THE REGULATION OF A PATHOGENIC MACROPHAGE POLARIZATION BY IL-10 IN POLYCYSTIC KIDNEY DISEASE

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ABSTRACT

Polycystic kidney disease (PKD) is a devastating genetic disorder that is one of the most common potentially fatal inherited diseases and is the fourth leading cause of end-stage renal disease (ESRD) in the US[5,6,7]. The most common form of the disease, autosomal dominant PKD (ADPKD) affects up to 1 in 500 and costs >$2 billion/year to care for those afflicted. As the name implies, PKD is characterized by the presence of large, fluid-filled cysts that expand within the kidney, distorting and damaging parenchyma and ultimately resulting in loss of kidney function. Currently, there are no FDA-approved treatments for PKD, but there is hope that a thorough understanding of disease pathogenesis will facilitate the development of treatment strategies that target key drivers of disease progression.

In this dissertation, I will summarize what is known about clinical and pathologic features of the disease, focusing on ADPKD and a previously underappreciated aspect of disease pathophysiology—the role of inflammation, especially involving innate immune cells known as macrophages. This work will then describe details of investigations undertaken to better understand the genesis of pathologic macrophages in the cystic kidney and their role in disease progression. These studies have revealed the regulatory cytokine IL-10 to play a key role in this process and thus have identified that cytokine and its signaling partners as potential targets for therapeutic intervention in PKD.

DEDICATION

This dissertation is dedicated, first and foremost, to my incredibly supportive family; parents James and Virginia Peda and sister Rebekah Joy Peda. They have always encouraged me to be my best self and yet accepted me at my worst, and nobody believed in my ability to accomplish this goal as much as they. Additional dedication goes to my understanding and long-enduring roommate, Andrew Reno. There are very few people who have the patience to deal with a neurotic doctoral candidate; and his willingness to escort me to lab no matter the late hour enabled me to properly carry out many crucial experiments.

This document, and the work it details, is made possible due to the love, encouragement, and support that I have received from family and friends.

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I have been blessed with a vast support system composed of so many different dynamics, and I thank God that he knew what he was doing when he placed each individual into my life. And at the end of the day, all of these individuals understood that some physical activity, a good glass of wine, and some solitary confinement was all that was necessary to encourage my scientific development.

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Chapter One

Introduction

ADPKD – CLINICAL PRESENTATION

ADPKD is a remarkably common genetic disease caused by mutations in one of two genes *PKD1* (polycystin 1, PC1; ~85% of patients) or *PKD2* (polycystin 2, PC2: ~15% of patients). For comparison, it is ~10x more common than Sickle Cell Disease, ~15x more common than Cystic Fibrosis, and ~20x more common than Huntington's Disease [8]. In the typical presentation of ADPKD, large fluid-filled cysts derived from non-functional tubules are detectable around age ~35, continue to expand throughout the lifetime of the patient, causing extensive damage to the surrounding normal parenchyma [9-11]. Diagnosis is usually made by ultrasound in patients with a family history or clinical suspicion [12]. In humans and animal models alike, cysts normally begin formation *in utero* and are detectable by age 30 in up to 70% of human patients [12, 13]. Patients typically develop hypertension, hematuria, and other features of chronic kidney disease (e.g., polyuria, sub-nephrotic proteinuria). An important clinical feature is intractable pain, which is unfortunately not uncommon and can be caused by cyst enlargement, rupture, blood clots, and nephrolithiasis. As hinted above, the majority of affected individuals reach End Stage Renal Disease/Failure (ESRD/ESRF) by the 5th to 6th decade of life, but there is marked variation in the disease course, including rare patients who present with early onset disease.

There are many factors that contribute to the severity and rate of progression of the disease, and these will be discussed later in this chapter.

Since the gene products involved in ADPKD are expressed in other tissues, non-renal manifestations are common. Approximately 80% of patients have liver cysts. Aneurysms are common. In fact, ruptured aneurysms in the Circle of Willis with accompanying subarachnoid hemorrhage accounts for ~5-10% of deaths in patients with ADPKD. Cardiovascular complications are common. Mitral valve prolapse and other valve abnormalities are seen in ~25% of patients. About 40% of patients die from cardiovascular-related complications [9, 14, 15].

ADPKD-PATHOLOGY

Kidneys are typically large and heavy (~2 kg) and architecture is dramatically distorted by numerous, variably-sized cysts. The cysts are typically unilocular and generally are filled with variable material: clear fluid, hemorrhagic fluid, gelatinous material, clotted material, cheesy, grumous material, etc. Cysts are most commonly derived from collecting duct, and lining cells are often flattened, but may show more cuboidal appearance [10, 16]. Micropapillary excrescences are also common. In the surrounding parenchyma, there is atrophy and fibrosis, with accompanying inflammation, including lymphocytes and macrophages. Any non-atrophic nephrons tend to show hypertrophy, with glomerular enlargement and enlarged proximal tubules [17].

ADPKD-TREATMENT

There are no currently FDA-approved specific treatments or therapy for PKD, though a recent clinical trial showed some efficacy for a drug that targets the vasopressin receptor [18], which will be discussed below. Thus, renal replacement therapy, usually with dialysis and then eventually transplantation is the most common outcome. A great deal of current research is focused on developing new therapies for PKD. This is a significant challenge, since therapies would have to be life-long and would be best if begun before a great amount of irreparable amount of damage has occurred in the kidney. To accomplish this, a thorough understanding of disease pathophysiology would be helpful to identify viable targets for therapy. Importantly, since PKD typically progresses very slowly, effective therapies, if begun early enough, would require only modest effects to be clinically significant. A modest ameliorative effect over decades could be sufficient to preserve renal function for the lifetime of the patient. Thus, understanding not only the genetics but also the factors that modify disease progression could be fruitful. Some of the work to date in this area is discussed below. Moreover, a focus of this dissertation is to advance our understanding of PKD pathophysiology for this purpose, and that work will be detailed in the ensuing chapters.

ADPKD-GENETICS AND PATHOPHYSIOLOGY

PKD1 encodes for PC1, which is a large 11 transmembrane protein associated with primary cilia. Mutations in PKD1 account for ~85% of ADPKD patients. PKD2 encodes for PC2, a 6 transmembrane domain protein that functions mainly as a cation channel. PC2 is related to Transient Receptor Potential Cation (TRPC) calcium channels and, importantly, interacts with the C terminal domain of PC1. PC2 is also associated with primary cilia; mutations in the encording gene account for ~15% of ADPKD patients. Finally, and perhaps most interestingly, ~10% of cases present with no family history of PKD, i.e., there is most likely *de novo* mutation. Mutations in either of the polycystins result in essentially identical ADPKD disease manifestations, although patients with PC2 mutations tend to have increased renal survival and fewer overall complications, due to slightly milder disease manifestations and a later onset of disease [5, 19].

Rodent models have provided important information regarding disease pathogenesis [20, 21 22 23]. Mutations in PC1 typically present as loss of function and include truncation, as well as missense mutations. Location at the primary cilia appears to be key, as mutations in PC1 and PC2 appear to disrupt ciliary function. Mutations in other ciliary genes also cause clinically distinct cystic diseases, some slightly more severe and all clearly distinguishable from ADPKD, e.g., ARPKD [24] and nephronophthisis [25, 26]. Thus, many investigators refer to cystic diseases collectively as “ciliopathies.”

The primary cilium is believed to be important in mechanosensation. In the tubule, this may be manifested as flow sensation. How this ciliary function relates to cyst formation, however, is incompletely understood. Nevertheless, abnormal cystic tubular epithelial cell proliferation appears to be an essential element of the resulting phenotype [19, 27]. A number of signaling pathways have been implicated in driving proliferation [27], though cAMP formation appears to be a particularly important event [9, 28-30]. Normal kidney epithelial cells regulate the response to cAMP using Ca^{2+} signaling, leading to a growth inhibition. With the loss of polycystin-driven Ca^{2+} regulation, ADPKD cyst cells respond in an aberrant fashion to cAMP, actively proliferating. In the collecting duct, the kidney segment from which most cysts originate, cAMP formation is primarily driven by vasopressin. This is the underlying rationale for PKD therapies that use tolvaptan, an antagonist of the V2 vasopressin receptor that is expressed in the collecting duct. Indeed, clinical trials using this drug showed slower kidney expansion in treated patients [18]. However, the treatment was not approved by the FDA due to concerns over toxicity.

ADPKD-MODIFIERS OF DISEASE

As mentioned above, the disease course for ADPKD is highly variable and can be influenced by many factors. Genetics of PKD can alter disease severity and age of presentation [15, 29, 31], especially the penetrance of the mutation and whether it is a truncating mutation. There are many mutations; to date,

approximately 314 different loss-of- function mutations in the *PKD1* gene have been identified in 400 families, 91 truncating mutations in *PKD2* from 166 families have been described, as well as additional missense mutations; making up about one fourth of the cystin mutations, and none of them are the same. Some mutations, such as homozygous hypomorphs, have been shown to be more severe clinically than others, like heterozygous hypomorphs [31, 32]. The severity of disease has been recently shown to correlate with the position of the mutation, and the subsequent disruption in signaling that occurs due to the mutational location. Dosage levels attributable to *PKD* genetic and allelic variability, as well as other possible gene modifier and environmental effects, likely underlie the large phenotypic variability among affected individuals. Either overexpression or lowering of *Pkd1* protein levels in murine models is sufficient to cause cystic disease, suggesting that dysregulation of polycystins, rather than complete loss of function, is what leads to PKD. It seems likely, though it is not yet known, that the level to which certain pathways are upregulated in cysts could affect the rate at which they progress.

Injury can accelerate the progression of PKD both in humans and in animal models [23]. Using an inducible Pkd1 knockout adult mouse model, it was recently discovered that renal injury resulting from ischemia/reperfusion (IR) accelerated PKD progression [33]. Another group showed that injury induced by the nephrotoxicant 1,2-dichlorovinyl- cysteine (DCVC) after *Pkd1*-gene inactivation accelerated adult cyst formation [34]. Furthermore, multiple groups have shown that *Pkd1* or *Pkd2* haplo-insufficiency alone was sufficient for

microcyst formation and increases in tubule dilation, only after acute injury [35, 36]. In many of these mice examples, the repair response to the injury was studied and thought to play a role in the development of PKD, though this will be discussed later.

THE INNATE IMMUNE SYSTEM AS A MODIFIER OF PKD

The role of inflammation and the immune system in PKD has become apparent in recent years. As mentioned previously, expanding cysts cause damage to the surrounding healthy tubules. This continuous damage brings in immune infiltrates, especially including innate immune cells known as macrophages. We and another lab have found that the abundant macrophages are present within the cystic kidneys in PKD [4, 37]. In these studies, some of which will be detailed in Chapter 2, macrophages were identified and quantified in both human PKD (ARPKD and ADPKD), as well as mouse models of disease. Importantly, using a non-orthologous model of PKD, *cpk* mice, which possess a recessive mutation in the cilia-associated *Cys1*, depletion of macrophages was shown to slow cystic epithelial cell proliferation and cyst growth and preserve renal function [4] (**Fig 1.1 and 1.2**). Similar results were observed in an orthologous mouse model of ADPKD [37], suggesting that macrophages play a role in PKD pathogenesis regardless of the underlying genetic origin of the disease.

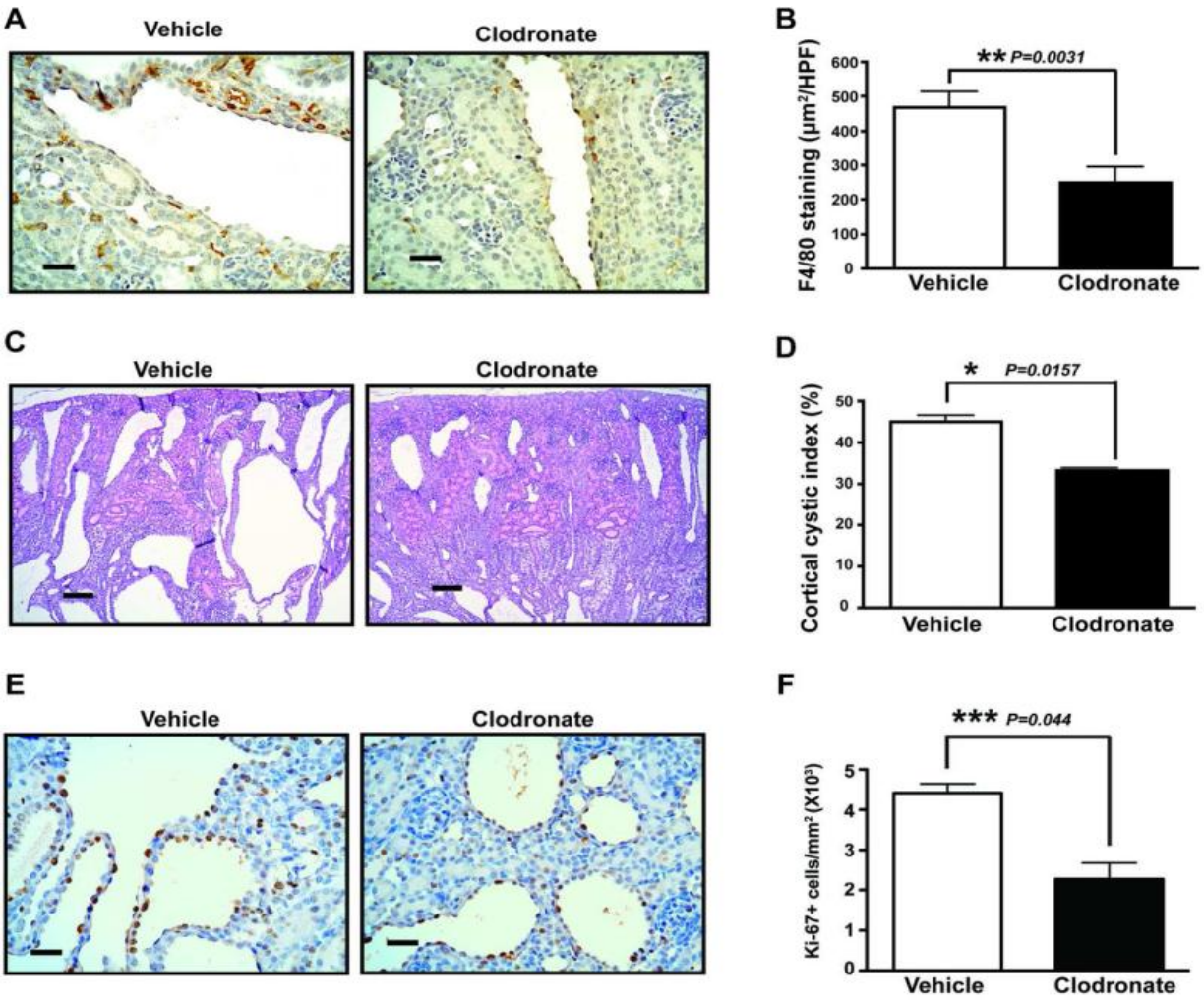


Figure 1.1: Clodronate liposome treatment of *cpk/cpk* mice reduces macrophage load, renal cortical cyst area, and renal cortical cell proliferation. (**A**) Formalin-fixed, paraffin-embedded kidney tissues from PN10 *cpk*/*cpk* mice treated with either vehicle (**left**) or clodronate liposomes (**right**) were sectioned and stained with hematoxylin and eosin. Each image is a representative field from one mouse of four examined per condition. Scale bars represent 100 µm. (**B**) Average cortical cystic index (cortical cyst area/total cortical area) was calculated from measurements of kidneys from animals treated with vehicle or clodronate liposomes, two per condition. (**C**) Formalin-fixed, paraffin-embedded kidney tissues from PN10 *cpk*/*cpk* mice treated with vehicle (**left**) or clodronate (**right**) were sectioned and stained by immunohistochemistry using a monoclonal antibody against F4/80. A representative image from one of two different mice for each condition is shown. Scale bars represent 25 µm. (**D**) The mean area (μm^2) of F4/80 staining per high-powered field (HPF) in sections of kidney cortex described in **C** was measured from 2 kidneys each of mice treated with either vehicle or clodronate liposomes. (**E**) Formalin-fixed, paraffin-embedded kidney tissues from PN10 *cpk*/*cpk* mice treated with vehicle (**left**) or clodronate (**right**) or were stained with an antibody to Ki-67. A representative image from one of two mice per condition is shown. Scale bars represent 25 µm. (**F**) Ki-67+ cells/mm^2tissue in PN10 *cpk*/*cpk* mice treated with either vehicle or clodronate.
Taken from: Swenson-Fields[4] *et al*.

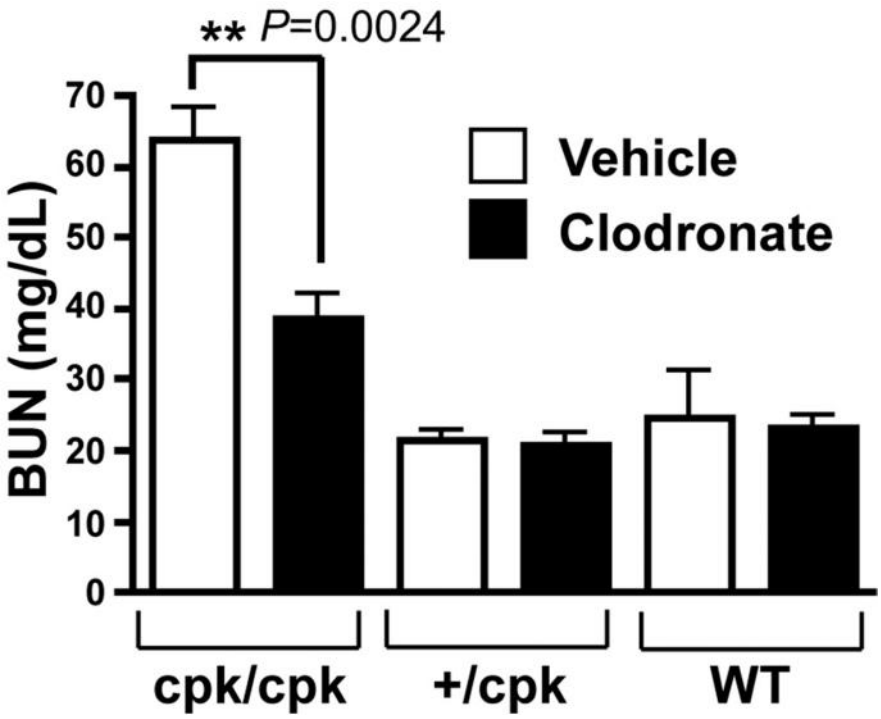


Figure 1.2: Clodronate treatment of cpk/cpk mice improves kidney function. Serum BUN concentration was measured in PN10 mice of the indicated genotypes (n=3-6 mice/condition) treated with either vehicle or clodronate, as indicated.

MACROPHAGES

Macrophages are complex components of the innate immune system that are known to play a role in both immune protection and a variety of disease states. To better understand the role of macrophages in the kidney and particularly in PKD, it is useful to understand their origin and biological characteristics, as well as their known functions in processes like tumor biology and tissue repair. Macrophages arise from mononuclear precursor cells, which develop in the bone marrow in the adult. Macrophages were originally discovered in the early 1900s, initially described as phagocytic cells that helped to eliminate pathogens from the host [38, 39]. Over the years it has become clear that their phagocytic functions are extensive and broad, including not only pathogens but also cellular debris and other foreign material.

Macrophage Polarization. Macrophages, however, are not simply unregulated eating machines. This began to become clear in the early 1980's, when it was discovered that macrophages could functionally alter their phenotype in order to respond to different stimuli [38-41]. That is, macrophages were found to take on different roles and abilities in response to cues from their surrounding microenvironment. This differentiation phenomenon ("polarization") and the role it plays in progression of PKD is the primary focus of this dissertation.

Macrophage polarization results in the generation of two main types of macrophages that were originally described: M1 and M2 [1, 40, 42-44]. This naming was based on the major T helper cell subtypes, Th1 and Th2, because cytokines

produced by Th1 and Th2 cells were found to promote M1 and M2 polarization, respectively (discussed in more detail below). Unlike their T cell counterparts, however, M1 and M2 phenotypes are not static, end-point states. Rather, macrophages show remarkable plasticity and can change quite rapidly, depending on the presence of activating stimuli in the immediate microenvironment [2, 44, 45]. This plasticity allows them to switch phenotypes into diverse functional states, including the artificially divided M1 or M2 state. There can even be a spectrum of macrophages that express mixed M2 or M1/M2 profiles *in vivo*, some of which can be poorly recapitulated *in vitro*. Macrophage plasticity is quite relevant to tissue homeostasis and disease, as is discussed below [42] and shown in **Figure 1.3**. The M1-M2 switch is particular relevant in kidney disease and will be demonstrated in the context of PKD in Chapter 2 of this document. Regulation of this switch is complex, but one pathway know to be important involves AMP Activated Protein Kinase (AMPK). Activation of this kinase enhances mitochondrial biogenesis, which increases the generation of M2 macrophages, which are characterized by oxidative metabolism and limited glycolysis [46].

M1 macrophages. M1 macrophages were first described in 1983 and called "classically activated [38, 39]. It was shown that Th1 type cytokines like IFNγ can drive their appearance. M1 macrophages are sometimes called "inflammatory" in part because they are characterized by the production of inflammatory mediators such as TNFα, iNOS, IL-6, and TGFβ. In the mouse, M1

macrophages are recognized by expression of markers: F4/80^{+}; Cd11c^{-}; MR^{-}; Ly6C^{hi}. Inflammatory stimuli like LPS, TNFα, or in some cases GM-CSF can drive their differentiation [38, 42, 43, 47]. M1 macrophages play an important role in the response to host infection from bacteria, parasites, or other foreign attackers [42]. M1 macrophages are also critical for host response to acute injury, especially by the release of effector molecules (reactive oxygen and nitrogen intermediates) and inflammatory cytokines (IL-1β, TNF, IL-6) that participate as inducer and effector cells in polarized Th1 responses [42]. M1 macrophages are capable of activating the adaptive immune system and mediate resistance against intracellular parasites and tumors [3, 43].

M2 macrophages. With the identification of M1 macrophages, it was found that macrophages could also respond to Th2 cytokines IL-4 and IL-13. These were termed "alternatively activated" or M2 macrophages and were shown to express markers such as *Arg1*, *Mrc1*/*CD206*, *Fizz1*, *Ym1*, and *IL10*). Alternatively activated macrophages generally show high expression of *Arg1*, mannose receptor (*MRC1*), and variable IL-10. M2 macrophages are detected in mice as F4/80^{+}; Cd11c^{-}; MR^{+}; Ly6C^{lo}. M2 macrophages have immunoregulatory capabilities and are critical regulators of tissue remodeling. They also are known to influence tumor progression. M2 macrophages can arise from signals such as apoptosis and can protect the host from excessive inflammation and initiate healing. M2s secrete anti-inflammatory mediators, promote tissue repair, and regulate immune response to infection [38, 39, 42]. Importantly, M2 macrophages

have been found to promote growth in many disease and injury models, due in part to the effect that IL-10 has on the surrounding immune and non-immune cells [48-50]. For example**,** M2 macrophages can be programmed by mesenchymal stem cells, increasing their IL-10 expression. The resulting M2s suppress the inflammatory response and promote proliferative repair phase of nearby epithelial cells [51]. This pro-proliferative capacity of M2 macrophages, driven in part by IL-10, is highly relevant to kidney disease, and this dissertation provides evidence that it is particularly relevant in PKD.

Because M2 macrophages were found to have such diverse functional roles (and markers), they have further been characterized into subcategories based on nuanced responsibilities, though some key characteristics remain the same. There are three general M2 subtypes (**Figure 1.3 (C)**) that are generally recognized in current literature: 2a, 2b, and 2c, categories that are largely based on their gene expression profiles [39, 52]. M2a are pro-fibrotic, often termed wound-healing, and are activated by a combination of IL-4 and IL-13. M2a macrophages also interact with the Th2 response to parasitic infections, and are the prototypic pro-fibrotic M2 subtype. M2b are induced via stimuli such as immune complexes, LPS, IL-1R ligands, and Toll-like receptor activation. M2b macrophages are the immune-regulating cells, secreting IL-10 among other cytokines to rein in the inflammatory response caused by M1 macrophages. M2c are regulatory and exhibit strong anti-inflammatory capabilities, and are most often involved in tissue

repair and remodeling. They are induced via a variety of factors, including IL-10, TGFβ, and glucocorticoids [1, 42, 52-54].

While this sub-classification is widely used and has some utility, some investigators feel that the rigid categorization does not capture the diversity of phenotypes that can exist, especially since macrophages are sometimes found that do not clearly fall into one single category [55]. In 2008 the idea that macrophages could exist along a spectrum of activation (**Figure 1.3 (B)**) was proposed, an idea particularly relevant to M2-like macrophages [38]. This idea is consistent with one of the defining characteristics of macrophages mentioned above, i.e., their plasticity. Macrophages are highly plastic and responsive to environmental stimuli *in vivo,* making a rigid classification scheme difficult to provide real-world utility [55]. It is perhaps more useful to think of macrophage polarization states as a continuum regulated by external signals and a continuity of expression of numerous effector genes. This paradigm extends not only to M2 subtypes but also the M1/M2 dichotomy, as mentioned above.

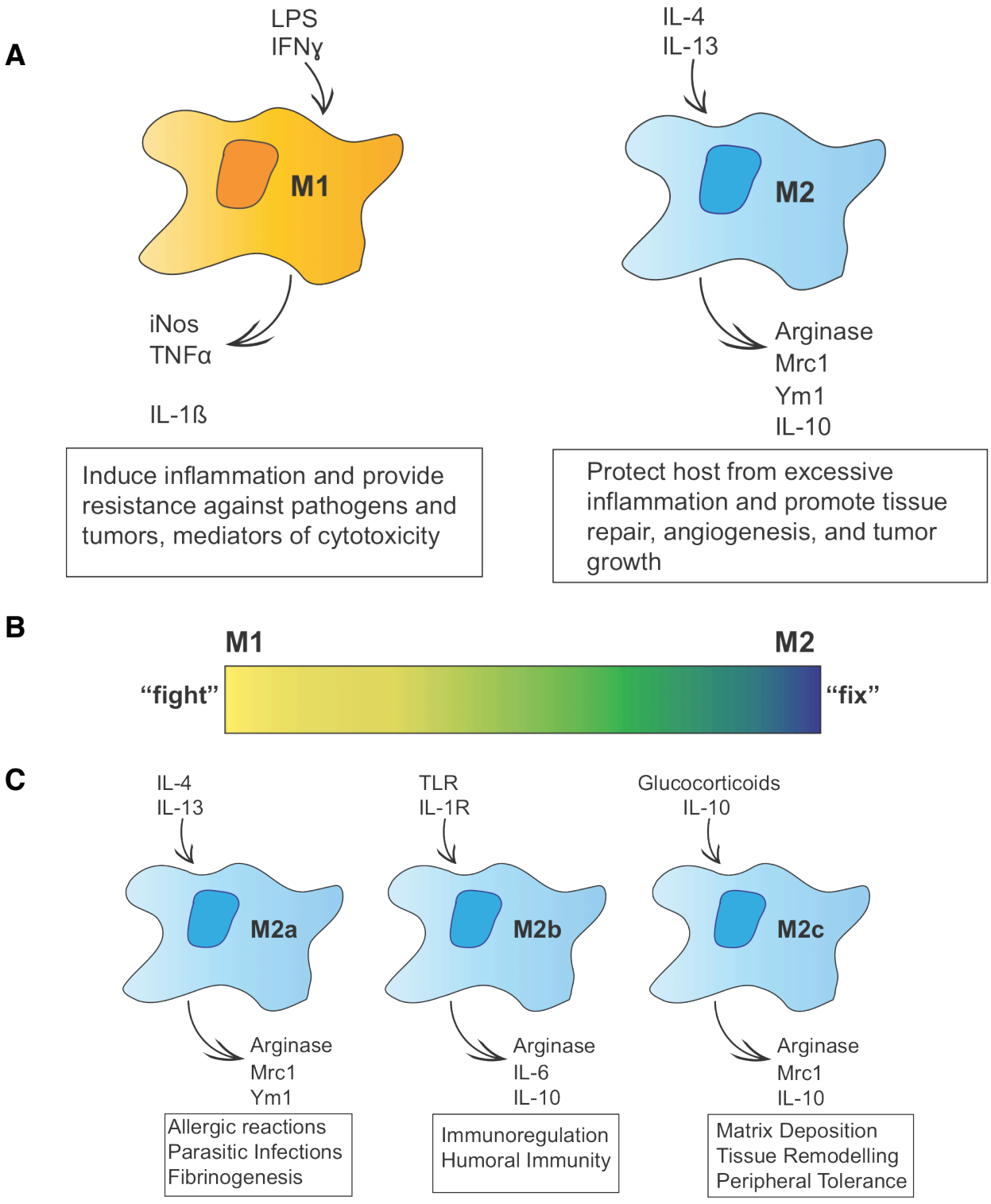


Figure 1.3: The M1/M2 paradigm and the sub-classifications of M2 macrophages. (A) The M1/M2 macrophage model indicates that M1 macrophages are activated by the presence of LPS or interferon in the microenvironment while M2 macrophages arise due to various other factors, most commonly IL-4 or IL-13. **(B)** Macrophages more accurately exist along a spectrum, depending on the cues found in their microenvironment. These highly plastic cells can have varying degrees of a fight or fix reaction, and can easily convert to adapt to the threat to the host. **(C)** Three subsets of M2 macrophages are commonly described, listed are the primary activators as well as the hallmark productions. Each box below lists the principal functions of each subtype.

MACROPHAGE POLARIZATION IN DISEASE

A full characterization of specific macrophage polarization states existing *in vivo*, including in any particular disease state, has not been achieved due to difficulties resulting from macrophage plasticity and the rate at which they change phenotypes when placed into a new environment [38, 42, 56, 57]. Nonetheless, "M1-like" macrophages found in *in vivo* settings, including the kidneys that share similar markers with the classically defined M1 macrophages have been shown to play an important role in the inflammatory response during chronic or acute injury. In turn, "M2-like" macrophages have been shown promote the tissue repair and regeneration of cells post-injury [58-60]. Loss of an M1 or M2 phenotype occurs *in vivo* in dividing macrophages isolated from bone marrow, while non-dividing macrophages from the spleen kept an M2 phenotype; this indicates that macrophages plasticity *in vivo* is based on programming factors in the microenvironment [56, 57, 61]. In this section, specific examples of macrophage polarization in disease will be discussed, ending with an emphasis on kidney disease.

Tumor Biology. Both M1-like and M2-like can promote tumor progression, depending on the stage of the tumor and the type of cancer [38, 62-64]. There is correlation between a high number of tumor-associated macrophages (TAMs) and poor prognosis in thyroid, lung and liver cancers [65]. TAMs very generally express *Arg1* (mouse models only), *Ym1*, *Fizz1* and *Mrc1*, however in each cancer there are distinct phenotypes of TAMs; with M1 and M2 as the

extremes to compare them to. This model, where there is no clearly defined or understood macrophage phenotype, is really where the continuum idea arose from. It seems that M1s, expressing NFκB, play a role in the initiation and promotion of tumors, while M2 cells play a role in the invasion and migration of tumor cells, as well as in assisting in hypoxic survival [38]. Extensive and long inflammation has been considered as a possible 7th marker of cancer, indicating the importance that macrophages are thought to have in tumor progression. However, a clear role for a specific phenotype has not yet been discovered, most likely because of the complexities of studying macrophages *in vivo* rather than *in vitro*, as well as the fact that mouse and human macrophages do not necessarily have overlapping prominent markers [62] [38]. M2 macrophages are associated with increased tumor growth, invasiveness and metastasis in Renal Cell Carcinoma; this is due to secretion of pro-angiogenic factors, induced cells migration, and modulation of the tumor response. The expression of CD163 and M-CSF was found to correlate with a worse prognosis in the setting of type 2 Papillary RCC [63, 65]. An increased infiltration of tumors by M2 macrophages decreases the chances of survival, mostly due to increased distant metastasis in both breast and pancreatic cancer [63, 65].

Cardiovascular Disease and Obesity. Macrophages are important in the pathophysiology of atherosclerosis. It is believed that in vessels macrophages take up cholesterol and become plaques. In particular, M1 macrophages are elevated in patients who experience a cardiac event related to atherosclerotic

disease. A specific contribution for M2 cells in atherosclerosis is still debated [38]. A recent study in mouse models of atherosclerosis has revealed that the balance of M1 and M2 macrophages in plaques can indicate disease progression (M1 majority) or regression (M2 majority) [66]. The relevance in human patients has yet to be studied, and the mechanism by which an M2 subtype may prevent disease progression has yet to be determined [66]. However, in patients with chronic kidney disease (CKD), the incidence of cardiovascular events was higher in those with high serum levels of the M2 cytokine IL-10. Macrophages may also play a role in hypertension, another vascular disease that increases risk of atherosclerosis. Depletion of macrophages and neutrophils eliminated the hypertensive response to Angiotensin II in mice, indicating a potential role for immunosuppression in hypertension treatment [67].

Macrophages can influence metabolic disease as well. The observed macrophages in obese mice are M1-like, while lean animals had macrophages that were skewed towards an M2 profile. In preliminary studies, the frequency of diabetes mellitus was also higher in IL-10 high patients, though further studies need to be done [68]. Obesity decreases IL-10 expression and synthesis, resulting in chronic kidney inflammation, but spleen-derived IL-10 can protect against obesity-induced inflammation due to altered macrophage function rather than a change in macrophages recruitment [69, 70]. IL-10 also has been show to attenuate glomerular fibrosis in high fat diet male mice, with improved blood pressure and decreased inflammation, while injury is worse yet salvageable in IL10KO mice [69].

Kidney Disease. Macrophages are key regulators and effectors in kidney disease, including both tubulointerstitial and glomerular disease. Macrophages come to reside in the kidney very early during development [71]. They are present in the kidneys before nephrons, suggesting that they may have a role in the prenatal stages of kidney development**.** Despite their early presence in the kidney, these so-called "resident" renal macrophages are also thought to be of bone marrow origin [71]. Kidney mononuclear cells have 5 subtypes based on surface marker expression and arise both from the yolk sac and the bone marrow, termed resident and migrating respectively [60]. However, no matter the surface markers, all of these macrophages react fairly similarly to cytokines. Renal epithelial cells have the ability to change macrophage phenotype from M1 to M2 [72, 73]. In the kidney the primary role of macrophages is ongoing immune surveillance, maintaining homeostasis and modulating physiological function [71, 74, 75]. Dysregulation of this role can be detrimental to the kidney in times of injury or disease, as will be discussed below.

Macrophages in Acute Kidney Injury. Macrophages undergo a phenotypic shift from M1 to M2 to help with repair after ischemia/reperfusion (IR), a common model for acute kidney injury (AKI). The precise cue that drives this switch is unknown, but tubular epithelial cells can induce alternative activation of BMDMs, even in the absence of IL4Rα or STAT6 [72]. Recovery takes much longer if the M1-M2 switch does not happen [76]. In IR, monocytes are recruited 24 hours after reperfusion, mature into macrophages, increase in number through day 7

after injury. In the first day or so the macrophage infiltrate is predominantly inflammatory (M1). The infiltrate helps to destroy and clear injured tissue. Thus, depletion of macrophages at the time of injury was shown to be protective [77]. Subsequently, the macrophages transition from pro-inflammatory state, which promotes tubular injury, to a reparative phenotype after 48-72 hours. In contrast, macrophage depletion during the repair phase worsens injury and delays tubule proliferation. Immediate adoptive transfer of M1 macrophages promoted injury, while injection of M1 macrophages 3 days after injury led to an induction in repair. When isolated from the injured kidney and sorted for additional study, however, it was discovered that the M1 cells had switched to a more M2-like phenotype [77, 78].

Further evidence of M2 macrophage involvement in AKI can be found by examining the appearance and function of the M2 cytokine IL-10. There is an increase in IL-10 production for 7 days after IR injury and this peaks at day 3, which is when IL10KO mice had greater tubular injury than WT as well as increased serum creatinine levels [79]. IL10KO mice also had an increased number of infiltrating macrophages that expressed high levels of IL-6 and TNFα, as well as less Ki67 staining, all reaching statistical significance on day 3 post injury [79, 80]. IL-10 injection pre- or immediately post-injury can protect from AKI, perhaps by upregulating netrin [48, 79, 81]. Moreover, IL10 overexpressing macrophages introduced at the time of acute injury not only reduced pro-inflammatory cytokines, but preserved renal function and limited tubular injury [82, 83]. In a

different model of AKI involving sepsis in the rabbit, kidney injury is reduced with hydrogen sulfide treatment, which promotes M2 polarization via unknown mechanisms. With this treatment there is increased IL-10 production, while NFκB and TNFα levels are reduced [51, 84]. Collectively, these studies indicate that IL-10 suppresses inflammation in acute kidney injury.

These studies highlight the idea that transition from M1 to M2 macrophages is critical and maximizes functional and structural recovery following injury [56]. As further evidence of this point, infusion of IL-4 treated macrophages promoted renal tubular cell proliferation after IRI, while infusion of IFNγ treated macrophages worsened disease [56, 72]. M1 or unprogrammed macrophages worsened injury in AKI mouse model [51]. *Socs3* KO mice had better renal function due to an increased tubular cell proliferation and limited initial damage. This was found to be due to a phenotypic switch from M1 to M2 leading to higher numbers of alternatively activated macrophages with elevated levels of IL-6 and IL-10. The result was better preservation of renal function [80]. While the M1-M2 phenotypic switch is essential for effective repair, persistent inflammation promotes tubular atrophy and fibrosis in AKI models, which is also driven by M2-like macrophages [71, 85]. Thus, M2-like macrophages can be either adaptive or pathologic, depending on the physiologic context.

Macrophages in Acute Glomerular Disease. In addition to AKI and CKD, macrophages also are key modifiers of glomerular disease. Macrophages are a major cellular component within glomeruli in many acute glomerular

diseases. For example, their presence correlates with a worsened outcome in lupus nephritis [86, 87]. Persistent pro-inflammatory macrophage presence also plays a role in forms of crescentic glomerulonephritis (GN). Ablation of macrophages has shown to improve the prognosis of crescentic GN resulting from lupus or other causes [88, 89]. Many studies have found that prolonged M1-like macrophages presence can exacerbate glomerular injury, while prolonged M2-like presence can lead to tubulointerstitial fibrosis and impaired renal function [46, 51, 75, 90]. Thus, in glomerular disease, as with tubulointerstitial disease, macrophages can have both adaptive and pathologic functions.

Macrophages in Chronic Kidney Disease. Chronic kidney disease (CKD) refers to disease that results from recurrent or progressive injury to components of the kidney, including glomeruli, tubulointerstitium, and vessels. There are many relevant models for CKD, including unilateral ureteral obstruction (UUO). In UUO obstruction is a significant component and ultimately results in CKD. Persistent presence of macrophages drives renal scarring and chronic fibrosis in UUO, which can result in organ failure. As with IRI, in UUO M1 ($Ly6C^{hi}$) macrophages are originally recruited to the injured kidney, and M2-like ($Ly6C^{lo}$), pro-fibrotic macrophages then develop from these [71, 91]. Systemic depletion of macrophages by administration of the bisphosphonate clodronate attenuated fibrosis in UUO [75, 90, 92]. This ameliorative effect is believed to be related to the loss of functional M2-like macrophages.

Macrophages in Polycystic Kidney Disease. As discussed above, the kidneys in PKD are infiltrated by macrophages, and we and others have shown that their presence can promote disease progression [4, 37]. In this dissertation, I will describe experiments that determine markers that define these pathogenic macrophages, as well as fully characterize their interactions with cystic epithelial cells (Chapter 2). Similar to other disease states described here, these macrophages take on an M2-like phenotype in PKD kidneys. I show that the polarization is driven by interaction with cystic epithelial cells, and these polarized macrophages display enhanced ability to promote cyst cell proliferation. As detailed above, interactions between epithelial cells and macrophages can be advantageous, by promoting repair after injury. However, these data suggest a model wherein reciprocal interactions between cystic epithelial cells and macrophages are pathogenic in PKD, since cyst cells are already hyperproliferative.

In Chapter 3, I explore signals responsible for pathogenic polarization, focusing on the role of IL-10. This cytokine has been implicated in repair in other settings. In the context of PKD, I provide evidence that its secretion is required for polarization. The IL-10 subsequently acts in an autocrine manner to promote differentiation to a macrophage phenotype that has enhanced pro-proliferative capabilities. In Chapter 4, I look at components of signaling pathways downstream of IL-10. In particular activation of Stat3 is shown to be required for acquisition of the pathogenic phenotype. Also, using gene expression data, I

describe likely pathways that set the IL-10-dependent differentiation process in motion. Collectively, these data imply that IL-10 and its signaling partners may be potential targets for therapy in PKD.

Chapter Two

The Phenotypic Characterization of Macrophages Present in Polycystic Kidney Disease

1. Introduction

Macrophages constitute an important part of the immune response to injury. In the kidney, macrophages are essential effectors of both repair and fibrosis following injury [58, 93]. Soon after acute kidney injury, monocytes are recruited to the kidney, where they differentiate into macrophages. As discussed in Chapter 1, these macrophages most commonly have an "inflammatory" or M1 phenotype, expressing factors such as IL-1, IL-6, TNF, iNOS [94, 95]. M2 macrophages appear subsequently as a result of either conversion of M1 due to stimulation by local environmental cues or the recruitment of new monocytes [95]. Persistence of the M2 and M2-like macrophages, which can result from sustained, ongoing injury, can promote fibrosis and scarring [95].

PKD is a genetic kidney disorder in which injury plays a prominent role. PKD results in the formation and slow expansion of numerous fluid-filled cysts within the kidney. These cysts dramatically distort the renal parenchyma and result in fibrosis and renal failure in most patients. The expansion of each cyst results in local injury by compressing surrounding tubules, vessels, and lymphatics. It is hypothesized that this persistent injury, in fact, is largely responsible for the scarring, fibrosis, and ultimate failure of the kidney in PKD [96].

Since both acute and chronic kidney injury play an important role in PKD pathophysiology, it stands to reason that the immune system, especially macrophages, would be an important component of the disease. In fact, several groups have documented the presence of macrophages in PKD kidneys [36, 97]. However, the specific phenotype of the macrophages was not investigated. This chapter examines both mouse models of PKD and human tissue to define macrophage phenotypes. This analysis reveals that a majority of macrophages existing in PKD kidneys of both study mice and human patients express M2 markers. Moreover, macrophage differentiation is influenced by either direct contact with primary ADPKD cells or exposure to ADPKD cell secretions in conditioned media. Signals from ADPKD cells not only influence naïve cells but are also sufficient to switch the expression profiles of previously polarized macrophages. Thus, the macrophage phenotype that is predominant in PKD is not only novel and distinct but arises due to factors produced by cystic epithelia.

2. Methods

Media: ADPKD media is DMEM/F-12 supplemented with 5% FBS, 15 mM HEPES, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (ITS, BD Biosciences) plus penicillin (100 U/ml), streptomycin (130 μg/ml) (Pen/Strep). THP-1 media is RPMI-1640 media containing 10% FBS, 2 μM L-glutamine and Pen/Strep. RAW media is DMEM with the addition of 10% FBS,

P/S, and 2 mM L-glutamine. XVIVO-10 (Bio Whittaker, Walkersville, MD) contains L-glutamine, gentamicin, phenol red, P/S, and 10 mM HEPES buffer.

Cells: THP-1 (#TIB-202) cells were obtained from ATCC (ATCC.org). The cells were passaged as suspension cells in THP-1 media until needed for assay. THP-1 cells were differentiated at a final density of 2.0×10^6 cells in 6-well dishes with the addition of 200 nM PMA (phorbol myristate acetate) to the media for 3 days, making them adherent. THP-1 cells were switched back into non-PMA media for 3 to 4 days after differentiation before being programmed for proliferation assays or protein analysis. Programming took place with the addition of ADPKD conditioned media (CM) made in XVIVO-10, XVIVO-10, and combined with various inhibitors, as indicated in the figures.

RAW 264.7 cells (TIB-71) were obtained from ATCC (ATCC.org) and were carried and used for up to 20 passages. Cells were seeded at a final density of 2.0×10^6 in 6-well plates for all protein and RNA analysis. Cells used for proliferation assays were seeded at a final density of 1.0×10^6 in 6-well plates in RAW media. Programming took place with the addition of ADPKD CM made in XVIVO-10 or RAW media, the appropriate controls, and any necessary inhibitors or cytokines, as indicated in the figures.

Primary cyst epithelial cells obtained from the cavities of cysts on the surfaces of ADPKD kidneys (ADPKD cells). Both these and non-cystic kidneys (NHK cells), which were derived from cortical tubule fragments of non-cystic kidneys, were supplied by the PKD Biomaterials Research Core laboratory at

KUMC and cultured up to 2 passages or 7 days, whichever occurred first. These cells were used to produce PKD CM, as well as in direct and transwell proliferation assays.

PCR: Total RNA was isolated from cells (macrophages or macrophages plus renal tubule cells) harvested after 18 h culture using RNeasy Miniprep kit (Qiagen) according to the manufacturer's instructions. 1.5 µg RNA was used as template for cDNA synthesis using High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA) in a 20 µl reaction. For semi-quantitative RT-PCR 1-2 *µ*L of the cDNA reaction was used in a 20 µl PCR mixture. Phusion High-Fidelity DNA polymerase was used and PCR carried out using the manufacturer's protocol (New England Biolabs; Ipswich, MA). The housekeeping gene *Gapdh* was used as the endogenous control. Cycle conditions and primers. PCR products after amplification were analyzed by 2% agarose gel electrophoresis in 1X TAE buffer. Real-time quantitative RT-PCR (qRT-PCR) was performed on Mx3005P using Sybr Green Master Mix (Agilent). The thermal cycler conditions were 95 °C for 10 m X 1, then 40 cycles of 95 °C for 30 s, 55 °C for 1 m, and 72 °C for 1 m, and completed with 1 cycle of 95 °C 1 m, 55 °C for 30 s, and 95 °C for 30 s. Expression of mouse gene products was normalized to mouse *Gapdh.* The following primers were used:

Arg1, 5'-CTCCAAGCCAAAGTCCTTAGAG-3' and

5'- GGAGCTGTCATTAGGGACATC-3';

iNos (Nos2), 5'-GTTCTCAGCCCAACAATACAAGA-3' and

5’–GTGGACGGGTCGATGTCAC-3’; *Mrc1*, 5'-CTCTGTTCAGCTATTGGACGC-3' and 5'-CGGAATTTCTGGGATTCAGCTTC-3';

Il6, 5'-CTGCAAGAGACTTCCATCCAG-3' and 5'-AGTGGTATAGACAGGTCTGTTGG-3';

Il10, 5'-GCAGCTCTAGGAGCATGTGG-3' and 5'-ACAGCCGGGAAGACAATAACT-3' and

Gapdh, 5’-CCACTCACGGCAAATTCAAC-3’ and 5’-GTAGACTCCACGACATACTCA-3’.

3. Results

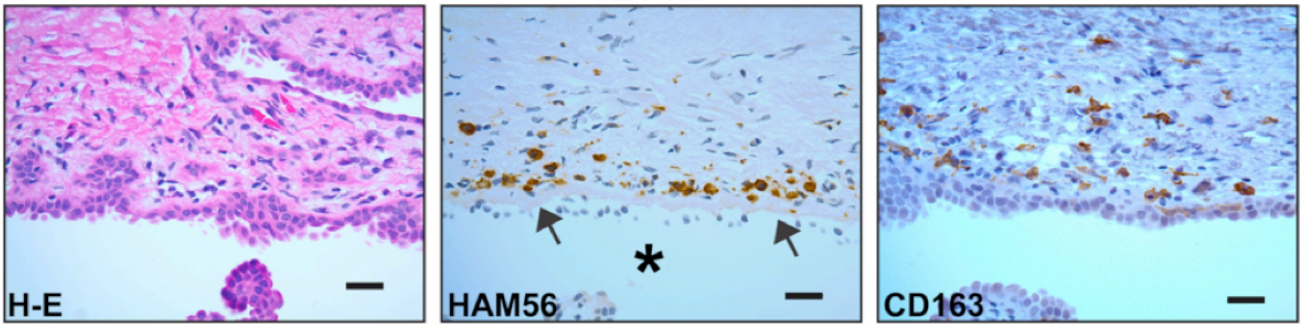
M2-like macrophages are present in ADPKD (Autosomal Dominant) and ARPKD (Autosomal Recessive) cystic kidneys.

To identify macrophages within human PKD kidneys, immunohistochemical staining was performed using the macrophage antibody HAM56 [98, 99]. Macrophages were scattered throughout the parenchyma in both ADPKD and ARPKD kidneys, including areas adjacent to cysts. Occasionally, macrophages were found infiltrating cysts. To determine whether the macrophages were M2-like, serial sections were stained with an antibody to CD163, a human M2 macrophage marker [100, 101]. The staining pattern was similar to that observed for HAM56 (**Figure 2.1 (A)**). The total number of CD163+ cells in each field was similar to the number of HAM56+ cells in the same field of the adjacent serial section (**Figure 2.1 (A)**), suggesting that most HAM56+

macrophages were also CD163+. These data confirm the presence of macrophages within both ADPKD and ARPKD kidneys and indicate that most exhibit an M2-like phenotype. Sections from non-cystic human kidneys (NHK) were also stained with HAM56 and anti-CD163 (**Figure 2.1 (B)**). While far fewer macrophages were present relative to PKD, the ratio of CD163+/HAM56+ cells was similar.

A similar analysis was performed on cpk mice, a non-orthologous, rapidly-progressing PKD model that phenotypically most closely resembles ARPKD. Flow cytometry was employed on cells isolated from cystic kidneys using F4/80 (murine monocyte/macrophage marker), Cd11c (dendritic cell marker), and Ly6C [102, 103]. Ly6C can distinguish M1-like ($Ly6C^{high}$) from M2-like ($Ly6C^{low}$) macrophages [59, 104, 105] in F4/80+Cd11c- cell populations. Renal macrophages ($F4/80^{+}Cd11c^{-}$) were elevated in cystic *cpk/cpk* kidneys compared to wild-type (WT) (20% versus 6% of total single cells; **Figure 2.2**)). Of these macrophages, most (66%) were M2-like ($F4/80^{+}Cd11c^{-}$ $Ly6C^{low}$). These data show that, as with human PKD, *cpk/cpk* kidneys contain elevated levels of macrophages, most of which are M2-like.

A. ADPKD



B. NHK

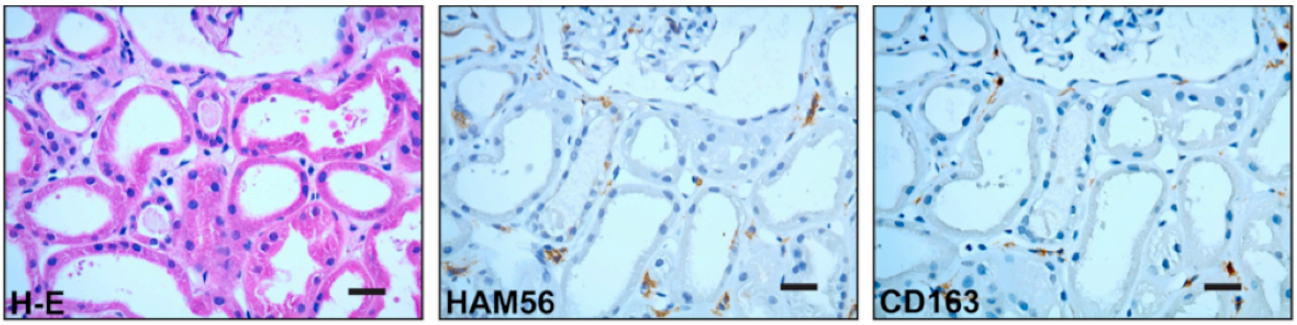
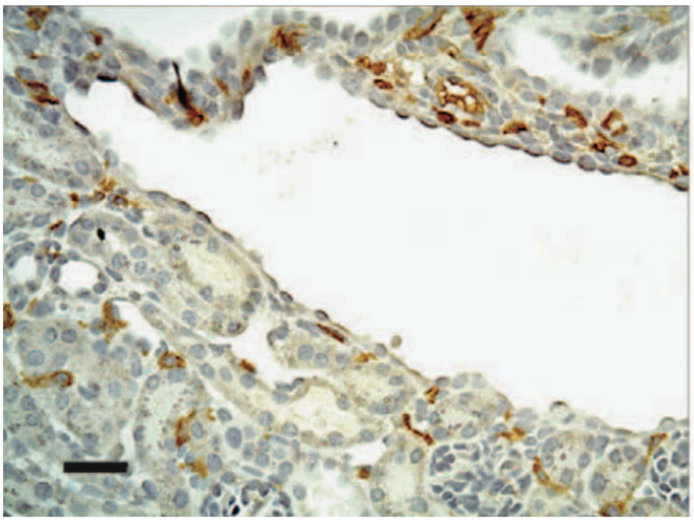


Figure 2.1: Macrophages are present in greater numbers in cystic kidneys, and are primarily of the M2 phenotype. Formalin-fixed, paraffin-embedded tissues from ADPKD (kidney K239) (**A**) as well as non-cystic kidney (**B**) were serially sectioned and consecutive sections stained by hematoxylin and eosin (**left**) or by immunohistochemistry using the macrophage antibody HAM56 (**middle**) or an antibody to CD163 (**right**). Scale bars represent 25 μm. Cystic space is indicated by *. The arrows indicate macrophage infiltration of cystic epithelium.

A. *cpk* sample



B. *cpk* macrophages

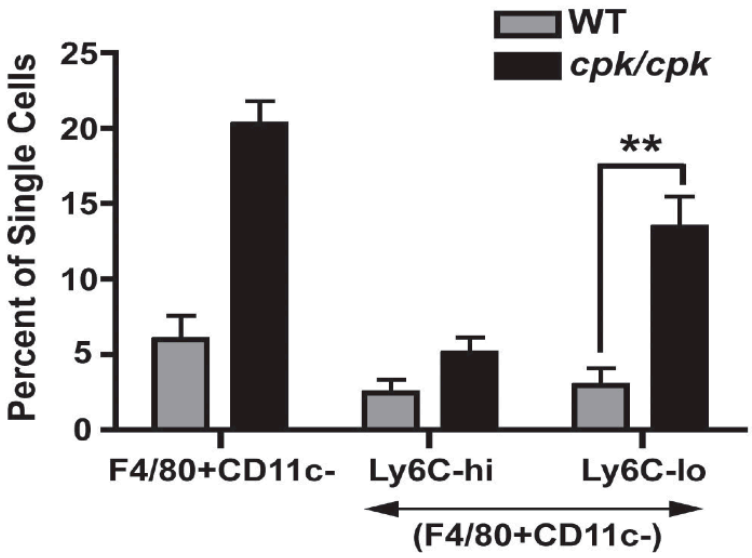


Figure 2.2: Cystic *cpk/cpk* kidneys contain elevated numbers of macrophages that are mostly M2-like. (A). Sections from *cpk/cpk* mice were cut and stained for the presence of macrophages using the mouse macrophage marker F4/80. **(B)**. Quantitative flow cytometry analysis of single, live cells isolated from WT (gray bars) and *cpk/cpk* (black bars) kidneys. Shown are the percentages of single cells that are macrophages ($F4/80^{+}CD11c^{-}$) (**left**), M1-like macrophages ($F4/80^{+}CD11c^{-}$ Ly6C-hi) (**middle**), and M2-like macrophages ($F4/80^{+}$CD11cLy6C-lo) (**right**). Data are presented as mean ± SEM. Kidneys from a total of 9 *cpk/cpk* and 8 WT mice were analyzed. ** denotes $P<0.01$.

Characterization of the M2-like PKD macrophage phenotype.

To further characterize the macrophage phenotype, primary ADPKD or NHK cells were co-cultured with a mouse macrophage-like cell line, RAW264.7 (RAW cells). This technique allowed us to design PCR primers that were mouse or human specific, so that the change in either cell type could be determined. These co-cultured cells were then checked for both M1 and M2 related markers, and the induction of each marker was compared in NHK vs ADPKD co-culture using end-point RT-PCR. In RAW cells co-cultured with ADPKD or NHK cells, there was a robust induction of the classic M2 gene *Arg1,* as well as M2 markers *CCL13* and *22* in THP-1 and ADPKD co-cultures. However, there was no stimulatory effect on *Mrc1*, which is also a prototypical M2 marker, while treatment with IL-4/IL-13 induced both *Arg1* and *Mrc1*, as expected. There was no change or a reduction of expression in M1 related genes such as iNos and IL-6 (**Figure 2.3 (A, C, D) and Table 2.1**), but these genes were induced by the M1 polarizing stimulus IFN-γ (**Figure 2.3 (A)**). We also measured expression of M2-like cytokine *Il10*. As with *Arg1*, *Il10* was upregulated in RAW cells following co-culture with either ADPKD or NHK cells, which was greater in ADPKD cell co-cultures (11-fold versus 6-fold in NHK co-cultures; **Figure 2.3 (B)**). These results indicate that both ADPKD and NHK cells can induce a macrophage phenotype that is M2-like but distinct from the classic, canonical M2 phenotype stimulated by IL-4/IL-13. As mentioned in Chapter 1, macrophages have been observed to exist as a spectrum rather than being strictly defined as M1 or M2.

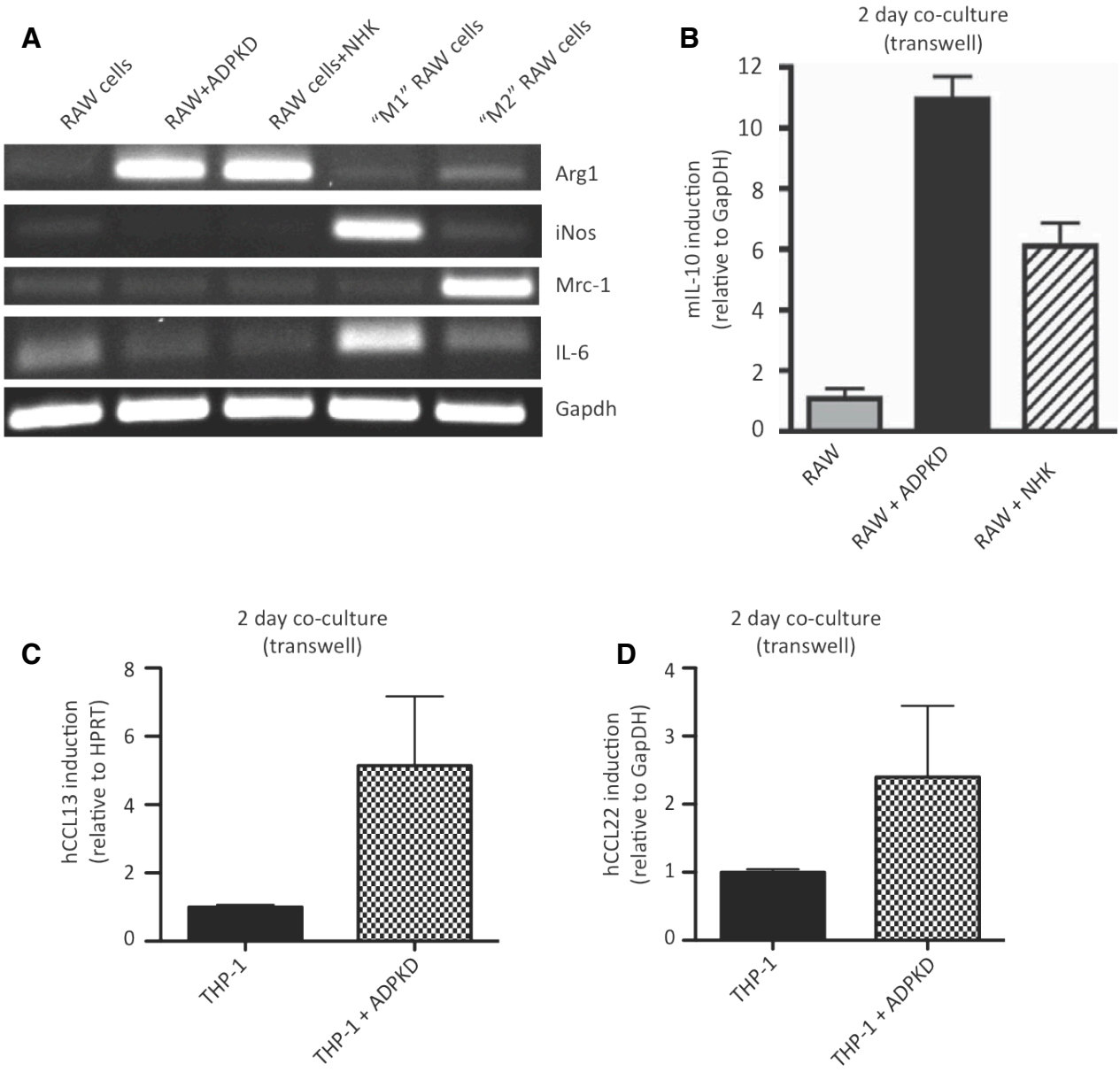


Figure 2.3: Co-culture with ADPKD cells induces an M2-like expression in MΦ. **(A)** RAW and kidney epithelial cells were cultured together for 2 days and end-point PCR was run to look at polarization markers. Co-cultured macrophages had a distinctly different expression profile when compared to cells exposed to IFNγ (M1) or IL-4/IL/13 (M2). **(B)** RAW macrophages were co-cultured with primary kidney epithelial cells for 2 days in a transwell setup. Il10 expression is significantly higher ($p < 0.05$) in RAW cells exposed to ADPKD epithelial cells when compared to NHK-exposed or naïve RAW cells. **(C)** THP-1 macrophages were co-cultured in a transwell setup with ADPKD epithelial cells for 2 days. The expression of CCL13 (M2 marker [1]) was upregulated significantly over baseline. **(D)** THP-1 macrophages were co-cultured with ADPKD epithelial cells in a transwell setup for 2 days. The expression of CCL22 (M2 marker [3]) was upregulated significantly over baseline. **B-D** were analyzed using quantitative RT-PCR.

Soluble factor(s) secreted by PKD cells are responsible for promoting M2-like macrophage polarization.

In the THP-1 co-cultures, transwells were used in order to ensure that only macrophage transcripts were analyzed. A cell culture well insert was used to separate the macrophages (seeded on the bottom plate) from the epithelial cells (seeded onto the insert). The insert has a membrane that allows free flow of soluble macromolecules but does not allow cell contact. These results indicate that polarization of macrophages by cystic epithelial cells is due to one or more soluble factors.

To determine whether physical contact between the epithelial and macrophage cells was necessary for PKD cell stimulated M2-like polarization, conditioned media (CM) taken from plated primary ADPKD or NHK cells was used to treat macrophage cells. Using this technique we were able to analyze both mouse cells (RAW264.7) and a human macrophage cell line, THP-1 cells. Treatment of RAW cells with ADPKD cell CM led to a significant increase in M2-related genes *Arg1, Il10, Fn-1* and *CCL22* when compared to cells treated with NHK conditioned media or those receiving control treatment (**Figure 2.4 (A, D)** and data not shown). Similarly, ADPKD CM treatment of THP-1 cells resulted in M2-related gene upregulation comparable to that observed in RAW cells (**Figure 2.4 (B, C)**). There was no significant change in M1-associated markers *Il6* or *iNos* (**Figure 2.4 (A)**).

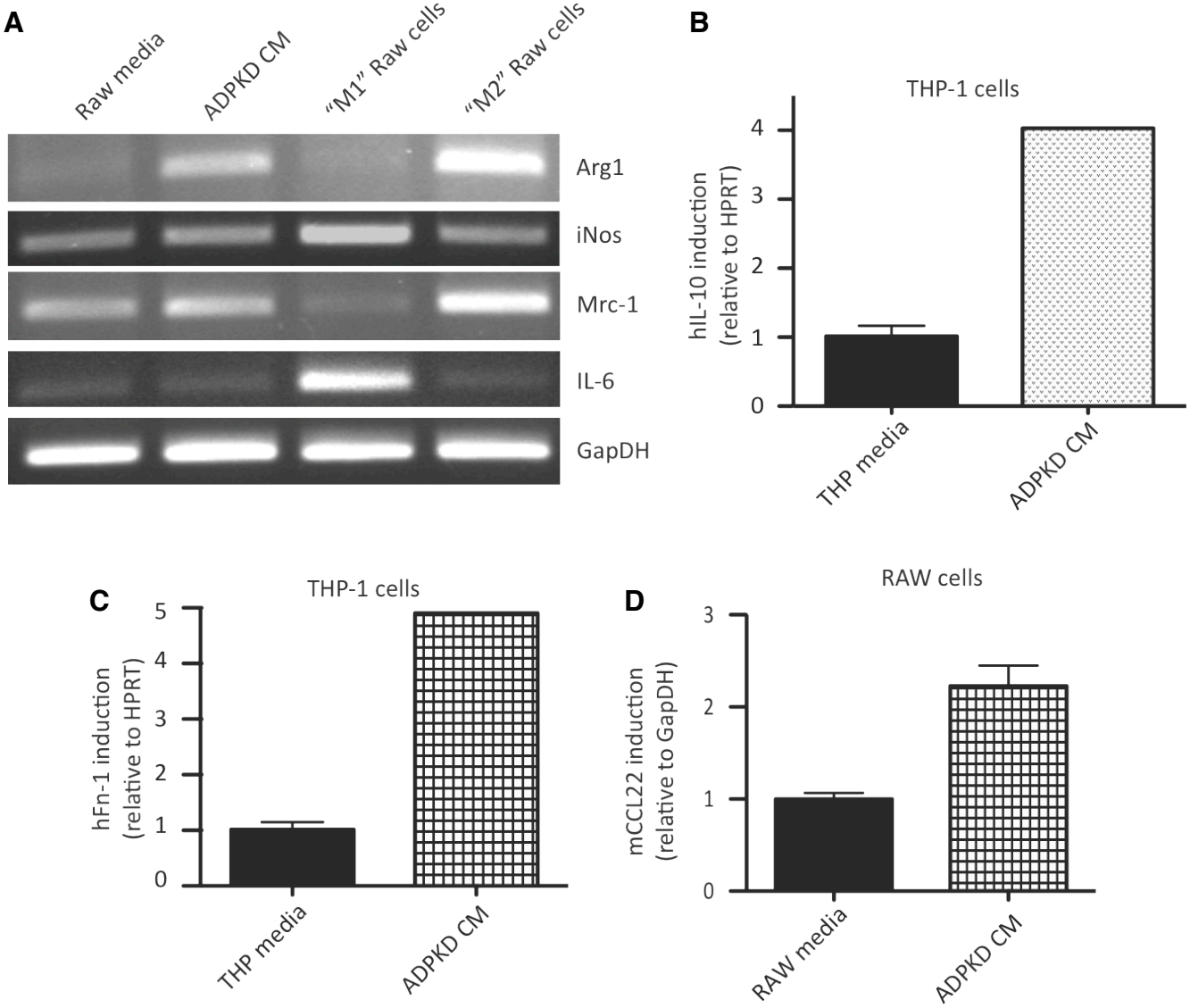


Figure 2.4: The MΦ polarizing effect of ADPKD cells is transmitted through CM. **(A)** RAW macrophages were treated with ADPKD CM for 18 hours and end-point PCR was run to look at polarization markers. CM-treated macrophages had a distinctly different expression profile when compared to cells exposed to IFNγ (M1) or IL-4/IL/13 (M2). **(B)** THP-1 macrophages were were treated with ADPKD CM for 18 hours. Il10 expression is significantly higher ($p < 0.05$) in THP-1cells exposed to ADPKD CM naïve RAW cells. **(C)** THP-1 macrophages were were treated with ADPKD CM for 18 hours. The expression of Fn-1 (M2 marker [2]) was upregulated significantly over baseline. **(D)** RAW macrophages were exposed to ADPKD CM for 18 hours. The expression of CCL22 (M2 marker [3]) was upregulated significantly over baseline. **B-D** were analyzed using quantitative RT-PCR.

Freezing or boiling the media had little effect on the polarization effect (data not shown). Collectively, these data indicate that soluble, heat-stable factor(s) secreted by ADPKD cells are responsible for the polarization of macrophages.

ADPKD cell secreted factors can polarize non-naïve macrophages.

Since macrophages that are recruited to the kidney in injury states have been shown to take on an M1 state, at least initially, the effects of cyst cell factors on non-naïve macrophages was assessed. To accomplish this, ADPKD cells were co-cultured with RAW cells that had been first polarized to M1 or M2 by treatment with either IFN-γ or IL-4/IL-13, respectively. For M1 macrophages, co-culture with ADPKD cells resulted in robust induction of *Arg1* and a diminishment in the M1 marker *Il6* (**Figure 2.5**, lane 4). For M2 macrophages, ADPKD co-culture enhanced *Arg1* and dramatically reduced *Mrc1* induction (**Figure 2.5**, lane 6). These results indicate that ADPKD cells can promote M2-like conversion of polarized, as well as naïve, macrophages and suggest that renal tubule cells may promote phenotypic conversion of previously polarized macrophages *in vivo.*

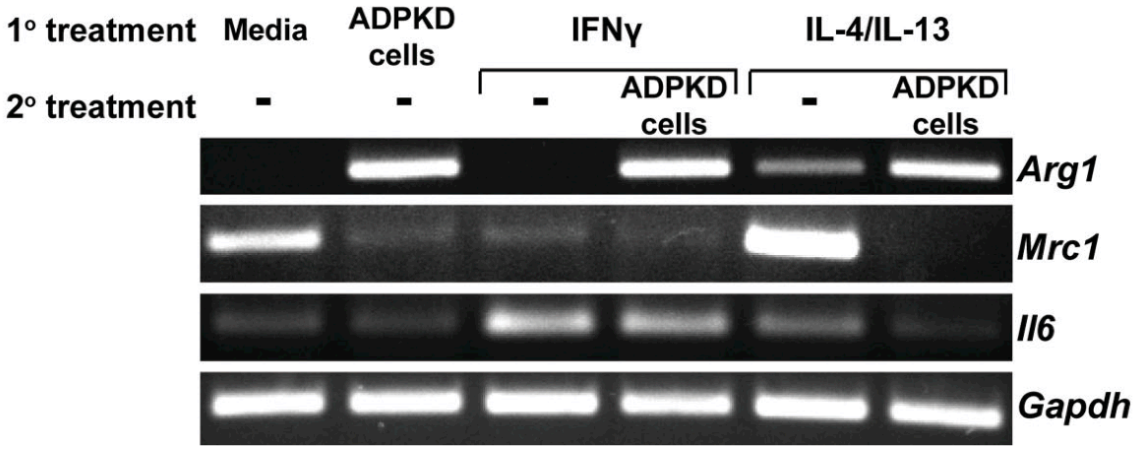


Figure 2.5: Previous MΦ stimulations can be reversed by the presence of ADPKD factors. Semi quantitative RT-PCR of the indicated mouse gene transcripts from lysed cell samples following 18 h culture of RAW macrophages with media (lane 1), with ADPKD cells (lane 2), or following treatment with either IFNγ (lanes 3-4) or IL-4 and IL-13 (lanes 5-6) for 18 h prior to either no further treatment (lanes 1-3 and 5) or a secondary treatment with ADPKD cell co-culture for 3 d (lanes 4 and 6).

MΦ Treatment:	ADPKD co-culture	ADPKD CM	"M2" stimuli	"M1" stimuli
Arg1	strongly ↑	strongly ↑	strongly ↑	strongly ↓
Il10	strongly ↑	strongly ↑	↑(data not shown)	↓(data not shown)
Mrc1	**no change**	**slightly ↑**	**strongly ↑**	**strongly ↓**
Fn1	not observed	strongly ↑	not observed	not observed
CCL13	strongly ↑	not observed	not observed	not observed
CCL22	slightly ↑	slightly ↑	not observed	not observed
iNos	strongly ↓	slightly ↓	strongly ↓	strongly ↑
Il6	strongly ↓	strongly ↓	strongly ↓	strongly ↑

Table 2.1: Summary of markers investigated in treated macrophages. This table is a summary of which markers were upregulated or downregulated, and to what relative extent, in the previous figures. *Mrc1* is highlighted to emphasize the difference in expression levels between classically M2-activated macrophages and ADPKD-activated macrophages.

4. Discussion

The studies in this chapter demonstrate that most macrophages in ADPKD, ARPKD, and mouse *cpk* kidneys express M2-like markers. Thus, M2-like macrophages are present regardless of the underlying genetic abnormality. Further, the *in vitro* experiments indicate that soluble molecules secreted by the cyst cells drive this differentiation. It should be noted that the expression profile of NHK and ADPKD exposed cells is similar but more pronounced with ADPKD cell exposure. While these isolated “normal” cells are not cystic, their harvest likely provides stresses to the cells. It is relevant that abundant M2-like macrophages are not found in NHK. The fact that NHK cells also elicit a response from macrophages indicates that the polarizing signal originating from the epithelial cells is not due to a mutation per se, but rather to a state of injury or stress that the cells are experiencing. This theory is further supported by the observation that PKD cells all elicit some type of macrophages response, some stronger than others yet still identical, despite the fact that the disease-causing mutations are different in each sample. Therefore, the data presented in this chapter shows that the state of chronic injury induced by gradual and constant cyst expansion exerts an influence on infiltrating macrophages, resulting in a similar yet distinct polarization state to that found in acute injury models.

Macrophages can express a variety of genes based on their microenvironment, thus leading them to be loosely grouped, as discussed in chapter 1. In fact, macrophage phenotypes may exist as a continuum, rather than

discrete steps. The exact function of all of the different expression profiles is unknown and most likely depends on the organ and disease in which they are found.

While the expression profile of macrophages *in vitro* does not always correlate to the observed macrophage function *in vivo*, there are often similarities that can be observed between the two. Often, the expression profile of immune cells *in vitro* can be helpful in directing researchers to examine previously studied similar profiles. For instance, though there is no human equivalent to *Arg1* expression in mouse macrophages, it is a meaningful and reliable marker that indicates an M2-like state, and often the loss of its expression correlates with a leaning towards an M1 state. Similarly, and perhaps more importantly, the expression of the M2 cytokine IL-10 has been extensively studied both *in vitro* and *in vivo*. Though *in vivo* studies rarely exactly recapitulate *in vitro* findings, observations made in cell culture have strongly correlated with those made in animal models and human patients with regards to IL-10.

The significance of IL-10 in disease was discussed extensively in Chapter 1, but due to the expression profile laid out in this chapter, a short summary would be useful. Essentially, in most cases, the loss of *Il10* expression or the absence of M2 macrophages leads to a slowed healing process and prolonged repair phase. Many studies indicate that the presence of M2 macrophages is essential for the formation of new tissue and the recovery of injured yet functional tissue. This is opposed to the presence of M1 macrophages, which play a critical

role in the case of infection or presence of pathogens. The extended presence of these M1s, however, is detrimental to recovery due to uninhibited inflammation. IL-10 plays a critical role in inhibiting inflammatory processes and in somehow contributing to epithelial proliferation. Examination of the role of this cytokine in PKD will be performed in chapter 3.

As stated above, in most cases of injury, this process is necessary for recovery. However, in the case of chronic injury in which the source of injury is never removed, and specifically in the case of a hyperproliferative environment such as PKD, the presence of macrophages which are secreting possible proliferative cytokines could be a confounding factor of disease progression.

Chapter Three

The Phenotypic Characterization of Macrophages Present in Polycystic Kidney Disease

1. Introduction

The pathological process of cyst enlargement in PKD is hypothesized to be largely the result of aberrant proliferation of cyst-lining epithelial cells, though fluid secretion also plays a role [106, 107]. The mechanisms that underlie the hyperproliferative phenotype of cyst cells are complex, but defects caused by mutations in PC1 and/or PC2 have been shown to be the primary drivers of the proliferation [108,107]. In particular, mutant collecting duct epithelial cells, which are the origin of most cysts in human ADPKD and ARPKD, show abnormal proliferative responses to cAMP [30, 107 109]. In the collecting duct, cAMP production is primarily driven by vasopressin *in vivo* [110, 111]. Other signaling abnormalities also are believed to contribute to proliferation, including responses to EGF, TGF, and Ca^{2+} [112, 113].

The proliferative tendency of these cystic cells has been the subject of much research, as agents that target this process are predicted to ameliorate disease. In fact, targeting proliferation has been shown to be a potentially successful approach for PKD therapy. Drugs that reduce cell proliferation, such as inhibitors of Src kinase, Erb-B1 (EGFR), mTOR, and MEK have all been shown to reduce cystic disease in mouse models [19, 27, 112, 114]. Additionally, a Raf

inhibitor was found to inhibit *in vitro* cAMP-dependent cyst proliferation and growth in human ADPKD cells [19, 107]. Antagonists of the V2-vasopressin receptor, which is highly expressed in collecting duct, also reduces cyst expansion in animal models of disease [110, 111]. One such antagonist, tolvaptan, has also recently been shown to have efficacy in slower cyst expansion in patients with ADPKD [18, 115]. Unfortunately, tolvaptan showed hepatic toxicity and other side effects that prevented its FDA approval. Thus, new strategies that target proliferation are needed.

As previously noted in Chapter 1, macrophages have been found to promote proliferation in a multitude of diseases. Specifically, in kidney injury models macrophages, especially M2-like macrophages, can promote proliferation as a part of the healing process. If these macrophages are removed, recovery is slower due to decreased proliferative capability, and the addition of M2-programmed macrophages promotes renal cell proliferation and accelerates recovery from injury [56, 72, 77, 80]. As described in chapters 1 and 2, M2-like macrophages are present in both human PKD and animal models of disease. Also, depletion of macrophages from animal models of disease results in slowed disease progression and lower proliferation [4, 37]. In this chapter, macrophages are shown to directly stimulate proliferation of cyst epithelial cells. Importantly, exposure of macrophages to cyst epithelial cells “programs” them, promoting differentiation to a phenotype that is particularly effective at promoting cyst cell proliferation. Further, we show that autocrine macrophage signaling of a

regulatory cytokine, IL-10, is critical for this programming step. These data lead me to conclude that a vicious cycle exists to promote disease progression: cystic epithelial cells promote the M2-polarization of macrophages and M2 polarized macrophages promote cystic epithelial cell proliferation, thereby promoting cyst growth.

2. Methods

Media: PKD_A media is DMEM/F-12 (Cellgro 15-090-CV, Mediatech Inc.; Manassas, VA) supplemented with 5% FBS, 15 mM HEPES, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml ITS, P/S, and 5 ng/ml EGF. XVIVO-10 (Bio Whittaker, Walkersville, MD) contains L-glutamine, gentamicin, phenol red, P/S, and 10 mM HEPES buffer. Minimal media is DMEM (D6429, Sigma-Aldrich) with P/S and 0.1% FBS. Maximal media is DMEM with P/S and 10% FBS. Basal media is DMEM with P/S and containing no FBS. ATP Depletion media is (glucose-free DMEM [11966, Life Technologies] containing 2% FBS,10 mM deoxyglucose and Pen/Strep. Bone Marrow (BM) media is DMEM containing 10% HIFBS, 2 mM L-glutamine, Pen/Strep, and 10% L-cell Conditioned Media. L929 media is DMEM (D6429; Sigma) media containing the following; 10% HIFBS (S11550,Atlanta Biologicals), 2mM L-glutamine, and Pen/Strep.

Conditioned media (CM): ADPKD CM was made by plating 3.7 x 10^6 ADPKD cells into a 15 cm plate. Media was replaced after 24 hours with 20 ml XVIVO-10 (Bio Whittaker, Walkersville, MD) and collected after 3 days. The media was then spun down at 600 RCF for 5 minutes, aliquotted, and stored at -

80°C until needed. LCCM (L-cell Conditioned Media) media is obtained by seeding L929 cells at 50% confluency in a 15 cm dish and allowing them to grow for 5 days in 25 ml of media. After 5 days the media is collected, spun down at 600 RPM for 5 minutes, filtered at 0.25 μm, and stored at -20°C.

Cells: PKD_A cells [116] (immortalized proximal tubule cell line) were used in proliferation assays because they respond to programmed conditioned media, though they are incapable of programming macrophages themselves. PKD_A cells were carried and used for 20 passages before being replaced, and were checked periodically for morphological or division abnormalities and changes. RAW cells were maintained in DMEM media containing 10% FBS, 200 uM L-glutamine, and Pen/Strep. THP-1 monocytes were maintained in RPMI-1640 media (R8758, Sigma-Aldrich, St. Louis, MO) containing 10% FBS, 200 µM L-glutamine and Pen/Strep.

ELISA: Macrophages were cultured for 24 hours before treatment, after which conditioned media were collected and centrifuged, stored at 4°C until all samples had been collected. The supernatant was analyzed for IL-10 using either the Mouse IL-10 ELISA Ready-SET-Go kit (eBioscience, cat #88-7105) in the case of RAW 264.7 cells or the Human IL-10 ELISA Ready-SET-Go kit (eBioscience, cat #88-7106) in the case of THP-1 cells.

Neutralizing Assays: Neutralization of mouse IL-10 was carried out using a mouse-specific anti-IL-10 antibody obtained from eBioscience (cat #16-7102-85) or the IgG isotype control (cat #14-4714-82). At the appropriate time, antibodies

were added directly to both the counted and designated macrophages cells in 1.5 ml eppendorf tubes as well as to the wells containing primary ADPKD cells without any pre-incubation period. The antibodies, contained in the media, were only removed once the experiment was collected for analysis.

Proliferation Assays:

BMDM. Transwell proliferation assays were conducted with primary ADPKD cells plated at a density of 2.0 x 10^5 cells per well in a flat-bottomed 6-well plate. Cells were incubated for 24 h then placed into an ATP depletion media for 24 hours, after which the PKD cells were placed into 2 ml of minimal media or maximal media. Transwells (0.4 μm) were then placed into the wells and BMDMs were plated onto the transwell in 1.0 ml of minimal media at a density of either 2.0 x 10^5 or 5.0 x 10^5. Mouse IL-10 (R&D) was added to both the top and bottom of the transwells at a final concentration of 20 μg/ml when appropriate. Once combined, cells were incubated for 3 days, after which the transwell was removed and the media was aspirated. The PKD cells were then collected into 100 μL of 2% PFA in PBS and stored at 4°C for 4-6 days, after which they were counted on a hemacytometer.

RAW 264.7. Direct co-culture proliferation assays were performed using RAW cells and an anti-IL-10 antibody (R&D) or IgG isotype control (R&D). ADPKD cells were plated at a density of 5.0 x 10^4 cells per well in a flat-bottomed 6 well dish, incubated for 24 hours, switched into ATP depletion media for 24 hours, and then RAW cells were plated on top at a density of 5.0 x 10^4 in minimal

media. Mouse-specific IL-10 neutralizing antibody (R&D) or the IgG control (R&D) was added to the appropriate wells at a concentration of 1 μg/ml. These co-cultures were collected in 2% PFA/PBS after 3 days, stored at 4°C for 4-6 days, and then manually counted on a Leica microscope (DMILFluorescence Microscope) using a hemacytometer. ADPKD and RAW cells were readily distinguished by morphology.

MΦ CM. All other proliferation assays were carried out without direct macrophage and ADPKD cells contact during the proliferation phase. PKD_A cells were plated in flat-bottomed 24-well plates at a final density of 1.2×10^4 cells. They were switched into ATP depletion media after growing overnight. After 24 hours, the media was replaced with programmed media, minimal media, or maximal media. All proliferation assays were collected after 3 days. The media was aspirated and the cells were washed once with 1X PBS then frozen at -80°C for 18-24 hours. Proliferation was then assayed using the Cyquant Cell Proliferation Assay Kit from Life Technologies, measuring fluorescence using a Synergy 2 from Biotek (BioTEK Instruments, Inc., Winooski, VT). Programmed media was made using RAW cells plated at a density of 1.0×10^6 or THP-1 cells plated at a final density of 2.0×10^6, placed in XVIVO or ADPKD CM (made in XVIVO) for the appropriate amount of hours (usually 18), and then switched into basal media for 24 hours. The programmed basal media was then collected and spun at 600 x g for 5 min, brought up to a final concentration of 0.1% FBS, and placed onto ATP-depleted PKD cells. The addition of inhibitors or cytokines took

place after the serum adjustment but before the media was placed onto the PKD cells.

Mice:

Strains and genotypes: All animal use was approved by the IACUC. Mice were housed according to LAR guidelines. Both the *cpk* [117] and *Il10* (Jackson Laboratories, C.129P2(B6)-*Il10t*^{m1Cgn}/J) mice were bred into and carried in a Balb/C background. All animals used for the IL-10 study were provided with Ciprofloxacin, supplementing their drinking water at a final concentration of 200 mg/l. This was done to prevent the early onset of intestinal irritation and subsequent severe bowel disease that occurs in *Il10 null* [118, 119] mice. In addition, all breeder mice were carried as double heterozygous pairs (*cpk* +/-: *Il10* +/-) as *cpk null* mice die before sexual maturity and *Il10 null* mice were only able to birth one litter before succumbing to bowel disease. Kidneys were collected and placed into RNAlater or formalin once blood had been collected from the thoracic cavity. All mice used for kidney collection were sacrificed at PN day 10. Sera was collected and stored for BUN analysis, the right kidney was collected and placed in RNAlater for RNA or protein analysis, and the left kidney was collected and placed in formalin for sectioning and IHC analysis.

Isolation of mouse Bone Marrow Derived Macrophages (BMDM): Balb/C mice were sacrificed and bone marrow was harvested from the tibias and femurs of male mice aged 6-8 weeks. Bones were removed from the mice, cleaned, stripped of all tissue with the use of forceps, placed in cold PBS, and

rinsed with 70% ethanol. Ends were cut from each bone and marrow was extracted using sterile technique with a 21 G needle and sterile PBS into a 50 ml conical tube. Cells were centrifuged at 4°C and 1200 RCF for 5 minutes, resuspended, and then plated at a density of 5.0×10^6 cells per plate in sterile non-TC coated dishes. Cells were plated in 10 ml of BM media and incubated at 37°C in 5% CO_2 for 3 days, after which 4 more ml of BM media were added and the cells were incubated for another 3 days (6 days total). After 6 days, all non-adherent cells were removed, and the adherent cells (macrophages) were placed into 5 ml of cold PBS and incubated at 4°C for 30 minutes. After incubation, the cells were scraped from the dish and centrifuged at 1200 RCF for 5 min. Cells were re-suspended in L929 media and the desired number (see Proliferation Assay Methods and figure legends) plated onto a transwell for use in proliferation assays.

3. Results

Macrophages promote cyst cell proliferation. (co-culture, transwell; RAW, THP-1, and primary BMDM)

To assess effects of macrophages on PKD cyst cell proliferation, primary ADPKD cells were cultured with a mouse macrophage cell line (RAW264.7 cells). Co-culture of the cyst cells with macrophages induced epithelial cell proliferation (**Figure 3.1**), as assessed by an increase in ADPKD cell number [4].

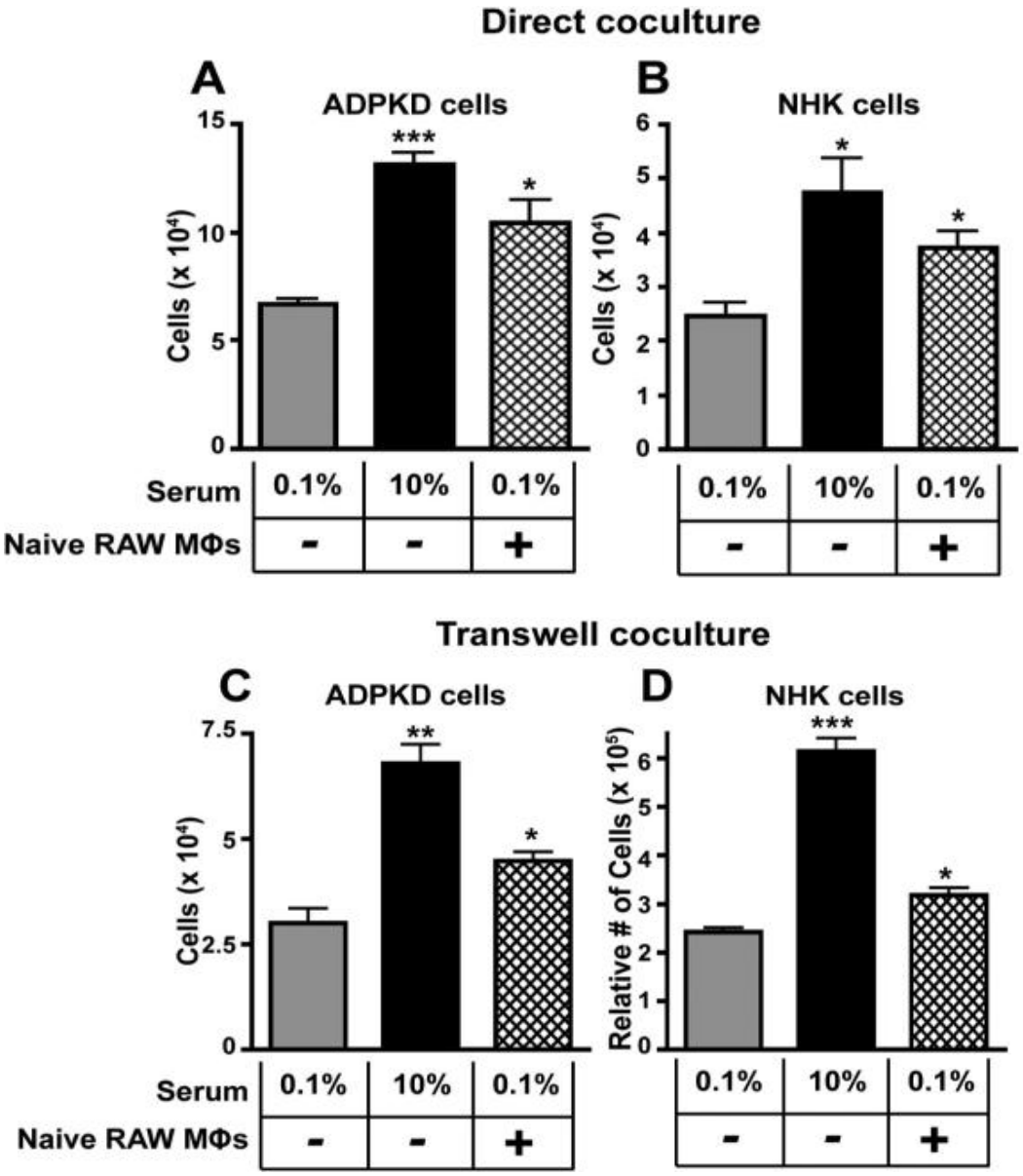


Figure 3.1: Macrophages elaborate soluble factor(s) that promote ADPKD cyst cell and NHK proliferation. **(A)** Primary ADPKD cyst epithelial cells (kidney K354) or **(B)** NHK cells (kidney K342) were incubated with low (1% FBS) or high (10% FBS) serum media or co-cultured with naïve RAW macrophages suspended in low serum media. After 72 h, cells were collected and fixed and the number of ADPKD cells determined by counting. The data are representative of four similar experiments for ADPKD cells from different kidneys. **(C and D)** Cell-impermeable transwell inserts were placed in tissue culture wells previously seeded with primary ADPKD (**C**) or NHK (**D**) cells (Kidneys K338 and K343, respectively). Naïve RAW macrophages in low serum, low serum media alone, or high serum media were placed in these inserts, and the kidney cells were collected after 72 h and counted (C) or relative number of cells determined by lysis and incubation with CyQUANT® GR dye (D). Data are presented as mean ± SEM. *, **, and *** denote $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Taken from Swenson-Fields[4] *et al.*

Similar results were observed when primary bone marrow-derived mouse macrophages (**Figure 3.5**) or human macrophage-like cell line (**Figure 3.1**) were incubated with the primary PKD cells. Additionally, media taken from plated naïve macrophages, when placed on primary ADPKD cells, increased their proliferative capacity over that of control (normal media) treated primary ADPKD cells. Macrophages promote ADPKD cell proliferation.

Cyst cells promote development of pathogenic (pro-proliferative) macrophage phenotype in PKD.

As shown in Chapter 2, PKD cyst cells elaborate factor(s) that promote differentiation of macrophages to a distinct M2-like phenotype, defined by increased expression of *Arg1*, *Il10* and *Fn1* and the absence of M1-associated cytokines such as *Il6* and *iNos*. While markers are useful tools to define differentiation, it is unknown whether this differentiation state influences macrophage function in PKD. A particularly relevant question is whether this differentiation state affects the ability of macrophages to promote cyst cell proliferation, since that activity would promote disease progression.

To address this hypothesis, macrophages were exposed to PKD cell secretions by incubation with ADPKD conditioned media ("programmed"), and their subsequent ability to promote PKD cyst cell proliferation was measured. The experimental design is shown in **Figure 3.2**.

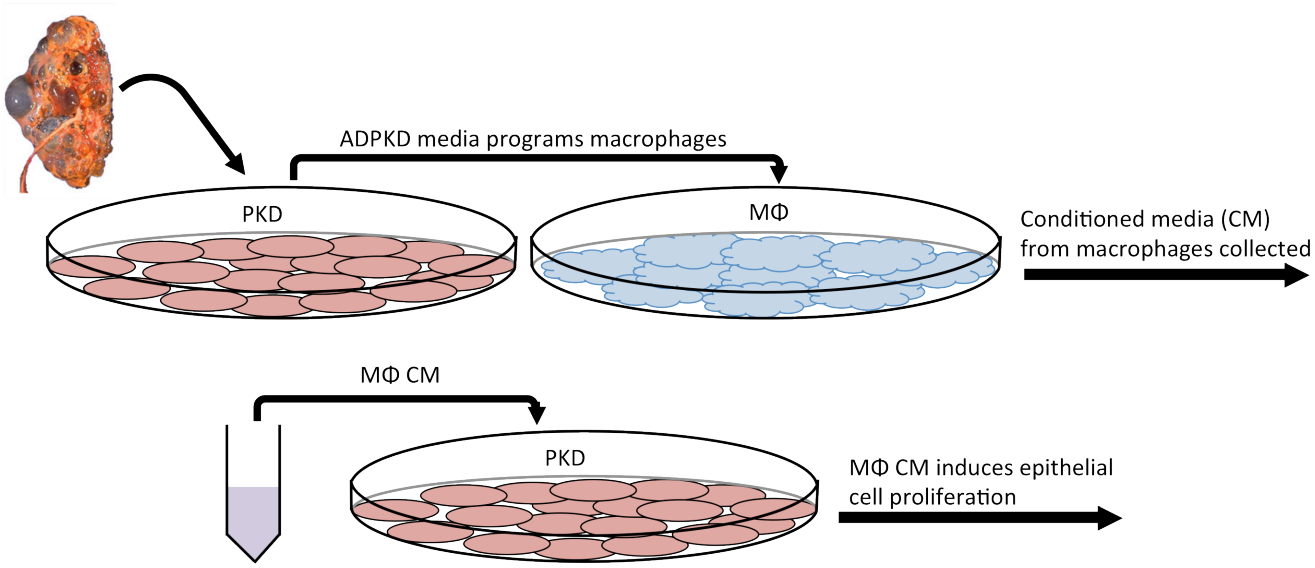


Figure 3.2: Model of CM Proliferation Assays. Primary ADPKD cells were seeded in their normal media overnight. Media was then replaced with XVIVO10 media and collected after 3 days. This is referred to as ADPKD CM. The ADPKD CM is then placed onto plated RAW or THP-1 macrophage cells for 18 hours to program the macrophages. After 18 hours, the ADPKD CM is replaced with 0.1% media (minimal media) for 24 hours. After 24 hours, the meida is collected off of the macrophages cells. This is referred to as MΦ CM. The MΦ CM is then placed onto PKD cells for 3 days, after which point the treated PKD cells are collected and proliferation is assayed.

ADPKD cells were seeded at a specific concentration (see Methods) and allowed to grow in their own media overnight. They were then switched into XVIVO or RAW media, depending on the experiments, for 72 hours, at which point the media was collected and stored (CM). This CM was then used to program macrophages for an appropriate amount of time (usually 18 hours), after which they were switched into basal media. After 24 hours, this media was collected and placed onto PKD-A cells, which were collected and assayed after another 72 hours.

As illustrated in **Figure 3.3**, mouse RAW macrophages programmed by ADPKD CM secreted factors that promoted cyst cell proliferation. Similar results were observed using human-derived macrophages (from THP-1 cells). These data indicate that cyst cells and macrophages are capable of reciprocal signaling in PKD: cyst cells promote differentiation of macrophages to an M2-like state, a pathogenic phenotype in PKD that is particularly effective at promoting cyst cell proliferation.

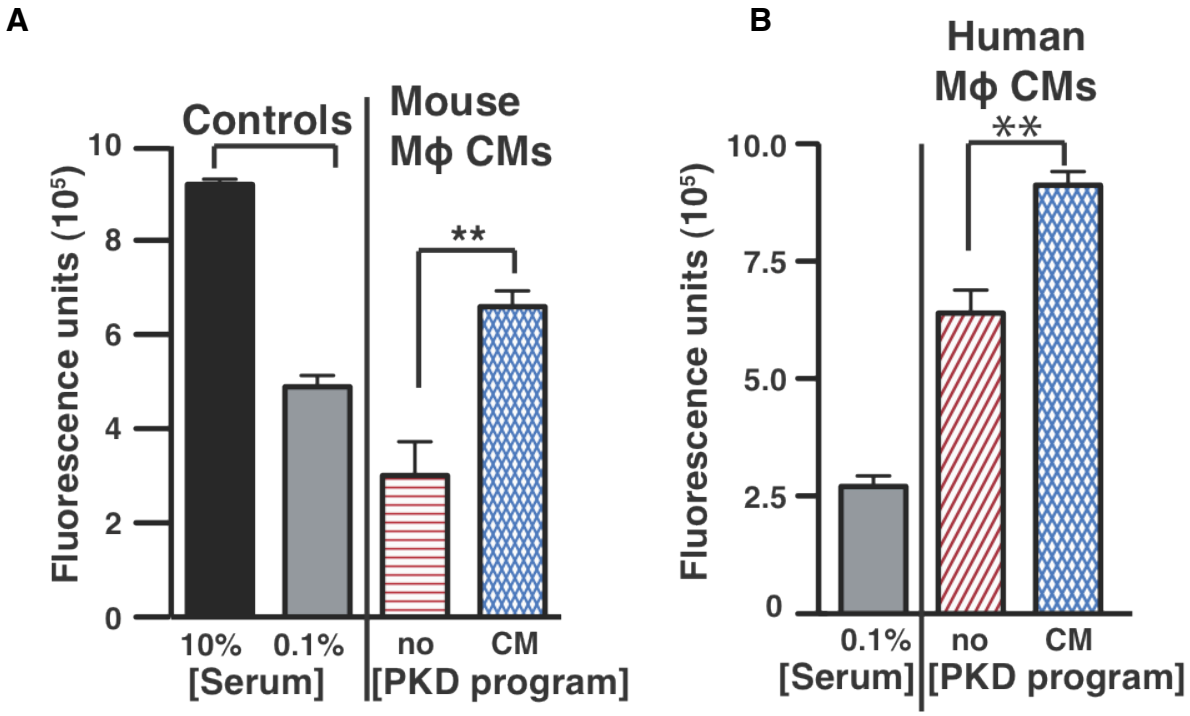


Figure 3.3: ADPKD CM induces a pro-proliferative MΦ reaction. PKD-A cells incubated for 72h with CMs obtained from **(A)** RAW macrophages or **(B)** THP-1 macrophages previously cultured alone (No program) or from those pre-programmed by incubation with ADPKD CM. FBS (0.1%) was added to CMs prior to assay. This experiment was repeated using CMs from ADPKD cyst cells prepared from more than 5 other kidneys with similar results. Data are presented as mean ± SEM. Asterisks ** denote $P<0.01$, respectively.

The M2 cytokine IL-10 is required for development of the pathogenic macrophage phenotype.

As discussed above, the pathogenic macrophage in PKD expresses a number of known M2-like markers. Of these markers, those that are critical for differentiation may be therapeutic targets for PKD. To identify crucial markers, attention was initially focused on IL-10, a regulatory cytokine that we have shown is upregulated in programmed macrophages (Chapter 2). Intriguingly, a recent report suggested that IL-10 expressing macrophages promote myoblast proliferation in the context of muscle injury [48].

To determine whether IL-10 was relevant to the pro-proliferative macrophage, the timing of induction of the pro-proliferative phenotype was compared to induction of IL-10 in RAW cells. Macrophages were exposed to ADPKD CM for increasing amounts of time, with the shortest collection occurring after 30 minutes and extending through 24 hours (**Figure 3.4**). Media was added in decreasing order, so that all of the media was collected from programmed macrophages at the same time and placed onto PKD-A cells. As shown in **Figure 3.4 (A** and data not shown**)**, significant macrophage pro-proliferative activity appeared after ~12 h of programming by ADPKD CM and was present through 24 h.

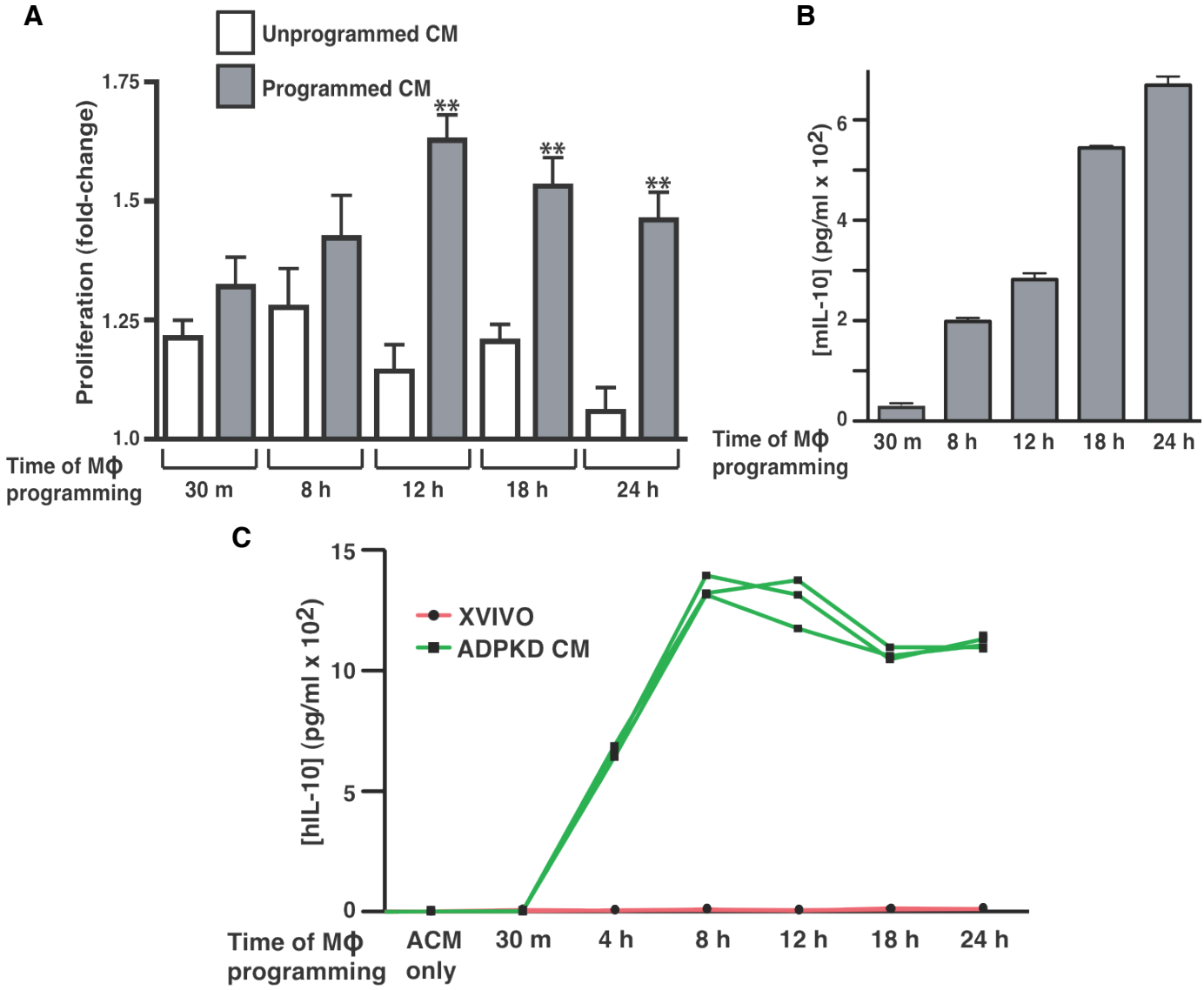


Figure 3.4: IL-10 secretion by MΦ correlates with the appearance of the pro-proliferative MΦ phenotype. (A) RAW macrophages were programmed for increasing time periods with ADPKD cyst cell CM, or were incubated with media alone. Media was collected on ice and cells were subsequently washed and incubated with basal media for an additional 24 h to produce either Programmed or Unprogrammed CM, which were then assayed for effects on proliferation of PKD-A cells. Data is plotted as fold-change relative to baseline proliferation values determined from PKD-A cells grown in 0.1% FBS in parallel. **(B)** Macrophage-produced IL-10 levels were measured in the ADPKD cyst cell CM used for programming described in (A), which was collected and stored on ice, by the use of an ELISA kit specific for mouse IL-10. **(C)** The levels of human IL-10 were measured in ADPKD cyst cell CM used for programming THP-1 macrophages for increasing time periods. The levels of human IL-10 present in ADPKD cyst cell CM prior to programming and after 30 m of programming, as well as those levels present in macrophages incubated in parallel in media only (data not shown) were below the detection limits (30 pg/ml) of the ELISA kit used.

To examine IL-10 induction, RAW cells or THP-1 cells were treated with conditioned media as mentioned above, and media not used for proliferation assays was collected and stored in siliconized tubes at 4°C for analysis. ELISA plates were coated with IL-10 capture antibody overnight to maximize sensitivity and then media was added and the assay performed. As shown in **Figure 3.4 (B)**, induction of significant IL-10 (~200 pg/ml) occurred by 8 h and increased throughout the programming time course. The induction of human IL-10 similarly required ~8 h (**Figure 3.4 (C)**). These data indicate that IL-10 induction preceded induction of the proliferative phenotype, suggesting that IL-10 could potentially contribute to its development.

To investigate the contribution of IL-10 directly, an IL-10 neutralizing antibody was used. ADPKD cells were seeded and grown overnight, at which point they were rinsed and placed into minimal media. RAW macrophages were brought up and counted in minimal media (0.1% media), at which point the correct treatment (IL-10 antibody or isotype control) was added. The treated and counted macrophages were then placed on top of the ADPKD cells and the cells were co-cultured for 72 hours before harvesting and fixing. The cells were then counted with the identity of the wells blinded to the evaluator. The distinct morphologies of RAW cells and ADPKD cells facilitated counting. The experiment was repeated twice with the appropriate number of technical replicates. As shown in **Figure 3.5**, anti-IL-10 but not the IgG isotype control significantly inhibited macrophage-stimulated PKD cell proliferation. Since mouse

macrophages were used in this experiment and mouse IL-10 does not have cross-species activity, it is most likely that IL-10 is affecting the macrophage phenotype rather than the epithelial cells directly. To formally address this possibility, mouse IL-10 was added to PKD cells directly and proliferation assessed (**Figure 3.5 (B)**). As illustrated, IL-10 does not have a pro-proliferative effect on PKD cells. Similar experiments were performed with human IL-10 (**Figure 3.5 (C)**) with comparable results, that is, human IL-10 did not stimulate PKD cyst cell proliferation.

To assess the contribution of IL-10 in primary, non-immortalized cells, bone marrow-derived macrophages (BMDM) from wild-type (WT) or IL-10 knockout (IL10KO) mice were incubated with ADPKD cyst cells and effects on proliferation measured. As shown above, WT BMDM stimulated proliferation (**Figure 3.6**); however, IL10KO macrophages showed no pro-proliferative activity. Strikingly, addition of exogenous IL-10 to the IL10KO macrophages completely rescued the pro-proliferative phenotype. These data indicate that IL-10 is a critical, autocrine regulator of pathogenic macrophage differentiation in PKD.

To determine whether IL-10 was sufficient for development of the pathogenic, pro-proliferative macrophage phenotype, macrophages were programmed with IL-10 alone. Interestingly, exogenous IL-10 alone did not induce the pro-proliferative phenotype (**Figure 3.6 (D)**). These data suggest that, while IL-10 is necessary, it is not sufficient for pathogenic macrophage differentiation in PKD.

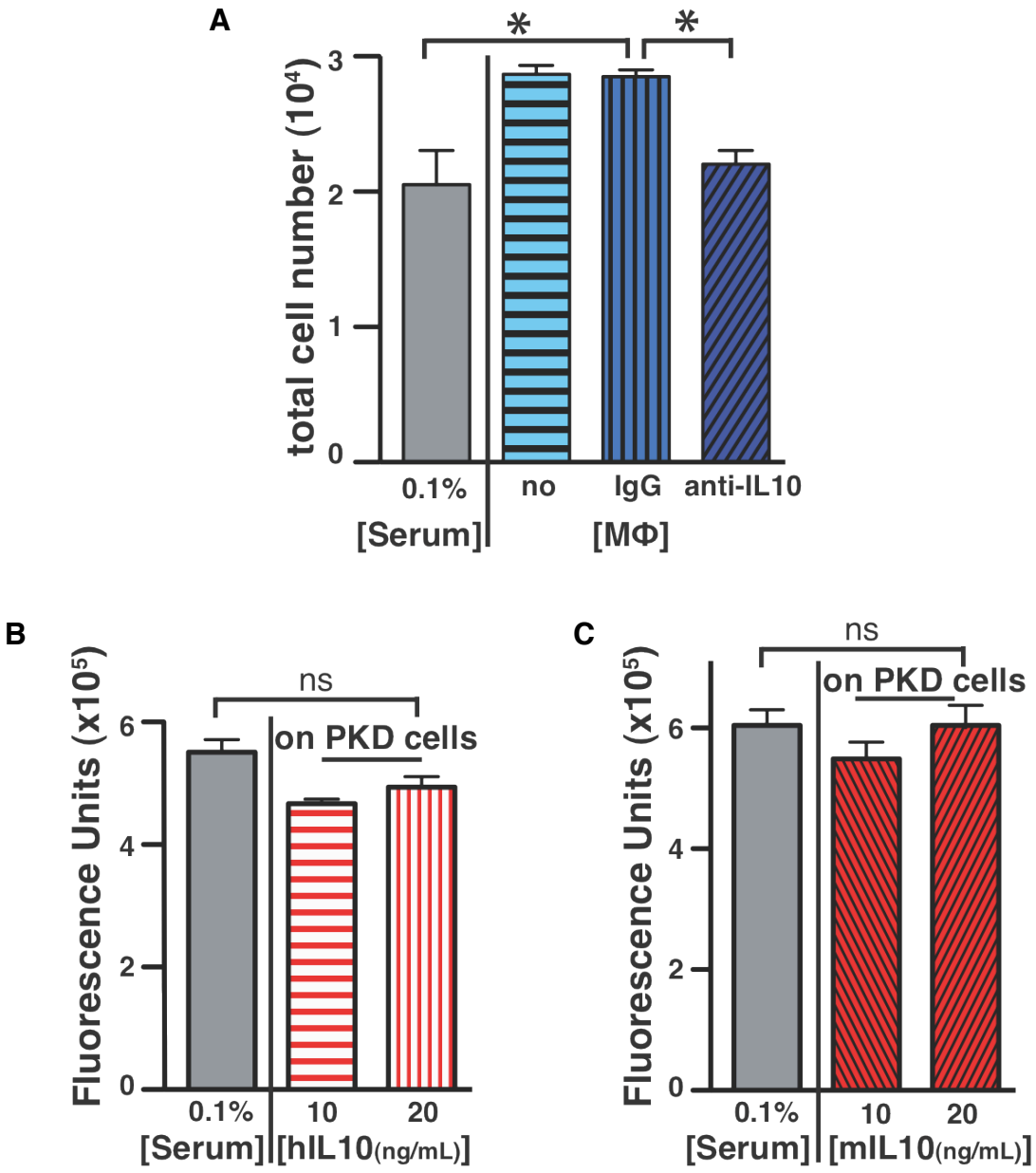


Figure 3.5: IL-10 is necessary for the proliferative MΦ phenotype but is not the proliferative factor acting upon the epithelial cells. **(A)** Proliferation assay of ADPKD cyst cells directly cocultured with RAW MΦs in the presence of 0.1% FBS and either a mouse-specific IL-10 neutralizing antibody (2 ug/ml), a non-immune IgG control antibody (2 ug/ml) or no additions (none). These cells also were incubated alone in parallel with 0.1% FBS to measure baseline proliferation, or 10% FBS to measure stimulated proliferation. After 72h, cells were trypsinized, fixed and the numbers of ADPKD cells determined by direct counting. This experiment was repeated using ADPKD cells from more than 5 different kidneys with similar results. Data are presented as mean ± SEM. **(B)** Proliferation assay of ADPKD cyst cells incubated with either 0.1% FBS media (Control) or the same media containing mouse IL-10 at the indicated concentrations. After 72h, cells were collected and assayed for relative proliferation following lysis and incubation with CyQUANT® GR dye. **(C)** Proliferation assay of ADPKD cyst cells carried out similarly and in parallel with assay described in **(B)** to test the direct effects of human IL-10 at the indicated concentrations.

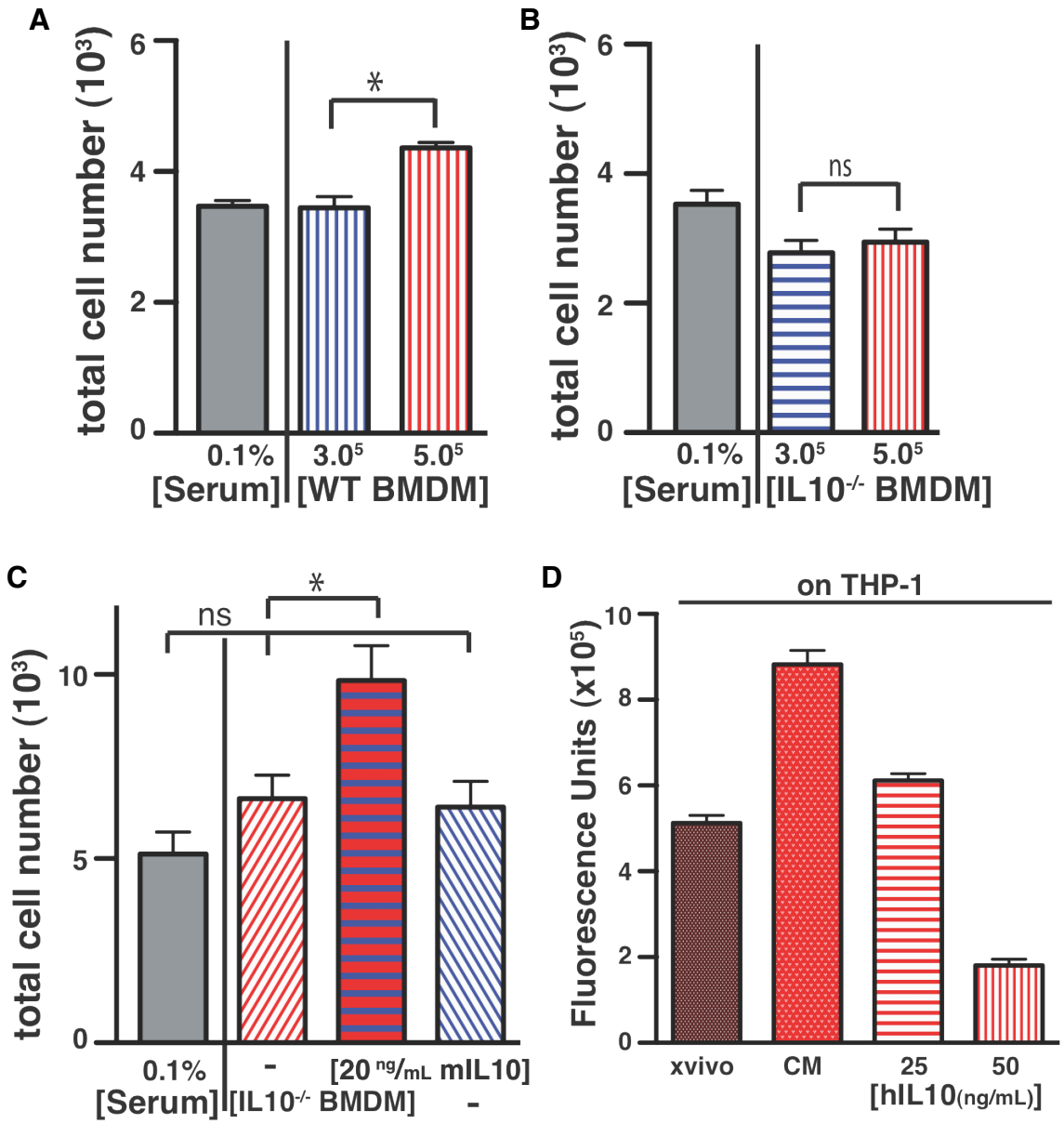


Figure 3.6: IL-10 is necessary for the proliferative MΦ phenotype, which can be restored with the addition of exogenous IL-10. Proliferation assay of ADPKD cyst cells indirectly cocultured (in Transwells) with BMDMs isolated from either **(A)** wild-type (WT) or **(B)** IL-10-deficient (IL-10-/-) mice. Cocultures were seeded in 0.1% FBS. ADPKD cyst cells also were incubated alone in parallel with 0.1% FBS. After 72h, cells were trypsinized, fixed and the numbers of ADPKD cells determined by direct counting. Data are presented as mean ± SEM. **(C)** Proliferation assay of ADPKD cyst cells indirectly cocultured (Transwells) with BMDMs isolated from IL-10-/- mice in the presence or absence of added recombinant mouse IL-10 (20 ng/ml) for 72h. ADPKD cyst cells also were incubated with IL-10 or 0.1% FBS only. Cocultures were seeded in 0.1% FBS. **(D)** Proliferation assay of ADPKD cyst cells incubated with CMs from THP-1 MΦs previously cultured alone (None), with ADPKD cyst cell CM or with human IL-10 in a programming step, prior to washing and a 24 h incubation in basal media. FBS (0.1%) was added to CMs prior to addition of ADPKD cells. In parallel, cells were also incubated with 0.1% and 10% FBS, to measure baseline and stimulated proliferation, respectively. After 72h, cells were collected and assayed for relative proliferation.

Heterozygous loss of IL-10 may slow disease progression in a rapid mouse model of PKD.

To assess the potential relevance of IL-10 *in vivo*, IL10KO mice were bred to cpk mice. IL10KO mice are known to develop inflammatory bowel disease [118, 119], a phenotype that complicated breeding strategies, since these mice were rarely successful in mating. Also, generation of *Il10*$^{-/-}$*; Cys1*$^{cpk/cpk}$ mice did not follow expected Mendelian frequencies, suggesting a deleterious effect of the presence of homozygous mutants in both loci on development. Thus, it was not possible to examine effects global IL10KO on cystic disease in this model.

However, cystic mice that were heterozygous at the IL-10 locus (*Il10*$^{+/-}$*; Cys1*$^{cpk/cpk}$*)* were generated and analyzed. Notably, cystic IL-10 heterozygotes showed a statistically significant decrease in kidney size (2 kidney/bodyweight ratio) compared to to IL-10 wild-type mice (**Figure 3.7 (C)**). Histologic analysis reveals a slight decrease in cystic area, but the results were not statistically significant (**Figure 3.7 (A, B)**). Similar findings were present in assay for renal function (BUN) (**Figure 3.7 (E)**). That is there is a small but not statistically significant decrease, also with a p ~0.1. While these data are not conclusive, they suggest a possible ameliorative effect of diminished IL-10 on cystic disease.

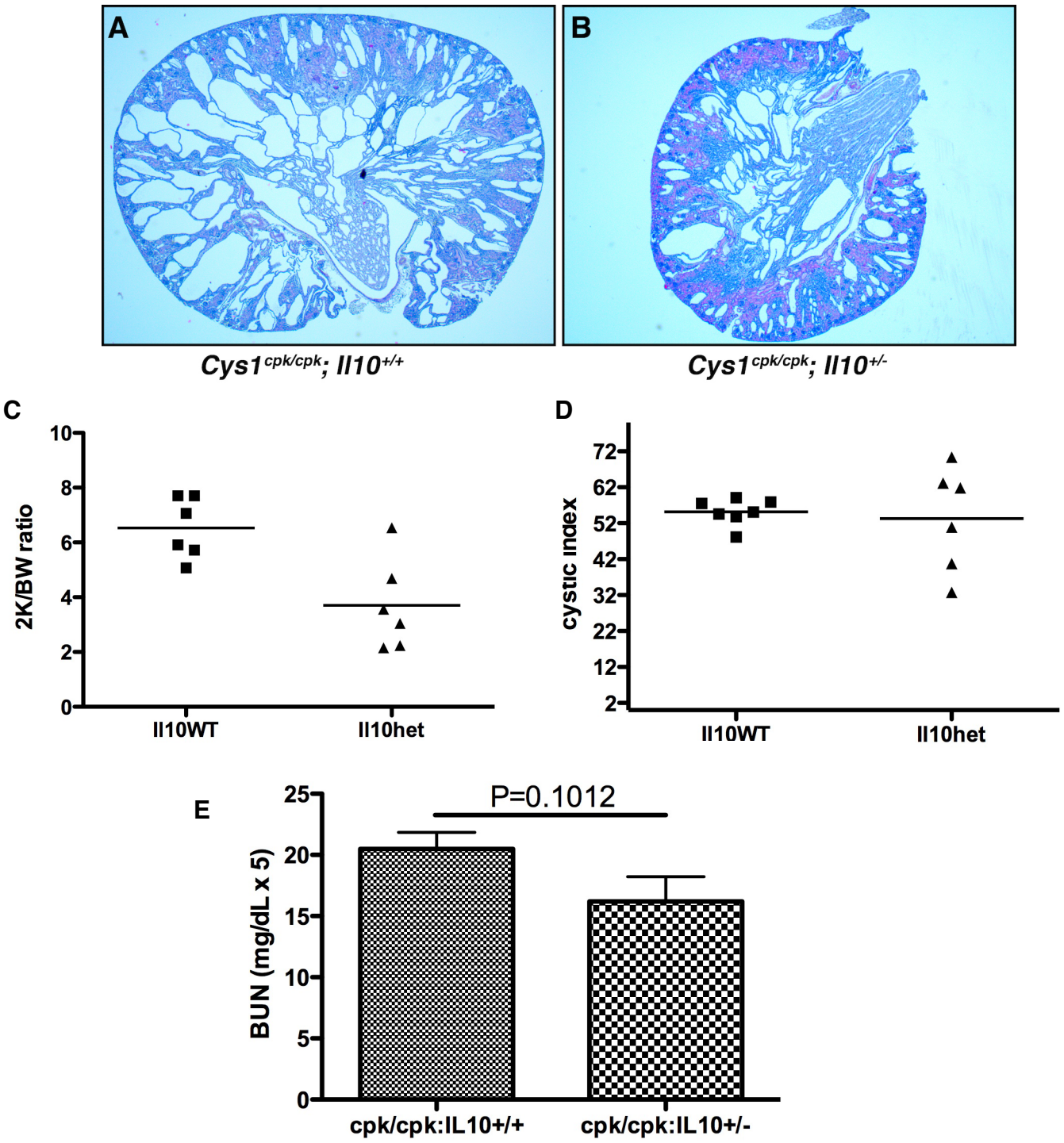


Figure 3.7: The effect of genetic depletion of *Il10* in a rapid cystic mouse model. IHC image of an **(A)** *Il10*$^{+/-}$ and a **(B)** WT control, both mice on the *cpk/cpk* background. This was repeated with 6 mice of each genotype, and these are representative of the two extremes from each group. **(C)** Mice were weighed and then the kidneys removed and weighed immediately after death. **(D)** Average cortical cystic index (cortical cyst area/total cortical area) was calculated from measurements of kidneys from animals on both backgrounds. 6 mice were studied for each genotype. **(E)** Blood was harvested upon euthanasia, spun down, and the isolated sera was frozen at -80 °C. BUN were measured as previously described, using a colorimetric assay.

4. Discussion

As previously discussed, IL-10 has been implicated as an important factor in the recovery of injury, caused either by disease or trauma, in many organs. Macrophages that are present during the repair and regenerative phase are typically classified as M2 macrophages and are commonly characterized by their elevated expression of IL-10. In a study focusing on muscle injury recovery, it was found that IL-10 levels were elevated from three to seven days post-injury. In fact, the macrophages *in vivo* did not reflect a clear M1 or M2 phenotype, but IL-10 was the only cytokine and macrophage marker clearly elevated after the injury occurred, indicating that it played a key role in the healing process [120]. In other studies of general wound healing, it was found that M2 macrophages dominated in response to injury and were the driving immune forces of tissue formation. The depletion of macrophages (characterized predominantly as M2s) during the healing phase delayed formation and maturation of new tissue, and actually led to detectable epithelial apoptosis [120]. During the characterization of these macrophages, IL-10 was found to be noticeably elevated, while there was no detectable change in *Ym1*, *Mrc1* or *Retnla*, classic M2 markers, which will be discussed in further depth in the next chapter.

Additionally, in research focused on spinal injury, which recovers in a pattern distinct from general wound healing, IL-10 was again found to be necessary during the proliferative phase to facilitate tissue remodeling [120, 121]. While persistent macrophage presence is recognized as a hallmark of chronic

injuries, these studies found that the macrophages that lingered throughout the proliferative and remodeling phases (one week to months) were M2-like. They secreted elevated levels of IL-10, CCL17, CCL22, and CCL18, all of which have been shown to promote production of extracellular matrix components and growth factors. The studies further found that the misregulation of macrophages, specifically the lack of IL-10 induction, played a role in extended and persistent spinal cord injury. Even more convincingly, skewing or boosting the macrophage phenotype to the M2b and M2c phenotypes (activated microglial cells) via injection of MCP-1, MIP-1α, or IL-1β adjacent to the injury site restored the proliferative phase of healing and led to an improved recovery [122].

As these varied studies show, IL-10 production and the macrophage phenotype that it leads to are both important in the healing process. However, in a disease such as PKD, the injury is chronic and persistent, worsening as time goes on. Further, the cystic cells have an intrinsic hyperproliferative phenotype. Hence, when the injury is unresolved, the presence of cells (in this case macrophages) that enhance and promote the proliferative phenotype of epithelial cells would be predicted to worsen the disease and accelerate progression. Consistent with this hypothesis, we and others have shown that depletion of macrophages from PKD mouse models diminishes cyst cell proliferation and slows disease progression. In this chapter, the data presented suggest that these macrophages take on a pathogenic phenotype that relies on IL-10 production and its autocrine effects. This cytokine, while often associated with anti-inflammatory

macrophage regulation, may have deleterious effects in PKD, indirectly promoting cyst growth and disease progression.

Chapter Four

IL-10 Signals in a Distinct and Autocrine Fashion to Induce a Pro-Proliferative Macrophages Phenotype

1. Introduction

IL-10 is an important cytokine that was originally identified as a potent inhibitor of Th1 polarized T cells [123] and has subsequently been shown to be a critical endogenous anti-inflammatory molecule affecting T cells as well as macrophages, dendritic cells, B cells, mast cells, and some epithelial cells [124]. Because of its broad immune effects, IL-10 signaling is known to play a role in a number of human diseases, including inflammatory bowel disease, diabetes, and rheumatoid arthritis [125-129]. IL-10 has also been found to play a role in kidney diseases, where its presence can improve recovery from injury or prevent inflammation due to obesity or other insults [70]. In Chapter 2, IL-10 was shown to be expressed in macrophages that infiltrate the cystic kidneys of PKD. Further, Chapter 3 demonstrated that this cytokine plays an essential role in generating a pathogenic macrophage phenotype in these kidneys, which promotes cyst cell proliferation. These findings suggest that modulation of IL-10 function, including its production and signaling mechanisms, could be a potential therapeutic strategy in PKD. In order to design an effective therapeutic intervention, it is critical to understand how IL-10 is produced and is signaling in the context of PKD.

IL-10 is expressed in all leukocytes, and its production is tightly controlled by a complex regulatory network [124]. While many pathways contribute to IL-10 production in a cell type specific manner, TLR stimulation results in a significant upregulation of *Il10* in macrophages, particularly that of TLR2/Myd88 and TLR4. Ligand binding to other TLRs has also been shown to induce *Il10*. In addition to direct ligand activation of TLRs, it is also possible for TLR-independent IL-10 stimulation to occur through NOD2 signaling, DC-SIGN, and Dectin-1. These signaling cascades are typically triggered by pathogens but TLR-independent production of IL-10 can occur because of exposure to other cytokines, such as IFNγ and IL-10 itself [130, 131]. Studies have revealed putative cAMP response elements (CRE) on the IL-10 promoter, indicating a possible regulatory interaction with CREB [132, 133]. NFκB family members such as RelA and p50 have also been associated with IL-10 regulation in LPS-stimulated macrophages [134].

The downstream pathways activated by IL-10 are also complex and can vary by cell type and numerous co-factors. However, it is widely accepted that signal transducer and activator of transcription 3 (Stat3) is activated by IL-10 receptor signaling. This activation is necessary and sufficient for the observed anti-inflammatory, properties and effects of IL-10 [49]. Importantly, it has previously been reported that Stat3 activation is elevated in both mouse models of PKD and in human patient samples [135]. Moreover, inhibition of Stat3 activation has recently been studied as a possible therapy for PKD with promising preliminary results in animals, effects that were believed to be due to effects on cyst epithelial cells [136].

In this chapter, the signaling network surrounding IL-10 production and function in PKD-polarized macrophages will be examined. The data suggest that signals activating NFκB are important upstream signals for the PKD-stimulated IL-10 production in macrophages. Also, evidence is presented indicating that Stat3 activation is an important downstream element of autocrine IL-10 signaling in PKD macrophages. These data suggest that NFκB and Stat3 could be potential targets for therapy in PKD.

2. Methods

Cells, macrophage programming, and proliferation assays were performed as described in previous chapters.

Western Blotting: RAW or THP-1 cells were seeded in 6-well plates and treated with ADPKD CM for the indicated times. Cells were washed once with 1X PBS and then harvested in an SDS lysis buffer. Protein concentration was determined using Bradford assay (Biorad, #5000006) according the manufacturer's instruction. Samples for Stat3 detection were processed on a 12% acrylamide gel at 100 V for 2 h. Samples probed for IL-10 were run on a 15% acrylamide gel at 120V for 2 h. Samples probed for all other proteins were run on 10% acrylamide gels at 100V for 2 h. Protein was transferred to nitrocellulose at 30V overnight at 4°C. Membranes were blocked in Licor reagent (Licor, P/N 927-40000) for 30 minutes then probed with primary antibodies (diluted at 1:1000) obtained from Cell Signaling for 1-2 hours. The following

primary antibodies were used: RhoGDI (#2564), Stat3 (#9139), phosph0-Stat3 (#9131), and IκBα (#4814). After 6X washing with Tris buffered saline with 0.05% Tween 20 (TBST), the membranes were probed with appropriate secondary antibodies (diluted at 1:15,000) conjugated to near-infrared fluorophores obtained from Licor. Probed membranes were scanned and analyzed using the Odyssey® (Licor).

ELISA: IL-10 was measured in culture supernatants using an ELISA kit (eBioscience #88-7104-22, San Diego, CA) according to the manufacturer's protocol. Media was collected from treated RAW cells at the indicated time points, centrifuged at 600 x g for 5 minutes, then stored at 4°C until all samples were collected and the assay could be run.

Immunohistochemistry*:* De-paraffinized sections were steamed in 0.01 M citrate buffer (pH 6.0) for 20 min (steamer #HS900, Black & Decker, Madison, WI). Sections were then incubated in 3% H_2O_2 followed by serum from the host animal in which the relevant secondary antibody was generated. Samples were incubated with an CD-163 antibody (Cat. No. CD163-MS-1103, ThermoFisher) or phosphor-Stat3 antibody (9145, Cell Signaling) overnight at 4°C. Appropriate secondary antibodies (ImmPRESS, Vector Laboratories, Burlingame, CA) were then applied for 30 min at room temperature prior to visualization by light microscopy. All IHC and Fluorescence Microscopy were done as previously described [4].

3. Results

Autocrine IL-10 Activates Stat3 in ADPKD-Programmed Macrophages

In the previous chapter, autocrine IL-10 was shown to be required for pathogenic macrophage polarization. Since IL-10 is known to activate Stat3 by signaling through the IL-10 receptor, Stat3 activation was examined in macrophages programmed by ADPKD cells. RAW macrophages were treated with ADPKD CM, and Stat3 activation was measured using phospho-specific antibodies to pTyr705 Stat3 and Western blot (**Figure 4.1 (A, B)**). This approach revealed two peaks of Stat3 activation: a transient one that peaked at 15 min and diminished to near baseline by 1 h and a second one that began ~8-12 and was maximal ~18 h. This latter peak roughly coincides with the macrophage production of IL-10 (see Chapter 3, **Figure 3.4**) suggesting this cytokine drives this later activation.

To confirm that autocrine IL-10 was responsible for the late Stat3 activation, an IL-10 neutralizing antibody was used. Neutralizing antibody but not control Ig blocked STAT3 activation in RAW MΦs in response to recombinant IL-10 treatment, as expected (**Figure 4.1 (C)**, left panel). Also, the neutralizing antibody but not control Ig prevented STAT3 activation by ADPKD CM after 18 h (**Figure 4.1 (C)**, right panel). These data indicate that MΦ IL-10 produced during programming with ADPKD CM is responsible for the late activation of the STAT3 pathway in MΦs.

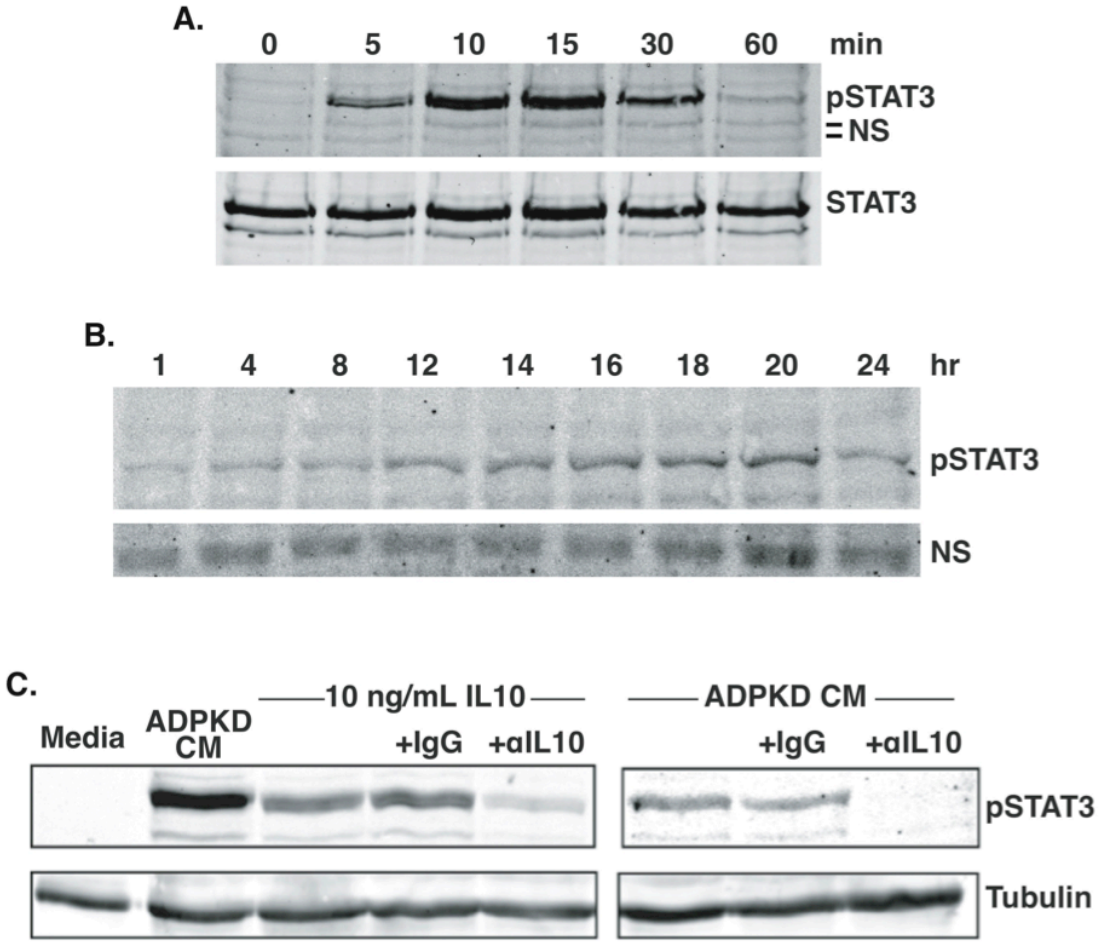
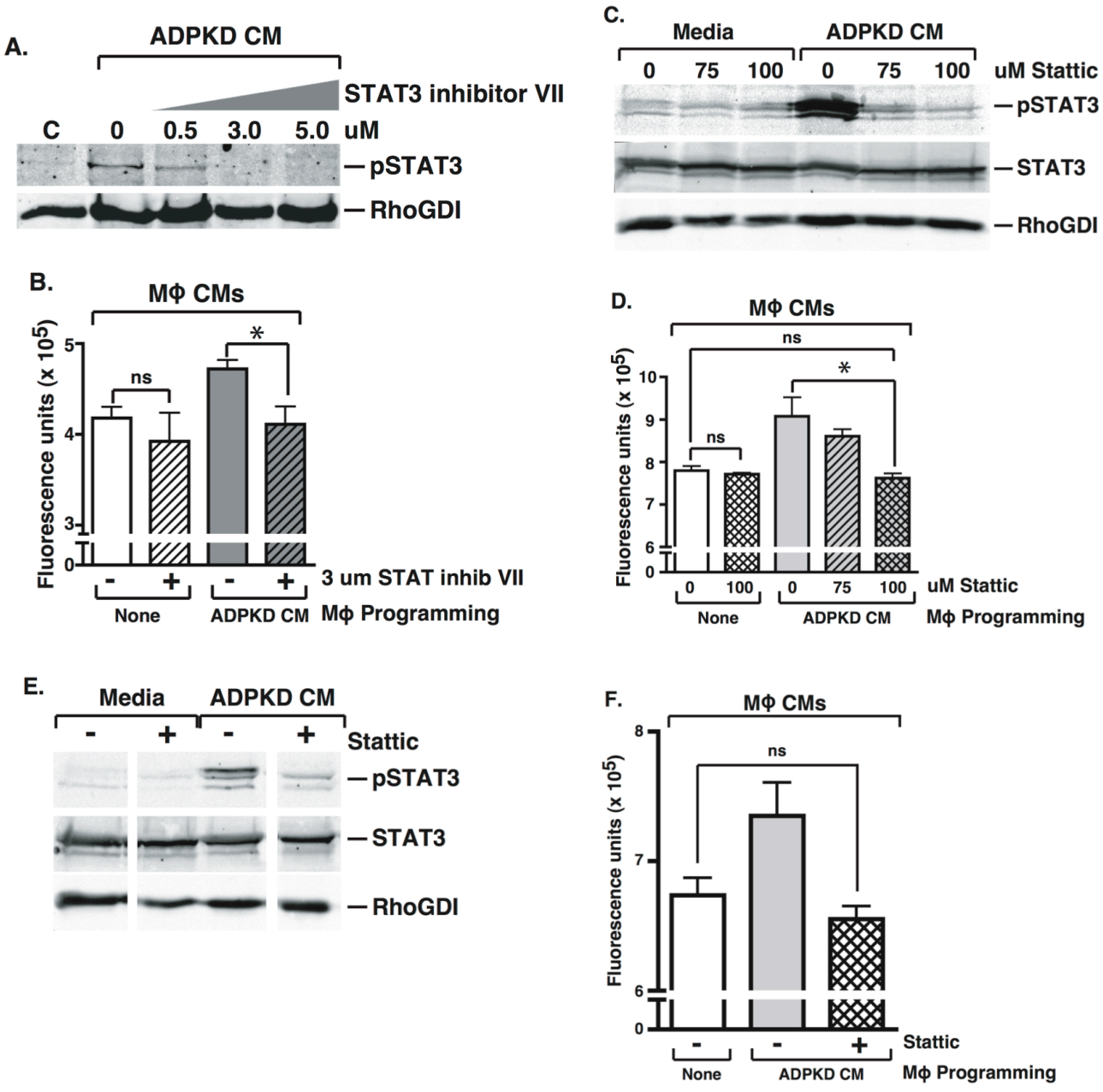


Figure 4.1: MΦ derived IL-10 activates autocrine Stat3 signaling.
A and B. RAW MΦs were incubated with ADPKD CM for increasing, successive time periods prior to lysis and immunoblot analyses of total protein using anti-phospho-STAT3 and anti-STAT3 antibodies for detection of activated and total STAT3, respectively. **(A)** Immunoblot of cell samples collected from 0-60 min. **(B)** Immunoblot of cell samples collected from 1-24 h. NS = non-specific bands, which serve as a control for protein loading. **(C)** RAW MΦs were incubated for 18 h with either mouse IL-10 (10 ng/ml), ADPKD CM or media alone, as indicated, in the presence of an anti-IL-10 neutralizing antibody (αIL10) or equal concentrations of a control antibody (IgG) prior to cell lysis. Total protein was subjected to immunoblot analyses using anti-phospho-STAT3 antibodies, as well as anti-tubulin antibodies for detection of a gel-loading control protein.

Autocrine IL-10-dependent activation of STAT3 is required for pathogenic MΦ differentiation

The next question was whether the IL-10/STAT3 pathway contributed to the generation of the pathogenic, pro-proliferative MΦ phenotype. Small molecule inhibitors of Stat3 were used to address this question. Using RAW MΦs, the concentration of Stat3 inhibitor VII [137](EMD Millipore, #573103) required to block Stat3 activation in response to ADPKD CM was determined (**Figure 4.2 (A)**). Using this concentration (3 μM), MΦs were then incubated in the presence or absence of Stat3 inhibitor VII during programming with ADPKD CM and the effects on the production of pro-proliferative activity measured (**Figure 4.2 (B)**). Stat3 Inhibitor VII also blocked the production of *Arg1*, an M2 marker studied in Chapter 2 (data not shown). While there was no significant effect of this drug on the proliferative activity of CM from unprogrammed MΦs, the Stat3 inhibitor completely blocked the induction of pro-proliferative activity caused by ADPKD programming (**Figure 4.2 (B)**). A second Stat3 inhibitor, Stattic [138](Santa Cruz Biotechnology, CAS 19983-44-9), also blocked STAT3 activation induced by ADPKD CM in human THP-1 MΦs (**Figure 4.2 (C)**). Importantly, this inhibitor also blocked MΦ production of enhanced pro-proliferative activity, while not affecting the proliferative activity in CMs collected from unprogrammed MΦs (**Figure 4.2 (D)**).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Stattic
None
ADPKD CM
MΦ Programming

Figure 4.2: Inhibitors of Stat3 activation attenuate proliferative effect of MΦs. (**A)** Concentration of STAT3 inhibitor VI required to block STAT3 activation in RAW MΦs during programming with ADPKD CM. RAW MΦs were incubated in either media (C) or ADPKD CM for 18h in the absence or presence of increasing concentrations of STAT3 inhibitor VII prior to cell lysis. Total protein was subjected to immunoblot analyses using anti-phospho-STAT3 antibodies, as well as anti-RhoGDI antibodies for detection of a gel-loading control protein. **(B)** Effects of STAT3 inhibitor VII on development of RAW MΦ pro-proliferative phenotype. MΦs were incubated in either media (None) or ADPKD CM for 18h in the absence or presence of STAT3 Inhibitor VII (3 uM). Media was then removed, and after a PBS wash, cells were incubated for a further 24h in basal media, which was then collected and tested for proliferative effects on ADPKD cells. FBS (0.1%) was added to CMs prior to addition of ADPKD cells. After 72h, cells were collected and assayed for relative proliferation. **(C)** Concentration of Stattic required to block STAT3 activation in THP-1 MΦs during programming with ADPKD CM. THP-1 MΦs were incubated in either media (C) or ADPKD CM for 18h in the absence or presence of increasing concentrations of STAT3 inhibitor VII prior to cell lysis. Total protein was subjected to immunoblot analyses using the indicated antibodies. **(D)** Effects of Stattic on development of THP-1 MΦ pro-proliferative phenotype. MΦs were incubated in either media (None) or ADPKD CM for 18h in the absence or presence of Stattic. Media was then removed, and after a PBS wash, cells were incubated for a further 24h in basal media, which was then collected and tested for proliferative effects on ADPKD cells. FBS (0.1%) was added to CMs prior to addition of ADPKD cells. After 72h, cells were collected and assayed for relative proliferation. **(E)** Stattic addition to THP-1 MΦ cultures after 1 h of programming blunts late activation of STAT3. THP-1 MΦs were incubated in either media (C) or ADPKD CM for 1h prior to addition (or not) of Stattic (100 uM) and were incubated further for 17 h. Cells were lysed and total protein was subject to immunoblot analyses using the indicated antibodies. **(F)**Stattic addition to THP-1 MΦ cultures after 1 h of programming blunts development of THP-1 MΦ pro-proliferative phenotype.

A small molecule inhibitor of Stat3 was also used to determine whether the early or late activation of Stat3 during programming was required for the pathogenic, pro-proliferative phenotype. Stattic was incubated with THP-1 MΦs starting at 1 h after the addition of ADPKD CM, so that the early activation of Stat3 would be unperturbed. The addition of Stattic at this time again blocked both the activation of Stat3 when measured at 18 h (**Figure 4.2 (E)**) and the induction of pro-proliferative activity (**Figure 4.2 (F)**). These results suggest that only the late Stat3 activation (stimulated by IL-10) is required for the generation of the pro-proliferative activity elaborated by MΦs in response to programming with ADPKD CM.

Activated STAT3 is present in MΦs in human ADPKD kidneys.

To determine whether MΦ STAT3 activation occurs in human disease, sections of human ADPKD cystic kidneys were stained using antibodies specific for both MΦs and phosphorylated STAT3. To stain MΦs, an antibody specific for CD163, a marker of M2-like MΦs that we previously showed reacted with over 90% of the MΦs present in these kidneys[4]. First, antibodies for CD163 and anti-phospho-STAT3 were used for immunohistochemical staining of adjacent serial sections of ADPKD cystic kidneys. In most MΦ-rich areas, nuclear phospho-STAT3 positive cells were detected, whereas few if any of these cells were detected in areas of interstitium without MΦs (**Figure 4.3 (A and B)**). Nuclear phospho-STAT3 staining could also be detected within scattered cyst epithelial

cells, which has been reported by others [136, 139]. Next, immunofluorescence co-staining for CD163 and phospho-STAT3 was performed. In these experiments, occasional CD163-positive cells also showed nuclear phospho-STAT3 staining (**Figure 4.3 (D)**). These results indicate that STAT3 activation is present in a subset of renal MΦs present in cystic kidneys *in vivo*.

NFκB Activation and Pathogenic Macrophage Programming

The data highlighted in Chapter 3 and thus far in this Chapter indicate that ADPKD cells secrete factor(s) responsible for pathogenic macrophage polarization. To identify the factors and their immediate signaling pathways, several approaches were taken. First, transcriptome data were analyzed. RNAseq expression data were obtained from THP-1 macrophages treated with ADPKD CM or control media (Sally Salah, unpublished observations). Forty-two genes were upregulated (ranging from 1.6 fold to 2951-fold) with p values <0.05 in programmed versus unprogrammed macrophages (Table 1). Due to variability in the biological replicates, no transcripts had significant q values (p values adjusted for false discovery rate). However, several genes from this small set were validated with antibody detection (Sally Salah, unpublished observations). Thus, the entire set of 42 genes was used to query a public database designed to find common upstream regulators of gene sets. This analysis, ChIP Enrichment Analysis (ChEA) [140], examines publicly available data to identify common factors that have been associated by chromatin immunoprecipiation (ChIP) with the gene

sets entered. This tool has been used successfully to help identify factors relevant in kidney fibrosis [141].

ChEA analysis revealed a striking result. The top hit for the 42 genes upregulated in programmed macrophages was RelA, with a p value of 0. RelA, also known as p65, is a component of the prototypical NFκB heterodimer (p65/p50), a transcription factor complex that regulates both innate and adaptive immunity [142]. To assess the potential involvement of NFκB in ADPKD-stimulated macrophage polarization, western blot analysis of IκB stability was performed. In macrophages treated with ADPKD CM, there was a rapid disappearance of IκB (**Figure 4.4**), indicating robust activation of the NFκB pathway. Activation was more pronounced than was observed for LPS, which is known to activate the pathway. To determine whether this activation was relevant to function of ADPKD-polarized macrophages, a small molecule NFκB inhibitor (JSH-23; Sigma-Aldrich, #J4455) was used. Macrophages were treated with ADPKD CM in the presence or absence of JSH-23. After programming and washout, macrophage CM was assessed for proliferative effects on ADPKD cells. As illustrated in **Figure 4.4**, NFκB inhibition resulted in a small but statistically significant diminution in the pro-proliferative phenotype. These data indicate that NFκB is activated in macrophages during programming by ADPKD cells and may be important for the generation of the pathogenic, pro-proliferative phenotype.

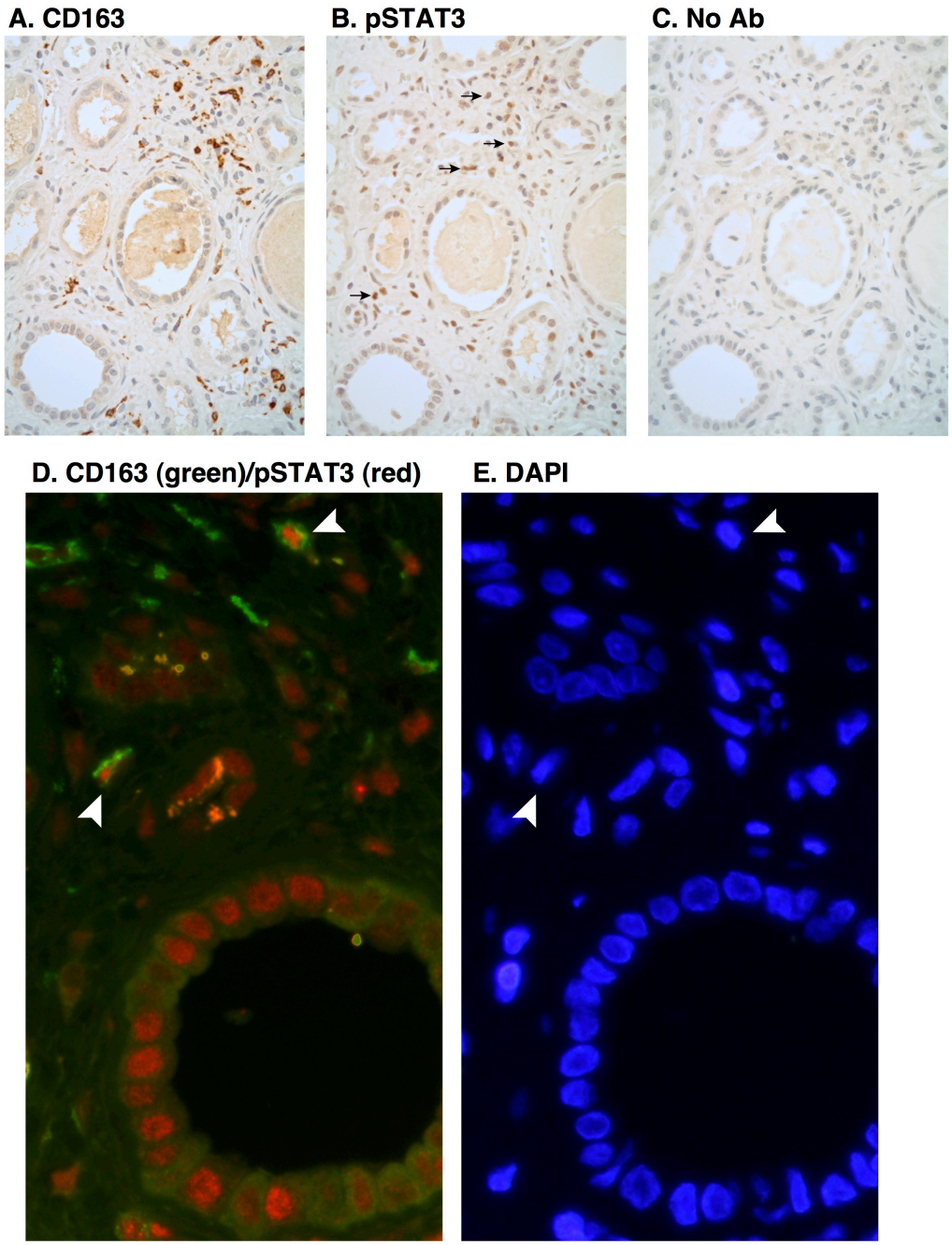

Figure 4.3: Phosphorylated STAT3 is present in macrophages of cystic ADPKD kidneys. Formalin-fixed, paraffin-embedded tissues from ADPKD kidneys were serially sectioned **(A-C)** and consecutive sections stained by immunohistochemistry using the M2 macrophage antibody, CD163 (**A**), the anti-phospho-STAT3 antibody **(B)** or buffer only **(C)** as a control for non-specific staining of the secondary antibody. Arrows indicate phospho-STAT3-positive cells in MΦ-rich regions of interstitium. **(D and E)** Section of similarly fixed and prepared sections of ADPKD kidneys co-stained with anti-CD163 (green), anti-phospho-STAT3 (red) antibodies followed by incubation with 2 distinct fluorescently-labeled secondary antibodies, each with the appropriate antibody specificity **(shown in D)**, and with DAPI **(shown in E)** (. Arrowheads indicate co-staining cells, which were present in the interstitium.

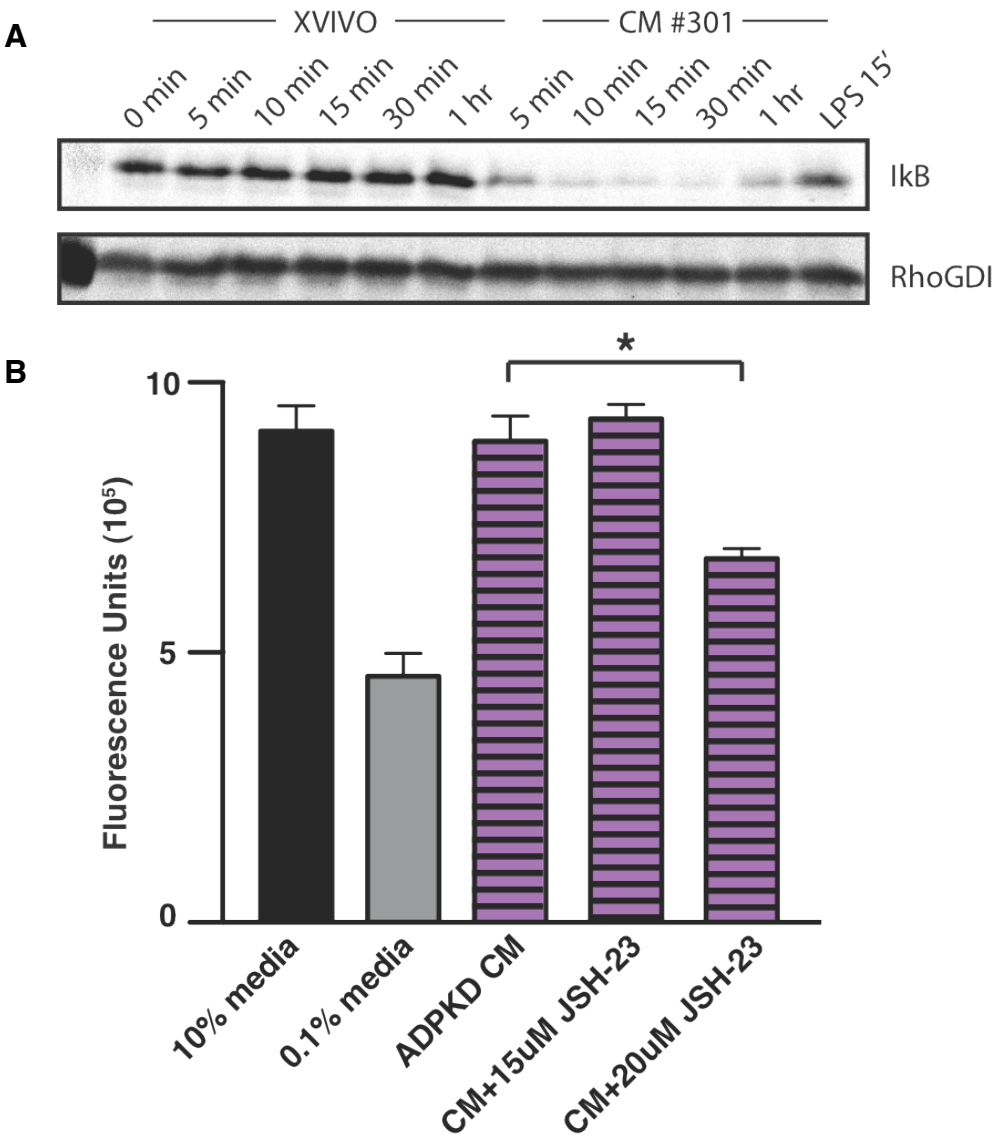

Figure 4.4: ADPKD CM treatment activates NFκB in MΦ and plays a partial role in induction of the pro-proliferative phenotype. (A) THP-1 macrophages were treated for the indicated times with either XIVVO or ADPKD CM. After the last treatment the cells were collected and processed for immunoblot analysis. The disappearance of IκB was observed over time, indicating the activation of NFκB in correlation. **(B)** THP-1 cells were programmed with XVIVO or ADPKD CM in the presence of the NFκB inhibitor JSH-23 for 18 hours, which was then washed out with PBS and DMEM. The MΦ CM was then collected and placed onto PKD cells for 72 hours. The treated PKD cells were collected and assayed for proliferation. JSH-23 had no effect on XVIVO-exposed cells (data not shown).

Other Potential Contributing Signaling Pathways in Programmed Macrophages

The observation that an NFκB inhibitor partially blocked the programming effect of ADPKD CM on macrophages implies that the pathway is important for the differentiation. However, since the effect is partial, it is certainly possible that other signals, ligands, and pathways may also contribute. The investigation of other potential contributing pathways is underway. One potential pathway is that controlled by purinergic signaling. Signals from ATP and adenosine have been shown to influence macrophage polarization and function [143], and extracellular ATP has been shown to promote cyst cell proliferation [144, 145]. Also, ATP is found in high levels in PKD cyst fluid [146], and cyst epithelial cells are known to secrete ATP 2-5X greater than non-cystic epithelial cells [146, 147].

To assess the function of extracellular ATP in pathogenic macrophage polarization, macrophages were incubated with increasing concentrations of ATP. Then, after washout, the macrophage CM was assessed for pro-proliferative activity on PKD cells. Neither 0.1 or 5 mM ATP had influenced the proliferation (**Figure 4.5**, blue bars). In parallel, macrophages were incubated with ADPKD CM plus ATP, and macrophage CM was collected as described above after a washout. In this experiment, incubation with ADPKD CM enhanced the pro-proliferative activity. However, ATP inhibited this enhancement. Collectively, these results suggest that extracellular ATP is not likely to be involved in programming pathogenic macrophages in PKD.

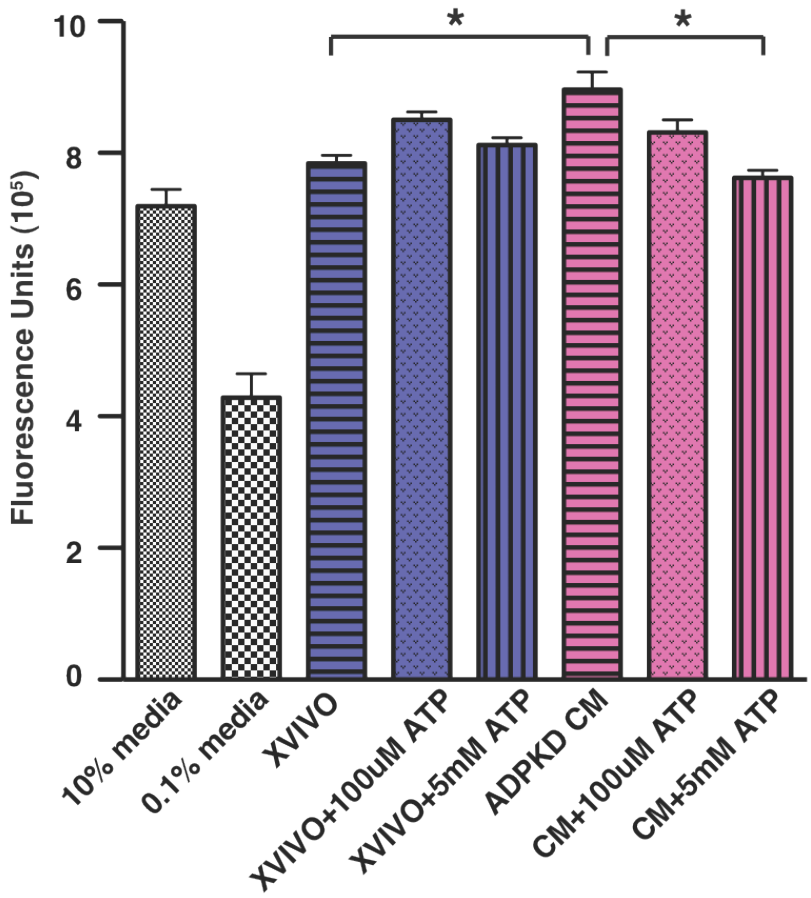

Figure 4.5: ATP inhibits the proliferative effect of MΦ on PKD epithelial cells. THP-1 cells were treated with ADPKD CM in the presence of 100 µM or 5 mM ATP for 72 hours. Treated PKD cells were then collected and assayed for proliferation.

4. Discussion

In earlier chapters, data were presented that support the hypothesis that macrophages and cyst epithelial cells communicate with one another in a reciprocal fashion. These interactions have functional consequences, namely, the cyst cell-promoted polarization of the macrophages enhanced their secretion of pro-proliferative factors. Autocrine IL-10 was shown to be essential for this process. In this chapter, signaling pathways both upstream and downstream of IL-10 were examined. Elucidation of these pathways could more clearly define specific components that could be targeted for therapy in PKD.

Not surprisingly, Stat3 was shown to be activated in the ADPKD-programmed macrophages as a result of IL-10 secretion. Data were also presented to support the hypothesis that Stat3 activation is critical for pathogenic macrophage polarization, enhancing their ability to produce pro-proliferative factors. Stat3 activation has been examined in PKD [136]. This group found sustained Stat3 activation in cystic epithelial cells of both human ADPKD and mouse models of disease. An inhibitor of Stat3 (pyrimethamine) slowed disease progression in a mouse model; the therapeutic benefit was attributed to its effects on cyst cells. Notably, interstitial cells with activated Stat3 were present in this study but not characterized. It is possible that some of the benefits of this drug may be the result of effects on these interstitial cells, which are quite possibly macrophages.

When considering the ADPKD cell-stimulated macrophage polarization process, responsible ligand(s) have not yet been identified. The polarizing activity is not sensitive to heat (not shown), so the ligand(s) are likely to be non-proteins (or perhaps small, heat-stable peptide). In this chapter, our data suggest that one candidate non-peptide ligand, ATP, which is highly secreted by ADPKD cells, is not the likely signal. Transcriptome analysis was used to identify relevant pathways activated during polarization and perhaps deduce upstream signaling events. This analysis suggested that NFκB activation is important for polarization. Examination of macrophage cell lysates demonstrated that NFκB is indeed robustly activated upon polarization by ADPKD CM. Potentially relevant to this observation is the proposed existence of a subtype of macrophage called M2d [148]. This subtype plays a specific crucial role in tissue formation and expresses high levels of IL-10 and VEGF but lacks the expression of other typical M2 markers such as Ym1, Mrc1, and Retnla. Interestingly, putative M2d macrophages arise independent IL-4 or IL-13 but rather require NFκB signaling. This phenotypic description sounds very similar to what this dissertation has described in PKD.

NFκB signaling can be initiated by numerous signals. In macrophages, pattern recognition receptors, especially toll-like receptors (TLRs), are most important for NFκB activation [149]. Future directions for this study will focus on TLRs as possible mediators of IL-10 production and subsequent pathogenic macrophage polarization.

Chapter Five

Summary, Conclusions, and Future Directions

Polycystic kidney disease (PKD) represents a family of genetic disorders characterized by renal cystic growth and progression to kidney failure. No FDA approved treatment is currently available for individuals with PKD. With the discovery of the genes *PKD1* and *PKD2*, mutations in which are the root cause of PKD, most research has focused on the subsequent cellular abnormalities that occur as a result of these mutations. Recently, however, there has been interest in studying non-cell autonomous factors that may affect disease progression. The research presented here focused on one such factor the involvement of the innate immune system.

A role for macrophages was first suggested when the observation was made that they were capable of promoting ADPKD cyst cell proliferation *in vitro*. The importance of macrophages in PKD was further supported by the discovery in our lab, as well as others, that chemical depletion of macrophages *in vivo* attenuated cyst growth and expansion in a murine model of ARPKD, the cpk mouse. With these observations, the environment of PKD kidneys bears a striking similarity to that of kidneys post-injury. In all cases of acute kidney injury, the presence of macrophages is initially detrimental to tissue regeneration, but once repair is the goal, macrophages play an important role in encouraging epithelial cell growth and healing. Importantly, though the role of macrophages

seems to be similar in AKI and PKD kidneys, it is important to note that in the case of PKD, the initial insult is never removed, leading to chronic injury. Thus, this work focused on characterizing the phenotype or polarization state of macrophages in PKD and determining the extent to which this state could contribute to disease state.

In this document, I presented data that supports the hypothesis that macrophages, once recruited, can be influenced by cystic PKD epithelia to differentiate to a harmful, pathogenic phenotype that promotes cyst expansion and disease progression. The hypothesis that follows this is that disease progression may be reduced if the signaling that leads to this macrophage polarization state or the subsequent proliferative influence on cystic epithelia could be interrupted. I further showed that IL-10 and its signaling pathway are critical for this process (**Figure 5.1**). Thus, the studies presented here suggest that Stat3 inhibitors, IL-10 neutralizing antibodies, or NFκB inhibitors could prove to be viable therapeutic interventions in PKD. Indeed, as mentioned above, pyrimethamine, which inhibits Stat3, has already been shown to be effective in animal models of PKD [136]. Another critical characteristic of a potential PKD therapy is that it must be safe over long periods of time. Notably, with the Stat3 inhibitor, this appears to be the case.

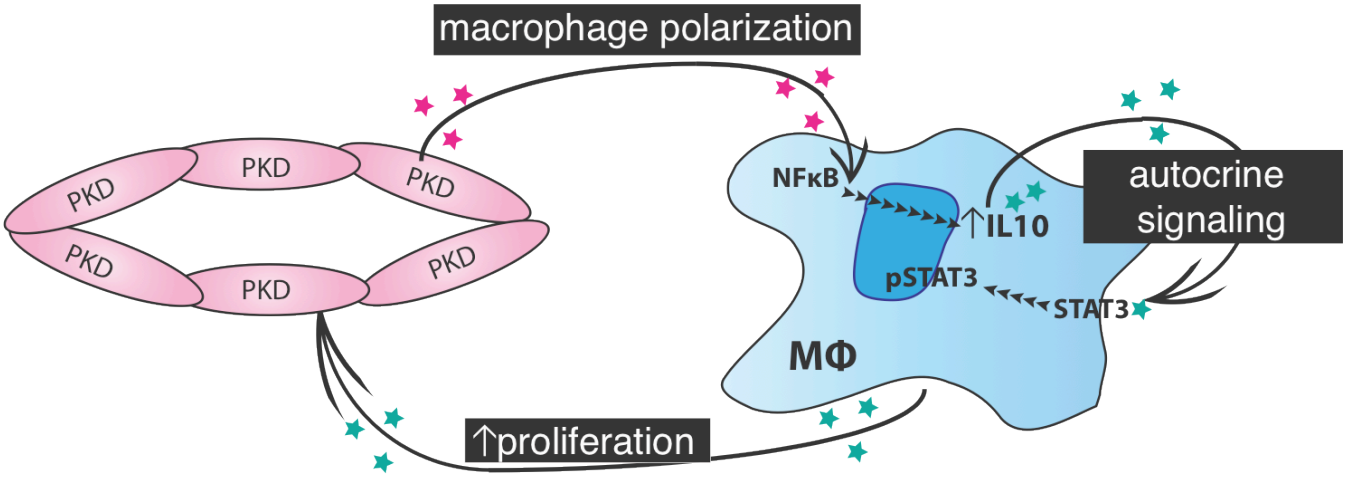

Figure 5.1: Proposed Model by which IL-10 regulates a pathogenic macrophage phenotype in response to ADPKD stimulation. ADPKD cells secrete soluble, heat-stabile factor(s), which induces macrophage IL-10 production in an NFκB-regulated fashion. IL-10 secreted from the macrophages signal in an autocrine fashion through Stat3 to induce a pathogenic macrophage phenotype. The ADPKD-polarized macrophages secrete one or more factors that promote accelerated proliferation of ADPKD cyst cells. The secreted factors from both the ADPKD cells and the macrophages are not yet known, but are currently being elucidated. However, this model indicates that there are three avenues by which to target the pathogenic macrophage conversion; inhibition or reduction of IL-10 signaling by direct targeting, upstream regulation via NFκB, and lastly regulation of Stat3 activation, directly downstream of IL-10.

Though we have begun to study the effects of a global knockout of IL-10, it may prove more informative to study macrophage-specific IL-10 depletion, as global knockouts have other problems, especially in the gut. These animals develop inflammatory bowel disease. It is possible this could influence kidney disease [118, 119]. The fact that we are presently unable to generate a double knockout (*cpk-/-*;*Il10-/-*) indicates that there may be a deleterious or fatal issue that arises in the case of total IL-10 loss in this particular mouse model. Small differences were detected in total kidney size in the heterozygotes compared to wild type, but we were unable to detect differences in total cystic index. The longevity or life expectancy of affected individuals may be different, so that would be important to study.

There are no specific treatments for PKD, but in the coming years, it may be most useful to consider combinatorial therapy rather than searching for one target. A two- or three-pronged attack may be the best approach, where a diseased individual would be treated with a cocktail of drugs: one that targets cell autonomous cyst growth, and another that targets the immune. Given the extensive time and investments that must be made to develop new drugs, it makes sense to look to drugs that are already in the stages of clinical trials. With this in mind, many of the targets examined in this study were chosen based on the knowledge of available drugs on the market. The hope is that the work presented here will contribute to the understanding of this complicated disease

and assist in the discovery of a viable, life-long treatment for those affected with Polycystic Kidney Disease.

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