REGULATION OF RENAL IMMUNITY AND IMMUNOPATHOLOGY BY INTERLEUKIN-17

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Integrated Masters of Science in Systems Biology

University of Hyderabad, 2011

Submitted to the Graduate Faculty of

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh
2016

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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University of Pittsburgh, 2016

Pro-inflammatory cytokine IL-17 has been implicated in tissue damage associated with various autoimmune diseases. IL-17 is also known to contribute in immunity against bacterial and fungal pathogens. Most research over the past decade focused on understanding how IL-17- producing cells are generated, but far less is known about the organ specific responses of IL-17. In this thesis, we delineate the specific roles for IL-17 in the kidney, an organ susceptible to both auto-inflammatory conditions and infection. With the help of murine models, we outline the IL-17-driven responses in infection and autoimmunity. We studied the role for IL-17 mediated protection in disseminated candidiasis, a fatal nosocomial infection associated with high mortality. We discovered a previously unappreciated connection between IL-17 and the kallikrein- kinin system in renal protection against disseminated candidiasis. Simultaneously, we defined the cellular and molecular events in IL-17 driven renal tissue damage in murine models of autoimmune glomerulonephritis and lupus nephritis. Our results suggest that the kidney specific role of IL-17 is in the regulation of innate immune cells associated with kidney pathology. In addition, we were the first to show that neutralization of IL-17 ameliorated renal pathology in the autoimmune glomerulonephritis model. The consequence of unchecked renal inflammation is irreversible damage in the kidney, leading to fibrosis. In the case of inflammation mediated end organ fibrosis, the role for pro-fibrotic or anti-fibrotic role for IL-17 is debatable. We focused our attention on the role for IL-17-mediates immune responses in the

kidney in a mouse model of fibrosis. We uncovered a surprisingly protective, anti-fibrotic role for IL-17 in the kidney that was driven by the IL-17-Klk1 axis. In summary, our findings have advanced the understanding of IL-17 mediated immunity and immunopathology and opens up the potential to develop therapeutic strategies based on the context of the disease.

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LIST OF ABBREVIATIONS

AKI – Acute Kidney Injury

CKD- Chronic Kidney Disease

ESRD- End Stage Renal Disease

BUN- Blood Urea Nitrogen

Th- T helper

IL- Interleukin

IL-17- Interleukin-17A

TNF- Tumor necrosis factor

IL-17R – Interleukin-17 receptor

IL-17RA – Interleukin-17 receptor A

RA- Rheumatoid Arthritis

MS- Multiple Sclerosis

SLE- Systemic Lupus Erythmatosus

i.v. – Intravenous

i.p. -Intraperitoneal

ANCA- Antineutrophil cytoplasmic autoantibodies

IFNγ - Interferon-γ

PRR – Pattern Recognition receptor

DAMP- Damage associated molecular patterns

TLR – Toll Like receptor

KKS- Kallikrein Kinin System

Klk1 – Kallikrein 1

Bdkrb1 - Bradykinin receptor β1

Bdkrb2 - Bradykinin receptor β2

RAS- Renin Angiotensin System

CFU –Colony Forming Unit

CMC – Chronic Mucocutaneous Candidiasis

OPC – Oropharyngeal Candidiasis

AGN – Autoimmune glomerulonephritis

EAGN- Experimental autoimmune glomerulonephritis

LuN – Lupus Nephritis

cGN- crescentic Glomerulonephritis

H&E – Hematoxylin and Eosin

PAS – Periodic Acid and Schiff

UUO – Unilateral Ureteral Obstruction

ECM – Extracellular matrix

MMP- Matrix metalloproteinase

TGFβ- Transforming growth factor β

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ACKNOWLEDGMENTS

The path to this PhD has been a fantastic experience and I am extremely thankful to my family, friends and colleagues for their support throughout this journey.

First and foremost, I am would like to thank my advisor, Dr. Partha Biswas. Your guidance and support in all my endeavors have been crucial to my development into the scientist I am today. The experiences I had in your laboratory have challenged me to learn skills both at the bench and away from it. For that, I am forever grateful, and I hope that we can maintain this relationship in the future.

The last four years have been productive and rewarding thanks to a lot of help from extremely talented people I have had the privilege to work with. I am grateful to all the past and present members in the Biswas lab, Sudesh, Kelly, Bianca and Chetan for all their help. I would also like to thank members of the Gaffen lab, Abhishek, Nilesh, Heather, Natasha, Erin, Akash, Michelle and Agustin, who always shared their resources and expertise when I was starting out in the lab. Many of these professional relationships have turned into friendships which I will cherish forever.

The work presented in this thesis has been a collaborative effort and a number of people have helped me with various experiments. I would like to thank Roderick Tan, Dong Zhou, Matt Henkel and Dr. Mandy McGeachy for technical and intellectual help.

I am extremely grateful to my thesis committee members Dr. Sarah Gaffen, Dr. Youhua Liu, Dr. Penelope Morel and Dr. Anuradha Ray. Your input and fruitful suggestions have been immensely helpful in advancing my thesis research. I really appreciate the time you have taken to attend the committee meetings. I would also like to thank Sarah for all her insightful wisdom and mentorship over the years on everything under the sun.

Last but not the least, I also want to thank my parents, for their love, care and support. My achievements are a testament to your encouragement and support over the years. A special mention to my brother Karun, for always reminding me to have fun. I would also like to thank all my friends. Grad school wouldn't have been half as much fun if it weren't for all the shenanigans we were always up to. Finally, I would like to thank Rahul for being there through thick and thin. I shall be forever thankful to you for being in my corner.

1.0 INTRODUCTION

The kidneys play a pivotal role in several physiological functions including blood pressure regulation, electrolyte balance, maintenance of cellular osmolarity and calcium homeostasis. Renal dysfunction in kidney diseases is associated with significant morbidity and mortality. As reported by the National Center for Health Statistics, more than 3.9 million adults (amounting to about 2% of the adult population) are affected by some form of renal disease. Mortality rates associated with renal diseases are high and it is reported as the 8th leading cause of death in the United States [1].These reports illustrate that renal diseases pose a grave health problem. Therefore, identifying underlying mechanisms of disease are crucial in making progress with therapy for renal diseases.

Kidney diseases can be categorized as either acute or chronic. Acute kidney injury (AKI) is defined as an abrupt decline in renal function over a period of hours to weeks. The clinical manifestations include an acute but reversible increase in levels of nitrogen waste products such as blood urea nitrogen (BUN) and serum creatinine. Acute kidney injury is usually associated with ischemia-reperfusion injury, bacterial infections or sepsis. On the other hand, chronic kidney disease (CKD) is the progressive loss of renal function over the course of months or years. CKD is associated with hypertension, diabetes, obesity or autoimmunity. Acute injury can lead to chronicity eventually progressing to end-stage renal disease (ESRD), a process that is

poorly understood. CKD is estimated to be a major economic and social burden especially in patients who progress to ESRD and require dialysis or even kidney transplants.

The Biswas laboratory focuses on the interlukin-17A (IL-17) mediated functions in kidney diseases. In this regard, several aspects including specific roles for IL-17 in renal autoimmunity and host defense against extracellular pathogens will be discussed in this introduction, as well as in subsequent chapters of this thesis.

1.1 IMMUNOLOGY OF THE KIDNEY

The kidneys are major targets of disorders involving the immune system. The damage seen in kidney disorders can result from abnormal renal function associated with either renal autoimmune diseases or infections. Autoimmune diseases that involve renal inflammation, including lupus nephritis, Sjögren's syndrome and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Kidneys are subject to infections via the ascending spread of pathogens like uropathogenic *Escherichia coli* (UPEC) and *Candida spp*. from the urinary bladder or through the hematogenous route. In addition, non-renal infections that can eventually manifest as renal infections include septic kidney injury, immune complex-mediated nephritis such as hepatitis-related immune complex glomerulonephritis and HIV nephropathy. In certain renal disorders, immune responses are a secondary mechanism causing renal inflammation and injury. These include disorders that affect the vascular system and affect the kidney as well such as in atherosclerosis, shock or hepato-renal syndromes. Obstructive and diabetic nephropathies also lead to immune responses occurring in the kidney.

The functional unit in a kidney is the nephron, which functions to remove toxic metabolic waste products from blood. Each nephron has one glomerulus that functions as a filter preventing the passage of molecules more than ~50 kDa into the blood. Low molecular weight compounds run through the glomeruli, enter the tubules and are excreted with urine except when reabsorbed by the tubular epithelium. Immune mediated injury can affect the glomeruli causing various forms of glomerulonephritis. Irreversible kidney damage is seen when inflammation spreads to the tubulointerstitium [2-4]. Immune related inflammation involved in this injury setting is attributed to the production of pro-inflammatory cytokines from inflamed glomeruli [5] along with the aberrant reabsorption of protein from the glomerular filtrate inducing a stress response in tubular epithelial cells [6]. This makes available glomerular antigens to dendritic cells (DCs) in the tubulointerstitium, providing stimulus to infiltrating T cells to secrete pro-inflammatory cytokines [7]. Tubulointerstitial immune cell infiltrates contribute to progressive tissue remodeling and scarring leading to kidney fibrosis - the final stage of CKD.

Immune-mediated CKD can be induced in a multitude of ways including contributions from immune complex deposition, innate immune cells as well as T cells that can potentially interact with resident kidney immune cells.

1.2 KIDNEY RESIDENT IMMUNE CELLS

For decades renal immunology has been largely overlooked by classical immunologists due to the inherent challenges involved in studying immune responses in the kidney. The poor regenerative capacity of nephrons, incidence of uremia and proteinuria, and hypoxia in the damaged kidneys present confounding factors in renal immune responses. Thus, keeping these in

mind, it is crucial to understand the immune landscape within the kidneys during renal disease. Inflammation and activation of the immune system are major underlying events in the development of both AKI and CKD. In steady state conditions, kidney resident immune cells include macrophages, DCs and some lymphocytes [8-11]. Macrophages are predominantly found in the renal medullary region [8] and function to maintain homeostasis and mediate repair [12]. Macrophages are activated by complement associated-immune complexes or by T cells and their cytokines. In AKI as well as CKD, increased macrophage numbers are seen in the kidney [13]. Macrophages are classified into subpopulations called M1 or M2. In the setting of renal disease, M1 macrophages, activated by inflammatory cytokines like IFNγ or TNFα, are commonly found in the kidneys [14]. These M1 macrophages contribute to the development of renal fibrosis by releasing more inflammatory cytokines and promoting oxidative stress [15]. Studies showed macrophage depletion [16] or blockade of monocyte chemoattractant protein (MCP-1) in mice made them resistant to renal disease [17], thereby supporting the pathogenic role for M1 macrophages. Increased infiltration of macrophages in the kidney causes local inflammation and renal injury leading to production of macrophage-derived cytokines like IL-1, IL-6, IL-23, and reactive oxygen species, all of which have been linked to compromised renal function [18, 19].

DCs are limited to the tubulointerstitium and are not found in the glomeruli [8, 9]. Murine kidney resident DCs bear the surface markers $CD11c^{+}CD11b^{+}F4/80^{+}CX_3CR1^{+}$, similar to DCs found in other non-lymphoid tissues [20, 21] and are derived from common DC precursors as well as monocytes. Mice deficient in chemokine receptor CX_3CR1 have reduced number of kidney DCs [22]. Increased expression of CX_3CL10, the ligand for CX_3CR1, is also seen in the kidney [23]. Kidney DCs are crucial in mounting an immune response during infections like bacterial pyelonephritis [10, 24]. DCs can also produce neutrophil-recruiting

chemoattractants [24]. Conversely, in renal ischemia and ureteral obstruction, kidney DCs contribute to tissue injury by inducing pro-inflammatory cytokines [25, 26].

Natural killer cells (NK) cells are responsible for activation of macrophages by releasing IFNγ [27]. NK cells were found to play a role in the pathogenesis of ischemia-reperfusion injury and to promote apoptosis in tubular epithelial cells [28]. Antigen presentation by DCs can also induce production of IFNγ by NK cells to aggravate renal disease progression [29]. In contrast, NK cells can destroy autoreactive T cells seen in autoimmune disease [30].

Apart from cells of the innate immune system, other immune mediators have been implicated in renal diseases including the complement system and pattern recognition receptors like toll like receptors (TLRs). Complement has contrasting roles in the progression of renal diseases. Dysregulation of complement has been shown to be responsible in CKD, where inhibition of complement components C3 and C5 lowered glomerular crescent formation and albuminaria in murine models of lupus nephritis [31, 32]. However, early complement components are crucial in clearing immune complexes and have a potential protective function in the kidneys in immune complex-mediated diseases. Indeed, humans with genetic deficiencies in either complement C1q or C4 have a greater risk of developing lupus nephritis [33].

Renal inflammation is also induced by intrinsic damage-associated molecular patterns (DAMPs) released either from dying parenchymal cells like tubular epithelial cells or endothelial cells [34-37]. TLRs are expressed on these parenchymal cells and have been reported in both AKI and CKD. TLR4$^{-/-}$ mice were resistant to developing cisplatin-induced AKI, and TLR inhibition provided protection against renal damage in a murine model of sepsis [38, 39]. In human patients with CKD, TLR expression in monocytes provided a correlation with

inflammation and disease [40, 41]. Similarly, inflammasome components have also been associated with induction of innate immune responses and renal inflammation [42].

1.3 ADAPTIVE IMMUNE CELLS AND RENAL DISEASE

The adaptive immune system comprises of T cells that control the cell mediated immune response and B cells that mount an antibody mediated immune response. In the renal context, B cells produce autoantibodies that contribute to kidney diseases including immunoglobulin A (IgA) nephropathy, systemic lupus erythematosus (SLE) and Goodpasture's syndrome [43, 44].

T cells are further classified into the $CD8^+$ cytotoxic cells and $CD4^+$ T helper cells. $CD8^+$ T cells have been found in the kidneys in lupus nephritis, causing local damage and inflammation with kidney autoantigens activating $CD8^+$ T cells [45]. However, most studies so far have focused on $CD4^+$ T cells. $CD4^+$ T helper cells (Th cells) are classified into subsets based on their cytokine profile and primary function. These include Th1 [46], Th2 [46], Th17 [47], and T regulatory cells (Tregs) [48]. Th1 cells mainly produce IFNγ and TNFα and drive activation of macrophages and $CD8^+$ T cells in glomerulonephritis [49]. The Th2 polarized subset of $CD4^+$ cells can promote B cell to produce pathogenic autoantibodies seen in autoimmune kidney disorders [49]. Crescentic glomerulonephritis, an acute form of nephritis that can rapidly descend into ESRD, is considered a Th1-dominant disease [50]. In contrast, membranous glomerulonephritis, a chronic form of nephritis caused by immune complexes deposition in response to autoantigens of the glomerular basement membrane, is a Th2-dominant disease [51].

A third subset of $CD4^+$ T cells that has garnered considerable attention over the past decade is Th17 cells, which produce IL-17, IL-21, and IL-22 [47, 52]. Th17 cytokines direct

renal inflammation partially by promoting the expression of TNFα and chemokines that are important for the renal recruitment of immune cells [53]. Th17 cells have been recognized to facilitate injury and inflammation associated with autoimmune-mediated nephritis. An in-depth discussion of various studies that prove a role for Th17-IL-17 mediated damage in renal diseases will be presented in the following chapters.

The Treg subset suppresses adaptive immune responses and promotes self-tolerance, thus protecting against autoimmune disease [54]. The immunosuppressive functions of Tregs are driven by cytokines such as transforming growth factor beta (TGF-β) and IL-10. Reduction or dysfunction in Tregs leads to development of inflammation and autoimmunity. The Treg-protective role in renal injury was demonstrated when an increase in the Treg population delayed the onset of renal inflammation and damage associated with autoimmune-induced nephritis [55].

1.4 TH17 CELLS AND IL-17 IN RENAL DISEASE

The prevalent paradigm in immune responses to diseases that existed for a long time was that they were mediated either by Th1 or Th2 cells [46]. Th1 cells produce IFNγ and differentiate in response to IL-12, and Th2 cells produce IL-4, IL-5 and IL-13 and develop in an IL-4- dependent manner. However, a little over a decade ago, this paradigm shifted with the discovery of Th17 cells. Two independent groups identified Th17 cells as a distinct population that produced the signature cytokine IL-17. Th17 cells develop independent of the cytokines and transcription factors necessary for either Th1 or Th2 cells [47, 56]. It was also discovered that Th1 and Th2 subsets could antagonize the development of the Th17 subset. Th17 cells differentiate from naïve $CD4^+$ T cells with cytokine cues from TGFβ, IL-6, IL-1β and require IL-23 for expansion in

vivo [57]. RAR-related orphan receptor gamma (RORγt) and signal transducer and activator of transcription 3 (STAT3) are the transcription factors essential for development of Th17 cells [58]. Besides IL-17, Th17 cells produce other cytokines including IL-17F, IL-21, IL-22 and a heterodimeric IL-17A/F.

The IL-17 cytokine family consists of six related members: IL-17A, IL-17B, IL- 17C, IL-17D, IL-17E (also known as IL-25), and IL-17F [59]. The IL-17R family has five receptor subunits, IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE [59]. While the IL-17A and IL-17F homodimers as well as IL-17A-F heterodimer bind to IL-17RA/IL-17RC [60], IL-17C binds to the IL- 17RA/IL-17RE complex. IL-17B binds to IL-17RB and IL-17E to the IL-17RA/IL-17RB receptor complex. IL-17RA is ubiquitously expressed and the major cell types responsive to IL-17 are epithelial cells, endothelial cells and fibroblasts, although DCs and macrophages have been shown to also be responsive. IL-17RC expression is lower in hematopoietic cells and high in the cells of non-hematopoietic origin such as cells of the kidney, liver joints and thyroid [60].

Upon stimulation by IL-17, the receptor complex consisting of IL-17RA and IL-17RC recruits the adaptor protein Act1. Act1 then associates with downstream signaling molecules such as TRAF6 leading to activation of IKK and NF-κB. IL-17-mediated Act1 signaling complex also activates MAPKs and induces C/EBP expression. IL-17 signaling cascades lead to the activation of transcription factors such as NF-κB, C/EBPs, and AP1 to induce gene transcription. An alternate signaling cascade includes the formation of another Act1-dependent complex through downstream mediators such as IKKi, TRAF2, TRAF5 and SF2. This signaling pathway mediates TRAF6-independent mRNA stability of certain chemokine and cytokine mRNA transcripts. In contrast, there are some negative regulators of the IL-17 signaling

pathways. TRAF3 is a proximal negative regulator of the IL-17R that interferes with the formation of the Act1-TRAF6 dependent receptor signaling activation complex [61]. There are other important regulators in the pathway which include proteins such as A20 and MCPIP1 that function as important feedback inhibitors of IL-17 receptor signal transduction [62] [63].

1.5 IL-17- SUPERHERO OR SUPERVILLAIN: ITS'S ALL IN THE CONTEXT

Th1 cells were previously considered the main agents of inflammation in autoimmunity, but it is now clear that Th17 cells are the prime drivers in the pathogenesis of autoimmune diseases. IL-17, the signature cytokine of Th17 cells plays a dichotomous role in mediating immune responses in various diseases. On one hand, IL-17 is essential for host defense against extracellular bacteria and fungi. On the other hand, IL-17 is pathogenic in autoimmune conditions [64]. The role of IL-17 in regulating immune responses to various pathogens and in autoimmune diseases will be described in this section.

1.5.1 IL-17 in protection against infection

Th17 cells and other innate producers of IL-17 are enriched at mucosal surfaces such as the gut, lung and oral cavity [65] Th17 cells bearing the CCR6 receptor are found to be enriched at mucosal sites [66, 67]. IL-17 is important in protection against infections by extracellular bacteria such as *Klebsiella pneumoniae* or *Citrobacter rodentium*, which is used to model for enteropathic *E.coli* infections in humans. Studies demonstrated that IL-17RA$^{-/-}$ mice infected with *K. pneumoniae* had high bacterial burdens in the lung and overall reduced survival.

Recruitment of neutrophils was also affected and diminished levels of chemokines such as CXCL1, CXCL2 and CXCL5 amongst others were noted. In *C. rodentium* infection, an abundance of IL-17 producing cells such classic Th17 cells as well as innate producers like γδ-T and natural killer T cells were found in the gut. IL-22, another Th17 cytokine is crucial in the initial stages of *C.rodentium* infection [68]. However, IL-17 was significant at later stages and this was shown using IL-17A$^{-/-}$ and IL-17F$^{-/-}$ which were more susceptible to gut infections compared to wild types [69].

IL-17 is particularly important in host defense against fungi, with most studies done primarily in *Candida albicans. C. albicans* exists as a commensal microbe, colonizing human mucosal surfaces such as oral mucosa, vaginal tract and gut. It can cause mucosal infections such as oropharyngeal candidiasis (OPC) and vulvovaginal candidiasis, but a graver form of the disease is disseminated candidiasis. Studies in murine models of OPC revealed the crucial role for IL-17 in the clearance of oropharyngeal infection [70]. IL-17 induces the expression of pro-inflammatory mediators such as IL-6, neutrophil recruiting chemokines CXCL1, CXCL2 and CXCL5 and monocyte recruiting chemoattractants like CCL2 and CCL7. IL-17 also strongly up-regulates expression of several anti-microbial peptides, such as lipocalin-2 and β-defensins in the oral mucosa in wild type mice infected with *C. albicans* [70]. The importance of IL-17 in host defense against various pathogens is apparent in humans with mutations in genes in this pathway. For instance, patients with Job's Syndrome/Hyper-IgE Syndrome (HIES) have autosomal dominant-negative mutations in STAT3 that correlate with significantly diminished Th17 cell numbers. These patients are susceptible to chronic mucocutaneous candidiasis (CMC) [71]. Similarly, mutations in STAT1 and caspase recruitment domain-containing protein 9 (CARD9) are also associated with CMC and reduced Th17 cell frequencies [72]. Autosomal-

recessive mutations in IL-17RA and ACT1 and autosomal-dominant IL-17F mutation are known to cause CMC [73]. Patients with an autosomal recessive IL-17RC deficiency were also susceptible to CMC [74]. The role for IL-17 in disseminated candidiasis was seen in studies that showed IL-17RA$^{-/-}$ mice were extremely susceptible to this systemic disease [75]. A more detailed discussion of existing evidence for the role of IL-17 in disseminated candidiasis is provided subsequently in **Chapter 2**.

1.5.2 IL-17 and Autoimmunity

IL-17 is a major driver of inflammation in several autoimmune diseases including psoriasis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), and inflammatory bowel disease (IBD) amongst others [64]. This has been established in multiple mouse models of disease including RA, the collagen induced arthritis (CIA), murine model of MS, experimental autoimmune encephalomyelitis (EAE) and in mouse models of SLE [56, 76, 77]. More evidence for the involvement of IL-17 in autoimmune diseases like psoriasis and RA came from human studies where elevated levels of IL-17 were detected in the serum of RA and psoriasis in patients [78, 79]. IL-17 signals through its receptors IL-17R which is ubiquitously expressed and targets cells mostly of non-hematopoietic origin, particularly epithelial and endothelial cells within diseased or inflamed tissues enhancing expression of pro-inflammatory markers to ultimately drive autoimmunity [59]. For example in EAE, IL-17 targets the stromal cells like astrocytes and microglial cells to produce inflammatory chemokines and cytokines [80], furthering the inflammatory response by recruiting innate and adaptive immune cells. Similarly in RA, IL-17 acts on synoviocytes driving chronic inflammation of synovial joints that leads to bone and cartilage damage [81].

Studies indicate that IL-17 is involved in SLE pathogenesis. Lupus-prone mice MRL.Fas^{lpr} mice showed enhanced IL-17-mediated tissue injury in an ischemia reperfusion model [82]. In another lupus-prone BXD2 mouse model, elevated numbers of IL-17-producing T cells were discovered and overexpression of IL-17 exacerbated the disease, whereas IL-17R blockade ameliorated disease [83]. In humans, elevated levels of serum IL-17 and IL-23 were seen in SLE patients compared to healthy controls [84]. IL-17 levels in the plasma showed a positive correlation with disease activity [84]. Frequency of IL-17-producing T cells was higher in peripheral blood of SLE patients. In the same study, in vitro stimulated lymphocytes from SLE patients produced higher levels of IL-17A compared to healthy controls [85]. The role for IL-17 in SLE and specifically in the clinical manifestation of lupus nephritis is discussed in **Chapter 3 and Chapter 4.**

1.6 SUMMARY

The pleiotropy of IL-17 is evident from its involvement in multiple contexts like infection or autoimmunity. In order to appreciate the specific function of IL-17 in these varied diseases, it is essential to define the mechanisms by which IL-17 mediates its downstream effects. Using murine models, we report the role for IL-17 in three diseases; (i) disseminated candidiasis (**Chapter 2)**, (ii) autoimmune glomerulonephritis (**Chapters 3 and 4)**, and (iii) renal fibrosis **(Chapter 5).** IL-17 is protective in disseminated candidiasis while it is pathogenic in autoimmune glomerulonephritis. In renal fibrosis, our data suggest a protective role. We show the local kidney specific IL-17 driven responses in all three diseases. Taken together, results in

this thesis have led to the discovery of novel mechanisms by which IL-17 drives immune responses in the kidneys.

1.7 STATEMENT OF PROBLEM

The kidneys can be affected either by infection, from the ascending spread of pathogen or via the hematogenous route, or due to insult from autoreactive immune cells in autoimmune renal diseases. As a result, the renal inflammation that ensues is driven by pro-inflammatory cytokines. Our particular interest is in the role of IL-17 as a driver of inflammatory responses in the kidney. Using different mouse models of disease for infection, autoimmunity and fibrosis, we seek to outline the mechanisms of IL-17 responses in renal immunity and immunopathology.

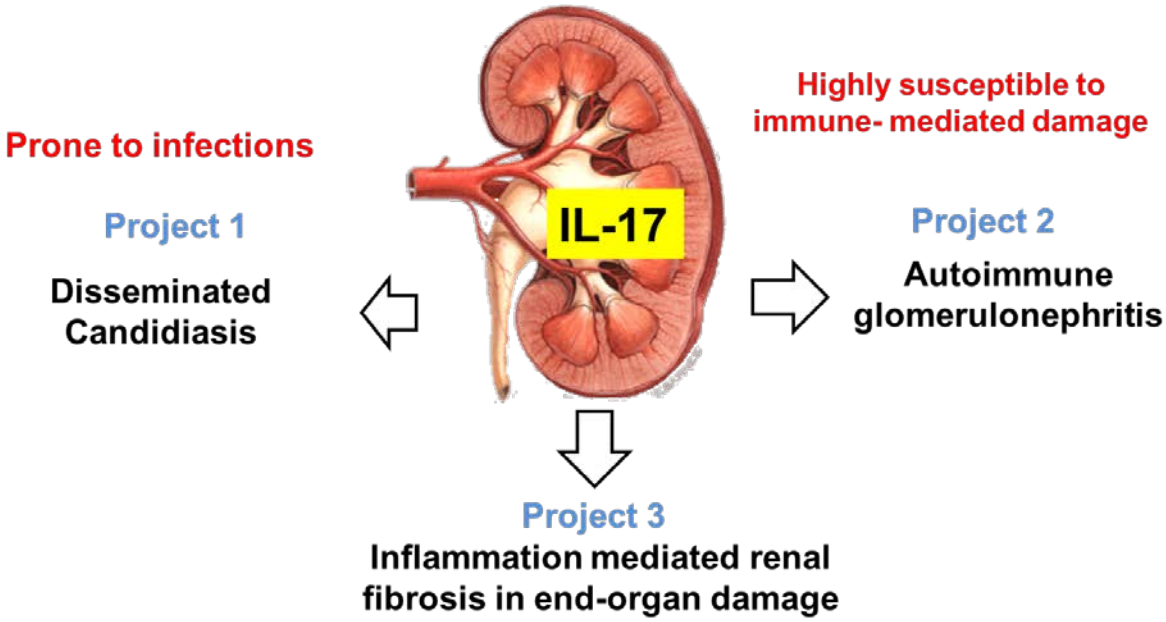

Figure 1. Thesis outline- studies in infection, autoimmunity and fibrosis

CHAPTER 2: THE KALLIKREIN-KININ SYSTEM: A NOVEL MEDIATOR OF IL-17-DRIVEN ANTI-CANDIDA IMMUNITY IN THE KIDNEY

This is the author's version of the work.

The definitive version will be published in

PLoS Pathogens, 12(11): e1005952. doi: 10.1371/journal.ppat.1005952

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2.0 SUMMARY

The incidence of disseminated *Candida albicans* infections is associated with hospitalized patients, with fatalities as high as 60%. Death from disseminated candidiasis in 30-40% adults and 50% neonates is due to fungal invasion of the kidney, leading to renal failure. Treatment of candidiasis is hampered by drug toxicity, the emergence of antifungal drug resistance and lack of vaccines against fungal pathogens. IL-17 is a key mediator of defense against candidiasis. The mechanisms of IL-17-mediated renal immunity have so far been studied only in the context of the regulation of antimicrobial mechanisms, particularly activation of neutrophils. Here, we identify an unexpected role for IL-17 in inducing the Kallikrein-Kinin System (KKS) in *C. albicans*-infected kidney, and we show that the KKS provides significant renal protection in candidiasis. Kallikrein1 (Klk1) was upregulated in the *C. albicans*-infected kidney in an IL-17-dependent manner. Overexpression of Klk1 or treatment with bradykinin rescued IL-17RA$^{-/-}$ mice from candidiasis. Therapeutic manipulation of IL-17-KKS pathways restored renal function and prolonged survival by preventing apoptosis of renal cells following *C. albicans* infection. Furthermore, combining a low dose of fluconazole with bradykinin markedly improved survival compared to either drug alone. These results indicate that IL-17 has a unique role in the prevention of tissue damage and preserves kidney function during disseminated candidiasis via the KKS. Since drugs targeting the KKS are approved clinically, these findings offer potential avenues for the treatment of this fatal nosocomial infection.

2.1 CANDIDA SPECIES

The *Candida* genus comprises around 200 species of fungi that can exist polymorphically, in either the yeast form (blastoconidia) or the filamentous form (hyphae and pseudohyphae). There are multiple *Candida* species, with *C. albicans* being the most common. Amongst other virulent *Candida* species are *C. glabrata, C. parapsilosis* and *C .tropicalis*. Other Candida spp. include *C. dubliniensis*, *C. krusei, C. keyfr* and *C. lusitaniae* that are less frequently involved in the clinical setting. *Candida* species can differ greatly in terms of their morphology, genetic composition and, importantly, their virulence in infections. *Candida* infections can broadly be classified as systemic or superficial. Systemic infections are usually seen in severely immunocompromised individuals and although these infections are relatively rare, they are associated with mortality as high as 40-60%. In contrast, superficial infections at mucosal surfaces, such as oral cavity and vagina are more common, but are less damaging to the host.

2.2 DISSEMINATED CANDIDIASIS

Candida species are fungi with a major yet underappreciated role in human pathology. In healthy people, *Candida* spp. are commensals that colonize mucosal surfaces and skin. However, when tissue homestasis is disrupted, *Candida* spp. can be pathogenic and cause an invasive infection. Amongst all *Candida* spp., *Candida albicans* is the most commonly isolated pathogen in patients with severe disseminated fungal infections [86]. Disseminated candidiasis is estimated to affect over 250,000 people worldwide annually [87, 88]. Disseminated candidiasis accounts for the fourth most common bloodstream infection in patients in intensive care unit and as the

seventh to tenth most common bloodstream infection in other patients according to population-based studies [89]. The incidence of disseminated candidiasis is highest in older adults and neonates, specifically those with low birth weight and preterm infants. Patients most susceptible to disseminated candidiasis include critically ill patients in the intensive care unit, patients with neutropenia, renal insufficiency, indwelling medical devices or those undergoing major abdominal surgery or solid-organ transplants [88]. Administration of glucocorticosteroids, chemotherapy, systemic antibiotics or hemodialysis also constitute the major risk factors for disseminated candidiasis [90]. *Candida* spp. invade sterile sites such as kidney, resulting in conditions such as ascending renal candidiasis [91]. Death due to disseminated candidiasis is seen in 30-40% of adults and 50% neonates. Although disseminated candidiasis is the most common manifestation of a bloodstream form of candidiasis, infections at other sites or organs such as joints, eyes, or central nervous system have been documented [91].

Disseminated candidiasis can also occur in patients who have variations in genes essential for anti-*Candida* immunity making them more prone to infection. Genome-wide clinical studies in patients have revealed that susceptibility to disseminated candidiasis was increased in those with SNPs in *TLR1*, *TLR2* and *TLR4* [92]. Similarly, SNPs in the *CD58*, *LCE4A-C1orf68* and *TAGAP* loci were identified and associated with an increased risk of disease [93]. Individuals who carried two or more alleles at these particular loci were prone to a 19-fold higher risk of developing disseminated candidiasis in the intensive care unit [93]. In another study, disseminated candidiasis in patients was associated with cytokine polymorphisms that led to either increased levels of anti-inflammatory IL- 10 or decreased levels pro-inflammatory IL-12 [94]. These results underscore the role for cytokines in the susceptibility to invasive candidiasis and in the clearance of the disseminated infection.

2.3 TREATMENT OPTIONS FOR DISSEMINATED CANDIDIASIS

There are currently no vaccines available against fungal pathogens. Antifungal drugs are the only option available for the treatment of disseminated candidiasis. These include azoles such as fluconazole or voriconazole, echinocandins such as caspofungin and anidulafungin, or amphotericin B [95]. However prolonged usage of azoles is associated with toxic effects in the host as well as emergence of drug resistant fungal strains [96]. This necessitates the development of novel antifungal drugs or improved therapeutic options to lower the doses of azoles in the treatment regimen for patients. Studies have provided reasonable support for the notion of combinatorial therapies of azoles with different compounds such as tacrolimus, cyclosporine A, amiodarone and retigeric acid B in both in vitro settings as well as in murine models of candidiasis [97-100]. These results are encouraging and suggest the potential for development of novel combination therapies with azoles to treat invasive fungal infections at lower administration doses or with a higher efficacy.

2.4 IMMUNE RESPONSES AGAINST CANDIDA

2.4.1 FUNGAL SENSING BY HOST

Host defense against invading fungi is dependent on the recognition of *C. albicans* through various pattern recognition receptors (PRRs), followed by the activation of an inflammatory response by the innate immune system. Most mechanistic studies so far have been done in mucosal candidiasis and far less is known about mechanisms of renal immunity in disseminated

candidiasis.

Toll-like receptors. Toll-like receptors (TLRs) were the first molecules identified as important receptors of the innate immune system that recognize pathogen associated molecular patterns (PAMPs) of microorganisms. TLR2 and TLR4 are the main TLRs implicated in *C. albicans* recognition [101]. TLR4 recognizes the mannan structures and induces the production of proinflammatory cytokines like TNFα and chemokines [102, 103]. TLR4 was also shown to be important in neutrophil recruitment to the infection site [104]. The ligand for TLR2 is phospholipomannan of *C. albicans* and induces pro-inflammatory cytokine production [105-107]. Other TLRs such as TLR6, TLR9 and TLR1 have also been implicated in anti-Candida immunity [108, 109].

C-type lectin receptors. C-type lectin receptors (CLRs) are a group of PRRs that recognize *C. albicans* fungal cell wall components. Dectin-1 is the most well characterized CLR, and recognizes β-glucans and is involved in ligand uptake, phagocytosis, and pro-inflammatory cytokines responses [110]. Dectin-2 is expressed on DCs, neutrophils and macrophages recognizes *C. albicans* α-mannan [111]. Dectin-2 is also known to modulate Th17 responses and formation of reactive oxygen species [112]. Dectin-2 in association with Dectin-3 leads to production of pro-inflammatory cytokines like TNFα, IL-1β and IL-6, during *C. albicans* infection [113]. Mannose-binding lectin, a soluble CLR, also binds mannan and regulates recruitment of phagocytes and pro-inflammatory anti-*Candida* responses [114]. Other CLRs with a role in anti-*Candida* host defense include scavenger receptors CD36 and SCARF1 [115].

Other PRRs and *Candida* immunity

- NOD-like receptors (NLRs) have also been implicated in disseminated candidiasis. Studies have reported NLRP3 inflammasome activation by *C. albicans* hyphae [116]. NLRP10-deficient mice were also found to be more susceptible to disseminated candidiasis [117].
- A recent study described the role for the RIG-I-like receptor MDA5, an intracellular RNA sensor, in the recognition of *C. albicans*. Polymorphisms in this receptor were shown to be linked to the susceptibility to disseminated candidiasis in humans [118].
- Complement receptor 3 (CR3) recognizes β-glucans found on neutrophils that can phagocytose and kill unopsonized *C. albicans* [119]. Mannans are another important cell wall component in *C. albicans* and are recognized by numerous CLRs. The mannose receptor, another PRR found on macrophages and monocytes can recognize N-mannans on the outer cell wall of *C. albicans* [120].
- Galectin-3 is expressed by macrophages and recognizes β-mannans to induce an antifungal response via secretion of TNFα. Mice lacking galectin-3 display increased susceptibility to disseminated candidiasis [121, 122].
- MINCLE is expressed on both neutrophils and monocytes, and initiates a protective antifungal response by inducing TNFα production [123].

2.4.2 INNATE MEDIATORS IN DISSEMINATED CANDIDIASIS

The next step upon recognition of fungal PAMPs by PRRs is the initiation of the cascade of downstream effector that culminate in a robust antifungal response. The outcome of the innate

sensing leads to the secretion of cytokines and chemokines that facilitate the influx of inflammatory cells into the infected kidney. Various components of the immune system contribute to the clearance of the fungal invasion.

Epithelial cells. An intact epithelial cell lining constitutes an important mechanical barrier against tissue invasion by *C. albicans*. Morphological changes from the yeast to hyphal form are crucial to tissue invasion. Epithelial cells express several PRRs including TLR 1-6 and TLR8-10, dectin-1 and galectin [109, 124, 125]. Infection of human epithelial cell with *C. albicans* downregulated most TLRs except TLR2 and TLR4, specifically in oral epithelial cells [104]. Studies have shown that epithelial cells can respond to colonization by *C. albicans* through recognition via TLR4 [125]. These data suggest that *C. albicans* may negatively regulate TLR-mediated sensing in oral epithelial cells. Additionally, epithelial cells help control *C. albicans* in a commensal state via Th17 cells or innate lymphoid cells (ILCs) leading to an IL-22 dependent production of antimicrobial peptides such as β-defensins [126, 127]. Epithelial cells secrete proinflammatory cytokines and chemokines after stimulation with *C. albicans*, including IL-1α/β, IL-6, G-CSF, GM-CSF, and TNFα, as well as the chemokines IL-8 and RANTES. IL-8 released by epithelial cells then recruits neutrophils into mucosal tissues and mediates fungal clearance.

Neutrophils. Neutrophils are essential in host defense against Candida infections. Epithelial cells and tissue-resident macrophages induce the production of chemokines necessary for the recruitment of neutrophils to the site of fungal infection [101]. Studies using neutropenic murine

models have delineated the role of neutrophils in clearance of disseminated candidiasis [128]. In humans, neutropenia constitutes a key risk factor in disseminated fungal infections [129, 130].

Monocytes and macrophages. Tissue-resident macrophages are crucial in antifungal defense. I*n vivo* studies in macrophage-depleted mice showed accelerated fungal proliferation in tissues and increased mortality [131] leading to potent fungicidal activity against *C. albicans* [132, 133]. In CX_3CR1-deficient mice, reduced accumulation of monocyte-derived macrophages in the kidney leads to renal failure and mortality [133]. In addition, increased susceptibility to disseminated candidiasis was noted in patients with a polymorphism resulting in diminished CX_3CR1 function [133]. Deficiency of CCR2, essential for monocyte recruitment to inflamed tissues, contributes to enhanced susceptibility to disseminated candidiasis [132].

Other innate mediators. Antimicrobial peptides (AMPs), produced in response to IL-17, are of particular relevance to fungal immunity. AMPs such as S100A8, S100A9, LL-37 and β-defensins (BDs) are known to be crucial in the clearance of candidiasis at mucosal surfaces like the oral cavity and skin [64]. IL-17 stimulates epithelial cells to induces AMPs like BDs, which have potent candidacidal activity. In mice, BD3 has been shown to be an IL-17-dependent gene induced during OPC [70]. BD1 also plays an important protective role in murine OPC [127]. Another class of AMPs with candidacidal activity are histatins, which are highly expressed in human salivary gland [134].

2.4.3 ADAPTIVE IMMUNITY IN DISSEMINATED CANDIDIASIS

Protective immunity against *C. albicans* is dependent on the activation of T cell based adaptive immune responses.

Th1 cells. Th1 cells are known to have protective role during invasive fungal infection. Th1 cells produce IFNγ, which is important for antifungal activity of neutrophils. IFNγ-deficient mice were highly susceptible to disseminated *C. albicans* infection [135]. Moreover, mice lacking IL-18, which augments the production of IFNγ, are also susceptible to disseminated candidiasis [136]. IFNγ also contributes to antifungal host defense by inducing nitric oxide production from macrophages and *Candida*-specific immunoglobulin production [137].

Th2 cells. Th2 responses are deleterious in fungal infection [138]. One report demonstrated that a balance is necessary between protective Th1 responses and detrimental Th2 responses in disseminated candidiasis. Overexpression of GATA-3, the master transcription factor of Th2 cells, causes an impairment of host defense against disseminated candidiasis, likely by limiting the production of IFNγ in Candida infection[139].

Th17 cells. The Th17 subset of cells have been implicated in host defense against *C.albicans*. Th17 cells produce IL-17 and IL-22, which are crucial for neutrophil recruitment and release of antimicrobial peptides such as β-defensins from epithelial cells. The first evidence for the involvement of Th17 cells in immunity against *C. albicans* came from a study that showed mice deficient in the IL-17 receptor subunit A (IL-17RA) were highly susceptible to disseminated *C. albicans* infection [75]. It was also observed that mice deficient in Th17 responses had enhanced

susceptibility to oropharyngeal candidiasis [70]. Additionally, IL-17RC$^{-/-}$, RORγt$^{-/-}$ and IL-17A$^{-/-}$ mice were also found to be susceptible to systemic *C. albicans* infection [75, 140-142]. In another study it was shown that dectin-2 induces IL-17A expression and is important in host antifungal defenses in disseminated candidiasis [111]. However, one recent study reported that IL-17 may also act on NK cells to drive production of GM-CSF, with protective activities in disseminated candidiasis [140]. The protective role for IL-17A and Th17 responses in *C. albicans* infection is seen in human patients as well. Patients who display chronic mucocutaneous candidiasis (CMC) or hyper-IgE syndrome and have a dysfunctional *Candida*-specific Th17 response, have also been found to be susceptible to mucosal *C. albicans* infections [143, 144]. Multiple studies have also implicated various CLRs such as dectin-1, dectin-2 and mannose receptor in the induction of Th17 responses in *Candida* infections. Unlike CMC, which affects individuals with compromised IL-17 signaling, systemic *C. albicans* infection normally impacts individuals with no known underlying genetic defects in IL-17 signaling pathways [86]. Systemic immunity against *C. albicans* has been extensively studied, however the mechanisms of local IL-17-mediated antifungal activities within the kidney still remain unclear.

2.5 KALLIKREIN-KININ SYSTEM

Kallikreins are made up of a family of fifteen serine proteases. Kallikrein1 (Klk1) specifically is known to play a key role in renal function and pathology [145]. Kallikreins catalyze the conversion of inactive kininogens to generate the active kinins, bradykinin and kallidin. For the purpose of this thesis, we focused on the role of bradykinin in particular. Bradykinin signals through two receptors; bradykinin receptor β1 (Bdkrb1), which is induced upon inflammatory

signals, and bradykinin receptor β2 (Bdkrb2), which is constitutively expressed. All these components of the signaling cascade constitute the Kallikrein-Kinin system (KKS) **(Figure 2).** The primary known function of the KKS is as a vasodilator in the regulation of blood pressure [145]. Additionally, studies in animal models have implicated the KKS in regulating inflammation, tissue repair and homeostasis in kidney injury [146]. The renal protective function of the KKS is mediated through upregulation of tissue repair proteins, inhibition of pro-fibrotic factors, and control of apoptosis [146]. In humans, polymorphisms in KKS-related genes such as *ACE*, *BDKRB2*, *NOS3*, *KLK1* are associated with an increased risk of acute and chronic renal injury [147-150]. While it is clear that the KKS protects the kidney in diseases associated with sterile inflammation, its role in renal immunity in infectious settings is poorly understood.

We sought to discover whether there was any link between the KKS and fungal infections caused by *C. albicans*. Kininogens have been shown to bind the *C. albicans* cell wall, causing the fungal protease SAP2 to induce the release of biologically active kinins [151, 152]. In addition, the KKS was recently implicated in IL-17-mediated skin inflammation and in an IL-17-dependent model of EAE [153, 154]. Nonetheless, to date there have been no links made between candidiasis, IL-17 and the KKS. Treatment of disseminated candidiasis with existing antifungal drugs have shown only partial success in improving prognosis. There is an impetus to identify new therapeutic avenues and we sought to identify the potential benefit of the IL-17-KKS axis in treatment of disseminated candidiasis.

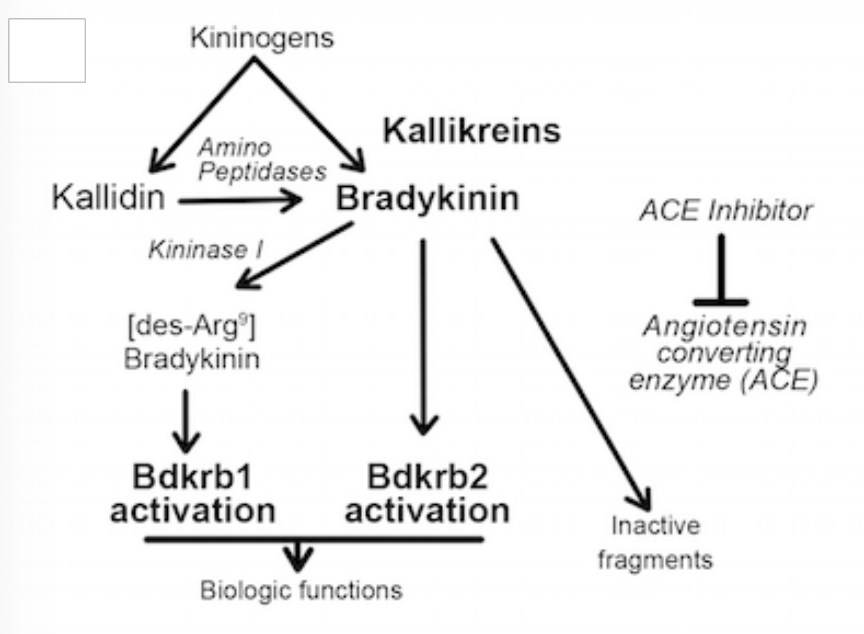


Figure 2. Kallikrein-kinin system

Overview of Kallikrein- Kinin system. Multiple proteins are involved in this pathway and have been explained in detail in this thesis.

2.6 MATERIALS AND METHODS

Mice

Wild type (WT) C57BL/6J mice were purchased from The Jackson Laboratory (Bar Habor, ME). IL-17RA$^{-/-}$ mice were kindly provided by Amgen (San Francisco, CA) and bred in-house. All mice were housed under specific pathogen-free conditions, and age-matched male mice were used for all experiments. Animal protocols were approved by the University of Pittsburgh IACUC (Protocol # 14094427), and adhered to the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

C. albicans culture and disseminated infection

C. albicans strains CAF2-1 or SC5314 were used as indicated. *C. albicans* was grown in YPD at 30^{o}C for 18-24 h. Mice were injected via the tail vein with PBS (sham-infected) or 1x10^{5} cfu (unless otherwise indicated) *C. albicans* yeast cells resuspended in PBS. Mice were weighed and monitored daily. Mice were sacrificed if they showed >20% weight loss or signs of severe pain or distress. Mice were evaluated for survival over a period of 14 days. At sacrifice on days 2, 3 and 7 p.i., as indicated, kidneys were weighed and homogenized in sterile PBS using a GentleMACS (Miltenyi Biotec, Cambridge MA). Serial dilutions of organ homogenates were plated on YPD agar with antibiotics, and fungal burden represented as colony forming units (cfu) per gram of tissue.

Measurement of serum creatinine and blood urea nitrogen

Serum was collected by retro-orbital bleeding at day 7 p.i. Creatinine and blood urea nitrogen levels were assessed using the QuantiChrom Creatinine Assay kit (BioAssay Systems, Hayward CA) and MaxDiscovery Blood Urea Nitrogen Enzymatic kit (Bioo Scientific Corp., Austin TX), respectively.

Measurement of vascular permeability

Vascular permeability in mice was assessed as described before [155]. Briefly, 100 µl sterile solution of Evans Blue dye (30mg/kg)(Sigma Aldrich., St Louis, MO) in PBS was injected intravenously. The stain was allowed to circulate for 30 min. After 30 min, mice were sacrificed and the hind feet were removed, blotted dry and weighed. The Evans blue was extracted from the feet with 1 ml of formamide overnight at 55° C and measured spectrophotometrically at 600 nm. Evans Blue stain was quantified according to a standard curve. The results are presented as ng of Evans Blue stain/mg of tissue.

In vitro stimulation of primary renal tubular epithelial cells and macrophages

Primary renal tubular epithelial cells (RTEC) from C57BL/6J mice (Cell Biologics, Chicago, IL) were cultured as per manufacturer's instructions. RTEC ($1x10^6$ cells/well) were treated with IL-17A (50 or 200 ng/ml) or TNFα (5 ng/ml) or IL-17C (50 or 200 ng/ml) or IL-17F (50 or 200 ng/ml) and IL-17 and TNFα in combination for 24 h. Recombinant murine IL-17A, IL-17C, IL-17F and TNFα were purchased from Peprotech (Rocky Hill, NJ).

Bone marrow derived macrophages (BMDM) from C57BL/6J mice were cultured for 7 days in the presence of L929 supernatants. BMDM and RTEC ($1x10^6$ cells/well) were treated with bradykinin (R&D Biosystems, Minneapolis MN) or left untreated for 24 h. LPS (Sigma Aldrich, St Louis, MO) was used as positive control. Supernatants were subjected to analyses using commercially available IL-6, IL-1β and TNFα ELISA kits (Ebiosciences, Dallas TX. Nitrite concentrations were measured by tri-iodide based reductive chemiluminescence as previously described [156]. Briefly, samples were injected into tri-iodine to reduce nitrite to NO gas that was detected by a Nitric Oxide Analyzer (Sievers, GE).

In vitro fungal killing assay

Neutrophils isolated from bone marrow using Neutrophil Isolation Kit (Miltenyi Biotech, San Diego, CA) were plated at 1×10^5 cells/well. Non-opsonized *C. albicans* was added to neutrophils at 0.5×10^5 yeast cells/well (ratio of 2:1). If indicated, bradykinin was added to the wells. Cultures were incubated with unopsonized *C. albicans* for 3 h and lysed in cold double-distilled H_2O. Killing was assessed by cfu counts in triplicate. The results are reported as percentage killing of *C. albicans* which is calculated as 1-[cfu of treatment group/cfu of control group] X 100. The same protocol was followed for bone marrow derived macrophages which were

cultured with L929 supernatants supplemented media for 7 days prior to carrying out the killing assay.

Gene expression profiling and quantitative real–time PCR analysis

At sacrifice, kidneys were stored at -80^{o}C. Total RNA was extracted from the homogenized kidney tissue with the RNeasy Micro Kit (Qiagen, Valencia CA) and submitted to Genomics Research Core at University of Pittsburgh. Gene expression analysis was performed using Mouse WG6 Gene Expression Bead Chip (Illumina). All test, normalization and transformation analyses were performed using caGEDA, a feely available informatics tool. The data sets were analyzed for differentially expressed genes. Efficiency analysis was performed by Random Resampling Validation using a Naïve Bayes Classifier and PACE analysis. The cluster analysis was performed by Unweighted Pair Group Method with Arithmetic Mean and similarity measure was determined by Euclidean distance. For real time PCR analysis, complementary DNA was synthesized with SuperScript III First-Strand (Invitrogen, Carlsbad CA). Gene expression was determined by qPCR with PerfeCTa SYBR Green FastMix ROX (Quanta BioSciences, Gaithersburgh MD) on a 7300 Real-Time PCR System (Applied Biosystems, Carlsbad CA). Primers were obtained from Quantitect (Qiagen, Valencia CA). The expression of each gene was normalized to *Gapdh.*

Flow cytometry

At sacrifice on day 7 p.i., kidneys were harvested following perfusion with PBS. Briefly, kidney homogenates were digested in PBS with 1 mg/ml collagenase type I (Worthington, Lakewood NJ) for 30 m at 37^{o}C. Cells were stained with the following antibodies: CD45 (BioLegend; clone 30-F11), Ly6G (BD Biosciences; clone IA8), F/480 (eBiosciences; clone BM8). For detection of apoptotic cells and cleaved Caspase-3 positive cells, cells were stained with Annexin V (BD

Pharmingen) and CaspGLOW Fluroscein active Caspase-3 staining kit (eBioSciences), respectively as per manufacturer's protocol. Samples were acquired and sorted on a Fortessa and FACS ARIA II, respectively (BD Biosciences, San Jose CA) and analyzed with FlowJo (Tree Star, Ashland OR).

Histology and Immunohistochemistry

Kidneys were fixed in formalin, dehydrated and paraffin embedded. Serial kidney sections were stained with H&E, Periodic-acid Schiff or Masson Trichrome stains for morphological analysis and determination of kidney injury.

Immunohistochemistry staining was done on formalin-fixed, paraffin embedded sections. Sections were rehydrated and antigen retrieval was performed with heated citrate. Primary antibodies against the following proteins were used: Klk1 (LifeSpan Biosciences, Seattle WA), NGAL (Santa Cruz Biotechnology, Dallas TX) and cleaved caspase-3 (Cell Signaling, Danvers MA). Secondary antibodies used were horseradish peroxidase coupled antibodies (Jackson ImmunoResearch, West Grove, PA). To detect apoptotic cells TUNEL staining was done on frozen kidney sections using the TUNEL apoptosis detection kit according to manufacturer's protocol (Millipore, Temecula CA). The number of $TUNEL^+$ cells was counted in 15 randomly selected high powered fields (400X) per slide. All images were obtained with EVOS FL Auto microscope (Life Technologies CA).

Western blots

Kidneys were homogenized in RIPA buffer. Concentration of protein was quantified by the BCA quantitation assay (Thermo Scientific, Pittsburgh PA). Equal amounts of sample were subjected to electrophoresis and transferred to PVDF membranes (Millipore, Billerica MA). After blocking with 5% milk in TBS, the blots were incubated with anti-mouse Klk1 (LifeSpan Biosciences,

Seattle WA), anti-mouse NGAL (R&D Biosystems, Minneapolis MN), anti-mouse cleaved Caspase-3 (Cell Signaling, Danvers MA) or anti-mouse beta-actin (Abcam, Cambridge, MA) overnight in 4°C. The blots were then washed and incubated for 1 hour at room temperature with individual secondary antibodies. Bands were detected using an enhanced chemiluminescence detection system (ThermoScientific, Pittsburgh PA) and developed with a FluorChem E imager (ProteinSimple, San Jose CA). Band corresponding to proteins of interest were analyzed by ImageJ software.

Adenoviruses

Adenoviruses expressing IL-17A (Ad-IL-17) and control vector (Ad-ctrl) were kindly provided by Dr. J. Kolls (U. Pittsburgh). Ad-Klk1 and corresponding Ad-ctrl vector were from Applied Biological Materials Inc. (Richmond, British Columbia, Canada). Mice were injected via the tail vein with $1x10^9$ pfu virus 72 h prior to induction of disseminated candidiasis.

Bradykinin receptor agonists and antagonists

Mice were injected with bradykinin **(**300 nmol/kg/day) (R&D Systems, Minneapolis MN) in a 200 µl volume i.p. Mice received i.p. injection of a combination of Bdkrb1 (R-715: 1 mg/kg/day) and Bdkrb2 (HOE-140: 1 mg/kg/day) antagonists (R&D Systems, Minneapolis MN). Untreated mice received equal volume of PBS.

Fluconazole treatment

C. albicans infected mice were treated with Fluconazole (FLC) (Diflucan: obtained from University of Pittsburgh Medical Center, Pittsburgh PA) as described before with minor modifications [157]. Briefly, mice were treated with 5, 10, 20 and 40 mg/kg body weight FLC by oral gavage at 2 h and 26 h post infection. Untreated mice received equal volume of PBS.

Statistics

Data were analyzed by Kaplan-Meier, ANOVA, Mann-Whitney or unpaired Student's *t* test using GraphPad Prism (La Jolla, CA). *P* values <0.05 were considered significant. All experiments were performed a minimum of twice to ensure reproducibility.

2.7 RESULTS

2.7.1 IL-17 triggers Klk1 expression in the kidney during disseminated candidiasis

In *C. albicans* intravenous challenge in mice, the kidney is the most heavily colonized organ [158]. With a higher inoculum (>10^6 cfu), mice succumb to infection within 48-72 h due to sepsis. However, mice infected with a low dose of *C. albicans* (10^5 cfu) exhibit progressive loss of renal function over a period of ~2 weeks, which more accurately reflects disease progression in humans [158]. During candidiasis, IL-17 is rapidly upregulated in the kidney, but its function there is unknown [75]. To understand how IL-17 mediates kidney-specific immunity, we performed Illumina microarray analyses comparing WT and IL-17RA$^{-/-}$ renal gene expression at 48 h p.i. Confirming previous reports, IL-17RA$^{-/-}$ mice demonstrated significantly increased kidney fungal burden in comparison to WT following infection with *C. albicans* (CAF2-1 or SC5314) **(Fig 3)** [63, 75, 141]. The classic IL-17 gene signature includes neutrophil-related genes and antimicrobial peptides (AMPs), such as CXC chemokines (*Cxcl1,2,5*), defensins (*Defb3*), calprotectin (*S100a8*) and lipocalin 2 (*Lcn2*) [70]. Although a few genes previously shown to be controlled by IL-17 were differentially expressed in the kidney during candidiasis, overall we saw a surprisingly distinct gene profile compared to analyses of IL-17-dependent genes in other settings **(Fig 4)**. Using the DAVID Gene Functional Classification algorithm

(which uses a gene-to-gene similarity matrix based shared functional annotation), we identified several functional groups with enrichment scores over 1.0. Most striking to us based on their known role in kidney physiology was the enrichment of genes encoding the KKS (**Fig 3B**).

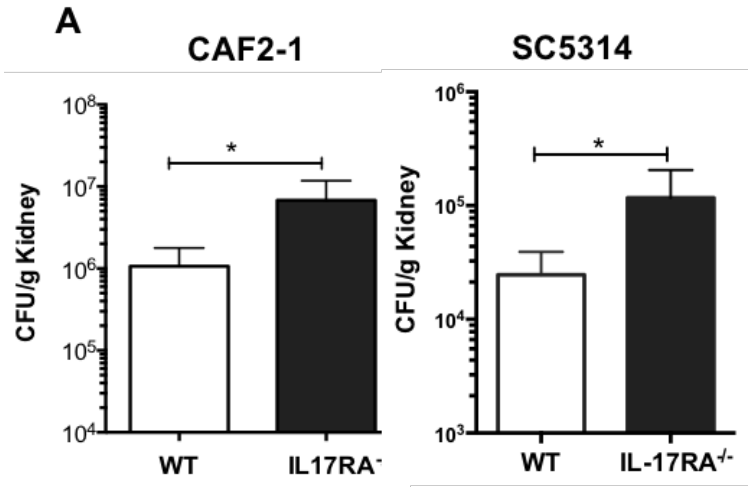


Figure 3. Increased fungal burden in IL-17RA$^{-/-}$ kidney following *C. albicans* infection

WT and IL-17RA$^{-/-}$ mice (n=4-6) were subjected to systemic *C. albicans* (CAF2-1 or SC5314) infection. After 48 h, kidneys were evaluated for fungal load. Data pooled from 2-3 independent experiments.

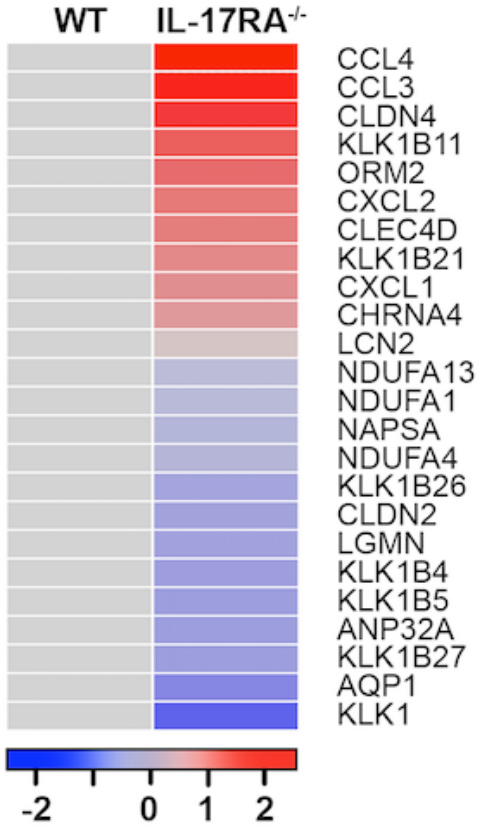
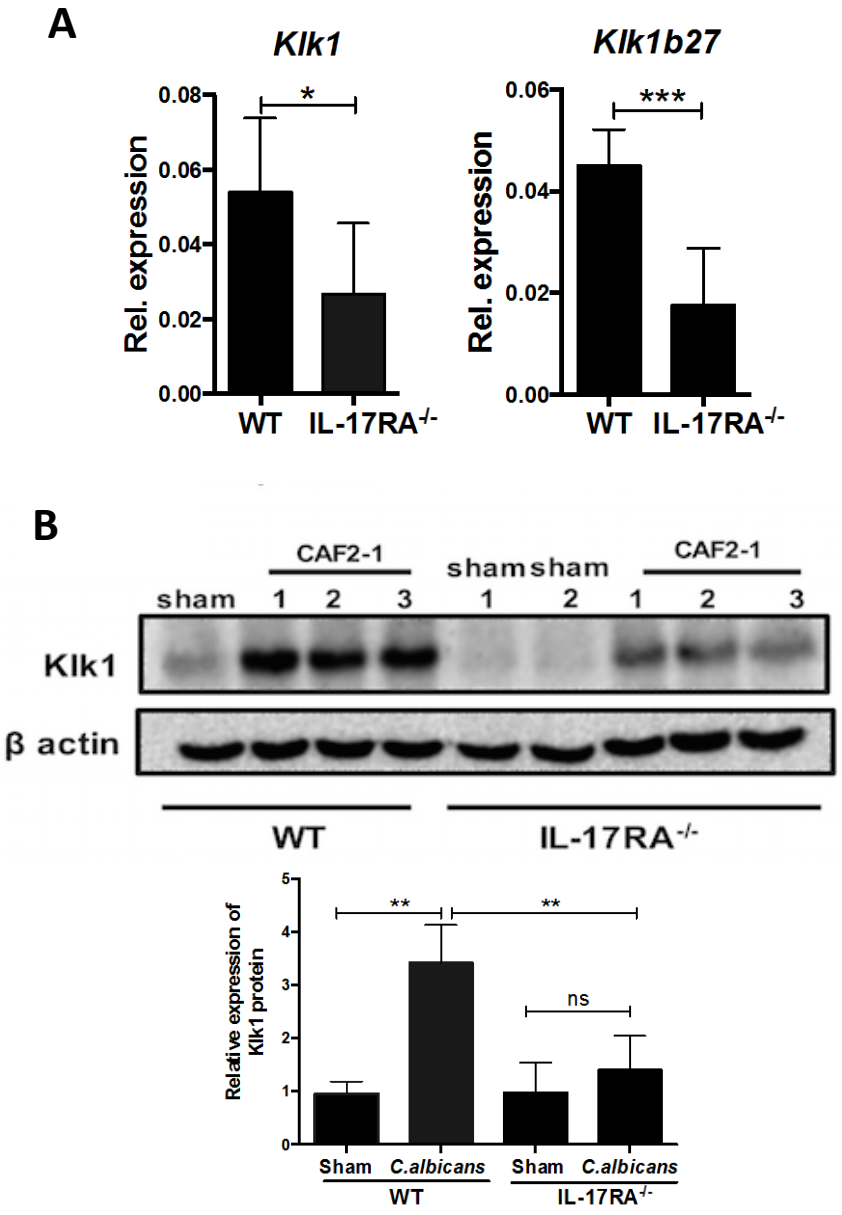


Figure 4. Increased fungal burden in IL-17RA$^{-/-}$ kidney following *C. albicans* infection

Heat map representing averaged intensity of expression of genes in WT and IL-17RA$^{-/-}$ kidneys (n=2) at 48 h p.i. RNA samples used for microarray courtesy Abhishek Garg.

Multiple *Klk* genes were suppressed in the kidney of IL-17RA$^{-/-}$ mice compared to WT following *C. albicans* infection. These results were verified by measuring the renal expression of *Klk1* and *Klk1b27* by qPCR **(Fig 5A)**. We further verified protein expression of Klk1 by immunoblotting. Klk1 was constitutively expressed at comparable, albeit low, levels in the sham-infected WT and IL-17RA$^{-/-}$ mice. However, Klk1 was upregulated in WT kidney following *C. albicans* infection. This observation was true upon infection with either the CAF2-1

or SC5314 strains of *C. albicans*. Confirming the gene expression data, we observed reduced expression of renal Klk1 in the absence of IL-17RA during systemic infection **(Fig 5B and 5C).** Collectively, these data indicate that IL-17 signaling in the kidney regulates Klk1 expression during disseminated candidiasis. To our knowledge, this is the first demonstration that renal Klk1 is upregulated in an infectious setting, and certainly first demonstration that the KKS is controlled by IL-17.



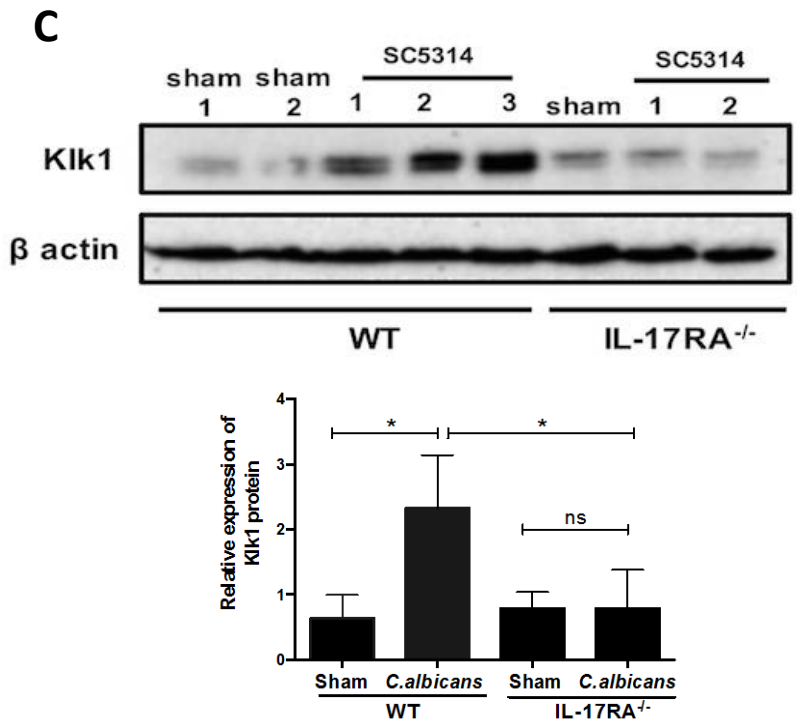


Figure 5. The expression of Klk1 is impaired in IL-17RA$^{-/-}$ kidney following *C. albicans* infection

Kidneys of WT and IL-17RA$^{-/-}$ mice (n=6) were evaluated for expression of *Klk1* and *Klk1b27* at 48 h p.i. Data pooled from 2 independent experiments. At 72 h p.i., whole cell extracts from WT and IL-17RA$^{-/-}$ kidneys (n=5-6) infected with *C. albicans* **(B)** CAF2-1 or **(C)** SC5314 were evaluated for Klk1 protein by western blotting. Sham infected mice received PBS. Images were quantified using ImageJ. Representative image of 1 of 2 independent experiments (**B** and **C**). For the bar diagram, data are combined from 2 independent experiments. Bars indicate mean ± S.D. P<0.05 (∗), <0.01 (**), <0.001 (***). ns, not significant.

2.7.2 Klk1 is required for IL-17-mediated renal protection against disseminated candidiasis

To understand the role of kallikreins in candidiasis, we focused on Klk1 based on its connection to IL-17-driven diseases including EAE and SLE [153, 154]. Kidney sections from *C. albicans*-infected WT mice were stained for Klk1 at 72 h p.i. Only kidney-resident cells, particularly renal tubular epithelial cells (RTEC), expressed Klk1 during infection **(Fig 6A).** These results agree with previous reports show that RTEC are the major producers of Klk1 in chronic kidney diseases [159, 160]. Although Klk1 controls vital kidney functions, its regulation and function under inflammatory conditions are not defined. We asked if changes in *Klk1* expression were a direct result of IL-17 signaling in RTEC, or a by-product of general renal inflammation due to increased fungal burden. We treated primary RTEC *in vitro* with IL-17 together with TNFα, with which IL-17 exhibits strong signaling cooperativity [161]. Indeed, IL-17 and TNFα triggered strong synergistic upregulation of *Klk1* and *Klk1b27* mRNA in RTEC **(Fig 6B),** revealing a previously unrecognized class of IL-17-dependent target

genes.

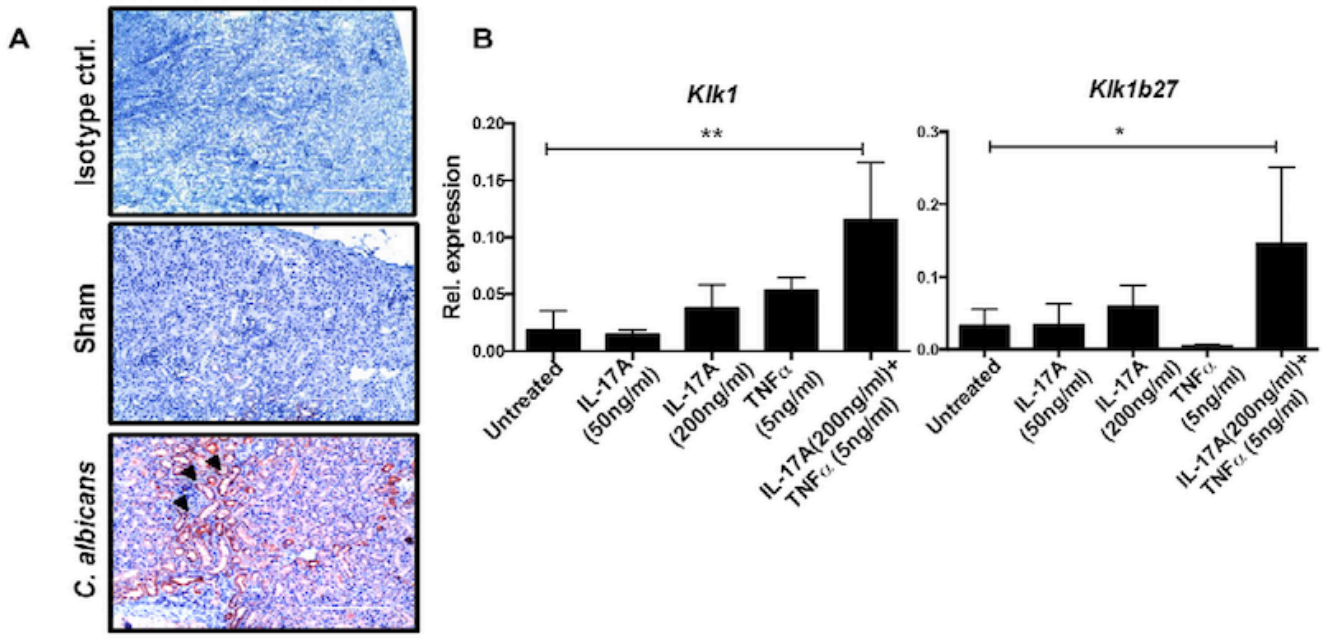


Figure 6. IL-17-driven Klk1 expression in the kidney is necessary for immunity to *C. albicans.*

(A) WT mice (n=5) were subjected to disseminated candidiasis. Sham-infected mice received PBS. After 72 h, kidney sections were stained with anti-Klk1 or isotype control Abs. Black arrows indicate Klk1 staining. Photomicrographs are representative of 2 independent experiments. Original magnification: 100X. **(B)** Primary RTEC from C57Bl6/J mice were treated ± IL-17 (50 ng/ml and 200 ng/ml), TNFα (5 ng/ml) or IL-17 (200 ng/ml) + TNFα (5 ng/ml) for 24 h. Expression of *Klk1* and *Klk1b27* was assessed by qPCR. Bars represent mean ± S.D. Data are representative of 4 independent experiments.

To verify the finding that IL-17 induces Klk1 in the kidney, we overexpressed IL-17 in WT mice using adenovirus (Ad-IL-17) [75]. RTEC stained positively for Klk1 following overexpression of IL-17. The expression of Klk1 was restricted to RTEC, as no staining could be detected in the glomerular and vascular compartments of the kidney **(Fig 7).** We therefore show that IL-17 induces the expression of Klk1 in RTEC following disseminated candidiasis. To

verify that IL-17 signaling was indeed required in the non-hematopoietic compartment we performed a bone marrow chimera experiment. We made bone marrow (BM) chimeric mice between WT and IL-17RA$^{-/-}$ mice and infected them with *C. albicans*. WT donors into IL-17RA$^{-/-}$ recipients as well as IL-17RA$^{-/-}$ donors into IL-17RA$^{-/-}$ recipients were susceptible to disseminated candidiasis as seen from the survival study. In contrast, IL-17RA$^{-/-}$ donors into WT recipients as well as WT donors into WT recipients displayed increased survival (**Fig 8**). These results indicate that IL-17 signaling in the non-hematopoietic compartments is crucial for the survival against disseminated candidiasis.

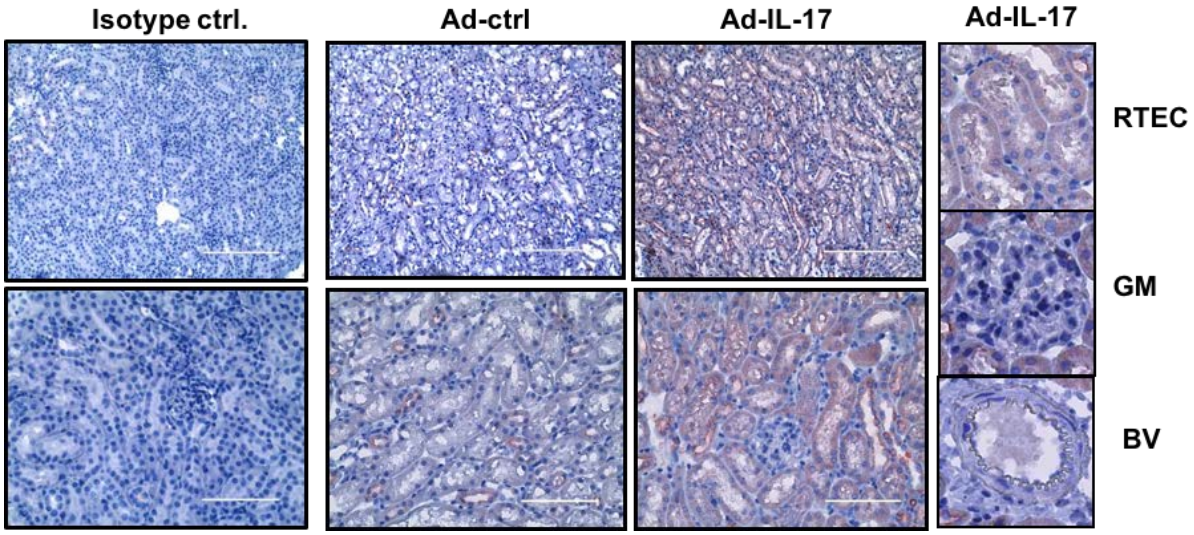


Figure 7. Expression of Klk1 is restricted to renal epithelial cells

WT mice (n=5) were injected with Ad-IL-17 or Ad-ctrl (1x10^9 pfu). Six days post-injection, kidney sections were stained with anti-Klk1 or isotype control Abs. Inset: Klk1 staining in RTEC and negative staining in glomerulus (GM) and blood vessels (BV). Photomicrographs are representative of 2 independent experiments. Original magnification: 200X (upper panel) and 400X (lower panel).

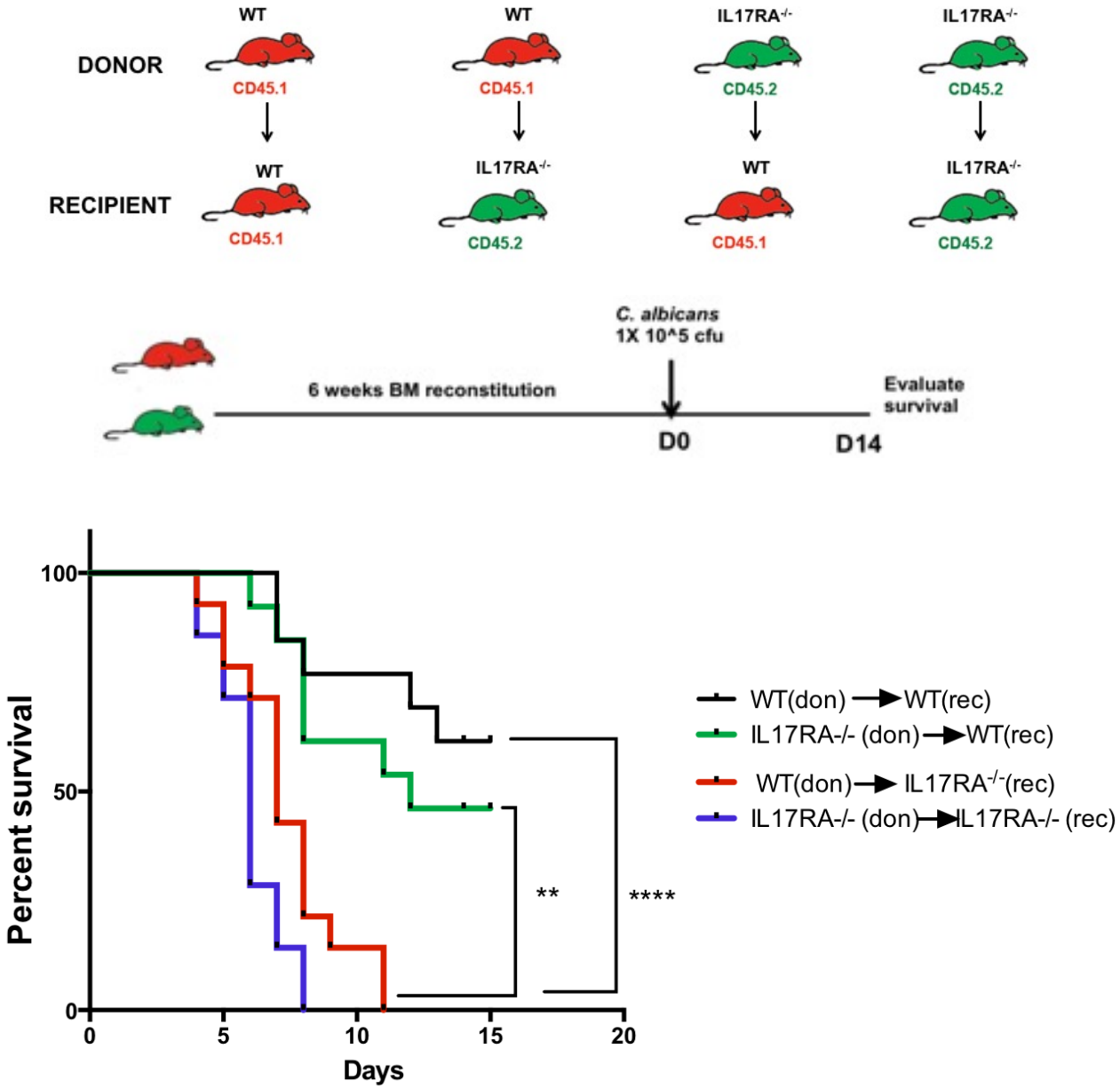
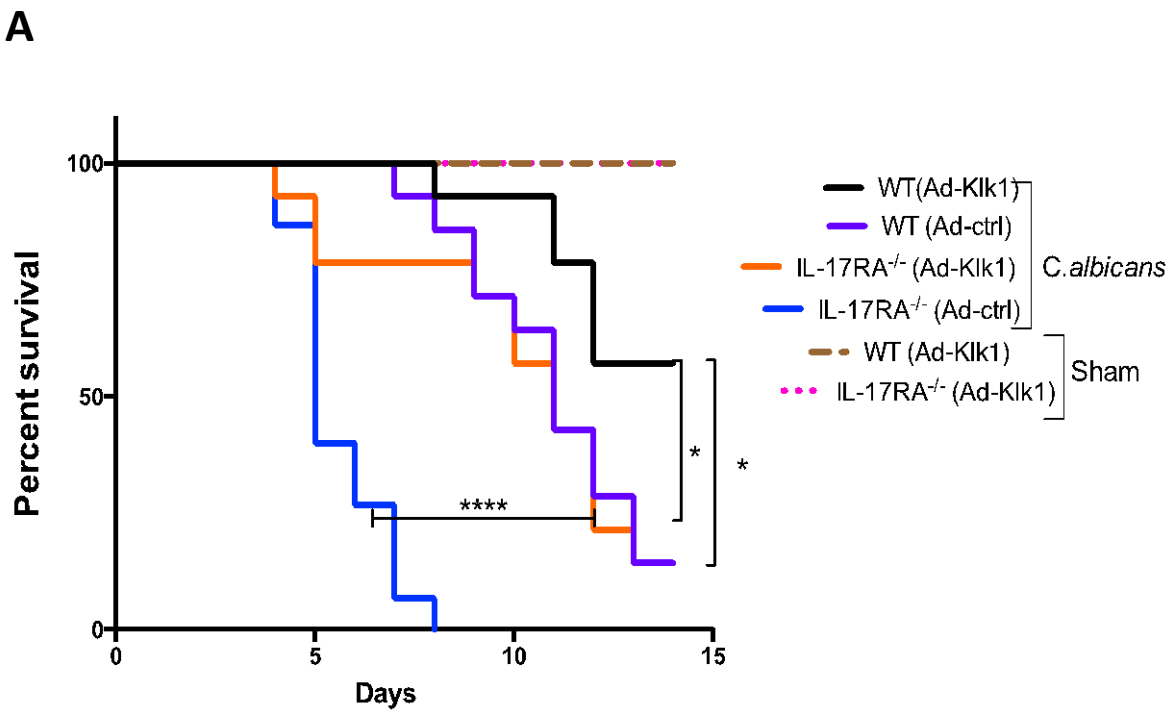


Figure 8. IL-17 signaling on non-hematopoietic cells is required for host defense against disseminated candidiasis

(A) WT (CD45.1) and IL-17RA$^{-/-}$(CD45.2) mice were sub-lethally irradiated and reconstituted with BM as indicated (n=4/group) **(B)** Experimental setup irradiated mice with transferred BM were let to reconstitute BM for 6 weeks after which thet were subjected to systemic *C. albicans* infection. (C) Survival was assessed over 14 d. Data pooled from 3 independent experiments $P < 0.05$ (*), <0.01 (**), <0.0001 (****).

Klk1 protects the kidney against acute and chronic disorders in sterile inflammation, but has not been linked to candidiasis or IL-17 signaling. To test the hypothesis that Klk1 plays a critical role in IL-17-driven renal protection against disseminated candidiasis, we overexpressed Klk1 with the adenoviral system (Ad-Klk1) and assessed disease susceptibility. Remarkably, overexpression of Klk1 significantly improved the survival of *C. albicans*-infected IL-17RA$^{-/-}$ mice and in WT mice **(Fig 9A).** A previous study suggested that Klk1 may induce inflammatory cytokines in human RTECs, at least *in vitro* [162]. However, we found that overexpression of Klk1 had very little impact on renal fungal load **(Fig 9B).** Overall, these data indicate that Klk1 enhances anti-*C. albicans* immunity in the kidney in an IL-17-dependent manner.



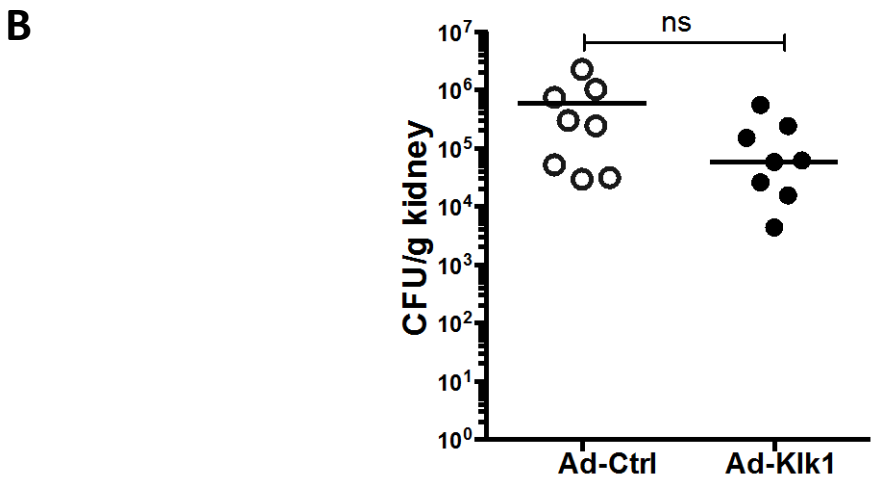


Figure 9. Overexpression of Klk1 prolongs survival in *C. albicans* infected IL-17RA$^{-/-}$ mice

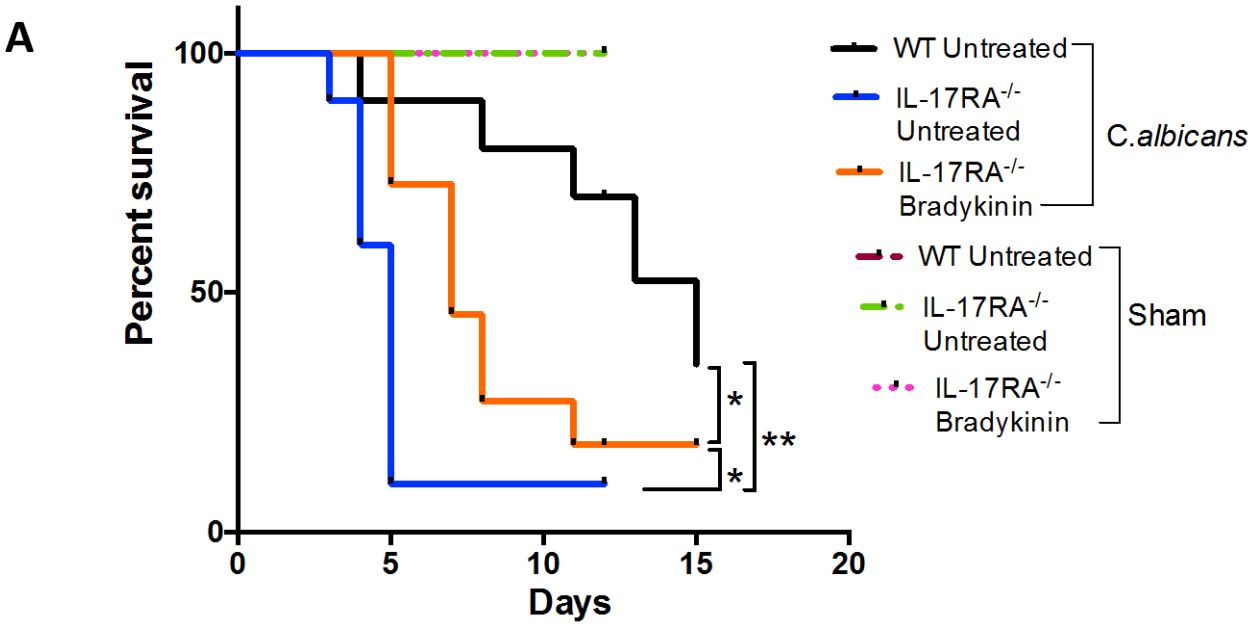
(A) WT and IL-17RA$^{-/-}$ (n=14) mice were injected with Ad-Klk1 or Ad-ctrl 72 h prior to systemic *C. albicans* infection. Sham-infected WT and IL-17RA$^{-/-}$ mice (n=3) received Ad-Klk1 only. Survival was assessed over 14 d. Data pooled from 3 independent experiments. **(B)** WT (n=8) mice were injected with Ad-Klk1 or Ad-ctrl 72 h prior to systemic *C. albicans* infection. Fungal burden was assessed at 72 h p.i. Each dot represents one mouse, and horizontal bars indicate mean. Data are combined from 2 independent experiments. $P < 0.05$ (*), <0.01 (**), <0.0001 (****). ns, not significant.

2.7.3 Activation of the bradykinin receptors is required for IL-17-Klk1 axis-driven protection against disseminated candidiasis

Klk1 mediates cleavage of kininogens to generate bradykinin, which signals through Bdkrb1 and Bdkrb2 **(Fig 2)** [146]. Additionally, Klk1 activates protease-activated receptors (PAR) such as PAR4 to trigger the release of inflammatory mediators from RTEC [162]. To define the role of Bdkrb activation in renal immunity, IL-17RA$^{-/-}$ mice were treated with bradykinin (300 nmol/kg) and survival evaluated following infection. As shown, 90% of the untreated IL-17RA$^{-/-}$ mice succumbed to infection by day 5 p.i., while mortality in IL-17RA$^{-/-}$ mice was delayed with

bradykinin treatment **(Fig 10A).** Additionally, untreated WT mice had a modestly increased survival benefit compared to bradykinin treated IL-17RA$^{-/-}$ mice, suggesting that there may also be a Bdkrb-independent pathway occurring in candidiasis.

Disseminated candidiasis typically impacts individuals with no known underlying immune defects [86]. Therefore, we assessed impact of Bdkrb signaling in mice with intact IL-17 signaling capacity and normal levels of Klk1. Accordingly, WT mice were treated with Bdkrb1 and Bdkrb2 antagonists and evaluated for survival following systemic *C. albicans* infection. Mice given Bdkrb1 and Bdkrb2 antagonists had significantly reduced survival compared to untreated controls **(Fig 10B).** Collectively, these results indicate participation of Bdkrb signaling in the renal host defense during disseminated candidiasis.



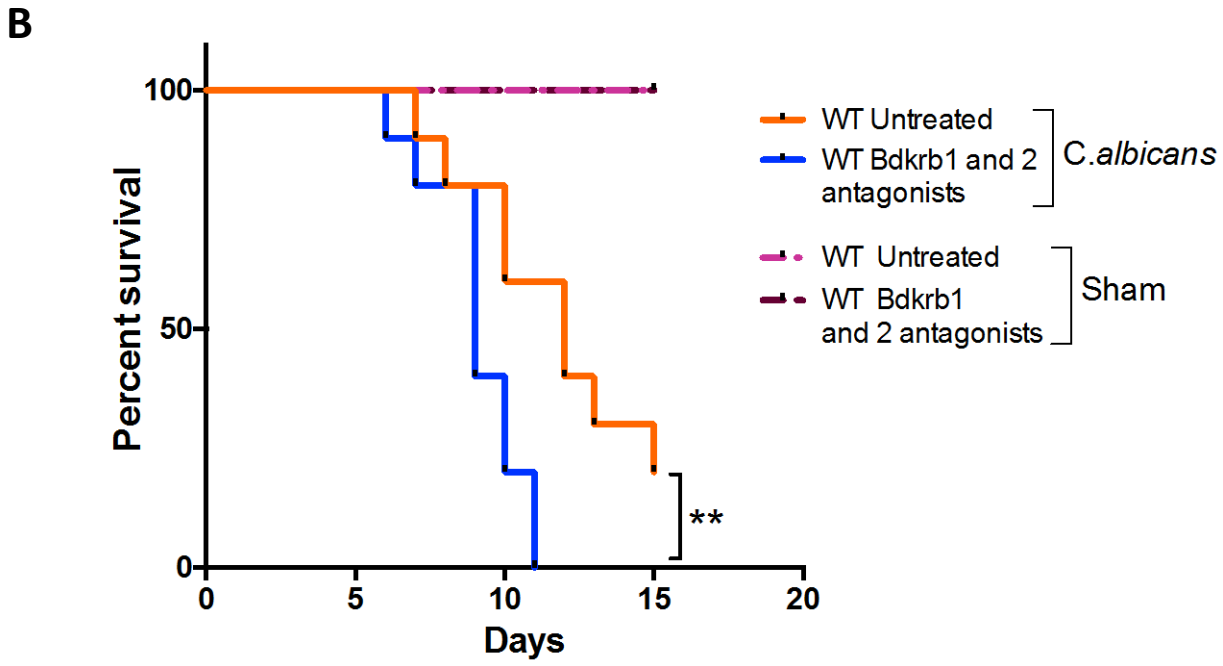


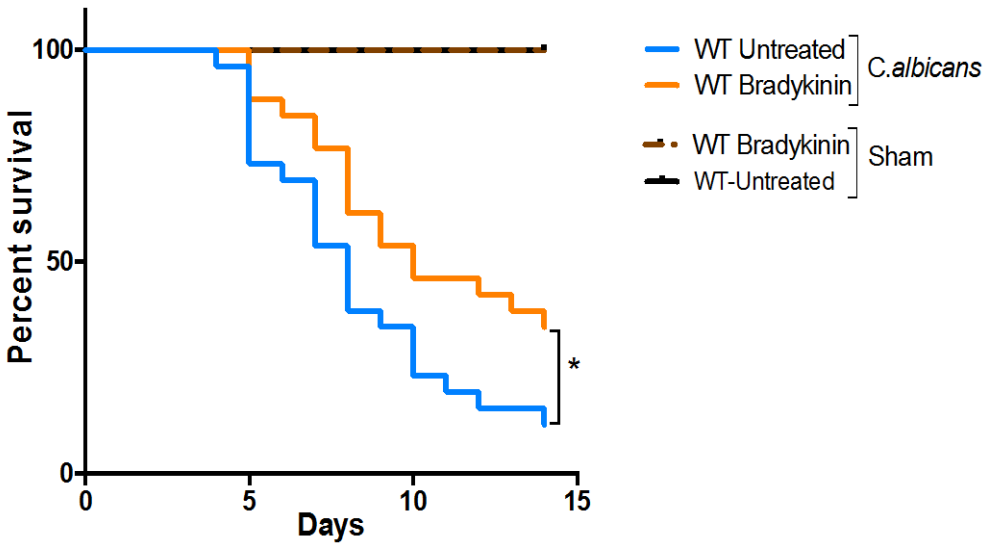
Figure 10. Bradykinin confers renal protection to IL-17RA$^{-/-}$ mice following disseminated *C. albicans* infection

(A) IL-17RA$^{-/-}$ mice (n=14) were treated with bradykinin (300 nmol/kg/day) or PBS starting on day -1 (relative to infection). On day 0, mice were administered *C. albicans* i.v. and evaluated for survival over 14 d. WT mice were infected with *C. albicans* and left untreated. Sham-infected IL-17RA$^{-/-}$ mice were given bradykinin only or left untreated (n=3). **(B)** WT mice (n=10-11) were treated with Bdkrb1 (R715; 1 mg/kg/day) and Bdkrb2 (HOE140; 1mg/kg/day) antagonists or PBS starting 1 day prior to infection and then daily for 14 d. Sham-infected WT mice were treated with the antagonists only (n=3). Mice were evaluated for survival over 14 d. Data are pooled from 2 independent experiments for **(A)** and **(B)**. *P* <0.05 (∗), <0.01 (**).

2.7.4 Bradykinin prevents renal damage and preserves kidney function in immunocompetent mice following disseminated candidiasis

Despite advances in antifungal therapy against disseminated candidiasis, mortality in patients with systemic *C. albicans* infection remains high [86]. Since IL-17 is implicated in controlling candidiasis in murine models, targeting downstream mediators of IL-17 signaling pathway is an attractive approach to treat disseminated candidiasis [86]. We hypothesized that treatment of infected mice with intact IL-17 signaling, with bradykinin would ameliorate candidiasis. To provide proof-of-principle, WT mice were treated with bradykinin and survival assessed upon infection two strains of *C. albicans* (CAF2-1 or SC5314*)*. Indeed, the bradykinin-treated cohort exhibited significantly delayed mortality compared to an untreated control group **(Fig 11A and 11B).**

A



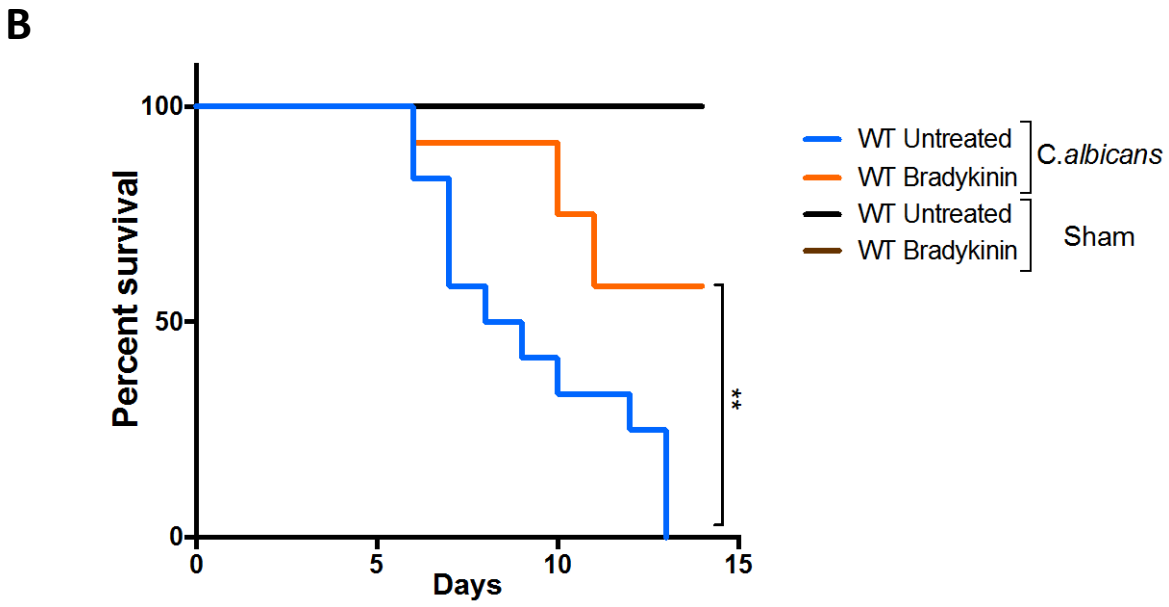


Figure 11. Delayed mortality in C. albicans infected WT mice treated with bradykinin

WT mice (n=14-20) were either left treated ± bradykinin (300 nmol/kg/day) starting day -1 (relative to infection). Mice were infected with *C. albicans* strains **(A)** (CAF2-1) or **(B)** (SC5314). Sham-infected WT mice were treated ± bradykinin (n=3-5). Survival was assessed over 14 d. Data are pooled from **(A)** 4 and **(B)** 2 independent experiments. P <0.05 (∗), <0.01 (**). <0.001 (***), <0.0001 (****). ns, not significant.

Measuring renal function is important to assess the extent of injury to the kidneys. Although bradykinin has been implicated in the development of angioedema [163], mice treated with bradykinin did not show any signs of angioedema at day 7 p.i. **(Fig 12A).** Clearance of *C. albicans* and timely repair of damaged tissues is crucial to preserve renal function. To understand the mechanisms by which bradykinin mediates renal protection during disseminated candidiasis, WT mice were treated with bradykinin starting on day -1 and then daily for 7 days. Mice treated with bradykinin demonstrated significantly diminished serum BUN and creatinine levels compared to untreated animals **(Fig 12B)**, indicating that bradykinin preserves normal renal function in systemic *C. albicans* infection.

A

B

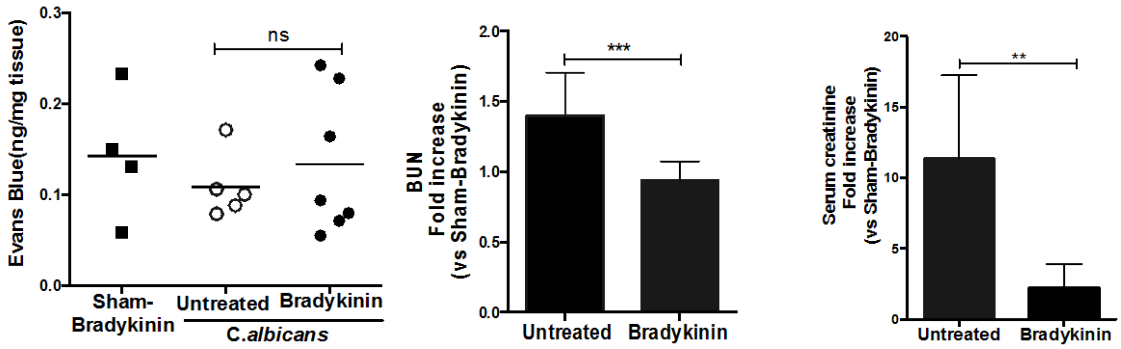
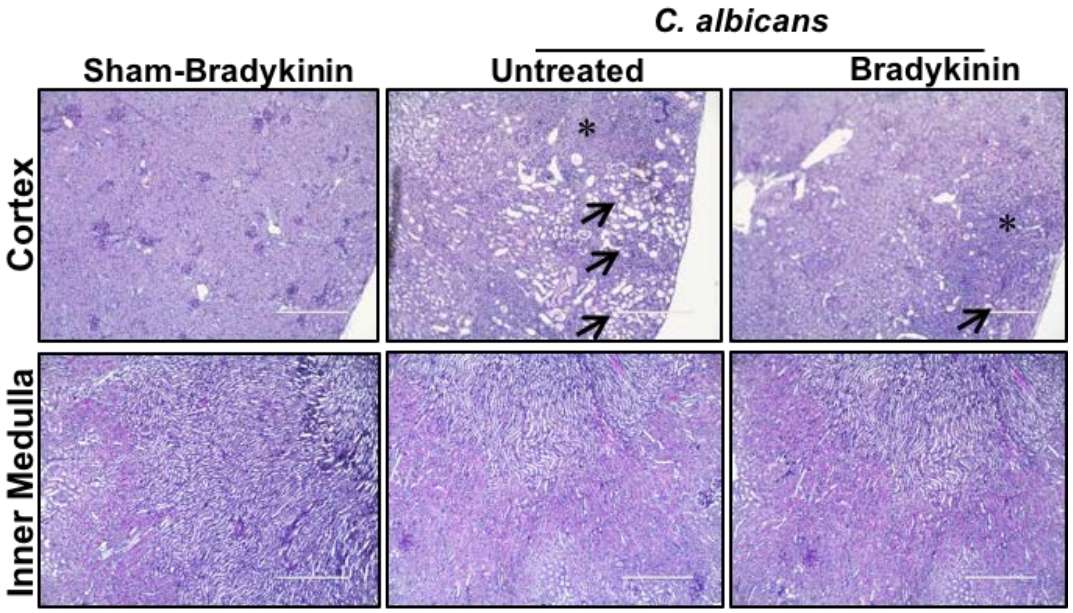


Figure 12. Renal function is preserved in infected WT mice treated with bradykinin

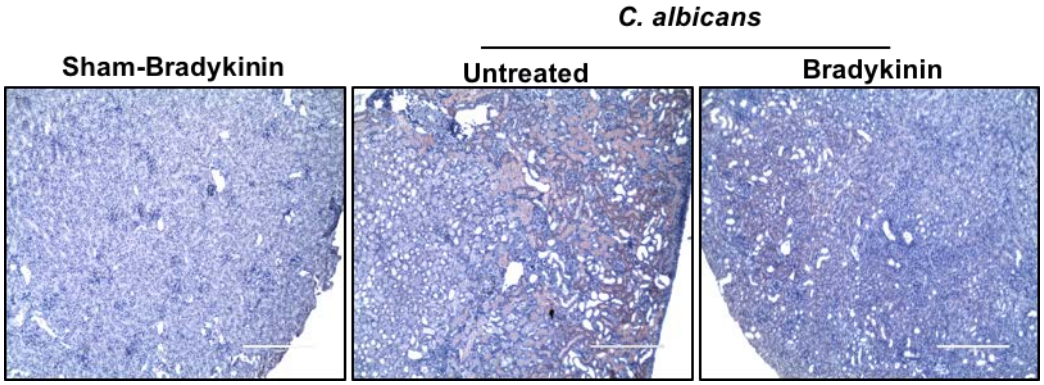
WT mice (n=14-20) were either left treated ± bradykinin (300 nmol/kg/day) starting day -1 (relative to infection). Mice were infected with *C. albicans*. At day 7 p.i., mice were evaluated for **(A)** angioedema development in the hind paw (n=4-7) and **(B)** serum BUN and creatinine levels (n=6-10). Data pooled from 2-3 independent experiments. Each dot represents one mouse and the bars indicate mean. P <0.05 (∗), <0.01 (**). <0.001 (***), <0.0001 (****). ns, not significant.

We further verified whether improved renal function in bradykinin treated mice was due to reduced damage of kidney parenchyma following fungal invasion. The renal parenchyma of infected mice showed overt pathological changes characterized by loss of brush border epithelium and tubular atrophy at day 7 p.i. compared to the sham mice. Moreover, the damage was primarily restricted to the renal cortex and outer medullary region. In line with the kidney function results, renal damage was ameliorated upon bradykinin treatment **(Fig 13A).** Furthermore, we observed significantly diminished expression of neutrophil gelatinase-associated lipocalin (NGAL), a prototypical kidney injury marker, in bradykinin treated animals **(Fig 13B and 13C).** Collectively, these results indicate that bradykinin prevents *C. albicans*-mediated renal damage and preserves renal function during disseminated candidiasis.

A


C. albicans
Sham-Bradykinin
Untreated
Bradykinin
Cortex
Inner Medulla

B


C. albicans
Sham-Bradykinin
Untreated
Bradykinin

C

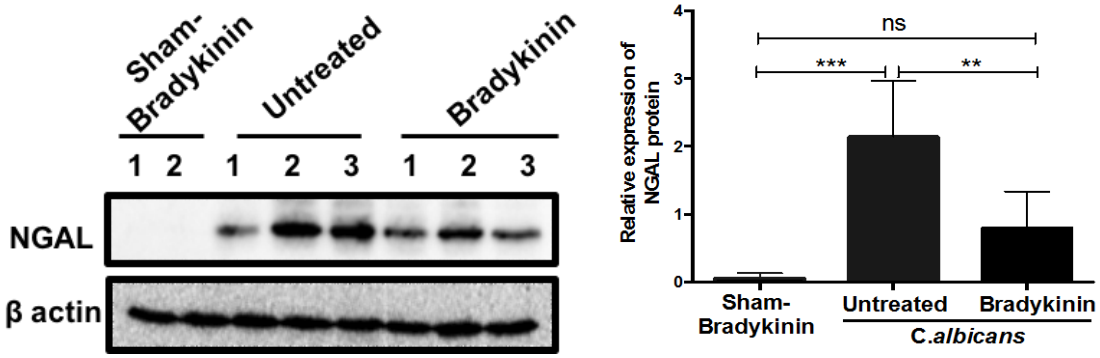

Sham-Bradykinin
Untreated
Bradykinin
1 2 1 2 3 1 2 3
NGAL
β actin
Relative expression of NGAL protein
ns
***
**
Sham-Bradykinin
Untreated
Bradykinin
C.albicans

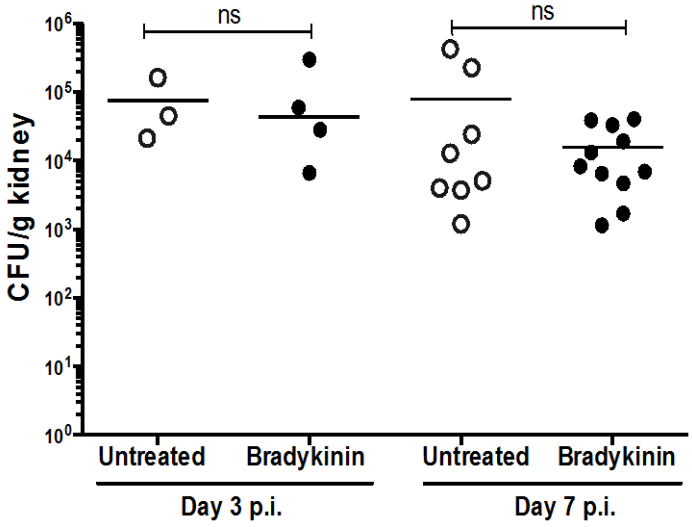
Figure 13. Bradykinin alleviates kidney injury in *C. albicans* infected mice

WT mice were either left treated ± bradykinin (300 nmol/kg/day) starting day -1 (relative to infection). Mice were infected with *C. albicans*. At day 7 p.i., kidney sections were evaluated for **(A)** histopathology and inflammatory cell influx by PAS staining (n=8) **(B)** NGAL expression by IHC (n=6-8). Black arrows indicate tubular damage and atropy; ∗ indicates inflammatory cell influx. Representative photomicrographs from 2 independent experiments. Original magnification: 100X. **(C)** Cell lysates of kidney homogenates (n=5-6) were evaluated for NGAL by western blotting. Images were enumerated using ImageJ. Representative image of 1 of 2 independent experiments. Bars indicate mean ± S.D and combined from 2 independent experiments. P <0.05 (∗), <0.01 (**). <0.001 (***), <0.0001 (****). ns, not significant.

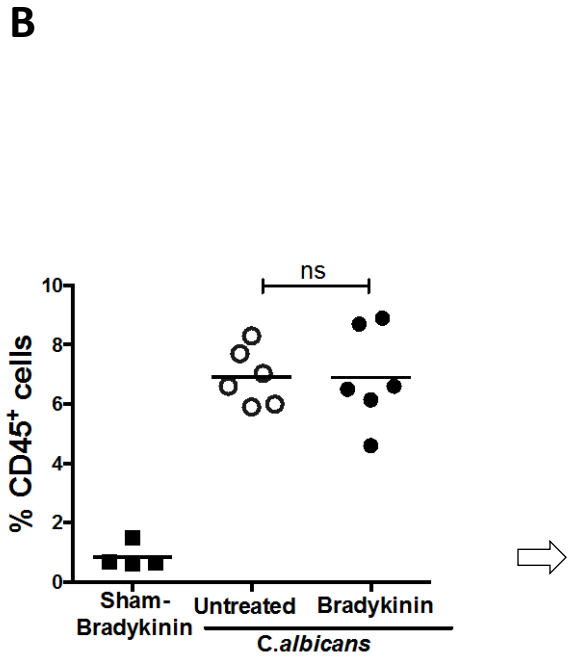
2.7.5 Bradykinin-mediated renal protection is independent of fungal clearance or inflammatory cell influx

To identify the effector mechanisms by which bradykinin prevents renal insufficiency, we evaluated fungal load and inflammatory cell influx at days 3 and 7 p.i. Although differences in kidney function were already evident early as day 7 p.i. **(Fig 12B)**, fungal loads were comparable between the bradykinin treated and untreated groups at these time points **(Fig 14A)**. Previous studies have shown that neutrophils and monocytes/macrophages mediate fungal clearance in candidiasis [133, 164]. Thus, we examined the frequency of infiltrating myeloid cells in kidney upon bradykinin treatment. In agreement with the fungal clearance rates, the percentages of kidney infiltrating inflammatory cells ($CD45^+$), neutrophils ($Gr1^+$) and macrophages ($F4/80^+$) were similar between groups **(Fig 14B).**

A


ns
ns
CFU/g kidney
Untreated
Bradykinin
Untreated
Bradykinin
Day 3 p.i.
Day 7 p.i.

B


ns
% CD45+ cells
Sham-Bradykinin
Untreated
Bradykinin
C.albicans

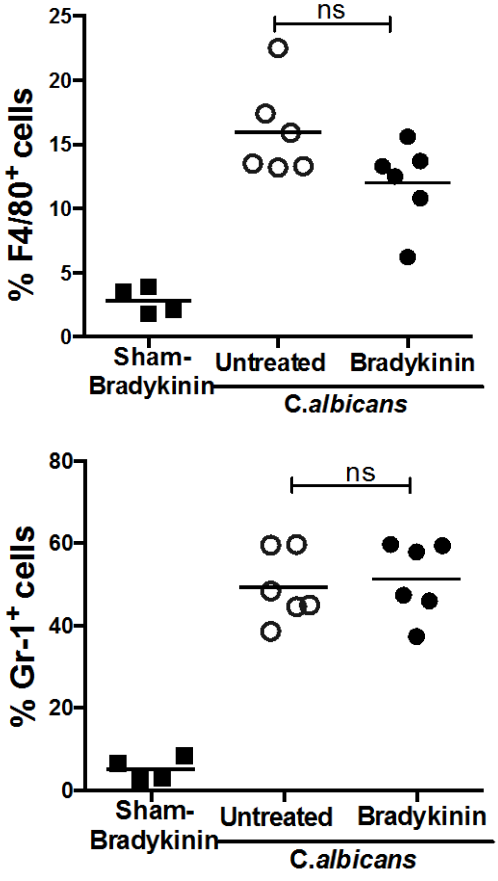
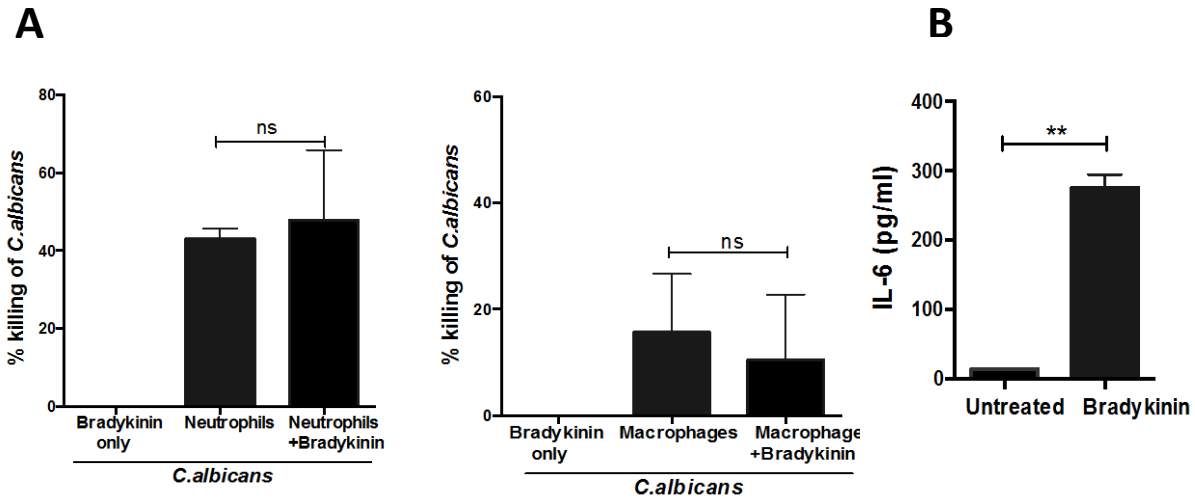

ns
% F4/80+ cells
Sham-Bradykinin
Untreated
Bradykinin
C.albicans
ns
% Gr-1+ cells
Sham-Bradykinin
Untreated
Bradykinin
C.albicans

Figure 14. Minimal impact of bradykinin treatment on fungal clearance and inflammatory cells influx in the *C. albicans* infected kidney

WT mice were treated ± bradykinin (300 nmol/kg/day) or PBS on day -1 (relative to infection). **(A)** Kidneys were evaluated for fungal load on days 3 (n=3-4) and 7 (n=8-11) p.i. (**B**) At day 7 p.i. (n=4-6), kidney infiltrating neutrophils ($Gr1^+$) and macrophages ($F4/80^+$) (gated on $CD45^+$) cells were evaluated by flow cytometry. Data are combined from 2 independent experiments for **(A)** and **(B)**. Each dot represents one mouse, and the bars indicate mean. $p < 0.01$ (**). ns, not significant.

We next assessed whether bradykinin impacted the candidacidal activity of innate cells. Bradykinin treatment of BM-derived neutrophils and macrophages (BMDM) did not alter their ability to kill unopsonized *C. albicans* yeasts, as determined by an *in vitro* fungal killing assay **(Fig 15A).** Additionally, RTEC and BMDMs were stimulated *in vitro* with bradykinin and culture supernatants evaluated for cytokines and nitrite production. Bradykinin induced IL-6 in RTECs **(Fig 15B),** but did not induce IL-6, TNFα or nitrite in BMDMs **(Fig 15C and 15D)**. Overall, these results indicate that bradykinin acts through a distinct mechanism independent of antimicrobial activities of renal infiltrating immune cells.



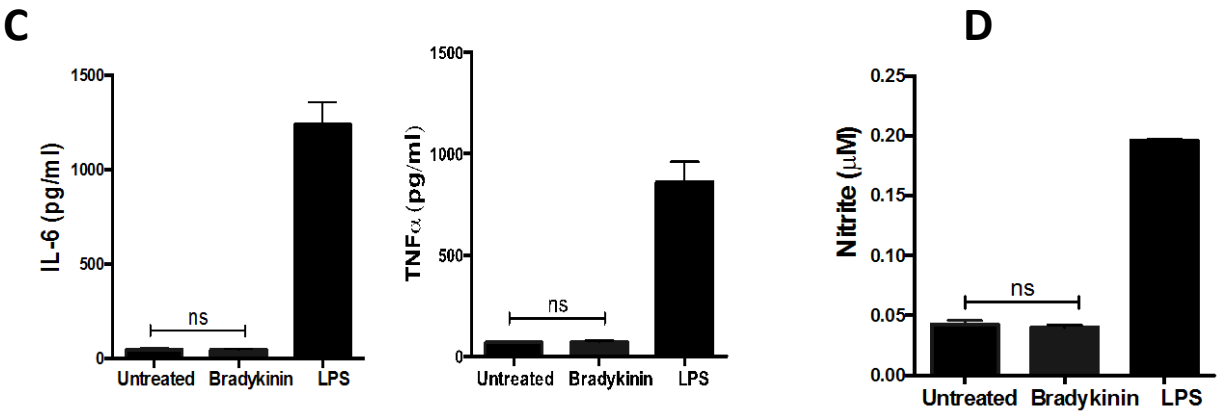


Figure 15. Renal infiltrating cells are functionally unaffected in mice treated with bradykinin

(A)BM-derived neutrophils and BMDMs from WT mice were incubated *in vitro* with unopsonized *C. albicans* yeast ± bradykinin for 3 h, and fungal load was determined by plating culture supernatants. Percentage of *C. albicans* killed by neutrophils and macrophages is shown. **(B)** IL-6 in RTEC conditioned media was assessed after 24 h bradykinin treatment. **(C)** IL-6 and TNFα and **(D)** nitrite levels in the supernatants of BMDMs treated ± bradykinin or LPS (1 ng/ml) for 24 h. Data are representative of 3 independent experiments. Bars indicate mean ± S.D. $p < 0.01$ (**). ns, not significant.

2.7.6 Bradykinin prevents apoptosis of kidney-resident cells during disseminated candidiasis

Activation of bradykinin receptors protects the kidney from end-stage renal damage by inducing tissue-protective growth factors and matrix-degrading enzymes [146]. Previous studies have described an anti-apoptotic function of bradykinin in kidney injury [165, 166]. Therefore, we assessed apoptosis in kidney-resident cells following *C. albicans* infection. At day 7 p.i., flow cytometry analysis revealed a significantly reduced frequency of both early ($AnnexinV^+PI^-$) and

late (AnnexinV^{+}PI^{+}) apoptotic kidney-resident cells (CD45^{-}) in bradykinin treated compared to untreated mice **(Fig 16).**

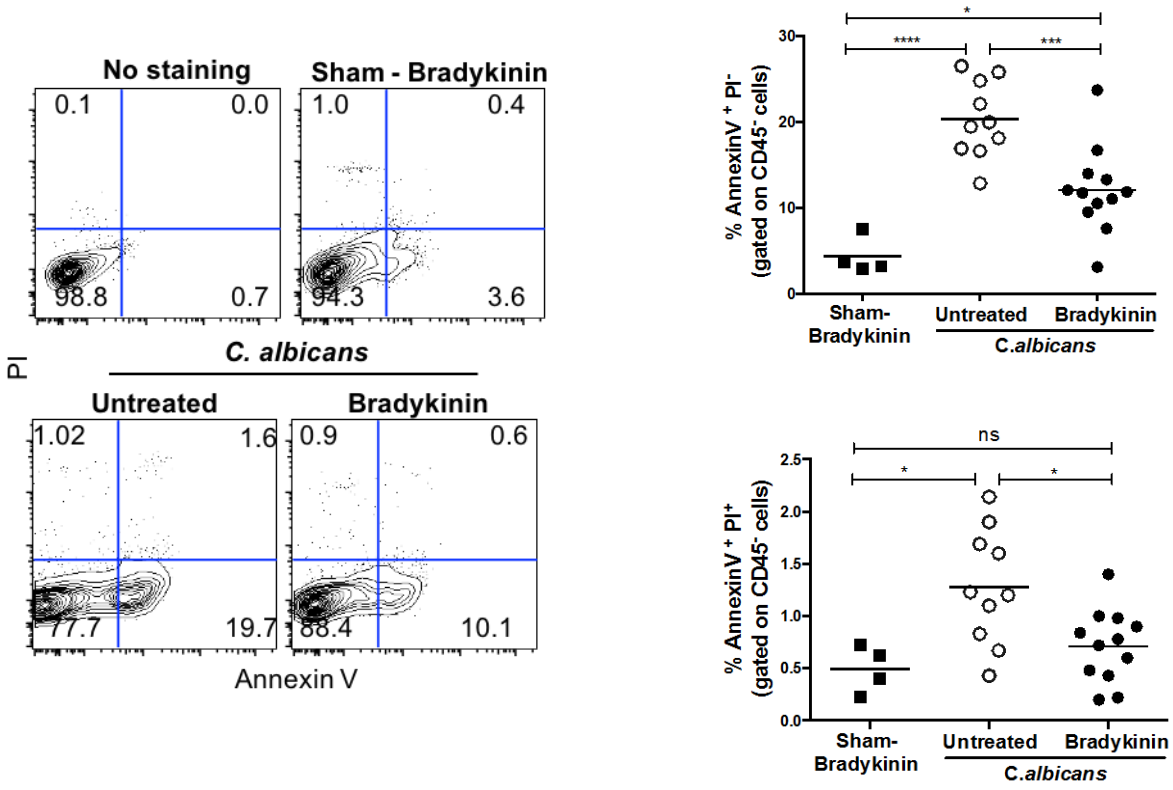
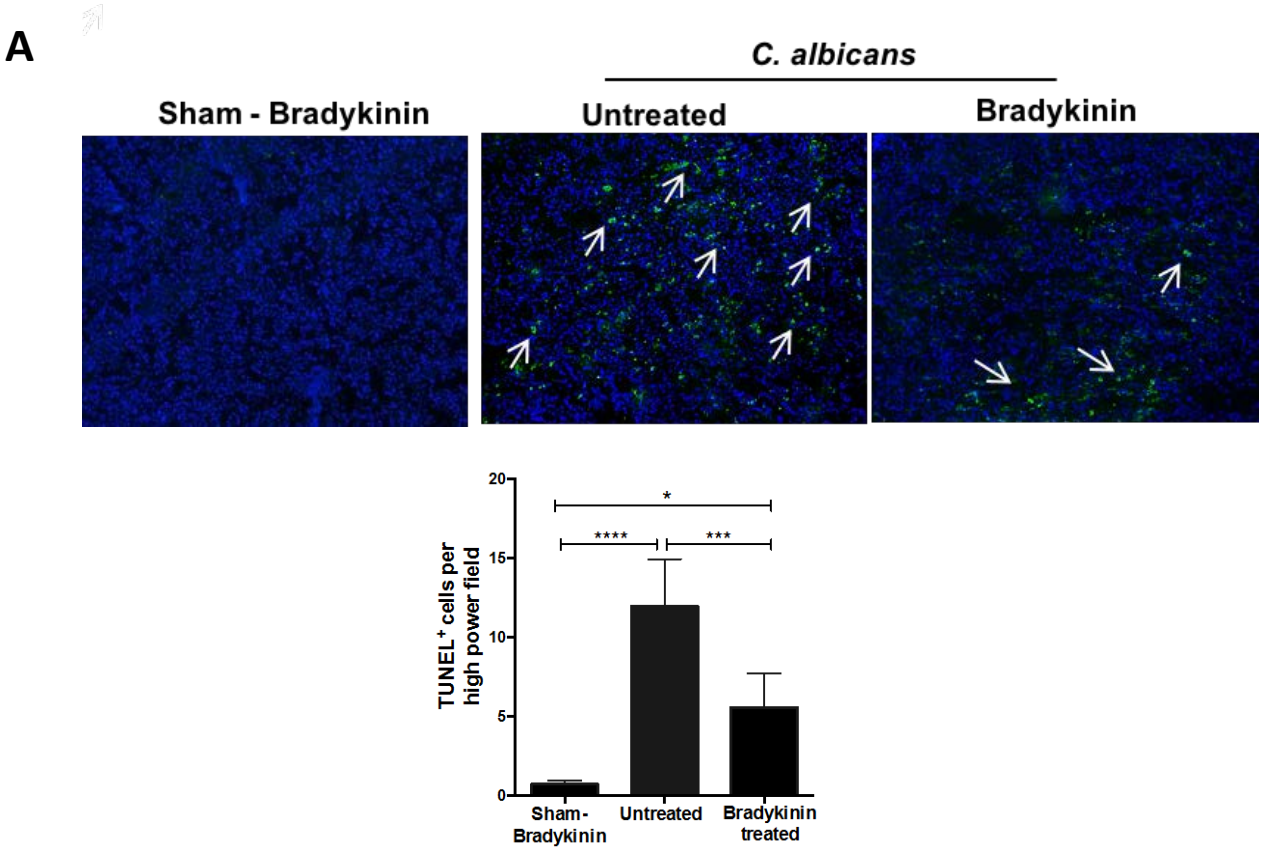


Figure 16. Apoptosis of kidney-resident cells is reduced in bradykinin treated mice in candidiasis

WT mice were treated with bradykinin (300 nmol/kg/day**)** starting 1 day prior to infection and daily for 7 days. Sham-infected WT mice were treated with bradykinin. At day 7 p.i. (n=4-12), early and late apoptotic kidney resident cells (gated on CD45^{-} cells) were quantified by AnnexinV and PI staining.

Concurrently, there was a significant reduction in the number of TUNEL positive cells in the renal cortex following bradykinin treatment **(Fig 17A).** *C. albicans* can regulate survival of kidney-resident macrophages via Caspase-3 [133]. In agreement with the reduced apoptosis, bradykinin treatment resulted in a significantly diminished number of cleaved Caspase-3^{+}

kidney-resident cells ($CD45^-$) **(Fig 17B).** The extent of cleaved Caspase-3 was markedly reduced following bradykinin treatment **(Fig 17C).** Although expression of *Bax* (a pro-apoptotic gene) in kidney-resident cells was comparable between the groups, there was an increase in expression of *Bcl-xL* (an anti-apoptotic gene) after bradykinin treatment **(Fig 17D).** This suggests that bradykinin preserves renal function during systemic fungal infection by limiting apoptosis of kidney-resident cells.



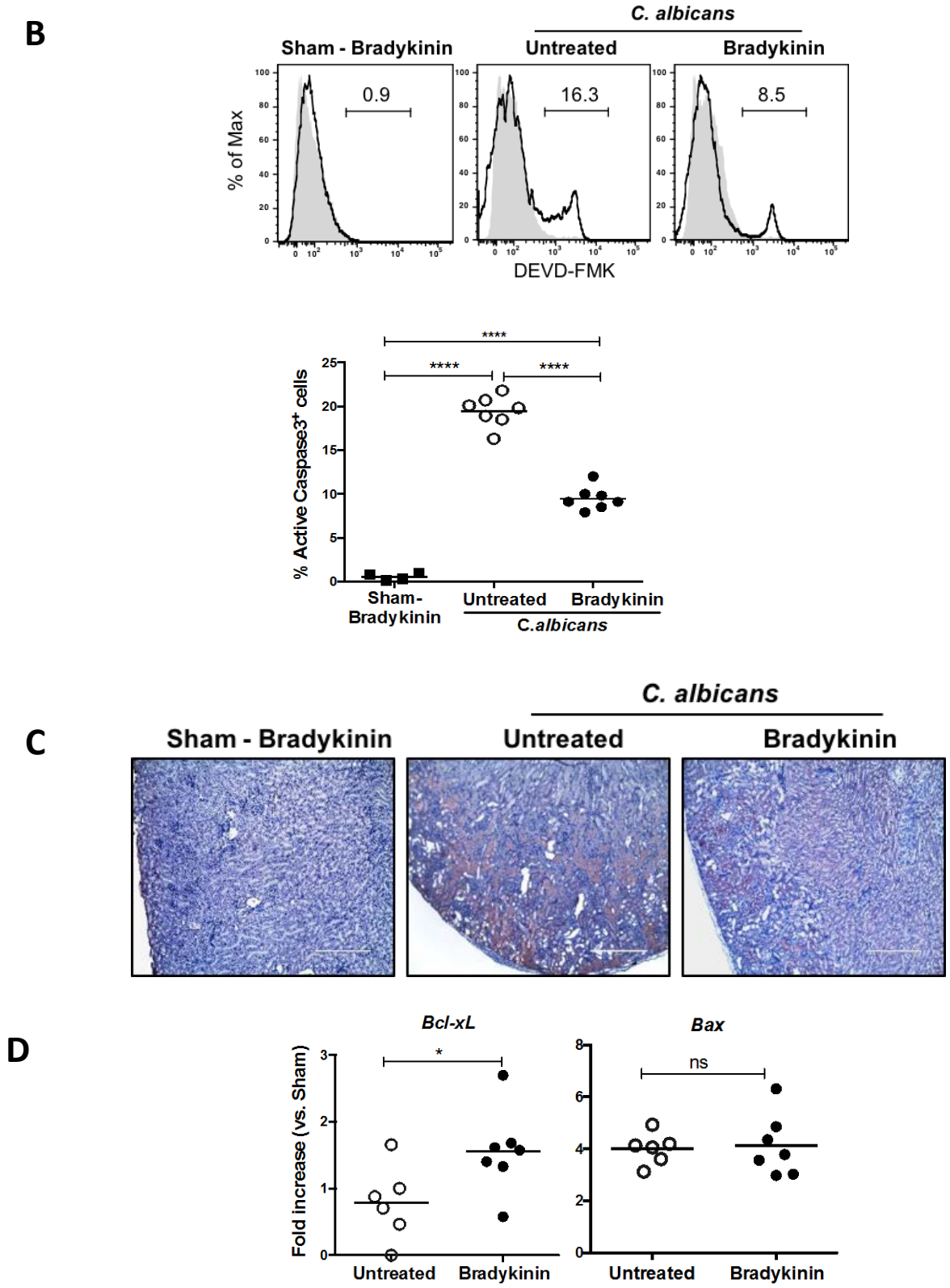


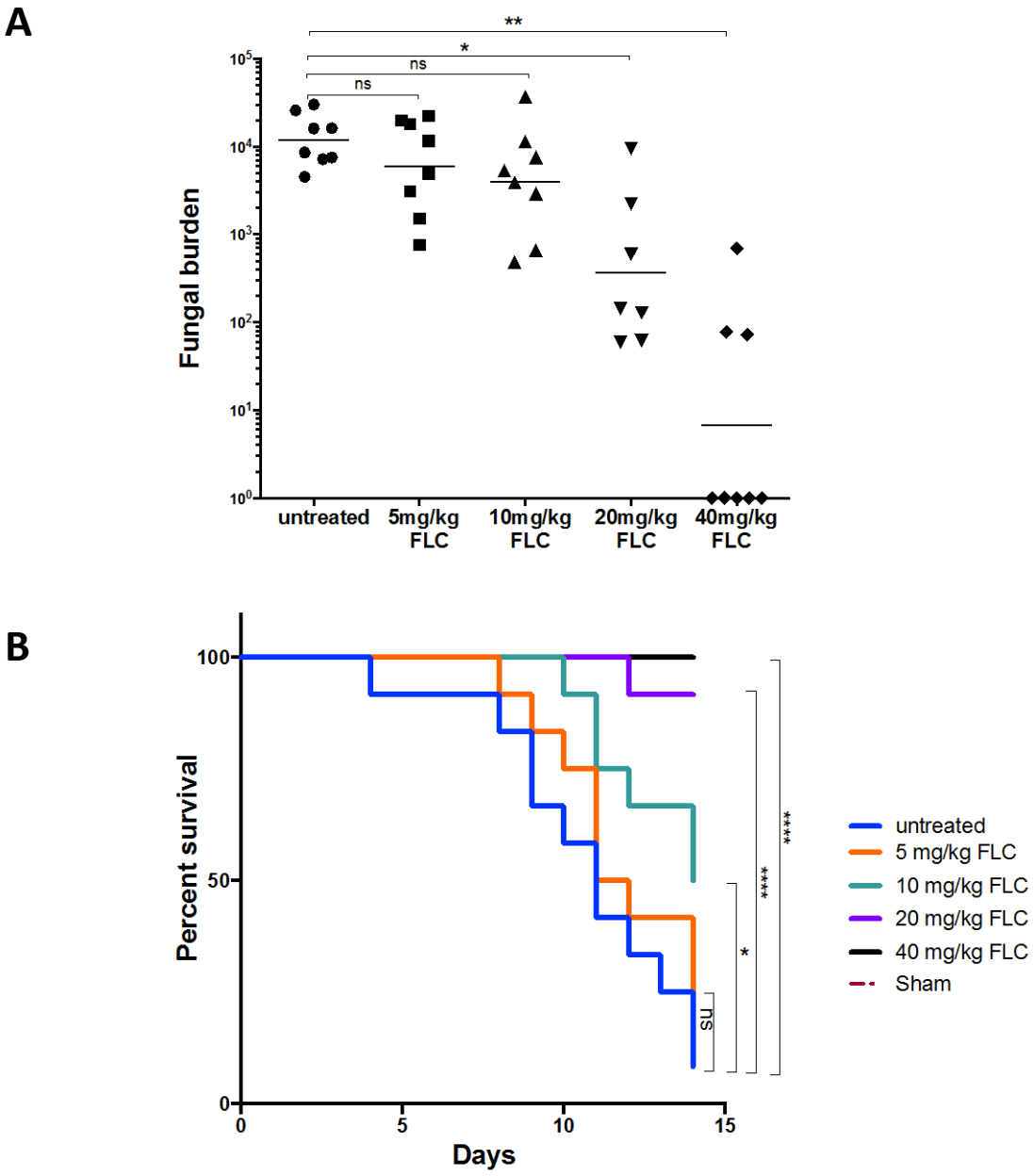
Figure 17. Apoptosis of kidney-resident cells is reduced in bradykinin treated mice in candidiasis

WT mice were treated with bradykinin (300 nmol/kg/day**)** starting 1 day prior to infection and daily for 7 days. Sham-infected WT mice were treated with bradykinin. **(A)** Frozen kidney sections (n=4-8) were subjected to TUNEL staining and counterstained with DAPI. White arrows indicate $TUNEL^+$ cells. The number of $TUNEL^+$ cells was quantified in 15 randomly selected high powered fields (400X). Original magnification: 100X. **(B)** Frequency of cleaved $Caspase\text{-}3^+$ kidney-resident cells (gated on $CD45^-$ cells) were quantified by flow cytometry (n=4-6). Solid histogram: Negative control; Open histogram: DEVD-FMK staining. **(C)** Serial kidney sections (n=4-6) were stained for cleaved Caspase-3. (**D**) Sorted kidney-resident cells ($CD45^-$) (n=6-7) were evaluated for the expression of *Bcl-xL* and *Bax* mRNA by qPCR. Data pooled from 2 and 3 independent experiments for (**A-C**) and **(D)**, respectively. Each dot represents one mouse and bars indicate mean. Bar indicates mean ± S.D. P <0.05 (*), <0.01 (**), <0.0001 (****). ns, not significant.

2.7.7 A minimally effective dose of fluconazole combined with bradykinin improved survival in disseminated candidiasis

There is an unmet clinical need to reduce the dosage of current antifungal drugs to overcome the problem of drug resistance and toxicity. Based on the renal protective function of bradykinin in disseminated candidiasis **(Fig 10A and 11A)**, we hypothesized that a minimally effective dose antifungal drug fluconazole (FLC) in combination with bradykinin would confer better protection against disseminated candidiasis than either agent alone. To test this hypothesis, we selected a minimally effective dose of 5 mg/kg dose of FLC to evaluate the impact of the combination therapy in disseminated candidiasis **(Fig 18A and 18B)**. *C. albicans* infected WT mice were treated with FLC and/or bradykinin and evaluated for survival over 14 d **(Fig 18C).** Strikingly, mice receiving the combination of bradykinin and FLC showed a significant increase in survival compared to untreated mice or mice given FLC or bradykinin alone **(Fig 18D).** As

expected, mice treated with bradykinin alone but not FLC demonstrated increased survival compared to untreated mice **(Fig 18D).** These data show that a combination of bradykinin and FLC confers better protection against disseminated candidiasis than either drug singularly. Consequently, use of bradykinin could potentially permit reducing the dose of antifungal drugs without compromising efficacy against fungal infection.



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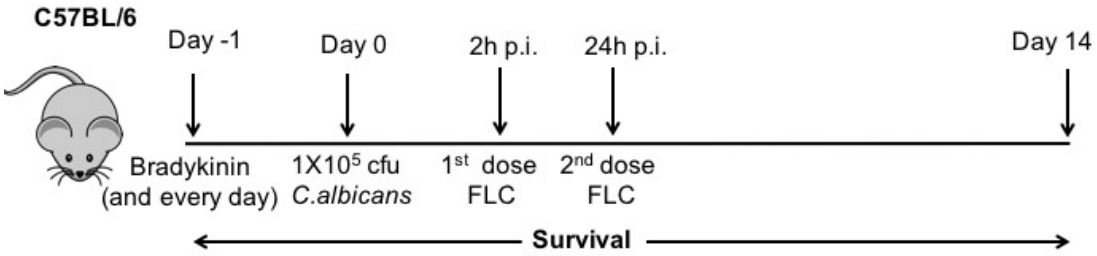

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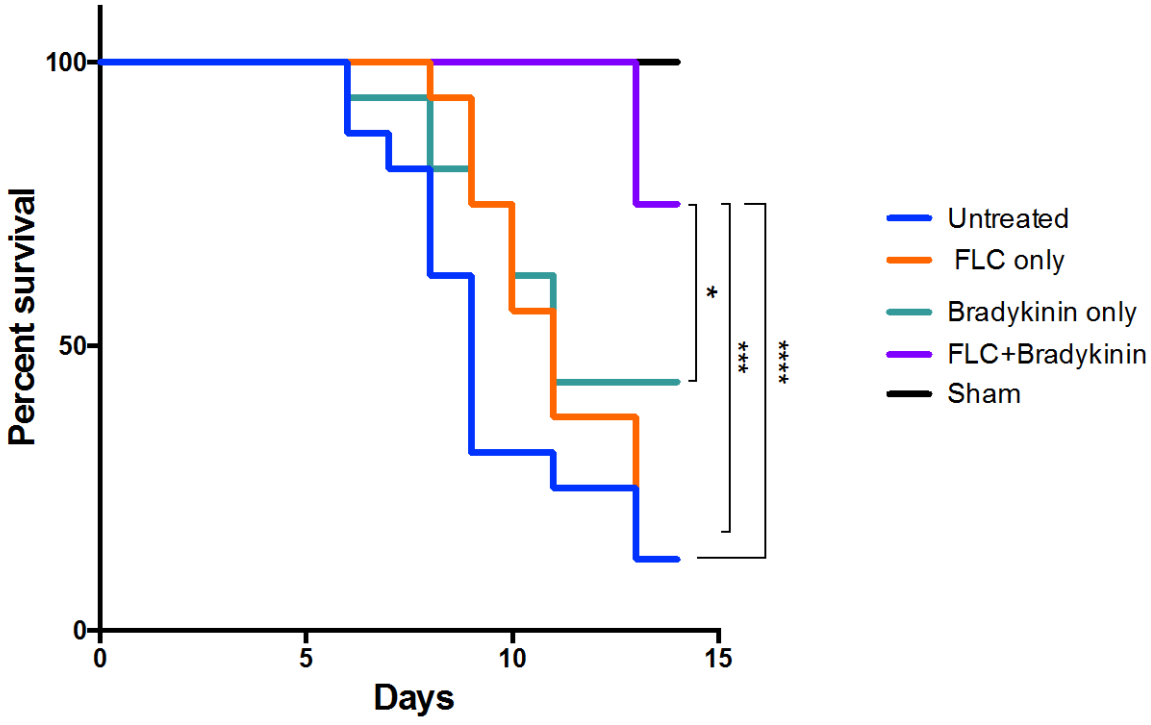

Figure 18. Combination of minimally effective dose of fluconazole and bradykinin increased survival of *C. albicans* infected mice

(A) WT mice (n=8) were infected with *C. albicans*. Infected mice were treated with doses of FLC only (5,10,20 and 40 mg/kg at 2 and 24 h p.i.). Fungal burden was measured on day 4 post infection. **(B)** WT mice (n=8) were infected with *C. albicans*. Infected mice were treated with doses of FLC only (5,10,20 and 40 mg/kg at 2 and 24 h p.i.). Survival was assessed over 14 d. **(C)** Experimental design for combinatorial fluconazole and bradykinin therapy strategy **(D)** WT mice (n=16) were infected with *C. albicans*. Infected mice were treated with two doses of FLC only (5 mg/kg at 2 and 24 h p.i.), bradykinin only (300nmol/kg starting 1 day prior to infection and daily for 14 days) or a combination of FLC and bradykinin. Sham-infected mice (n=3) were treated with FLC, bradykinin or both. Survival was assessed over 14 d. Data are combined from 2 **(A-B)** and 3 **(D)** independent experiments. $p<0.05$ (*), $p>0.001$ (***), $p>0.0001$ (****).

2.8 DISCUSSION

Kidneys in a healthy state are sterile. Renal infections can occur via hematogenous routes or from ascending spread from the bladder or urethra [167]. In recent years, considerable evidence suggests a role for IL-17 in immunity against disseminated candidiasis [75, 141]. However, it is unclear how IL-17 regulates renal immunity as the kidney is the most heavily colonized organ in disseminated *C. albicans* infection. To elucidate this question, we have identified renal-protective kallikreins as novel IL-17 target genes in disseminated candidiasis, revealing a previously unknown connection between IL-17 and KKS in renal defense. Therapeutic manipulation of the IL-17-KKS pathways protects mice from early mortality in disseminated candidiasis. Our data provide important and potentially translatable insights into the renal functions of IL-17 in the context of disseminated candidiasis.

Renal immune responses are mediated by both kidney-infiltrating immune cells as well as kidney-resident cells. Although IL-17RA is ubiquitously expressed, most documented IL-17R signaling occurs in cells of non-hematopoietic origin, particularly epithelial and mesenchymal cells [61, 64]. However, a recent study surprisingly suggested a role for IL-17RA signaling in NK cell development in the context of disseminated candidiasis [140]. Notably, this report conflicts with a study showing that NK cells are redundant for antifungal defense in immunocompetent hosts [168]. Consistent with the former finding, we and others have observed upregulation of transcripts encoding IL-17A and IL-17-responsive genes in the kidney [75, 169]. We also showed that kidney-resident cells express the IL-17R and are responsive to IL-17 [169]. Taken together, these results support a kidney-specific role of IL-17 in immunity to disseminated candidiasis.

Unlike mucocutaneous candidiasis, disseminated candidiasis typically occurs in individuals with no known defects in IL-17 signaling pathways. We discovered a distinct gene expression profile in *C. albicans*-infected kidney compared to prior studies in mucosal tissues like the tongue that were infected by *C. albicans* [70]. These results highlight the fact that different cell types interpret IL-17 signals differently, with context-dependent patterns of gene expression. Therefore, inferences from studies of anti-*C. albicans* immunity at mucosal sites do not always apply to renal immune responses in a disseminated infection setting. Although we show that IL-17 in synergy with TNFα induces *Klk1* in primary RTEC, there is little known about *Klk1* gene regulation at the transcriptional level. In preliminary analyses, conserved CCAAT Enhancer Binding Protein (C/EBP)-β binding sites within the putative proximal promoters of the *Klk1* gene hinting at IL-17- C/EBPβ-dependent regulation of *Klk1* expression. This is supported by the fact that C/EBPβ$^{-/-}$ mice are susceptible to disseminated candidiasis

[170, 171]. Another avenue that can be explored is to assess *Klk* gene expression in the kidney of TNFα-/- mice, since TNFα is required for protection against disseminated candidiasis [172].

One of the key events in the KKS is the cleavage of kininogens by Klk1 to form bradykinin, a process known as the bradykinin-dependent pathway [145]. We have demonstrated the role for this pathway in our studies in the disseminated candidiasis model. There also exists a bradykinin-independent pathway, in which Klk1-mediated activation of protease activated receptor 4 (PAR4) induces cytokine production and prevents apoptosis in RTEC [162]. Mice deficient in specific PARs or treated with PAR antagonists exhibited compromised renal inflammatory changes [173, 174]. However, the contribution of PARs in IL-17-Klk1-mediated renal protection is unknown. In addition, as bradykinin can signal through two separate receptors, the relative contributions of Bdkrb1 and Bdkrb2 in renal defense against candidiasis needs to be delineated.

Our data show that bradykinin treatment only delayed mortality, despite improved renal function. This outcome may indicate potential side effects of the bradykinin treatment in mice. Although bradykinin has been implicated in angioedema [37], mice treated with bradykinin did not show any signs of angioedema following fungal infection **(Fig 4C)**. Bradykinin at the dose used in this study did not cause hypotension. Therefore, these data argue against the likelihood of side effects to bradykinin treatment in mice. However, comprehensive toxicity studies to investigate these questions are necessary to assess the beneficial impact of IL-17-KKS axis, without compromising the safety, in the treatment for disseminated candidiasis.

An antibody targeting IL-17 (secukinumab and ixekizumab) was approved in 2016 to treat moderate-severe plaque psoriasis [175]. Other antibodies against IL-17/IL-17RA are in clinical trials to treat various autoimmune conditions [176]. However, an obvious concern with blocking IL-17 is compromising IL-17-driven antifungal immunity. Although systemic *C.*

albicans infection has not been reported thus far with secukinumab, patients on this medication stand the risk of developing disseminated candidiasis if other predisposing factors exist, such as an indwelling catheter, abdominal surgery or long-term antibiotic use. Our data show that activation of the KKS pathway restored protection in IL-17RA$^{-/-}$ mice. Thus, drugs targeting the IL-17-KKS axis may be considered to treat or prevent disseminated *C. albicans* infection in patients receiving anti-IL-17 therapy.

Amphotericin B, azoles and echinocandins are used to treat systemic *C. albicans* infections, but there are concerns due to drug-resistance and toxicity. Here, we show proof-of-concept that combination therapy with bradykinin and low dose FLC is effective in treating candidiasis in mice. Notably, the survival rate of mice treated with the combination therapy was similar to the survival rate in mice given four times the FLC dose. This approach could be valuable with antifungals like amphotericin B due its intrinsic renal toxicity. Future studies to test the efficacy of combination therapy with amphotericin B and bradykinin are warranted, provided access to oral or injectable form of amphotericin B suitable for administration in mice are found. In addition, the efficacy of ACE inhibitors, known to increase the levels of bradykinin and routinely used to treat patients with obstructive nephropathy, can present alternative strategies to evaluate in pre-clinical animal models of disseminated candidiasis. Overall, the novel convergence between IL-17 and the KKS pathways in renal defense against fungal infection represents a major advance in our understanding of IL-17 signaling in the kidney inflammation.

2.9 MODEL

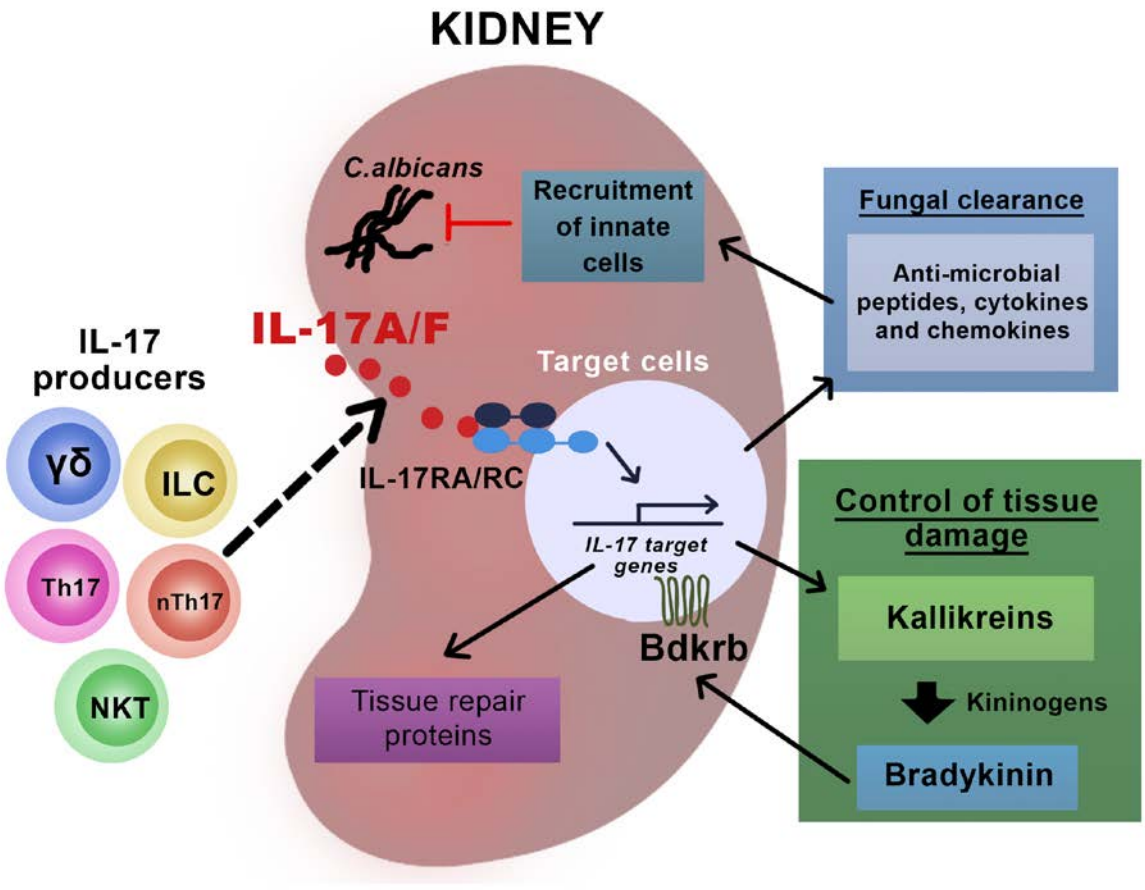

Figure 19. The IL-17-KKS-axis confers renal protection against disseminated candidiasis

In response to disseminated candidiasis, kidney infiltrating innate and adaptive IL-17-producing cells is the major source of IL-17. IL-17 in turn binds its receptor (IL-17RA/RC) on kidney- resident target cells, activating downstream signaling events leading to expression of IL-17- responsive cytokines, chemokines and AMP genes. Innate effectors (neutrophils, macrophages) recruited in response to IL-17-induced signals facilitate fungal clearance. IL-17 also induces expression of kallikreins in target cells. Kallikreins cleave kininogens to form bradykinin. Activation of bradykinin receptors (Bdkrb) on renal cells prevents apoptosis and controls of tissue damage.

CHAPTER 3: AN ESSENTIAL ROLE FOR INTERLEUKIN-17 RECEPTOR SIGNALING IN THE DEVELOPMENT OF AUTOIMMUNE GLOMERULONEPHRITIS

The definitive version was published in

Journal of Leukocyte Biology, September 2014, Volume 96, Issue 3, 463-72

" An essential role for interleukin-17 receptor signaling in the development of autoimmune glomerulonephritis"

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and

Cytokine, January 2016, Volume 77, 238-244

"Emerging roles of Th17-IL-17 axis in glomerulonephritis"

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3.0 SUMMARY

Studies have indicated that pro-inflammatory cytokines in the nephritic kidney appear to contribute to the pathogenesis of autoimmune glomerulonephritis (AGN). The complex inflammatory cytokine network that drives renal pathology is poorly understood. IL-17 promotes autoimmune pathology in various settings, has also been identified in acute and chronic kidney diseases. However, the mechanism of IL-17-mediated renal damage in the nephritic kidney has not been elucidated. Here, with the use of a murine model of experimental AGN (EAGN), we showed that IL-17RA signaling is critical for the development of renal pathology. Despite a normal systemic autoantibody response and glomerular immune-complex deposition, IL-17RA$^{-/-}$ mice exhibit a diminished influx of inflammatory cells and kidney-specific expression of IL-17 target genes correlating with disease resistance in EAGN. IL-17 enhanced the production of pro-inflammatory cytokines and chemokines from RTECs. Finally, we were able to show that neutralization of IL-17A ameliorated renal pathology in WT mice following EAGN. These results demonstrated that IL-17RA signaling significantly contributes to renal tissue injury in EAGN and suggest that blocking IL-17RA may be a potential therapeutic strategy for the treatment of proliferative and crescentic glomerulonephritis.

3.1 INTRODUCTION

T helper (Th) cells are one of the central players in mounting antigen specific adaptive immune responses. Antigen-activated naive Th cells differentiate into functionally distinct subsets that are categorized by the effector cytokines they secrete. Th1 cells predominantly produce interferon- γ (IFNγ) and are crucial in mediating cellular immunity against intracellular pathogens whereas Th2 cells produce IL-4, IL-5 and IL-13 [46]. The most recently defined Th cell subset, Th17, is characterized by the production of IL-17A and other cytokines, including IL-17F, IL-21, and IL-22. Apart from protective immune responses, Th cells, if not properly regulated can cause autoimmune conditions. Specifically in the kidney, dysregulated Th responses can cause inflammation and lead to glomerulonephritis (GN). The Th1-Th2 paradigm was initially used to outline the immunopathology associated with GN. Different forms of GN are associated with different Th subset responses. For example, autoimmune responses in crescentic GN and anti-glomerular basement membrane GN are thought to be Th1-mediated. In contrast, Th2 immunopathology is associated with membranous GN. However, in certain auto-inflammatory kidney diseases such as lupus nephritis, it is difficult to ascribe the tissue damaging response to be purely Th1 or Th2 based [177]. The heterogeneity in the immune responses is has now been correlated with the presence of Th17 cells [178].

3.2 ROLE OF TH17-IL-17 AXIS IN PATHOGENESIS OF GN

Originally, glomerular diseases were thought to be Th1-mediated. However, studies have shown that Th17 cells are also implicated in the induction of proliferative GN. Reintroduction of ova-specific Th17 or Th1 cells induced proliferative GN [179]. Th17 cell-mediated injury was noted earlier in disease progression and was crucial in the increased recruitment of neutrophils, while Th1 cell-mediated GN was delayed and was associated with enhanced activation of macrophages. These studies suggest that Th17 cells could be a dominant factor in neutrophil dependent glomerular diseases. However, evidence also exists to show a role for Th17 cells in macrophage recruitment in autoimmune renal diseases.

Anti-GBM GN. Anti-glomerular basement membrane (anti-GBM) antibody disease is a rare autoimmune disorder in which circulating antibodies are generated against the alpha-3 chain of type IV collagen, an autoantigen normally present in the glomerular and alveolar basement membrane. This results in the development of rapidly progressive GN [180-182]. In many patients, GN is associated with pulmonary hemorrhage and is then referred to as Goodpasture syndrome [183]. Studies with various experimental models have demonstrated the importance of humoral and cellular immune components in anti-GBM GN, with $CD4^+$ Th cells crucial to both arms of effector function [49, 177]. For decades, it was considered that Th1 cells were the predominant $CD4^+$ T cells required for anti-GBM GN [3]. Th1-associated IgG antibody subclasses (IgG1 and/or IgG3) and delayed-type hypersensitivity (DTH) effectors were prevalent in the kidney [184, 185]. More recently, evidence for IL-17-dependent but IFNγ-independent autoimmunity against collagen was noted in humans [186]. Mice deficient in IFNγ were not

protected from anti-GBM GN [187]. IL-12p40$^{-/-}$ mice (lacking IL-23 and IL-12) and IL-12p19$^{-/-}$ (lacking IL-23 with intact IL-12), but not IL-12p35$^{-/-}$ (lacking IL-12 with intact IL-23) were resistant to the development of anti-GBM GN [188]. These data implicated IL-23 in the pathogenesis anti-GBM GN and is consistent with other autoimmune conditions including psoriasis, rheumatoid arthritis and multiple sclerosis, where the IL-23-driven Th17 cell subset has been implicated in end organ pathology [189]. Other experimental models have also been used to study the role of Th17/IL-17 axis in GN. For example, in a sheep anti-mouse GBM antibody-induced GN, IL-17A$^{-/-}$ and IL-23p19$^{-/-}$ mice were resistant to the development of glomerular damage caused by the deposition of anti-GBM antibodies [169, 190]. In this system, chemokine receptor CCR6^{+}Th17 cells infiltrate the kidney in response to CCL20 and act as major producer of IL-17A. IL-17A induces the expression of chemokines including CCL2, CCL3 and CCL20 in kidney-resident cells and facilitated the influx of innate cellular effectors in the kidney [191].

Lupus nephritis. Systemic lupus erythematosus (SLE) is a complex autoimmune disorder that develops in genetically prone individuals under the influence of various environmental factors [192]. Lupus nephritis (LuN) is a clinical manifestation of SLE and affects 60% of lupus patients [192]. Several lines of evidence link the Th17/IL-17-axis to the pathogenesis of LuN. In lupus-prone MRL.Fas^{lpr} mice, a significant number of IL-17-producing cells in the nephritic kidney belong to a unique population of CD4-CD8-double-negative T cells (DN T cells), which are selectively expanded in these mice [193]. Further confirmation for IL-17 involvement in renal damage came from an additional study in the MRL.Fas^{lpr} model that showed ‘classical’ Th17 cells also contribute to the IL-17-producing T cell subset in the nephritic kidney [194]. B6.Fas^{lpr}

mice deficient in IL-23 signaling were resistant to the development of LuN [195]. Adoptively transferred IL-23-stimulated B6.Fas^{lpr} lymph node cells were able to transfer LuN into Rag1$^{-/-}$ mice [193]. In the NZB/WF1 murine model, a deficiency of both TNF receptors (TNFR1 and TNFR2) led to increased numbers of memory Th17 cells leading to accelerated LuN, indicating a crucial role for TNF signaling in regulation of the systemic Th17 response [196].

Studies in other models of lupus-prone mice also provide supportive evidence for participation of Th17/IL-17 pathway in autoimmune kidney injury. The BXD2 mouse is characterized by the development of arthritis, glomerulonephritis, aberrant autoantibodies and spontaneous germinal center (GC) development. BXD2 mice exhibit high IL-17 levels in serum [197, 198]. The significance of IL-17 in GC formation was further demonstrated when overexpression of IL-17 in pre-diseased BXD2 mice induced spontaneous GC formation [197]. Concordantly, formation of GC and production of anti-DNA and anti-histone antibodies were abrogated in BXD2 mice deficient in IL-17R signaling, suggesting a role for IL-17 signaling in B cells in the development of LuN [197]. Further evidence supports the role for IL-17 in B cells, by demonstrating that Th17 T cells aid B-cell class switching [199]. Genetic deficiency or blockade of IL-21, another cytokine produced by Th17 cells, ameliorated exaggerated B cells response, autoantibody production and prevented the development of LuN in BXSB-Yaa and MRL.Fas^{lpr} mice [200-205]. In addition to the spontaneous model of LuN, studies from our group as well as others have identified a pathogenic role for IL-17 in the renal damage associated with pristane induced LuN [206, 207].

Pauci-immune ANCA-associated glomerulonephritis. Th17 cells are crucial in neutrophil recruitment. IL-17A promotes neutrophil accumulation in anti-neutrophilic cytoplasmic antibody

(ANCA)-associated GN, supporting a potential role in ANCA disease. Accordingly, mice lacking IL-17A were protected from ANCA-associated GN [208]. IL-17A promoted neutrophil recruitment to glomeruli and enhanced adaptive autoimmune response to myeloperoxidase injected into the kidney [208].

Table 1. IL-17 in AGN associated with auto-inflammatory kidney diseases

Disease	**Mouse model**	**Disease phenotype**
Lupus	BXDIL-17RA$^{-/-}$	Diminished GC and auto-antibody levels
	MRL.Fas^{lpr}	Susceptible to AGN
	NZWBF1	Susceptible to AGN
	B6.Fas^{lpr}IL-23R$^{-/-}$	Resistant to AGN
	MRL.Fas^{lpr}IL-21R$^{-/-}$	Resistant to AGN
	FcγRIIB$^{-/-}$Act1$^{-/-}$	Resistant to AGN
	FcγRIIB$^{-/-}$IL17A$^{-/-}$	Resistant to AGN
ANCA associated AGN	IL-17A-/-	Resistant to AGN
Goodpasture disease	IFNγ$^{-/-}$	More severe AGN
	IL-12p40$^{-/-}$	Resistant to AGN
	IL-12p19$^{-/-}$	Resistant to AGN

3.3 TH17-IL-17 AXIS IN HUMAN GLOMERULONEPHRITIS

The initial studies linking the Th17/IL-17 axis to renal inflammation were performed in renal transplant recipients during allograft rejection. These reports demonstrated that IL-17 mRNA and

protein could be detected in rejected renal allografts, whereas pre-transplant biopsies and normal kidneys were negative [209, 210]. Currently, there is only limited evidence for an involvement of the Th17/IL-17-axis in the pathogenesis of GN in humans. To date, no direct link between the Th17/IL-17-axis and SLE has been found from genetic studies, although copy number amplifications have been seen for IL-17F, IL-21 and IL-22 genes in patients with SLE [211]. In line with murine studies, sporadic IL-17$^+$CD3$^+$ cells are found in the tubulointerstitial infiltrates in kidney biopsies from patients with active renal diseases [85]. Single cell analyses from renal biopsies of patients with LuN revealed a positive correlation between percentage of IL-17$^+$TCR$^+$ cells within the kidney and hematuria and disease activity scores in LuN [212]. In a separate study, SLE patients showed upregulation of IL-17A mRNA expression in the urine sediment [213]. Peripheral blood T cells of patients with SLE also contain increased level of IL-17A and a higher percentage of IL-17$^+$ T cells compared to healthy controls [84, 214, 215]. Although transcripts encoding IL-17A, IL-23 and IL-6 were upregulated in the PBMC of SLE patients, some but not all studies have found correlation between serum IL-17A level with disease activity scores and renal involvement [84, 216]. Multiple reports suggest an association of IL-17A in the pathogenesis of other forms of human GN. The patients suffering from minimal change nephrotic syndrome and IgA nephropathy exhibited increased IL-17A in the urine [217]. Increased levels of serum IL-17A, IL-23 and myeloperoxidase specific Th17 cells are present in patients with ANCA-associated vasculitis [218]

Since the discovery of IL-17, several studies have helped realize the clinical potential of targeting IL-17 in chronic inflammatory diseases. There is substantial experimental evidence to support the idea that the Th17/IL-17-axis contributes to the pathogenesis of GN. However, the

underlying cellular and molecular mechanisms of Th17/IL-17-axis-mediated GN are poorly understood and require future investigation. Keeping this in mind we addressed the role for IL-17 in the pathogenesis of autoimmune glomerulonephritis using an experimental mouse model.

3.4 MATERIALS AND METHODS

Mice

C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). IL-17RA$^{-/-}$ mice were kindly provided by Amgen (San Francisco, CA) and bred in-house. All mice were housed and bred under specific pathogen-free conditions. Experimental mice were age- and sex-matched. All mice were maintained and used in accordance with approved University of Pittsburgh IACUC guidelines.

Experimental Autoimmune Glomerulonephritis (EAGN)

The protocol for induction of EAGN has been previously described [77]. Briefly, rabbit IgG (0.1 mg/ml) in CFA (2.5 mg/ml) (Sigma, St.Louis, MI) was injected i.p. on day −3 (relative to start of experiment). Controls were injected with PBS in CFA. On day 0, 100 µl of heat-inactivated mouse anti-GBM serum (Lampire Biological Laboratories, Everett, PA) was injected i.v. Mice were sacrificed on day 14 and the spleen, kidneys and blood were collected for further analyses. For the neutralization studies, EAGN was induced as per protocol and the mouse anti-IL-17A and isotype control antibodies (Janssen Pharmaceuticals, Titusville, NJ) were injected as indicated.

Histological analyses and immunofluorescence staining

Kidneys were fixed in 10% formalin and embedded in paraffin. Tissue sections were stained with H&E or Periodic acid–Schiff (PAS) and histological scores were evaluated blindly as described before [188].

Frozen sections (5 μm thickness) were fixed in acetone, and blocked with 1% BSA in PBS. Immune complex deposition was evaluated by staining with FITC conjugated mouse anti-IgG antibody (Jackson Immunoresearch, West Grove, PA). Slides were mounted with Vectashield without DAPI (Vector Labs, CA) and visualized using a Leica AF6000LX fluorescence microscope.

Real time PCR

Total RNA was isolated from kidney tissue by using the RNeasy Micro Kit according to the manufacturer's instructions (Qiagen, Valencia CA). cDNA was synthesized using SuperScript III First-Strand (Invitrogen, Carlsbad CA). Gene expression was measured by qPCR with PerfeCTa SYBR Green FastMix ROX (Quanta BioSciences, Gaithersburgh MD) on 7300 Real-Time PCR System (Applied Biosystems, Carlsbad CA). Gene expression levels were then determined for mouse IL-17A, IL-17F, IL-6, CXCL1, CXCL2 and CCL20 using commercially available Quantitect primers (Qiagen, Valencia CA) by real-time PCR.The expression of each gene was normalized to that of *Gapdh.*

$CD4^+$ T cell isolation

Naive $CD4^+$ T cells were isolated from spleen and lymph nodes of WT and IL-$17RA^{-/-}$ mice using CD4+ magnetic bead sorting (Miltenyi Biotech, San Diego, CA).

Flow cytometry

Single-cell suspensions from spleen were RBC-lysed and stained with following surface markers - fluorochrome-conjugated B220 (clone RA3-6B2, eBioScience), CD138 (clone 281-2,

eBioScience), CD4 (clone L3T4, eBioScience), CD62L (clone Ly-22, eBioScience), CD44 (clone IM7, eBioScience), Ly6G (clone IA8, eBioScience), Ly6C (clone AL-21, eBioScience), CD11b (clone M1/70, eBioScience), F4/80 (clone BM8, eBioScience) and GL-7 (clone Ly-77, BD Biosciences), CD95 (clone Jo2, BD Biosciences). For intracellular Foxp3 staining, cells were fixed and permeabilized, with mouse Foxp3 staining kit (eBioscience, San Diego, CA) as per the manufacturer's instructions. For intracellular cytokine staining, splenocytes or renal cells were ex-vivo stimulated with PMA and Ionomycin (Calbiochem, La Jolla, CA) in the presence of Monensin (BD Pharmingen, CA) for 4h. Cells cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and were intracellularly stained for IL-17A (clone TC11-18H10, BD Pharmingen) and IFN-γ (clone XMG1.2, BD Pharmingen) followed by flow cytometry. Cell staining was analyzed on a FACS Aria (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo (Tree Star, OR).

Absolute neutrophil count of peripheral blood smears

Blood was collected by saphenous venous puncture or cardiac puncture and collected in EDTA-containing tubes (Microvette Tubes, Sarstedt, Newton, NC, and Microtainer Tubes, BD, Franklin Lakes, NJ). Total WBC counts were determined by trypan blue exclusion method. The percentage of neutrophils was determined by enumeration of a blood smear stained with Giemsa (Fisher Scientific, Fair Lawn, NJ). Two hundred cells were counted per sample and the absolute neutrophil count was determined by multiplying the percentage of neutrophils (mature and band forms) by total WBC.

ELISA

Cell culture supernatants were assayed for cytokine production by murine ELISA kits; IL-17A, IFN-γ and IL-4 (eBioscience, San Diego, CA). Mouse anti-rabbit IgG antibody titers were

measured from sera collected on day 14 after induction of EAGN. Briefly, ELISA plates were coated with 100 µl of 100 µg/ml rabbit IgG (Sigma, St. Louis, MI) in carbonate–bicarbonate buffer and incubated overnight at 4°C. Blocking was carried out with 1% BSA in Tris-buffered saline (Sigma, MI), following which the plates were incubated with serial dilutions of serum (1:100 to 1: 12,500) for 1 h at room temperature. Bound mouse IgG was detected using peroxidase-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) at 1:1000, 3,3′,5,5′-tetramethylbenzidine peroxidase substrate. Absorbance was measured at 450 nm on a spectrophotometer. Ig isotypes (IgG1 and IgG2a,) were also measured by ELISA. Serum BUN levels were determined using MaxDiscovery Blood Urea Nitrogen Enzymatic kit (Bioo Scientific Corp., Austin TX) according to manufacturer's instructions.

***In vitro* stimulation of primary renal tubular epithelial cells**

Primary renal tubular epithelial cells (RTEC) from C57BL/6J mice (Cell Biologics, Chicago, IL) were cultured as per manufacturer's instructions. RTEC cells ($1x10^6$ cells/well in a 6 well plate) were treated with IL-17A (50 or 200 ng/ml) or TNFα (5 ng/ml) and IL-17 and TNFα in combination for 24h. Recombinant murine IL-17A and TNFα were purchased from Peprotech (Rocky Hill, NJ).

Transwell chamber migration assays

Neutrophils were isolated from bone-marrow of 8-10 week old C57BL/6J mice using Neutrophil isolation kit (Miltenyi Biotech, Billerica, MA). Purity of isolated neutrophils was greater than 95% in all the experiments as determined by flow cytometry. Migration of neutrophils was assayed using a 5.0 µm pore size trans-well chamber (Corning, Corning, NY) in the presence of supernatant from RTEC cultures either left untreated or treated with IL-17 and/or TNF-α.

Statistical analyses

Results are expressed as mean ± SD. Differences between groups were calculated for statistical significance using 2-tailed paired Student's *t* tests. A *p* value less than 0.05 was considered significant.

3.5 RESULTS

3.5.1 IL-17A and IL-17A-responsive inflammatory genes are upregulated in the kidneys of mice with EAGN

Based on the prior connection of IL-17A in the pathogenesis of glomerulonephritis [77], we sought to examine the presence of IL-17A in the kidneys of mice subjected to an experimental model of glomerulonephritis. C57BL/6 (WT) mice were pre-immunized with rabbit IgG in CFA 3 days prior to the administration of nephrotoxic rabbit serum (NRS). Control mice received PBS in CFA. We evaluated transcript expression of IL-17 cytokines such as IL-17A and IL-17F in the kidney cortex during EAGN by qPCR. IL-17A mRNA was significantly upregulated at early (day 7) and late (day 14) stages of EAGN development (**Fig 20A**). However, there was no difference in the levels of IL-17F transcripts between nephritic and control mice **(Fig 20B)**. Additionally, flow cytometry analyses on day 14 revealed an increased frequency of IL-17A producing cells within the $CD3^+CD4^+$ and $CD3^+CD4^-$ compartments **(Fig 20C)**. These results indicate Th17 and innate IL-17-producing cells as cellular sources of IL-17A in the nephritic kidney.

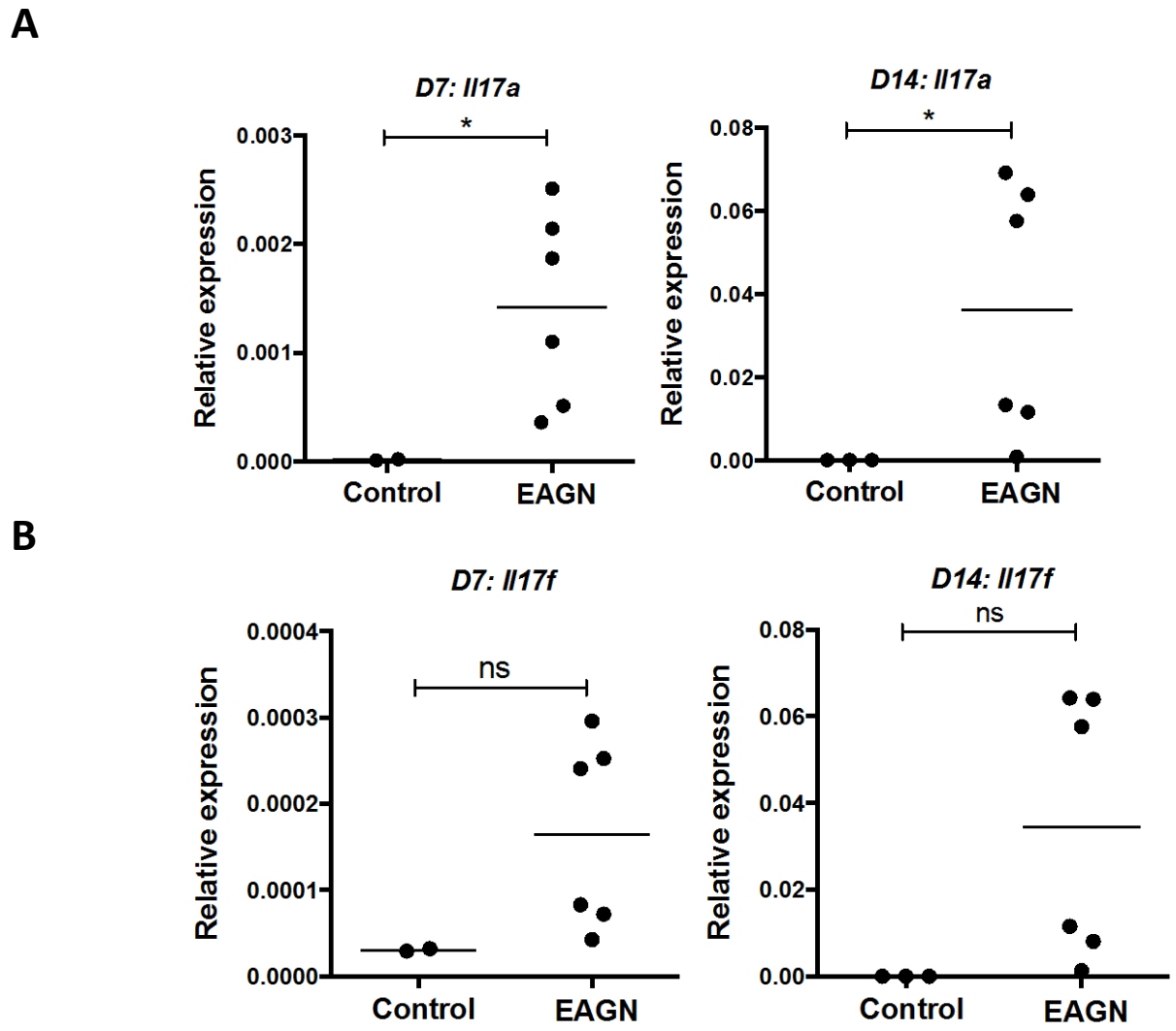


Figure 20. Increased level of IL-17A in the nephritic kidney of EAGN mice

C57BL/6 mice were subjected to AGN, as described in Materials and Methods. Mice were sacrificed at Day 7 [*n*=7 for EAGN, and *n*=3 for control (Ctrl)] and Day 14 (*n*=7 for AGN, and *n*=3 for control), and kidney cortex was dissected out carefully. **(A)** IL-17A and **(B)** IL-17F mRNA expression was evaluated by qPCR. *$P < 0.05$; *ns*, not statistically significant.

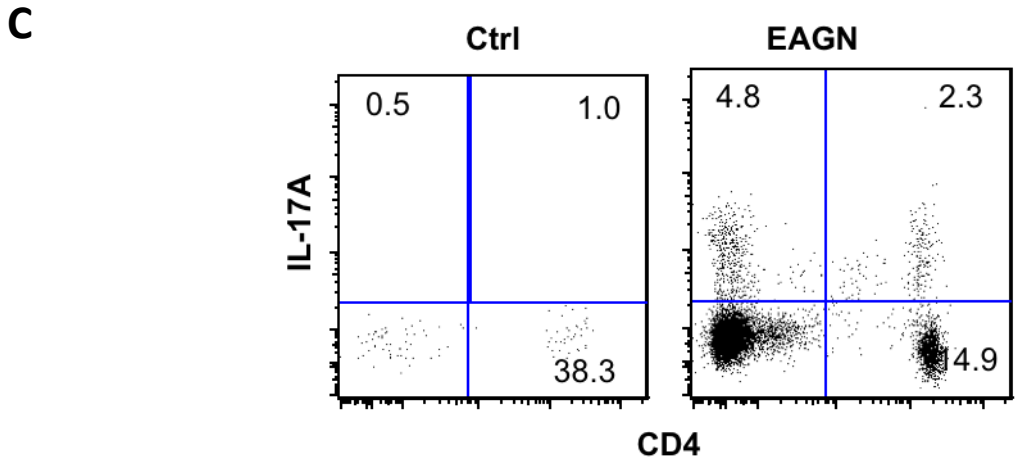


Figure 20. Increased level of IL-17A in the nephritic kidney of AGN mice.

C57BL/6 mice were subjected to AGN, as described in Materials and Methods. Mice were sacrificed at Day 7 [*n*=7 for EAGN, and *n*=3 for control (Ctrl)] and Day 14 (*n*=7 for AGN, and *n*=3 for control **(C)** At Day 14, leukocytes isolated from kidney were stimulated with PMA/ionomycin for 4 h and intracellularly stained for IL-17A, followed by FACS analyses (gated on $CD3^+$ cells). Numbers in the dot plot represent percentage of cells.

In order to determine the downstream effects of IL-17A expression, we assessed the IL-17-dependent cytokine and chemokine gene expression in the kidney cortex of nephritic mice. While there was no difference in the IL-17-responsive genes between the groups at day 7, expression of *Il6, Ccl20, Cxcl1* and *Cxcl2* were significantly increased in the nephritic kidney compared to control at day 10 **(Fig 21).** These data provide evidence that IL-17A and IL-17 responsive inflammatory genes were upregulated in the late stages of renal inflammation.

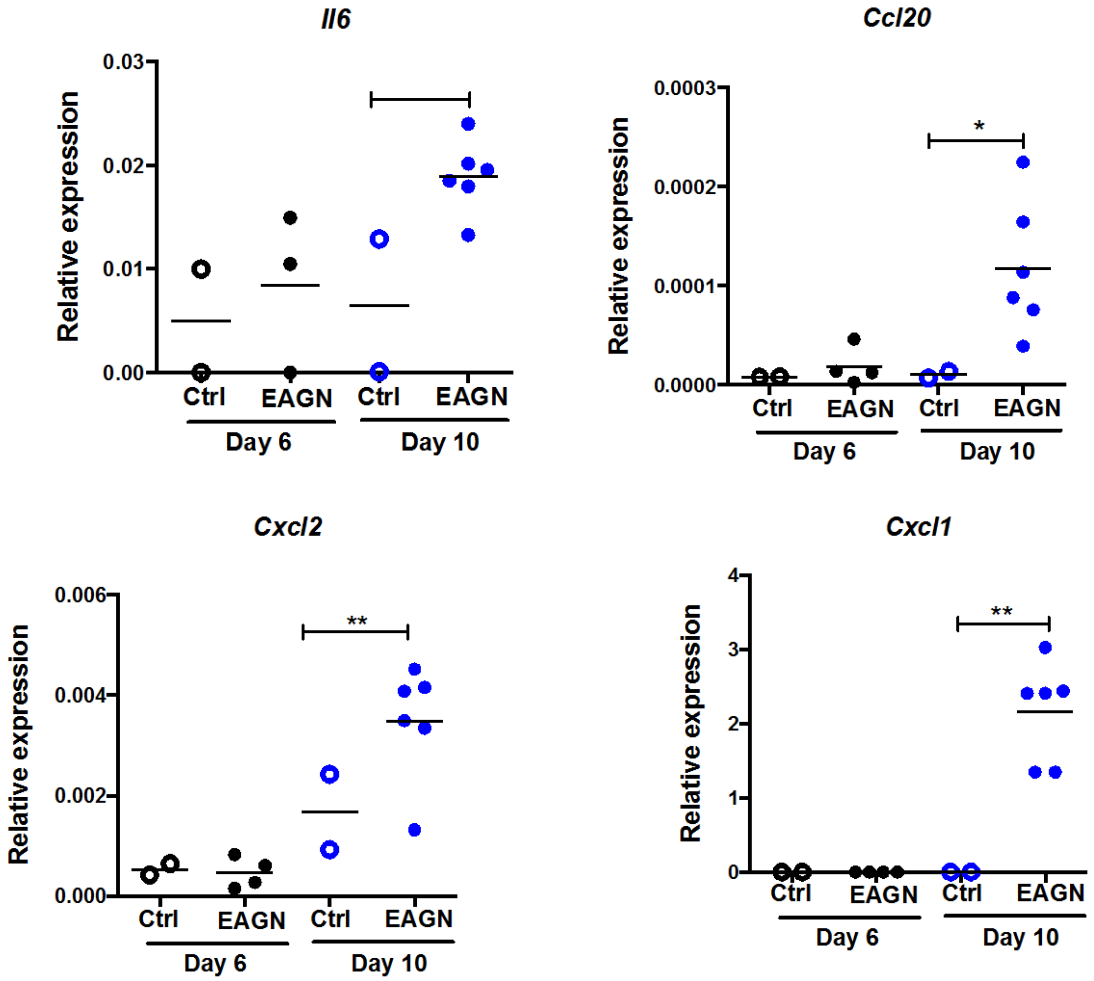


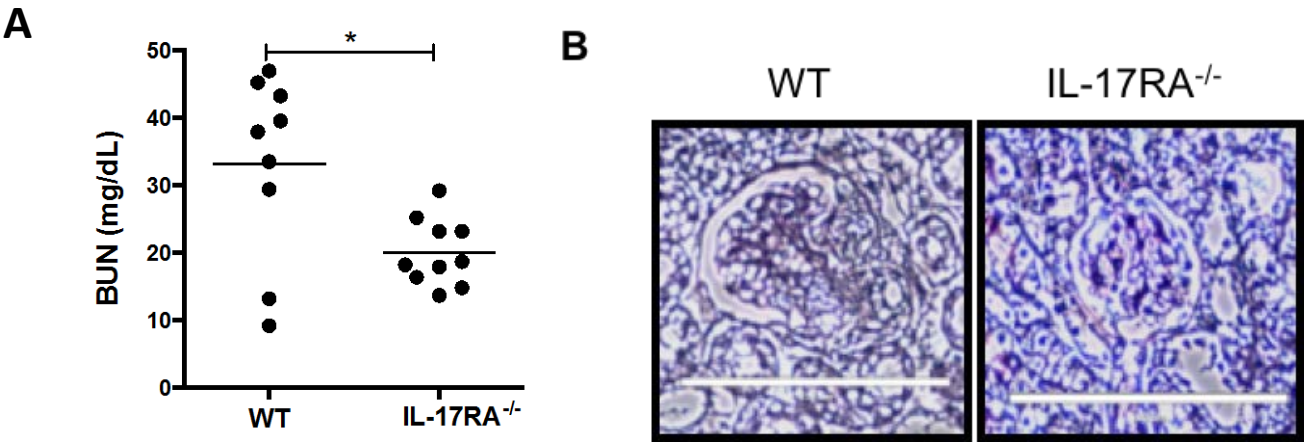
Figure 21. Increased level of IL-17A- target genes in the nephritic kidney of EAGN mice

IL-17-responsive genes (*Il6*, *Ccl20*, *Cxcl1*, and *Cxcl2*) were measured by qPCR. Each dot represents individual mice, and the horizontal bars indicate mean for each group. The scattered plot is the pooled data from two individual experiments. *$P < 0.05$; **$P >0.001$; *ns*, not statistically significant.

3.5.2 Mice with IL-17RA deficiency are protected from renal dysfunction in EAGN

To test whether signaling through IL-17RA contributes to nephritis, we induced EAGN in WT and IL-17RA$^{-/-}$ mice and evaluated for EAGN development. At day 14, WT mice demonstrated significant loss of kidney function as seen from the increased levels of blood urea nitrogen (BUN) when compared to IL-17RA$^{-/-}$ mice **(Fig 22A)**. Examination of H&E and PAS-stained

kidney sections of nephritic WT mice at day 14 showed severe focal glomerular and tubular damage with destruction of regular tissue structures. Glomerular changes included hypercellularity and formation of cellular crescents, capillary aneurysms, and intra-glomerular deposition of PAS-positive material **(Fig 22B).** In addition to massive leukocyte infiltrates, the tubulointerstitial compartment showed tubular dilation, necrosis and atrophy, and protein casts. In contrast, glomerular and tubulointerstitial tissue damage was less severe in nephritic IL-17RA$^{-/-}$ mice **(Fig 22B)**. To quantify renal tissue damage, PAS-stained kidney sections were blindly evaluated for the presence of crescents, glomerular sclerosis, and tubulointerstitial injury. At day 14, the frequency of abnormal glomeruli was reduced in nephritic IL-17RA$^{-/-}$ mice compared to WT mice **(Fig 22C)**. Furthermore, nephritic kidneys of IL-17RA$^{-/-}$ mice showed decreased crescent formation and diminished tubulointerstitial inflammation as indicated by a reduction in the interstitial area **(Fig 22C)**. However, glomeruli of IL-17RA$^{-/-}$ and WT mice demonstrated comparable immune complex deposition as revealed by immunofluorescence staining for mouse IgG **(Fig 22D)**. Collectively, these results indicate an essential role for IL-17RA signaling in renal pathology associated with EAGN.



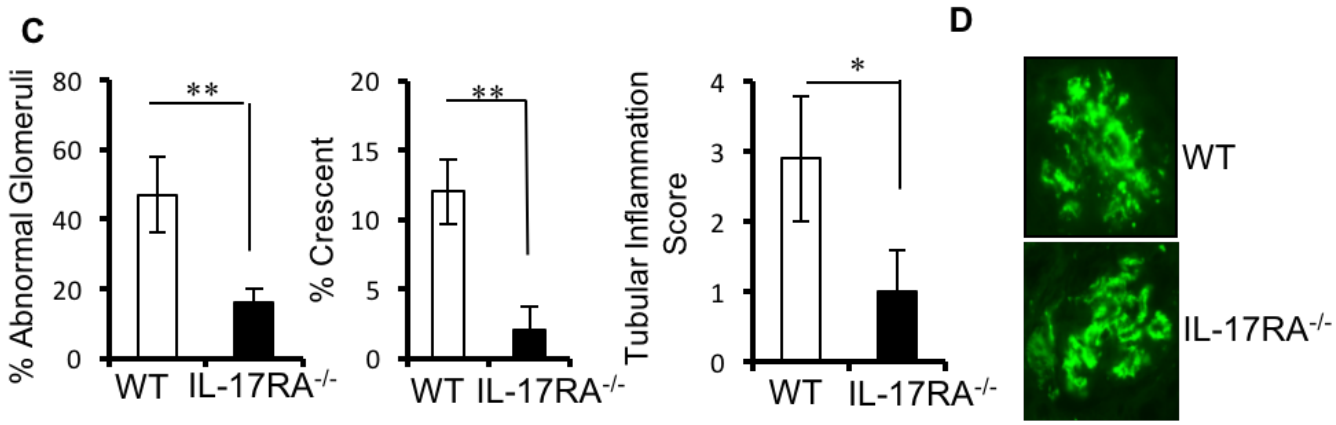


Figure 22. IL-17RA$^{-/-}$ mice were protected from EAGN

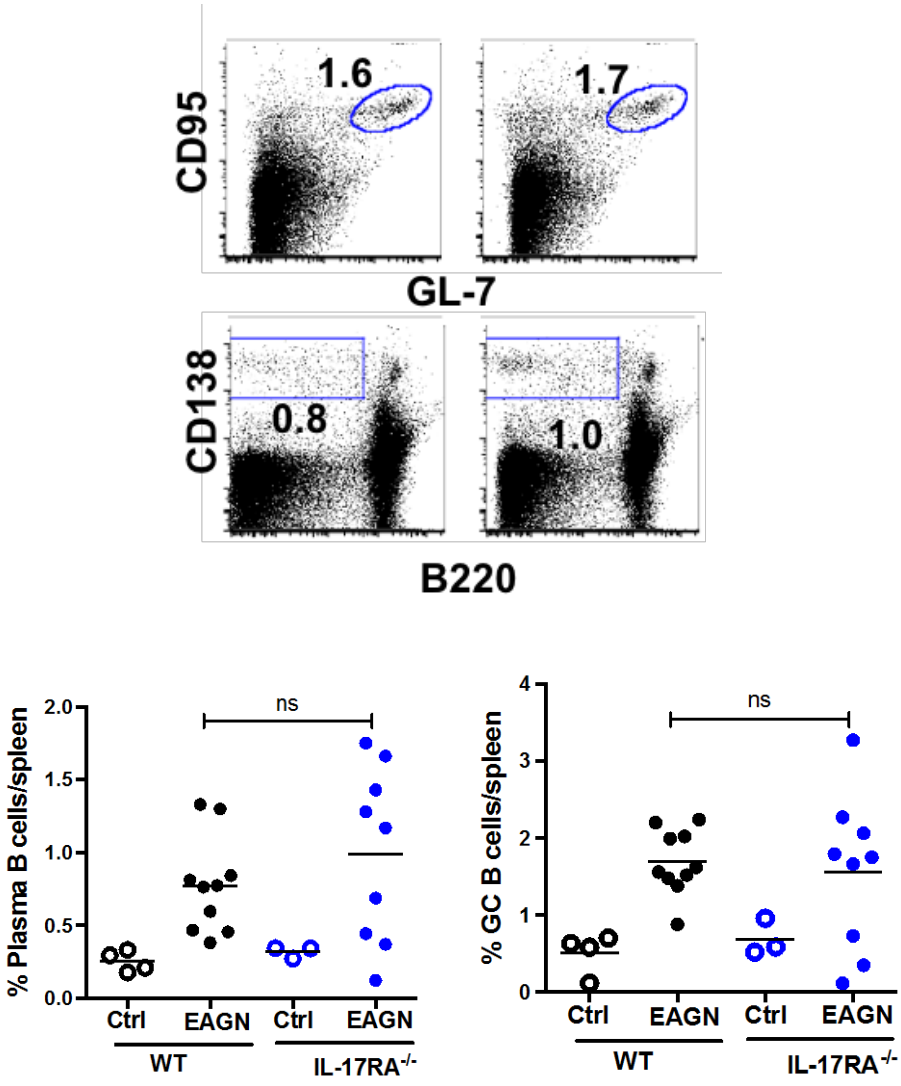
C57BL/6 mice (*n*=9) and IL-17RA$^{-/-}$ mice (*n*=10) were subjected to AGN. Renal dysfunction at Day 14 was assessed by determination of the **(A)** serum BUN level. Each dot represents individual mice, and the horizontal bars indicate mean for each group. **(B)** Representative photographs of PAS-stained kidney sections of nephritic C57BL/6 and IL-17RA$^{-/-}$ mice at Day 14 (original magnification, 400×). **(C)** Renal pathology was blindly evaluated and scored for percentage of abnormal glomeruli, percentage crescent formation, and tubular inflammation in C57BL/6 and IL-17RA$^{-/-}$ mice. **(D)** Representative photographs of mouse IgG deposition in the kidney glomeruli of C57BL/6 and IL-17RA$^{-/-}$ mice at Day 14. Bar diagram represents mean ± SD. Data are pooled from two independent experiments. *P < 0.05; **P >0.001.

3.5.3 Systemic immune response is unaltered in absence of IL-17RA

Previous studies have suggested a role for IL-17R signaling in B and T cell responses [83, 219]. Therefore, to evaluate whether IL-17RA deficiency induces alteration in systemic humoral and cellular immune response against rabbit IgG, we performed detailed phenotypic characterization of splenic B and T cells response in WT and IL-17RA$^{-/-}$ mice. Percentages of germinal center (GC) B cells (B220^{+}GL-7^{+}CD95^{+}) and plasma cells (B220^{lo}CD138^{+}) were comparable between

the WT and IL-17RA$^{-/-}$ mice **(Fig 23A).** Furthermore, antigen specific humoral immune response were measured in the sera of WT and IL-17RA$^{-/-}$ mice by assaying total IgG and isotype pattern of mouse IgG antibody response directed against rabbit IgG. There was no significant difference in total mouse IgG antibody titers to total rabbit IgG at day 14 between the groups **(Fig 23B).** Additionally, the analysis of IgG isotypes revealed no bias for either Th1 (IgG2a) or Th2 (IgG1) antibody production in the absence of IL-17RA signaling **(Fig 23B).**

A



B

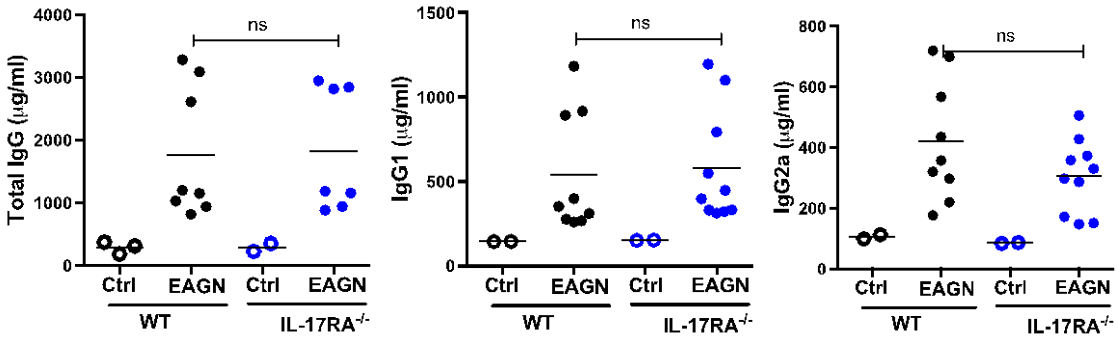


Figure 23. Comparable B cell responses between C57BL/6 and IL-17RA$^{-/-}$ mice

(A) Flow cytometric analysis of splenic cells from C57BL/6 (*n*=9) and IL-17RA$^{-/-}$ (*n*=10) was performed at Day 14 to determine the frequency of GC B cells (B220^{+}GL-7^{+}CD95^{+}) and plasma cells (B220^{lo}CD138^{hi}). Numbers in the dot plot represent percentage of cells. **(B)** At Day 14, serum titers of mouse anti-rabbit total IgG, IgG1 isotype, and IgG2a isotype were measured by ELISA in C57BL/6 (*n*=9) and IL-17RA$^{-/-}$ (*n*=10) mice.

Since CD4^{+} T cells play a major a role in the pathogenesis of EAGN, we characterized the splenic and renal T cell response in WT and IL-17RA$^{-/-}$ mice. At day 14, the frequency of effector CD4^{+} T cells (CD4^{+}CD62L^{lo}CD44^{hi}) as well as natural Tregs (CD4^{+}CD25^{+}Foxp3^{+}) **(**in the spleen was comparable between the groups **(Fig 24A)**. To assess the cytokine production from CD4^{+} T cells, splenocytes were *ex vivo* stimulated with PMA/Ionomycin and then intracellularly stained for IL-17A and IFN-γ. There was no difference in the frequency of splenic CD4^{+}IL-17A^{+} and CD4^{+}IFN-γ^{+} cells in WT and IL-17RA$^{-/-}$ following EAGN **(Fig 24B)**. These data indicate that systemic B and T cells responses were largely unaffected in the absence of IL-17RA signaling in mice with EAGN.

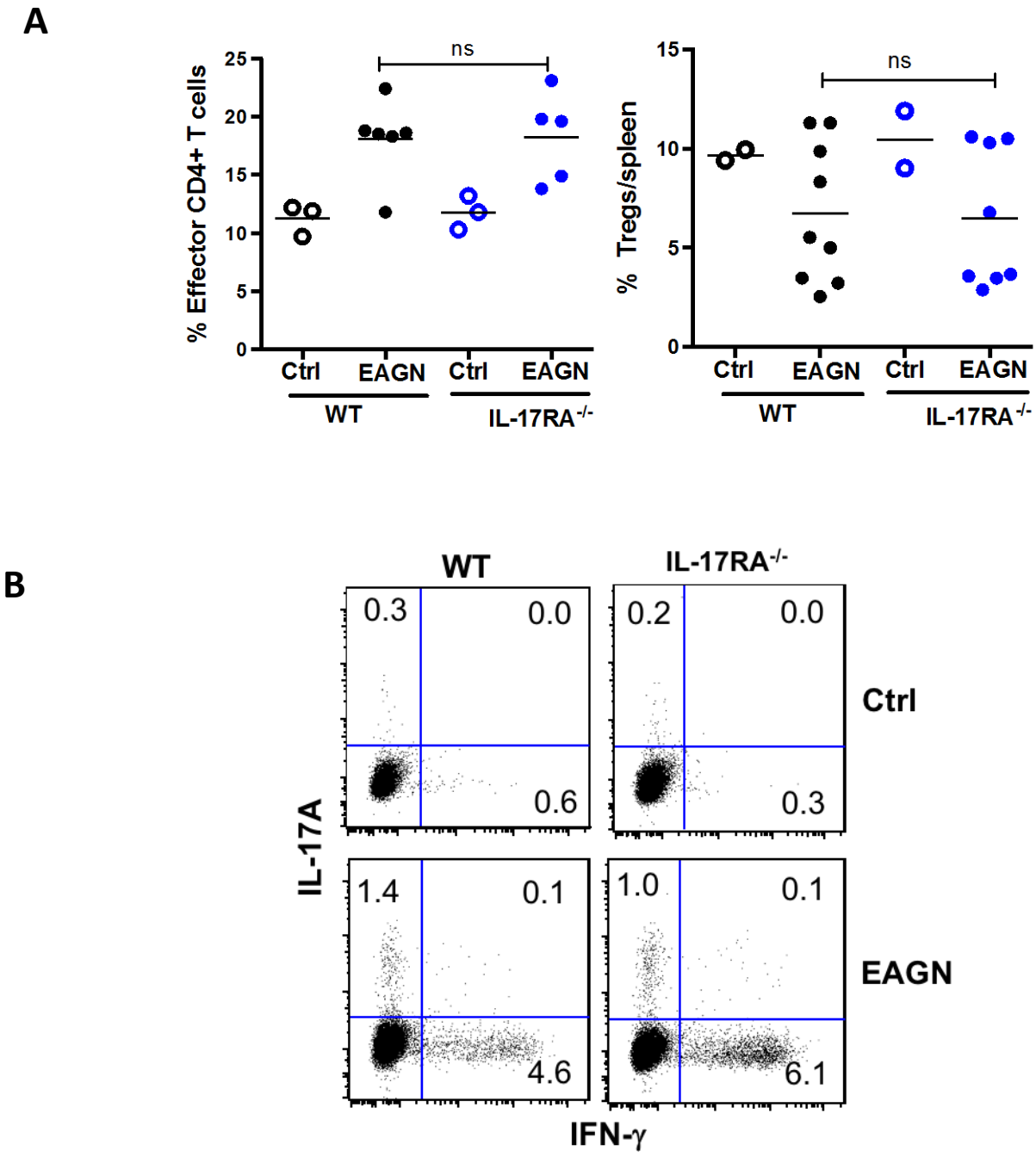


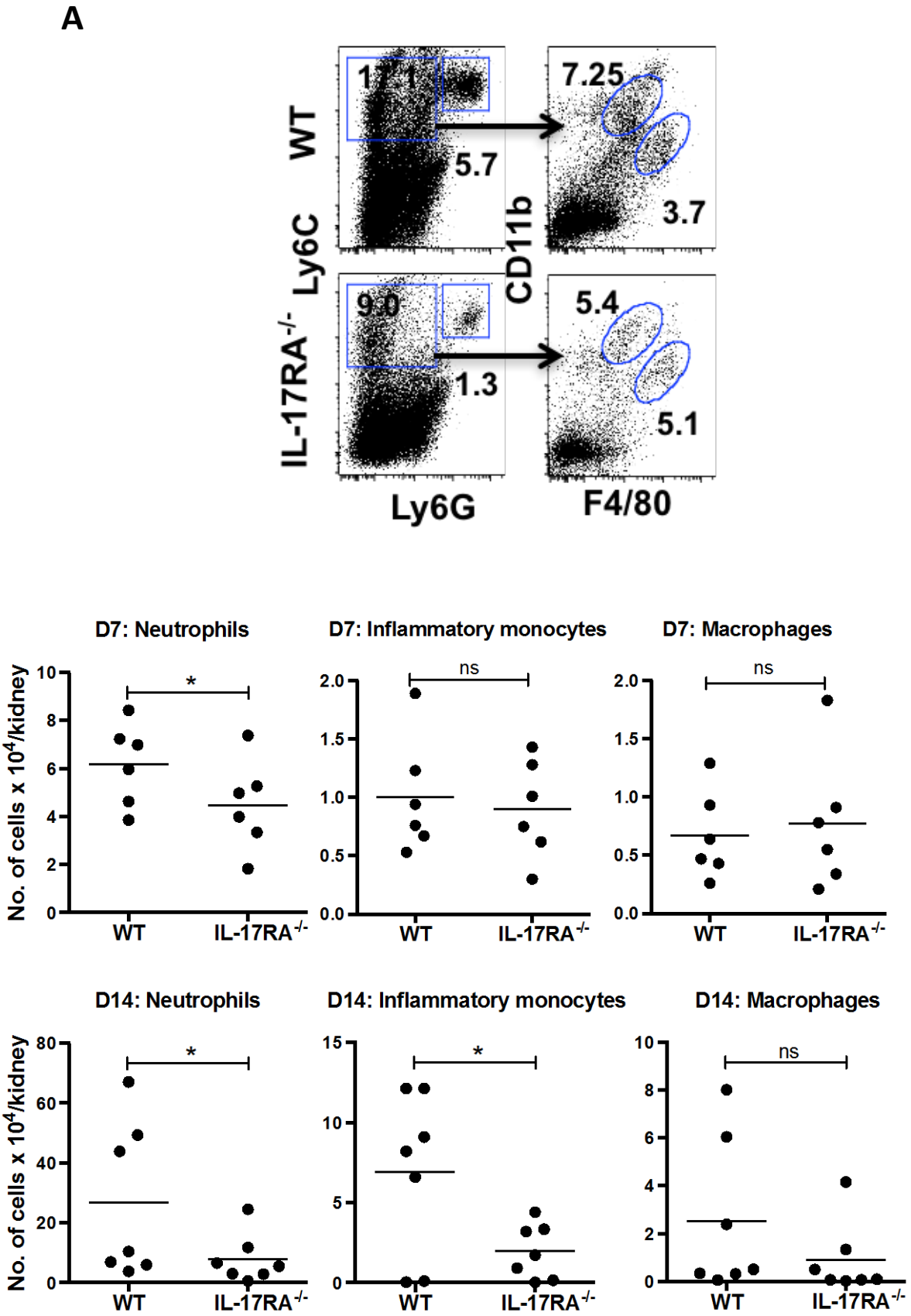
Figure 24. Comparable systemic immune response between C57BL/6 and IL-17RA$^{-/-}$ mice

Flow cytometric analysis of splenic cells from C57BL/6 (n=9) and IL-17RA$^{-/-}$ (n=10) was performed at Day 14 to determine the frequency of **(A)** effector CD4^{+} T cells (CD4^{+}CD62L^{lo}CD44^{hi}) and T_{regs} (CD4^{+}CD25^{+}Foxp3^{+}). Each dot represents individual mice, and the horizontal bars indicate mean for each group. Data are pooled from two independent experiments.

Flow cytometric analysis of splenic cells from C57BL/6 (*n*=9) and IL-17RA$^{-/-}$ (*n*=10) was performed at Day 14 **(B)** Splenocytes were stimulated with PMA/ionomycin for 4 h and intracellularly stained for IL-17A and IFN-γ, followed by FACS analyses (gated on CD4^{+} cells). Numbers in the dot plot represent percentage of cells.

3.5.4 Renal inflammation is alleviated in the absence of IL-17RA signaling

To evaluate the contribution of IL-17RA signaling in the local inflammatory response in the kidney, the frequency of kidney infiltrating innate effectors at early (day 7) and late (day 14) stages of EAGN development was measured by flow cytometry. At day 7, the frequency of neutrophils (Ly6G^{+}Ly6C^{-}) was significantly diminished, but the percentages of kidney infiltrating inflammatory monocytes (Ly6G^{-}Ly6C^{+}CD11b^{+}F4/80^{lo}) and macrophages (Ly6G^{-} Ly6C^{+}CD11b^{+}F4/80^{hi}) were comparable between the IL-17RA$^{-/-}$ and WT mice **(Fig 25A).** However, at day 14 of EAGN, the number of kidney infiltrating neutrophils and inflammatory monocytes were significantly reduced in absence of IL-17RA signaling. Although the frequency of macrophages at day 14 was also reduced in IL-17RA$^{-/-}$ kidney compared to WT mice, it did not achieve statistical significance. To determine whether IL-17RA$^{-/-}$ mice are neutropenic, we enumerated the absolute number of neutrophils in the blood of WT and IL-17RA$^{-/-}$ mice by Giemsa staining. There was no significant difference in the number of neutrophils between WT and IL-17RA$^{-/-}$ mice **(Fig 25B).** These results suggest that infiltration of neutrophils at early and late stages of EAGN is IL-17RA-dependent, whereas monocytes and macrophages require IL-17RA signaling to infiltrate the kidney at a later stage.


A
WT
IL-17RA-/-
Ly6C
CD11b
Ly6G
F4/80
17.1
5.7
7.25
3.7
9.0
1.3
5.4
5.1
D7: Neutrophils
D7: Inflammatory monocytes
D7: Macrophages
D14: Neutrophils
D14: Inflammatory monocytes
D14: Macrophages
No. of cells x 10^4/kidney
*
ns
WT
IL-17RA-/-

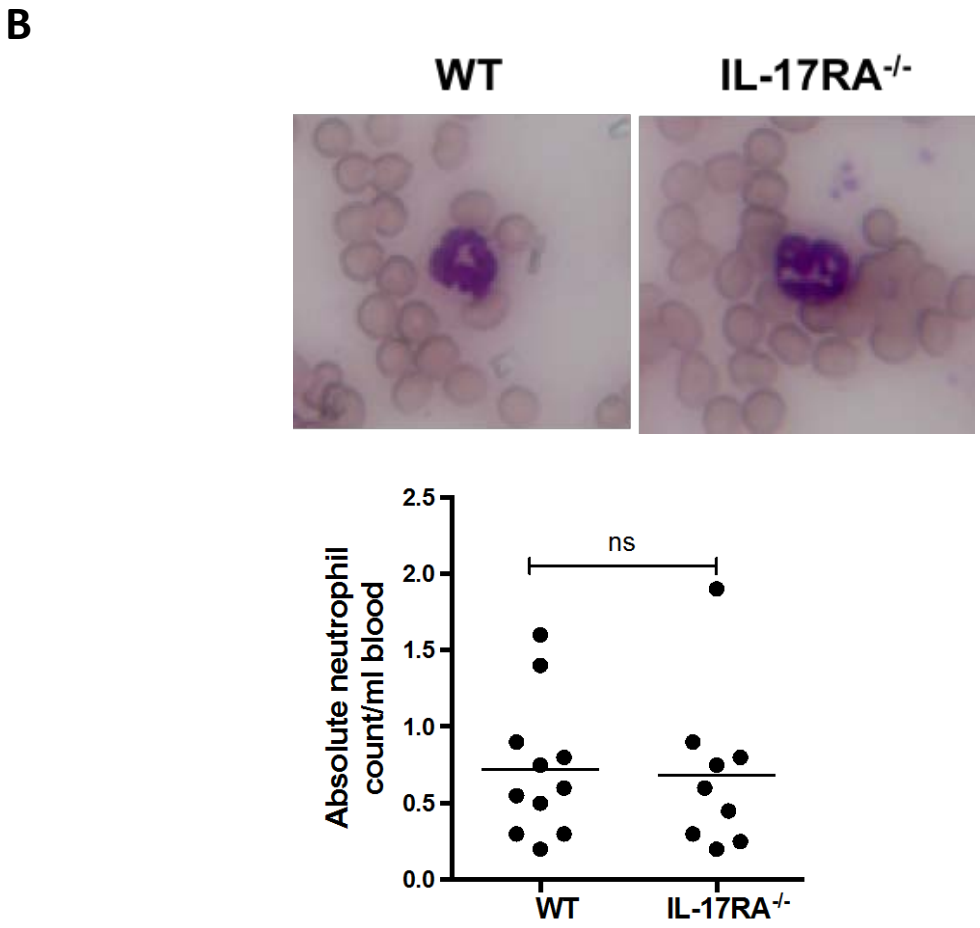


Figure 25. Diminished frequency of renal infiltrating cells in the absence of IL-17RA

(A) Flow cytometric analysis of isolated renal leukocytes from C57BL/6 and IL-17RA$^{-/-}$ mice were performed at Days 7 (*n*=6) and 14 (*n*=9–10) to determine the number of neutrophils (Ly6G^{+}Ly6C^{-}), inflammatory monocytes (Ly6G^{-}Ly6C^{+}CD11b^{+}F4/80^{lo}), and macrophages (Ly6G^{-}Ly6C^{+}CD11b^{+}F4/80^{lo}). Numbers in the dot plot represent percentage of cells. The scattered plots represent absolute number of cells/kidney. **(B)** Peripheral blood smears of WT (*n*=11) and IL-17RA$^{-/-}$ (*n*=9) mice were stained with Giemsa stain and microscopically evaluated for the number of neutrophils/ml blood. **P* < 0.05; ***P* <0.01.

IL-17 drives production of cytokines and chemokines from target cells which leads to infiltration of innate effector cells in the inflamed organs [64]. To determine whether diminished number of

infiltrating innate effectors correlate with reduced kidney specific expression of inflammatory mediators, we measured the transcript expression of IL-17 responsive cytokine and chemokine genes in the kidney of WT and IL-17RA$^{-/-}$ mice by qPCR. At day 10, kidney specific expression of *Il6*, *Cxcl2* and *Cxcl1* mRNA was significantly reduced in IL-17RA$^{-/-}$ mice compared to WT mice. However, there was no difference in the *Ccl20* mRNA expression between the groups **(Fig 26)**. These results demonstrate that IL-17 driven chemotactic signals in the nephritic kidney are required for infiltration of innate effector cells in the pathogenesis of EAGN.

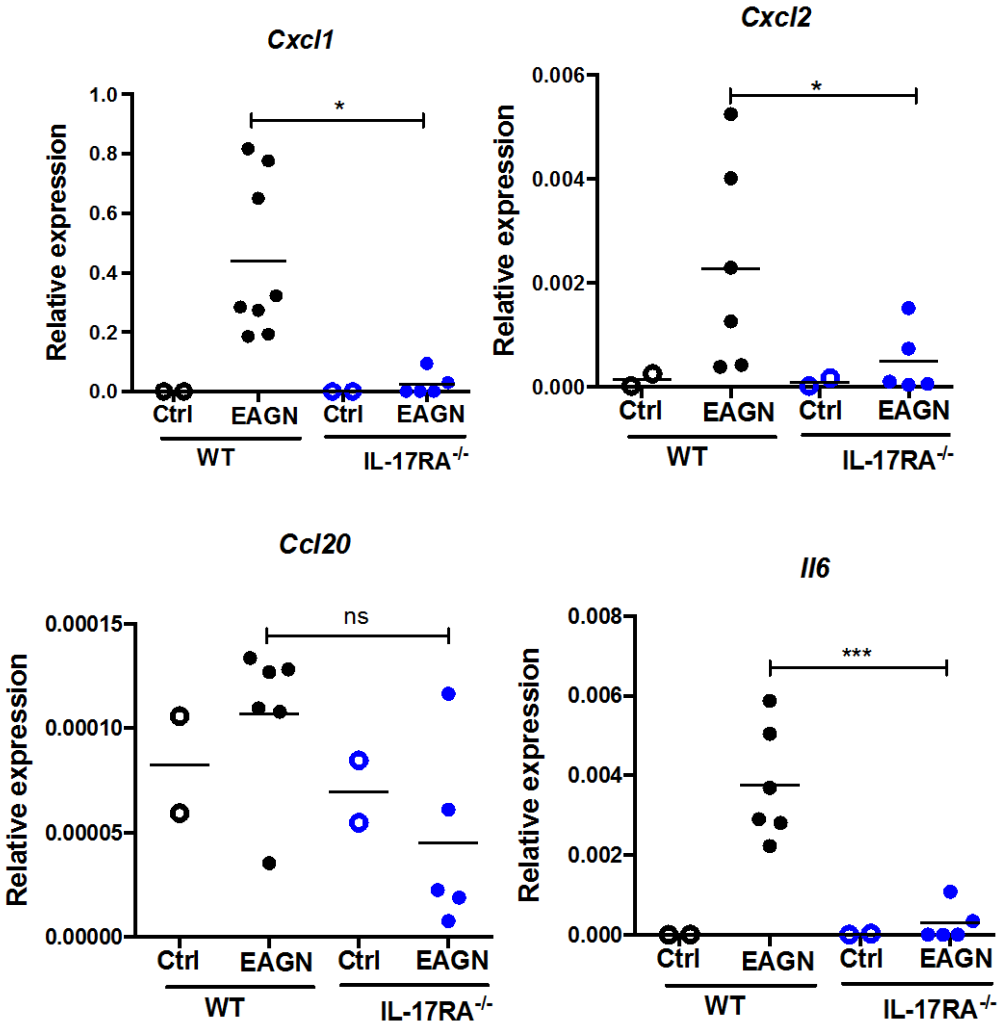


Figure 26. Reduced expression of IL-17 target genes in the kidney in the absence of IL-17RA

qPCR analysis of renal cytokine and chemokine mRNA expression in C57BL/6 (*n*=3 for control; *n*=9 for AGN) and IL-17RA$^{-/-}$ (*n*=3 for control; *n*=10 for AGN) mice at Day 10. Each dot represents individual mice, and the horizontal bars indicate mean for each group. The scattered plot is the pooled data from two individual experiments. *$P < 0.05$; **$P < 0.01$.

3.5.5 IL-17 induces pro-inflammatory cytokine and chemokine expression in murine renal tubular epithelial cells

Previous studies in murine models of acute and chronic kidney injury showed that interactions between inflammatory cytokines and tubular epithelial cells activate a signaling cascade that precede induction of local inflammatory events in the kidney. Renal tubular epithelial cells (RTECs) express functional IL-17R and constitute approximately 70% of the total cells in the kidney cortex [190]. We sought to identify the potential regulatory effects of IL-17 on the expression of the cytokines and chemokines in renal inflammation. Mouse primary RTECs were stimulated with IL-17 in the presence or absence of TNFα, a synergistic partner of IL-17 [27]. We found that mRNA expression of *Il6*, *Cxcl1*, *Cxcl2* and *Ccl20* transcripts was significantly induced by a combination of IL-17 and TNFα, whereas IL-17 or TNFα alone had minimal effect in RTECs **(Fig 27A).**

To ascertain whether the IL-17-responsive chemokines produced by RTECs can induce chemotactic signals to facilitate migration of neutrophils, we performed transwell chamber migration assays with supernatant from *in vitro*-treated or untreated RTECs cells. Bone marrow-derived neutrophils from WT mice demonstrated a significant increase in chemotactic migration in the presence of supernatants from RTECs stimulated with IL-17 and TNFα compared to untreated group **(Fig 27B).** Consistent with the mRNA expression patterns of cytokine and

chemokine genes in IL-17 stimulated RTECs, we found no difference between the migration of neutrophils in the presence of supernatant from RTECs treated with IL-17 or TNFα alone and untreated groups **(Fig 27B).**

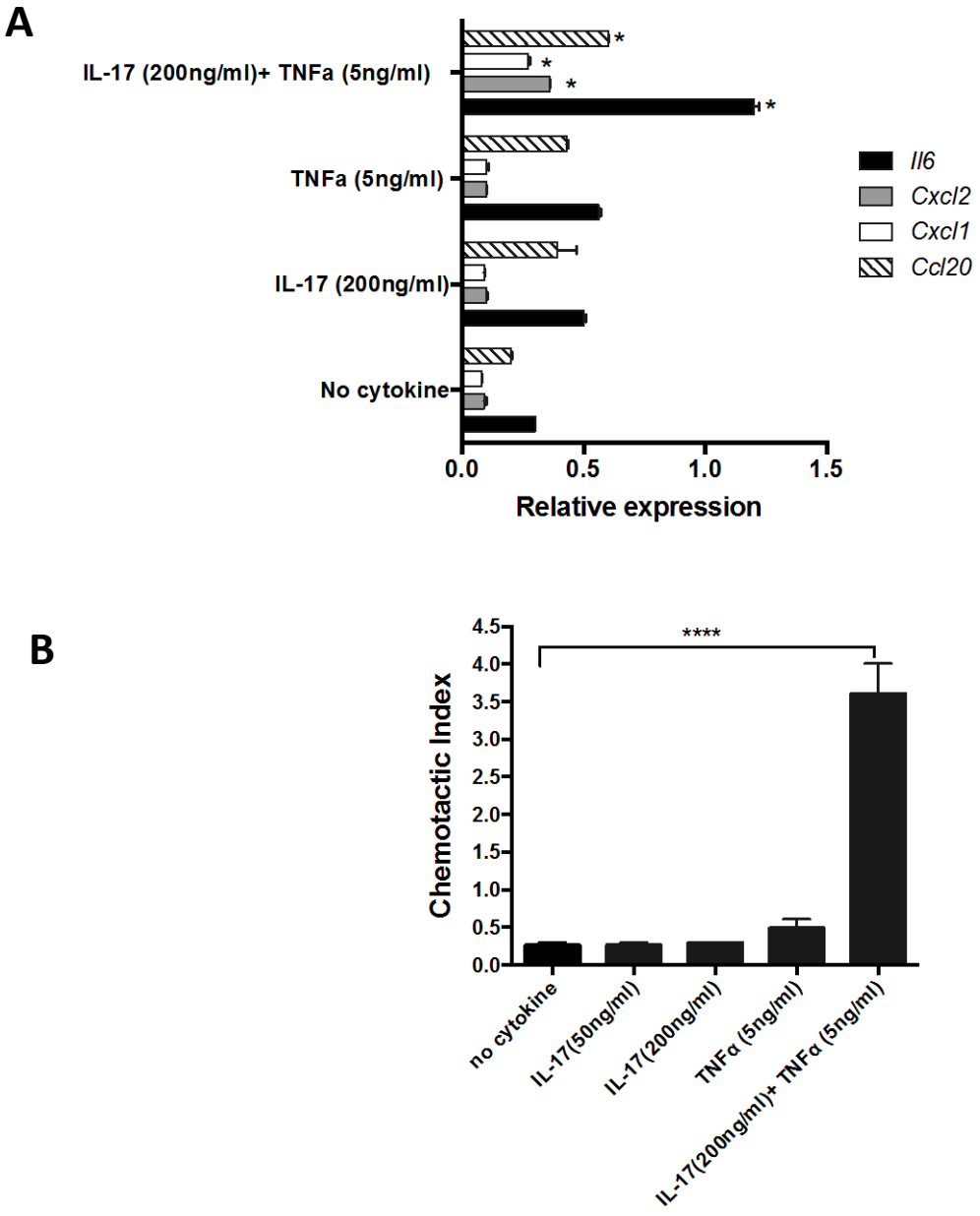


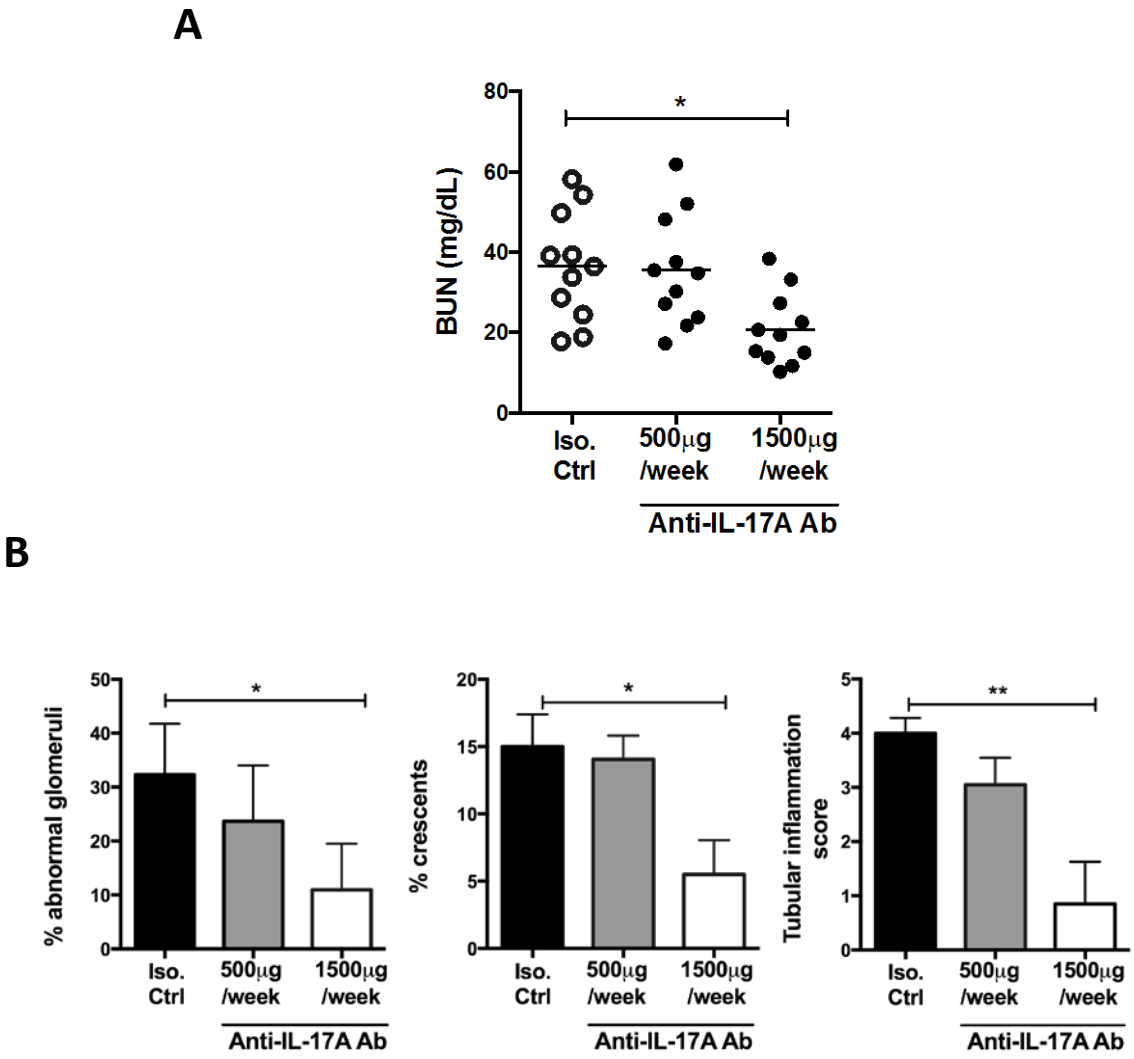
Figure 27. IL-17A induces cytokine and chemokine gene expression in RTECs

(A) Mouse RTECs were left untreated (Untr) or treated with IL-17 (50 ng/ml and 200 ng/ml), TNFα (5 ng/ml), or IL-17 (200 ng/ml) + TNFα (5 ng/ml). After 24 h of stimulation, cells were evaluated for cytokine and chemokine gene expression by qPCR. **(B)** Following 24 h of stimulation, cell culture supernatant from tECs was used to perform in vitro transwell membrane migration assays using bone marrow-derived neutrophils as responding cells. The bar diagram represents mean ± sd. The data represent one of three independent experiments with similar results. $*P < 0.05$; $**P < 0.01$; $***P < 0.0001$.

3.5.6 Neutralization of IL-17A ameliorates EAGN in wild type mice

We showed that the absence of IL-17RA diminished renal pathology and inflammatory response in EAGN. Based on this finding, we hypothesized that neutralizating of IL-17A may impede the development of AGN. To this end, 3 groups of WT mice were either treated with a low (500 µg/once a week) or high dose (500 µg/thrice a week) of anti-IL-17A or isotype control (500 µg/thrice a week) antibodies following induction of EAGN. Consistent with the disease resistant phenotype in IL-17RA$^{-/-}$ mice, on day 14, we observed significantly lower levels of BUN in the serum in mice treated with high dose of anti-IL-17A compared to control groups **(Fig 28A).** There was no difference between the group of mice treated with the low dose of anti-IL-17A and control mice **(Fig 28A).** PAS-stained kidney sections of WT mice treated with high dose of anti-IL-17A had significantly fewer abnormal glomeruli, crescentic glomeruli as well as diminished tubular inflammation compared to isotype treated group (**Fig 28B).** We observed a dose dependent effect of IL-17A neutralization on the glomerular and tubular inflammatory changes indicating that low dose of anti-IL-17A was insufficient to neutralize IL-17A in the nephritic kidney. We further analyzed whether the resistance to EAGN observed in mice treated with anti-

IL-17A correlated with diminished innate cell influx in the nephritic kidney. On day 14, mice treated with high dose of anti-IL-17A had fewer kidney infiltrating neutrophils. There was a trend noted in the frequency of inflammatory monocytes and macrophages which were reduced in anti-IL-17A treated mice compared to control group **(Fig 28C).** These results point to a potential prophylactic role for the neutralization of IL-17A in the development of renal pathology associated with EAGN.



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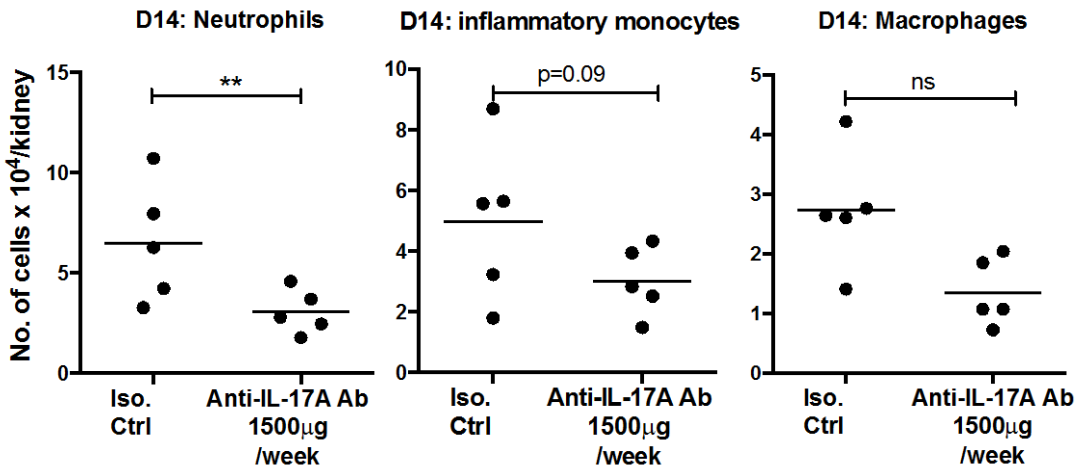

Figure 28. Neutralization of IL-17A attenuated EAGN development in WT mice

Three groups of C57BL/6 mice (*n*=10) were treated with a lower dose (500 μg/week) or higher dose (1500 μg/week) of anti-IL-17A or isotype (Iso.) control (500 μg, three times/week) antibody following induction of AGN. At Day 14, mice were evaluated for the development of AGN. Renal dysfunction at Day 14 was assessed by determination of the **(A)** serum BUN level. Each dot represents individual mice, and the horizontal bars indicate mean for each group. **(B)** Renal pathology was blindly evaluated and scored for percentage of abnormal glomeruli, percentage crescent formation, and tubular inflammation in C57BL/6 and IL-17RA$^{-/-}$ mice. Bar diagram represent mean ± sd. Data are pooled from two independent experiments. (**C)** Flow cytometric analysis of isolated renal leukocytes from anti-IL-17A antibody and isotype control antibody-treated C57BL/6 mice was performed at Day 14 (*n*=5) to determine the number of neutrophils (Ly6G^{+}Ly6C^{-}), inflammatory monocytes (Ly6G^{-}Ly6C^{+}CD11b^{+}F4/80^{lo}), and macrophages (Ly6G^{-}Ly6C^{+}CD11b^{+}F4/80^{lo}) following AGN. The scattered plots represent absolute number of cells/kidney. The horizontal bars represent mean for each group. *P < 0.05; **P < 0.01.

3.6 DISCUSSION

T cells infiltrate the kidney and are crucial in mediating tissue damage in the pathogenesis of inflammatory diseases including glomerulonephritis. However, the role of different Th subsets, particularly the Th17 cells and their signature cytokine IL-17, is poorly defined in autoimmune glomerulonephritis. Using a murine model of EAGN, we showed that mice deficient in IL-17RA signaling exhibited diminished renal inflammation and infiltration of the innate effector cells in the kidney, correlating with the reduced expression of IL-17 responsive cytokine and chemokine genes. *In vitro*, IL-17 enhanced the production of proinflammatory cytokines and chemokines from RTECs, which are implicated in the recruitment of innate effector cells in the kidney. Finally, neutralization of IL-17A ameliorated renal pathology in wild type mice following induction of EAGN. These results establish the contribution of IL-17RA signaling in renal tissue injury. These data implicating IL-17RA signaling in EAGN suggest that blocking IL-17RA signaling may be a promising therapeutic strategy for the treatment of glomerulonephritis associated with multiple auto-inflammatory kidney disorders.

Although most documented IL-17R signaling occurs in non-hematopoietic cells [64], some reports indicate a role for IL-17 in hematopoietic cells as well [64, 83, 219-221]. IL-17 promotes granulopoiesis and neutrophil survival by stimulating the expression of G-CSF and neutrophil attracting chemokines in inflamed organs [220]. In macrophages, IL-17 indirectly induces the expression of TNFα and IL-1 [221]. While the role of IL-17 on innate cells is well established, some evidence suggests a direct role of IL-17 in promoting germinal center formation and auto-antibody production from B cells [77, 83]. In contrast, loss of IL-17 resulted in reduced IL-17 responsive gene expression and infiltration of inflammatory cells in the nephritic kidney [77]. These data point to a direct role for IL-17 on cells of non-hematopoietic

origin in mediating renal inflammation. In support of these findings, our data show absence of the receptor IL-17RA protected mice from EAGN. IL-17RA$^{-/-}$ mice exhibit diminished renal inflammation, despite an abnormal systemic immune response, correlating with disease resistance. These data indicate of the fact that IL-17R signaling in kidney-resident cells plays a central role in driving renal inflammation in autoimmune glomerulonephritis. Identifying the target cells of IL-17 and blocking IL-17R signaling in these cells could be potentially therapeutic in controlling autoimmune glomerulonephritis.

The immune response in AGN is controlled by two types of cells in the kidney – (i) the infiltrating cellular and humoral effectors and (ii) resident cells. While the injurious role of infiltrating immune cells has been emphasized, the contribution of kidney resident cells is less understood. Studies in chronic kidney injury models have shown that inflammatory cytokines induce kidney resident cells to activate signaling pathways that drive local inflammatory events resulting in renal injury and fibrotic events. The major kidney resident cells implicated in the autoimmune glomerulonephritis are podocytes, mesangial cells and proximal and distal tubular epithelial cells [222-224]. So far, most IL-17R signaling experiments have involved *in vitro* approaches using fibroblast and epithelial cell lines. Data to delineate pathological consequence of IL-17R signaling *in vivo* in the nephritic kidney are currently lacking. To investigate the potential contribution of IL-17 in inducing inflammatory mediators from kidney resident cells, we showed that IL-17 in synergy with TNF-α induced the expression of cytokine and chemokine genes from renal epithelial cells. Since, cell types interpret IL-17 signals differently, with overlapping but non-identical patterns of gene expression, the contribution of IL-17R signaling in the other kidney-resident cells types such as mesangial cells, podocytes and glomerular endothelial cells in the pathogenesis of AGN remains to be defined.

Given that IL-17 plays a central role in the pathogenesis of AGN, therapeutic neutralization of IL-17 to diminish the severity of AGN has never been tested in mouse models of auto-inflammatory kidney diseases. Lupus-prone mice treated with anti-IL-6 (IL-6 is required for Th17 differentiation) or IL-21RF$_C$ (IL-21 is produced by Th17 cells) resulted in diminished glomerulonephritis [200, 225]. However, given the pleiotropic effect of IL-6 and IL-21 on other immune cells such as B cells and myeloid cells, it is difficult to assess the therapeutic efficacy of anti-IL-6 and anti-IL-21 therapies in diminishing the Th17 response. Administration of IL-17RF$_c$ in autoimmune-prone BXD2 mice resulted in diminished germinal center reaction and autoantibody production. But the clinical consequence of IL-17R blocking in renal inflammatory response is largely unknown [83]. We showed for the first time that administration of anti-IL-17A attenuates renal pathology and inflammation significantly improving renal function in EAGN.

Multiple clinical trials targeting IL-17 pathway and signaling mechanisms are ongoing, and positive results have been obtained in psoriasis, rheumatoid arthritis and ankylosing spondylitis [176]. The 2 FDA approved anti-IL-17 drugs is in clinics being used to treat psoriasis. Our results provide a strong rationale for future clinical trials to test the therapeutic effectiveness of anti-IL-17R in patients suffering from autoimmune glomerulonephritis associated with auto-inflammatory kidney disorders.

3.7 MODEL

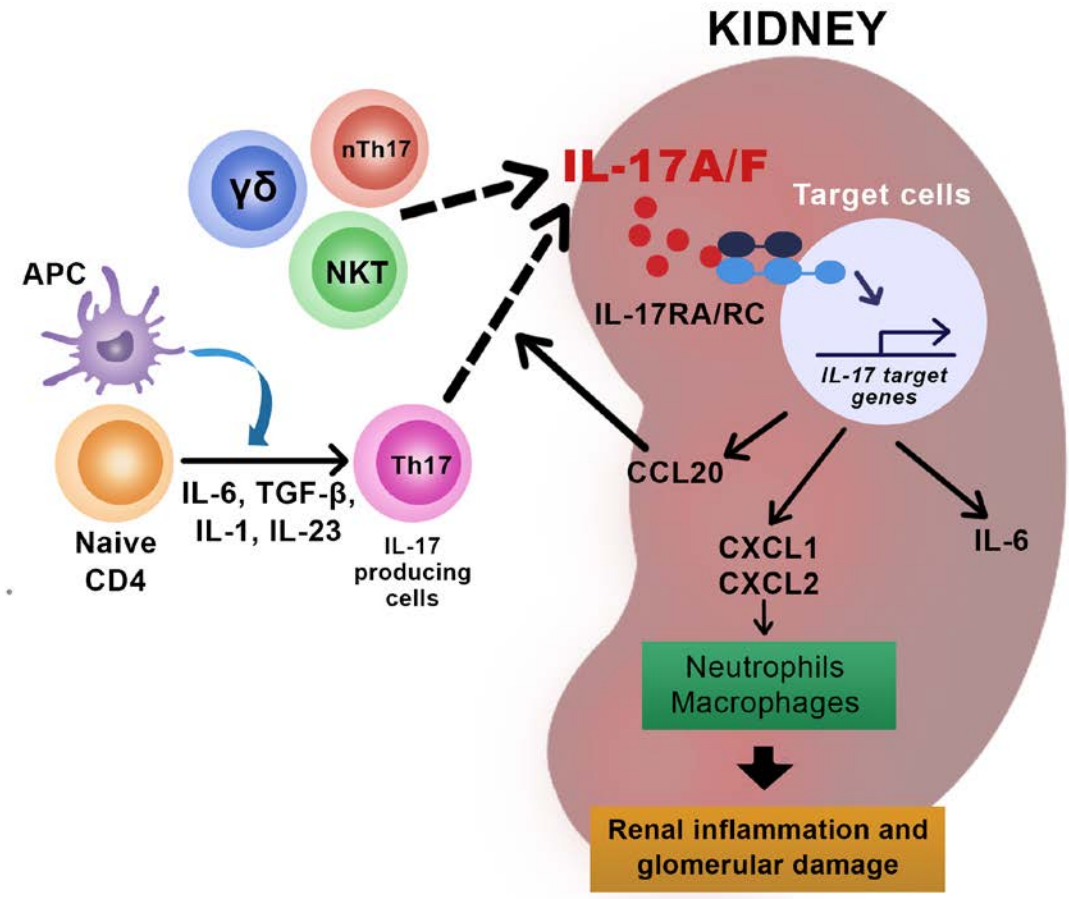

Figure 29. IL-17 drives local immune responses in the kidney in autoimmune glomerulonephritis

IL-17 drives renal inflammation by upregulating pro-inflammatory cytokines and chemokines.

CHAPTER 4: INTERLEUKIN-17 SIGNALING DRIVES TYPE I INTERFERON INDUCED PROLIFERATIVE CRESCENTIC GLOMERULONEPHRITIS IN LUPUS-PRONE MICE

The definitive version was published in

Clinical Immunology, January 2016, Volume 162, 31-36

"Interleukin-17 signaling drives type I interferon induced proliferative crescentic glomerulonephritis in lupus-prone mice"

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4.0 SUMMARY

Membranoproliferative glomerulonephritis is a common feature in patients with SLE and associated with increased morbidity and mortality [226]. The severity of renal failure correlates with the percentage of formation of cellular crescents leading to crescentic glomerulonephritis (cGN) [226]. cGN is associated with severe glomerular injury and is an indicator of poor renal outcome [226]. Although the initiation and progression of membranoproliferative glomerulonephritis has been extensively studied, our understanding of the pathogenesis of cGN in SLE is rudimentary.

Emerging evidence suggests a critical role for IL-17 in the pathogenesis of membranoproliferative glomerulonephritis in lupus. However, the role of IL-17 receptor (IL-17RA) signaling in cGN is unknown. Here, we developed a model of poly I:C-induced type I interferon (IFN-I)-dependent cGN in B6.MRL-Faslpr/J (B6.lpr) mice. B6.lpr mice deficient in IL-17RA were protected from IFN-I-dependent cGN. While systemic response was unabated, renal infiltration of alternatively activated macrophages was severely impaired in IL-17RA$^{-/-}$ mice. We also show that IL-17 in association with IFN-I differentially regulates the expression of macrophage chemoattractant genes in RTECs and macrophages. These results suggest that neutralization IL-17 may confer better protection in SLE patients with high IFN-I gene signature and cGN.

4.1 TYPE I INTEFERONS IN LUPUS NEPHRITIS

Type I interferons (IFN-I) drive aberrant systemic autoimmune responses in SLE [227]. IFN-I are primarily important in anti-viral defense and are produced by several cell types though the major source is the plasmacytoid dendritic cell (pDC) [228]. Endogenous nuclear particles can induce IFN-I expression after the engagement of endosomal nucleic acid recognition receptors such as TLR3,TLR7 and TLR9 [229]. Direct effects of IFN-I on renal tissue damage in the nephritic kidney have been well documented [230, 231]. The role for IFN-I in SLE was discovered when enhanced expression of a group of IFN induced genes known as the IFN gene signature was found in PBMCs from lupus patients [227]. The IFN gene signature can be induced in healthy PBMCs by SLE plasma. Anti-IFNα antibodies can block the induction of the gene signature establishing IFNα, in particular, as the cytokine driving the pathogenesis of SLE [232]. IFN-I promote autoimmunity through the activation of dendritic cells and auto-reactive T and B cells [233]. In lupus nephritis, pDCs accumulate in the kidney where they can interact with renal immune complexes and increase IFNα levels. Resident renal cells including glomerular endothelial and mesangial cells can also participate in nucleic acid immune recognition. This can result in the secretion of high levels of IFNα and other pro-inflammatory cytokines [234, 235]. Studies using lupus prone mouse models have further elucidated the role for IFN-I in lupus nephritis. IFN-I receptor (IFNAR1) deficiency reduces autoimmunity in lupus prone mice [236]. Conversely, administration of exogenous IFNα, or a TLR3 ligand, polyinosinic: polycytidylic acid [poly (I:C)], triggered the development of accelerated cGN in lupus-prone mice after onset of systemic autoimmunity, but prior to clinically penetrant lupus nephritis [230, 237, 238]. In line with this finding, sustained activation of TLR3 signaling in four week old lupus prone B6.MRL-Faslpr/J(B6.lpr) mice and before the onset of systemic autoimmunity, resulted in a

dramatic aggravation of renal disease, an effect completely abrogated in the absence of IFNAR signaling [239]. These studies highlight the essential contribution of IFN-I in the pathogenesis of cGN in SLE.

4.2 IL-17 IN LUPUS NEPHRITIS

Increasing evidence suggests a role for IL-17 and IL-17-producing cells in the development of lupus nephritis [240]. IL-17 was found to be elevated in the serum of many SLE patients, correlating with disease severity [241]. In addition to Th17 cells, $CD4^{-}CD8^{-}$double-negative (DN) T cells were found to be major producers of IL-17 in SLE and infiltrate kidneys in patients with lupus nephritis [85]. Studies in murine models of SLE have supported a pathogenic role for IL-17 in lupus nephritis. High levels of IL-17 and IL-17-producing T cells have were seen in BXD2 mice, which spontaneously develop lupus-like features [83]. In IL-23R-deficient B6.lpr mice the generation of DN T cells was abolished and these mice were resistant to the development of membranoproliferative glomerulonephritis [195]. $Fc\gamma RIIB^{-/-}$ mice deficient in IL-23R signaling or lacking IL-17 signaling components were resistant to the development of nephritis [77]. We also showed that the loss of IL-17R signaling resulted in reduced expression of IL-17 responsive genes and infiltration of inflammatory cells in the nephritic kidney, correlating with disease resistance [169]. In ANCA-associated vasculitis, IL-17 drives the pathogenesis of cGN in the kidney [208]. Since spontaneous lupus-prone mouse models do not faithfully develop cGN, role of IL-17R signaling in the development of crescent formation in SLE is unknown.

4.3 CO-EXISTENCE OF IL-17 AND TYPE I INTERFERONS IN LUPUS NEPHRITIS

In the literature, the interplay of IFN-I and IL-17 has been suggested. Although, IFN-I has been shown to limit Th17 cell development by inducing IL-27 [242], various studies have shown that pathogenic Th17 cells and IFN-I can co-exist in autoimmune diseases like psoriasis [243] and SLE [244]. This dichotomy can be explained by the differential signaling via IFNAR [245, 246]. Canonical signaling by IFN-I is mediated by the phosphorylation of STAT1 and STAT2 [247]. In contrast, non-canonical IFNAR signaling activates STAT3 in T cells [248, 249]. Since STAT3 is critical for the generation of Th17 cells [250], non-canonical IFNAR mediated STAT3 activation can potentially support the presence of Th17 cells. In addition, it is also possible that IFN-I may support IL-17 production by participating in the induction of the production of cytokines important for Th17 cell differentiation such as IL-6 [251]. Recently a report from our laboratory showed that activation of complement component 5a recepte (C5aR) on murine macrophages blocked IFN-I–mediated IL-27 production, thus permitting the development of Th17 cells [206]. C5aR activation is inhibitive for the production of IFN-I. Activation of C5aR blocks IFN-I–mediated IL-27 production, thus permitting the development of Th17 cells. Concomitantly, C5aR-deficient mice induced with lupus showed an increased IFN-I signature. This demonstrated that IL-17 and IFN-I could co-exist in lupus nephritis. However, the caveat with the use of C5aR deficient mice is that they are not normally lupus prone. To clarify the co-existence of IL-17 and IFN-I we decided to use the B6.lpr mice that develop lupus nephritis spontaneously. Since it was found that IL-17 and IFN-I exist at sites of inflammation in SLE we hypothesize that there might be possible synergy between these cytokines in driving cGN.

4.4 MATERIALS AND METHODS

Mice

B6.lpr mice were obtained from Jackson Laboratory (Bar Harbor, ME). The IL-17RA-/- mice were obtained from Amgen (San Francisco, CA) and bred in-house. All mice were housed and bred under specific pathogen-free conditions and age and sex matched mice were used for all the experiments. All animal protocols were approved by the University of Pittsburgh IACUC, and adhered to the guidelines in the Guide for the Care and Use of Laboratory Animals of the NIH.

Induction of accelerated Poly I:C induced lupus nephritis

12-15 week-old female B6.lpr or B6.lpr IL17RA$^{-/-}$ mice were treated i.p. with 200 μg polyinosinic: polycytidylic acid [poly (I:C)] (Invivogen, San Diego,CA) in sterile endotoxin- free water or control PBS , 3 times a week for 10 weeks. Induction of poly I:C induced accelerated cGN at the end of 10 weeks' post-treatment for renal pathology studies and at 6 weeks for macrophage studies. Proteinuria was evaluated using dip-stick method (Roche, San Francisco, CA). Kidneys were fixed in 10% formalin and embedded in paraffin. Tissue sections were stained with H&E or Periodic Acid-Schiff (PAS) and histological scores were evaluated blindly.

Flow cytometry

The spleen and lymph nodes were harvested and single-cell suspensions were surface stained with the following antibodies: anti-F4/80-PE (BM8), -Gr-1-FITC (RB6-8C5), -CD11b-PeCy7 (M1/70) Ly6C-PerCP-Cy5.5(HK1.4), (eBioscience, San Diego,CA) –CD11c-BV21 (HL3), -B220-APC (RA3-6B2), -CD4-V450 (RM4-5), -CD8a-PerCP(53-6.7) (BD Biosciences, San Jose, CA) , CD206-FITC (C068C2) (Biolegend, San Diego, CA). Intracellular staining of Foxp3 was performed with mouse Foxp3 staining kit (eBioscience, San Diego,CA). Data were analyzed

using FlowJo (Tree Star, OR) software. For flow cytometry of kidney, perfused kidneys were harvested, processed and stained for flow cytometry.

Real time qPCR analyses

Total RNA was isolated, reverse transcribed and gene expression was measured by real time qPCR. The primers for various target genes were obtained from QuantiTect Primer Assays (Qiagen, CA). The expression of each gene was normalized to the expression of mouse GAPDH.

***In vitro* stimulation of primary mouse tubular epithelial cells (RTEC) and bone marrow-derived macrophages (BMDM) cultures**

Primary mouse renal tubular epithelial cells (RTECs) were cultured according to manufacturer's instructions (Cell Biologics, IL). For BMDM cultures, bone marrow cells were extracted from the femur and tibia of mice and cultured in DMEM with L929 cell supernatants for 7 days. Cells were either stimulated with IL-17A (200 ng/ml), TNFα (5 ng/ml) or IFN-α (250U/ml) and all three cytokines in different combinations or left unstimulated for 24h.

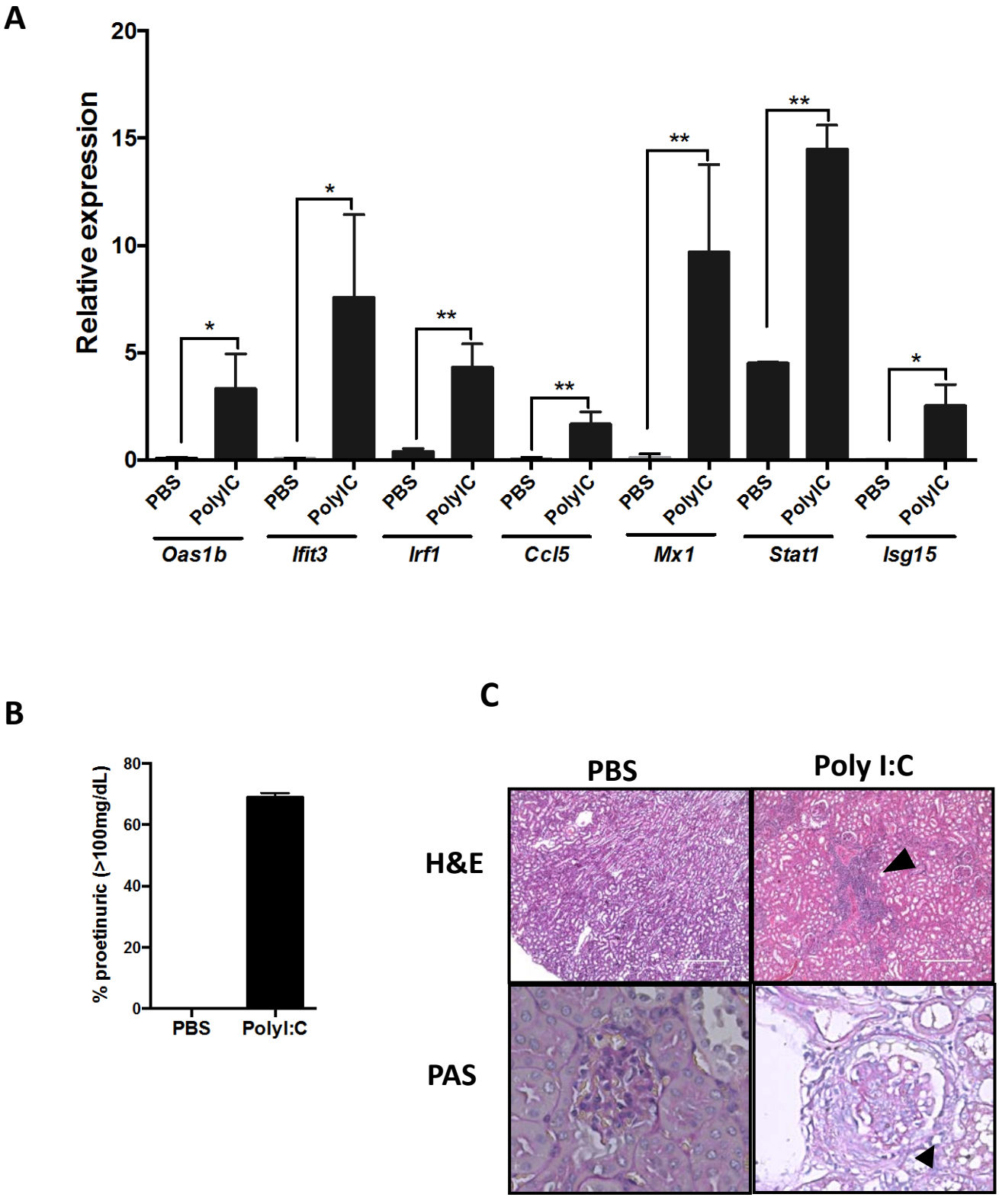
Statistical analyses

Results are expressed as mean ± SD. Differences between groups were calculated for statistical significance using 2-tailed paired Student's *t* tests and ANOVA as appropriate. A *p* value less than 0.05 was considered significant.

4.5 RESULTS

4.5.1 Administration of poly I:C accelerates IFN-I dependent cGN in B6.lpr mice

B6.lpr mice spontaneously develop mild membranoproliferative nephritis at 40-45 weeks of age in an IL-23-Th17 axis dependent manner. Both Th17 cells and innate cells are a major source of IL-17 [280], but the role of IL-17 in the pathogenesis of IFN-I-dependent cGN is poorly understood. We used the poly I: C induced IFN-I dependent accelerated cGN model in lupus-prone B6.lpr mice. We injected B6.lpr mice that are 12-15 weeks old with poly I: C thrice a week for 10 weeks. At this age, B6.lpr mice develop systemic autoimmune responses but renal disease is not apparent. Poly I: C induced the expression of multiple IFN-I responsive genes in the kidney and spleen of B6.lpr mice within 6h post-injection **(Fig 30A).** Ten weeks post treatment, poly I: C injected mice displayed an increased incidence of proteinuria, glomerular abnormalities, crescent formation and tubular damage compared to PBS injected controls **(Fig 30B-D)**. These results confirmed that poly I: C stimulates the expression of renal IFN-I induced genes and served as a good model to accelerate the development of cGN.


A
Relative expression
20
15
10
5
0
PBS
PolyIC
Oas1b
Ifit3
Irf1
Ccl5
Mx1
Stat1
Isg15
B
% proetinuric (>100mg/dL)
80
60
40
20
0
PBS
PolyI:C
C
PBS
Poly I:C
H&E
PAS

D

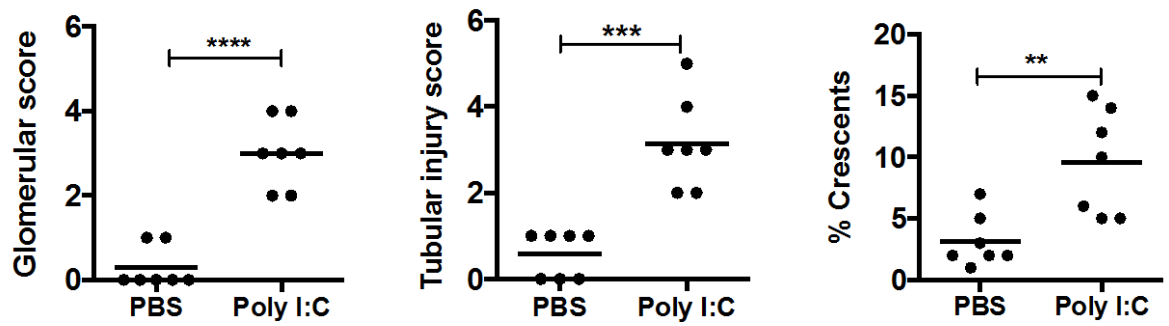


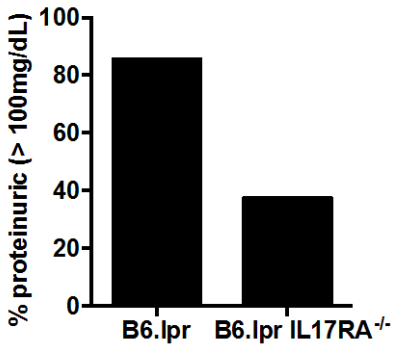
Figure 30. Poly I:C can induce IFN-I dependent renal damage associated with cGN

12–15 week old female B6.lpr mice (n = 10) were either injected with poly I: C or PBS for 10 weeks**. (A)** Six hours' post-treatment, poly I: C treated and untreated mice were evaluated for transcript expression of multiple IFN-I-responsive genes. **(B)** At 10 weeks' post-treatment, mice were assessed for proteinuria. **(C)** Serial kidney sections were stained for H&E and PAS stain to evaluate kidney inflammation and renal pathology. Black arrows indicate tubulointerstitial inflammation (upper panel) and crescent formation (lower panel). **(D)** Kidney pathology was blindly evaluated and scored for glomerular damage, crescent formation and tubular injury.

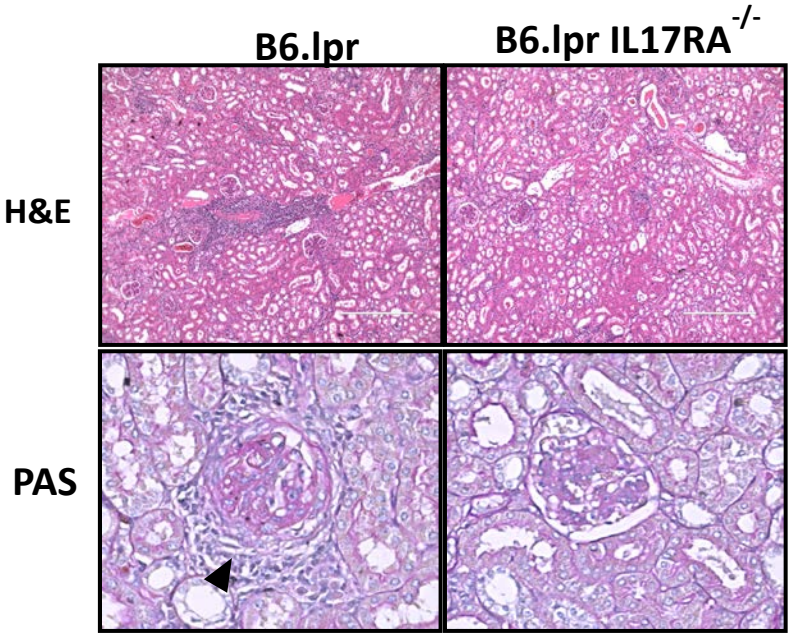
4.5.2 Lupus –prone mice that lack IL-17RA were protected from IFN-I dependent cGN

To define the role of IL-17RA signaling in IFN-I induced cGN, we generated B6.lpr mice lacking IL-17RA (B6. lprIL-17RA$^{-/-}$). B6. lprIL-17RA$^{-/-}$ and control B6.lpr mice were subjected to poly I:C-induced cGN and evaluated for renal pathology. The B6. lprIL-17RA$^{-/-}$ mice had significantly reduced proteinuria and less glomerular damage, crescent formation and tubular inflammation at the end of 10 weeks with poly I:C treatment **(Fig 31A-D)**. These data point to a previously unappreciated role for IL-17R signaling in IFN-I induced cGN in lupus-prone mice.

A


% proteinuric (> 100mg/dL)
100
80
60
40
20
0
B6.lpr
B6.lpr IL17RA-/-

B


B6.lpr
B6.lpr IL17RA-/-
H&E
PAS

C

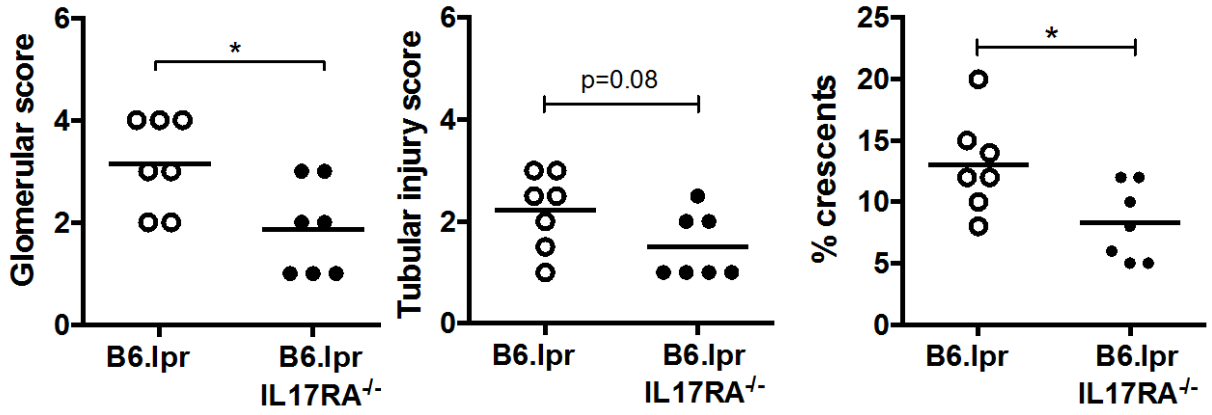

Glomerular score
6
4
2
0
*
B6.lpr
B6.lpr IL17RA-/-
Tubular injury score
p=0.08
% crescents
25
20
15
10
5

Figure 31. B6. lprIL-17RA$^{-/-}$mice are resistant to IFN-I-induced cGN

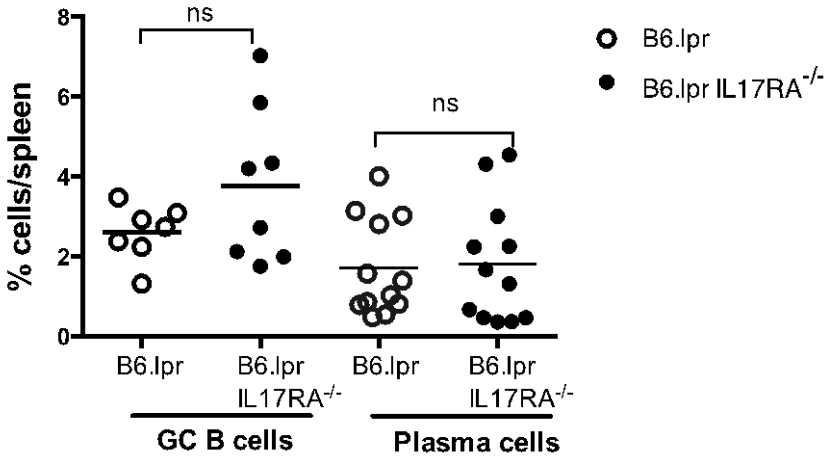
12–15 weeks old female B6.lpr and B6. lprIL-17RA$^{-/-}$ mice (n = 10–13) were subjected to poly I:C-induced accelerated cGN. 10 weeks' post-treatment, mice were evaluated for **(A)** proteinuria, **(B)** renal pathology in H&E and PAS stained slides and **(C)** renal pathology was blindly evaluated and scored. Bar diagrams indicate mean ± S.D. Each dot represent individual mice and horizontal bar indicate mean for each group. P value < 0.05 (*), 0.01 (**)

4.5.3 Reduced frequency of kidney infiltrating alternatively activated macrophages in the absence of IL-17R signaling

Most documented IL-17R signaling occurs in cells of non-hematopoietic origin. However, some studies suggest a significant role for IL-17 in the hematopoietic compartments, particularly B cells and T cells. Percentages of germinal center (GC) B cells, plasma cells, effector CD4$^+$ and CD8$^+$ T cells, γδ T cells, Tfh cells and Tregs were comparable between B6. lprIL-17RA$^{-/-}$ and B6.lpr mice in the spleen **(Fig 32A and 32B)** and kidney **(Fig 33A and 33B)**, indicating that IL-17R signaling in B and T cells is dispensable for IFN-I induced renal pathology. Macrophage infiltration into the glomeruli during inflammation leads to renal damage. An essential role for alternatively activated macrophages also known as M2-macrophages (M2-MΦ) has been documented in the pathogenesis of IFN-I induced cGN in NZWB/F1 mice [227]. In this system, TGFβ and MMP9 produced from the kidney infiltrating M2-MΦ triggered cGN following poly I: C treatment [227]. At 6 weeks with poly I: C treatment, percentages of these renal infiltrating M2-MΦ (CD11b^{hi}Gr1^{lo}F4/80$^+$CD206$^+$) was significantly lower in B6.lprIL-17RA$^{-/-}$ compared to B6.lpr mice (**Fig 33C).** Consequently, the renal transcript expression of *Tgfb* but not *Mmp9* was significantly lower in the absence of IL-17R signaling **(Fig 33D)**. This observation is in line with

cGN development in murine models of ANCA vasculitis and anti-GBM accelerated glomerulonephritis. In these models, the consequence of IL-17 signaling deficiency was the diminished recruitment of macrophages and neutrophil in the nephritic kidney [227], suggesting common cellular and molecular events in the pathogenesis of crescent formation in these renal inflammatory conditions.

A



B

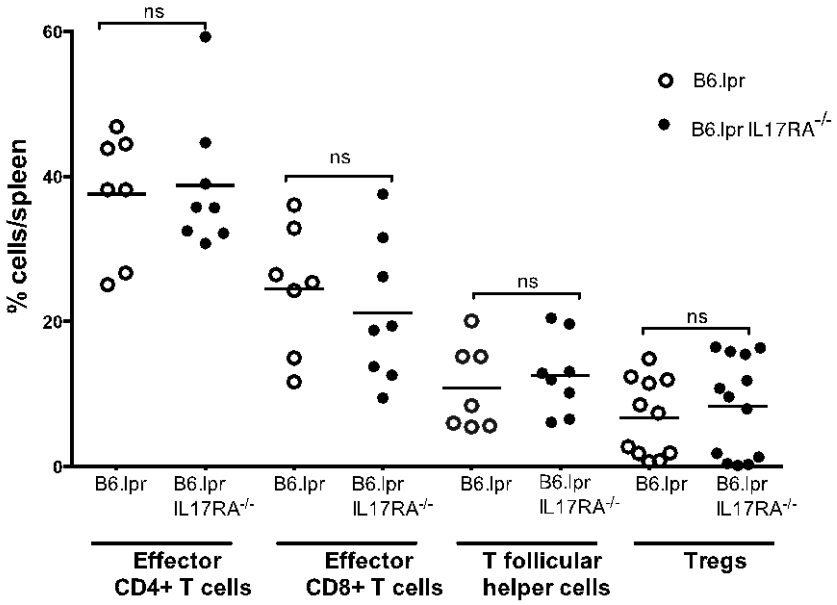
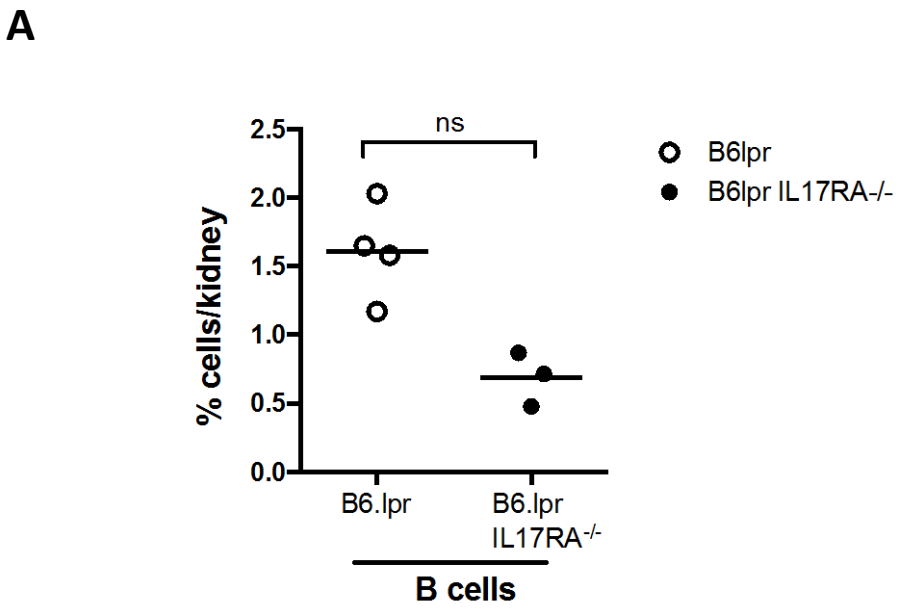
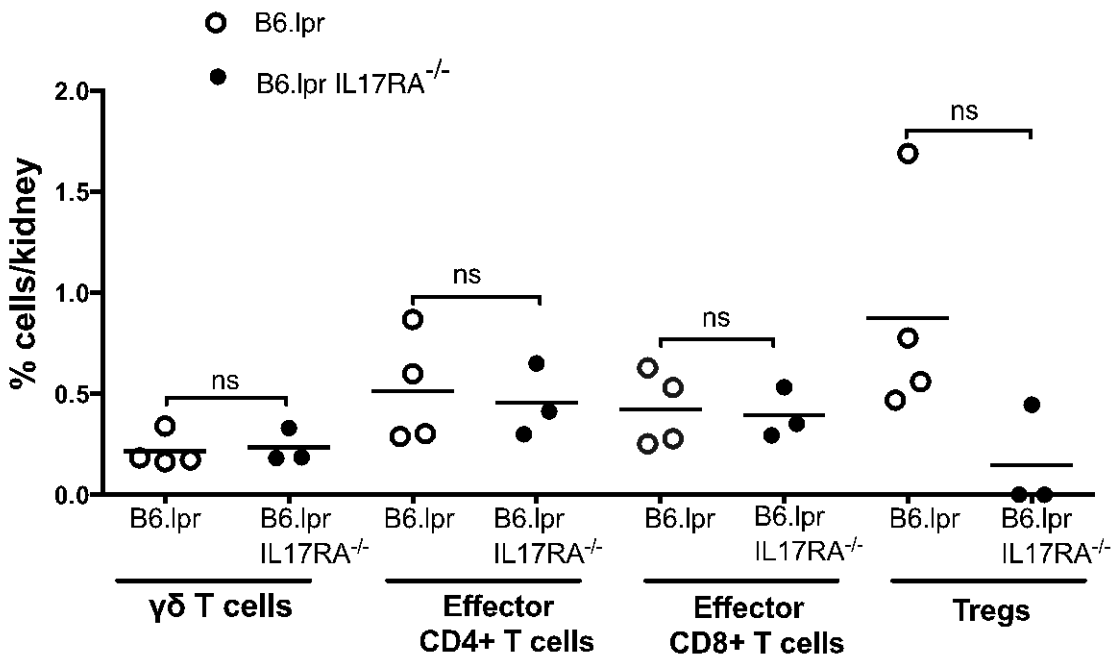


Figure 32. Systemic response is comparable between B6.lpr and B6. lprIL-17RA$^{-/-}$ mice

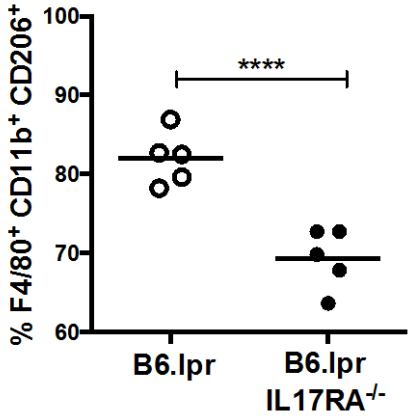
12–15 weeks old B6.lpr and B6. lprIL-17RA$^{-/-}$ mice (n = 10–13) were subjected to poly I: C-induced cGN. At 10 weeks' post-treatment, splenic frequency of **(A)** germinal center B cells (GL-7+ cells, gated on total B220+ cells) and plasma cells (B220lowCD138+ cells, gated on total live cells). **(B)** Effector CD4+ T cells (CD62LlowCD44high cells, gated on total CD4+ T cells), effector CD8+ T cells (CD62LlowCD44high cells, gated on total CD8+ T cells), T follicular helper cells (PD-1+ CXCR5+ cells, gated on total CD4+ B220− T cells) and T reg cells (Foxp3+ cells, gated on total CD4+ T cells) were determined by flow cytometry.



B


B6.lpr
B6.lpr IL17RA-/-
% cells/kidney
2.0
1.5
1.0
0.5
0.0
ns
ns
ns
ns
B6.lpr
B6.lpr IL17RA-/-
γδ T cells
Effector CD4+ T cells
Effector CD8+ T cells
Tregs

C


% F4/80+ CD11b+ CD206+
100
90
80
70
60
****
B6.lpr
B6.lpr IL17RA-/-

D

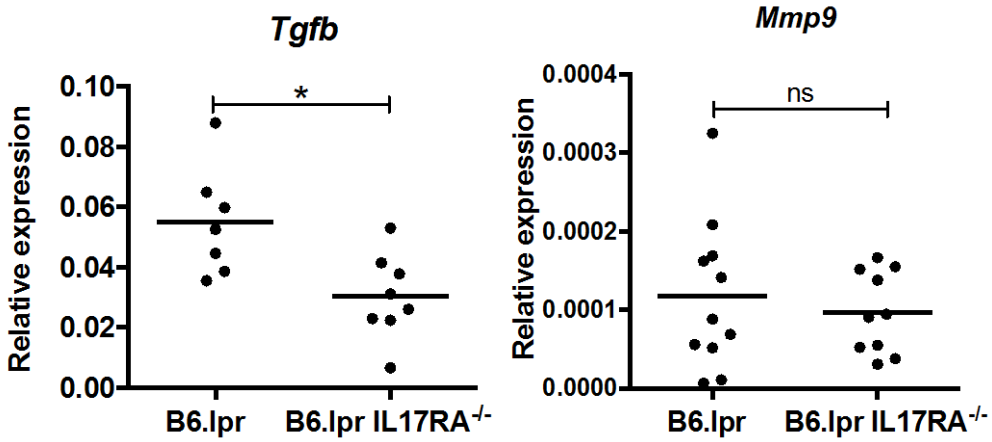

Tgfb
Mmp9
Relative expression
0.10
0.08
0.06
0.04
0.02
0.00
*
0.0004
0.0003
0.0002
0.0001
0.0000
ns
B6.lpr
B6.lpr IL17RA-/-

Figure 33. Renal infiltration of alternatively activated macrophages is diminished in B6. lprIL-17RA$^{-/-}$ mice following IFN-I-induced cGN

At 6 weeks' post-poly I: C treatment, kidney frequency (n = 4) of **(A)** total B cells **(B)** γδ T cells, effector CD4+ T cells, effector CD8+ T cells and T reg cell were determined by flow cytometry. **(C)** At 6 weeks' post-poly I: C treatment, frequency of kidney (n = 5) infiltrating F4/80+ Gr-1− CD11b+ CD206+ macrophages were evaluated by flow cytometry. **(D)** Renal expression of *Tgfb* and *Mmp9* mRNA (n = 7) was assessed by qPCR. $p < 0.05$ (*), and 0.0001 (****). Ns: not statistically significant.

4.5.4 IL-17R signaling in hematopoietic and non-hematopoietic cells is critical for inducing chemotactic signals for renal infiltration of macrophages in cGN

The mechanisms of renal recruitment of M2-MΦ in cGN are unknown. Since IL-17RA is expressed on both hematopoietic and non-hematopoietic cells, we determined if IL-17R signaling, in association with IFN-I, could stimulate the expression of chemokines required for the renal migration of macrophages. Accordingly RTECs and BMDMs were either stimulated with IL-17A, TNFα (a cytokine with which IL-17 exhibits strong signaling cooperativity) or IFNα in different combinations for 24h. RTECs stimulated with IL-17A and TNFα significantly up-regulated the transcript expression of *Ccl2* and *Spp1* (gene encoding osteopontin) compared with stimulation with IL-17 or TNFα or IFNα alone **(Fig 34A)**. IL-17 and IFNα synergistically induced the transcript expression of *Ccl2* and *Spp1* in BMDMs **(Fig 34B)**. Collectively these data indicate that IL-17R signaling in the hematopoietic and non-hematopoietic cells differentially regulate the expression of chemotactic signals in renal inflammation associated with IFN-I induced cGN.

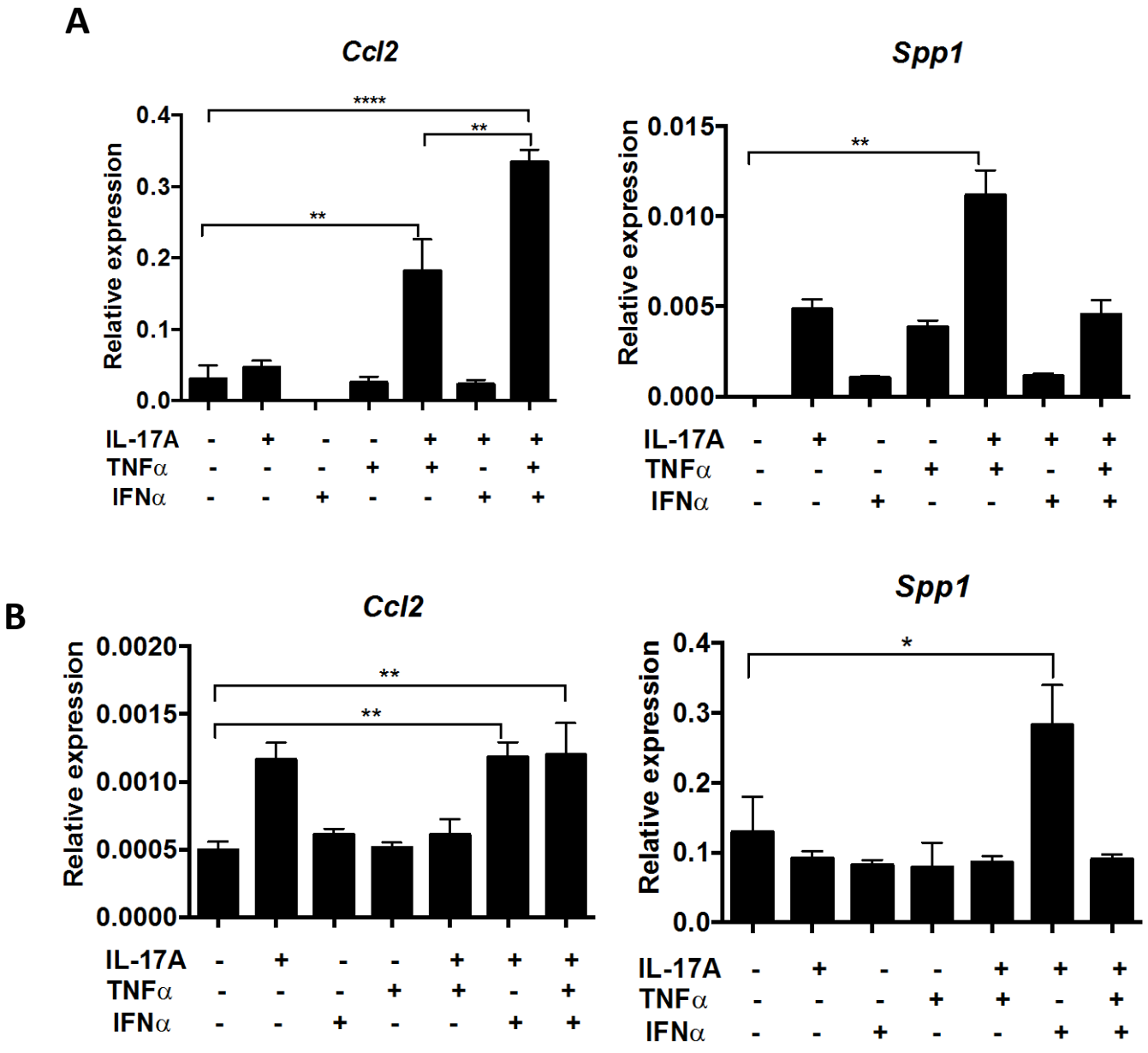


Figure 34. . IL-17R signaling in RTEC and BMDM differentially regulates Ccl2 and Spp1 expression in IFN-I-induced cGN

(A) Primary RTEC and **(B)** BMDM were stimulated with IL-17A (IL-17) (200 ng/ml) or TNFα (5 ng/ml) or IFNα (250 U/ml) and all three cytokines in different combinations or left unstimulated for 24 h and transcript encoding Ccl2 and Spp1 were evaluated by qPCR. Data are representative of three independent experiments with similar results. $p < 0.05$ (*), 0.01 (**), 0.001 (***) and 0.0001 (****).

4.6 DISCUSSION

We show that IL-17R signaling is essential for the pathogenesis of IFN-I-induced accelerated cGN in lupus-prone mice. IL-17R signaling in immune and non-immune cells differentially regulates the expression of chemokines necessary for renal infiltration of pathogenic alternatively activated macrophages during IFN-I induced cGN. The effect of IL-17 and IFN-I signaling on chemokines gene upregulation in macrophages is surprising. It is known that IFN-I inhibits the development of Th17 cells through the induction of IL-27 from dendritic cells and macrophages [252]. However, previously published studies from the lab have demonstrated IFN-I induced IL-27 production is blocked by complement 5a (C5a) via activation of C5a-receptor, thus permitting the co-existence of IFN-I and Th17 cells in SLE patients and lupus-prone mice [77, 206, 253]. The cross talk between IFN-I and IL-17 signaling pathways in renal damage associated with cGN is unclear and awaits future investigation. Further studies could lead to the potential of therapeutic neutralization of IL-17 so as to have better renal outcomes in SLE patients with high IFN-I gene signature and severe cGN.

4.7 MODEL

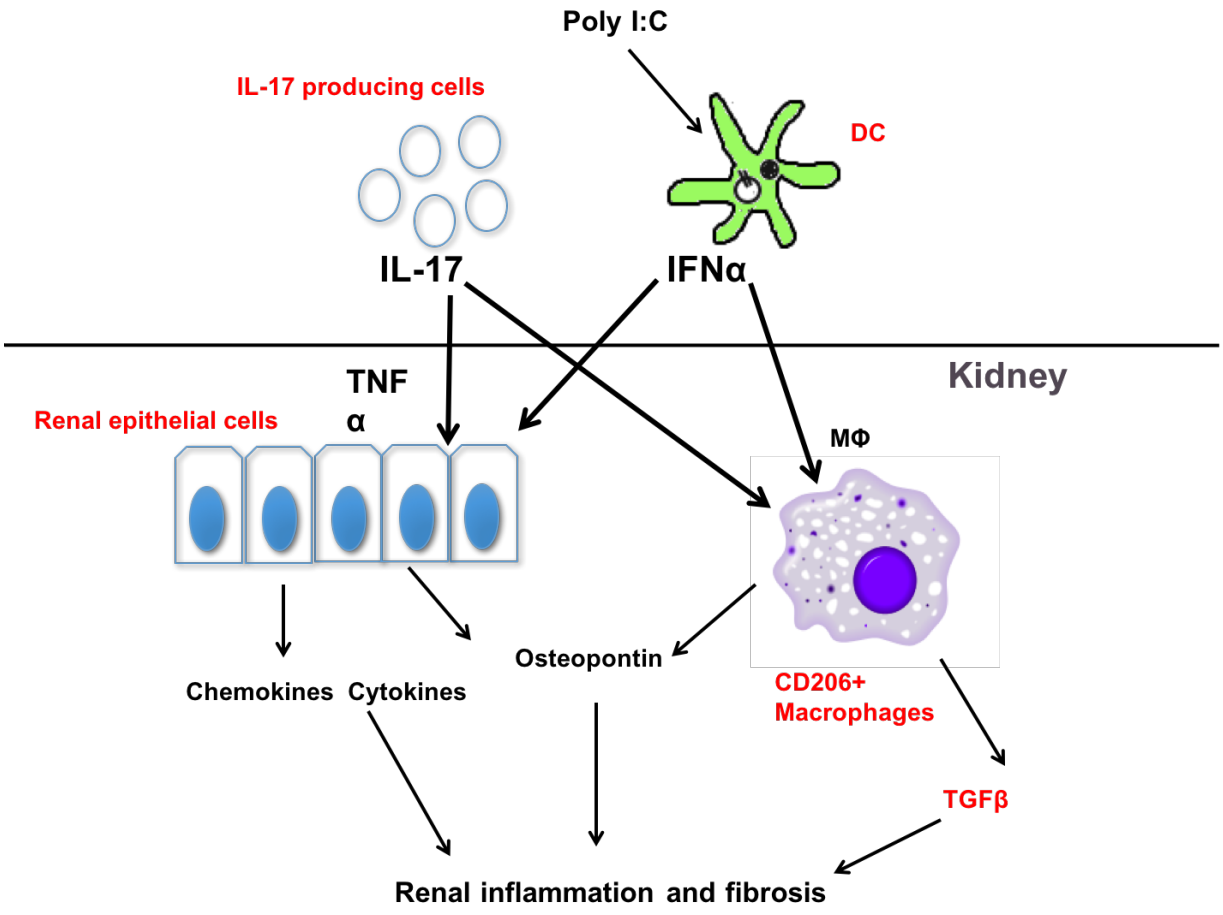


Figure 35. Role of IL-17 in type I interferon mediated renal damage associated with lupus nephritis

CHAPTER 5: DISRUPTION OF INTERLEUKIN-17 RECEPTOR SIGNALING AGGRAVATES RENAL FIBROSIS IN OBSTRUCTIVE NEPHROPATHY

5.0 SUMMARY

Renal fibrosis is the final outcome of obstructive nephropathy and a major cause of chronic renal failure. Recently, IL-17 emerged as a key cytokine in lung, skin and liver fibrosis. Although IL-17 producing cells were detected in the obstructed kidney, the role of IL-17 in renal fibrosis is unknown. We sought to define the contribution of IL-17 in renal fibrosis in obstructive nephropathy. Mice deficient in IL-17 receptor signaling (IL-17RA$^{-/-}$) were assessed for renal fibrosis following unilateral ureteral obstruction (UUO), a well-characterized mouse model of obstructive nephropathy. We determined IL-17 receptor signaling is required for protection against renal fibrosis following UUO. IL-17RA$^{-/-}$ mice developed severe morphological injury and displayed increased deposition of collagen in the obstructed kidney compared to controls. We observed a significant decrease in the level of matrix degrading enzymes in the kidney of IL-17RA$^{-/-}$ mice, despite normal ECM synthesis, correlating with increased fibrosis. Overexpression of IL-17 increased the level of matrix degrading enzymes and protected mice from renal fibrosis. We also show that IL-17R signaling in tubular epithelial cells up regulated multiple genes of Kallikrein-kinin system, known to play a critical role in renal protection against kidney fibrosis. Our data clearly indicate a previously unappreciated role of IL-17 in renal protection against fibrosis. These results offer the opportunity for the development of potential therapies in the treatment of chronic kidney diseases.

5.1 INTRODUCTION

The prevalence of fibrosis related diseases including chronic kidney disease (CKD), liver fibrosis, intestinal fibrosis, and pulmonary fibrosis is significantly rising and amount to a major public health concern. Approximately 45% deaths associated with diseases are attributed to fibrosis. In the developed world, according to recent estimates fibrosis thus is a major cause of morbidity and mortality [254]. Although fibrosis is associated with almost every kind of organ disease, a lack of information on the clear mechanisms underlying the fibrotic process pose a major challenge to the development of targeted therapies against fibrosis. The incidence of CKDs worldwide is an estimated 8–16% and this number is fast increasing [255]. The prevalent etiologies of CKDs and kidney fibrosis are hypertension and diabetes mellitus in adults and obstructive nephropathy in children [254]. Moreover, CKDs are associated with an inflammatory component, displaying a robust correlation with the progression of renal fibrosis and the eventual decline in kidney function [255].

Fibrosis is a complex process that offers a conundrum of whether it is an inflammatory or a non-inflammatory response. Usually in an organ injury, repair mechanisms are preceded by a robust inflammatory response, which is crucial to counter the potential for infection at a site where a barrier is breached. Adult tissue repair can lead to the formation of a fibrotic tissue upon wound healing [256]. From recent studies, it is apparent that the inflammatory response may be, at least in part, responsible for fibrosis at sites of tissue repair. Various fibrotic diseases demonstrate shared features such as chronic inflammation associated with progression of the fibrotic conditions. The local fibrotic response in injured organs includes the chemokine-mediated recruitment of immune cells such as neutrophils and macrophages. The immune infiltrating cells produce pro-inflammatory cytokines that trigger the activation of effector cells.

These cells proceed to remodel the injured tissue that aids the regeneration of the tissue. However, in chronic fibrotic diseases there is a perturbation in the balance between production and degradation of extracellular matrix (ECM) components [257]. The continuously activated effector cells synthesize an excessive amount of ECM resulting in the formation of excessive connective tissue. This unabated pathogenic tissue remodeling contributes to the destruction of organ architecture and function [258]. Since inflammation is a major contributor to the progression of fibrosis, strategies that target inflammatory pathways could offer therapeutic benefits.

5.2 T-CELLS IN FIBROSIS

$CD4^+$ T cells have a crucial role in fibrosis. The opposing effects of Th1- and Th2-cytokine responses in fibrosis have been studied [256]. Studies using different cytokine-deficient mice showed that fibrosis is linked to the development of Th2 response involving IL-4, IL-5 and IL-13 whereas Th1 cells, which produce IFNγ completely attenuate the development of tissue fibrosis. Specifically, the fibrotic role of the Th2 response can be ascribed to its role in the regulation of ECM remodeling. Th2 responses activate collagen deposition, whereas Th1 responses inhibit this process, indicating opposing roles for Th1 and Th2 responses in tissue repair. In fact, the balance between the pro and anti-fibrotic nature of the Th1/Th2 paradigm determines the outcome of end-stage fibrotic disease [259].

There is evidence for the role of IL-17 cytokine family in the crucial events of renal fibrosis [26]. IL-17 is the most studied in the context of fibrosis but whether it has a pro-fibrotic or an anti-fibrotic role is under debate. IL-17 has been implicated in a pro-fibrotic role in various

organs including the intestine [260], lung [261] and liver [262, 263]. The pro-fibrotic effect of IL-17 was noted in cases of skin inflammation and fibrosis [264]. In a bleomycin model of skin fibrosis, IL-17 knockout mice displayed decreased fibrosis [265]. Similarly in a murine model of scleroderma, an IL-17 deficiency diminished skin thickening associated with the disease [265]. An increased level of IL-17 were found in human intestine during fibrosis associated with Crohn's disease [260]. There is an IL-17 dependent induction of collagen and the expression of matrix metalloproteinase-3 (MMP-3), MMP-12, and tissue inhibitor of MMPs (TIMP)-1 in the colon, which have significant effects on the ECM remodeling and tissue architecture [258]. IL-21 has also been known to promote tissue fibrosis by meditating the differentiation of naive CD4+ T cells to Th17 cells [266]. Moreover, IL- 17 can induce nitric oxide synthase and play a significant role in the prevention of tissue damage, which is a common element of chronic fibrotic diseases [261].

Recently, a connection between renal fibrosis and IL-17 has been suggested. It was demonstrated that upon the onset of unilateral ureteral obstruction (UUO), a mouse model of kidney fibrosis, severe inflammation and renal fibrosis developed in the obstructed kidney of IL-17 deficient mice compared to wild type controls. Following UUO, the IL-17 deficient mice displayed diminished infiltration of inflammatory cells and down regulation of pro-inflammatory chemokine RANTES in the obstructed kidney. In accordance with the observation that IL-17 deficiency reduced fibrosis, neutralization of IL-17A in wild type mice yielded the same results confirming the pro-fibrotic role for IL-17 in CKDs [267].

Diabetes mellitus is another major cause for renal failure, with estimates of >45% of new cases requiring dialysis [257]. Diabetic nephropathy is generally characterized by chronic inflammation and fibrosis. Although IL-17A has mostly been implicated as pro-fibrotic, there is

data from groups including ours to suggest that IL-17A can be anti-fibrotic. Diminished IL-17A levels were observed in urine and plasma samples of patients with advanced diabetic nephropathy. Administration of a low dose therapy of IL-17A prevented and reversed established nephropathy in genetic models of diabetes. Additionally, renal tubular epithelial cellular overexpression of IL-17A suppressed the diabetic nephropathy. Moreover, this protective effect was noted with the administration of low doses of other IL-17 cytokine family members such as IL-17F but not with IL-17C or IL-17E [268].

5.3 KALLIKREIN-KININ SYSTEM IN RENAL FIBROSIS

It is known that the renin-angiotensin system (RAS) is a major regulator of blood pressure and homeostasis and that the kallikrein kinin system (KKS) counteracts RAS. However, with the presence of active peptides and receptors of the RAS and KKS in various tissues, it has been identified that these systems could have a pleiotropic role in mediating biological functions. Advances in the field have found a link between IL-17 and angiotensin-II (Ang-II), one of the main effectors of the RAS. $CD4^+$ T cells can up-regulate Ang-II levels and augment the production of IL-17. In a rat model for acute kidney injury that leads to fibrosis, injured rats were given an Ang II antagonist significantly attenuated renal fibrosis [147]. ACE inhibitors reduce the progression of renal fibrosis in both mice and human models. Accelerated renal fibrosis was attenuated by ACE inhibition in a UUO-induced fibrosis model [269]. One study demonstrated that ACE inhibitors via bradykinin can activate matrix degrading enzymes and reduce ECM deposition thereby attenuating fibrosis [270]. Similarly, the role for various components of the KKS in protection against fibrosis has been shown. Mice lacking $Bdkrb2^{-/-}$, $Bdkrb2^{-/-}$ $Bdkrb1^{-/-}$

exhibit increased renal pathology and mortality in acute or chronic kidney disease models including UUO [269]. In humans, polymorphisms in KKS-related genes *ACE, BDKRB2, KLK1* are associated with an increased risk of developing renal disorders [271]. These observations indicate an essential role of KKS, particularly bradykinin, in renal protection against acute or chronic injury.

Based on the evidence for IL-17 in renal fibrosis as well as the KKS in renal fibrosis independently, we asked if there was any connection between these different components. From our studies in the disseminated candidiasis model, we identified the important tissue protective role for Klk1 in preserving renal function. Since Klk1 is a direct target of IL-17 signaling, we hypothesized a renal-protective role for the IL-17-Klk axis is kidney fibrosis.

Table 2. Kallikreins in renal damage and fibrosis

Renal disease	**Intervention**	**Phenotype**
Unilateral ureteral obstruction	Bdkrb2-/- mice	Increased interstitial fibrosis
Diabetic nephropathy	Bdkrb2-/- Akita diabetic mice	Increased fibrosis and mortality
Ischemia-reperfusion injury	Bdkrb2-/- mice Bdkrb1-/-Bdkrb2-/- mice	Increased renal damage and mortality
Autosomal dominant polycystic kidney diseases	Administration of Ad-Klk1	Reduced albuminaria
Heymann nephritis and Alport syndrome	Treatment with ACE inhibitor	Reduced proteinuria and fibrosis
Aminoglycoside-induced renal injury	Administration of Ad-Klk1	Protection of kidney function

5.4 MATERIALS AND METHODS

Mice

C57BL/6 wildtype (WT) mice were obtained from Jackson Laboratory (Bar Harbor, ME) IL-17 receptor A-deficient (IL-17RA$^{-/-}$) mice on the C57BL/6 background were generously provided by Amgen (San Francisco, CA) and bred in-house. 8- to 12-week-old male mice were used for experiments. All studies were carried out under approved protocols of the University of Pittsburgh Institutional Animal Care and Use Committee adhering to the guidelines in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health.

Unilateral ureteral obstruction model of kidney fibrosis

Unilateral ureteral obstruction (UUO) was executed as described. Briefly, under anesthesia, the left ureter was isolated and ligated 2-4 mm below its origin. 7 days' post-surgery, mice were sacrificed, and the obstructed and contralateral unobstructed kidneys were harvested for analysis. Bradykinin treated mice received a dose of 300nmol/kg/day 24 hours prior to UUO and continuing until organ harvest on day 7 of UUO.

Morphological and histological analysis for UUO

Portions of kidney were fixed in formalin, dehydrated and paraffin-embedded. Sections were stained with hematoxylin and eosin (H&E) and periodic-acid Schiff (PAS) for morphological analysis and determination of the extent of renal injury. To visualize kidney fibrosis via detection of total collagen, serial kidney sections were stained with Picrosirius red and Masson trichrome (Sigma Aldrich, St. Louis, MO).

Immunofluorescence was performed using the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA). To evaluate collagen deposition, kidney sections were incubated with an antibody to against α-smooth muscle actin (Abcam, Cambridge MA). Images were examined with EVOS FL Auto microscope (Life Technologies, CA)

Renal function assays

Serum was collected by retro-orbital bleeding at day 7 post UUO induction. Blood urea nitrogen was measured using a commercial kit (Bioo Scientific Corp., Austin TX).

Isolation of RNA and real-time PCR

Total RNA was isolated from kidney tissue by using the RNeasy Micro Kit according to the manufacturer's instructions (Qiagen, Valencia CA). cDNA was synthesized using SuperScript III First-Strand (Invitrogen, Carlsbad CA). Gene expression was measured by qPCR with PerfeCTa SYBR Green FastMix ROX (Quanta BioSciences, Gaithersburgh MD) on 7300 Real-Time PCR System (Applied Biosystems, Carlsbad CA). Gene expression levels were then determined for *Cxcl1, Cxcl2, Ccl20, Il6, Collagen1, Collagen3, tPA, Mmp9* using commercially available Quantitect primers (Qiagen, Valencia CA) by real-time PCR.The expression of each gene was normalized to that of *Gapdh.*

Adenoviruses

Adenoviruses expressing IL-17A (Ad-IL-17) and control (Ad-ctrl) were kindly provided by Dr. J. Kolls (U. Pittsburgh). Ad-Klk1 and corresponding Ad-ctrl were from Applied Biological Materials Inc. (Richmond, British Columbia, Canada). Mice were injected via the tail vein with $1x10^9$ pfu 72 hours prior to UUO surgery.

Bradykinin receptor agonists

Mice were injected i.p. with 200 μl Bradykinin (300 nmol/kg/day) (R&D Systems, Minneapolis MN). Control mice received equal volumes of PBS. For experiments with selective agonists, mice received i. p. injection of Bdkrb1 (1mg/kg/day) or Bdkrb2 (750nmol/kg/day) selective agonists as indicated (R&D Systems, Minneapolis MN). Control mice received equal volume of PBS.

Western blots

Kidney tissues (10mg) were homogenized in RIPA buffer (Thermo Scientific, Pittsburgh PA). Concentration of protein was quantitated by the BCA quantitation assay (Thermo Scientific, Pittsburgh PA). Equal amounts of sample were subjected to electrophoresis and transferred to PVDF membranes (Millipore, Billerica MA). After blocking with 5% milk in TBS, the blots were incubated with anti-mouse Klk1 (LifeSpan Biosciences, Seattle WA) or anti-mouse beta-actin (Abcam, Cambridge MA) overnight in 4°C. The blots were then washed and incubated for 1 hour at room temperature with individual secondary antibodies accordingly. Bands were detected using an enhanced chemiluminescence detection system (Thermo Scientific, Pittsburgh PA) and developed with a FluorChem E imager (ProteinSimple, San Jose CA). Band corresponding to proteins of interest were analyzed by ImageJ software.

Hydroxyproline assay

Hydroxyproline is a component largely seen only in collagen. Measurement of hydroxyproline levels can therefore be used as an indicator of collagen content.). 10mg of kidney tissue was homogenized with 12M HCL at 120°C overnight. Hydroxyproline levels were quantified using a commercially available hydroxyproline assay kit (Chondrex, Redmond WA) following manufacturer's instructions. The samples were finally read at 560 nm. Values were represented as μg Collagen/ mg kidney tissue.

Statistical analyses

Results are expressed as mean ± SD. Differences between groups were calculated for statistical significance using 2-tailed paired Student's *t* tests and ANOVA as appropriate. A *p* value less than 0.05 was considered significant.

5.5 RESULTS

5.5.1 UUO-induced fibrosis promotes expression of IL-17 –responsive inflammatory genes

We used the well-established unilateral ureteral obstruction (UUO) model of renal fibrosis in wild type mice. 7 days post-surgery, we assessed the expression of IL-17 in the kidney. Quantitative analysis of transcripts in obstructed kidney of WT mice confirmed the increase in *Il17a* **(Fig 36A)** but a decrease in *Il17f* **(Fig 36B)** compared to the unobstructed kidney. mRNa levels of lL-17 responsive genes like *Cxcl1, Cxcl2, Ccl20* and *Il6* were elevated in the obstructed kidneys as well **(Fig 36C).**

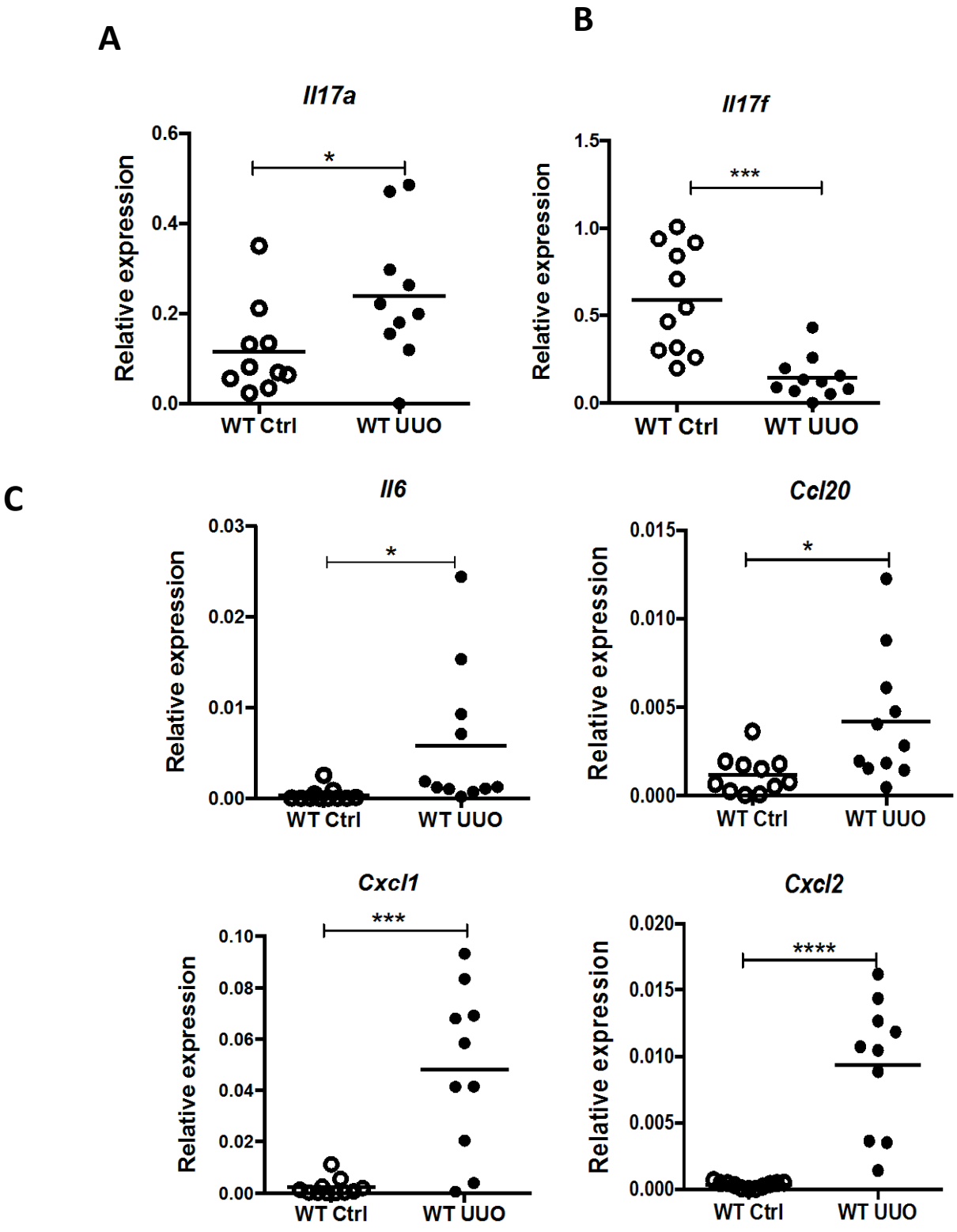


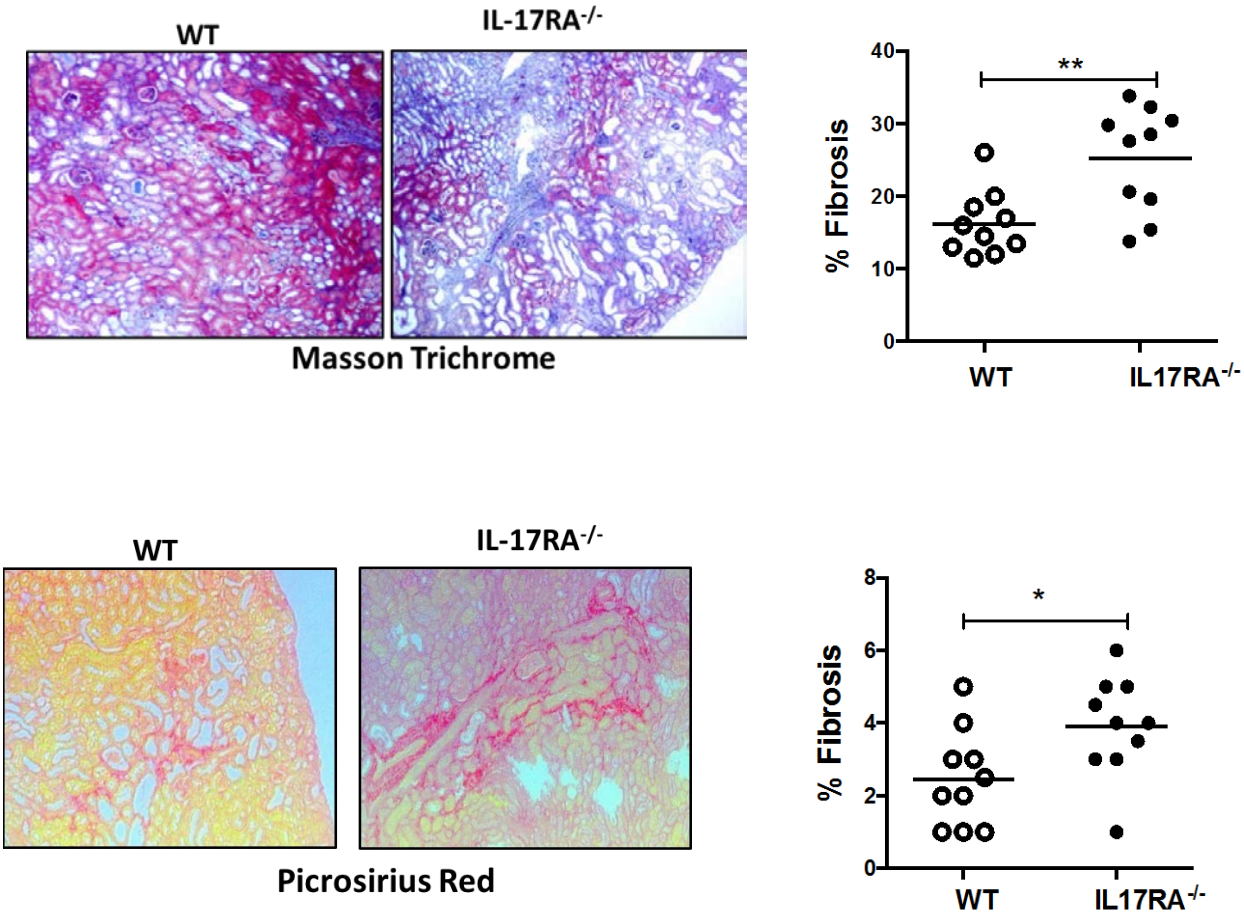
Figure 36. IL-17 and IL-17 target gene expression is increased in obstructed kidney of WT mice

C57BL/6 mice were subjected to UUO. Mice were sacrificed at Day 7 [n=10 per group] and kidney cortex isolated **(A)** IL-17A mRNA expression **(B)** IL-17F mRNA expression **(C)** IL-17-responsive genes *(Il6*, *Ccl20*, *Cxcl1*, and *Cxcl2*) were Each dot represents individual mice. Pooled data from two individual experiments. p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***).

5.5.2 IL-17RA deficiency exacerbates UUO-induced fibrosis

Since a pathogenic role was shown for IL-17 in fibrosis of other organs such as lung, liver and skin, we hypothesized a similar role for IL-17 in kidney fibrosis. To assess the contribution of signaling through IL-17RA in renal fibrosis, we chose to use the mice lacking IL-17RA (IL-17RA$^{-/-}$). To our surprise, we found that in the IL-17RA$^{-/-}$ mice, upon UUO induction, fibrosis was exacerbated. We analyzed collagen deposition at 7 days post-surgery after UUO by Masson Trichrome and Sirius red staining and found that kidneys from IL-17RA$^{-/-}$ mice were damaged from the development of overt fibrosis **(Fig 37A).** There was an increase in collagen deposition that indicates more fibrosis in the obstructed kidneys from IL-17RA$^{-/-}$ mice compared to obstructed kidneys from WT **(Fig 37B).** We confirmed the extent of fibrosis by quantifying hydroxyproline, an amino acid found predominantly in collagen. The levels of hydroxyproline were increased in the kidneys of the IL-17RA$^{-/-}$ mice in comparison to the wild type cohort **(Fig 37C)**.

A


WT
IL-17RA-/-
Masson Trichrome
% Fibrosis
40
30
20
10
0
**
WT
IL17RA-/-
WT
IL-17RA-/-
Picrosirius Red
% Fibrosis
8
6
4
2
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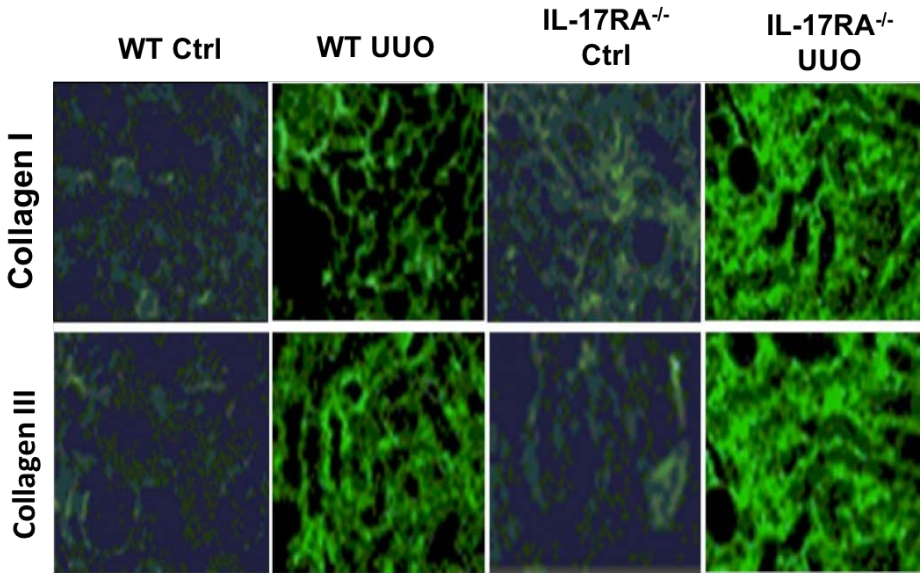
*
WT
IL17RA-/-

B



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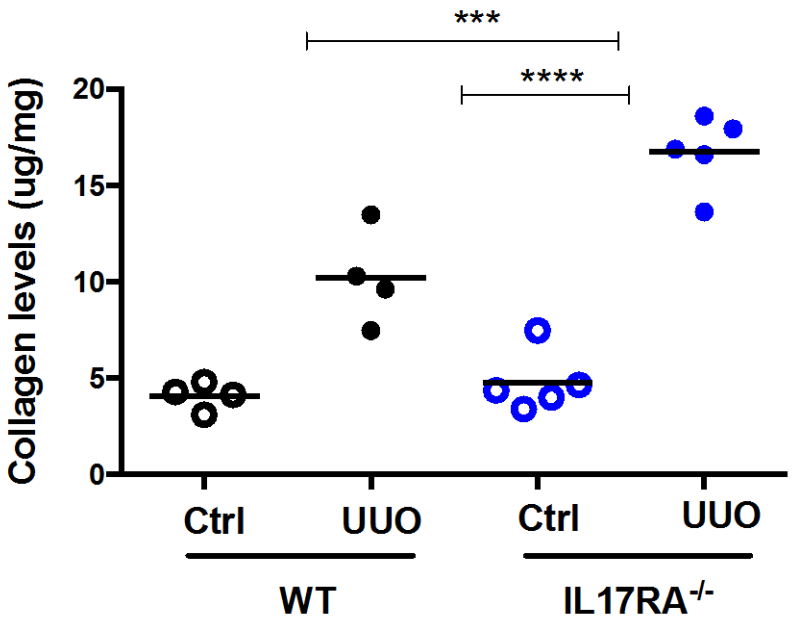


Figure 37. Enhanced fibrosis and collagen deposition in obstructed kidneys of IL-17RA$^{-/-}$ mice

(A) Masson trichrome staining and Picrosirius red staining shows collagen deposition as a measure for fibrosis. (n=10 for each group). Collagen stains blue in Masson trichrome staining and stains orange in the Picrosirius red staining protocols. The collagen deposition was quantified and represented as percentage fibrosis. The data are pooled from two independent experiments. p value ≤0.05 (*), ≤0.01

(**), ≤0.001 (***). **(B)** Collagen deposition in kidney mice was determined by immunofluorescence staining. (n=4-5 for each group). **(C)** Collagen levels in the control vs obstructed kidney was quantified using a hydroxyproline assay. Each dot represents individual mouse (n=4-5 for each group). The data are pooled from two independent experiments. p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***).

5.5.3 Decrease in level of matrix degrading enzymes in the absence of IL-17RA despite normal ECM synthesis

In renal health and disease, the balance between the synthesis and degradation of extracellular matrix (ECM) governs the local accumulation of matrix. Tissue plasminogen activator (tPA) is a serine protease that has matrix-degrading properties and can cleave plasminogen to plasmin. Plasmin has a role in the degradation of ECM proteins, and can also activate members of the matrix metalloproteinase (MMP) family. Studies have shown altered levels of these enzymes in the kidney and they can facilitate accumulation of ECM in conditions associated with renal fibrosis. We induced UUO in WT and IL-17RA$^{-/-}$ mice and measured the transcript levels of ECM components. The transcript levels of collagen1 (*Col1a*) and collagen3 (*Col3a*) in the obstructed kidneys of both WT and IL-17RA$^{-/-}$ mice showed no difference **(Fig 38A)**. In contrast, the transcript levels of *tPA* and *Mmp2* were reduced in the obstructed kidneys of IL-17RA$^{-/-}$ mice compared to WT mice **(Fig 38B)**.

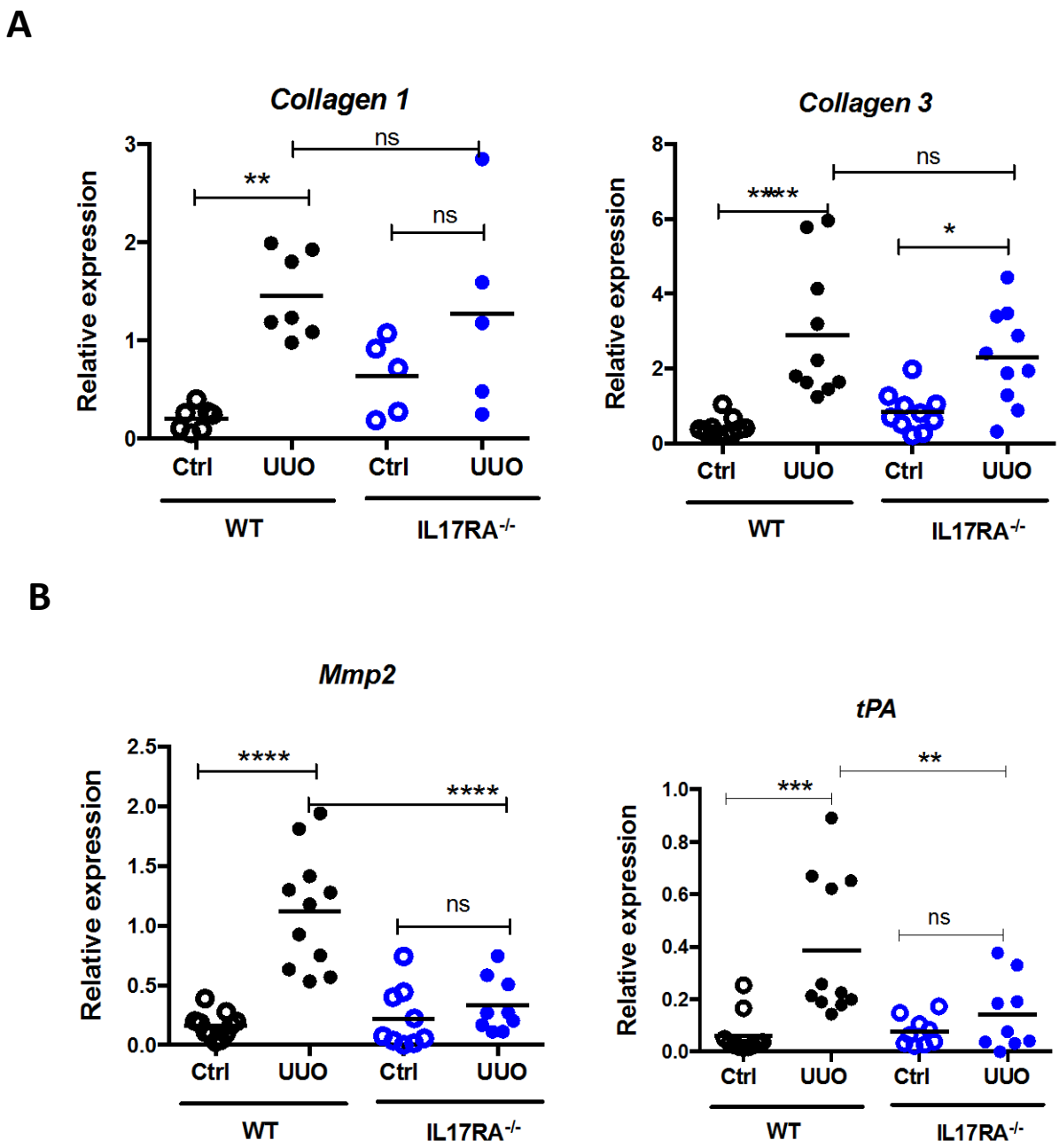
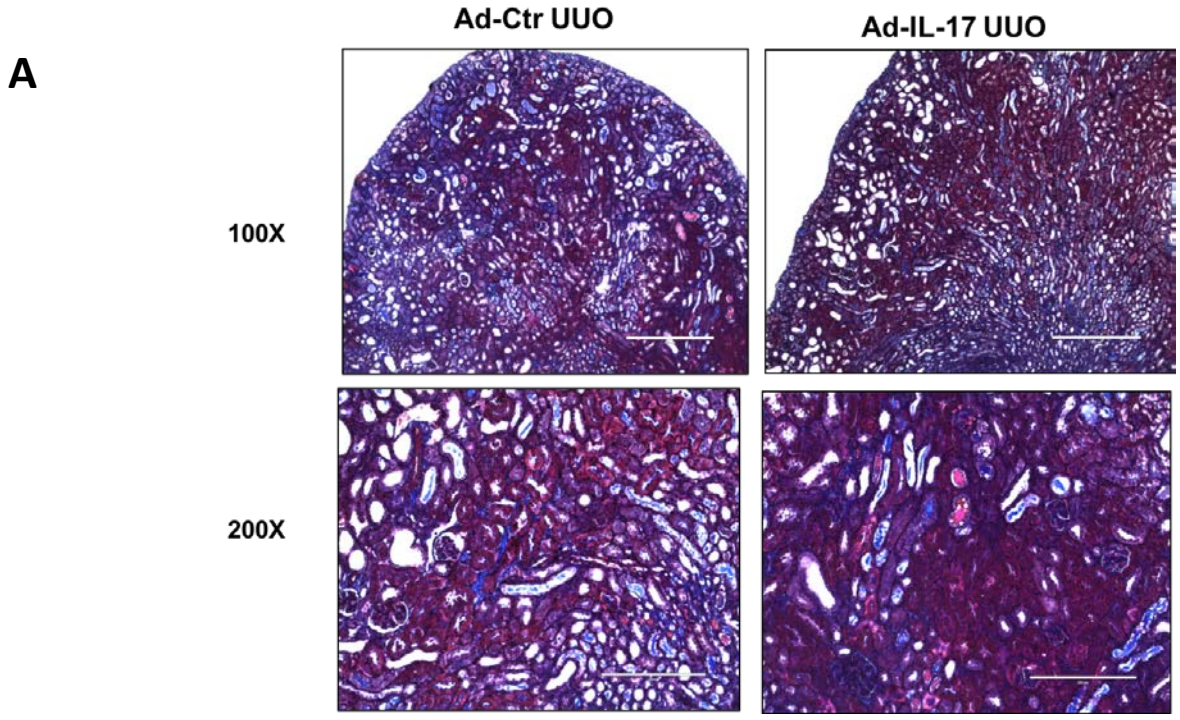


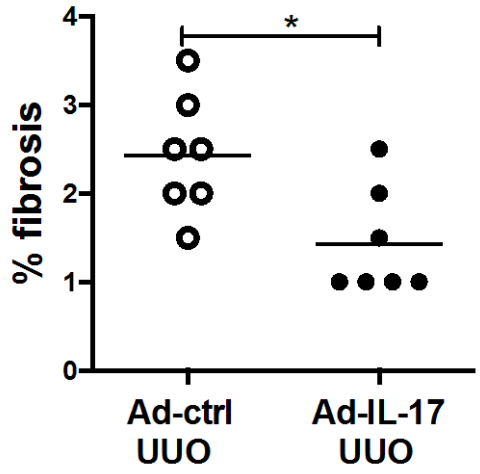
Figure 38. Reduced expression of matrix degrading enzymes in the absence of IL-17RA signaling

(A) UUO was performed on WT and IL-17RA$^{-/-}$ or a control vector. Collagen synthesis was unaffected in both groups. (n=4-5 for each group). **(B)** *Mmp2* and *tPA* are known to degrade matrix proteins and these genes were down-regulated in the absence of IL-17 receptor signaling. (n=10 for each group). The data are pooled from two independent experiments. p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***).

5.5.4 Overexpression of IL-17 protected mice from renal fibrosis

To establish the role for IL-17 in renal fibrosis, we overexpressed an adenovirus expressing IL-17 or a control adenovirus in WT mice and then induced UUO. Day 7 post-surgery, we assessed various parameters to determine extent of fibrosis. The overexpression of IL-17 protected the mice from fibrosis as determined by collagen levels estimated by Masson Trichrome staining **(Fig 39A).** Upon quantification of collagen deposition, it revealed the percentage of collagen in the kidney was lower in the mice with overexpression of IL-17 **(Fig 39A)**. Hydroxyproline estimation confirmed the seemingly protective role for IL-17 in ameliorating renal fibrosis **(Fig 39B).**





B

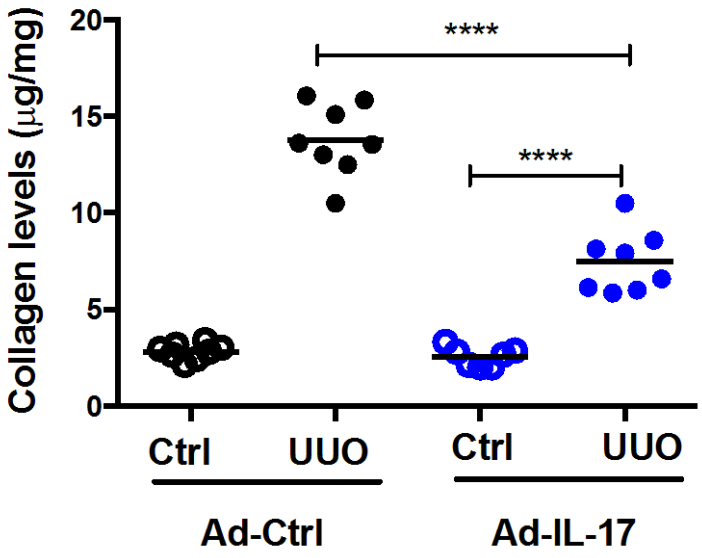


Figure 39. Over expression of IL-17 protected mice from renal fibrosis

WT mice were treated with adenovirus expressing IL-17A or a control vector and UUO was performed. Masson trichrome staining for collagen deposition in the obstructed kidneys of the mice given Ad-IL-17A. (n=8 for each group). **(A)** Percentage fibrosis in Ad-ctrl vs Ad-IL-17A obstructed kidneys. **(B)** Collagen levels were quantified using a hydroxyproline assay in control vs obstructed kidneys in Ad-ctrl

vs Ad-IL17A treated WT mice. (n=8 for each group). The data are pooled from two independent experiments. p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***).

5.5.5 IL-17 induces the expression of kallikrein in renal tubular epithelial cells

To identify the downstream mediators involved in the IL-17 mediated protection in renal fibrosis, we looked whether family of serine proteases called kallikreins had any role. We have previously noted a tissue protective role for kallikreins in the kidney in disseminated candidiasis. The protein levels of Klk1 were elevated in the obstructed kidney in WT mice compared to the unobstructed kidneys. It was also noted that the levels of Klk1 were diminished in the obstructed kidneys of IL-17RA$^{-/-}$ mice in contrast to the WT mice **(Fig 40).**

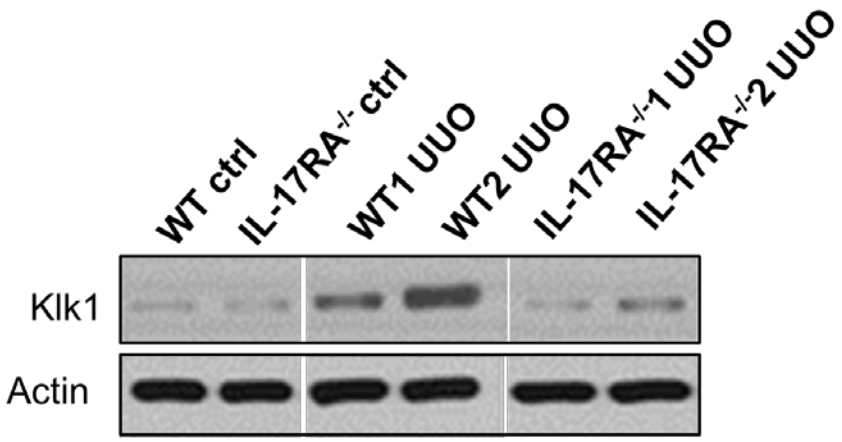


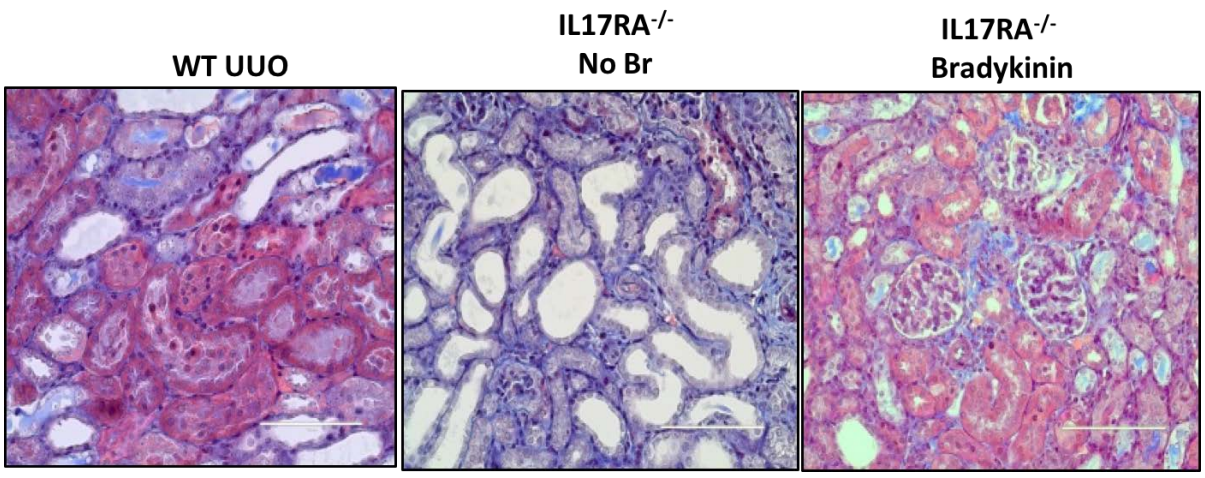
Figure 40. Diminished expression of Klk1 in the obstructed kidney in the absence of IL-17RA signaling

Klk1 protein expression in WTs IL17RA$^{-/-}$ mice from obstructed or control kidneys.

5.5.6 Fibrosis is ameliorated in IL-17RA$^{-/-}$ mice treated with bradykinin

Kallikreins are proteases, which cleave inactive kininogens into active kinin molecule-bradykinin. Bradykinin acts through its receptors Bdkrb1 and Bdkrb2 to mediate its downstream effects. We hypothesized that administering bradykinin would confer protection against development of renal fibrosis in mice subjected to UUO. This was indeed the case when IL-17RA$^{-/-}$ mice were subjected to UUO and treated with bradykinin for the duration of 7 days post-surgery or left untreated. Mice treated with bradykinin exhibited lower accumulation of collagen deposits **(Fig 41A)**. Hydroxyproline levels were also reduced in the treated group as compared to untreated group **(Fig 41B).**

A



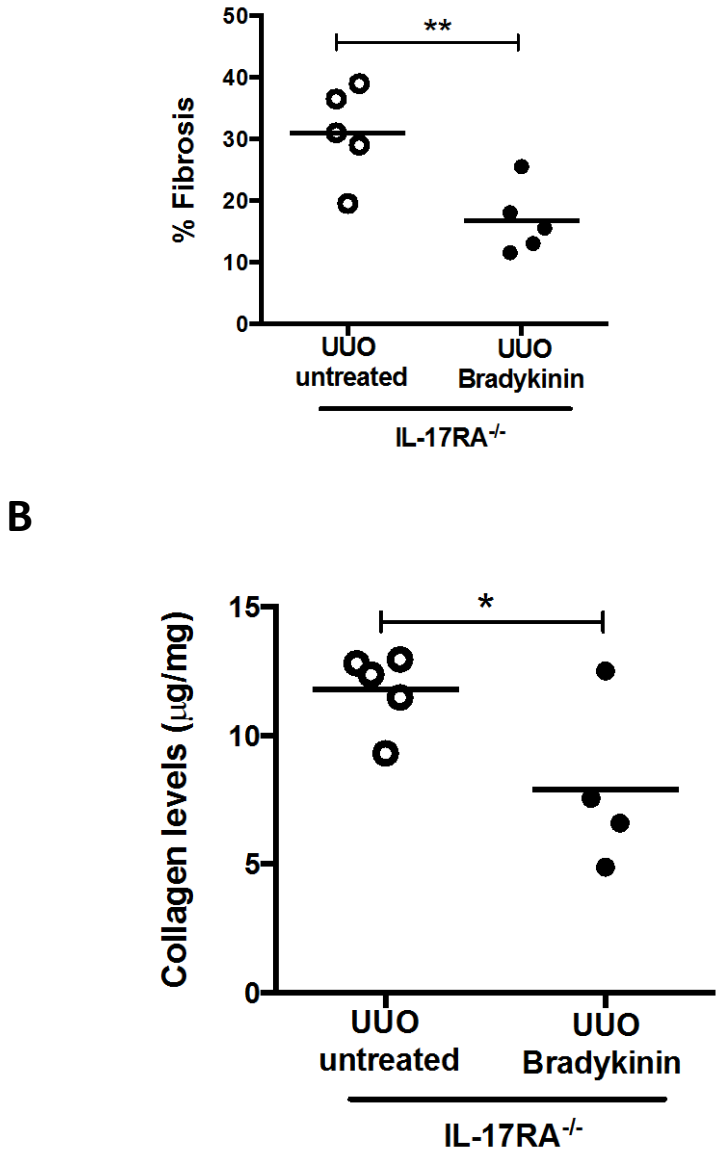


Figure 41. Diminished fibrosis in obstructed kidneys of IL-17RA$^{-/-}$ mice treated with bradykinin

(A) Reduced collagen deposition in the obstructed kidneys of mice treated with bradykinin. Quantification of % fibrosis from Masson trichrome staining (n=4-5 for each group). Collagen is stained blue in Masson trichrome staining **(B)** Collagen levels as measure by hydroxyproline amounts in obstructed kidneys in IL17RA$^{-/-}$ mice treated with bradykinin vs mice left untreated. (n=4-5 for each group). The data are pooled from two independent experiments. p value ≤0.05 (*), ≤0.01 (**), ≤0.001 (***).

5.6 DISCUSSION

CKD is seen in conditions such as urinary obstruction, unresolved inflammation or autoimmunity [272], eventually progressing to end-stage renal disease that could result in requirement of dialysis or renal transplants. No specific therapy is currently available to improve organ function, and the cellular mechanisms that drive fibrosis are still under debate [273]. Pro-inflammatory cells and cytokines have been identified as key effectors in pathogenesis of CKD [258], but the mechanisms of cytokine-driven role in renal fibrosis remain poorly deciphered. Identifying the link is crucial to the devise new therapeutic strategies that could deter the progression of CKD. We used a murine unilateral ureteral obstruction model to induce fibrosis to provide evidence for the contribution of IL-17 and its downstream effectors in the protection against renal fibrosis.

With respect to the renal fibrosis, we show that (i) expression of IL-17 is induced in the obstructed kidney (ii) in the absence of IL-17 receptor signaling there is an enhanced fibrotic response leading to the progression of the disease. Fibrosis is promoted due to an impaired extracellular matrix degradative mechanism and an imbalance in the accumulation of ECM components. In the absence of IL-17R signaling, the matrix synthesis was unaffected but matrix degradative mechanisms were impaired. While mRNA expression of ECM matrix proteins like collagen I, and III were unaffected, expression of degradative enzymes such as MMP-2 and tPA was hampered. We also report an amelioration of renal fibrosis when we overexpress IL-17 using an adenovirus vector. Therefore, our results highlight the importance of IL-17 in protection against renal fibrosis. Interestingly, these results are in contrast with the previously identified pathogenic role for IL-17 in fibrosis in other organs like the lung, skin or liver[261, 262, 265].

We have previously reported the importance of the IL-17-KKS axis in renal immunity against disseminated candidiasis **(Chapter 2).** The KKS promotes vasodilation, an effect mediated by nitric oxide induction that counteracts the renin-angiotensin system. The KKS also functions to regulate inflammation and tissue homeostasis during acute or chronic kidney injury. The renoprotective effect of KKS is primarily mediated by upregulation of a number of tissue repair proteins, inhibiting profibrotic factors, and preventing apoptotic and necrotic changes [146, 274]. Mice lacking components of the KKS or subjected to pharmacological inhibition of this system exhibit exacerbated pathology and mortality in various acute or chronic kidney disease models such as unilateral ureteral obstruction as well as ischemia reperfusion. Klk1is the prototypical member of the kallikreins and we proceeded to study it in the context of fibrosis. We found that Klk1 expression was down regulated in the damaged obstructed kidneys compared to the normal unobstructed kidney in the absence of IL-17R signaling. This points to a potential protective role for the IL-17 signaling cascade in fibrosis.

Klk1 cleaves kininogens to form active bradykinin. Bradykinin acts via its receptors Bdkrb1 and Bdkrb2 and mediates its downstream functions. In a studies in both rodents and humans, bradykinin was found to be a potent stimuli of tPA release in vasculature [275].In vivo bradykinin treatment decreased collage deposition in the obstructed kidneys of mice deficient in IL-17RA. This provides an avenue for further research into the specific mechanism by which bradykinin mediates its protective function in fibrosis. Another interesting question to address is the specific contributions of the individual bradykinin receptors.

As such, treating with IL-17 could be counterproductive since it can lead to unwarranted systemic inflammation. In contrast, focusing on understanding the role for downstream targets of IL-17 could provide a better option in ameliorating at the end stage in organ fibrosis. In

summary, manipulation of the IL-17-Klk1 axis can be regarded as a safe strategy for ameliorating fibrosis, especially for fibrotic processes in which inflammation plays an important part in the progression of the disease.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

The prevalence of CKD is increasing globally and is associated with high mortality and morbidity. The annual report released by the U.S. Renal Data System reported kidney diseases are a major cause for concern in the USA and nearly 10% of cases progress to end stage renal damage (ESRD) [276]. In addition, kidney inflammation leads to progressive kidney damage that eventually results in loss of glomeruli, tubular damage and fibrosis. Therefore, it is imperative to gain insight into molecular pathways contributing to persistent renal inflammation. This can aid in deciphering the pathogenesis of various renal diseases and to develop better and novel targeted therapeutic strategies to prevent ESRD.

The kidney is the archetypal organ of homeostasis and it is noteworthy that its role now extends to the immune system. Several discoveries have brought the field of renal immunology into focus. Significant progress has been made to understand why the kidneys are frequent targets of systemic autoimmunity or infection. This includes learning about anatomical and physiological features that render kidneys susceptible to distinct forms of immune-mediated injury such as high osmolarity in the renal compartments, or activation of protein catabolism by tubular epithelial cells which exposes them to effector T cell functions [272].

The past decade has erupted with data pertaining to the role for IL-17 in various settings. On one hand, it is beneficial to the host in defense against extracellular bacteria and fungi,

mounting a potent immune response leading to the clearance of the infection. On the other hand, it plays a pathogenic role driving adverse immune responses associated with autoimmune diseases such as psoriasis, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus amongst many others. IL-17 is a versatile cytokine that has both direct and indirect effects in carrying out its immune related functions. We have tackled the diverse roles of IL-17 in renal immunity and immunopathology using different mouse models for diseases that range across the entire spectrum from infection to autoimmunity and ultimately fibrosis.

In our first study, we sought to delineate mechanisms of IL-17 mediated renal immunity against a fungal pathogen, *C. albicans* in a bloodstream disseminated candidiasis model. We discovered that the gene expression pattern in response to IL-17 was distinct from the pattern observed in other forms of the disease, mainly the mucosal forms such as OPC or CMC. This begged the question whether kidney specific IL-17 driven immune responses in the protection against disseminated candidiasis were different from infections at mucosal surfaces. We proceeded to identify a distinct set of genes- the kallikreins which were differentially regulated in the absence of IL-17RA signaling in disseminated candidiasis. Kallikreins encode a family of fifteen serine proteases, are a part of the Kallikrein kinin system (KKS) known to play a critical role in renal function and pathology. Kallikreins act on inactive kininogens to generate active kinins, bradykinin and kallidin **(Figure 2).** Bradykinin signals through two G-protein coupled receptors: Bdkrb1 that is inducible upon inflammatory signals and Bdkrb2 which is constitutively expressed. To our knowledge, this is the first report that IL-17 directly regulates members of the kallikrein family of which Klk1 is the prototypical member. We focused our studies on Klk1 and its role in immunity to disseminated candidiasis. Bradykinin, which is a product of the cleavage of inactive kininogens into active form, is the main mediator in the

increased survival in mice with disseminated candidiasis. The increased survival benefit was attributed to reduced apoptosis of kidney resident cells and the preservation of kidney function upon bradykinin treatment.

Current treatment regimen for disseminated candidiasis includes the use of azole compounds like fluconazole. However, higher doses of azoles can lead to renal toxicity [95] and there exists a growing concern about the emergence of drug resistant strains. To this end, we provide evidence in a proof-of-concept study for the therapeutic efficiency of a combinatorial regimen of low dose fluconazole along with bradykinin. We found a significant survival benefit in infected mice treated with the combination of fluconazole and bradykinin in comparison with cohorts treated with either drug singularly. Thus our data reveal a previously unappreciated link between IL-17, KKS and renal protection against systemic candidiasis **(Chapter 2).**

Although *C. albicans* is the major species driving candidiasis, it accounts for only half the isolated strains detected in clinical settings [95]. Non-albicans strains such as *C. glabrata* and *C. parapsilosis* have gained importance as pathogens in invasive candidiasis. These species have differing susceptibilities to azoles and echinocandins [95]. Thus, there is an urgent need to discover new therapeutic strategies. Based on our observations with the combination strategy of antifungals with bradykinin, we venture to suggest a potential to extend therapeutic strategy in treating candidiasis caused by other strains of Candida. More studies are warranted in this direction to determine dosage and related benefits.

The KKS and the renin angiotensin system (RAS) are intertwined and have opposing physiological functions **(Figure 2).** Angiotensin converting enzyme (ACE) degrades bradykinin [277] and ACE inhibition can considerably increases bradykinin levels [278]. Numerous clinical and experimental studies have shown the therapeutic benefits of ACE inhibitors in delaying the

progression of various kidney diseases [279, 280]. These studies allude that bradykinin could be a factor in the beneficial effects of ACE inhibitors, but not many studies have looked into it specifically. This is another avenue of investigation that would prove useful to pursue as ACE inhibitors are routinely used in the clinic.

$CD4^+$ T cell responses can be categorized as protective or pathogenic. While Th17 cells producing IL-17 can provide protection against fungi and extracellular bacteria, they can also drive autoimmune diseases [64]. Previously, Th1 cells were considered pathogenic in the case of human and experimental glomerulonephritis. However, multiple studies have brought the role for the Th17-IL-17 axis to the fore [178]. To analyze whether the IL-17 signaling was crucial in renal autoimmune diseases, we used an experimental murine model of autoimmune glomerulonephritis. Our findings showed that mice deficient in IL-17R signaling were resistant to the disease. Interestingly, we observed that local immune responses in the kidney were directly dependent on the IL-17R signaling whereas the systemic immune responses were unabated. This suggests that the specific inflammatory environment in the target organ, i.e. the kidney can directly influence IL-17 mediated response, but more studies are needed to better understand the underlying mechanisms. Upon showing that IL-17 was pathogenic in EAGN, we sought to therapeutically interfere with IL-17 using neutralizing antibodies. The therapeutic benefit of this intervention resulted in the reduction of kidney pathology and restoration of renal function leading to amelioration of EAGN. These data collectively show that signaling through the IL-17 receptor in mice may be a causal factor in renal injury in glomerulonephritis. This provides the basis for interventions that modulate Th17-IL-17 axis to inhibit the pro-inflammatory immune responses leading to autoimmunity **(Chapter 3).**

Although the pathogenic role of the Th17/IL-17 axis in glomerulonephritis has been clearly established, most studies focus exclusively on IL-17A. IL-17A is the prototypical member of the IL-17 family and has been implicated in tissue injury and, ultimately, in the loss of renal function [53, 178, 272] However, lymphocytes that produce IL-17A can also produce IL-17F, another member of the IL-17 cytokine family that shares closest homology with IL-17A [281, 282] The cytokines are secreted either as homodimers or as IL-17A/F heterodimers by innate cells like $\gamma\delta$ T cells and group 3 innate lymphoid cells (ILC3) and adaptive immune cells like $CD4^+$ T helper cells [59, 283, 284]. IL-17A and IL-17F signal through the same heterodimeric IL-17RA and IL-17RC receptor complex [60]

So far, some published studies have shown that IL-17F has a role host defense against pathogens [69, 285], whereas its role in autoimmune diseases remains unclear. IL-17A and IL-17F mediate their biologic functions by activating the same heterodimeric IL-17RA/RC receptor complex [60]. However, IL-17F binds IL-17RC with higher affinity than it does IL-17RA, while IL-17A has the opposite affinity for the receptors. Thus, cells with higher levels of IL-17RC expression may respond better to IL-17F than to IL-17A, whereas cells with higher IL-17RA expression might be more responsive to IL-17A [69]. This could result in a non-redundant and unique role for IL-17F and further analysis of the molecular mechanisms employed by IL-17F in its downstream functions is warranted.

Studies have reported distinct phenotypes of mice deficient in either IL-17A or IL-17F in murine models of asthma and colitis [282]. It is known that IL-17RC expression is limited to non-hematopoietic cells while IL-17RA is more widely expressed [61]. This raises the question whether the distinct cell- and tissue-specific expression profiles of IL-17RA and IL-17RC and the differing affinities for the respective receptor subunits, contribute to diverse biological

functions of IL-17F and IL-17A. More studies to dissect the individual roles of IL-17A and IL-17F to autoimmune glomerulonephritis would answer these questions.

Previous reports from our lab have provided evidence for the co-existence of type I interferons (IFN-I) and IL-17 in the kidney in lupus nephritis in mouse models as well as human studies [206, 253]. Since we identified a crucial role for IL-17 in the kidney in the context of glomerulonephritis, we focused on gaining an understanding on the synergy between IFN-I and IL-17 in driving the pathogenesis of glomerulonephritis. In an IFN-I dependent glomerulonephritis model, we found that although systemic immune responses are similar, kidney infiltrating immune cells like macrophages were dependent on IL-17R signaling. Moreover, we discovered that *in vitro* IFN-I and IL-17 collaborate to drive the expression of chemokines like MCP-1 and osteopontin from renal epithelial cells as well as macrophages **(Chapter 4).**

Osteopontin (Opn) expression is significant in autoimmune disease in mouse models as well as several human autoimmune diseases, including lupus nephritis, multiple sclerosis and rheumatoid arthritis. Opn has been previously associated with glomerular crescent formation, fibrosis, glomerular sclerosis and the knockdown of Opn reduces scarring and fibrosis [286]. Studies in wound healing show that elevated levels of Opn contribute to inflammation-associated fibrosis [286]. In murine lupus nephritis, alternatively activated macrophages known to participate in tissue repair, express Opn and mediate formation of lesions in the glomeruli of the kidney and enhance crescent formations [230]. It is also critical to develop biomarkers that could obviate the need for biopsies to assess lupus nephritis activity or kidney damage, and that could accurately predict an impending exacerbation of renal disease [287]. Current markers for renal disease include urinary protein to creatinine ratio and this may not be sufficiently sensitive to

detect nephritis [288]. Opn could serve as a potential biomarker for end organ kidney damage in lupus nephritis patients. This leads to interesting questions about the role for Opn in mediating renal inflammation and fibrosis associated with lupus nephritis.

Persistent inflammation and progressive renal damage results in fibrosis in the kidney. This signals an irreversible change in the kidney, impacts function tremendously and eventually causes ESRD. Given the association of IL-17 responses in fibrosis of other organs [261, 262, 265, 289, 290], we hypothesized a pathogenic role for IL-17 in kidney fibrosis. However, unexpectedly, IL-17 proved to be protective in renal fibrosis. Using the UUO mouse model to induce renal fibrosis, we discovered that mice lacking IL-17RA had increased fibrosis and it was ameliorated when IL-17 was reintroduced. To identify the mediators downstream of IL-17 in protection against kidney fibrosis, we wondered if the kallikreins would play a role. Kallikreins have a range of biological functions that include blood pressure regulation, where they promote vasodilation [146, 274]. They have additional roles in regulation of inflammation, apoptosis and fibrosis [145]. Kallikreins have been implicated in the control of inflammation and maintenance of tissue homeostasis in acute or chronic kidney injury. Mice deficient in various components of the KKS exhibit exacerbated pathology and mortality in various acute or chronic kidney disease models including UUO-induced fibrosis, lupus nephritis and ischemia reperfusion [147-150]. The renal protective effect of KKS is primarily mediated by upregulation of tissue repair proteins, inhibition of pro-fibrotic factors, and preventing apoptotic changes [145, 146]. With these data from multiple studies we hypothesized that IL-17-Klk1 axis mediated protection against renal fibrosis. Consistent with our hypothesis we found that treatment of mice with bradykinin led to decreased collagen deposition **(Chapter 5).**

While these studies offer a good start in uncovering the mechanism in the protection,

more studies are necessary to delineate the significant contribution of the IL-17 driven Klk1 downstream cascade. Specific attention should be paid to determining the individual contributions of the bradykinin receptors, Bdkrb1 and Bdkrb2 in modulating the progression of fibrosis. While there are studies pointing in this direction using receptor knockout mice [269], proving the connection between IL-17 and the KKS is a novel finding. Receptor agonists and antagonists for the bradykinin receptors are in clinical trials for brain tumors and cryptococcal meningitis [ClinicalTrials.gov: NCT00005602, NCT00019422, NCT00002316, NCT00001502]. Extending our understanding for their role in renal fibrosis can lead to potential for these drugs to be tested for renal fibrosis too.

SUMMARY

Current efforts to control kidney disease are aimed at the development of new drug regimen. Therefore, a better understanding of the factors that influence CKD in seemingly distinct diseases like autoimmunity and infection, is paramount. In this work, we have demonstrated for the first time that IL-17 has a new distinct set of target genes that encode the kallikrein family. In particular, the kallikreins mediate important renal tissue protective functions. Collectively, all our observations indicate an essential role of KKS in protection against acute or chronic renal injury. An essential consideration is that it will be important to understand if treatments involving this pathway could be realistically introduced to tackle CKD. Thus, while the full impact of IL-17 on the severity of kidney disease and the development of renal immunity remains an open question, there is persuasive evidence, supported by our data, to warrant investigation of the IL-17-KKS nexus as a relevant factor for the control of kidney diseases.

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