDCs from EHHD patients as compared to HVs (58.98 ± 17.08% vs. 15.35 ± 19.74%, $P = 0.015$). However, there was no significant difference in the percentage of MDDCs expressing CD40 and CD83 between the different dialysis populations. Otherwise, MDDCs from all study cohorts expressed similar levels of MHC class II (HLA-DR), CD80, and CD86 molecules (Figure 7A-E).

We also determined the receptor density of co-stimulatory and MHC class II molecules on MDDCs by measuring the median fluorescence intensity (MFI) of each marker. The MFIs of CD80, CD86, CD83, and HLA-DR were comparable in HVs and HD patients (Figure 8).

Figure 7: CD83 and CD40 expression is increased on a larger percentage of MDDCs from CHD patients compared to EHHD patients and HVs. Monocytes were cultured with IL-4 and GM-CSF for 6 days to yield MDDCs, which were then terminally differentiated with 48 hours LPS treatment. Mature MDDCs from HVs (n=19), EHHD patients (n=8), and CHD patients (n=5) were stained with fluorochrome-conjugated antibodies against CD80 (A), CD86 (B), CD83 (C), CD40 (D), and HLA-DR (E). Expression of maturation markers was determined by flow cytometry and reported as percent positive cells. Bars and whiskers represent mean ± standard deviation (*$P < 0.05$, **$P < 0.001$).

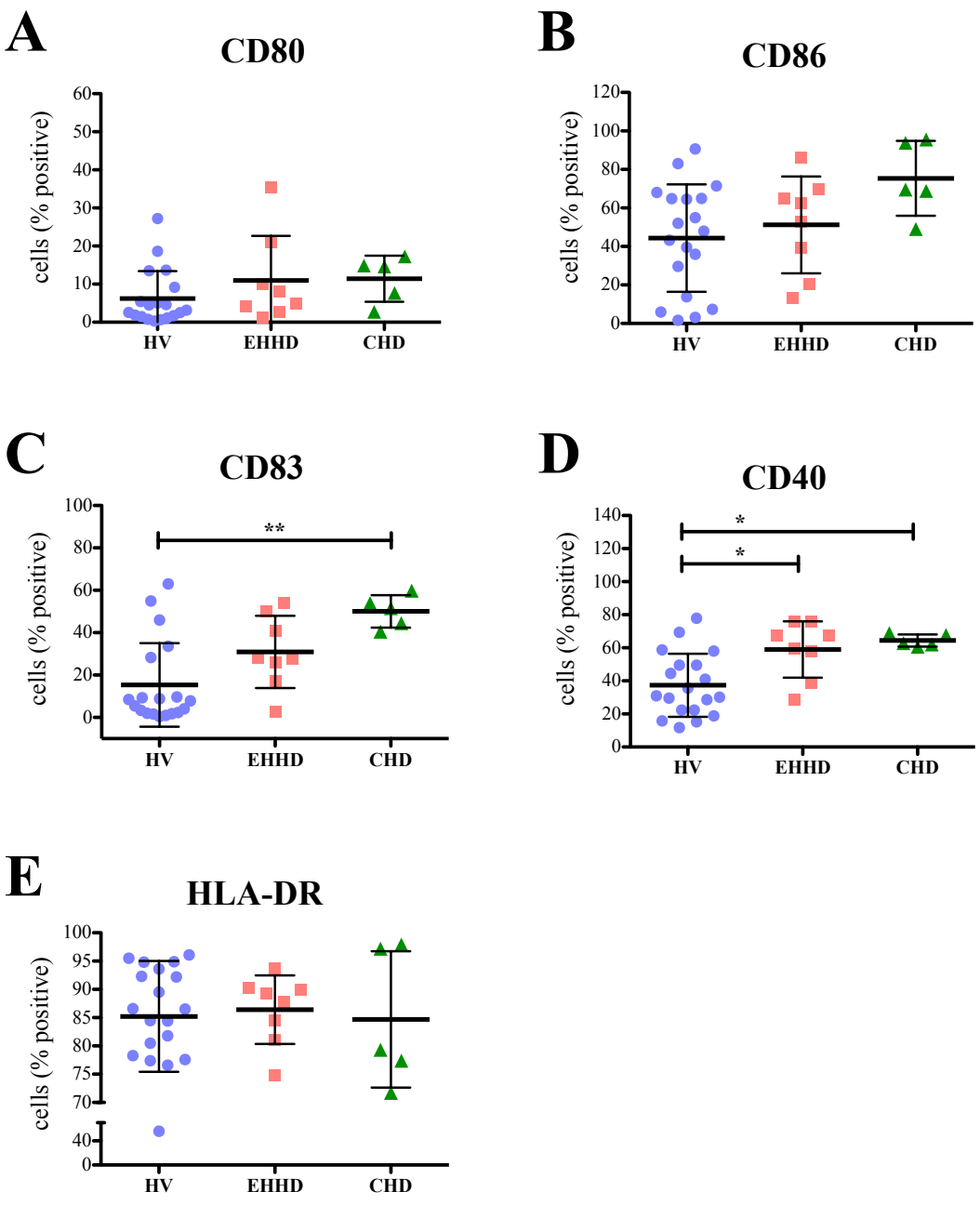
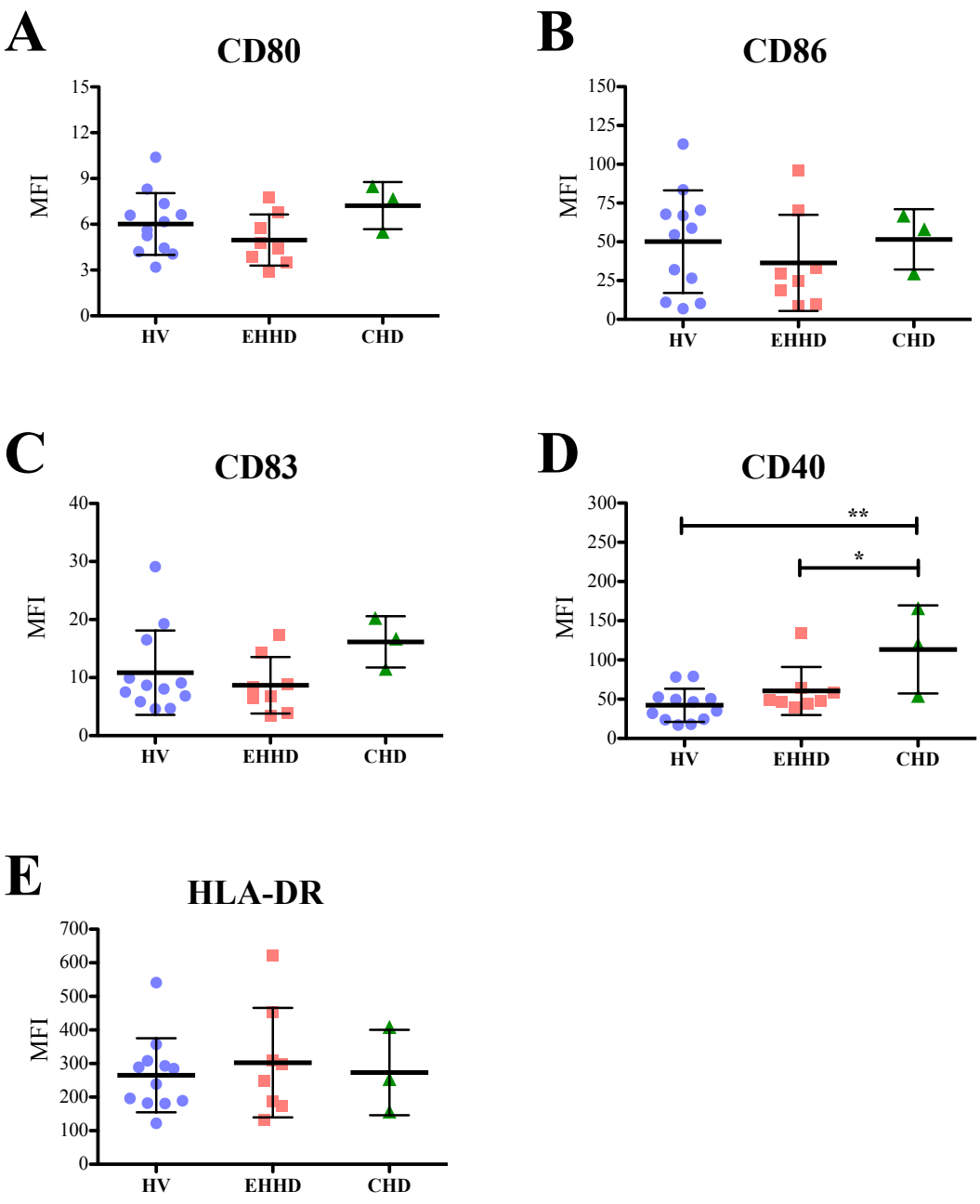

A
CD80
cells (% positive)
60
50
40
30
20
10
0
HV
EHHD
CHD
B
CD86
cells (% positive)
120
100
80
60
40
20
0
HV
EHHD
CHD
C
CD83
cells (% positive)
100
80
60
40
20
0
**
HV
EHHD
CHD
D
CD40
cells (% positive)
140
120
100
80
60
40
20
0
*
*
HV
EHHD
CHD
E
HLA-DR
cells (% positive)
100
95
90
85
80
75
70
40
0
HV
EHHD
CHD

Figure 8: CD40 receptor density is increased on MDDCs from CHD patients compared to EHHD patients and HVs. Monocytes were cultured with IL-4 and GM-CSF for 6 days to yield MDDCs, which were then terminally differentiated with 48 hours LPS treatment. Mature MDDCs from HVs (*n*=12), EHHD patients (*n*=8), and CHD patients (*n*=3) were stained with fluorochrome-conjugated antibodies against CD80 (A), CD86 (B), CD83 (C), CD40 (D), and HLA-DR (E). Expression of maturation markers was determined by flow cytometry and reported as median fluorescence intensity. Bars and whiskers represent mean ± standard deviation (*$P < 0.05$, **$P < 0.001$).


A
CD80
MFI
15
12
9
6
3
0
HV
EHHD
CHD
B
CD86
MFI
150
125
100
75
50
25
0
HV
EHHD
CHD
C
CD83
MFI
40
30
20
10
0
HV
EHHD
CHD
D
CD40
MFI
300
250
200
150
100
50
0
**
*
HV
EHHD
CHD
E
HLA-DR
MFI
700
600
500
400
300
200
100
0
HV
EHHD
CHD

However, CD40 expression was increased on MDDCs from CHD patients (113.5 ± 56.09 MFI) compared to both HVs (42.28 ± 21.17 MFI) and EHHD patients (60.55 ± 30.70 MFI) (Figure 8D). To ensure that MFI values were comparable between study participants, the flow cytometer was calibrated with fluorescent beads before each experiment (Table 6).

Table 6: Flow cytometer calibration with FlowSet™ Pro Fluorospheres

	HV (*n* = 6)	EHHD (*n* = 7)	CHD (*n* = 3)
FL1 (FITC, AF488)	113.2 (3.60%)	112.8 (6.79%)	113.0 (4.06%)
FL2 (PE)	249.7 (2.22%)	250.6 (4.82%)	251.0 (1.74%)
FL6 (PB)	76.3 (3.14%)	74.5 (4.87%)	74.6 (0.95%)
FL8 (APC)	82.3 (3.71%)	82.5 (4.16%)	82.9 (7.14%)

Data is shown as average MFI with CV (%). HV healthy volunteers, EHHD extended home hemodialysis, CHD conventional hemodialysis, MFI median fluorescence intensity, CV coefficient of variation, FL-1,2,6,8 photomultiplier tube detector and fluorochromes used.

3.4 Endocytic Capacity of MDDCs in HD Patients

3.4.1 MDDC FITC-Dextran Endocytosis Is Similar in All Study Participants

Immature MDDCs efficiently capture antigen via receptor-mediated endocytosis and/or macropinocytosis in order to survey the antigenic environment. In contrast, mature MDDCs capture antigen poorly and are more specialized for antigen presentation and T-cell activation. We tested the ability of immature and mature MDDCs from HVs and HD patients to uptake FITC-dextran from their culture media in order to determine whether dialysis therapy affects endocytic capacity of antigen presenting cells. We found no difference in the percentage of immature MDDCs engulfing FITC-dextran in HVs, EHHD patients, and CHD patients (22.18 ± 18.96% vs. 27.89 ± 13.12% vs. 30.50 ± 18.79% respectively) (Figure 9A).

Likewise, the MFI of engulfed FITC-dextran was comparable in all study participants (Figure 9A). Mature MDDCs were generally less endocytic compared to immature MDDCs, but there was no difference in FITC-dextran uptake between controls and HD patients (Figure 9B). Since we could not detect any significant differences in total FITC-dextran uptake between our study participants, we also looked at each individual's change in dextran uptake after LPS stimulation (immature vs. mature MDDC uptake). All study participants showed a similar decrease in dextran uptake after LPS stimulation and we could not detect any statistically significant differences in endocytosis between controls and HD patients (Figure 9C).

3.4.2 Expression of Endocytic Mannose Receptors Are Elevated in HD Patients

The macrophage mannose receptor (CD206) enables MDDCs to capture mannosylated protein antigens derived from many bacteria and fungi by receptor-mediated endocytosis. High expression of CD206 is a hallmark of immature MDDCs whereas low / no expression of this receptor is common on monocytes and mature dendritic cells. FITC-dextran is engulfed through the mannose receptor; thus, we measured dextran uptake in mature MDDCs (Figure 9B) as well as the expression of CD206 (Figure 10) to confirm the maturation status of MDDCs in HD patients. We found similar dextran uptake by mature MDDCs but, interestingly, a larger percentage of mature MDDCs tended to express CD206 in EHHD (51.01 ± 16.50%) and CHD (56.24 ± 10.46%) patients compared to controls (33.83 ± 20.88%). This trend, however, did not reach statistical significance. In addition, CD206 expression positively correlated with the percentage of mature MDDCs engulfing FITC-dextran ($P = 0.0001$) (Figure 11).

Figure 9: MDDC FITC-dextran endocytosis is similar in all study participants. Monocytes were cultured with IL-4 and GM-CSF for 6 days to yield MDDCs. The cells were either untreated (A) or terminally differentiated with 48 hours LPS treatment (B). FITC-dextran (1 μg/mL) was added to MDDC cultures for 1 hour and cells were stained with 7-AAD. Live MDDC FITC-dextran endocytosis was determined by percent positive cells (HV *n*=19, EHHD *n*=8, CHD *n*=5) and median fluorescence intensity (HV *n*=12, EHHD *n*=8, CHD *n*=3). We then calculated each individual's percent change in dextran uptake after LPS stimulation (immature vs. mature MDDC uptake) (C). Bars and whiskers represent mean ± standard deviation.

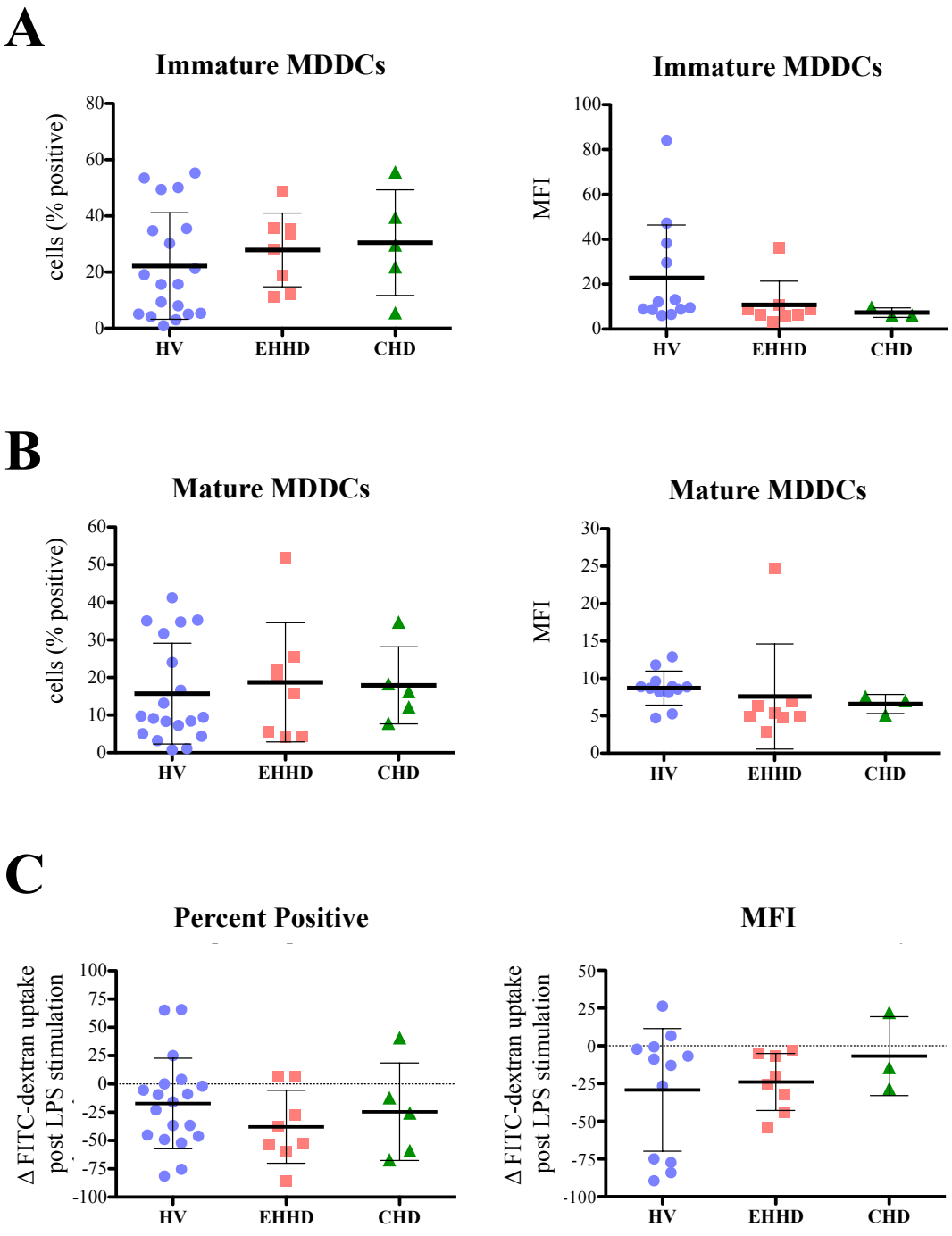

A
Immature MDDCs
cells (% positive)
80
60
40
20
0
HV
EHHD
CHD
Immature MDDCs
MFI
100
80
60
40
20
0
HV
EHHD
CHD
B
Mature MDDCs
cells (% positive)
60
50
40
30
20
10
0
HV
EHHD
CHD
Mature MDDCs
MFI
30
25
20
15
10
5
0
HV
EHHD
CHD
C
Percent Positive
Δ FITC-dextran uptake post LPS stimulation
100
75
50
25
0
-25
-50
-75
-100
HV
EHHD
CHD
MFI
Δ FITC-dextran uptake post LPS stimulation
50
25
0
-25
-50
-75
-100
HV
EHHD
CHD

Figure 10: CD206 expression is slightly higher on mature MDDCs from HD patients versus healthy controls. Monocytes were cultured with IL-4 and GM-CSF for 6 days to yield MDDCs, which were then terminally differentiated with 48 hours LPS treatment. Mature MDDCs were stained with fluorochrome-conjugated antibodies against CD206 and expression was measured by percent positive cells (A) (HV *n*=19, EHHD *n*=8, CHD *n*=5) and median fluorescence intensity (B) (HV *n*=12, EHHD *n*=8, CHD *n*=3). Bars and whiskers represent mean ± standard deviation.

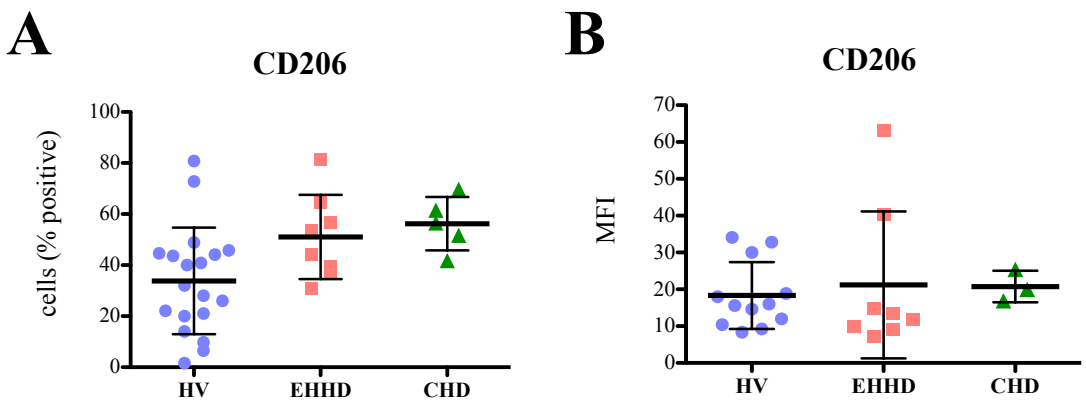
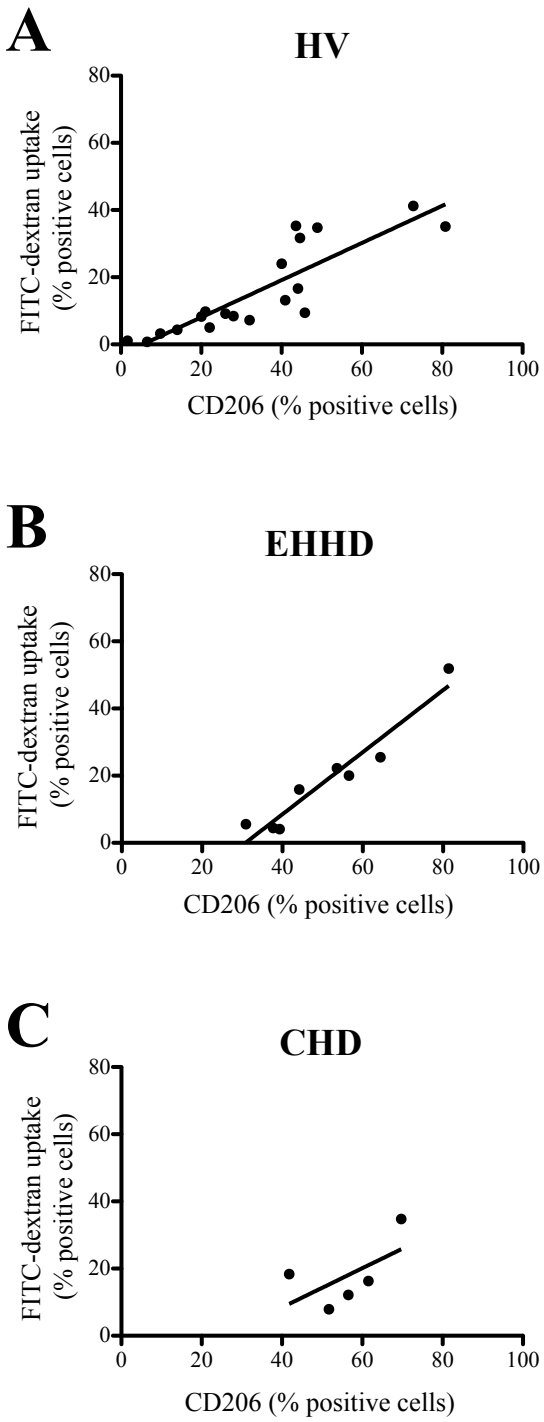

A
CD206
cells (% positive)
100
80
60
40
20
0
HV
EHHD
CHD
B
CD206
MFI
70
60
50
40
30
20
10
0
HV
EHHD
CHD

Figure 11: Expression of CD206 correlates with FITC-dextran uptake. Monocytes were cultured with IL-4 and GM-CSF for 6 days to yield MDDCs, which were then terminally differentiated with 48 hours LPS treatment. Mature MDDCs from HVs (*n*=19), EHHD (*n*=8), and CHD (*n*=5) patients were stained with fluorochrome-conjugated antibodies against CD206 or incubated with FITC-dextran (1 μg/mL) for 1 hour. For each study participant, the percentage of CD206-expressing MDDCs was plotted against the percentage of FITC-dextran positive cells. Data was analyzed by Spearman correlation (****$P = 0.0001$).


A
HV
FITC-dextran uptake (% positive cells)
CD206 (% positive cells)
B
EHHD
FITC-dextran uptake (% positive cells)
CD206 (% positive cells)
C
CHD
FITC-dextran uptake (% positive cells)
CD206 (% positive cells)

3.5 Activation and Proliferation of T-Lymphocytes in HD Patients

3.5.1 MDDC-Induced T-Lymphocyte Activation in Allogeneic MLRs Is Similar in Controls and HD Patients

Mature MDDCs are highly specialized for T-cell activation; thus, we tested the ability of these APCs to activate and induce proliferation of allogeneic T-lymphocytes in a mixed leukocyte reaction. T-cell proliferation was measured at various MDDC:T-cell ratios based on the dilution of CFSE fluorescence intensity (Figure 12). Cells that divided for one or more generations were defined as proliferating. The percentage of proliferating T-cell increased in proportion to the MDDC:T-cell ratio (Figure 13A). However, there was no difference in percent proliferating T-cells between study cohorts. The proliferation index, or the average division number per proliferating T-cell, was slightly higher in HVs (3.31 ± 0.40 divisions) compared to EHHD (3.11 ± 0.59 divisions) and CHD (2.81 ± 0.19 divisions) patients but it was not considered statistically significant (Figure 13B). Thus, MDDCs from controls and HD patients exhibited similar T-cell activation capacity.

3.5.2 T-Lymphocyte Proliferation Is Similar in Controls and HD Patients

Since MDDCs from HD patients were functionally similar to controls, we also assessed the proliferative capacity of T-lymphocytes from study participants. CFSE-labeled PBMCs were stimulated with serial dilutions of anti-CD3 antibodies and proliferation of live $CD3^+$ T-cells was measured by CFSE dilution (Figure 14). T-lymphocyte proliferation was extensive in all study participants and we could not detect any differences in the percentage of T-cells responding to anti-CD3 stimulation in controls versus HD patients (Figure 15A). The average number of T-cell divisions was marginally higher in EHHD (2.17 ± 0.53 divisions)

Figure 12: Representative histogram plots of T-cell proliferation in an allogeneic mixed leukocyte reaction. CFSE-labeled T-cells from one healthy donor were cultured for 5 days with LPS-matured MDDCs from study participants at various MDDC:T-cell ratios (indicated on graphs). Cells were then stained with fluorochrome-conjugated antibodies against CD3 and with the viability dye 7-AAD. Proliferation of live $CD3^+$ T-cells was assessed by CFSE dilution (filled histograms). Non-stimulated (NS) cells served as negative controls and PMA + ionomycin stimulated cells as positive controls for proliferation. Open histograms represent background autofluorescence.

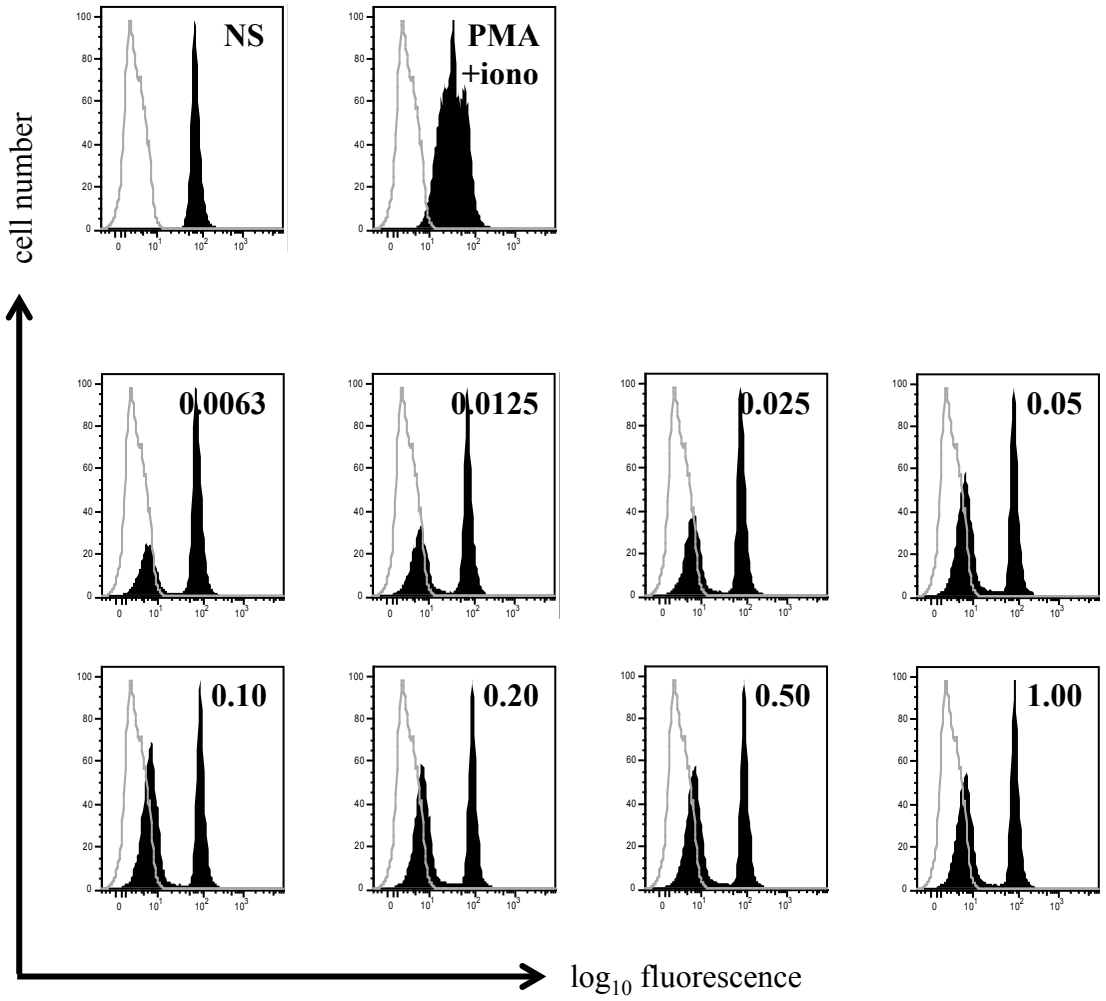
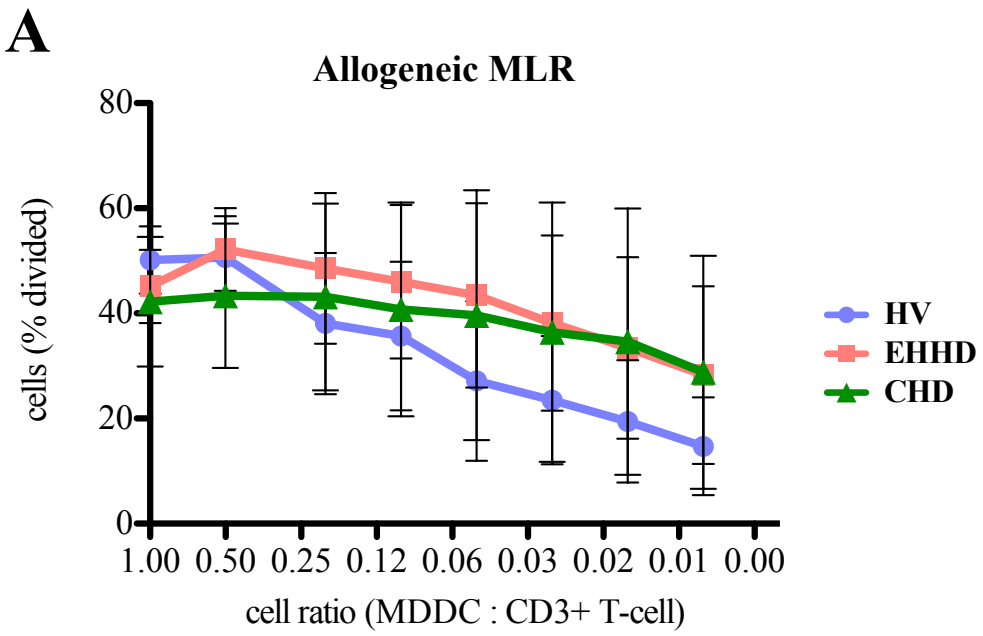

NS
PMA
+iono
0.0063
0.0125
0.025
0.05
0.10
0.20
0.50
1.00
cell number
log10 fluorescence

Figure 13: T-Lymphocyte activation in allogeneic MLRs is similar in controls and HD patients. CFSE-labeled T-cells from one healthy donor were cultured for 5 days with LPS-matured MDDCs from study participants at various MDDC:T-cell ratios. Cells were then stained with fluorochrome-conjugated antibodies against CD3 and with the viability dye 7-AAD. Proliferation of live $CD3^+$ T-cells was assessed by CFSE dilution. The percentage of dividing cells (one or more cells divisions) (A) and the proliferation index (average division number of proliferating cells) (B) were measured in HVs (*n*=14), EHHD (*n*=9), and CHD (*n*=5) patients.


A
Allogeneic MLR
80
60
40
20
0
cells (% divided)
1.00 0.50 0.25 0.12 0.06 0.03 0.02 0.01 0.00
cell ratio (MDDC : CD3+ T-cell)
HV
EHHD
CHD

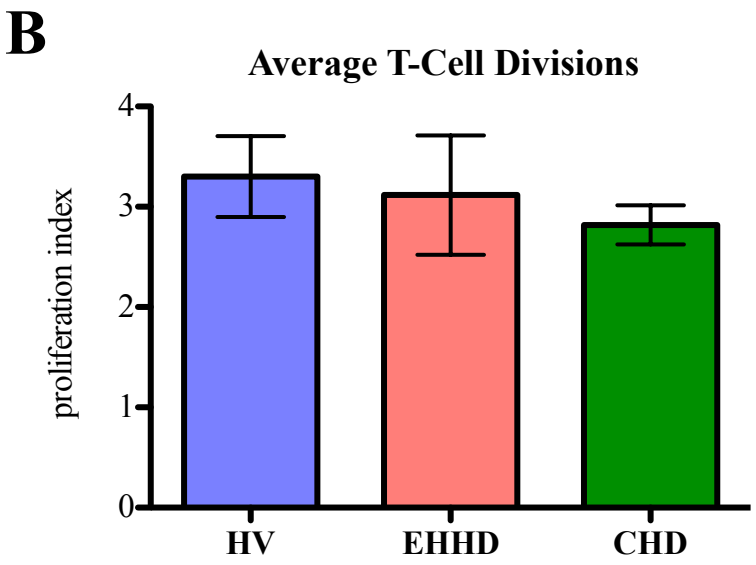

B
Average T-Cell Divisions
4
3
2
1
0
proliferation index
HV
EHHD
CHD

Figure 14: Representative histogram plots of T-lymphocyte proliferation in response to anti-CD3 stimulation. CFSE-labeled PBMCs from study participants were cultured for 3 days with serial dilutions of plate-bound anti-CD3 antibodies. Cells were then stained with fluorochrome-conjugated antibodies against CD3 and with the viability dye 7-AAD. Proliferation of live $CD3^+$ T-cells was assessed by CFSE dilution (filled histograms). Non-stimulated (NS) cells served as negative controls and PMA + ionomycin stimulated cells as positive controls for proliferation. Open histograms represent background autofluorescence.

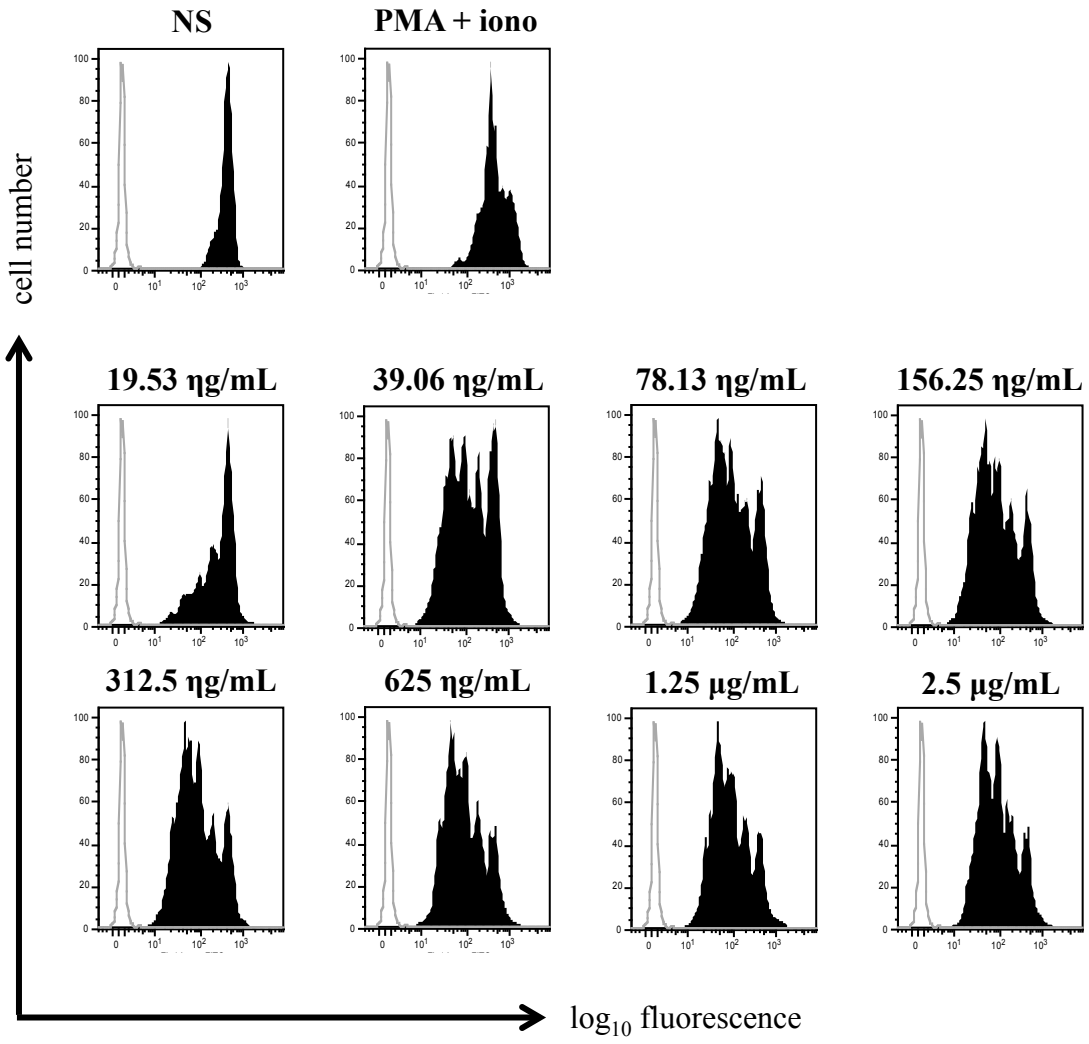
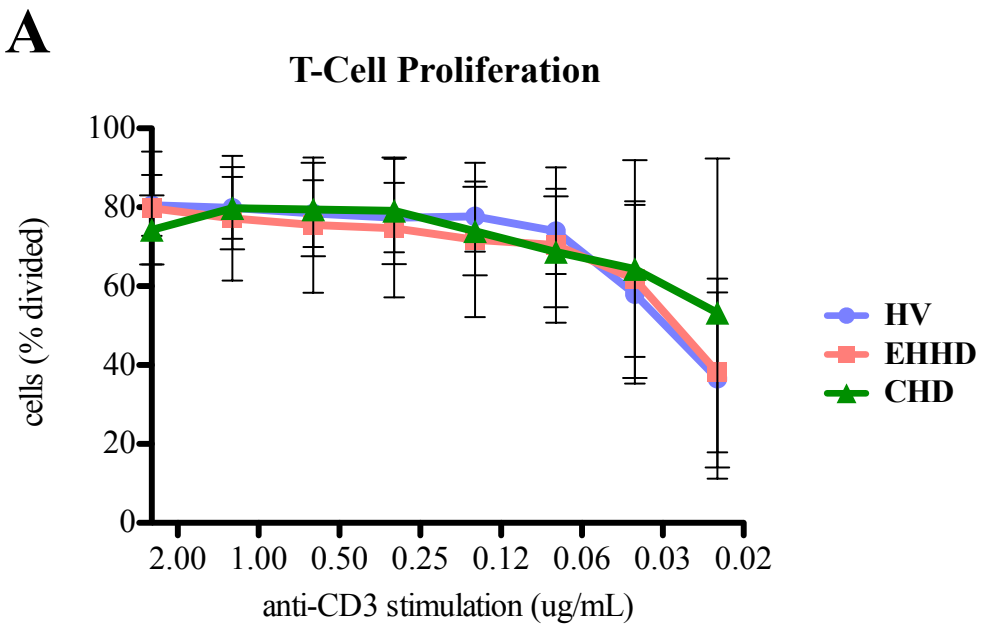
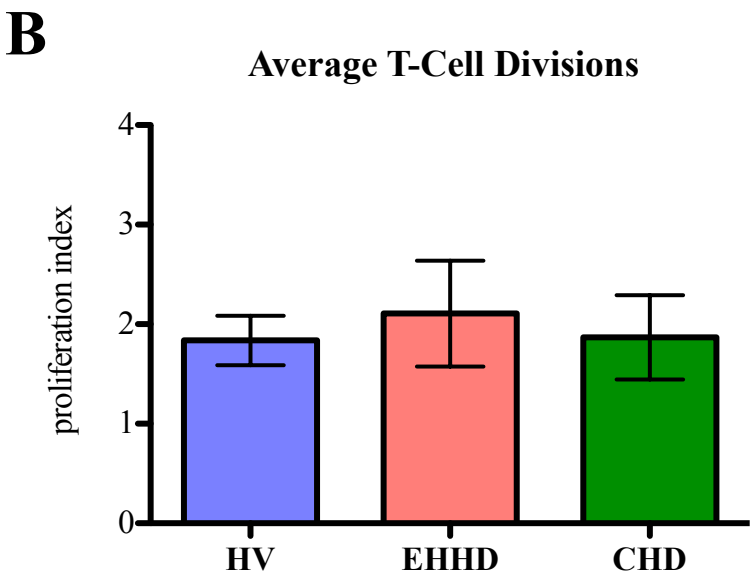

NS
PMA + iono
19.53 ηg/mL
39.06 ηg/mL
78.13 ηg/mL
156.25 ηg/mL
312.5 ηg/mL
625 ηg/mL
1.25 µg/mL
2.5 µg/mL
cell number
log10 fluorescence

Figure 15: T-Lymphocyte proliferation is similar in controls and HD patients. CFSE-labeled PBMCs from study participants were cultured for 3 days with serial dilutions of plate-bound anti-CD3 antibodies. Cells were then stained with fluorochrome-conjugated antibodies against CD3 and with the viability dye 7-AAD. Proliferation of live $CD3^+$ T-cells was assessed by CFSE dilution. The percentage of dividing cells (one or more cells divisions) (A) and the proliferation index (average division number of proliferating cells) (B) were measured in HVs (*n*=17), EHHD (*n*=9), and CHD (*n*=4) patients.


A
T-Cell Proliferation
cells (% divided)
100
80
60
40
20
0
2.00
1.00
0.50
0.25
0.12
0.06
0.03
0.02
anti-CD3 stimulation (ug/mL)
HV
EHHD
CHD


B
Average T-Cell Divisions
proliferation index
4
3
2
1
0
HV
EHHD
CHD

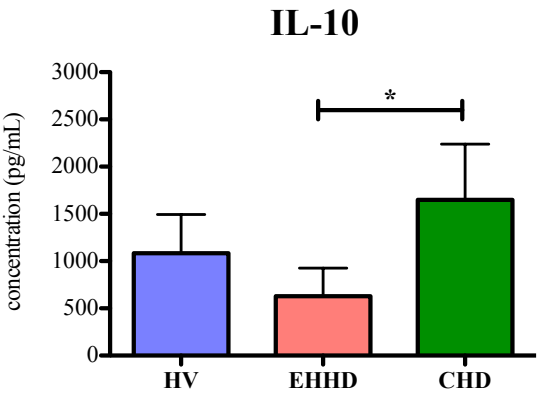
compared to CHD patients (1.87 ± 0.42 divisions) and HVs (1.83 ± 0.25 divisions) but this difference was not deemed statistically significant (Figure 15B).

3.5.3 T-Cells from CHD Patients Produce More IL-10 Compared to EHHD Patients

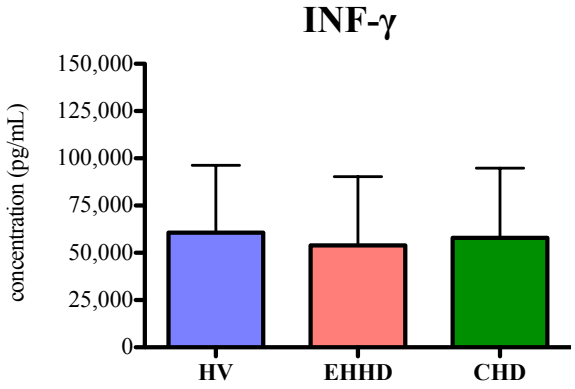
To determine the effector function of proliferating T-cells in HVs and HD patients, we measured levels of pro- and anti- inflammatory cytokines in PBMC cultures stimulated with anti-CD3 antibodies. T-lymphocytes from CHD patients produced significantly more IL-10 compared to EHHD patients (1648 ± 592 pg/mL versus 628 ± 296 pg/mL, P=0.0164) (Figure 16A). However, there was no difference in levels of INF-γ, predominantly produced by Th1 cells in response to intracellular viral and bacterial challenges, between HVs (60.6 ± 35.7 μg/mL), EHHD (53.9 ± 36.4 μg/mL), and CHD (57.9 ± 36.8 μg/mL) patients (Figure 16B).

Figure 16: T-lymphocyte INF-γ production is similar in all study participants but IL-10 production is increased in CHD patients. PBMCs from study participants were stimulated with anti-CD3 antibodies for 3 day to induce T-cell proliferation. Culture supernatants were reserved and ELISAs were used to measure concentrations of IL-10 (A) and INF-γ (B) in HVs (n=7), EHHD patients (n=4), and CHD patients (n=3). Bars and whiskers represent mean ± standard deviation (* $P < 0.05$).

A


IL-10
concentration (pg/mL)
3000
2500
2000
1500
1000
500
0
*
HV
EHHD
CHD

B


INF-γ
concentration (pg/mL)
150,000
125,000
100,000
75,000
50,000
25,000
0
HV
EHHD
CHD

4

DISCUSSION

4.1 Summary of Results

Chronic inflammation and functional immunodeficiency pose substantial problems for HD patients and contribute significantly to the high morbidity and mortality rates of these patients. While extended dialysis treatment improves many health aspects, it is unknown whether immune function is improved in patients choosing this type of dialysis modality. Therefore, we designed a matching-cohort clinical research trial to assess immune function in patients receiving longer more frequent hemodialysis at home. We measured the baseline serum markers of inflammation and the functional capacity of antigen presenting cells and T-lymphocytes in EHHD patients compared to healthy controls and conventional HD patients.

We found that both serum inflammatory and anti-inflammatory biomarkers were low in HVs and significantly increased in dialysis patients. IL-6, CRP, and IL-10 were also lower in EHHD compared to CHD patients although only CRP was significantly different between the two groups. In addition, we did not find many statistically significant differences in MDDC function between controls and HD patients. First, immature and mature MDDCs from HD patients engulfed similar amounts of FITC-dextran compared to HVs, and all study participants showed a comparable decrease in endocytosis following LPS maturation. Second, MDDC terminal differentiation in response to LPS was robust in controls and HD patients. These cells expressed high levels of co-stimulatory molecules and were considered phenotypically mature. However, the co-stimulatory markers CD83 and CD40 were significantly elevated in dialysis patients, which suggests that MDDCs from HD patients might be slightly pre-activated and more phenotypically mature compared to HVs. Third, mature MDDCs from HVs and HD patients both induced strong T-cell proliferation in allogeneic MLRs and no substantial differences were detected between the study cohorts. In addition, we did not find any major differences between MDDC phenotype and/or function in patients on conventional versus home hemodialysis. We were also unable to find any differences in T-lymphocyte proliferation in response to *in vitro* stimulation between the different study cohorts. Despite equal cellular proliferation, T-cells from conventional HD patients produced significantly greater amounts of immunosuppressive IL-10 compared to both EHHD patients and HVs. These results / trends are summarized in Table 7. The findings in bold represent statistically significant differences between HVs and HD patients.

Table 7: Summary of results and observed trends in HVs and HD patients.

	HV	EHHD	CHD
Serum biomarkers:			
Inflammatory	IL-6 (-) CRP (-)	**IL-6 (+)** CRP (+)	**IL-6 (++) CRP (++)**
Anti-inflammatory	IL-10 (-)	**IL-10 (+)**	**IL-10 (++)**
MDDC phenotype post LPS maturation:	$CD80^+$ / $CD86^+$ / $CD83^+$ / $CD40^+$ / $HLA\text{-}DR^{high}$	$CD80^+$ / $CD86^+$ / $CD83^+$ / **$CD40^{++}$** / $HLA\text{-}DR^{high}$	$CD80^+$ / $CD86^+$ / **$CD83^{++}$** / **$CD40^{high}$** / $HLA\text{-}DR^{high}$
Dextran endocytosis:			
Immature MDDCs	22% (++ uptake)	28% (+ uptake)	30% (low uptake)
Mature MDDCs	CD206 (+)	CD206 (++)	CD206 (+++)
MDDC dependent T-cell proliferation:	CFSE dilution (+) P. index (3.3)	CFSE dilution (++) P. index (3.1)	CFSE dilution (++) P. index (2.8)
T-cell proliferation:	CFSE dilution (++)	CFSE dilution (++)	CFSE dilution (++)
Cytokine production:	IL-10 (+) INF-γ (+)	IL-10 (+) INF-γ (+)	**IL-10 (++)** INF-γ (+)

HV healthy volunteer, EHHD extended home hemodialysis, CHD conventional hemodialysis, MDDC monocyte-derived dendritic cell, IL interleukin, CRP c-reactive protein, INF interferon, LPS lipopolysaccharide, HLA human leukocyte antigen, CD cluster of differentiation, CFSE carboxyfluorescein succinimidyl ester, P. index (average division number of proliferating cells).

4.2 Serum Inflammatory/Anti-Inflammatory Biomarkers

The most significant finding of this study was the change in serum inflammatory biomarkers in our study participants. As expected, levels of IL-6 and CRP were markedly elevated in CHD patients compared to healthy controls. Extended home dialysis therapy decreased concentrations of both molecules, although decreases in CRP levels were the most profound.

Inflammatory biomarkers are generally elevated in patients with renal failure and levels of IL-6 and CRP, in particular, are strongly correlated with poor outcomes in patients on

chronic intermittent hemodialysis (Danesh et al., 2004; Honda et al., 2006). Both of these acute-phase proteins are associated with chronic inflammation and are independent risk factors for cardiovascular disease, atherosclerotic disease and death (Honda et al., 2006). With results consistent with our own, Yuen *et al.* demonstrated that serum inflammatory biomarkers were lower in patients undergoing 6-8 hour nocturnal hemodialysis at home five times per week (Yuen et al., 2005b). IL-6 was significantly decreased in patients on nocturnal hemodialysis compared to those on conventional in-hospital hemodialysis. CRP was similarly decreased although it did not reach statistical significance. Because elevated levels of both IL-6 and CRP are associated with prolonged inflammation, cardiovascular stress and poor patient outcomes, decreases in inflammatory biomarkers may signify prospective health improvements in patients choosing longer more frequent dialysis therapy.

Elevated serum CRP levels are observed in patients with atherosclerosis, hypertension, aneurysms, and a number of other vascular diseases (Danesh et al., 2004). Excess C-reactive protein likely contributes to local inflammation and enhanced cardiovascular damage (Liu et al., 2010). CRP activates the immune system by binding to phosphocholine, which is expressed on the surface of dead/dying cells and on some bacterial cells. It subsequently activates the classical complement pathway and enhances phagocytosis of microbial and host ligands by interacting with Fc receptors on phagocytes. CRP also augments the synthesis of pro-inflammatory cytokines and chemokines through NFκB activation in immune cells and local endothelial cells (Liu et al., 2010). Furthermore, CRP levels are increased significantly by rises in IL-6 and LPS as well as mechanical stretch of vascular smooth muscle cells (Huang et al., 2009). CRP is, therefore, synthesized in response to stress and infection and further augments immune activation and local tissue inflammation.

Likewise, IL-6 is a pleiotropic cytokine produced by many cell types and, although it has both pro- and anti- inflammatory properties, high levels of this cytokine are most often associated with severe and/or chronic inflammatory conditions. It has been implicated in the disease progression of diabetes, atherosclerosis, Alzheimer's, rheumatoid arthritis, depression, and much more (Fulghesu et al., 2011; Gabay, 2006; Robak et al., 1998). IL-6 induces production of many cytokines and chemokines, aids in the recruitment of immune cells, and stimulates synthesis of CRP. Elevated levels of IL-6 correlate strongly with increased morbidity and mortality in HD patients. In fact, IL-6 was shown to be a stronger predictor of cardiovascular disease and death in this population than even C-reactive protein (Jug et al., 2009; Panichi et al., 2004).

It is unclear if lower levels of IL-6 and CRP in EHHD patients are a result of increased molecular clearance, decreased local/systemic synthesis, or both. But, in any case a reduction in serum inflammatory biomarkers likely diminishes inflammation in these patients and contributes to improved cardiovascular health. In accordance, many studies showed that extended dialysis therapy decreases left ventricular mass (associated with cardiac hypertrophy), increases survival and proliferation of vascular smooth muscle cells, stabilizes blood pressure, and ameliorates anemia (Walsh et al., 2005). Furthermore, in a controlled cohort study, Bergman *et al*. showed that conversion to nocturnal home hemodialysis reduced cardiovascular-related hospital admissions (Bergman et al., 2008). The observed decrease in serum inflammatory biomarkers in our study is consistent with other findings in the literature and with our predicted health improvements in the extended home hemodialysis population.

We also found that serum anti-inflammatory markers are elevated in HD patients compared to healthy controls. IL-10 levels followed the same trend as IL-6 and CRP and were almost undetectable in controls and slightly elevated in CHD versus EHHD patients. Agrawal *et al.* and Verkade *et al.* also found similar results. In both studies, IL-10 concentrations were low in healthy volunteers and elevated in patients on chronic intermittent hemodialysis (Agrawal et al., 2010; Verkade et al., 2007b). However, very little is known about IL-10 levels in patients on extended / nocturnal hemodialysis and no studies have currently reported changes in concentrations of serum anti-inflammatory markers in these patients. In our study, IL-10 levels in CHD and EHHD patients were not significantly different but tended to be slightly lower in EHHD patients most likely due to enhanced clearance of molecules in longer dialysis runs.

IL-10 is mostly produced by monocytes and lymphocytes and has several anti-inflammatory properties. First, IL-10 decreases transcription of pro-inflammatory cytokines (Brunet et al., 1998). Interestingly, Kim *et al*. showed that, despite elevated basal inflammation in HD patients, the fold-increase in monocyte cytokine production before and after LPS treatment was much more robust in healthy controls than in dialysis patients (Kim et al., 2011). Second, IL-10 down-regulates the expression of T_H1 cytokines and dampens cellular immunity while promoting antibody production. The poor immunization success in HD patients is often attributed to defects in cell-mediated immunity and T-lymphocyte function (Bauer and Jilg, 2006). Satisfactory antibody titers to vaccines like diphtheria, tetanus, and influenza can be achieved in HD patients but these responses wane significantly over time without strong memory T-cells. Thus, elevated levels of IL-10 in conventional HD patients may partially explain some of the immune dysfunctions in this population. Extended home

hemodialysis, on the other hand, may improve immune function as a result of increased clearance of excess pro- and anti- inflammatory markers, which can cause several perturbations of the immune system.

4.3 Monocyte-Derived Dendritic Cells

Like other antigen presenting cells, MDDCs have relatively complex functions that change based on their maturation stage. Immature dendritic cells function as sentinels that survey the antigenic environment and alert lymphocytes of changes. They reside in non-lymphoid tissues where they uptake and process considerable amounts of antigen for presentation. Signaling through pattern-recognition receptors (TLR, NOD, etc.) and/or cytokine receptors induce the maturation of dendritic cells. Mature dendritic cells migrate to the lymph nodes and function as potent activators of lymphocytes. They lose their antigen capturing capacity, express co-stimulatory molecules, and produce cytokines to ensure strong activation of leukocytes. Mature MDDCs are terminally differentiated and have a limited lifespan after interacting with lymphocytes in order to prevent excessive immune activation (Bertho et al., 2002).

In this study, we found few differences in MDDC function between controls and HD patients. We incubated immature MDDCs with FITC-dextran and found that the percentage of cells engulfing dextran was similar in HD patients and healthy controls. Lim *et al.* reported a decrease in FITC-dextran uptake in dialysis patients and further showed that culturing healthy MDDCs in uremic serum decreased their endocytic capacity (Lim et al., 2007). However, other studies found that immature MDDCs from controls and HD patients

were able to uptake similar amounts of antigen, which is consistent with our results (Verkade et al., 2007b). However these studies used FITC-albumin, which is internalized by macropinocytosis, compared to FITC-dextran, which is internalized by both macropinocytosis and receptor-mediated endocytosis (Sallusto et al., 1995). At this time, it is unclear whether MDDC-mediated antigen uptake (by either endocytosis and/or macropinocytosis) is different in healthy individuals and the dialysis population.

Recently, Verkade *et al.* found that MDDCs from HD patients continue to uptake antigen even after maturation (Verkade et al., 2007b). We found similar endocytosis in controls and HD patients but slightly higher expression of CD206 in conventional and extended HD patients. FITC-dextran is internalized through the mannose receptor (Sallusto et al., 1995) and CD206 expression on MDDCs correlated with an increased percentage of FITC-positive cells. These results suggest that mature MDDCs from HD patients may retain their endocytic capacity due to the expression of the mannose-macrophage receptor.

Several studies have reported impaired MDDC maturation in HD patients. Lim *et al.* showed that MDDCs from dialysis patients express fewer co-stimulatory molecules, which are necessary for strong T-cell activation (Lim et al., 2007). Furthermore, they showed that expression of these co-stimulatory molecules was severely impaired when cells were cultured in medium containing 10% uremic serum. They also found similar results when cells were cultured with urea, although impaired MDDC maturation was only significant at the highest urea concentration (40 mmol/L). Similarly, Verkade *et al.* found that CD86 expression was significantly lower on MDDCs from HD patients that were non-responders to the hepatitis B vaccine (Verkade et al., 2007a). T-cell activation depends on the interaction

with antigen presenting cells; thus, a decrease in the surface expression of co-stimulatory molecules can contribute to impaired immune function in HD patients.

We did not see impaired MDDC maturation in our study participants. On the contrary, a higher percentage of LPS-matured MDDCs from HD patients expressed the co-stimulatory molecules CD83 and CD40 as compared to controls. The receptor densities (determined by median fluorescence intensity) of these molecules were also elevated in HD patients. No differences were seen in expression of HLA-DR, CD80 and CD 86 between cohorts. CD83 and CD40, which are important for activation and differentiation of T-cells, were especially elevated in CHD patients compared to EHHD patients and HVs. This mature MDDC phenotype is not entirely unexpected in dialysis patients because cytokines, like LPS and CD40L, induce MDDC terminal differentiation *in vitro.* The elevated serum cytokine levels in HD patients likely contribute to the mature MDDC phenotype we observed.

Our results might differ from previous studies because of experimental differences. For instance, we supplemented our culture media with 5% sterile-filtered fetal calf serum while other studies chose 10% heat-inactivated AB^+ pooled human serum. It is possible that FCS contains additional cytokines and/or molecules that cause activation of MDDCs. Despite these differences our experimental protocols are valid since MDDCs can be cultured in a variety of ways. However, this also suggests that experimental differences can contribute significantly to the overall phenotype of these cells. Several other studies by Choi *et al.* and Agrawal *et al.* were unable to detect differences in MDDC maturation in controls versus HD patients (Agrawal et al., 2010; Choi et al., 2011). Thus, there is no current consensus in the literature regarding terminal differentiation of human MDDCs. Our data suggests that

MDDCs from dialysis patients exhibit a mature phenotype when treated with LPS, although the effect of dialysis modality on dendritic cell maturation was not clear.

Consistent with the observed mature phenotype, MDDCs from CHD patients, EHHD patients, and HVs induced strong T-cell proliferation our allogeneic mixed leukocyte reactions. However, Lim *et al*. noted that allogeneic T-cell proliferation was increased in HD patients and uremic culture media (Lim et al., 2007). They attributed this high MDDC activation capacity to increased IL-12 production. Thus, elevated levels of IL-12 in uremic cultures may enhance overall T-cell proliferation, even if uremic MDDCs express fewer co-stimulatory molecules.

However, other studies by Verkade *et al.* and Girndt *et al.* found impaired MDDC maturation in HD patients and decreased allogeneic T-lymphocyte proliferation in mixed leukocyte reactions (Girndt et al., 2001; Verkade et al., 2007b). These results are difficult to compare to our work because of differences in experimental technique. First, those studies used traditional allogeneic MLRs, which consist of co-culturing PMBCs from two different donors. We, on the other hand, cultured purified T-cells from one healthy donor with purified MDDCs from study participants. In our assay, we directly tested the activation capacity of MDDCs because no other cells were present to induce T-cell proliferation. Second, we used CFSE dilution and flow cytometry to determine T-cell proliferation instead of the tritiated thymidine assay. Both are acceptable assays for cell proliferation but one measures the dilution of covalently bound intracellular fluorescent molecules whereas the other measures the incorporation of radioactive nucleotides in newly synthesized cellular DNA. Thus, they are very different assays that measure cellular proliferation in distinct ways. Our results are

not directly comparable with other studies to date. However, they are consistent with the mature MDDC phenotype and elevated cytokine levels measured in our study participants.

4.4 T-Lymphocytes

T-lymphocytes are the main effector cells of the adaptive immune system. T-cell subsets can kill abnormal or infected cells, induce immunologic tolerance, or activate other immune cells like macrophages and B-lymphocytes. A strong T-cell response is essential for cell-mediated immunity as well as for antibody production and long-term immunologic memory. Thus, we measured T-cell proliferation in response to *in vitro* stimulation and T-cell cytokine production. We found a high degree of T-cell proliferation in all study cohorts. The proliferative capacity of T-lymphocytes was not compromised in either CHD or EHHD patients. In contrast, Verkade *et al.* found that T-cell proliferation in the presence of autologous MDDCs and recall antigen (*tetanus toxoid* and *Candida albicans*) was significantly reduced in HD patients (Verkade et al., 2007a). However, these experiments differed from our own by measuring proliferation of memory T-cells, thus inferring prior antigenic exposure to Tetanus toxoid and Candida albicans by the patients. It was not clear whether these patients had previously been vaccinated or exposed to these "recall" antigen. This is especially important considering that HD patients have poor immunization success to tetanus. Hence, it is not altogether surprising that memory T-cells in HD patients showed a low proliferative capacity in response to these antigen.

We stimulated PBMCs with plate-bound anti-CD3 antibodies to determine T-cell proliferation. The T-cell receptor recognizes specific antigen presented in the context of

MHC molecules but requires additional CD3 interactions to induce a signaling cascade. Anti-CD3 antibodies and co-stimulation from autologous APCs engage the T-cell receptor directly to induce cell proliferation. Thus, our stimulation was non-specific and capable of activating both naïve and memory T-lymphocytes. Our results indicate that T-cells from HD patients have a similar proliferative capacity to those from healthy controls. However, we cannot dismiss the possibility that certain impairments may exist in different T-cell subsets.

In our study, proliferating T-cells from all study cohorts produced similar levels of INF-γ. INF-γ has many anti-viral properties and promotes cellular immunity by enhancing T_H1 differentiation. Although no defects in INF-γ production were observed, T-cells from CHD patients did produce significantly more immunosuppressive IL-10 compared to EHHD patients and HVs. Previously, Lisowska *et al.* found that PBMCs from dialysis patients produced more IL-10 compared to healthy controls after stimulation with anti-CD3 antibodies (Lisowska et al., 2013). They also showed that recombinant human erythropoietin, which is prescribed to many dialysis patients for the treatment of anemia, contributed to the rise in IL-10 production. However, there may not be a difference in erythropoietin use between CHD and EHHD patients. A recent retrospective clinical study by the Frequent Hemodialysis Network (FHN) found that patients on nocturnal and conventional daily hemodialysis required similar doses of erythropoietin stimulating agents to achieve target hemoglobin levels (Ornt et al., 2013). Therefore, further investigation is necessary to determine why IL-10 levels were significantly elevated in our CHD versus EHHD patients. There may be less clearance of IL-10 in CHD versus EHHD patients. Nevertheless, increased levels of immunosuppressive cytokines, such as IL-10, might contribute to impaired cellular immunity in HD patients. Ratios of T_H1:T_H2 $CD4^+$ T-cell subsets (and newer subsets

including T_H17 and regulatory T cells) and their contributions to immunity in dialysis still need to be clarified.

4.5 Study Limitations and Confounding Factors

The low number of recruited patients was a major limitation in our study. All patients were recruited from The Ottawa Hospital Nephrology Program and only a small percentage of these individuals were on home dialysis modality. The small home dialysis population plus the extensive eligibility and exclusion criteria for this study severely limited the numbers of patients recruited into the different study cohorts. In addition, matching CHD patients to EHHD patients was difficult due to the inherent differences in these two populations. In general, EHHD patients are younger, more independent, and have less co-morbidity, which allows them to comply with this form of self-administered dialysis.

Like all clinical trials, our study had several confounding variables. First, the patients were not randomized to their respective dialysis modality. Patients were only assigned to extended home hemodialysis if they were medically eligible for this form of treatment and/or if they had a personal preference for this modality. Therefore, EHHD represents a more select patient population that may differ in health and overall wellbeing from CHD patients. Despite attempting to match EHHD and CHD patients to minimize any differences not attributed to dialysis modality, residual confounding variables may still exist. In addition, patients on home dialysis often transition from another dialysis modality – either conventional HD or peritoneal dialysis. On average, the EHHD patients in our study had

been diagnosed with CKD and were on dialysis for a longer time period than CHD patients. This factor may limit any potential difference in immunity between CHD and EHHD.

Second, we did not control for any lifestyle variables such as diet, exercise, or obesity. Many studies demonstrated a link between the pro-inflammatory effects of adipose tissue and poor patients outcomes (Coresh et al., 2007; Nteeba et al., 2013). But on the other hand, a larger body size with more muscle mass has also been associated with a higher survival rate for patients receiving long-term HD (Kalantar-Zadeh et al., 2010).

Lastly, levels of vitamin D (calcitriol), erythropoietin (EPO), parathyroid hormone (PTH), and other hormones with immune modulatory properties can confound our study. Vitamin D deficiency was previously linked to cardiovascular disease and early mortality in dialysis patients as well as increased inflammation (Inaguma et al., 2008; Levin et al., 2013; Ojaimi et al., 2013). Vitamin D supplements are recommended for most dialysis patients but supra-physiologic or constant administration of calcitriol can also cause immune suppression and impaired T-lymphocyte proliferation (Cantor, 2009; Teng et al., 2003; Zarrabeitia et al., 1990). Likewise, recombinant human EPO improves anemia and reduced the need for blood transfusions in dialysis patients but affects both lymphocyte proliferation and cytokine production (Barany et al., 1992; Jungers et al., 2001; Steffensen et al., 1996). Over secretion of parathyroid hormone due to vitamin D deficiency also causes several immune impairments in CKD (Ori et al., 1999). For example, elevated PTH levels increase intracellular calcium and decrease activation of T-cells (Alexiewicz et al., 1996; Ori et al., 1999). Ca^{2+} influx into the cell is necessary for T-cell activation / proliferation; thus, elevated

levels of cytosolic calcium affect normal cellular processes and impair overall immune responses.

As noted, several uncontrolled variables that modulate immune function exist in our study and might confound the results presented in this thesis. A larger clinical trial would allow us to control for some of these confounding variables through multivariate statistical analysis, although all observational clinical trials are subject to residual confounding. Since EHHD patients are few in numbers and highly selected by modality choice, a larger randomized clinical trial would not be feasible.

Despite the known risk of confounding, our results do indicate that differences in immune function exist between CHD and EHHD patients. But because of our study design, we cannot definitively conclude that these differences are entirely due to dialysis modality.

5

CONCLUSION

5.1 Proposed Model of Immune Dysfunctions in CHD and EHHD Patients

In this study, we hypothesized that extended dialysis therapy improves immune function in patients with end-stage renal disease. We used serum pro- and anti- inflammatory markers as indications of overall health status in dialysis patients. Serum inflammatory markers are strong clinical predictors of morbidity / mortality and it is reasonable to assume that changes of these markers reflect global health improvements. Our results indicate that CRP levels are decreased in extended home hemodialysis. This decrease correlates with previously reported improvements in cardiovascular health and overall quality of life.

Serum pro- and anti- inflammatory markers also have a direct impact on overall immunity. An inflammatory environment is typical in dialysis patients because cytokines accumulate as a result of decreased renal clearance and increased cellular activation triggered by the biologic activity of uremic toxins, dialysis membranes, potential exposure to endotoxin from dialysate, and osmotic changes caused by solute and fluid retention. A high concentration of pro-inflammatory cytokines leads to pre-activation of immune cells and has significant impacts on immunity. In our study, immature dendritic cells had a similar endocytic capacity compared to healthy controls while LPS-matured dendritic cells expressed equally high levels of co-stimulatory molecules and induced strong T-cell proliferation. However, we noticed a trend towards pre-activated dendritic cells in HD patients because these expressed the highest levels of the co-stimulatory molecules CD83 and CD40. In addition, activated dendritic cells synthesize more cytokines and augment the existing inflammatory environment. Although we did not notice statistically significant differences between the dialysis modalities, the observations that dendritic cells from CHD patients tended to be the most mature and pro-inflammatory markers were highest in this population suggest that pre-activation may cause immune dysfunctions. Extended hemodialysis might improve immunity by decreasing levels of pro-inflammatory cytokines.

Clinical observations and laboratory investigations suggest that T-lymphocytes and cell-mediated immunity are the most severely affected branches of the immune system in dialysis patients. Cell-mediated immunity involves activation of T_H1 cells, phagocytes and cytotoxic T-cells for direct clearance of pathogens. Although we did not find any differences in T-cell proliferation, serum anti-inflammatory markers and T-cell IL-10 synthesis were elevated in CHD patients. IL-10 dampens immune responses and decreases cell-mediated immunity. It

inhibits NFκB activation and decreases the expression of INF-γ and IL-2, which are essential for the growth and differentiation of T_H1 cells and overall viral immunity. Patients on EHHD have lower levels of this immunosuppressive cytokine and, as a result, may have improved immunity.

Overall, it is possible that extended home hemodialysis therapy improves immunity by increasing the clearance of both pro- and anti- inflammatory cytokines. A healthy immune response constitutes a balance between immune activation and suppression. Abnormal cell function and decreased immunity is likely the product of cytokine accumulation and/or excess solutes / fluids that exert biologic functions on immune cells. Extended dialysis therapy removes solutes more efficiently and creates a more physiologic environment, which can enhance overall immune function in patients with renal failure.

5.2 Clinical Significance of Study

Cardiovascular disease and infections are the leading causes of death in dialysis patients. Both are associated with increased solute / fluid retention and elevated cytokine levels. Therefore, hemodialysis modalities that create a more physiologic environment will improve patient outcomes by decreasing the risk of developing cardiovascular disease and improving immunity. Extended home hemodialysis has already been shown to significantly improve solute clearance, stabilize blood pressure, decrease hospitalizations, and improve quality of life (Walsh et al., 2005). In this study, we showed a decrease in serum pro- and anti-inflammatory markers as well as trends towards improved immune cell functions. Additional patient recruitment is necessary to confirm these results and many questions regarding

immunity in this population still need to be addressed. However, these results are encouraging and point towards improved immune function in patients on extended dialysis therapy. Extended home hemodialysis may not be appropriate for all patients with renal failure but improved immunity, fewer cardiovascular complications, and a better quality of life are compelling reasons to raise awareness about this form of hemodialysis for eligible patients.

5.3 Future Directions

It is widely accepted that chronic inflammation plays a key role in morbidity and mortality rates in dialysis patients. However, the underlying mechanism leading to this inflammatory environment is still unclear. Better patient outcomes will likely depend on a good understanding of the "uremic" environment and its cellular / systemic effects. As a future direction, we would like to explore the mechanism leading to chronic inflammation as well as how extended dialysis therapy contributes to the decease in serum inflammatory markers.

Cytokines might accumulate in dialysis patients as a result of decreased renal clearance of these molecules. However, proteomic urine and dialysate fluid analyses indicate that the native kidneys and dialysis machines do not play a substantial role in their direct removal (Molina et al., 2005; Rossing et al., 2008). Cytokines, like other signaling molecules, are likely degraded rapidly by the liver or recycled by cells after they have fulfilled their biologic function to prevent aberrant signaling (Tarakcioglu et al., 2003). Hence, an inflammatory environment in dialysis patients suggests continuous cellular activation and production of cytokines. It was hypothesized that increased exposure to synthetic membranes and dialysate

fluid cause cellular activation (Urbani et al., 2012) but in our study we did not notice increased inflammation in EHHD patients, who, on average, were exposed to double the amount of weekly dialysis compared to CHD patients. Therefore, cellular activation might be caused predominantly by solute and fluid retention. The European Uremic Toxin Work Group (EUTox) identified over 95 "uremic toxins" in CKD patients and many more remain unidentified (Neirynck et al., 2013). These retention solutes are significantly elevated in patients with renal failure compared to healthy controls and include a variety of small water-soluble compounds (MW <500 Da), protein-bound solutes, and middle molecules (MW > 500 Da) (Vanholder et al., 2003). It is currently unclear to what extent these molecules exert biological / biochemical activity and contribute to the uremic syndrome. Extended dialysis therapy removes solutes more efficiently and might decrease inflammation through the removal of uremic toxins, although this hypothesis remains to be addressed.

In addition, fluid volume overload and hyperosmotic stress might enhance cellular activation in dialysis patients (Paniagua et al., 2010; Vicente-Martinez et al., 2004). Cells respond to osmotic stress by accumulating compatible organic osmolytes within the cytoplasm. Renal medullary cells, which are often exposed to hypertonic environments during urine formation, increase their intracellular concentrations of sorbitol, betaine, inositol, taurine, and glycerophosphocholine via activation of the transcription factor NFAT5 (nuclear factor of activated T-cells) (Lopez-Rodriguez et al., 2001). It appears that immune cells respond to hypertonicity in a similar manner and NFAT5 activation contributes to increased cellular activation (Go et al., 2004). Daily hemodialysis therapy maintains a more stable fluid volume environment and decreases fluctuations in body weight between dialysis runs. It is

reasonable to hypothesize that extended dialysis therapy might reduce inflammation by decreasing osmotic stress in patients with renal failure.

Our immediate short-term goal is to recruit additional patients into our clinical trial in order to strengthen our conclusions. We will then begin to examine the role of chronic inflammation in immune cell dysfunction.

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CONTRIBUTIONS OF COLLEAGUES

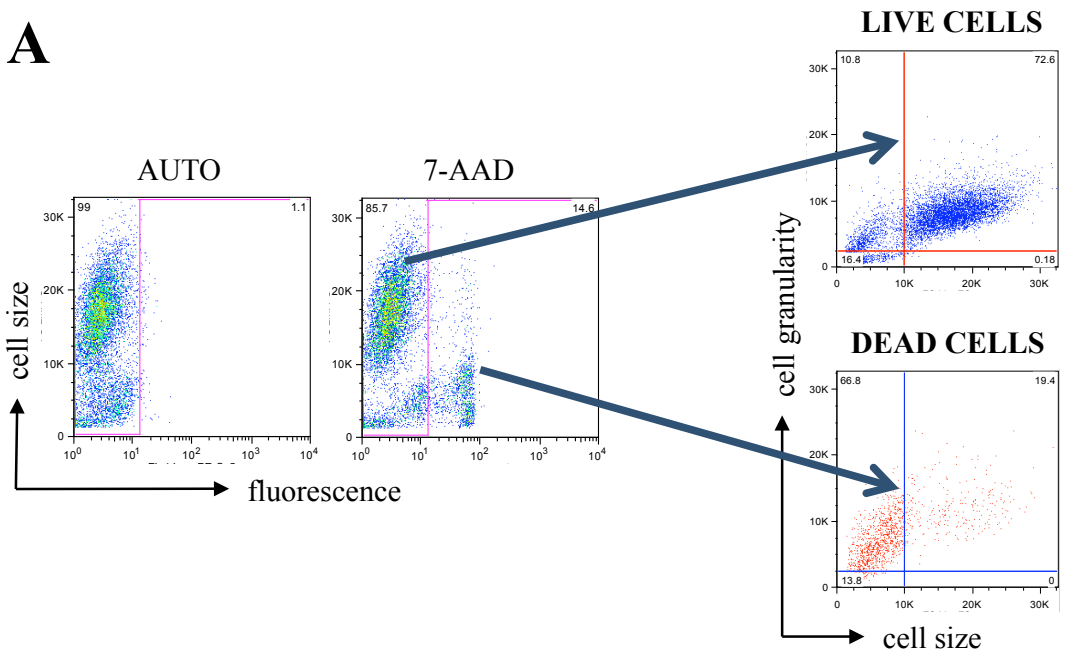
Drs. Todd Fairhead, Deborah Zimmerman, and Greg Knoll of The Ottawa Hospital Nephrology Program devised the initial concepts of this clinical study in accordance with The Ottawa Hospital Research Ethics Board.

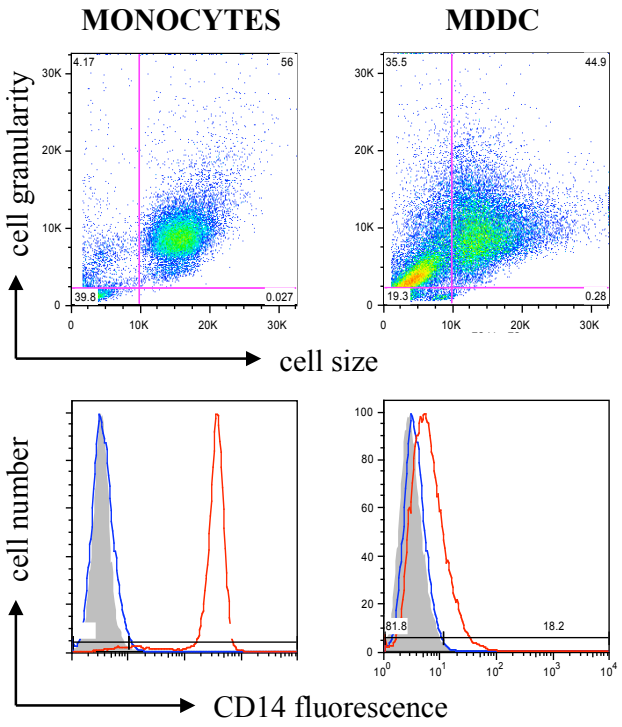
Jessica Wagner, Edita Delic, and Dr. Todd Fairhead screened, matched, and obtained written informed consent from all the EHHD and CHD patients recruited into this study while Hemodialysis Unit nurses completed the blood draws for these patients.

Judy Cheeseman, Valerie Cronin, and Dr. Todd Fairhead recruited, obtained written informed consent, and performed the blood draws for all the healthy volunteers in this study.

Andreea Slatculescu developed, performed, analyzed, and interpreted all the laboratory experiments in this study with approval and guidance from Dr. Todd Fairhead. She is also the sole author of this thesis.

APPENDIX I





MDDC Gating Strategy:

A) The MDDC gate was set based on forward scatter (size) and side (granularity) scatter parameters. Viability staining with 7-AAD was used to confirm this gate.

B) Freshly isolated monocytes and MDDCs were stained with FITC-conjugated anti-CD14 antibodies for confirmation of gating strategy and MDDC differentiation.

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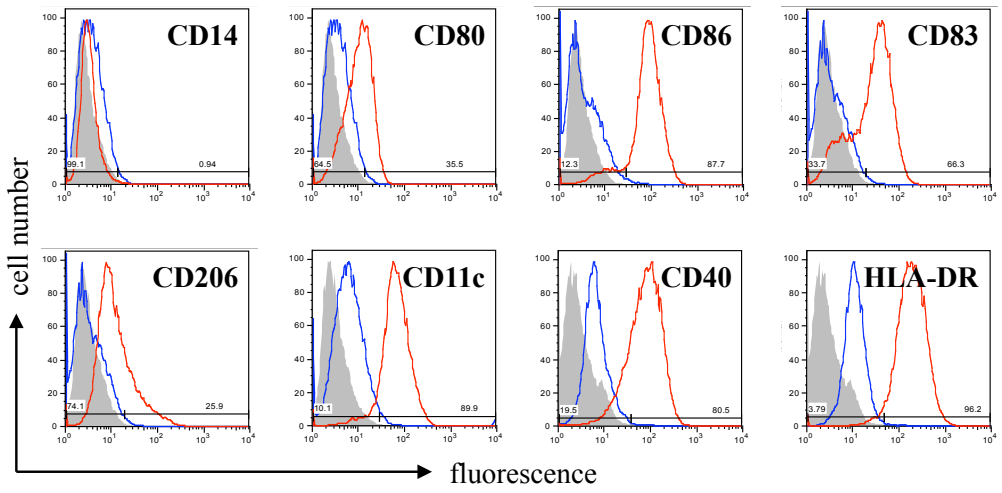
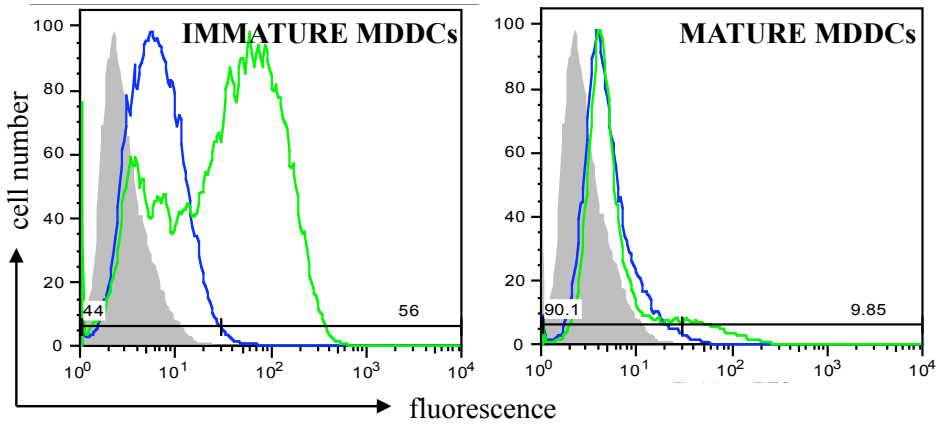
C



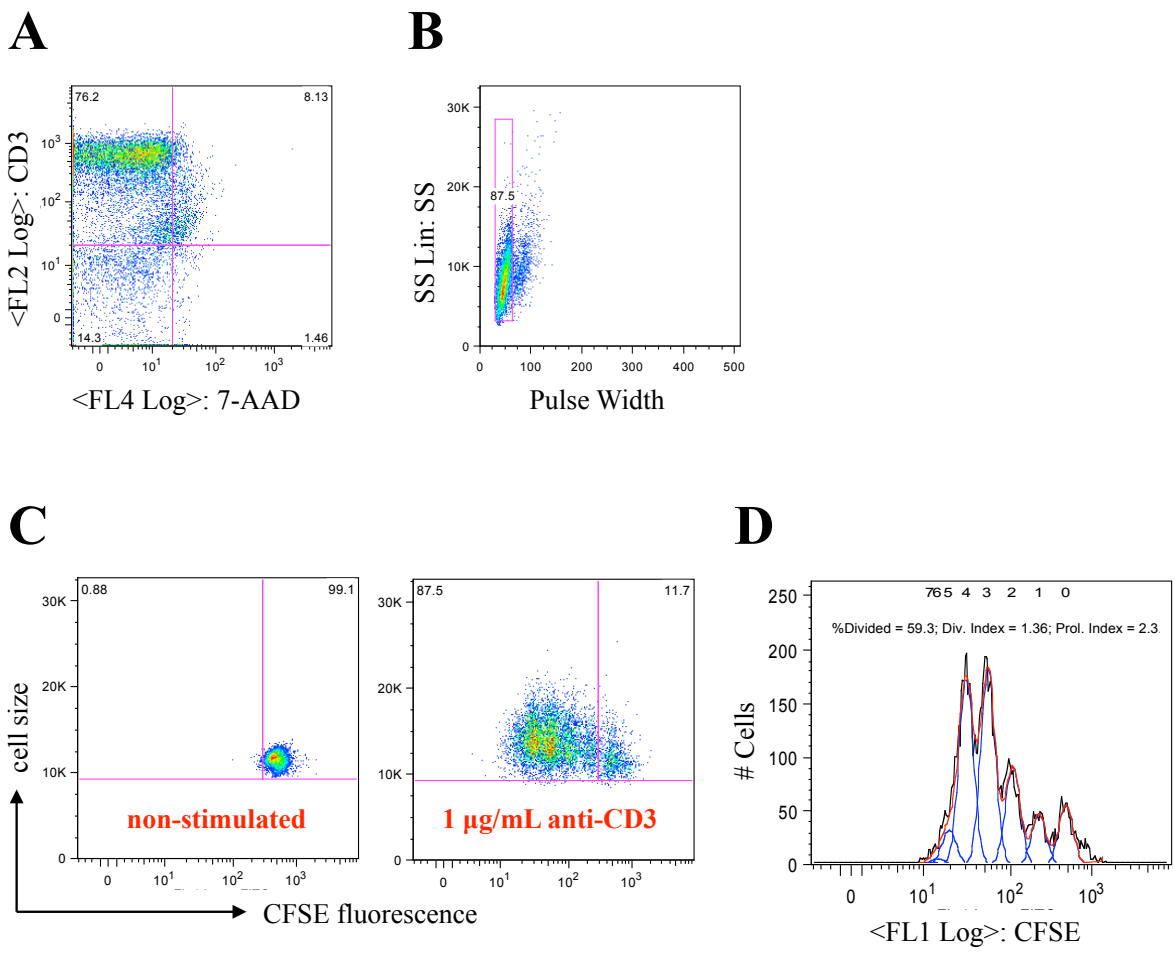
D



C) For phenotype analysis, MDDCs were stained with fluorochrome-conjugated antibodies against CD14, CD80, CD86, CD83, CD206, CD11c, CD40, and HLA-DR (red histograms). Appropriate isotype controls (blue histograms) were used to set the percent positive gates indicated on each graph. For each marker, the median fluorescence intensity was calculated for the entire MDDC population and was standardized before each experiment using fluorescent beads. The filled histograms show background fluorescence.

D) For endocytosis analysis, MDDCs were incubated with FITC-dextran for 1h at 37°C (green histograms). Cells incubated at 4°C (blue histograms) were used to set the percent positive gates as indicated on each graph. MFI was calculated as mentioned above.

APPENDIX II



T-Cell Proliferation Gating Strategy:

A) The T-cell gate was set based on cell viability with 7-AAD and expression of CD3 as indicated on the graph.

B) Doublet cells, which can affect CFSE fluorescence intensity, were removed from the T-cell gate based on their side scatter parameters and pulse width.

C) Non-stimulated T-cells were used to set the gate for dividing cells (defined as CFSE low cells that underwent at least one division).

D) FlowJo analysis software was used to assess the proliferation index (the average number of divisions per proliferating cells).

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