THE INFLUENCE OF INTERLEUKIN 21 AND T FOLLICULAR HELPER CELLS IN HYPERTENSION AND VASCULAR DYSFUNCTION

By

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This body of work is dedicated to my parents, Barbara and Bill, for always believing in me, even if I took the road less travelled and forever engraining in me the name B. Dale is to be earned, even if you are born with it.

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systolic blood pressure (SBP)
diastolic blood pressure (DBP)
blood pressure (BP)
renin-angiotensin system (RAS)
sympathetic nervous system (SNS)
Angiotensin converting enzyme (ACE)
angiotensin II (Ang II)
sodium- hydrogen antiporter 3 (NHE3)
vascular smooth muscle cell (VSMC)
nitric oxide (NO)
smooth muscle cell (SMC)
endothelial NO synthase (eNOS)
tetrahydrobiopterin (H_4B)
reactive oxygen species (ROS)
superoxide ($O2^{\cdot-}$)
pulse wave velocity (PWV)
coronary artery disease (CAD)
Cerebral blood flow (CBF)
deoxycorticosterone acetate (DOCA)
L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride)
cardiovascular disease (CVD)
SPRINT (Systolic blood Pressure Intervention Trial)
PATHWAY trial
common myeloid progenitor (CMP)
common lymphoid progenitor (CLP)
Toll-like receptors (TLRs)
lipopolysaccharide (LPS)
inducible nitric oxide synthase (iNOS)
antigen presenting cells (APCs)
dendritic cells (DCs)
natural killer (NK)
major histocompatibility complex I (MHC I)
major histocompatibility complex II (MHC II)
T cell receptor (TCR)
B cell receptor (BCR)
immunoglobulin (Ig)
cluster of differentiation 3 (CD3)
helper T (Th)
T regulatory (Treg)
T follicular (Tfh)
Interleukin (IL)
B cell lymphoma protein 6 (BCL6)
CX chemokine receptor 5 (CXCR5)
CX chemokine ligand type 13 (CXCL13)
secondary lymphoid organ (SLO)
germinal center (GC)
somatic hypermutation (SHM)
class switch recombination (CSR)
nicotinamide adenine dicucleotide phosphate (NADPH)
cytotoxic lymphocyte antigen 4 (CTLA4)
recombinase activating gene 1 (Rag1)
Genome wide association studies (GWAS)
lymphocyte adaptor protein (LNK)
B-cell activating factor receptor (BAFF-R)
Na-Cl co-transporter (NCC)
interferon gamma (IFNγ)
tumor necrosis factor alpha (TNFα)
forkhead box P3 (Foxp3)
rheumatoid arthritis (RA)
type 1 diabetes (T1D)
multiple sclerosis (MS)
systemic lupus erythematosus (SLE)
peripheral helper T cells (Tph)
pulmonary arterial hypertension (PAH)
peripheral blood mononuclear cells (PBMCs)
spontaneously hypertensive rats (SHR)

CHAPTER 1: INTRODUCTION

Classical Concepts of Hypertension and Blood Pressure Regulation

Hypertension is an elevation in systolic blood pressure (SBP) greater than 130 mmHg or diastolic blood pressure (DBP) greater than 80 mmHg.[1] High blood pressure (BP) is considered the greatest burden on global disease and effects nearly 50% of Americans.[1-3] Diagnosis of hypertension is significantly associated with the development of vascular disease, chronic renal failure, and heart disease.[4, 5] While effectively lowering BP reduces the overall incidence of cardiovascular events,[6] those individuals with regulated BP remain at a higher risk for cardiovascular events.[7, 8] The etiology of essential hypertension, elevated BP in the absence of a secondary indication, is unknown in 90% of diagnosed individuals. The absence of disease causation and co-manifestation with other metabolic disorders has led to the wide use of pharmacological therapies, such as, vasodilators, diuretics, and anti-sympatholytic drugs that only address the physiological symptoms of hypertension rather than the underlying cause of the disease.[9] Unfortunately, it is estimated close to 30% of patients have resistant hypertension, failing to achieve regulated blood pressure on 3 or more antihypertensive drugs, thus highlighting the need to understand the fundamental biology behind the disease.[10, 11]

Classically, hypertension is identified as a disease effecting the vasculature, central nervous system, and kidneys, with one or all of these contributing to sustained high BP. The renin-angiotensin system (RAS) in thought to play the most central role in regulation of BP. Renin is produced predominantly by the juxtaglomerular cells of the kidney in response to the glomerular being under perfused, or in response to stimulation by the

sympathetic nervous system (SNS). Renin's essential role is in converting the pro-hormone angiotensinogen to angiotensin I. Angiotensin converting enzyme (ACE) rapidly converts angiotensin I to angiotensin II (Ang II). Ang II is a potent active hormone that can directly act on the vasculature via the angiotensin type I receptor, inducing smooth muscle contraction, systemic vasoconstriction, increased renalvascular resistance, all of which lead to an increase in BP. Ang II can enhance sodium reabsorption by increasing the activity of sodium- hydrogen antiporter 3 (NHE3) and other transporters by stimulating the release of aldosterone from the adrenal gland, resulting in changes in sodium and water retention, furthering the rise in BP.[12] Additional effector functions of aldosterone has been shown to include endothelial dysfunction, vascular smooth muscle cell (VSMC) proliferation, vascular remodeling, fibrosis, and oxidative stress.[13, 14]

The endothelium itself is also a major source for regulating vascular tone through the vasoactive substance nitric oxide (NO). In healthy endothelial cells, NO is produced and released in response to shear-stress, inducing smooth muscle cell (SMC) relaxation via the generation of intracellular cyclic guanosine monophosphate by guanylate cyclase.[15] Disruption of endogenous NO production through inhibition of endothelial NO synthase (eNOS) or uncoupling of eNOS (through the absence of the critical cofactor tetrahydrobiopterin (H_4B) and substrate L-arginine) results in an increase in BP and hypertension in humans and animals.[16, 17] In fact, a decrease in whole body production of NO has been observed in hypertensive patients compared to normotensive volunteers.[18, 19] The literature currently suggests the uncoupling of eNOS leads to a buildup of reactive oxygen species (ROS) like superoxide ($O2^{\cdot-}$). Excess superoxide anions react with NO,

decreasing the bioavailability of NO, and generating the pro-inflammatory molecule peroxynitrite. A decrease bioavailability of NO is a critical factor promoting vascular remodeling, endothelial dysfunction, and hypertension.[20].

There is evidence that also implicates the SNS in BP regulation, as an observed increase in sympathetic outflow is a universal finding in essential hypertension.[21] Baroreceptors that function in sensing alterations in vascular tone are localized within the arterial tree, one key location being the bifurcation of the carotid artery. When BP is elevated, the artery is stretched and this stretch is sensed by the baroreceptor. The innervation of the artery transduces a signal to the brain to decrease sympathetic outflow in order the decrease BP. Interestingly, common risk factors associated with hypertension, such as obesity and age, also exhibit greater SNS activity.[22, 23] The kidney also has direct communication to the brain. Increased renal sympathetic activity can increase local renin levels and promote sodium retention via tubular epithelial cells.[24] The increase in renin further exacerbates the conversion of angiotensinogen to angiotensin I, fueling the renin angiotensin system.[25]

Cardiac output is the volume of blood pumped by the heart each minute. Thus, anything that increases cardiac output (such as increased blood volume) also increases blood pressure. Early hypertension results in vascular remodeling, vascular rarefaction, and vasoconstriction, thus altering vascular resistance. Blood pressure is the product of systemic vascular resistance and cardiac output, therefore, one or both of these must be altered during chronic hypertension. Under homeostasis an increase in systemic vascular

resistance is concurrently normalized by cardiac output. This phenomenon was defined by Guyton in 1987, as “pressure-natriuresis” curve where he described the relationship between BP, water excretion and salt.[26] During homeostasis salt balance in maintained by a counterbalance of natriuretic and anti-natriuretic systems. Under normotensive circumstances there is an abrupt instance of diuresis in response to elevated BP or increased blood volume, resulting in the maintenance of normal BP. However, in order to sustain hypertension, the curve must shift to the right. This shift is a result of decreased renal capacity to excrete sodium and water. Reabsorption of sodium and water are regulated along the nephron through numerous proximal and distal transporters such as NHE3. Insult to the regulation of this system, such as increased activity or expression of transporters, will result in elevated BP.

Pathophysiology of Hypertension

The pathophysiological mechanisms that govern essential hypertension are complex, however, the effects if high blood pressure goes untreated or unregulated are well documented. Chronic elevation of BP may result in systemic changes in the vasculature. Some of these involve microvascular rarefaction (loss of capillaries and resistance vessels), vascular remodeling (narrowing of the lumen and hypertrophy of the media), and enhanced vasoconstriction (such as due to loss of NO bioavailability, enhanced local production of Ang II, or increase ROS) leading to increased vascular dysfunction and systemic vascular resistance (**Figure 1-1**).[25] The remodeling process that occurs can also result in aortic stiffening, where the vessels ability to buffer the pressure induced during systole is decreased. This capacitance characteristic is known as the “Windkessel effect”.

Clinically, this capacitance ability can be measured as pulse wave velocity (PWV).[27] Further, the physiological stresses of high BP can lead to left ventricular hypertrophy and local production of ROS which contribute to coronary artery disease (CAD), ultimately leading to heart failure.[28]

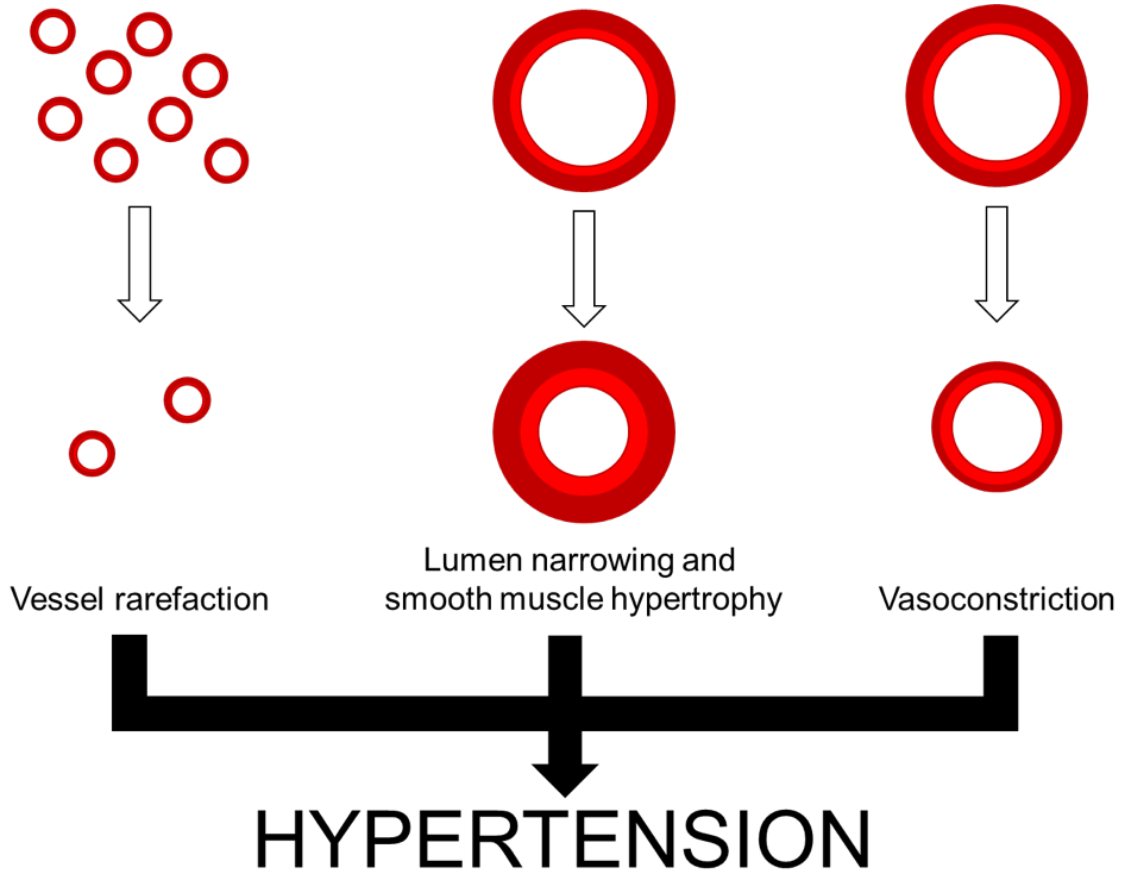

Figure 1-1. Alterations in vessel architecture leading to and as a consequence of hypertension.

The brain is also a major target of high BP. Hypertension is the leading cause of cognitive decline and the number one risk factor for stroke.[29] In fact, for every 1 mmHg increase in SBP the likelihood of stroke mortality increases by 2%.[30] Stroke can result from the occlusion of a major artery (ischemic) or a rupture of arterioles (hemorrhagic). Cognitive impairment is thought to be caused by the occlusion of arterioles that essentially cause a

disruption in neural signaling involving memory.[31, 32] Cerebral blood flow (CBF) increases when a brain region is activated. Vasoactive agents are released to induce vasodilation during this brain activity.[33] Interestingly, CBF can be autoregulated independent of mean arterial pressures <150 mmHg.[34] To counteract arterial pressure, cerebral arterioles constrict when pressure increases and relax when pressure decreases. Cerebral vascular endothelial cells are essential for exerting the changes in vascular tone by releasing vasoconstrictors (endothelin, Ang II, endothelium-derived constriction factor) and vasodilators (NO, prostacyclin, bradykinin). Unfortunately, hypertension alters CBF autoregulation due to vascular remodeling, lumen narrowing, endothelial dysfunction, and hypertrophy resulting in increased cerebrovascular resistance and higher perfusion pressures to maintain the same level of CBF.[34-37]

Hypertension remains the second leading cause of end-stage renal disease.[38] The kidneys are a highly vascularized organ and help filter waste and fluids from the blood via the nephron that is comprised of the glomerulus and its tubule. Long-term exposure to high BP results in increased intraglomerular pressure which manifests as impaired glomerular filtration. Due to this damage, hypertensives experience increased protein filtration (albuminuria/proteinuria) and an altered albumin-to-creatinine ratio.[39] As with CBF, the kidney has means to protect itself from large swings in blood pressure via autoregulatory mechanisms, however, if the BP goes above the regulatory range, vascular injury ensues and autoregulatory responses become compromised.[40] In addition, vascular remodeling in the renalvascular infrastructure is important in the maintenance of hypertension.[41] Under increased mechanical stress, the vessel walls

respond by increasing the medial thickness and a narrowing of the lumen, further magnifying the pathological effects of increased BP. The thickening of the vascular wall increases the distance oxygen must diffuse across the vessel leading to ischemic injury. Moreover, hypertensive stress on the vessel increases local ROS production and the induction of tubule-interstitial fibrosis, among other downstream effects. Collectively, the pathophysiology that ensues due to hypertension results in severe renal damage.[42]

Experimental Model Systems of Hypertension

There are multiple methods in which to study experimental hypertension in small mammals. One of the most common methods involves the chronic infusion of a pressor dose of Ang II with the use of a subcutaneous implantation of an osmotic mini pump. Once the pumps are surgically placed, Ang II is constantly infused over the time period desired.[43, 44] Another model used to induce hypertension is the deoxycorticosterone acetate (DOCA)-salt model.[45] In the DOCA-salt model, a uninephrectomy is performed, a DOCA pellet is implanted subcutaneously, and the animal is supplemented with 1% NaCl drinking water. This model is typically carried out for 21 days. This model is thought to be a “low Ang II” model of hypertension. DOCA is an aldosterone derivative and can affect the kidney by altering water reabsorption and salt retention. The third model utilizes a compound called L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) and induces hypertension primarily via inhibiting NO production, thus leading to significant vascular dysfunction.[46]

Clinical Trials and Treatment of Hypertension

The link between hypertension and cardiovascular disease (CVD) underscores the necessity of treating high BP. On the coat tail of the results published by SPRINT (Systolic blood Pressure Intervention Trial), indicating intensive BP treatment significantly reduced CVD incidences,[6] the American Heart Association and US affiliates lowered the recommended blood pressure guidelines to define hypertension as SBP >130 mmHg and/or DBP >80 mmHg.[1]

Controlled randomized trails performed in hypertensive humans have demonstrated a reduction in sodium consumption is positively correlated with a decrease in BP.[47] Compelling evidence for this was provided by the DASH-sodium (Dietary Approaches to Stop Hypertension) Trial, where the consequence of varying amounts of salt intake were tested independently and concurrently with two diets: the standard American diet, and the DASH diet (low in fat, cholesterol and high in fruit and vegetables). The study determined a reduction in sodium intake resulted in a reduced BP not only in hypertensive volunteers, but also healthy controls. This study interestingly also provided evidence that an increase in potassium consumption, during reduced salt intake, is correlated with reduced BP. There is additional literature demonstrating reduced sodium intake can prevent hypertension and aid in controlling hypertension, therefore reducing the need for pharmacological intervention.[48-50] Thus, altering lifestyle habits to reflect the recommendation of The American Heart Association and American Society of Hypertension, to 3.8g/day salt, could have beneficial results in BP regulation.[51, 52]

Antihypertensive classes of drugs have proved significantly effective in improving CVD morbidity and mortality.[53] Antihypertensive pharmacotherapy is typically initiated using a monotherapy or a combination treatment if the patient presents with aggressively high BP.[54] Pharmacological therapy typically includes ACE inhibitors, Ang II receptor blockers, calcium channel blockers, and thiazide diuretics.[55] Beta-blockers are also utilized in patients with history of heart attack, heart failure, or reduced left ventricular ejection fraction.[56] In most patients, combination therapy is necessary to control BP, however the optimal combination is determined based on additive effects on BP, side-effects and patient comorbidities. In the worst cases, 4 or more medications may be necessary to reduce BP. These patients are considered to have resistant hypertension, and in some cases never obtain controlled BP.[11] The prevalence of resistant hypertension is not well documented, but is estimated at about 13% of those diagnosed with hypertension in the US.[57] The PATHWAY trial investigated if adding a fourth or fifth drug could improve hypertension and found mineralocorticoid antagonism to be the most beneficial choice.[58] Lastly, a less well defined area of treatment involving device-based treatments have been developed and evaluated in cases of severe resistant hypertension.[59] Renal nerve ablation, carotid body denervation, deep brain stimulation, among others, are thought to lower BP via inhibition of the SNS.[60-62]

Ultimately, BP control comes down to lifestyle changes, efficacious drug intervention and compliance by the patient. Although cardiovascular pharmacological reagents are effective, nearly 50% of those diagnosed with hypertension still have poorly controlled BP and 12-15% have uncontrolled BP, suggesting that current treatments on the market are

insufficient to address the etiology of the disease and highlighting the need to identify additional therapeutic targets. Over the past decade, it has become increasingly evident that the immune system plays an essential role in establishing and maintaining hypertension,[25] thus, defining a potential area of unchartered therapeutic opportunity.

Immune system brief

Immune cells arise from hematopoietic stem cells and diverge at the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). The immune system is comprised of two major arms, the innate (myeloid lineage) and the adaptive (lymphoid lineage) immune systems.

The innate immune system is thought to be the first line of defense against invading pathogens, while the adaptive immune system is understood to provide support if the pathogen is not cleared. The innate immune system is comprised of many components such as the epidermis, the respiratory epithelium, mucosal surface, and vascular endothelium.[63] When pathogens invade the body, they can be identified by pattern recognition receptors and Toll-like receptors (TLRs) which recognize pathogen related motifs like, unmethylated CpG, lipopolysaccharide (LPS) or double stranded RNA. When innate immune cells bind these motifs via receptors, a cascade of intracellular signaling occurs including synthesis of cytokines and chemokines, inducible nitric oxide synthase (iNOS) expression, and production of ROS.[64] Components of the innate immune system include antigen presenting cells (APCs) like macrophages and dendritic cells (DCs), neutrophils, granulocytes, and natural killer (NK) cells. APCs are important as they act as

a key mediator between the innate and adaptive immune systems. APCs present foreign proteins via major histocompatibility complex I and II (MHC I and MHC II) to T cells (of the adaptive immune system) in order to elicit a pathogen specific response.[65]

The adaptive immune response is designed quite differently than the innate, in that it can respond to foreign antigens specifically. If an invader survives the innate immune response the adaptive immune response is activated. The adaptive immune system is comprised of T cells, B cells, and NK T cells. APCs of the innate system present antigen to T cells generating a specific response. The MHC complex on the APC presents the pathogenic peptide to the T cell receptor (TCR). If the TCR has specificity for the antigen, the signal induces T cell activation resulting in an array of downstream effector functions. These may include T cell maturation, proliferation, and cytokine secretion which result in changes in T cell function and phenotype.[65] The B cells recognize antigen via the B cell receptor (BCR), which are membrane embedded immunoglobulin (Ig) molecules. When B cells are activated they can help generate a pathogen specific response via secretion of Igs.[66] Activation of the adaptive immune system can also result in memory T and memory B cells, to be called upon a secondary encounter with the pathogen.[65, 67]

T cells are a major component of the adaptive immune system. T cells have a TCR composed of 2 variable chains and cluster of differentiation 3 (CD3) subunits. The variable chains of most T cells are random arrangements of the alpha and beta genes, giving rise to conventional T cells. T cells can also rearrange the gamma and the delta genes, giving rise to $\gamma\delta$ T cells. When not specified, conventional $\alpha\beta$ T cells will be referred

to as T cells. T cells migrate from the bone marrow to the thymus where they develop and exit as naïve CD4 or CD8 T cells. T cells can then circulate in the periphery or reside in secondary lymphoid organs (SLOs) in a naïve state until interaction and activation with an APC.

Antigen presentation to T cells is canonically performed by DCs, macrophages, and B cells. T cells respond to stimulation via interaction with APCs. DCs are thought to be the predominate population to present antigen via MHC to T cells. Two signals are required for the immunological synapse. The first being the recognition of the peptide/MHC complex by the TCR. APCs expressing MHC I activate a subset of T cells called CD8 T cells, and APCs expressing MHC II activate a subset of T cells called CD4 T cells. The second interaction provides additional necessary stimulation. Classically, this is the interaction of B7 ligands (CD80 and CD86) on APCs and CD28 on the T cell. In the absence of a second signal, T cell activation is blunted, however, in response to these two conditions being met T cells can respond.[64]

CD3 broadly labels all T cells which can be categorized more specifically as CD8 or CD4 T cells. CD8 T cells are commonly referred to as cytotoxic T cells. Once activated, a CD8 T cell is programmed to destroy a pathogen via secretion of granzymes and perforin, effecting the integrity of the cell membrane, and ultimately leading to cell destruction or apoptosis.[68] CD4 T cells are a major subset of T cells characteristically defined as “helper” T (Th) cells due to their ability to directly provide “help” to B cells and other cells types via immunological synapses and cytokine production.

Naïve CD4 T cells become polarized towards a Th subset lineage dependent on many factors such as TCR signal strength, but a key driver of lineage polarization is the cytokine milieu present in the microenvironment. Helper T cell subsets are further defined based on their cytokine profile and transcriptional machinery and include, Th1, Th2, Th17, T regulatory (Treg), T follicular (Tfh), Th22, and Th9. Each helper T cell subset serves a specific function in the role they play in host defense. An imbalance or unbridled expansion of a helper T cell population, such as Th17 or Tfh cells, can lead to pathogenesis.[43, 66, 67, 69-74]

On interest, Tfh cells are a subset of CD4 T cells identified to function primarily to provide B cell help.[71] Early Tfh cell development is reliant on the interaction of the TCR with a DC antigen presenting cell and the presence of interleukin (IL)-6 which induces B cell lymphoma protein 6 (BCL6) expression.[75] The transcription factor BCL6 is required for Tfh cell differentiation, establishment, and maintenance of the specific lineage.[76-78] BCL6 upregulates surface expression of CX chemokine receptor 5 (CXCR5).[79, 80] CXCR5 binds the CX chemokine ligand type 13 (CXCL13) produced by germinal center (GC) B cells, helping localize the subset to GC containing follicles.[80, 81] Additionally, Tfh cells exert a majority of their effector function via production of IL-21, with IL-21 being the most potent inducer of GC B cell proliferation and differentiation (**Figure 1-2**).[82-84]

Within a secondary lymphoid organ (SLO) primed Tfh cells interact with GC B cells in a transient structure called a germinal center (GC). When a BCR is engaged by physical T

cell help, and cytokine signaling in the “light zone”, the B cell localizes to the “dark zone” in the tissue and starts to proliferate. GC B cells can be detected by surface expression of CD19, B220, Fas, and GL7 (**Figure 1-2**).[83, 85 86-88].

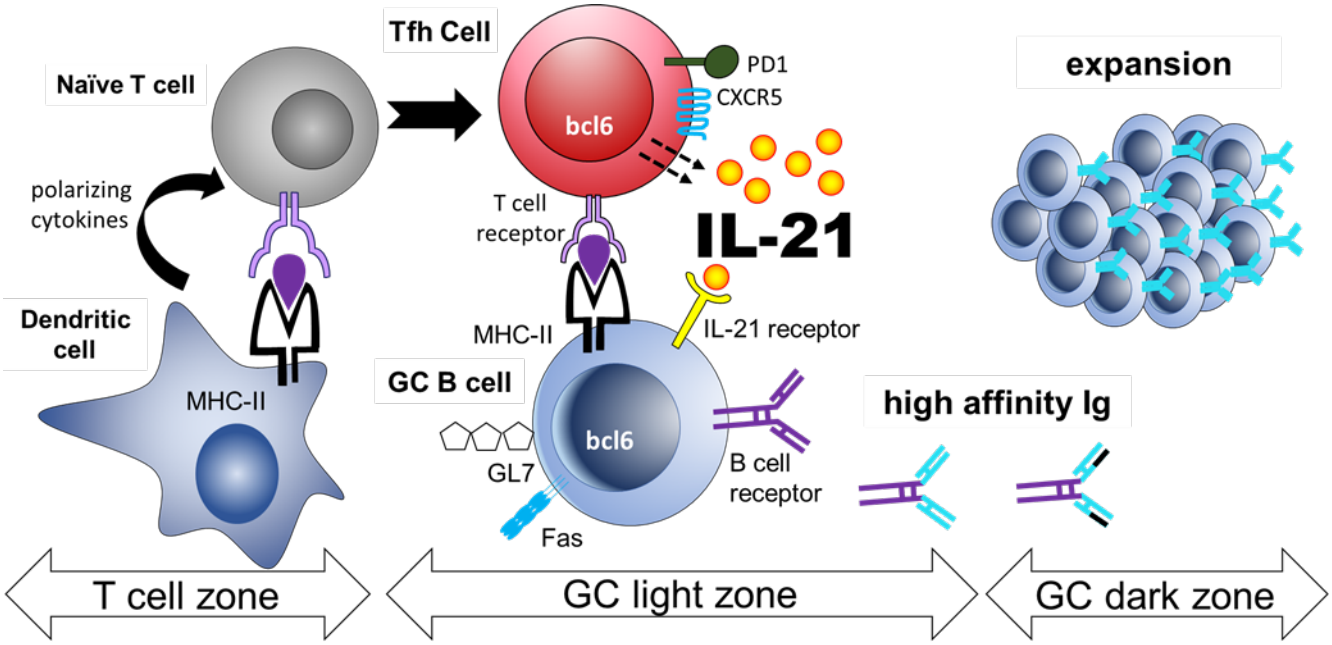

Figure 1-2. Anatomy of signaling within a follicle. The conventional location where T cells provide B cell help is within secondary lymphoid organs, such as the lymph node and spleen. Immune cells form macro structures called follicles, and within follicles, T and B cells organize forming transient structures called germinal centers. On the border of the germinal center is the T cell zone, where dendritic cells prime naïve T cells via antigen presentation and cytokine polarization. Some of these naïve T cells will polarize to T follicular helper cells. These cells are characterized by the transcription factor bcl6 and surface expression of PD1 and CXCR5. CXCR5 helps localize the Tfh cells to the germinal center light zone, where it interacts with germinal center B cells. Germinal center B cells can be characterized by the transcription factor bcl6, and surface expression of GL7 and Fas. Upon T cell interaction, B cells can undergo class switch recombination and somatic hypermutation to generate high affinity immunoglobulins, and/or expand within the germinal center. IL-21 has been shown to be the most potent inducer of the germinal center reaction

As a result of the GC reaction, B cells modify their receptors using a process called affinity maturation. During this process, the stimulated B cell makes irreversible alterations to its DNA via somatic hypermutation (SHM) and immunoglobulin class switch recombination (CSR). SHM produces single point mutations in the BCR and CSR alters the heavy chain

isotype. The 5 heavy chain isotypes of immunoglobulin (Ig) are IgM, IgD, IgG, IgA, and IgE. IgG is the major serum isotype and is comprised of four different subclasses (IgG1, IgG2a/c, IgG2b, and IgG3 in mice and IgG1, IgG2, IgG3, and IgG4 in humans).[89] Each of these subclasses have different capacities to activate downstream immune responses. These mutations together produce unique and antigen specific high affinity BCRs, may result in a non-functional BCR or even decrease the affinity to bind antigen.[86] Due to this, only a small portion of B cells will survive the GC reaction and be selected for survival.

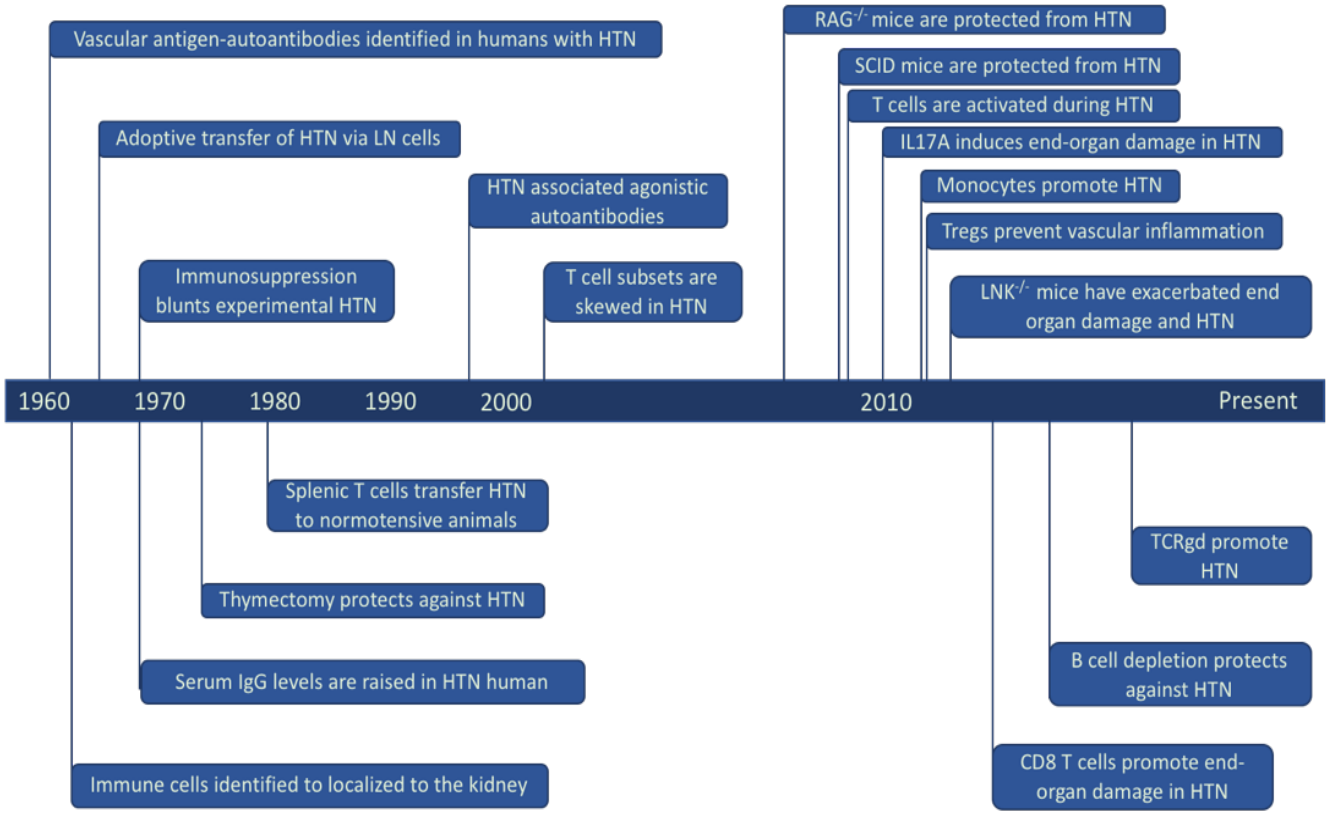

Figure 1-3. Timeline of publications connecting hypertension and the immune system

The Immune System in Hypertension

The idea that the immune system is involved in the pathogenesis of hypertension is not

new. As early as the mid 20th century lymphocyte infiltration was observed in the kidneys of humans with elevated blood pressure, with an expansion in publications in the late 2000's (**Figure 1-3**).[90] Following the initial studies, White and Grollman et al. demonstrated that BP can be lowered with immunosuppression in rats with partial renal infarction, and that these animals developed autoantibodies to renal tissue.[91] In a follow-up, Okuda and Grollman et al. demonstrated that an allotransplant of lymph nodes from rats with renal infarction raised BP in normal recipient rats, suggesting for the first time that unknown circulating factors or cells can be transferred and cause hypertension.[92] An additional early study by Olsen et al. described the periadventitial accumulation of immune cells within the vasculature of humans with hypertension.[93] While these studies set the foundation for the field of immunocardiovascular studies in hypertension, little progress was made until the early 21st Century when advanced models of genetics and immunological methods had advanced enough to study the immune system in more depth.[94-97] Studies over the past decade conducted by the Madhur laboratory and colleagues suggest the innate and adaptive immune systems play key roles in the development and maintenance of hypertension (**Figure 1-4)**.[25, 64, 98]

Components of the innate immune system are relevant to cardiovascular disease. In 2005, De Ciuceis et al. studied mice lacking macrophage colony stimulating factor (m-CSF), commonly referred to as Op/Op mice, which exhibit severe deficiency in their monocyte and macrophage populations.[99] They found that these animals have blunted hypertension in response to Ang II infusion, preserved vascular function, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Further studies

on the Op/Op mice demonstrated they were also resistant to DOCA-salt induced hypertension.[100] To build on these findings, Wenzel et al., showed that hypertension induced aortic infiltration of monocytes and macrophages and when upon depletion of monocytes animals had attenuated hypertension, preserved vascular endothelial function, and decreased superoxide production. Further, they showed that the hypertensive phenotype was fully restored upon adoptive transfer of wild type monocytes into monocyte depleted recipients.[101] Additionally, Krishnan et al. demonstrate that hypertension is blunted in animals lacking key components of the inflammasome, an element of the innate immune system activated by TLR signaling.[102]

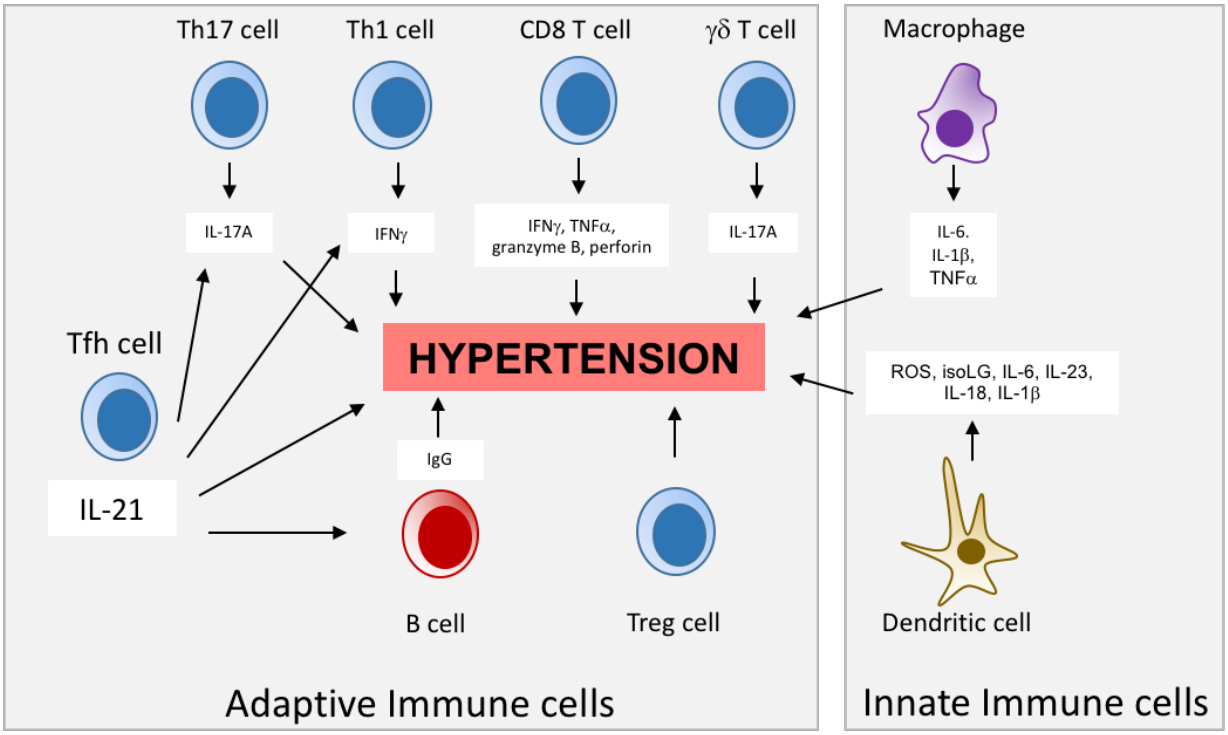

Figure 1-4. Innate and adaptive immune cells that have been shown to play a critical role in hypertension. Adaptive immune cells: $CD8^+$ T cells, $CD4^+$ cells (Th1, Th17, and T reg cells), T cells, and B cells produce factors that promote or inhibit hypertension. Innate immune cells: Macrophages, microglia, monocytes, DCs, and MDSCs also produce cytokines and ROS, which promote or inhibit hypertension. The NLRP3 inflammasome in monocytes and DCs plays a key role in hypertension. γδ T cells function on the border of innate and adaptive immunity.(modified from Norlander et al.)[25]

Another component of the innate immune system, the complement system, also functions to enhance the effector functions of immune cells during hypertension. The complement system is comprised of a set of proteins which are circulating in the body.[65] One of these protein complexes, C1q, can bind antigen-antibody complexes, initiating a cascade of events involved in the complement pathway. Ang II induced hypertension is associated with an increase in C1q expression and promotes vascular smooth muscle hypertrophy and proliferation. Further, inhibition of C1q, prevents vascular remodeling in hypertensive rodent models.[103, 104] Additional complement components, such as C3 and byproduct C5a, increase within the perivascular fat during hypertension where it can induce further vascular inflammation and promote remodeling.[105]

T Cell Activation in Hypertension

During hypertension, APCs infiltrate into the kidney and perivascular fat of vessels where they can take up and process antigen to present to T cells. As previously discussed, this interaction is dependent on the synapse between CD28 on T cells, and B7 ligand on APCs. Upon activation T cells upregulate cytotoxic lymphocyte antigen 4 (CTLA4), which binds to B7 ligands, preventing their interaction with CD28, thus regulating further T cell activation. The concept that this immunological synapse is involved in hypertension was tested using the drug Abatacept, a CTLA4-Ig reagent used in autoimmune disease and organ transplantation. The investigators found Abatacept treatment or B7-deficiency had diminished hypertensive and immunological responses to Ang II or DOCA-salt induced hypertension.[106] Further findings by the Harrison laboratory have also revealed

hypertension can stimulate conversion of monocytes to DCs, DCs have increased ROS production, and hypertensive DCs are more efficient at stimulating T cells to proliferate.[107, 108]

The Adaptive Immune System in Hypertension

The knowledge that the immune system plays a role in hypertension expanded alongside the development of genetic mouse models and the advancement of immunological methods. A key genetic model used to help establish a role for T cells in hypertension were recombinase activating gene 1 (*Rag1)* deficient animals. Mice lacking either *Rag1* or *Rag2* fail to develop T and B cell repertoires. In 2007, a seminal study by Guzik et al. investigated whether mice deficient in *Rag1* were protected from experimental hypertension. He found that these animals are profoundly protected from Ang II or DOCA-salt induced hypertension.[96] Additionally, they reported *Rag1* knockouts had less endothelial dysfunction compared to WT controls. Importantly, adoptive transfer of WT T cells into the *Rag1* knockouts restored the hypertensive phenotype, further suggesting T cells play a substantial role in hypertension. These studies were followed up using additional models of immunodeficiency in mice and rats. Severe combined immunodeficient mice had a blunted hypertensive response and less cardiac hypertrophy in response to Ang II infusion.[97] Further, mutation of the Rag1 gene in Dahl salt-sensitive rats protected against salt-induced hypertension, and renal damage.[109]

Genome wide association studies (GWAS) have enabled the identification of disease associated alterations in the genome. The lymphocyte adaptor protein (LNK) was

identified as a key driver gene in blood pressure regulation by GWAS.[110] The function of LNK in hypertension was validated by our laboratory. Saleh et al. described that LNK knockout animals had severe inflammation, even without hypertensive stimuli, and worsened inflammation after Ang II-induced hypertension, thus indicating that LNK likely functions as a brake for lymphocytes during proliferation and cytokine signaling.[44]

Hypertension has long been associated with elevated levels of Igs,[111, 112] although only a few to date have directly questioned the role for B cells in hypertension. In 2015 Chan et al. revisited the role that B cells play during Ang II-induced hypertension using two different models of B cell deficiency.[113] This study found that B-cell activating factor receptor (BAFF-R) knockout mice, which lack circulating mature B cells, or animals treated with a murine anti-CD20 (analogous to human rituximab), resulting in B cell depletion, have blunted hypertension and reduced aortic IgG deposition in response to Ang II infusion. Additionally, upon reconstitution of WT B cells, the hypertensive phenotype was restored. Further the investigators demonstrated purified hypertensive IgG induces the production of the profibrotic cytokine TGFβ in cultured macrophages.[113] These findings collectively highlight the necessity to further understand the interface between T and B cells during chronic hypertension.

T Cell Subsets and Cytokines in Hypertension

Due to the heterogeneity of the T cell repertoire, several studies have attempted to determine what types of T cells are relevant to hypertension.[114] As the most basic level αβ T cells can be classified as CD8 and CD4. One of the earliest studies used global CD8

or CD4 deficiency to determine which T cell subset was most relevant. Trott et al. demonstrated cytotoxic CD8 T lymphocytes localize to the kidney of hypertensive mice and are antigenically activated, as determined by TCR gene spectratyping, revealing an oligoclonal expansion of CD8 cells within the kidney. They further went on to show CD8 deficiency protects against hypertension and renalvascular end-organ damage.[115] It has more recently been shown that CD8 T cells can interact directly with renal tubules during DOCA-salt induced hypertension, resulting in the upregulation of the Na-Cl co-transporter (NCC), thus influencing BP via salt and water reabsorption.[116]

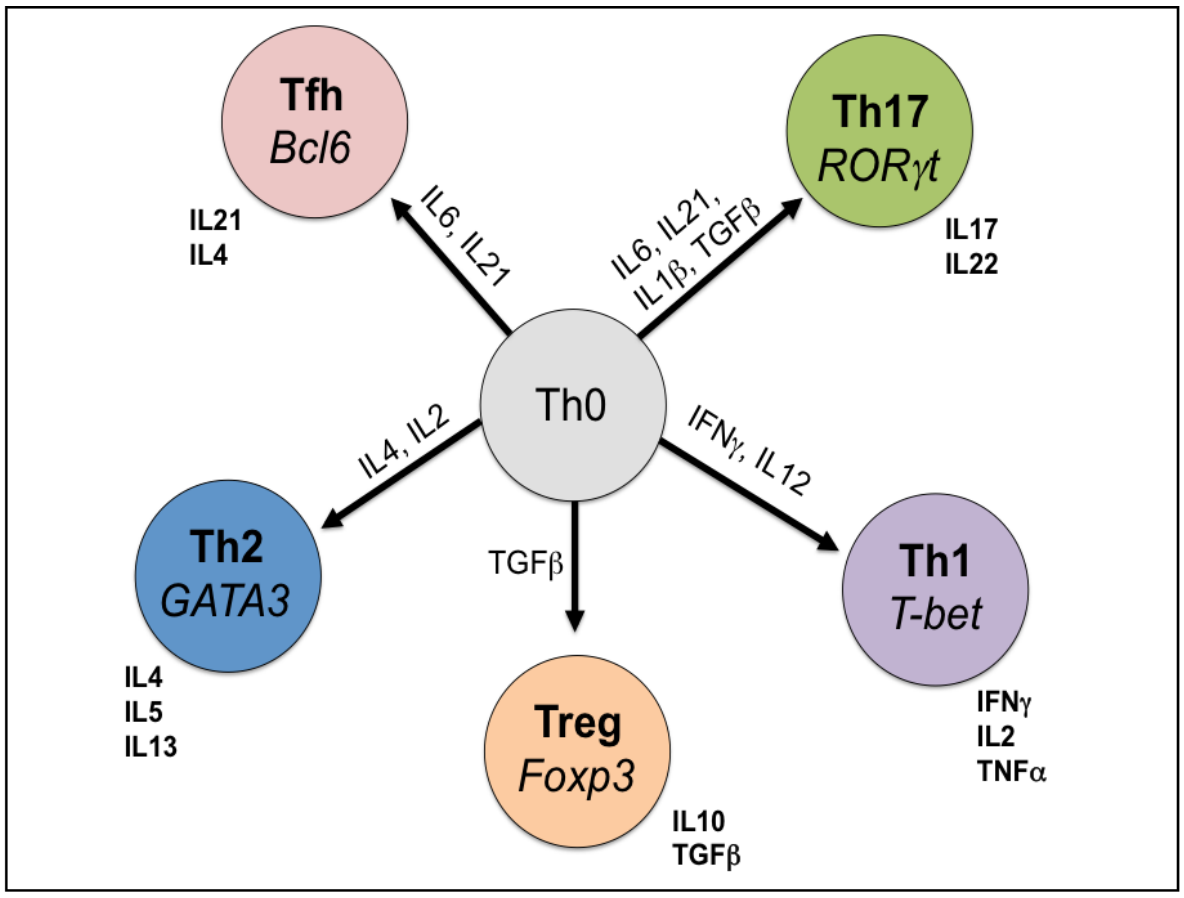

Figure 1-5. T cell subsets and their characteristic transcription factors and associated cytokines.

There is a large body of literature providing evidence that CD4 T cells and their derived cytokines are pathogenic in hypertension.[25] As discussed previously, CD4 Th cells are categorized into subsets by their transcription factor and cytokine profile. T cell plasticity, characterization, and phenotyping is complex, consequently thorough characterization of these subsets and their effector functions is important to understanding their role in disease pathogenesis (**Figure 1-5**).

Th1 cells are characterized by the transcription factor *Tbet* and signature cytokine interferon gamma (IFNγ). In a study conducted in our laboratory by Saleh et al., found that IFNγ producing CD4 and CD8 T cells increase during Ang II-induced hypertension and that IFNγ knockout animals lack the ability to establish and sustain the full hypertensive phenotype.[44] Marko et al. also observed IFNγ receptor knockout mice, while they don't have an observable BP phenotype, have less severe cardiac damage and a decrease in cardiac inflammation.[117] A proposed mechanism by which IFNγ promotes hypertension is its ability to induce angiotensinogen expression in hepatocytes and renal proximal tubular cells. Nevar et al. has shown that local elevated levels of Ang II can occur in the kidney where it acts locally by promoting sodium and water reabsorption by the proximal and distal nephron. This action has been reported to be mediated in part by NHE3.[118]

Another cytokine characteristic of Th1 cells is tumor necrosis factor alpha (TNFα). Guzik et al. found that the TNFα agonist etanercept blunts hypertension and vascular superoxide production after Ang II infusion.[96] Zhang et al. investigated the effect of Ang

II infusion on TFNα knockout mice. The authors observed blunted hypertension and reduced end-organ damage.[119] Interestingly, these mice also had enhanced eNOS expression and NO bioavailability suggesting that TNFα signaling may regulate BP acting through the NO pathway.

A particularly important cytokine associated with many chronic inflammatory diseases is IL-17.[73] IL-17 exists in 6 different isoforms (A-F), with IL-17A and F being the most closely related.[120] IL-17A notably is produced largely by CD4 Th17 cells, and CD4 production of IL-17A increases with hypertension in rodents and humans.[43, 121] My mentor, Dr. Meena Madhur, was the first to establish a significant role for IL-17A in Ang II hypertension showing mice lacking IL-17A were unable to sustain the hypertensive phenotype, have less T cell inflammation, and have preserved vascular function.[43] IL-17A can promote hypertension by directly effecting the bioavailability of NO. Direct infusion of IL-17A raises BP in wild type mice, and induces an inhibitory phosphorylation event on eNOS (Thr495) which facilitates impairment of the endothelium-dependent relaxation.[122] More recently, our laboratory demonstrated pharmacological neutralization of IL-17A but not IL-17F prevents a further increase in BP as well as renal injury and inflammation and that IL-17A can regulate sodium and water balance in the kidney via altering the expression of renal transporters.[123, 124] Lastly, $\gamma\delta$ T cells contribute to hypertension by production of a IL-17 as well.[125] Aortic and renal infiltration of IL-17 producing $\gamma\delta$ T cells has been reported in hypertensive mice.[126, 127] Mice lacking $\gamma\delta$ T cells have a marked protection in response to Ang II-induced hypertension.[127] These studies collectively provide valuable insight into IL-17A as a potential therapeutic target in hypertension.

Tregs are characterized by the transcription factor forkhead box P3 (Foxp3), surface expression of CD25 and production of IL-10. Generally, Tregs are considered an immunological protection mechanism that counterbalance the effects of expanding pathogenic immune populations.[67] Hypertension is associated with a decrease in Treg numbers.[128] In fact, adoptive transfer of $CD4^+$ $CD25^+$ T cells but not $CD4^+$ $CD25^-$ T cells blunts Ang II-induced hypertension, vascular dysfunction, and aortic infiltration of macrophages and T cells, while Treg depletion using a CD25 monoclonal antibody exacerbates the development of hypertension and end-organ damage.[129-131]

Lastly, a unique function of the adaptive immune system is the formation of a memory response. Using a model of repeated hypertensive stimuli, Itani et al. reported a substantial increase in renal IL-17A and IFNγ producing T effector memory cells and showed that mice lacking CD70 (an essential signal in the generation of memory T cells) do not accumulate these populations in the kidney.[132] In this same study, upon repeated exposure to hypertensive stimuli, memory T cells residing in the bone marrow were reactivated and migrating back to the kidney. These data established an undescribed phenomenon previously not known to occur in hypertension.

Clinical Significance of IL-21 and Tfh Cells

IL-21 is a promiscuous cytokine predominately produced by Tfh cells and to an extent NKT and Th17 cells and has been demonstrated to have effector functions across the immune system (**Figure 1-4**).[74, 133] The IL-21 receptor is expressed on lymphoid cells, but

has also been detected on nonlymphoid cells, such as, epithelial cells and endothelial cells.[134, 135] Through receptor ligation, IL-21 can promote the proliferation and differentiation of Tfh and Th17 cells, skew T helper cell subsets, and induce B cell differentiation into Ig producing plasma cells. Since the effector functions of IL-21 can influence numerous immune responses, it comes as no surprise that IL-21 plays a role in autoimmune and chronic inflammatory diseases.[82, 136]

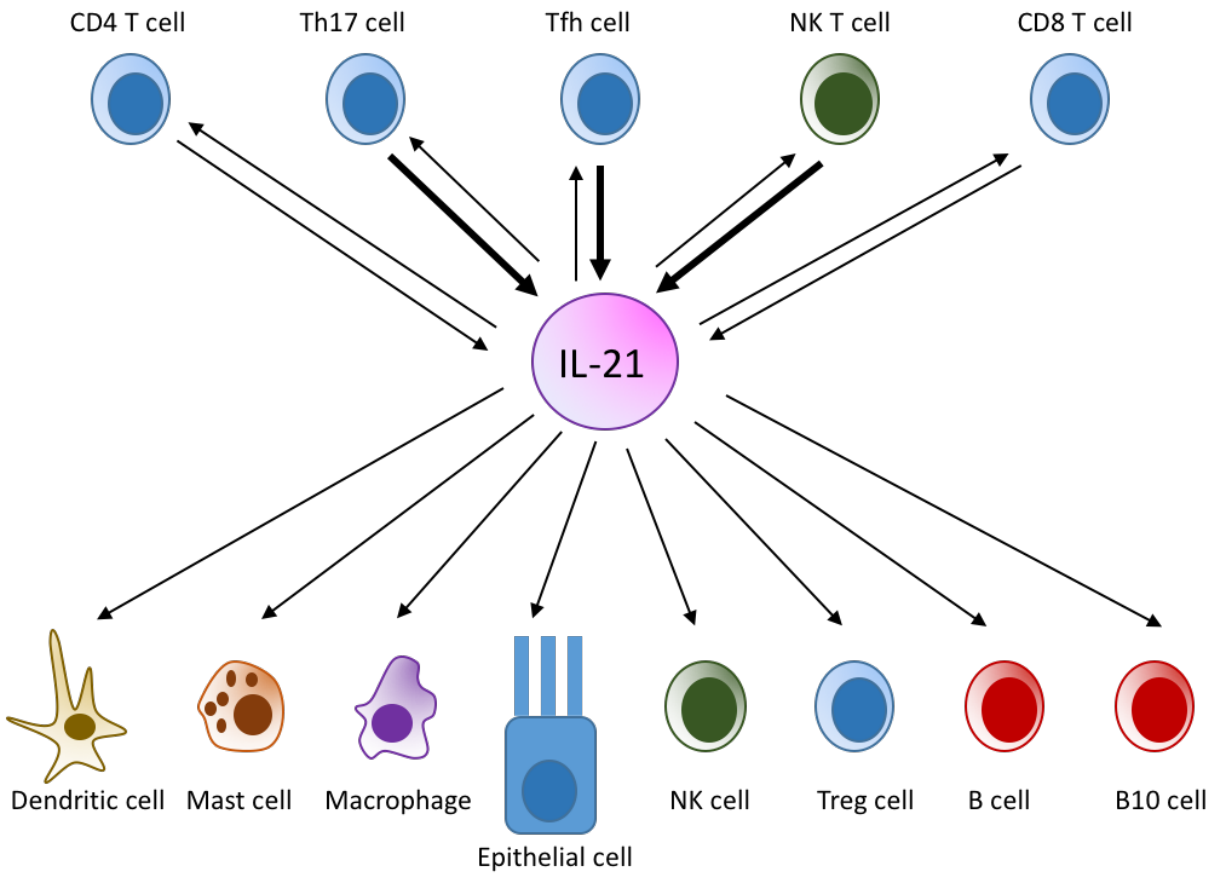

Figure 1-6. IL-21 production and signaling. Interleukin-21 (IL-21) is produced by $CD4^+$ T cell populations, with the highest production by T follicular helper (T_{FH}) cells and T_H17 cells, and slightly lower levels produced by natural killer T (NKT) cells (see bold arrows). $CD8^+$ T cells can also produce IL-21. IL-21 exerts actions on multiple lymphoid and myeloid populations as well as on epithelial cells. (modified from Spolski and Leonard)[74]

IL-21 and TfhC cells In Autoimmunity

Elevated levels of IL-21 have been found in the blood and tissue of numerous autoimmune diseases,[137] and perturbations to the effector populations of IL-21, such as Tfh cells and GC B cells, are also associated with initiation and/or progression in diseases such as rheumatoid arthritis (RA), type 1 diabetes (T1D), psoriasis, multiple sclerosis (MS), and systemic lupus erythematosus (SLE).[138] Clinical trials are currently underway to evaluate neutralization of IL-21 using monoclonal antibodies in RA and T1D.[74] Phase I and II trials have been completed and have established this treatment to be safe and pharmacologically effective.[139]

Aberrant expansion and presence of "germinal center-like" cells have also been found in peripheral tissues in cases of chronic inflammation.[140] Specifically, a population of Tfh-like cells called peripheral helper T cells (Tph) are reported to produce relatively high levels of IL-21 and operate under alternative transcriptional machinery than conventional Tfh cells. These cells were shown to functionally mimic Tfh cells (however are characterized as $PD1^+$ $CXCR5^-$), thus inducing pathology via secretion of IL-21 in peripheral tissues.[140]

IL-21 and Tfh cells in Cardiovascular Disease

Since its discovery, IL-21 has been associated with more than one facet of CVD. In a 2014 study, Ding et al. reported elevated levels of serum IL-21 in patients with CAD and levels of IL-21 proportionally increased with the number of vessels affected. Of note, when the CAD patients were stratified based on hypertensive status, those with hypertension

had significantly higher serum IL-21 levels. These data are suggestive that IL-21 is closely linked with CAD development and progression and suggest and indication in hypertension.[141] In a separate study, Hashimoto-Kataoka et al. demonstrated IL-21 promotes pulmonary arterial hypertension (PAH) via polarization of macrophages to stimulate pulmonary artery smooth muscle cell proliferation.[142] Additionally, Th cells are essential during the acute phase of ischemia reperfusion injury. Using a mouse model of transient focal brain ischemia, Clarkson et al. showed Th cells have enhanced production of IL-21 and that IL-21 deficient animals or animals treated with an IL-21 receptor Fc fusion protein have blunted reperfusion injury and end-points.[143] Taken together, these studies support the notion that IL-21 may serve as a novel therapeutic target in CVD although the mechanism is poorly understood.

Techniques to study immune cells

A key tool to study the immune system is that of genetically modified rodents. In many cases investigating the role of immune cells in hypertension, this method is utilized. Rodent models are useful in that a global genetic knockout or a cell specific genetic knockout can help reveal the function of a gene of interest.[45] While a downfall to either of these approaches is an altered baseline due to developmental compensation, animal studies allow for us to study tissues not readily accessible from human volunteers, such as, the kidney, blood vessels, and SLOs. Though rodent models are useful, the immune system does not translate perfectly between species. It remains important to have access to human peripheral blood mononuclear cells (PBMCs) from whole blood to validate rodent model findings.

Fluorescent flow cytometry is a technique commonly used to phenotype single cells. The use of antibodies conjugated to a fluorophore allow the detection and analysis of specific cell populations by lasers.[144] While this technology has continued to evolve with the addition of more lasers, fluorophore conjugates, and advanced software, there are pitfalls that remain. For instance, due to overlapping emission spectra, a limited number of fluorophores can reliably be used simultaneously, limiting the number of markers used to phenotype cells. Additionally, the user can adjust the laser voltage, therefore the fluorescence intensity is only a semi-quantitative analysis.[145] If a well-defined cell population utilizing less than ~12 markers is achievable this would not be of concern, however, if aiming to identify a new undefined cell population additional markers may be warranted.[144] The inovation of mass cytometry has helped eliminate the issue of broad spectral overlap and the semi-quantitative data output. Mass cytometry uses Cytometry by Time Of Flight (CyTOF) to identify and measure markers of single cells. This method utilizes antibodies conjugated to heavy metal isotopes rather than fluorophores.[146] As opposed to fluorescent flow cytometry, intensity is not relative since there are no laser voltages to adjust, thus resulting in quantitative data. Additionally, since this method eliminates the use of fluorescently tagged antibodies, antibody panels of greater than 30 markers can be built to accurately phenotype cell populations.[147] Thus, CyTOF is a powerful tool for the study of immunophenotyping.

Thesis objective and specific aims

Despite evidence that T and B cells play a role in hypertension, the precise T cell subsets and immune mediators, such as Tfh and IL-21, are poorly understood. This dissertation

aims to investigate the role of IL-21 and the Tfh-B cell axis on the development and maintenance of hypertension. In **Chapter 2** I test the hypothesis that IL-21 deficiency or Tfh deficiency abrogates hypertension, end-organ damage, and vascular dysfunction and inflammation and that the Tfh-B cells axis is induced during hypertension in an IL-21 dependent manner. I further present findings demonstrating the direct effect of IL-21 neutralization on blood pressure, vascular function and vascular inflammation after the onset of experimental hypertension. Lastly, I provide evidence that IL-21 is linked to hypertension in humans. In **Chapter 3** I present data demonstrating the use of mass cytometry for the identification of T cell cytokine production. The findings from these studies have elevated our understanding of the direct and non-direct effects of IL-21 and Tfh cells on the vasculature and immune system as well as cutting edge technologies we can harness to investigate novel immune cell populations. Future directions and implications of my findings are discussed in **Chapter 4**. Collectively, the establishment for a causal role of IL-21 and Tfh cells in hypertension further contributes to unraveling the role of T cell subsets in this disease as well as the revelation of a novel therapeutic target to help lessen the end-organ damage and vascular inflammation and dysfunction associated with chronic hypertension.

CHAPTER 2: CRITICAL ROLE OF INTERLEUKIN 21 AND T FOLLICULAR HELPER CELLS IN HYPERTENSION AND VASCULAR DYSFUNCTION

*contents of this chapter have been submitted to the journal of *JCI Insight*

Dale
IL-21 and Tfh cells in hypertension

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INTRODUCTION

Hypertension affects nearly one-half of all adults in the United States [1]. As a key contributor to vascular disease, chronic kidney disease, and heart failure, hypertension is the leading risk factor for global mortality [148]. Unfortunately, nearly 50% of people with hypertension have uncontrolled blood pressures despite current pharmacological therapies, highlighting the need for a better understanding of the pathophysiology of this disease and identification of novel therapeutic targets [149].

A potential role for immune cells and elevated serum immunoglobulins in hypertension was noted over 50 years ago [91, 92], but we are still far from understanding the key immune cell subsets and pathways that lead to hypertension and hypertensive end-organ damage. In 2007, Guzik et al. demonstrated that mice deficient in T and B cells develop blunted Ang II-induced hypertension, with the hypertensive response restored by adoptive transfer of T cells [96]. Our group and others demonstrated pathogenic effects of specific T cell subsets, most notably interleukin 17A (IL-17A) producing Th17 cells and interferon gamma (IFNγ) producing Th1/Tc1 cells, and protective effects of T regulatory (Treg) cells in hypertension [25]. IL-17A and IFNγ induce vascular dysfunction, glomerular injury, and renal salt and water reabsorption [25, 150]. In 2015, Chan et al. demonstrated that pharmacological or genetic depletion of B cells protects against experimental hypertension [113]. The specific T helper subset that provides help to B cells is T follicular helper (Tfh) cells that produce IL-21, and yet the role of IL-21 and Tfh cells in hypertension is unknown. Importantly, in addition to promoting Th17 and Th1 cells, IL-21 from Tfh cells drives a germinal center (GC) reaction in secondary or tertiary lymphoid organs resulting

in GC B cell immunoglobulin (Ig) class switching (e.g. IgM → IgG) and high affinity antibody production [74, 135, 151, 152]. Whether hypertension is associated with a germinal center response is not known.

Here we report the novel finding that hypertension is associated with increased Tfh and GC B cells in the aorta and increased GC B cells in secondary lymphoid organs along with increased plasma IgG1. Using genetic and/or pharmacological inhibition of IL-21 and Tfh cells, we demonstrate a previously undefined role for IL-21 in hypertension pathogenesis and vascular dysfunction, suggesting that IL-21 and Tfh cells may serve as novel therapeutic targets for this disease.

METHODS

Animals and Experimental Hypertension

Wild type C57BL/6J mice, CD4-cre transgenic mice (*Tg*^{CD4cre}), and *Bcl6*$^{flox/flox}$ mice were purchased from Jackson Laboratories. IL-21$^{-/-}$ mice were generated as previously described [153]. These mice were backcrossed greater than 10 generations to C57BL/6J mice. All protocols were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. Age and gender matched mice between 10-12 weeks of age were used. Experimental hypertension was induced using the Ang II model or deoxycorticosterone acetate (DOCA)-salt treatment. Mice were anesthetized with isoflurane via nose cone or ketamine/xylazine (90-120 mg/kg + 10mg/kg; 1:1 volume) via intraperitoneal injection. For Ang II-induced hypertension, 28-day (Alzet, DURECT Corporation, model 2004) or 14-day (Alzet, DURECT Corporation, model 2002) osmotic minipumps were implanted subcutaneously containing Ang II (490 ng/kg/min) (Sigma Catalog #A2900) or vehicle (0.08 M sodium chloride + 1% acetic acid solution). For DOCA-salt treatment, uninephrectomy was performed, a DOCA pellet (100mg; Innovative Research of America) was implanted subcutaneously, and the drinking water was supplemented with 1% NaCl for 21 days. Animals were euthanized after 7, 14, 21, or 28 days as indicated by CO_2 inhalation.

Blood pressure (BP) measurement

BP was measured twice weekly and averaged using a noninvasive tailcuff platform (Hatteras) or invasively by carotid radiotelemetry as previously described [45]. Mice were

allowed to recover for 10-14 days post-telemetry implantation prior to obtaining baseline BPs and implantation of osmotic minipumps.

Murine T cell isolation, culture, and cytokine quantification

Spleen homogenates were filtered through a 40 μm cell strainer followed by depletion of red blood cells using red blood cell lysis buffer (eBioscience). $CD4^+$ and $CD8^+$ T cells were isolated from splenic single-cell suspensions using Miltenyi cell separation kits according to the manufacturer's instructions and sorted using an AutoMACS magnetic cell sorter (Miltenyi Biotec). Cells were plated in RPMI 1640 media + 10% FBS+ 1% penicillin/streptomycin + 50μM β-mercaptoethanol at a density of 200,000 cells/100μl on a non-tissue culture treated 96-well plate coated with mouse anti-CD3 (2μg/ml) and mouse anti-CD28 (2μg/ml) antibodies (BD Biosciences) for 72hours. Murine IL-17A, IFNγ, interleukin 10 (IL-10) and IL-21 were measured from cell culture supernatants by ELISA Ready-Set-Go! Kits (eBioscience).

RNA isolation and qRT-PCR

RNA was isolated from cells using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA concentration was measured using a DS-11 spectrophotometer (DeNovix). A High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used according to the manufacturer's instructions to make cDNA. The Taqman assay system (Thermo Fisher Scientific) was used to detect *Il21*, *Bcl6*, and *Gapdh* (endogenous control). Relative quantification was determined using the comparative CT method with data normalized to *Gapdh* and calibrated to the average of the control group.

Immunohistochemistry, histological staining, and imaging

10% formalin-fixed, paraffin-embedded tissue sections of aorta or spleen from WT or IL-21$^{-/-}$ mice treated with 28 days of Ang II or vehicle were sectioned at 5 micron thickness. Slides were labeled with anti-CD3 (ab16669, Abcam, 1:250 dilution), anti-B220 (553086, BD Pharmingen, 1:20,000 dilution), or PNA (B1075, Vector, 1:250 dilution) to detect T cells, B cells, and GC B cells, respectively. For collagen staining, slides were stained with Masson's Trichrome Blue or Picrosirius Red as previously described [44, 154]. Slides were imaged at 20x using a Leica SCN400 Slide Scanner and acquired and exported using Leica Biosystems.

Vascular reactivity studies

Isometric tension studies were conducted using 2-mm segments of third-order mesenteric arterioles dissected free of perivascular fat. Studies were performed in a small vessel horizontal wire myograph (Danish Myo Technology, models 610M and 620M) as previously described [45]. Vessels were pre-constricted with norepinephrine prior to treatment with increasing doses of acetylcholine (Ach) or sodium nitroprusside (SNP). For *ex vivo* IL-21 treatment experiments, vessels were incubated with recombinant murine IL-21 (100 ng/ml; Peprotech) or vehicle (phosphate buffered saline (PBS)) in the organ chamber for 1 hr between assessments of endothelium-dependent relaxation to Ach.

Mesenteric vessel superoxide measurement

Superoxide levels were measured from mesenteric arterioles by quantification of 2-hydroxyethidium (2-OH Eth) from dihydroethidium (DHE) by high performance liquid chromatography. The 2-OH Eth product specifically reflects the interaction of DHE with

superoxide as previously validated [155].

Fluorescent flow cytometry staining of lymph node and aortic leukocytes

Mesenteric lymph nodes free of surrounding fat were homogenized and filtered through a 40 μm cell strainer. Single-cell suspensions of whole aorta with adjacent perivascular fat were isolated as previously described [156]. Samples were stained for viability and surface markers using the following reagents for the *T cell panel*: LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), brilliant violet 510 (BV510) anti-CD45 (Biolegend, clone 30-F11), peridinin chlorophyll protein-cyanin-5.5 (PerCp-Cy5.5) anti-CD3 antibody (BioLegend, clone 17A2), allophycocyanin (APC) anti-CD8 antibody (eBioscience, clone 53-6.7), allophycocyanin-cyanin-7 (APC-Cy7) anti-CD4 antibody (Biolegend, clone GK1.5), phycoerythrin-cyanin-7 (PE-Cy7) anti-CXCR5 antibody (BD Pharmingen, clone 2G8), and fluorescein isothiocyanate (FITC) anti-PD1 (eBioscience, clone J43); *B cell panel*: LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), BV510 anti-CD45 (Biolegend, clone 30-F11), PerCp-Cy5.5 anti-CD45R/B220 antibody (BD Biosciences, clone RA3-6B2), APC anti-IgD antibody (eBioscience, clone 11-26), APC-Cy7 anti-CD138 antibody (Biolegend, clone 281-2), PE-Cy7 anti-CD19 antibody (BD Pharmingen, clone 1D3), phycoerythrin (PE) anti- GL7 (Invitrogen, clone GL-7), FITC anti-Fas (eBioscience, clone 15A7); *Innate immune cell panel*: LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), BV510 anti-CD11b (Biolegend, clone M1/70), PerCp-Cy5.5 anti-CD3 antibody (BioLegend, clone 17A2), APC anti-NK1.1 antibody (Biolegend, clone PK136), APC-Cy7 anti-F4/80 antibody (Biolegend, clone BM8), PE anti-Ly6G (Biolegend, clone 1A8) and FITC anti-CD45 (Biolegend, clone 30-F11). Staining was performed in 100μl. All antibodies were used at 1:100 dilution. Samples were

acquired on a BD FACSCanto II system and analyzed using CytoBank. Gates were applied using fluorescence minus one (FMO) controls. A known quantity of counting beads (123count eBeads, eBioscience) were added to each sample prior to acquisition. Results were normalized using the bead count.

Antibody treatments

Mice were implanted with 28-day osmotic minipumps containing Ang II. After 14 days of Ang II infusion, mice were randomized to either an isotype control group (eBioscience, rat IgG2 k isotype control functional grade purified, clone eBRa) or mouse anti-IL-21 treatment group (eBioscience, anti-mouse anti-IL-21 functional grade purified, clone FFA21). Treatment was administered by intraperitoneal injection with 100μg of antibody twice a week for the last 2 weeks of Ang II infusion (days 16, 19, 22, and 25). This regimen was chosen based on prior studies [157].

Plasma immunoglobulin isolation and quantification

Mice were euthanized, and whole blood was collected by direct cardiac puncture into an EDTA coated syringe. Blood was immediately centrifuged, and plasma stored at -80°C. Plasma immunoglobulin was quantified using LEGENDplex Mouse Immunoglobulin Isotyping Panel (Biolegend) according to the manufacturer's instructions.

Dendritic cell (DC)-CD4$^+$ T cell co-culture

Splenic single cell suspensions were generated as described above. Dendritic cells (CD11c$^+$) and naïve CD4$^+$ T cells (CD44$^-$) were enriched using Miltenyi cell separation kits (Cat no 130-108-338 and 130-104-453) according to the manufacturer's instructions and sorted using an AutoMACS magnetic cell sorter (Miltenyi Biotec). 200,000 cells were

plated in duplicate at a 1:5 ratio (DC:T) in RPMI 1640 media + 10% FBS+ 1% penicillin/streptomycin + 50μM β-mercaptoethanol + 1mM Sodium Pyruvate + 1% L-glutamine at a density of $1x10^6$ cells/ml in a tissue culture treated 96-well round bottom plate for 72 hours.

Nitric oxide measurement from cultured endothelial cells

Human aortic endothelial cells were purchased from Lonza (CC-2535; Lot No. 0000297640) and grown to 90% confluency in endothelial cell growth medium (PromoCell). The cells were detached using trypsin, resuspended in endothelial cell media, and allowed to rest for 30 minutes at 37 degrees. Cells were loaded for 30 minutes with 1μM of 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM diacetate; ThermoFisher) dissolved in DMSO. Cells were then incubated with 100 ng/mL of recombinant human interleukin-21 (PeproTech) or vehicle (PBS). After 15 minutes, the cells were acquired via flow cytometry. Gating and data analysis were performed in FlowJo.

Human Subjects

Blood samples were obtained as part of Vanderbilt's American Heart Association Strategically Focused Research Network protocol, which was approved by the Vanderbilt Institutional Review Board (IRB#141382) and conforms to the standards set forth by the US Federal Policy for the Protection of Human Subjects. All volunteers provided written informed consent. Inclusion criteria were age 30-80 years, systolic blood pressures 110-150 mmHg and/or diastolic blood pressure 80-99 mmHg on the day of the study visit. Blood pressure (BP) was measured in the seated position after a 10-15 min rest period

by an automated cuff (GE CRITIKON Dinamap Pro 1000). Exclusion criteria were pregnancy, intolerance to study protocols, acute cardiovascular events within the previous 6 months, impaired renal function (estimated GFR < 45 ml/min/1.73m^2), current or recent (within 1 month) treatment with systemic glucocorticoid therapy, current use of hypertensive drugs (except calcium channel blockers and beta blockers), pharmacologically treated diabetes mellitus, morbid obesity (BMI >45), prior adverse reaction to thiazide or spironolactone, contraindications to MRI, impaired hepatic function (aspartate amino transaminase and/or alanine amino transaminase > 1.5x upper limit of normal range), current illicit drug use, and sexually active women of childbearing potential not conforming to use of birth control.

Human T cell isolation, culture, and cytokine quantification

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll plaque centrifugation (GE Life Science). CD4$^+$ T cells were isolated from the Ficoll buffy coat using Miltenyi cell separation kits according to the manufacturer's instructions and sorted using an AutoMACS magnetic cell sorter (Miltenyi Biotec). Cells were plated in RPMI 1640 media + 10% FBS+ 1% penicillin/streptomycin + 50μM β-mercaptoethanol at a density of 200,000 cells/200μl on a non-tissue culture treated 96-well plate coated with human anti-CD3 (2μg/ml) and human anti-CD28 (2μg/ml) antibodies (BD Biosciences) for 72 hours. Human IL-21 and IL-17A was measured from cell culture supernatants by LEGENDplex bead assay (Biolegend).

Statistics

All data are expressed as mean ± SEM or as box and whisker plots as indicated. Data were analyzed in GraphPad Prism or R using Student's *t*-test (for 2 group comparisons) or 2-way ANOVA (for multiple group comparisons). Nonparametric 2 group data were analyzed by Mann Whitney test. Vascular relaxation studies were compared using area under the curve analysis. Human cytokine data were log transformed for Pearson's correlation analysis. $P < 0.05$ was considered significant.

RESULTS

Hypertension is associated with increased CD4$^+$ T cell production of IL-21, and IL-21 deficiency attenuates the rise in blood pressure (BP) in response to hypertensive stimuli. To determine the role of IL-21 in hypertension, we first isolated splenic CD4$^+$ T cells from C57/Bl6J wild type (WT) mice infused with vehicle or Ang II for 4 weeks and cultured them in the presence of anti-CD3 and anti-CD28 antibodies for 72 hrs. We found that CD4$^+$ T cells from hypertensive mice display increased *Il21* mRNA expression (**Figure 2-1A**) and increased IL-21 secretion (**Figure 2-1B**). We then investigated the effect of IL-21 deficiency on Ang-II induced hypertension. Following 4 weeks of Ang II (490 ng/kg/min) infusion, IL-21$^{-/-}$ male mice develop a systolic BP approximately 20 mmHg lower than WT animals by tail cuff (**Figure 2-1C**) and invasive radio telemetry (**Figure 2-1D**). Diastolic BP was similarly reduced in IL-21$^{-/-}$ mice compared to WT controls with no change in heart rate (**Figures 2-1E and 1F**). To determine if there are gender differences in the effect of IL-21 on hypertension, we studied female mice and found that both the hypertensive response and effect of IL-21 deficiency were similar to those observed in male mice (**Figure 2-S1A**). To confirm that the BP protection was not unique to the Ang II model, we investigated the effect of IL-21 deficiency in a salt-sensitive hypertension model characterized by uninephrectomy, implantation of a deoxycorticosterone acetate (DOCA) pellet, and 1% NaCl in the drinking water and observed a similar 20 mmHg reduction in systolic BP (**Figure 2-S1B**). All further studies were conducted in male mice using the Ang II hypertension model.

Loss of IL-21 protects against Ang II-induced vascular remodeling and endothelial dysfunction. Hypertension is associated with increased aortic collagen deposition, vascular smooth muscle cell hypertrophy, and microvascular endothelial dysfunction. To determine the effect of IL-21 deficiency on vascular remodeling and endothelial function, WT and IL-21$^{-/-}$ mice were infused with vehicle or Ang II for 4 weeks. Importantly, IL-21$^{-/-}$ mice thoracic aortas exhibit significantly less aortic collagen deposition and reduced medial hypertrophy (**Figures 2-2A-C**). Representative aortic cross-sections are shown in **Figures 2-2A-B** with quantification in **Figure 2-2C**. Resistance artery endothelial function was assessed by measuring endothelium-dependent and -independent relaxation of third-order mesenteric arterioles. A modest baseline impairment in endothelium-dependent relaxation to acetylcholine (Ach) is present in IL-21$^{-/-}$ mesenteric arterioles, but the key finding is that the IL-21$^{-/-}$ mice are completely protected from further endothelial dysfunction in response to Ang II infusion, while WT vessels exhibit a severe impairment in endothelium-dependent relaxation in response to Ang II. There was no effect of Ang II or IL-21 deficiency on endothelium-independent relaxation to sodium nitroprusside (SNP) (**Figure 2-2D**). Since vascular reactivity can be mediated by alterations in superoxide levels, we measured superoxide production in isolated mesenteric arterioles. Interestingly, IL-21$^{-/-}$ mice exhibit increased superoxide levels at baseline, consistent with their baseline impairment in vascular reactivity, but no further increase in response to Ang II infusion. In contrast, WT mice exhibit a marked upregulation of superoxide production in response to Ang II-induced hypertension (**Figure 2-2E**).

IL-21 deficiency blunts early vascular infiltration of natural killer (NK) T cells and macrophages. We previously showed that during the initial phase of Ang II-induced hypertension (day 7), there is an increase in total aortic leukocytes composed primarily of innate immune cells such as F4/80^{+} monocytes/macrophages [45]. Here we quantified total leukocytes (CD45^{+}), NK T cells (CD3^{+}NK1.1^{+}), macrophages (F4/80^{+}Ly6G^{-}), and neutrophils (F4/80^{-}Ly6G^{+}) in the aorta of WT and IL-21$^{-/-}$ mice following 7 days of vehicle or Ang II infusion. Flow cytometry gating strategy and fluorescence minus one controls are shown in **Figure 2-S2**. Macrophages and neutrophils were gated from CD11b^{+} cells and NK T cells were gated from CD3^{+} cells (**Figure 2-S2**). Representative biaxial flow cytometry dot plots are shown for each group (**Figure 2-3A**). We found that Ang II-induced hypertension is associated with an early increase in total leukocytes, NK T cells, and macrophages, and this increase is abrogated in IL-21$^{-/-}$ mice (**Figure 2-3B**). Neutrophils were not increased by Ang II infusion or affected by IL-21 deficiency (**Figure 2-3B**). Thus, IL-21 deficiency protects from early innate immune cell infiltration into the aorta.

Hypertension induces GC-like cells in the vasculature and increased splenic T cell cytokine production in an IL-21 dependent manner. Peripheral GC-like cells have been found in inflamed tissues in autoimmune conditions such as multiple sclerosis, rheumatoid arthritis, and lupus nephritis [140, 158, 159]. Since IL-21 is a key driver of the GC reaction and produced predominantly by Tfh or peripheral helper T (Tph) cells (which do not express the lymphoid follicle homing chemokine receptor CXCR5 but still retain PD1) [140], we determined whether the number of these cells and GC B cells are altered in the aorta during experimental hypertension. WT and IL-21$^{-/-}$ mice were infused with vehicle

or Ang II for 4 weeks. Flow cytometry gating strategy and fluorescence minus one controls are shown in **Figures 2-S3-S4**. Total leukocytes, $CD4^+$ T cells, and $CD19^+B220^+$ B cells increase in WT mice in response to 4 weeks of Ang II infusion, and this increase is blunted in IL-$21^{-/-}$ mice (**Figures 2-S3-S4**). Tfh cells and Tph cells are gated from $CD4^+$ T cells, and GC B cells are gated from total B cells that are also IgD^- and $CD138^-$. Representative biaxial flow cytometry dot plots for Tph cells ($PD1^+CXCR5^-$), Tfh cells ($PD1^+CXCR5^+$), and GC B cells (Fas^+GL7^+) are shown for each group (**Figures 2-4A-B**). We found that Tph, Tfh, and GC B cells accumulate in the aorta of WT, but not IL-$21^{-/-}$ animals, in response to Ang II-induced hypertension (**Figure 2-4C**). We previously showed that hypertension is associated with increased T cell production of the pro-inflammatory cytokines IL-17A and IFNγ [43, 44]. IL-21 can potentially modulate the balance of Th17, Th1, and Treg subsets in disease [135, 160]. Therefore, we investigated the effect of IL-21 deficiency on T cell production of IL-17A, IFNγ, and IL-10 in hypertension. Consistent with our prior results, we detected an increase in $CD4^+$ T cell production of IL-17A and $CD8^+$ T cell production of IFNγ in splenic T cells cultured from hypertensive vs normotensive mice, and these hypertension-induced changes were completely abrogated in IL-$21^{-/-}$ mice. Neither Ang II nor IL-21 deficiency affected $CD4^+$ T cell production of IL-10 (**Figure 4D**). Thus, hypertension is associated with increased aortic accumulation of Tph, Tfh, and GC B cells and increased splenic T cell production of IL-17A and IFNγ in an IL-21 dependent manner.

Hypertension induces an IL-21 dependent GC response, tertiary lymphoid development, and increased IgG production. The primary effector function of Tfh cells is to stimulate GC B cells in secondary and tertiary lymphoid organs to undergo cycles of proliferation and selection leading to the production of class switched and/or high affinity antibodies and long-lived plasma cells [161]. Although B cells have recently been implicated in the pathogenesis of hypertension [113], it is not known whether hypertension is associated with a GC reaction as observed in the setting of infection, vaccination, and some autoimmune diseases [162]. Due to their proximity to mesenteric resistance vessels in which IL-21 deficiency was shown to play a protective role, we isolated mesenteric lymph nodes from WT and IL-21$^{-/-}$ mice infused with vehicle or Ang II for 4 weeks and quantified Tfh and GC B cells. Tfh cells were expressed as percent of total CD4^{+} T cells, and GC B cells were expressed as percent of total B cells. Ang II infusion did not alter the percentage of Tfh cells in mesenteric lymph nodes of WT mice, but Tfh cells were reduced both at baseline (vehicle infusion) and after Ang II infusion in IL-21$^{-/-}$ mice (**Figure 2-5A**). Interestingly, the percentage of GC B cells increased in mesenteric lymph nodes of WT mice in response to Ang II-induced hypertension. In contrast, IL-21$^{-/-}$ mice had fewer GC B cells at baseline (vehicle infusion) and exhibited no increase in these cells after Ang II infusion (**Figure 2-5B**). Of note, neither hypertension nor IL-21 deficiency is associated with changes in overall immune cell numbers or composition in the mesenteric lymph nodes as total CD45^{+} leukocytes, percent of CD4^{+} T cells, and percent of total B cells were unchanged in all four groups (**Figure 2-S5A**). We then investigated follicular architecture and germinal centers in the spleens of WT and IL-21$^{-/-}$ mice following 4 weeks of vehicle or Ang II infusion. Interestingly, and consistent with the findings of Drummond and

colleagues [113], we observed an increase in follicle number and decrease in follicle size in spleens of hypertensive WT mice, and this finding was attenuated in IL-21$^{-/-}$ mice. Furthermore, PNA staining to detect germinal centers demonstrated almost complete absence of germinal center B cells in IL-21$^{-/-}$ spleens (**Figure 2-5C**). To determine whether tertiary lymphoid organs (TLOs) form in hypertensive aortas, we performed whole mount immunohistochemistry of aortas from WT mice infused with 4 weeks of Ang II. We found evidence of T and B cell clusters resembling TLOs as well as sites of unstructured T and B cell aggregates (**Figure 2-5D**) in hypertensive aortas. We then quantified plasma immunoglobulins in WT and IL-21$^{-/-}$ mice infused with 4 weeks of vehicle or Ang II. Ang II-induced hypertension is associated with an increase in total IgG, driven primarily by the IgG1 subclass, in WT but not IL-21$^{-/-}$ mice. IgG2a was not significantly increased by Ang II infusion but was higher in Ang II-infused WT vs IL-21$^{-/-}$ mice. There was no change in the other IgG subclasses or IgM between all four groups (**Figures 2-5E and 2-S5B**). Taken together, these data suggest that hypertension is associated with an IL-21-dependent germinal center response.

Hypertensive DCs induce Tfh cell polarization, and Tfh cells play a critical role in hypertension. Hypertensive stimuli have been shown to activate DCs which in turn stimulate T cell proliferation through presentation of isolevuglandin-modified peptides [107]. To determine if hypertensive DCs promote naïve T cells to polarize to Tfh cells, we utilized a co-culture assay. Splenic DCs from mice infused with vehicle or Ang II for 2 weeks were isolated and co-cultured with naïve CD4^{+} T cells at a 1:5 ratio for 72 hours (**Figure 2-6A**). Hypertensive DCs induced a 2-fold increase in Tfh cell polarization compared to

normotensive DCs (**Figure 2-6A**). Since BCL6 is an essential transcription factor for the Tfh cell differentiation program, we generated mice with Tfh cell deficiency by crossing floxed BCL6 mice (*Bcl6*$^{fl/fl}$) with mice transgenic for cre recombinase under the control of the CD4 promoter (*Tg*^{CD4cre}). To confirm T cell deletion of BCL6, we isolated CD4^{+} T cells from the spleen of *Bcl6*$^{fl/fl}$ *Tg*^{CD4cre} mice and cre negative *Bcl6*$^{fl/fl}$ littermate controls and demonstrated a significant reduction in *Bcl6* expression by qRT-PCR in *Bcl6*$^{fl/fl}$*Tg*^{CD4cre} mice (**Figure 2-S6A**). To determine the effect of Tfh cell depletion on hypertension, we infused Ang II for 4 weeks in *Bcl6*$^{fl/fl}$*Tg*^{CD4cre} mice and *Bcl6*$^{fl/fl}$ littermate controls. Although the initial rise in systolic BP was similar in both groups of mice, Tfh deficient mice were unable to sustain the hypertensive response, reaching a systolic BP approximately 20 mmHg lower than floxed control mice by day 28 (**Figure 2-6B**). We then assessed aortic smooth muscle cell hypertrophy, mesenteric endothelial function, and vascular inflammation in these mice. Aortic media thickness was significantly decreased in the Tfh cell deficient mice following Ang II infusion with no change in endothelium-dependent relaxation (**Figures 2-6C-D and 2-S6B**), similar to that seen in IL-21 deficient mice. In contrast to IL-21$^{-/-}$ mice, there was no change in the overall number of aortic total leukocytes, CD4+ T cells, B cells, and Tph cells, but there was a specific reduction in aortic Tfh and GC B cells (**Figure 2-6E**). This intermediate phenotype observed in Tfh deficient mice compared to IL-21 deficient mice suggests that there may be additional sources of IL-21, such as Tph cells, that are relevant in hypertension or a compensatory increase in other T cell subsets, such as Th17 cells. Indeed, we isolated splenic T cells and quantified IFNγ and IL-17A production from Tfh deficient and floxed control mice following 4 weeks of Ang II infusion and found no change in IFNγ production, but

increased IL-17A production (**Figure 2-S6C**). Taken together, these results highlight the critical role played by Tfh cells in hypertension independent of alterations in other T cell subsets.

Anti-IL-21 treatment lowers blood pressure and reverses endothelial dysfunction and vascular inflammation. To determine whether pharmacologic targeting of IL-21 may be a beneficial therapeutic strategy, WT mice were infused with Ang II for 4 weeks and randomized to receive twice weekly injections of isotype control or an anti-IL-21 neutralizing antibody (100μg/injection) during the last 2 weeks. Mice that received anti-IL-21 treatment exhibit an approximately 15 mmHg reduction in BP (**Figure 2-7A**). Importantly, anti-IL-21 treatment restores endothelium-dependent relaxation to Ach to levels comparable with vehicle infused WT animals (**Figure 2-7B**). Endothelium-independent relaxation to SNP was similar in both groups (**Figure 2-S7**). In addition, anti-IL-21 treatment decreases aortic inflammatory cells, including Tph, Tfh, and GC B cells, to levels comparable to vehicle infused WT animals (**Figure 2-7E**). Thus, inhibition of IL-21 lowers BP and reverses endothelial dysfunction and vascular inflammation.

Recombinant IL-21 impairs endothelium-dependent relaxation ex vivo and decreases nitric oxide production from cultured human aortic endothelial cells. To determine whether IL-21 has direct vascular effects independent of T and B cells, we isolated third order mesenteric arterioles from WT mice and assessed endothelium-dependent relaxation to Ach before and after 1 hour of incubation with recombinant IL-21 protein (100 ng/ml) or vehicle (PBS). IL-21 but not vehicle treatment resulted in impaired endothelium-

dependent relaxation (**Figures 2-8A-B**). We have previously shown that vascular NO levels are decreased in WT mice in response to Ang II infusion [44]. To determine the effect of IL-21 on endothelial NO production, we cultured human aortic endothelial cells (HAECs) with vehicle or recombinant IL-21 and assessed NO production using flow cytometry with the DAF-FM dye. IL-21 decreased NO production from these cells (**Figure 2-8C**). Thus, IL-21 has direct detrimental effects on the vessel wall independent of alterations in T and B cells.

$CD4^+$ T cell production of IL-21 correlates with systolic BP and IL-17A in humans. To determine the relevance of our findings to human hypertension, we quantified IL-21 and IL-17A production from cultured $CD4^+$ T cells isolated from the peripheral blood of 37 subjects (**Figure 2-9A and Table 2-S1)**. Systolic BP (SBP) was measured on the day of blood draw and significantly correlated with IL-21 levels (**Figure 2-9B**). In addition, when stratified by SBP less than or greater than or equal to 130 mmHg, IL-21 production from $CD4^+$ T cells was significantly higher in those with SBP greater than or equal to 130 mmHg (**Figure 2-9C**). In this sample set, IL-21 levels did not differ significantly between blacks and whites or males and females and did not correlate with BMI or age. Furthermore, T cell production of IL-21 strongly correlated with IL-17A production (**Figure 2-9D**), and this correlation remained significant in multivariate analysis after controlling for race, gender, age, and BMI. This finding suggests that the same or similar cells are producing IL-21 and IL-17A in human hypertension and/or that one cytokine strongly regulates the other. As described above, IL-21 is known to promote IL-17A production. Taken together, these data suggest that IL-21 and the cells that produce it are an attractive therapeutic target

for hypertension and its vascular complications.

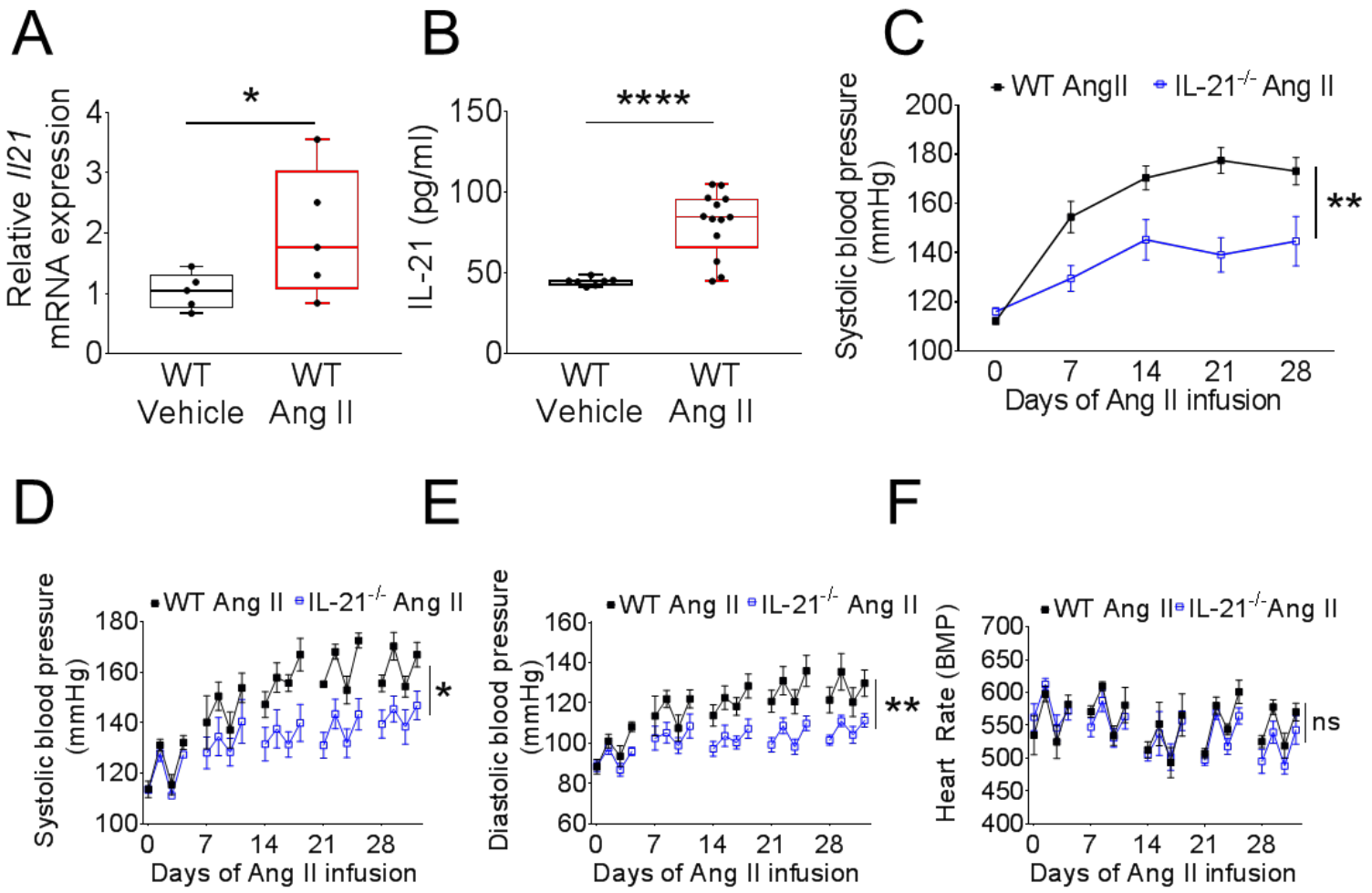

Figure 2-1. Hypertension is associated with increased CD4+ T cell production of interleukin 21 (IL-21), and IL-21 deficiency blunts the hypertensive response to Ang II infusion. (**A**) Relative il21 mRNA expression by quantitative real time PCR from splenic CD4+ T cells cultured for 72 hours with anti-CD3/anti-CD28 coated plates (n=5). (**B**) IL-21 protein was quantified in culture supernatants by ELISA (n=7-13). (**C**) Systolic blood pressure was measured by tail-cuff weekly over 28 days of Ang II infusion in wild type (WT) and IL-21-/- mice (n=8-9). (**D**) Systolic blood pressure, (**E**) diastolic blood pressure, and (**F**) heart rate were measured invasively weekly using carotid radiotelemetry over 28 days of Ang II infusion in WT and IL-21-/- mice (n=5-8). Data are expressed as box and whisker plots (**A-B**) or mean ± SEM (C-F); *P<0.05, **P<0.01, ****P<0.0001 by Student's t-test (**A-B**) or 2-way ANOVA with repeated measures (**C-F**).

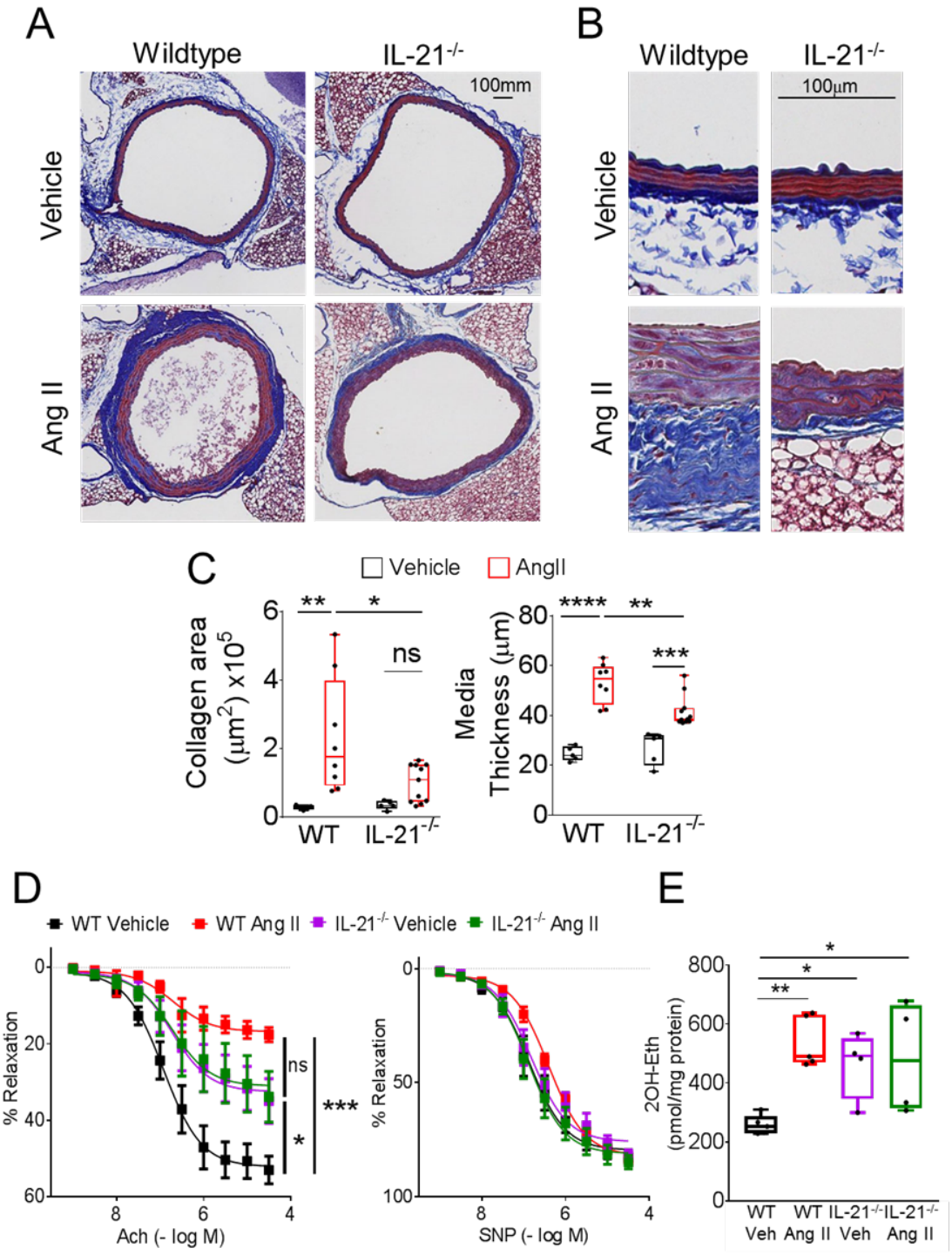

Figure 2-2. Loss of IL-21 protects against Ang II-induced vascular remodeling and endothelial dysfunction. WT and IL- $21^{-/-}$ mice were infused with Ang II or vehicle for 28 days. Representative images of (**A**) bright field aortic collagen deposition and (**B**) media thickness by Masson's trichrome blue staining. (**C**) Quantification of aortic collagen deposition area and media thickness (n=5-11). (**D**) Endothelium-dependent relaxation in response to increasing doses of acetylcholine (Ach) (left) and endothelium-independent relaxation in response to increasing doses of sodium nitroprusside (SNP) (right) were measured in isolated mesenteric arterioles (n=6-8). (**E**) Superoxide levels from mesenteric arterioles as measured by quantification of 2-hydroxyethidium (2OH-Eth) (n=4-5). Data are expressed as box and whisker plots (**C and E**) or mean ± SEM (**D**); **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 by 2-way ANOVA (**C-E**).

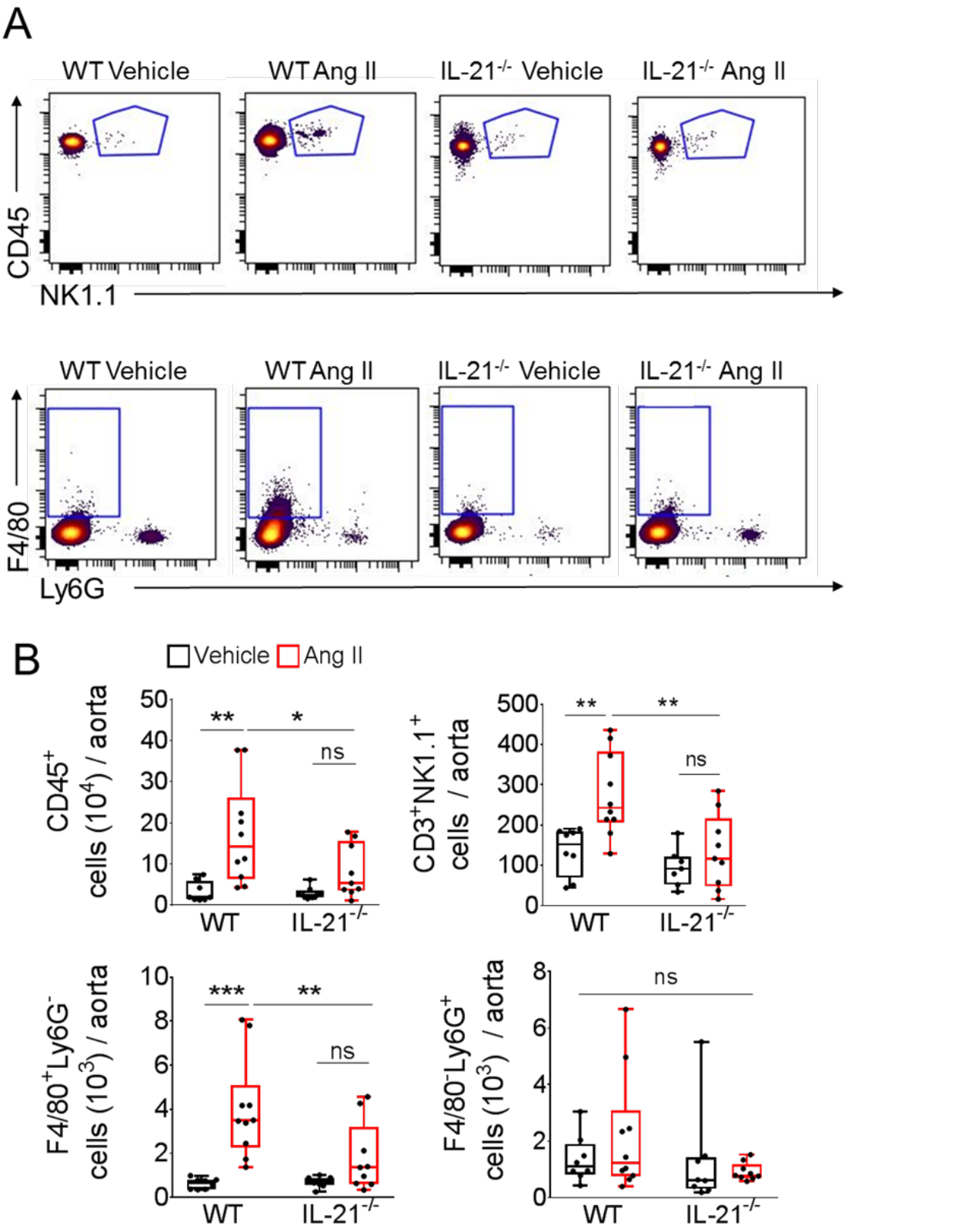

Figure 2-3. IL-21 deficiency blunts early vascular infiltration of natural killer (NK) T cells and macrophages. (**A**) Representative flow cytometry bi-axial plots for NK T cells ($NK1.1^+$), macrophages ($F4/80^+Ly6G^-$), and neutrophils ($F4/80^-Ly6G^+$) in the aorta of WT and IL-$21^{-/-}$ mice infused with Ang II or vehicle for 7 days. (**B**) Summary quantification of leukocytes ($CD45^+$ cells), NK T cells ($CD3^+NK1.1^+$ cells), macrophages ($F4/80^+Ly6G^-$), and neutrophils ($F4/80^-Ly6G^+$) (n=7-10). Data are expressed as box and whisker plots; **P*<0.05, ***P*<0.01, ****P*<0.001 by 2-way ANOVA.

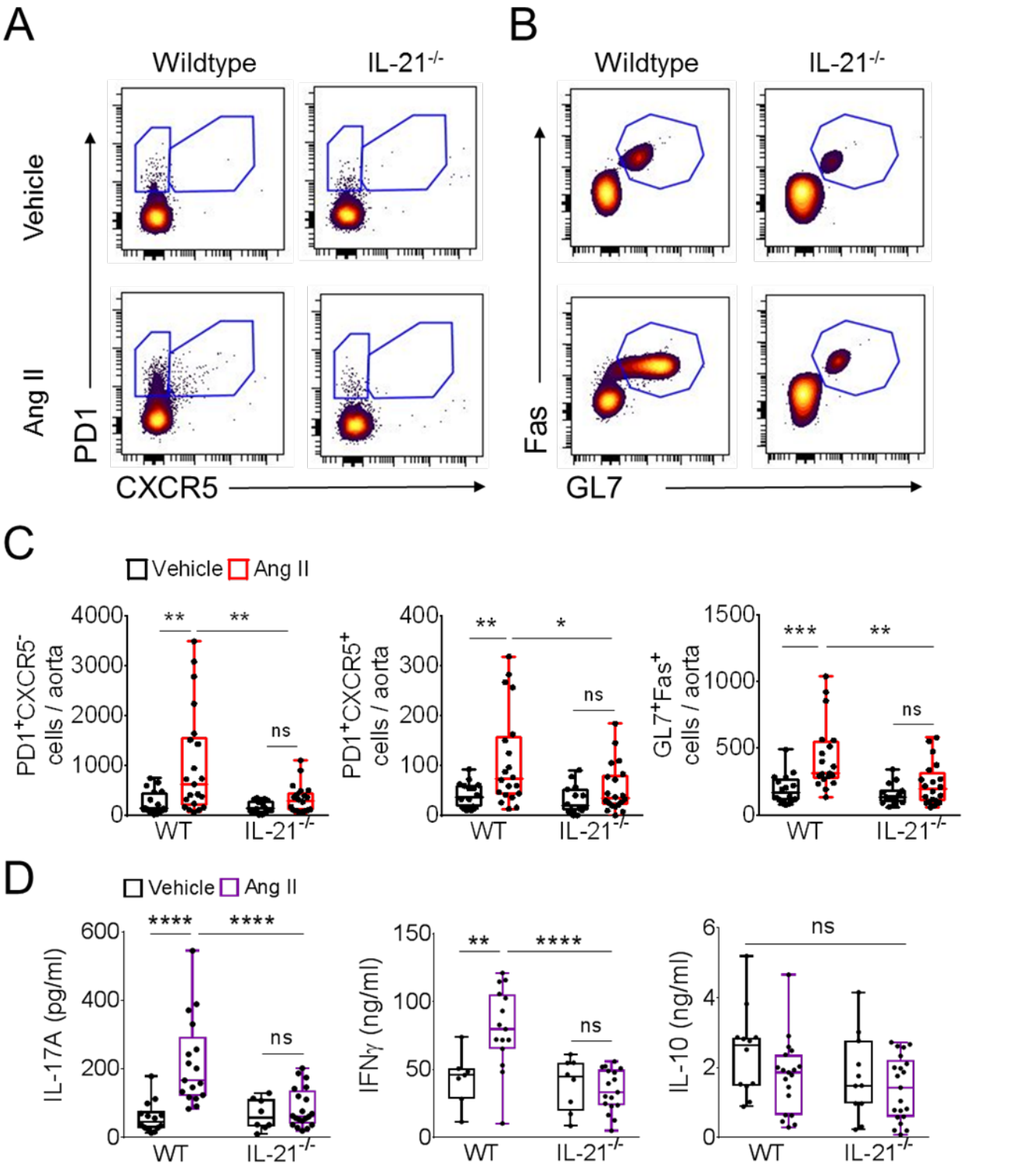

Figure 2-4. Hypertension induces peripheral T helper (Tph), T follicular helper (Tfh), and germinal center (GC) B cells in the aorta in an IL-21 dependent manner. (**A**) Representative flow cytometry bi-axial plots for Tph ($PD1^+CXCR5^-$), Tfh ($PD1^+CXCR5^+$) cells, and (**B**) GC B cells ($GL7^+Fas^+$) in the aorta of WT and IL-$21^{-/-}$ mice infused with Ang II or vehicle for 28 days. (**C**) Summary quantification of Tph, Tfh, and GC B cells (n=14-20). (**D**) Splenic $CD4^+$ T cell production of interleukin 17A (IL-17A) and interleukin 10 (IL-10) and $CD8^+$ T cell production of interferon gamma (IFNg) from WT and IL-$21^{-/-}$ mice infused with Ang II or vehicle for 28 days quantified by ELISA (n=7-21). Data are expressed as box and whisker plots; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001 by 2-way ANOVA.

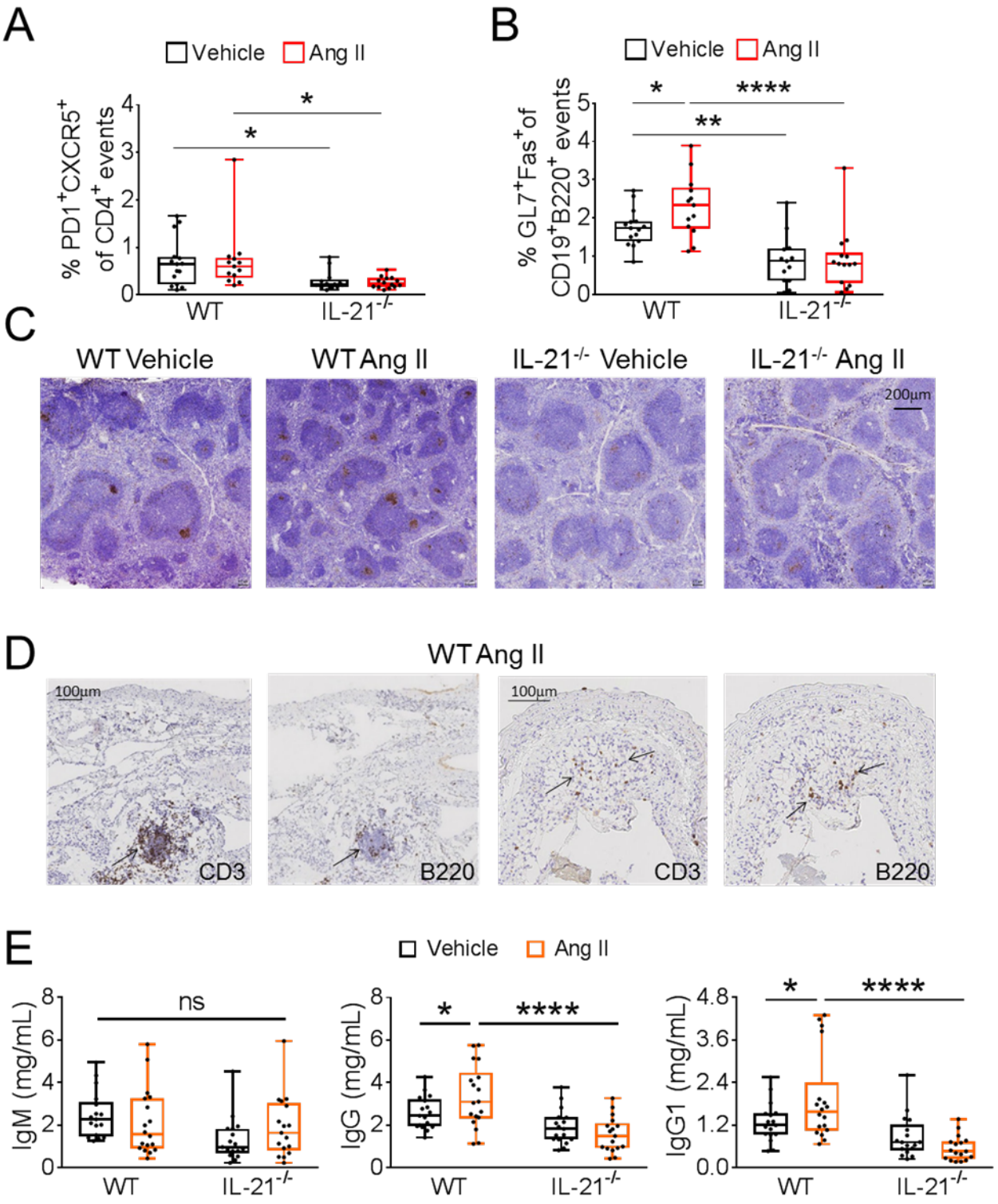

Figure 2-5. Hypertension induces an IL-21 dependent germinal center (GC) response, tertiary lymphoid development, and increased IgG production. WT and IL- 21$^{-/-}$ mice were infused with Ang II or vehicle for 28 days. (**A**) Percent Tfh cells (PD1^{+}CXCR5^{+}) of CD4^{+} T cells and (**B**) percent GC B cells (GL7^{+}Fas^{+}) of total CD19^{+}B220^{+} B cells from mesenteric lymph nodes (n=13-15). (**C**) Bright field images of splenic cross sections stained for the GC B cell marker PNA (representative of n=4-7). (**D**) Example bright field images of aortic sections stained for CD3 (T cells) and B220 (B cells). (**E**) Plasma immunoglobulin concentration of IgM, IgG, and IgG1 (n=17-18). Data are expressed as box and whisker plots; *P<0.05, **P<0.01, ****P<0.0001 by 2-way ANOVA.

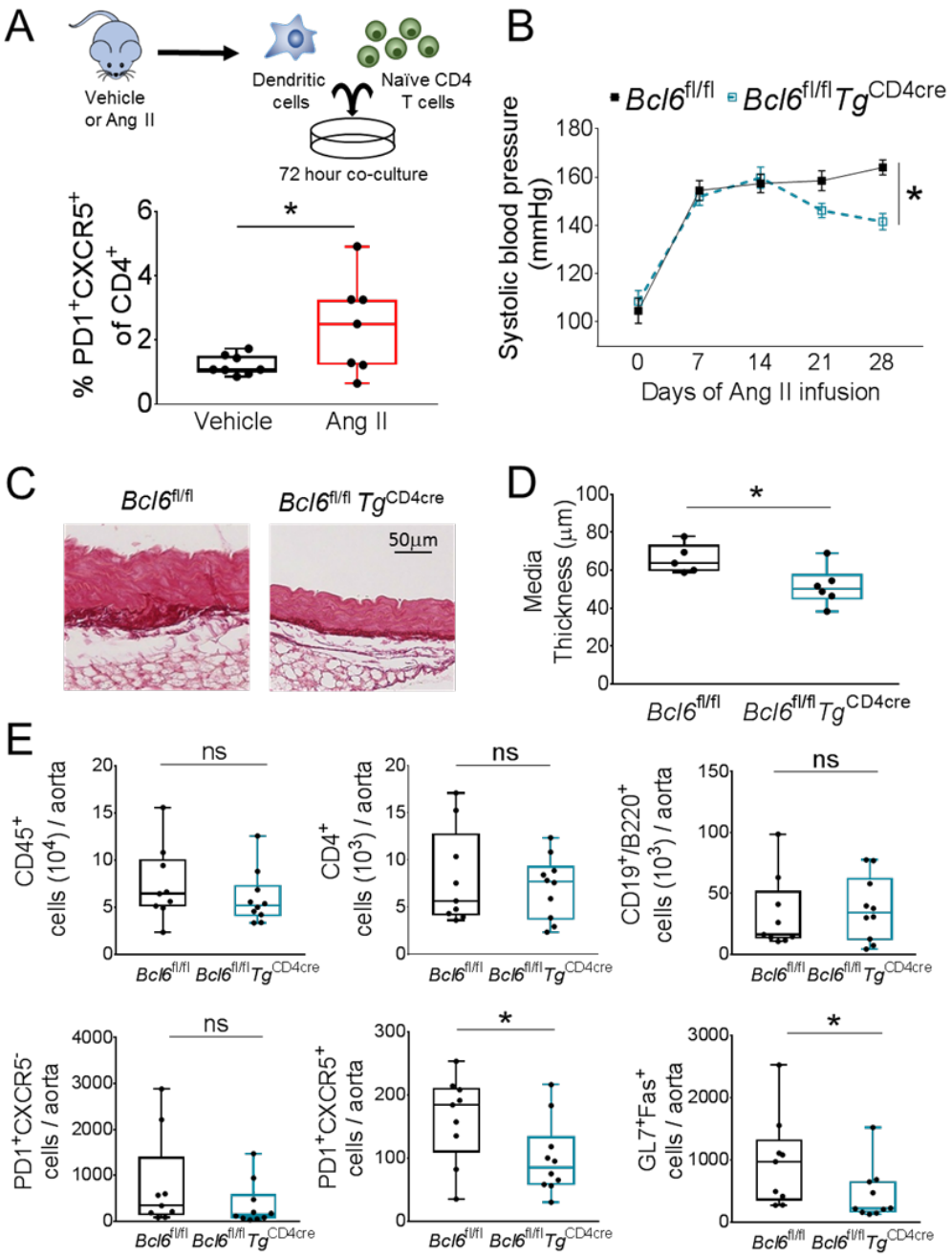

Figure 2-6. Hypertensive DCs induce Tfh cell polarization, and Tfh cells play a critical role in hypertension. (**A**) DCs were isolated from the spleen of WT mice infused with Ang II or vehicle for 14 days and co-cultured with splenic naïve CD4$^+$ T cells from vehicle infused WT mice (top). Percent Tfh cells (PD1$^+$CXCR5$^+$) of CD4$^+$ T cells was determined by flow cytometry (n=7-8) (bottom). (**B**) Systolic blood pressure was measured by tail-cuff weekly over 28 days of Ang II infusion in *Bcl6*$^{fl/fl}$*Tg*^{CD4cre} and control *Bcl6*$^{fl/fl}$ littermates (n=9-11). (**C**) Representative images of bright field aortic wall thickness by Picrosirius Red staining. (**D**) Quantification of aortic wall thickness (n=5-6). (**E**) Summary quantification of total leukocytes (CD45$^+$), T helper (CD4$^+$), B (CD19$^+$B220$^+$), Tph (PD1$^+$CXCR5$^-$), Tfh (PD1$^+$CXCR5$^+$), and GC B (GL7$^+$Fas$^+$) cells in the aorta from *Bcl6*$^{fl/fl}$*Tg*^{CD4cre} and control *Bcl6*$^{fl/fl}$ littermates infused with Ang II for 28 days (n=9-10). Data are expressed as box and whisker plots (**A, D-E**) or mean ± SEM (**B**); **P*<0.05 by Student's *t*-test (**A, D-E**) or 2-way ANOVA with repeated measures (**B**).

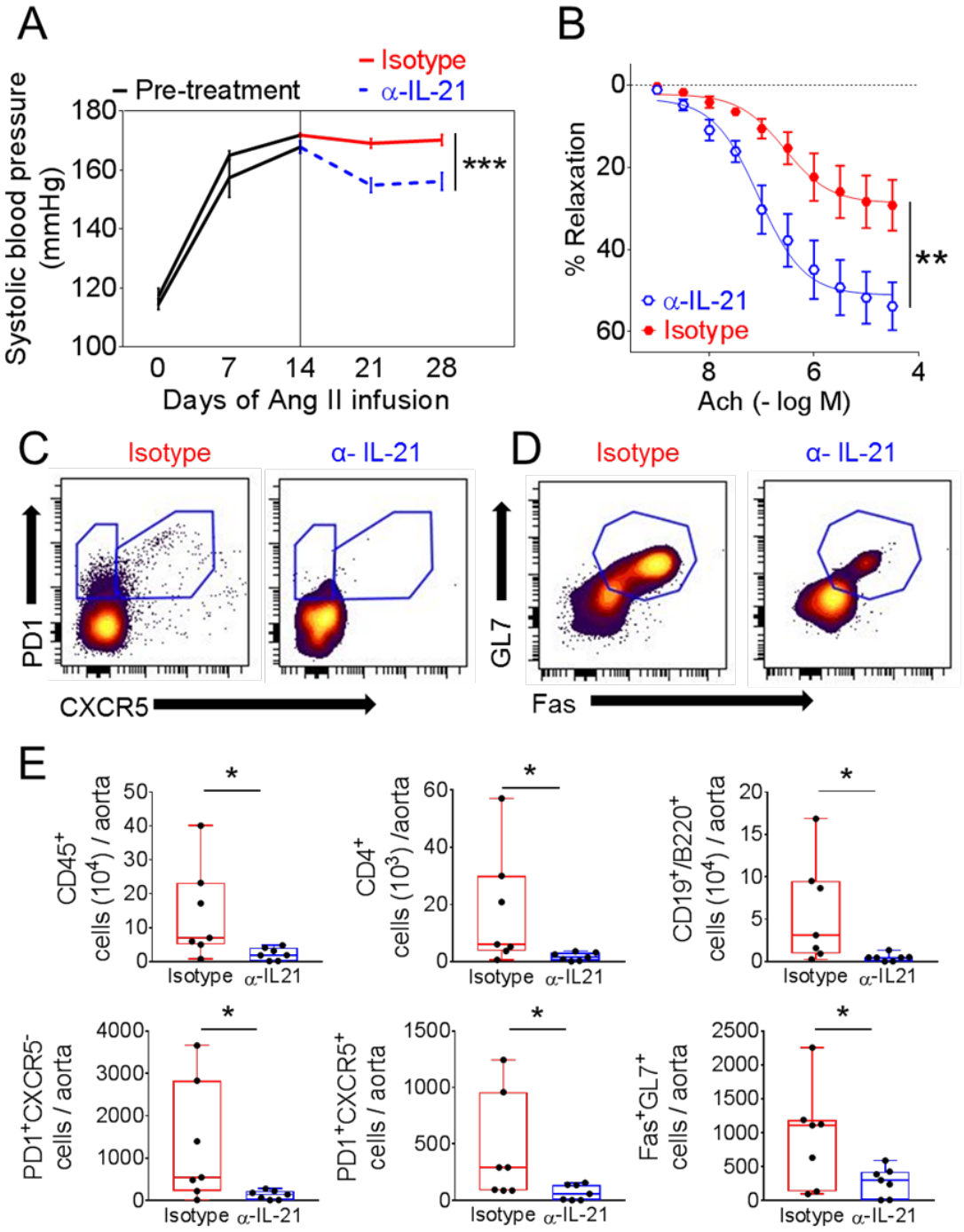

Figure 2-7. Anti-IL-21 treatment lowers blood pressure and reverses endothelial dysfunction and vascular inflammation. (**A**) Systolic blood pressure was measured by tail-cuff weekly over 28 days of Ang II infusion. Isotype control or IL-21 neutralizing antibodies were administered twice weekly during the last 2 weeks of Ang II infusion (n=7-8). (**B**) Endothelium-dependent relaxation in response to increasing doses of Ach was measured in both groups (n=7-8). (**C**) Representative flow cytometry bi-axial plots for Tph ($PD1^+CXCR5^-$), Tfh ($PD1^+CXCR5^+$), and (**D**) GC B ($GL7^+Fas^+$) cells in the aorta from both groups. (**E**) Summary quantification of total leukocytes ($CD45^+$), T helper ($CD4^+$), B ($CD19^+B220^+$), Tph, Tfh, and GC B cells from both groups (n=7). Data are expressed as mean ± SEM (**A**-**B**) or box and whisker plots (**E**); **P*<0.05, ***P*<0.01, ****P*<0.001 by 2-way ANOVA with repeated measures (**A**) or Student's *t*-test (**B, E**).

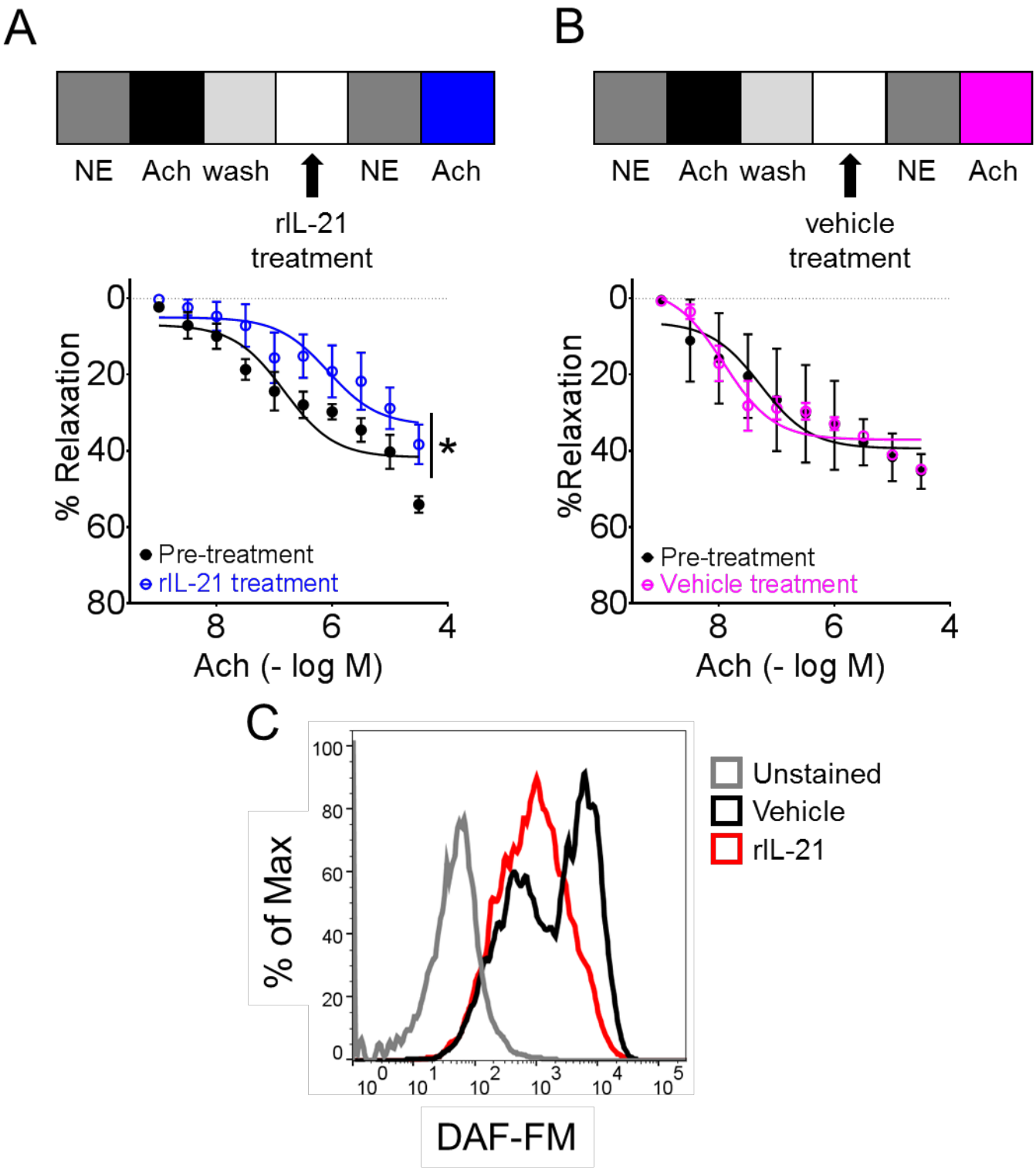

Figure 2-8. Recombinant IL-21 impairs endothelium-dependent relaxation *ex vivo* and decreases nitric oxide production from cultured human aortic endothelial cells. (**A-B**) Schematic of *ex vivo* treatment of WT mesenteric arterioles (top) and endothelium-dependent relaxation in response to increasing doses of acetylcholine (Ach) before and after treatment with recombinant IL-21 (**A**, n=5) or vehicle (**B**, n=3). NE=norepinephrine. (**C**) Histogram of 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM) fluorescence in cultured human aortic endothelial cells treated with recombinant IL-21 or vehicle (representative of n=4). Data are expressed as mean ± SEM (**A-B**); **P*<0.05 by paired Student's *t*-test (**A-B**).

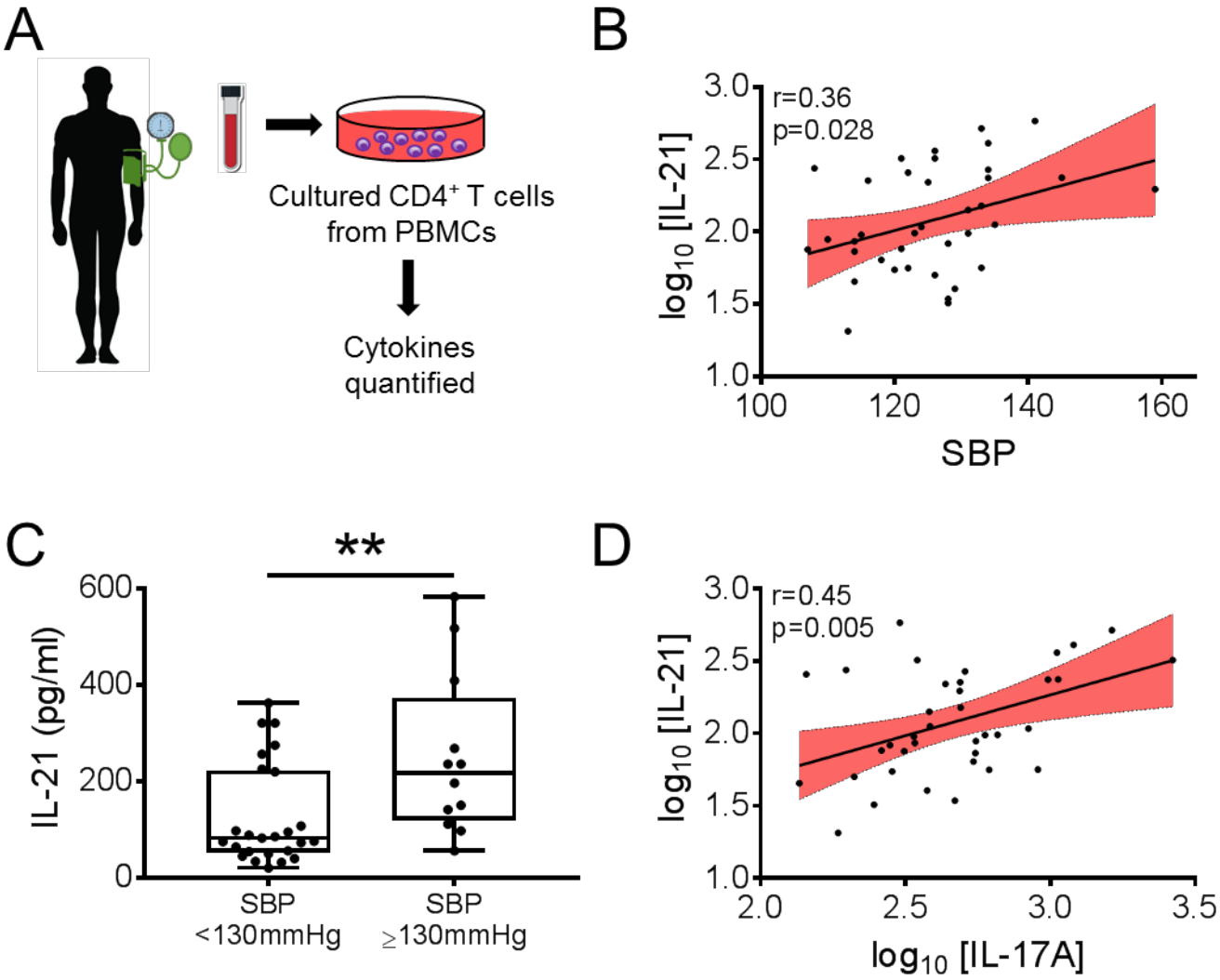

Figure 2-9. CD4⁺ T cell production of IL-21 correlates with systolic blood pressure (SBP) and IL-17A in humans. (**A**) SBP was determined at the time of blood draw in human subjects. CD4+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) and cultured for 3 days. Cytokines were then quantified from the supernatants. (**B**) CD4⁺ T cell production of IL-21 vs. systolic blood pressure in humans (n=37). (**C**) Human CD4⁺ T cell production of IL-21 dichotomized by systolic blood pressure (SBP) <130 mmHg or ≥ 130 mmHg (n=12-25). (**D**) CD4⁺ T cell production of IL-21 vs. IL-17A in humans (n=37). Pearson's correlation co-efficient and corresponding p-value are shown on the graph (**B and D**). Data are expressed as box and whisker plots (**C**); $^{**}P<0.01$ by Student's *t*-test (**C**).

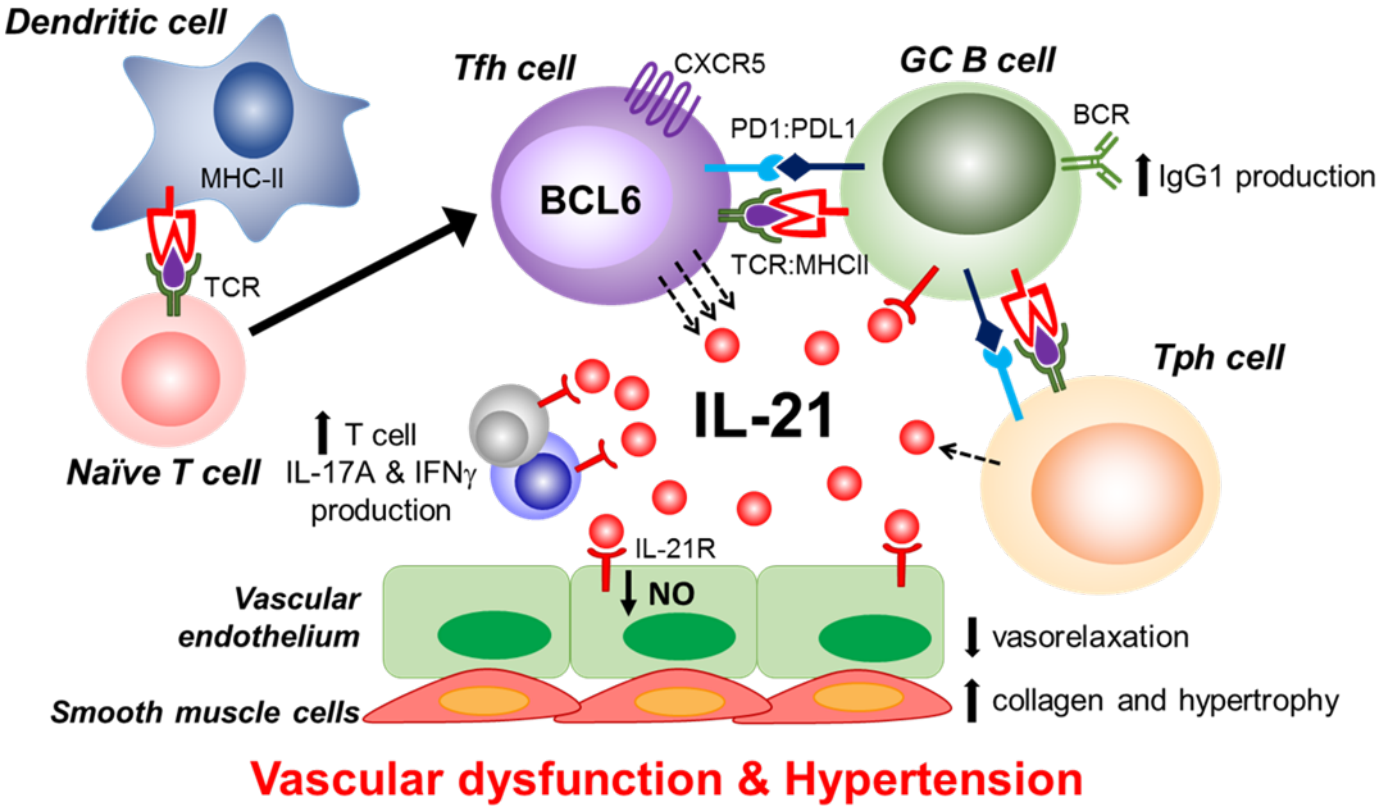

Figure 2-10. Working model of how IL-21 functions as a master cytokine in hypertension coordinating T, B, and somatic cell responses. TCR = T cell receptor; MHCII = major histocompatibility complex II; BCR = B cell receptor; NO = nitric oxide.

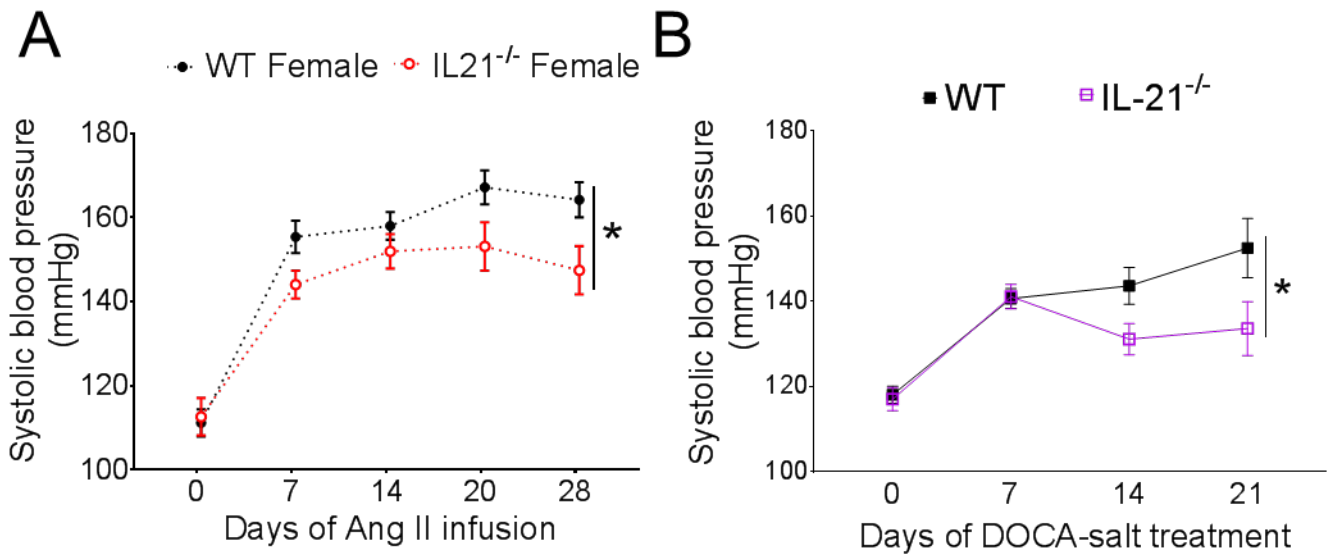

Figure 2-S1. IL-21 deficiency blunts Ang II-induced hypertension in female mice and the hypertensive response to DOCA-salt treatment. (**A**) Systolic blood pressures were measured by tail-cuff weekly over 28 days of Ang II infusion in WT and IL-21$^{-/-}$ female mice (n=9). (**B**) Systolic blood pressures were measured by tail-cuff weekly over 21 days DOCA-salt treatment in WT and IL-21$^{-/-}$ mice (n=6-8). Data are expressed as mean±SEM; *P<0.05 by 2-way ANOVA with repeated measures.

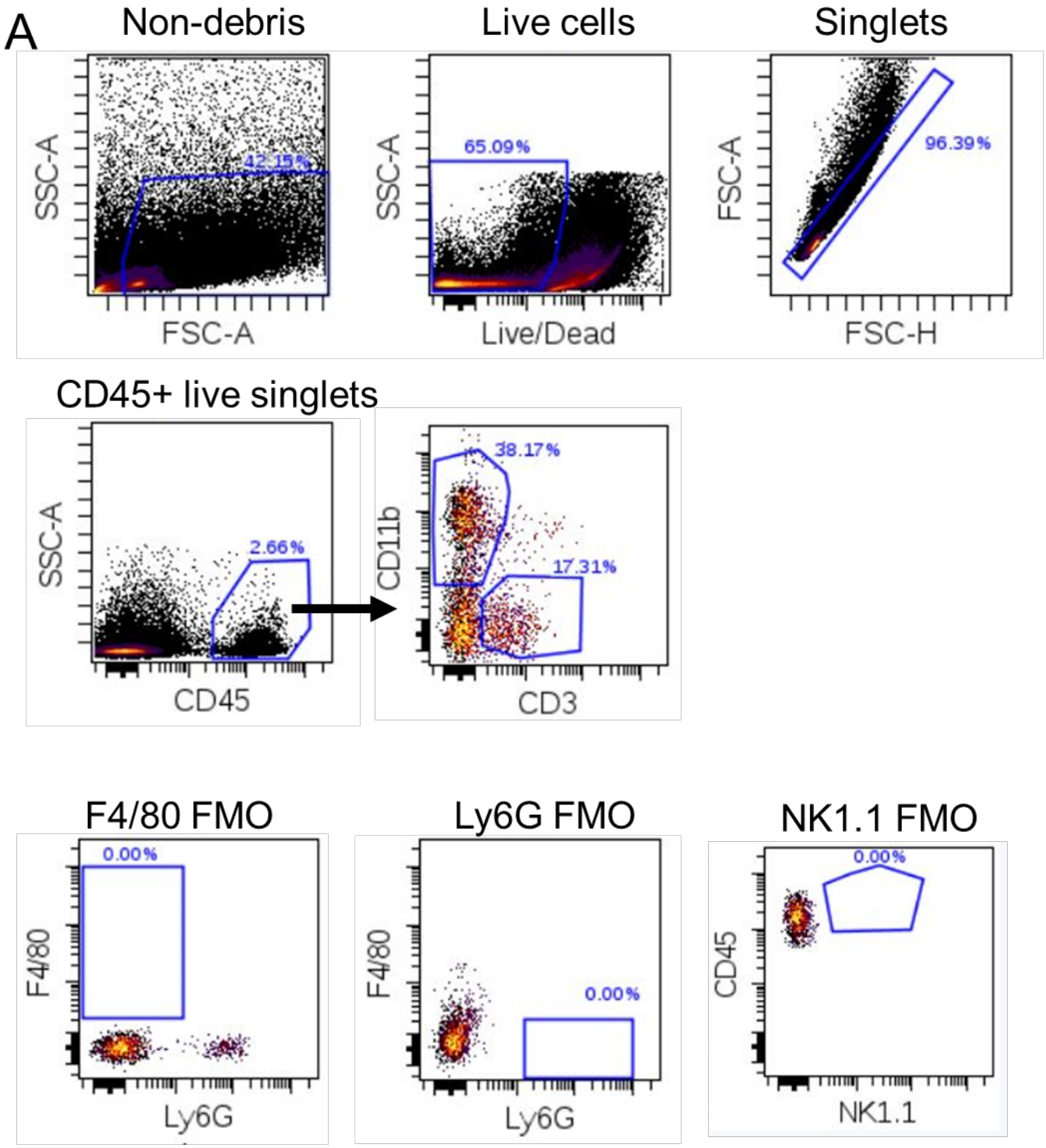

Figure 2-S2. Innate panel gating strategy and fluorescence minus one (FMO) controls. Gating strategy to identify total CD45$^+$ leukocytes from live singlets in aortic single cell suspensions. CD11b$^+$ and CD3$^+$ cells were gated from CD45$^+$ cells. Macrophages (F4/80$^+$Ly6G$^-$) and neutrophils (F4/80$^-$Ly6G$^+$) were gated from CD11b$^+$ cells and natural killer (NK) T cells (NK1.1$^+$) were gated from CD3$^+$ cells. FMO controls for each terminal marker are shown below.

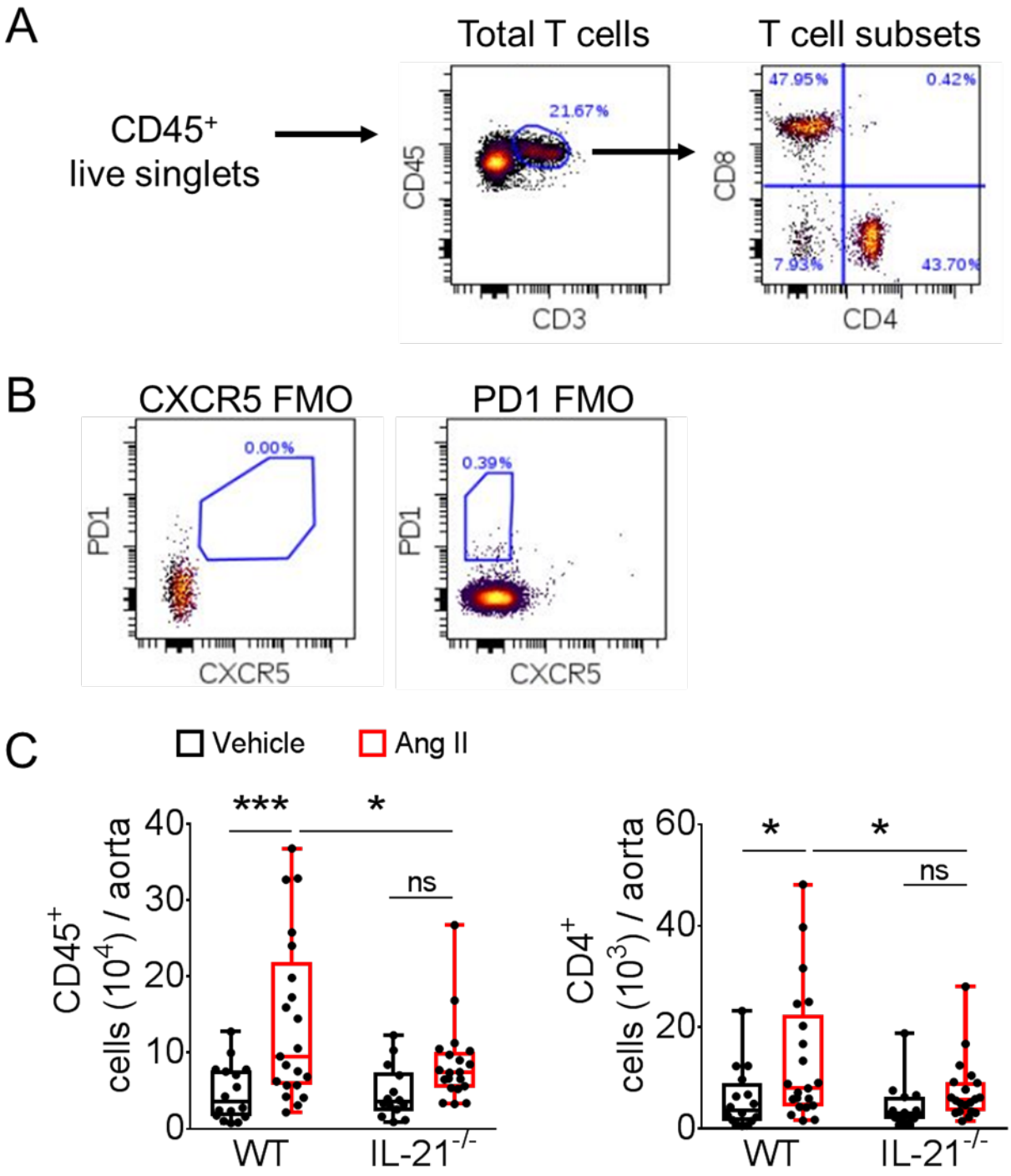

Figure 2-S3. T cell panel gating strategy and FMO controls. (A) Gating strategy to identify total $CD3^+$ leukocytes from live CD45+ singlets in aortic single cell suspensions. CD4+ and CD8+ cells were gated from CD3+ cells. Tfh cells ($PD1^+CXCR5^+$) and Tph cells ($PD1^+CXCR5^-$) were gated from $CD4^+$ cells. (**B**) FMO controls for each terminal marker. (**C**) Summary quantification of $CD45^+$ total leukocytes and $CD4^+$ T cells from WT and IL-$21^{-/-}$ mice after 28 days of Ang II or vehicle infusion (n=14-20). Data are expressed as box and whisker plots; *P<0.05, ***P<0.001 by 2-way ANOVA.

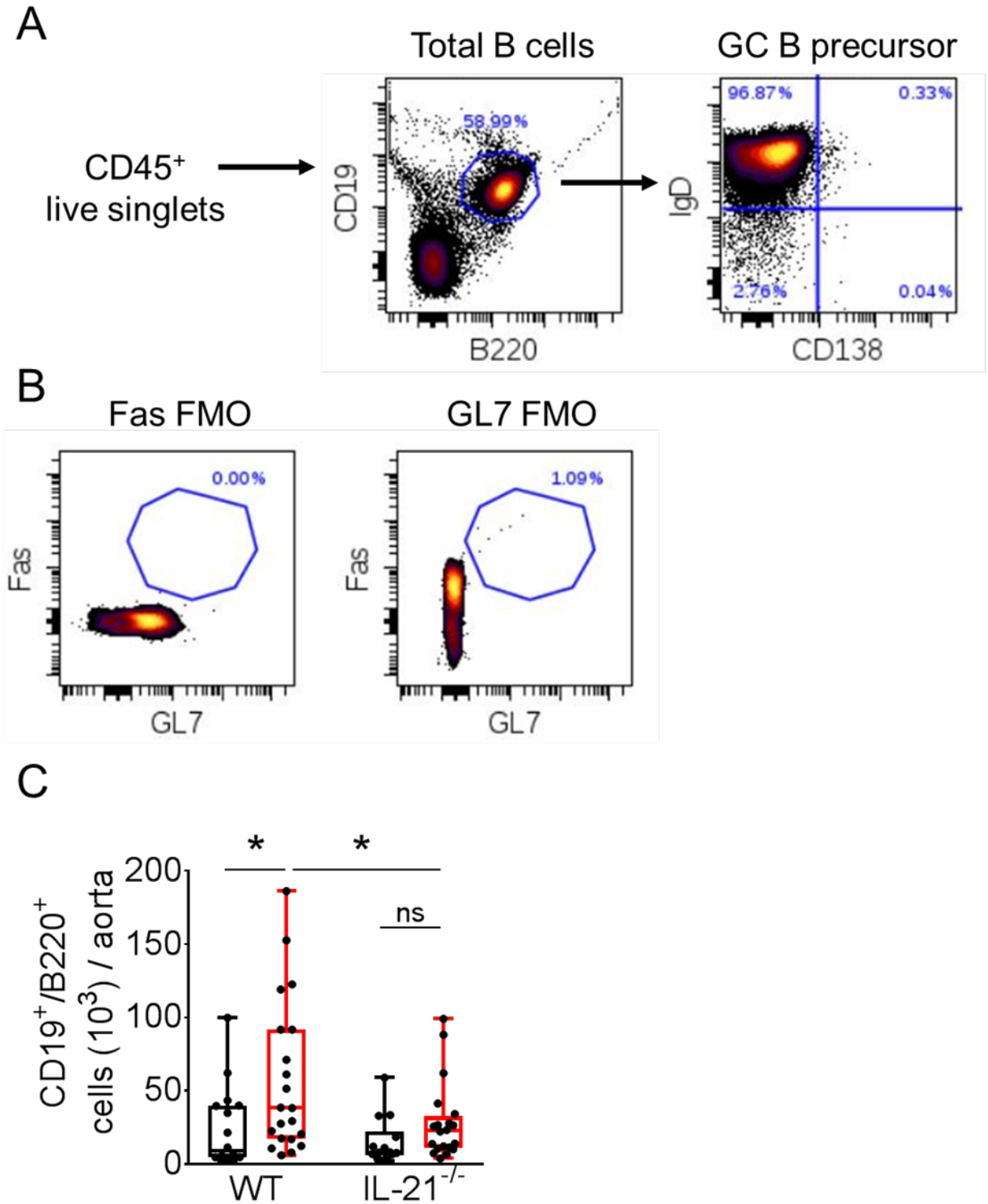

Figure 2-S4. B cell panel gating strategy and FMO controls. (A) Gating strategy to identify total $CD19^+B220^+$ B cells from live CD45+ singlets in aortic single cell suspensions. IgD^-CD138^- cells were gated from $CD19^+B220^+$ B cells. GC B cells (Fas^+GL7^+) were gated from IgD^-CD138^- cells. (**B**) FMO controls for each terminal marker. (**C**) Summary quantification of total $CD19^+B220^+$ B cells from WT and IL-$21^{-/-}$ mice after 28 days of Ang II or vehicle infusion (n=14-20). Data are expressed as box and whisker plots; *P<0.05 by 2-way ANOVA.

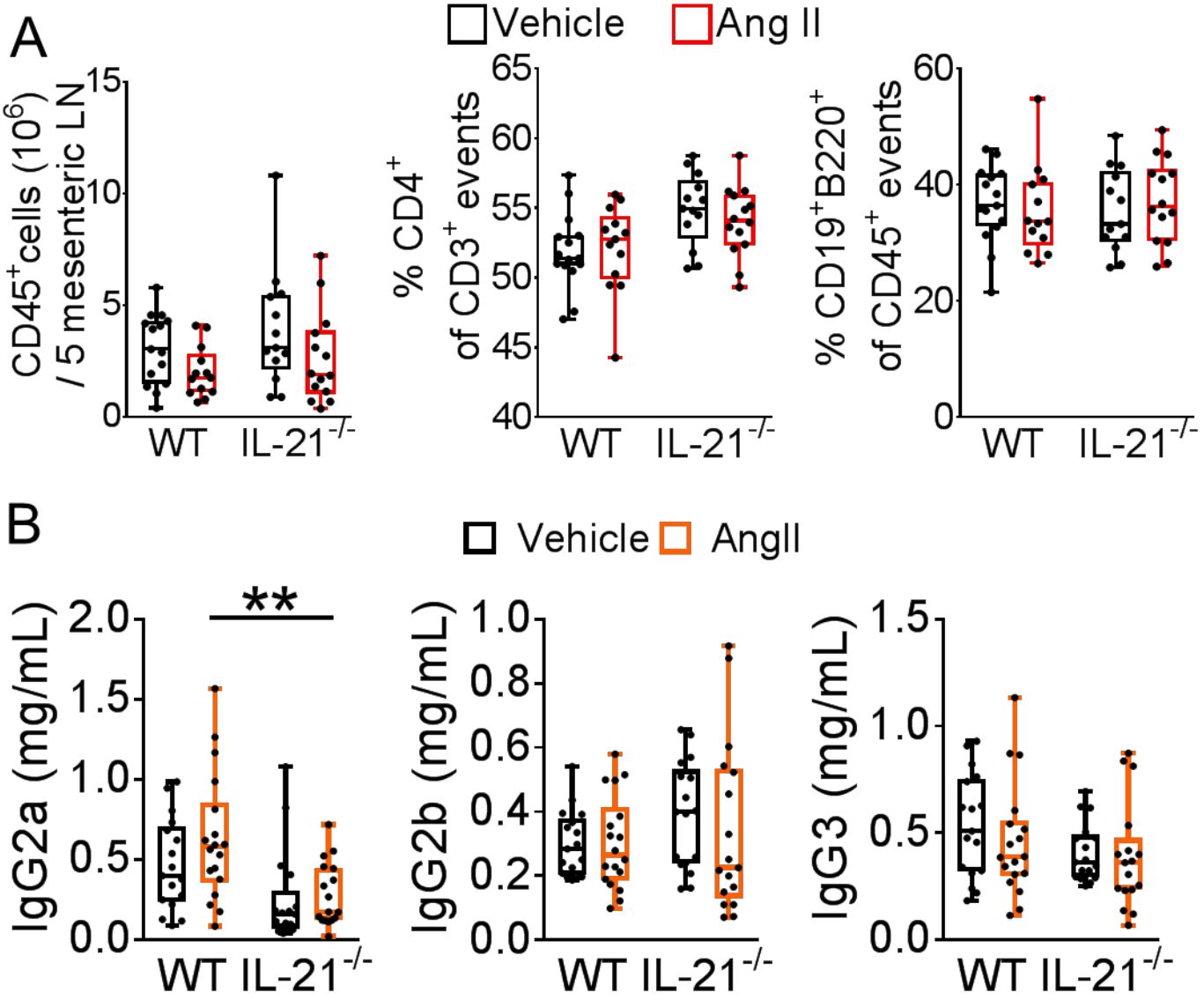

Figure 2-S5. Flow cytometry of mesenteric lymph nodes and plasma immunoglobulin quantification. WT and IL- 21$^{-/-}$ mice were infused with Ang II or vehicle for 28 days. (**A**) Summary quantification of CD45+ total leukocytes, percent CD4+ T cells, and percent CD19^{+}B220^{+} B cells in mesenteric lymph nodes (n=13-15). (**B**) Plasma immunoglobulin concentration of IgG2a, IgG2b, and IgG3 (n=17-18). Data are expressed as box and whisker plots; $^{**}P<0.01$ by 2-way ANOVA.

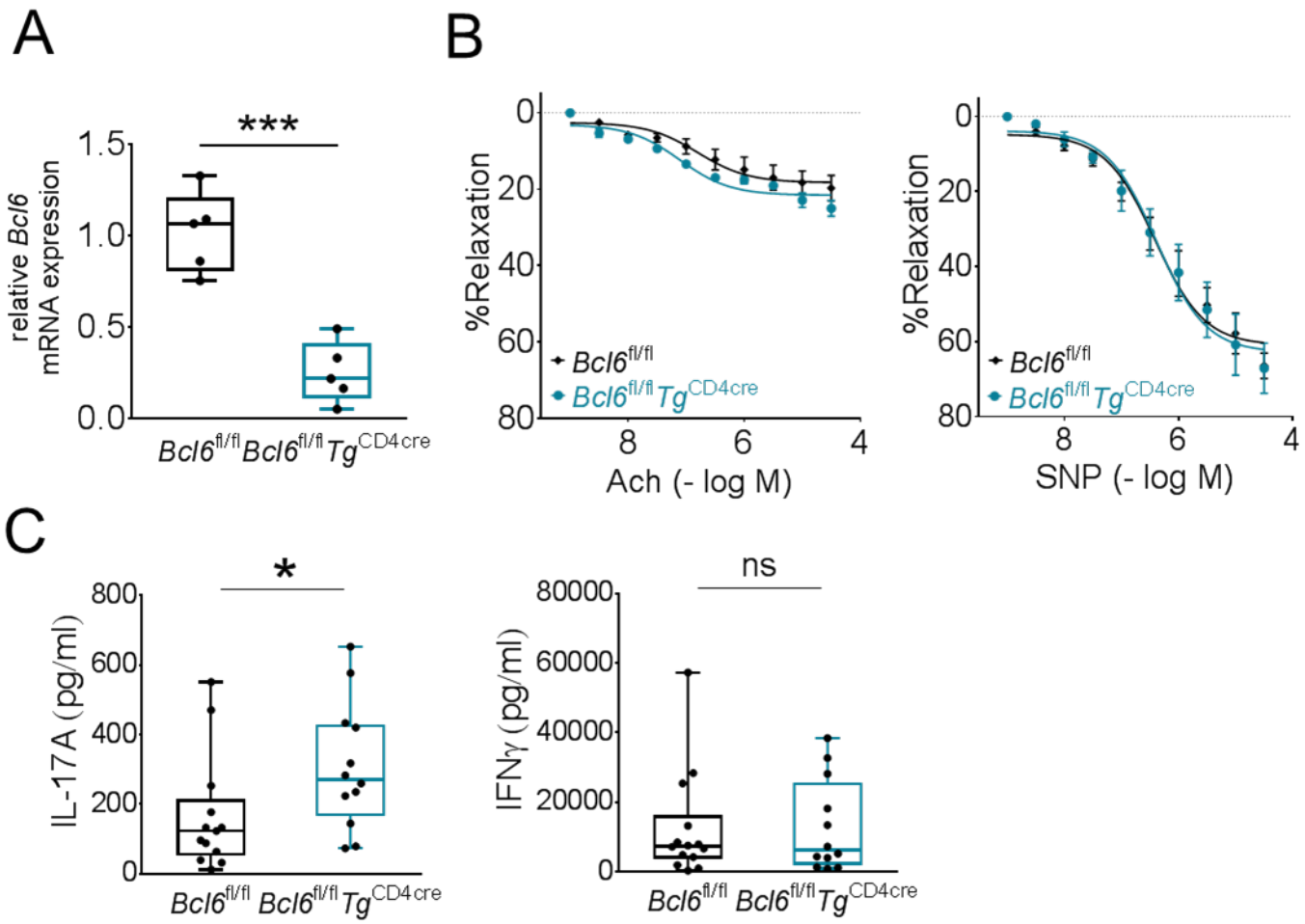

Figure 2-S6. CD4+ T cell BCL6 expression, vascular reactivity, and splenic cytokine production from *Bcl6*$^{fl/fl}$ and *Bcl6*$^{fl/fl}$*Tg*^{CD4cre} mice. *Bcl6*$^{fl/fl}$*Tg*^{CD4cre} and control *Bcl6*$^{fl/fl}$ littermates were infused with Ang II or vehicle for 28 days. (**A**) Relative *Blc6* mRNA expression by quantitative real time PCR of splenic CD4$^+$ T cells (n=5). (**B**) Endothelium-dependent relaxation in response to increasing doses of acetylcholine (Ach) (left) and endothelium-independent relaxation in response to increasing doses of sodium nitroprusside (SNP) (right) were measured in isolated mesenteric arterioles (n=5-6). (**C**) Splenic CD4$^+$ T cell production of interleukin 17A (IL-17A) and CD8$^+$ T cell production of interferon gamma (IFNg) quantified by ELISA (n=12-14). Data are expressed as box and whisker plots (**A,C**) or mean±SEM (**B**) ;*P<0.05 by Mann Whitney test (**C**); ***P<0.001 by Student's t-test (**A**).

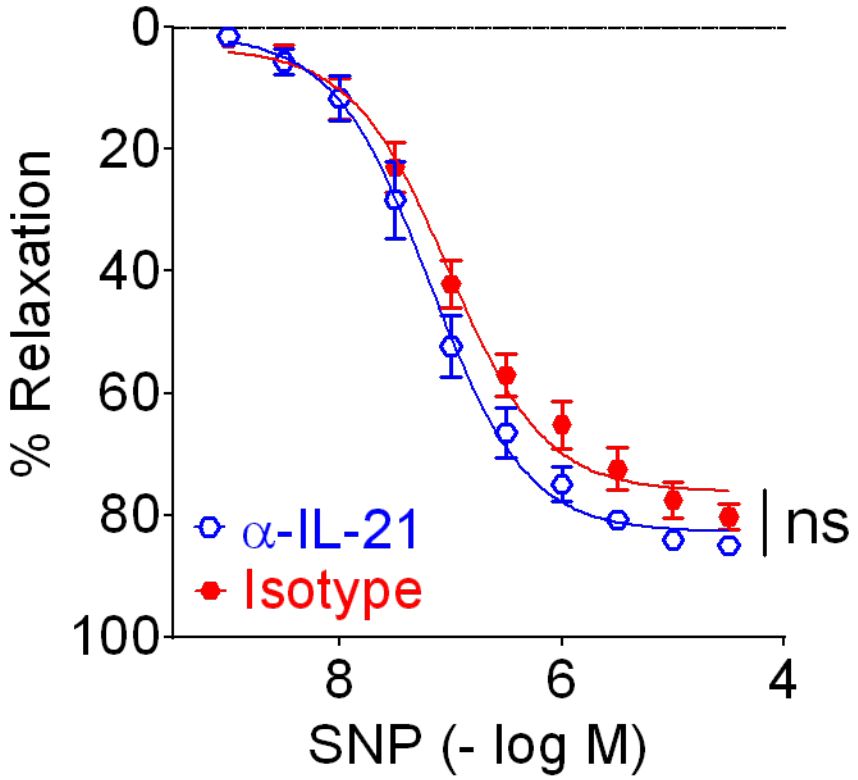

Figure 2-S7. Endothelium-independent relaxation to sodium nitroprusside (SNP) is not affected by anti-IL-21 treatment. WT mice were infused with Ang II for 4 weeks. Isotype control or IL-21 neutralizing antibodies were administered twice weekly during the last 2 weeks of Ang II infusion. Endothelium-independent relaxation in response to increasing doses of SNP was measured in isolated mesenteric arterioles from both groups (n=7-8). Data are expressed as mean±SEM.

Table 2-S1. Characteristics of the participants (n=37)

Age (yrs, x±SEM)	44.9 ± 1.9
Sex (F/M, n/n, %/%)	26/11, 70.3/29.7
Race (W/B, n/n, %/%)	14/23, 37.8/62.2
SBP (mmHg, x±SEM)	125.4 ± 1.8
DBP (mmHg, x±SEM)	75.0 1.3
HR (bpm, x±SEM)	67.7 ± 1.6
BMI (Kg/m^2, x±SEM)	29.0 ± 1.1

F=female, M=male, W=white, B=black, SBP=systolic blood pressure, DBP=diastolic blood pressure, HR=heart rate, BMI=body mass index

DISCUSSION

It is now evident that hypertension is an inflammatory disease with key roles played by most innate and adaptive immune cells including DCs, monocytes/macrophages, gamma delta ($\gamma\delta$) T cells, $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, and B cells [25]. Modest elevations of serum immunoglobulins have also been observed in both experimental and human hypertension [163, 164], but whether hypertension is associated with a GC reaction is not known. Here we show that genetic or pharmacological inhibition of IL-21 results in blunted hypertension and reduced vascular inflammation/dysfunction. Since IL-21 is produced primarily by Tfh cells whose main effector function is to promote a GC reaction, our data strongly suggest that hypertension is associated with a GC response. Indeed, we show accumulation of GC-like cells in the aorta and mesenteric lymph nodes and increased IgG1 production in hypertensive WT but not $IL21^{-/-}$ mice. Moreover, genetic deficiency of Tfh cells results in protection from chronic hypertension. Finally, we show that BP in a cohort of humans correlates with $CD4^+$ T cell production of IL-21. This latter finding is consistent with a previous report showing that among patients with coronary artery disease, serum IL-21 was upregulated in those with concomitant hypertension compared to those without [141].

IL-21 is a pleiotropic cytokine with effects on innate and adaptive immune cells as well as non-immune cells [74, 82]. IL-21 has been shown to promote Th17 and Th1 cells and inhibit Treg cells [135, 160]. We and others demonstrated that hypertension is associated with an increase in IL-17A and IFNγ production, and that these cytokines play important roles by

modulating renal sodium transporters, vascular fibrosis, and endothelial function [124, 160, 165]. Here, we show that loss of IL-21 abrogates hypertension-induced T cell production of IL-17A and IFNγ. IL-21 is also the most potent cytokine known to induce GC B cell proliferation and differentiation into antibody secreting plasma cells [160]. In keeping with this, we found that hypertension is associated with increased accumulation of GC-like cells in the vasculature and secondary lymphoid organs in an IL-21 dependent manner. Finally, we show that IL-21 has direct effects on vascular endothelial cells, altering bioavailability of nitric oxide and promoting endothelial dysfunction. Future studies are needed to determine mechanisms by which IL-21 alters superoxide and NO levels and to investigate whether IL-21 has direct effects on vascular smooth muscle cells and fibroblasts to increase chemokine expression and collagen synthesis. In line with this, it has been shown in inflammatory bowel disease that colonic myofibroblasts and epithelial cells respond to IL-21 by secreting matrix metalloproteases and chemokines that recruit other inflammatory cells [82]. These potential effects of IL-21 in hypertension are not mutually exclusive, and thus we propose that IL-21 may indeed function as a “master” cytokine in hypertension, orchestrating responses of T cells, B cells, and parenchymal cells (**Figure 10**).

The finding that hypertension is associated with a GC reaction has important therapeutic implications. The primary purpose of a GC reaction is to mount a specific and effective immune response in the setting of infection or vaccination. GCs are transient microanatomical sites in secondary or tertiary lymphoid organs where somatic hypermutation (to generate high affinity antibodies) and isotype class switching (e.g. IgM

→ IgG) occurs. After multiple rounds of affinity maturation, B cells exit GCs as memory B cells or long-lived plasma cells. B cells can also differentiate along an extrafollicular pathway and produce IgM or class-switched antibodies with a low degree of somatic mutation. Although autoimmune diseases may use either pathway, there is emerging data that a GC response is the predominant pathway at least in some autoimmune diseases [162, 166-168]. Interestingly, Clement et al. recently demonstrated that experimental atherosclerosis is associated with an overactive Tfh-GC B cell axis in secondary lymphoid organs and increased development of tertiary lymphoid organs in the aorta. Moreover, these authors demonstrated the presence of Tfh cells within tertiary lymphoid organs of human atherosclerotic aneurysmal arteries [72]. We found that experimental hypertension is associated with both structured and unstructured T and B cell aggregates, increased accumulation of aortic Tfh and GC B cells, and an increased percent of lymph node GC B cells. To determine the role of Tfh cells in hypertension, we created mice with T cell specific deletion of the Tfh cell lineage determining transcription factor BCL6. Mice with T cell deletion of BCL6 had blunted accumulation of Tfh cells and GC B cells in the aorta and were unable to sustain the hypertensive response to chronic Ang II infusion, suggesting a critical role for a GC reaction in hypertension.

Of note, mice with T cell deletion of BCL6 exhibited a delayed protection from Ang II induced BP elevation and were not protected from vascular accumulation of other immune cells in contrast to IL-21$^{-/-}$ mice. One explanation for this is that other immune cells such as NK T cells and/or Th17 cells are also important sources of IL-21 in hypertension or are able to compensate in the presence of Tfh cell deficiency. Indeed, we

detected an increase in splenic CD4+ T cell production of IL-17A in the Tfh cell deficient mice following Ang II infusion. Another explanation is that our model did not deplete the Tfh-like "peripheral helper T cells" or Tph cells which do not express prototypic Tfh markers like CXCR5 or BCL6 but still produce factors important in B cell help like IL-21. Recent studies demonstrate that these Tfh-like cells cooperate with B cells in unstructured aggregates of inflamed tissues and may be important in the local differentiation of B cells into antibody-secreting cells [169]. For example, Rao and colleagues used mass cytometry to analyze T cells isolated from the joints and blood of patients with rheumatoid arthritis. They identified an expanded population of $PD1^{hi}CXCR5^{-}CD4^{+}$ T cells which they named Tph cells, distinct from $PD1^{hi}CXCR5^{+}CD4^{+}$ Tfh cells, and showed that these cells express IL-21 and chemokine receptors such as CCR5 that direct migration to inflamed sites [140]. Indeed, when we quantified $PD1^{hi}CXCR5^{-}CD4^{+}$ T cells in our animal models, we detected an increase in Tph cells in the aorta of WT but not $IL21^{-/-}$ mice (**Figure 2-4C**). In addition, as expected, these cells were not reduced in mice with T cell deletion of BCL6 (**Figure 2-6E**). It is not clear to what extent Tph-B cell interactions in peripheral tissues are similar to classic Tfh-GC B cell interactions in secondary lymphoid organs. Further studies are needed to elucidate the relative role of Tph vs Tfh cells in hypertension.

We and others have elucidated the role of various T effector and regulatory cell subsets in hypertension pathogenesis [25]. Recently Chan et al. demonstrated that mice with genetic or pharmacological depletion of B cells develop blunted Ang II-induced hypertension [113]. It has also been shown that DCs are activated in hypertension and

present immunogenic isolevuglandin-modified peptides to T cells that promote T cell proliferation [107]. A missing link has been how these various immune cells produce a coordinated and orchestrated response. Based on our results, we propose that hypertensive DCs induce naïve T cells to differentiate into Tfh cells. Tfh cells then induce a GC reaction in the aorta and secondary lymphoid organs leading to increased IgG production. IL-21 production from Tfh cells, and perhaps other sources such as Tph and Th17 cells, promotes the production of pathogenic IL-17A and IFNγ producing T cell subsets and may have direct effects on parenchymal cells leading to hypertension and end-organ dysfunction (**Figure 2-10**). Further studies are necessary to more precisely define the nature and mechanism of action of B cells and immunoglobulins in hypertension.

Our data reveal some subtle differences between IL-21$^{-/-}$ animals and acute depletion of IL-21 with neutralizing antibodies. Anti-IL-21 antibody treatment appears to be more effective at reducing vascular inflammation. In addition, while endothelium-dependent relaxation to Ach is impaired at baseline in IL-21$^{-/-}$ mice, anti-IL-21 antibody treatment restores endothelial function to the levels seen in normotensive WT mice. When we assessed mesenteric arteriolar superoxide production in the IL-21 deficient mice, it was elevated at baseline compared to WT animals. This may explain the baseline impairment in vascular function and suggests that IL-21 may have some protective effects at early developmental stages or at low levels. Furthermore, compensatory changes may have occurred in the mice deficient in IL-21 from birth. Nevertheless, these complementary approaches for deleting IL-21 demonstrate the critical role played by this cytokine in

hypertension and end-organ damage and suggest that anti-IL-21 treatment may be a promising therapeutic strategy for hypertension.

Phase I or II clinical trials using monoclonal anti-IL21 antibodies for lupus, rheumatoid arthritis, and Crohn's disease are either completed or in progress [74]. Hypertension is highly prevalent in patients with autoimmune diseases, and thus it would be interesting to determine how this therapy affects BP and vascular dysfunction. However, the risk-to-benefit ratio of such therapeutics should be carefully considered since IL-21 also has potent anti-tumor and anti-viral activities. In fact, IL-21 is currently being investigated as a therapeutic agent in solid tumors and hematopoietic malignancies. Boosting IL-21 levels may also be beneficial for viral infections such as Hepatitis B and C and HIV. Thus, tissue-restricted targeting of a specific cellular source of IL-21, such as Tfh or Tph cells, if feasible, would be a more attractive therapeutic option.

In summary, we show that both experimental and human hypertension is associated with increased $CD4^+$ T cell production of IL-21. Mice deficient in IL-21 exhibit blunted hypertension and vascular end-organ dysfunction. Pharmacological inhibition of IL-21 after the onset of hypertension lowers BP and reverses endothelial dysfunction and vascular inflammation. Furthermore, hypertension is associated with increased aortic Tfh and GC B cells, and Tfh cell deficiency protects from chronic Ang II-induced hypertension. To our knowledge, this is the first study implicating a GC reaction in hypertension and suggests that inhibition of IL-21 or specific depletion of IL-21 producing cells may be a novel therapeutic strategy for the treatment of hypertension and its micro- and

macrovascular complications.

CHAPTER 3: DETECTION OF NOVEL CYTOKINE PRODUCING CELLS BY CYTOF

INTRODUCTION

Early studies in rodents, performed when little was known about the regulation of the immune system, suggested immune cells were involved in the pathophysiology of hypertension.[94, 170] However, it is only in the past decade, through pioneering work from our group and others, that we have a better understanding of the cell types and cytokines that are involved in the genesis of hypertension and particularly the end-organ damage associated with hypertension. Yet, most of these studies have been conducted in mice and rats leaving us with little information about whether immune dysfunction contributes to hypertension in humans. Since hypertension affects almost 70 percent of adults by the age of 70 and is a leading global burden of disease,[171] it is imperative that we perform studies to better define the underlying mechanisms of this disease in humans.

An emerging paradigm from our laboratory and others is that hypertension is an autoimmune disease mediated by both innate and adaptive immune cells.[25] A prominent role for T cells in hypertension was demonstrated by Guzik et al. using mice lacking both T and B lymphocytes (*Rag1*$^{-/-}$ mice).[96] These mice exhibited blunted hypertension and preserved vascular function in response to multiple hypertensive stimuli. Adoptive transfer of T cells, restores vascular dysfunction and the hypertensive response to angiotensin II infusion. We recently showed that mice deficient in the adaptor molecule, LNK/SH2B3, are predisposed to hypertension, and that bone marrow transplantation from LNK$^{-/-}$ mice into wild type mice completely recapitulates the hypertensive response.[44] Similarly,

Santisteban et al. transplanted bone marrow from spontaneously hypertensive rats (SHR) into normal Wistar-Kyoto rats and showed that this raised blood pressure as well as central and peripheral inflammation.[172] Thus, bone marrow derived cells play a critical role in hypertension.

Specialized subsets of T cells and innate immune cells produce pro-inflammatory cytokines such as IL-17A and IFNγ, which we have shown contribute to angiotensin II-induced hypertension, vascular dysfunction, and salt/water retention in the kidney.[43, 123, 124, 157] Youn et al. found that hypertension in humans was associated with an accumulation of senescent $CD8^+$ T cells that produce pro-inflammatory and cytotoxic factors such as perforin and granzyme B.[173] We recently performed RNA-seq analysis of monocytes isolated from normotensive and hypertensive humans and found that hypertension is associated with changes in the expression of 60 genes, many of which are involved in pro-inflammatory pathways and T cell activation.[174] Thus, a thorough and exhaustive effort to characterize the T cell immune profile of humans with hypertension is of great interest.

CyTOF (Cytometry by Time of Flight) is a relatively new and unique multiplexing technique not widely available in many institutions which can be used to simultaneously evaluate a large number of cell parameters between multiple populations.[175] The focus of the panel will be to successfully detect T cell production of IL-4, IL-17A, IL-10 and IFNγ. Further, T cell populations which produce multiple cytokines will also be investigated. Employing CyTOF to characterize cytokine producing T cells is a novel approach to

investigate immune cell populations that may be altered in hypertension.

METHODS

Healthy human donor samples

Blood samples were obtained as part of the *The Vanderbilt Immune Mechanisms of Hypertension and Coronary Artery Disease Study* project (IRB#130979) and conforms to federal standards. The volunteer provided informed consent prior to blood draw. The donor was considered a healthy donor with a systolic and diastolic BP < 120/80, lack of autoimmune disease, recently vaccinated, and not currently on steroid or immunosuppression pharmaceuticals.

PBMC isolation

PBMCs were isolated as described in **Chapter 2** from whole blood by Ficoll plaque gradient, and frozen at a concentration of 10^6 cells/ml (Bambanker, Bulldog Inc) prior to staining.

Lymphocyte ex vivo stimulation and staining by mass cytometry

Briefly, cells were thawed, and re-suspended at a concentration of $2x10^6$ cells/ml in RPMI+5% FBS. 1 million cells were plated per well using a tissue culture treated 6-well plate (CoStar). All cells were treated with golgi blocker (Brefeldin A, 10ug/ml). Additional wells receiving stimulation were also treated with PMA-ionomycin cocktail without Brefeldin A and used according to the manufacturers protocol (Biolegend). Cells were removed from culture dishes, washed and live/dead stained with cisplatin prior to surface

staining, fix/perm, and intracellular staining. The detailed protocol is provided in the **Appendix**.

Acquisition and analysis

Samples were acquired on the CyTOF at the Vanderbilt Flow Cytometry Shared Resource Core. ViSNE multidimensional analysis and expert gating was performed using CytoBank.

RESULTS

ViSNE analysis of BFA and PIB treated CD45+ cells versus the 32-marker panel. A comprehensive T cell focused panel included 28 surface markers and 4 intra-cellular cytokines (IL-4, IL-17A, IFNγ, and IL-10) was curated to capture major T cell subsets already known to play a role in hypertension, as well as encompass enough markers to capture new novel cell populations (**Table 3-1**). PBMCs isolated from healthy normotensive human volunteers were acquired from consenting individuals and stimulated with and without PMA/ionomycin for 3 hours prior to surface and intracellular staining and acquisition (**Figure 3-1**). *Ex vivo* stimulation of PBMCs resulted in an almost complete loss of $CD16^+$ cells, as well as a shift in $CD14^+$ density (**Figure 3-2A**). T cell markers including CD3, CD4 and CD8 have been reported to be downregulated after *ex vivo* stimulation, therefore viSNE islands were visualized for surface marker expression with BFA and PIB treatment to evaluate differential effects after stimulation. In addition to the gross loss of a CD16+ population, CD3 and CD4 expression was decreased, and to a lesser extent CD8 and CD11a. While a decrease in signal was noted, cell populations remained identifiable (**Figure 3-2B**).

Expert gating and cytokine distribution across pan T cells by intracellular staining by mass cytometry. To evaluate the effectiveness of the *ex vivo* stimulation, gross changes in percent cytokine positive cells were examined in pan $CD3^+$ T cells identified from the gating strategy in **Figure 3-3A**. Post PIB stimulation there is a drastic visual increase in IFNγ positive cells, followed by IL-17A, and IL-4, with little to no signal picked up for IL-10

(**Figure 3-3B**). Analysis of BFA versus PIB stimulation demonstrates an almost 500-fold increase in IFNγ signal and 100-fold increase in IL-17A. Staining for IL-4 revealed a modest increase after PIB stimulation, with no change in IL-10 signal (**Table 3-2**).

T cell subsets have differential staining for IL-17A and IFNγ. Helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells are established producers of IL-17A and IFNγ, respectively. Naïve/effector T cells and memory helper $CD4^+$ and cytotoxic $CD8^+$ T cells were expert gated (**Figure 3-4A**) and analyzed for IL-17A and IFNγ to confirm cell staining specificity (**Figure 3-4A and B**). In fact, memory helper $CD4^+$ T cells were identified to have a higher percent of IL-17A cells (3.28%) than the four other cell types, whereas memory $CD8^+$ cytotoxic T cells had the highest expression of IFNγ (65.38%) producing cells (**Table 3-3**).

Identification of IL-17A, IFNγ double positive T cells by mass cytometry. Vascular infiltrating CD4 T cells have been shown to express both IL-17A and IFNγ and are an active area of research, therefore it was important that we be able to identify such a population with the panel created.[176] When naïve/effector and memory cells were analyzed for both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells only memory $CD4^+$ helper T cells produced a population that was double positive for both cytokines (**Figure 3-5**).

Table 3-1: Mass cytometry antibody panel for immunophenotyping of human hypertension

Isotope	Antigen	clone	Cell Identification Note
141Pr	CD196 (CCR6)	G034E3	T cell subsets
142Nd	CD11a	HI111	All leukocytes, important in lymphocyte co- stimulation
143Nd	CD5	UCHT2	T cells
144Nd	***IL-4****	MP4-25D2	
145Nd	CD4	RPA-T4	T cell subsets
146Nd	CD8a	RPA-T8	T cell subsets
147Sm	CD7	CD7-6B7	Mature T cells
148Nd	CD14	RMO52	Macrophages, Neutrophils, Dendritic cells
149Sm	CD25 (IL-2R)	2A3	T and B cell subsets
150Nd	CD44	IM7	Lymphocyte activation
151Eu	CD2	TS1/8	T cells, NK cells
152Sm	TCRgd	11F2	Gamma delta T cells
153Eu	CD194 (CCR4)	L291H4	T cell subsets
154Y	CD45	HI30	leukocytes
155Gd	CD27	L128	T cell activation
156Gd	CD195 (CCR5)	NP-6G4	T cell subsets, macrophages, Dendritic cells, Eosinophils
158Gd	***IFNγ****	B27	
159Tb	CD161	HP-3G10	Th17, NK cells
160Gd	CD28	CD28.2	T cell co-stimulation
161Dy	***IL-17A****	BL168	
163Dy	CD183 (CXCR3)	G025H7	T cell activation, B cells, Macrophages, Dendritic cells
164Dy	CD45RO	UCHL1	Memory T cells
165Ho	CD16	3G8	Macrophages, Neutrophils, NK cells, monocytes
166Er	***IL-10****	JES3-9D7	
167Er	CD197 (CCR7)	G043H7	Naïve T cells
169Tm	CD45RA	HI100	Naïve T cells
170Er	CD3	UCHT1	Pan T cells
171Yb	CD185 (CXCR5)	51505	T cell subsets, GC homing
172Yt	CD57	HCD57	T cell senescence
173Yt	HLA-DR	L243	APCs
175Le	CD279 (PD-1)	EH12.2H7	T cell activation
176Yb	CD127 (IL-7R)	A019D5	T cell differentiation

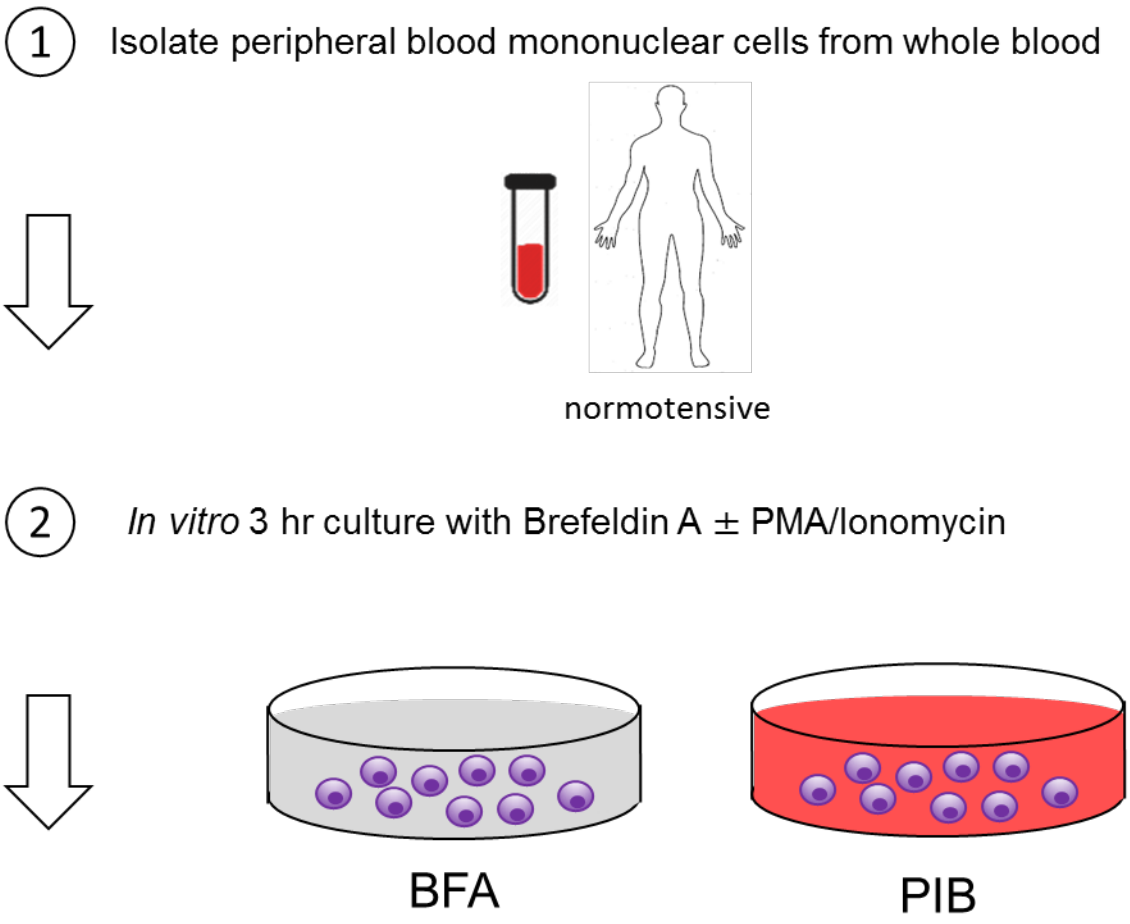

Figure 3-1. Study schematic. The study will recruit normotensive subjects. PBMCs will be isolated from whole blood and frozen for future processing. Samples will be thawed and stimulated with Brefeldin A alone (BFA) or PMA/Ionomycin/Brefeldin A (PIB) for 3 hours prior to staining. Cells will be stained using heavy metal isotope labeled antibodies and detected by CyTOF. Cytobank and associated programs will be used for analysis.

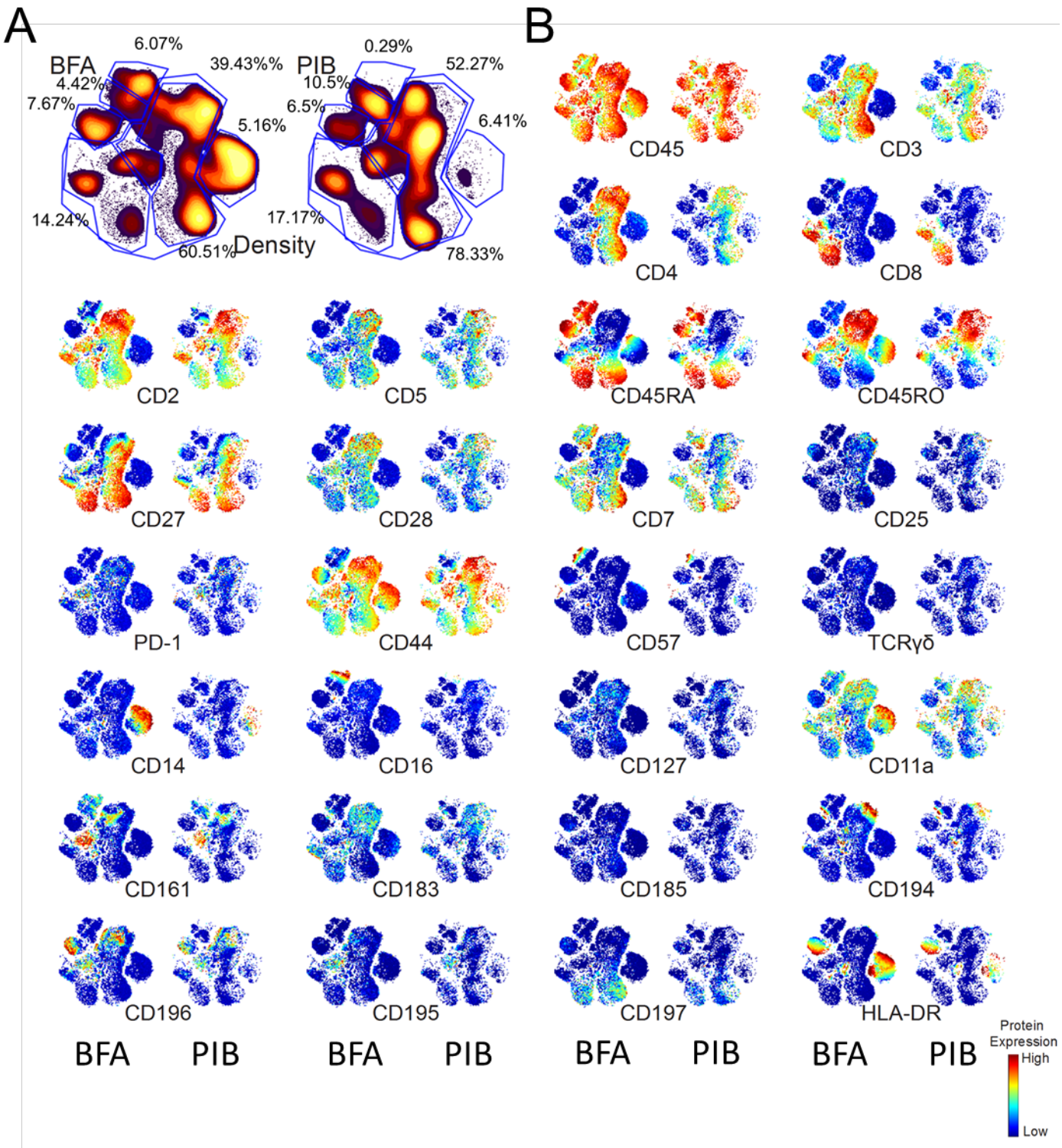

Figure 3-2. viSNE analysis of BFA and PIB treated CD45+ cells versus the 32-marker panel. Density distribution (**A**) and cell marker heat overlaid (**B**) without (left) and with (right) stimulation.

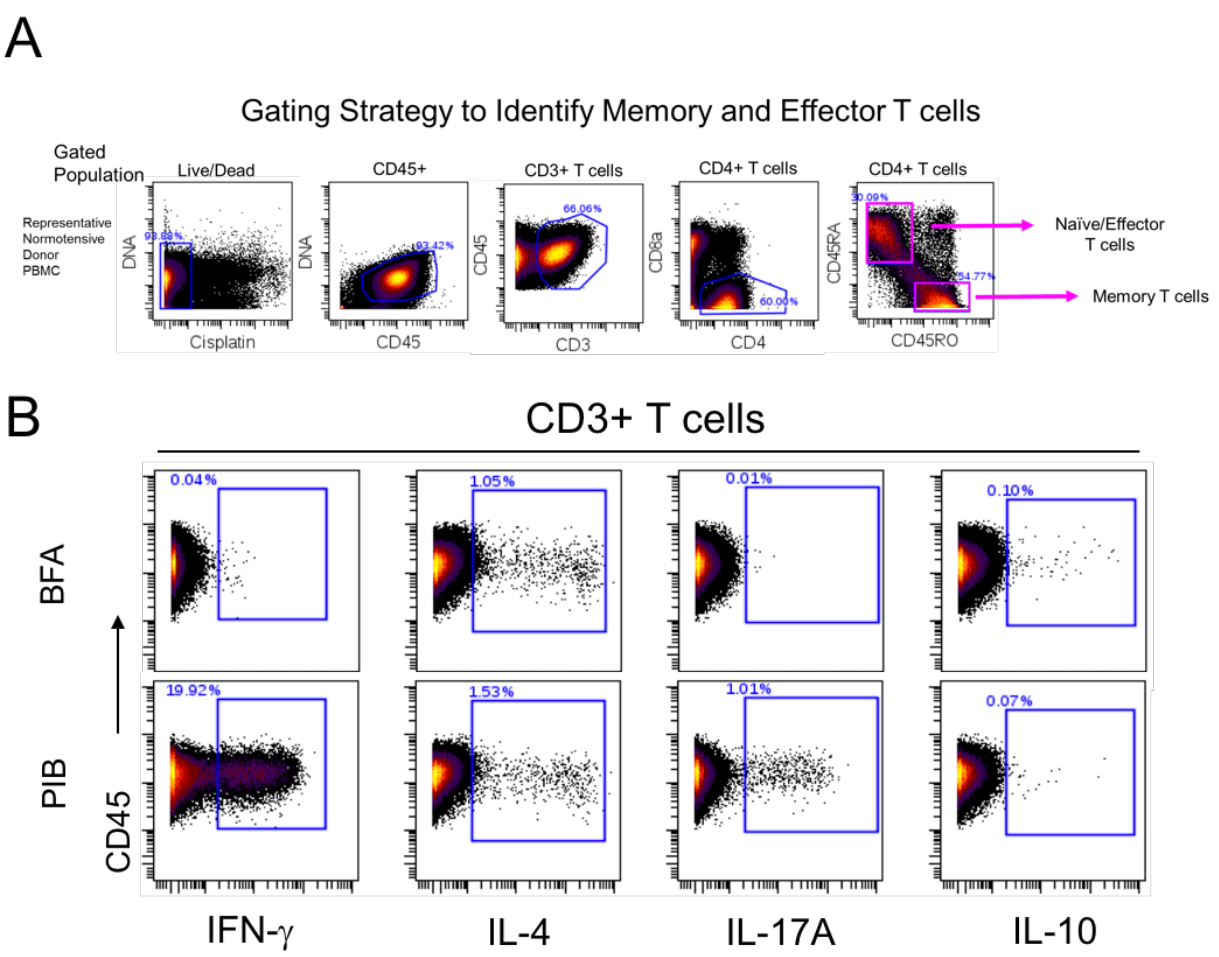

Figure 3-3. Expert gating and cytokine distribution across pan T cells by intracellular staining by mass cytometry (**A**) Expert gating strategy from PBMCs to identify pan T cells ($CD3^+$), Helper T cells ($CD4^+$), Cytotoxic T cells ($CD8^+$), naïve/effector T cells ($CD45RA^+$), and Memory T cells ($CD45R^+$) (**B**) Bi-axial plots of intracellular staining for IFNγ, IL-4, IL-17A, and IL-10 for $CD3^+$ T cells treated with BFA or PIB.

Table 3-2 Distribution of cytokine positive $CD3^+$ cells pre and post stimulation				
	IFNγ	IL-4	IL-17A	IL-10
BFA	0.04%	1.05%	0.01%	0.10%
PIB	19.92%	1.53%	1.01%	0.07%
fold increase	498	1.45	101	not applicable

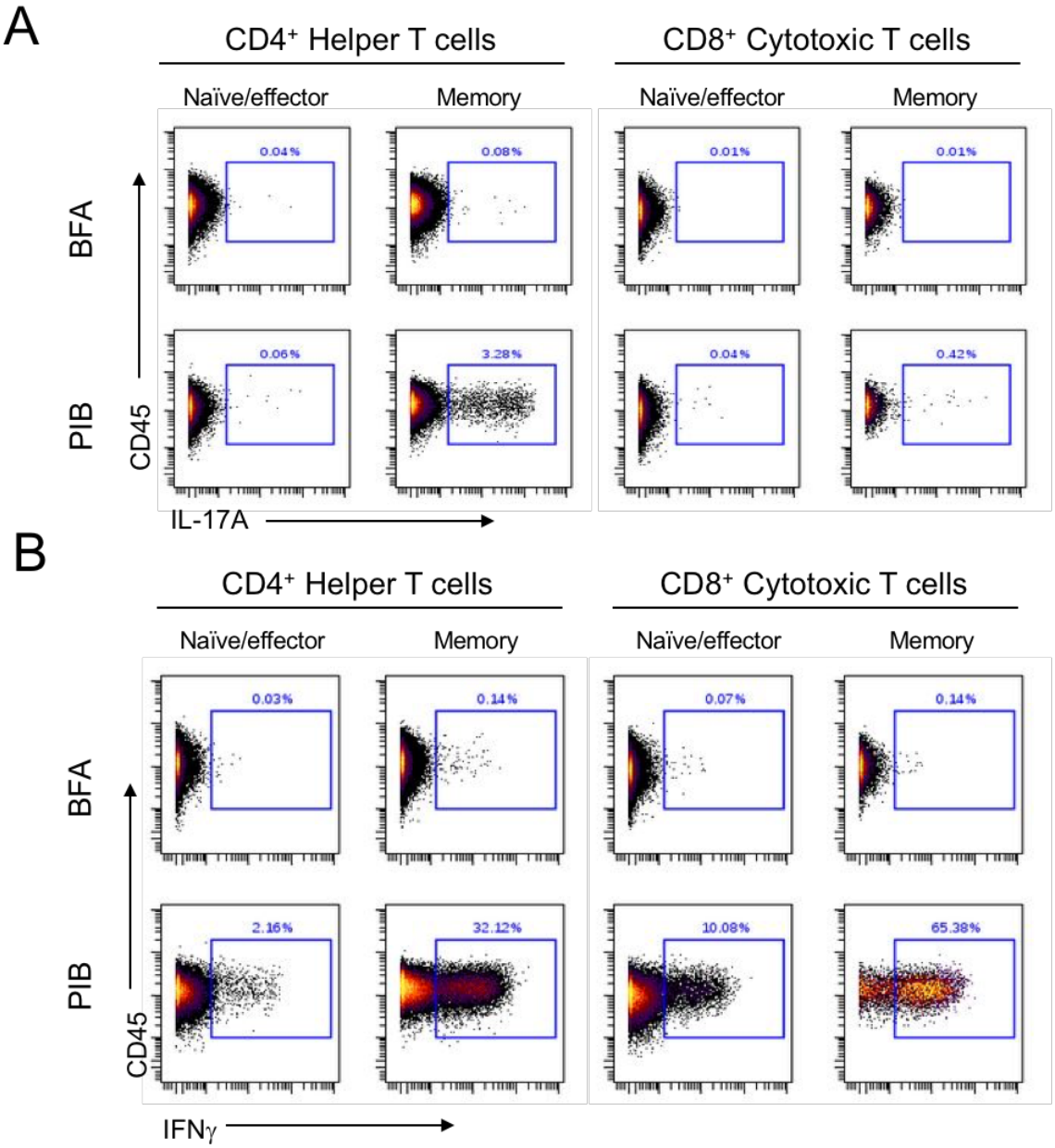

Figure 3-4. T cell subsets have differential staining of IL-17A and IFNγ. Bi-axial plots for Naïve/effector and Memory, $CD4^+$ Helper T cells and $CD8^+$ Cytotoxic T cells stained for (**A**) IL-17A and (**B**) IFNγ from Naïve/effector and Memory, $CD4^+$ Helper T cells and $CD8^+$ Cytotoxic T cells after PIB.

Table 3-3 Distribution of IL-17A and IFNγ positive T cell subsets with stimulation (PIB)				
	Naïve/effector CD4	Memory CD4	Naïve/effector CD8	Memory CD8
IL-17A	0.06%	3.28%	0.04%	0.42%
IFNγ	2.16%	32.12%	10.08%	65.38%

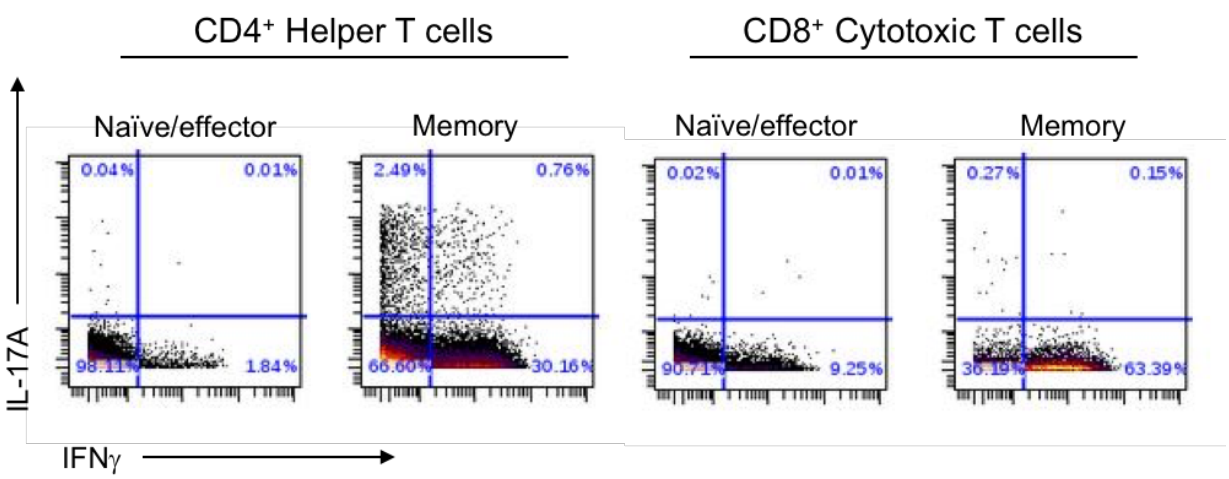

Figure 3-5. Identification of IL-17A, IFNγ double positive T cells by mass cytometry.

DISCUSSION

Identification of alterations in the immune system is a vibrant area of research that has come of age due to the progress made in our ability to detect and define highly specific immune cell populations in disease.[177, 178] With the understanding that hypertension is a disease that is mediated by the immune system, comprehensive immunophenotyping is essential to capturing a systemic view, rather than one cell type at a time. Elevated levels of IL-17A and IFNγ have both been noted in essential hypertension and shown to contribute to hypertension and end-organ damage.[25, 43, 44] thus the ability to identify producers of these cytokines is of importance.

While CyTOF has advantages over traditional fluorescent flow cytometry (namely quantity of markers and lack of spectral overlap) there are considerations to be made when initiating a new investigation. One of the most notable characteristics of a CyTOF study is the panel design. This is because the markers on your panel will ultimately serve as a roadmap during expert gating analysis and unsupervised multidimensional analysis. A significant amount of time should go into the curation of a novel panel, although the use of a predetermined panel will be most time effective. For example, during the preliminary analysis of this study multiple surface markers had yet to be optimized, such as $\gamma\delta$TCR and chemokine receptors (used to further subset T cells), and therefore Th1 versus Th17 production of cytokines could not be determined. While creation of a custom panel would be more novel, the time you are willing to wager to accomplish this task should be

considered.

The design of a comprehensive CyTOF T cell panel allows us to confirm observations made in rodent models, as well as capture novel T cell populations that may not translate effectively between rodent models of hypertension and humans with hypertension. Here I present data suggesting a novel method to characterize T cells, not only by their surface markers, but by their functional ability to produce cytokines known to promote hypertension. This will serve as an invaluable tool to observe changes not only in T cell populations, but also their effector functions.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Study Synopsis

Over the past 25 years the prevalence of hypertension has nearly doubled to 46% of the United States adult population.[1] Importantly, hypertension is the leading cause of global burden of disease, increasing risk of stroke, heart failure, myocardial infarction, and chronic kidney disease.[3] Despite effective pharmacological treatment, BP remains uncontrolled in nearly 50% of people with hypertension, and even those with relatively good control still have an elevated risk of adverse cardiovascular events.[29] These statistics suggest that novel therapeutics to undescribed targets contributing to disease pathophysiology are needed to control this disease and the end-organ effects.

While it was over 50 years ago that Grollman demonstrated BP can be lowered with immunosuppression in rats, nearly 40 years passed before hypotheses generated during this time were tested with emerging technologies.[96, 179] Since then, evidence has accrued over the past decade demonstrating that hypertension is an inflammatory disease with autoimmune characteristics.[25] T cell production of IL-17A by Th17 cells and IFNγ by Th1/Tc1 cells has been shown by my laboratory to contribute to hypertension and the end-organ damage.[43, 44] In addition to promoting the germinal center reaction, IL-21 has been reported to promote T cell production of IL-17A and IFNγ, thus acting as a master cytokine of the T cell response.[135] Furthermore, due to its recent discovery in 2000[133], the role of IL-21 and Tfh cells was previously unknown in hypertension. The overall goal of this dissertation was to determine the role of IL-21 in hypertension and end-organ damage and determine if Tfh cells play a critical role in hypertension. Through completion of my

studies I report the following conclusions:

1. IL-21 production is significantly elevated during hypertension and contributes to the hypertensive phenotype via alterations in immune cell effector functions as well as direct effects on the vasculature.
2. Pharmacological neutralization of IL-21 effectively lowers blood pressure, reverses endothelial dysfunction, and abrogates vascular inflammation.
3. Hypertension is associated with the accumulation of germinal center-like cells in the vasculature and Tfh cells are necessary to sustain hypertension.
4. T cells from humans with hypertension produce more IL-21 and this positively correlates with SBP and IL-17A production.
5. Novel cytokine producing cell populations can be identified through the use of CyTOF. This may aid in identifying highly specific cell populations as potential targets for therapeutic intervention.

Discussion

My investigation of the role of IL-21 and Tfh cells in hypertension has revealed multiple findings that warrant further discussion. How IL-21 signals in hypertension remains unknown, however canonically, IL-21 signals through its receptor, IL-21R and the signal is transduced by the Janus Kinase (JAK) /STAT signaling cascade. IL-21R is expressed on all hematopoietic cells as well as epithelial and endothelial cells.[134, 180] IL-21R is a member of the common gamma chain family of receptors. Upon ligation by IL-21, JAK1 and JAK3 are activated, and can phosphorylate transcription factors STAT1 and STAT3 and to a lesser extent STAT5. In most cases, STAT3 dimerizes and induces changes in

gene expression by localizing to the nucleus. If the signal activates STAT3 in B cells, this will induce the transcription factor BCL6, promoting the GC B cell phenotype, while if it signals via STAT5 in B cells will induce Blimp-1 and promote differentiation towards a plasma cell phenotype. In addition to B cells, IL-21 signals to an array of other cells such as T cells and endothelial cells. During T cell differentiation IL-21 also signals through STAT3 to promote the Th17 phenotype, by targeting RORγt, the transcription factor which stabilizes the Th17 effector function. IL-21 signaling has been investigated in T and B cells for nearly a decade, however less is known about how IL-21 signaling occurs in non-immune cells. To date it is understood IL-21 signals via JAK3 and STAT3 in endothelial cells and independently IL-6 induced STAT3 signaling in endothelial cells has been shown to inhibit eNOS transcription.[134, 181] Therefore, the chronic effect of IL-21 on vessels may be through regulation of eNOS expression, however the acute effects are likely through modulation of eNOS phosphorylation. Collectively, IL-21 can modulate the immune system and the vasculature independently, and in concert promoting inflammation and vascular dysfunction.

Immune cells express the angiotensin type-1 receptor (AT1R). Whether the immune system is activated in response to a specific antigen, tissue damage, or directly by Ang II is a contemporary topic. One school of thought revolves around the premise there is increased oxidative stress in hypertension, which results in isoketal modified proteins being presented by APCs to T cells, as neoantigens, promoting T cell proliferation and effector function.[107] A potential alternative hypothesis is the immune response is antigen specific. This response could be initiated due to an initial insult to the vascular wall

integrity from exposure to chronic shear stress and/or mechanical stretch. Endothelial cell damage would result in exposure, processing, and presentation of self-antigens by APCs to T cells, which don't normally detect such peptides. These T cells could then communicate with B cells within the tissue or circulate back through the lymphatic system to a SLO and select for B cells with BCRs which cross-react with the auto-antigen, resulting in a specific mounted antibody response. Lastly, none of the above may occur, and the immune system is activated directly by Ang II. This phenomenon has been reported in monocytes, where direct stimulation with Ang II induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling and release of TNF.[182] Interestingly, further *in vivo* studies of T cells and macrophages have provided evidence AT1R signaling on immune cells actually inhibits progression of the pro-inflammatory phenotype.[183-185] Interestingly, *in vitro* T cells from animals that lack AT1R have impaired cytokine production, and transfer of these T cells into RAG1$^{-/-}$ animals only partially restores the hypertensive phenotype.[96, 186] This observed phenomenon for immune AT1 receptors to dampen the inflammatory response may be a mechanism that evolved to lessen the inflammatory response that occurs in hypertension.

In **Chapter 2** I present data using two different models of IL-21 depletion, a global genetic knockout as well as the use of an IL-21 neutralizing antibody. While both models resulted in a 15mmHg decrease in blood pressure and reduced inflammation, there were some observed discrepancies. First, the global knockout animals have a baseline endothelial dysfunction which does not manifest in a hypertensive phenotype. Studies have shown that IL-21R is present on endothelial cells (ECs) and IL-21 signaling via STAT3 plays a

key role in neoangiogenesis.[134] I also report the IL-21$^{-/-}$ vessels have an elevated level of SO as baseline, which is a precursor of peroxide. Increased peroxide has been shown to enhance vascular endothelial growth factor (VEGF) signaling.[187] It is possible the global deletion of IL-21 from birth manifests in a vascular phenotype due to alternative mechanisms required for angiogenesis, such as enhanced VEGF signaling. This suggests some amount of IL-21 is necessary for healthy vessel development and function. While this may help explain the baseline dysfunction, the anti-IL-21 treated vessels demonstrated improved function when IL-21 was neutralized, suggesting *some* level of IL-21 is pathogenic. Additionally, the effects of IL-21 neutralization were observed after 2 weeks of treatment, which resulted in a drastic decrease in inflammation. Therefore, whether the effect of IL-21 on the vessels is through immune modulation or a direct effect on the vessel could not be assessed from this specific study. I followed this study up with an acute *ex vivo* treatment using rIL-21 prior to vascular function studies and found this impairs endothelial dependent relaxation and *in vivo* studies where HAECs were treated with rIL-21 resulted in a decrease in NO. The precise mechanism as to how this is occurring remains unknown, however based on the current literature, I postulate the acute response it is likely mediated through an alteration in the inhibitory phosphorylation status of eNOS, as cytokines such as IL-17A have been demonstrated to enhance eNOS inhibitory phosphorylation.[122]

As introduced in **Chapter 1**, multiple T cell subsets and their associated cytokines have been associated, and demonstrated to contribute to the hypertensive phenotype.[25] An interesting finding of my studies was the identification that global deletion of IL-21 in mice

results in a blunted hypertensive cytokine profile, namely IL-17A and IFNγ (suggesting IL-21 acts upstream of IL-17A and IFNγ) although, interestingly, Tfh deficient animals have more IL-17A and similar levels of IFNγ. It has also been shown that when IL-17A or IFNγ are independently knocked out, animals are protected from hypertension and end-organ damage. These finding call into question if all T cell subsets/cytokines contribute equally to hypertension or if there is an immunological hierarchy at play since whenever a "pro-inflammatory" cytokine is altered in the system there is a protective effect. If a hierarchy can become established then the earliest event mediated by the immune system could be targeted pharmacologically to prevent the downstream effects from persisting. Consideration should be made while using this information for therapeutic target identification since targeting a molecule or cell population too far up the cascade of events could have global immunosuppressive effects. The goal in determining a hierarchy would be to identify a branch point where the result of intervention of a target can be sensitive and specific. Due to the observed immunosuppressive effects of anti-IL-21 on all immune cell subsets quantified, IL-21 may be too broad of a target highlighting the need to target a specific cell population producing IL-21, such as Tph cells or Tfh cells. Studies addressing this question will be invaluable to translating findings, like those presented here, into clinical practice.

Immunoglobulin levels have long been associated with hypertension in humans and animal models, although the studies presented here are the first to establish a role of Tfh cells, presence and elevation of peripheral Th cells, and extrafollicular localization of germinal center-like cells in the vasculature in hypertension. Elevated IL-21 serum levels,

peripheral Th cell numbers, and aberrant GC reactions are reported in autoimmune diseases.[83, 137, 169] Interestingly, there is an increased incidence of resistant hypertension in those with autoimmune disorders, such as SLE, versus the general population.[188] During SLE, IL-21 is known to promoter plasma cell generation, and auto-antibody production, leading to disease pathology, such as kidney disease. Additionally, endothelial dysfunction is a marker of SLE pathology and anti-IL-21 treatment has been shown to ameliorate disease severity.[189, 190] The established literature from both of these disease models can contribute to one another. While the understanding of how autoantibodies contribute to SLE is well documented, little is known of the effect of autoantibodies on hypertension. Additionally, as the effect of IL-21 on the vasculature are unraveled in cardiovascular disease, lessons learned using hypertension models may also translate to experimental SLE.

Future directions

Collectively my studies have added to the growing body of work investigating the role of T cells and their associated cytokines in hypertension and vascular dysfunction. Whether T cell activation and cytokine production cause hypertension or are a result of an initial insult to the RAAS system remains a matter of debate. Our current understanding revolves around a novel mechanism suggesting that there is an initial insult to the endothelium resulting in the activation of the immune system. This occurs because elevated blood pressure can increase shear stress and mechanical stretch within the vasculature resulting in endothelial insult, generation of ROS, and recruitment of immune cells.[108] APCs recruited to the endothelium take up antigen and present to T cells, which

may resulting in T cell polarization such as the Tfh cell polarization shown in **Chapter 2**. Tfh cells can then elicit their effector function within SLOs, the periphery via direct effect on the vasculature, or indirect effector function through communication with other immune cells, such as other T cell subsets and B cells. Although I have defined a role for IL-21 and Tfh cells in the setting of hypertension, there remain many unknowns as to the mechanism-of-action for IL-21 and Tfh cells in hypertension.

I demonstrate $CD4^+$ T cells produce IL-21 in hypertension and the Tfh deficient animals cannot sustain the hypertensive response, however the precise cell population remains unknown. IL-21 can be produced by many $CD4^+$ T helper cells, such as Th17, Tfh, and the less studied Tph, in addition to NK T cells and NK cells.[140, 169, 180] I have shown Tfh, Tph and NK T cell numbers are elevated in the vasculature during hypertension. In addition to these cell types, it is plausible that IL-21 may be produced by the endothelium, as endothelial cells have been described to be known sources of cytokines.[108, 191] Future studies should aim to identify the key producers of IL-21 during hypertension. One valuable method to test this would be through the use of an IL-21 reporter mouse line. IL-21 is notoriously difficult to perform intracellular staining for, and thus these data often go unreported in studies. Cells from the vasculature of these animals after the onset of hypertension should be sorted by FACS and subjected to CyTOF staining and analysis, or RNAseq to obtain thorough cell marker phenotyping and gene expression profiles. These studies would help create a more comprehensive view of $IL\text{-}21^+$ cells in hypertension.

I have shown for the first time that hypertension is associated with an increase in mesenteric LN GC activity, the accumulation of GC-like cells in the vasculature, and the formation of TLOs. Moreover, I show hypertension results in an increase in serum IgG in an IL-21 dependent manner. B cells have been shown to serve an obligatory role in hypertension,[113] and IL-21 has been reported as the most potent inducer of B cell differentiation, Ig class switching, proliferation, and antibody production.[82-84] While hypertension has been associated with antibodies that antagonize the RAAS for many decades, whether hypertensive IgGs are functionally relevant in the pathophysiology of essential hypertension is undetermined.[163] In the context of autoimmune disease, antibodies are often a product of aberrant GCs and ultimately cause organ damage due to antibody deposition and cellular damage.[138] Our laboratory has preliminary evidence suggesting adoptive transfer of purified hypertensive IgG but not normotensive IgG results in the accumulation of $CD8^+$ T cells and $F4/80^+$ macrophages in the vasculature (data not shown). IgG deposition can recruit $CD8^+$ T cells and $F4/80^+$ macrophages via their expression of Fc receptors. Through engagement of these receptors, $CD8^+$ T cells can elicit their cytotoxic function and initiate cellular damage via secretion of perforin and granzymes. Further, $F4/80^+$ macrophages may uptake the damaged cells and present intracellular antigens viewed as foreign via MHC to T cells, enhancing the autoimmune response.[65] Future studies should be conducted to determine if hypertensive IgG are specific to the vasculature such as by running a western blot of homogenized vessel or pure cell lines and probing the blot with hypertensive IgGs. An additional approach could utilize antibody-dependent or independent cytotoxicity assays to test if hypertensive antibodies can fix complement or enhance cytotoxicity of effector cells, such as $CD8^+$ T

cells. Since it is possible the antibodies are specific to a endothelial marker, HAECs or endothelial cells sorted from murine aorta and mesenteric vessels could be used as the target tissue.

It is clear IL-21 is a pleotropic cytokine with many effector functions.[180] In keeping with that, I found that IL-21 likely plays more than one role in promoting hypertension. One route is through orchestrating the T cell cytokine response. During hypertension, T cells become activated and produce significant amounts of IL-17A ($CD4^+$ T cells)[43] and IFNγ ($CD8^+$ T cells)[44] and I show animals that are IL-21 deficient lack the ability to enhance the production of these cytokines. Renal damage and dysfunction are a trademark of hypertension.[12] Our group showed that IL-17A promotes hypertension through regulation of renal sodium transporters in a serum glucocorticoid regulated kinase-1 dependent pathway, resulting in enhanced salt retention and water reabsorption.[124] Additionally, IFNγ has been shown to promote the production of angiotensinogen from cultured renal proximal tubule cells, shifting the dynamics of the RAS system.[192] Thus future studies should be considered to investigate whether IL-21, by virtue of promoting IL-17A and IFNγ or independently has an effect on renal damage, as well as alter the production of RAS components such as renin and angiotensinogen. Mice which lack IL-17A or IFNγ could be infused with rIL-21 to determine if end-organ damage is altered. If IL-21 deficient animals are only protected due to IL-21 acting upstream, the effect of IL-21 infusion on end-organ damage could help determine if this is the case.

In addition to an orchestration of T cells, I show that IL-21 deficient animals have no change in endothelial function as a result of Ang II-induced hypertension and further demonstrate a direct effect of IL-21 on the vasculature independent of T cells. Using *ex vivo* treatment of resistance vessels and subjecting them to vascular relaxation studies, I show acute treatment with rIL-21 impairs endothelial dependent relaxation and through a separate experimental setup that this may be through altering NO bioavailability. NO bioavailability can be altered by phosphorylation status. Endothelial nitric oxide synthase (eNOS) has an inhibitory phosphorylation site (Thr495) and a stimulatory phosphorylation site (Ser1177). Of interest, IL-17A has been shown to have direct effects on the endothelium through alterations in NO bioavailability via promoting Thr495 phosphorylation in a RhoA kinase dependent manner.[122] Furthermore, IL-6 directly results in a decrease in eNOS expression via STAT3 inhibition of the eNOS promoter.[181] While an acute treatment does not provide sufficient time to alter gene expression and protein translation, future studies investigating whether IL-21 is altering eNOS phosphorylation via STAT3 or being promoted endogenously by RhoA kinase would be a valuable addition to the current mechanism. Additionally, an *in vitro* cell culture system using aortic endothelial cells co-cultured with T cells from Ang II-infused WT and IL-21$^{-/-}$ animals could help decipher if IL-21 can also act indirectly via T cells on endothelial cells.

A physiological characteristic noted in the vascular wall during hypertension is hypertrophy of the vascular smooth muscle medial layer, accumulation of collagen deposits in the adventitia, and increased aortic stiffness.[12, 27] I found that IL-21 deficient animals have blunted media hypertrophy and collagen deposition. IL-21 has been shown

to stimulate pulmonary artery smooth muscle cell proliferation indirectly through macrophages production of C-X-C motif chemokine 12,[142] as well as promote fibrosis via $CD8^+$ T cell production of interleukin-13.[193] To elicit a mechanism of how IL-21 deficiency protects against medial hypertrophy and fibrosis, future studies should examine the direct effect of IL-21 using an *in vitro* cell culture system of vascular smooth muscle cells and aortic fibroblasts ability to produce collagen.

While I show IL-21 is *necessary* to achieve WT end-points of experimental hypertension, I did not test if IL-21 or IL-21 producing cells are *sufficient* to cause hypertension. Our group has previously performed adoptive transfer studies demonstrating that pan T cell transfer into *Rag1*$^{-/-}$ mice is sufficient to restore the hypertensive phenotype.[96] Future studies should determine if adoptive transfer of IL-21^+ cells from hypertensive animals back into the IL-21 deficient animals is sufficient to restore the WT response. Additionally, adoptive transfer of IL-21^+ cells into WT mice infused with a subpressor dose of Ang II would address if the expansion of the cells is sufficient to induce hypertension. Completion of these studies would help address if IL-21 producing cells are sufficient to restore and/or cause hypertension. Alternatively, infusion of rIL-21 should also be performed. This would be of interest as rIL-21 infusion is currently being used for the treatment of advanced melanoma and in Phase 1and 2 studies for metastatic melanoma and renal cell carcinoma, among other cancer types.[194-196] Interestingly, patients infused with rIL-21 have enhanced anti-tumor $CD8^+$ T cell function as measured by elevated mRNA levels of perforin, granzyme, and IFNγ, all of which are known to play active roles in the pathophysiology of hypertension and end-organ damage.[115, 136, 197, 198] With these

findings, I hypothesize rIL-21 infusion would be sufficient to induce hypertension through enhanced $CD8^+$ T cell effector function.

I also report anti-IL-21 treatment after the onset of Ang II-induced hypertension can lower SBP, reverse endothelial dysfunction, and abolish vascular inflammation. Anti-IL-21 treatment interventions are currently in Phase I and II clinical trials for RA, Chron's disease, and lupus.[74] While our findings in hypertension are encouraging and should be pursued, considerations need to be made concerning the use and frequency of anti-IL-21 monoclonal antibodies to abrogate the effects of elevated levels of IL-21 in disease, as IL-21 exerts effector function across a wide array of cell types.[180 136, 153, 199]

Conclusions

Taken together, the studies presented and discussed in this thesis have established a critical role for IL-21 and Tfh cells in hypertension. Further our findings suggest IL-21 likely mediates disease progression through direct effects on the vasculature, as well as serving as a "master cytokine" of T cell subsets in hypertension. Moreover, the use of CyTOF and multidimensional analysis will be a vital asset to identify and phenotype IL-21 producing cells in the future. In summary, these data collectively suggset anti-IL-21 monoclonal antibody treatment as a novel therapeutic strategy for hypertension and vascular disease.

APPENDIX

CyTOF Protocol for cell stimulation, surface stain and intracellular staining

Media: warm RPMI+10% FBS
Staining Media: cold PBS+1% BSA
DAY 1: make stains
DAY 2:

1. **Thaw** cells in warm water
2. Add 1ml warm media to thawed cells and transfer into a 15ml conical
3. Bring volume of media up by adding 8 ml of media
4. spin down at 200g, at room temp for 5 minutes
5. re-suspend and count cells
6. set cell concentration to 2 million cells/ml in RPMI+5% FBS
7. In a 6 well cell culture plate, plate 1 million cells/well.
    a. For non-stim wells: add Brefeldin A only. Stock (5mg/ml). Use at 1:50 dilution, (10ug/ml in PBS).
    b. For Stim wells: Add PMA/Ionomycin cocktail at 2ul/ml
8. **Stim** for 3 hours.
9. After stimulation, collect cells and spin at 200g at RT for 5 min
10. **Live Dead Stain**
    a. Cisplatin at 1ul/ml for 5 min at RT
    b. Add 4 ml PBS+1% BSA and spin at 200g, at RT, for 5 min.
11. **Live surface stain**
    a. Transfer 30ul of cells to new FACS tube
    b. Add 20 ul of premade surface stain
    c. Stain at RT for 30 min
    d. Wash with 2 ml PBS+BSA
    e. Spin at 200g, at RT, for 5 min
    f. Repeat wash
12. **Fix**
    a. Decant
    b. Vortex tubes while adding 1ml of 1x BD TF Fix/Perm
    c. Incubate for 45 min at 4C
    d. Add 1ml Perm/Wash and vortex
    e. Spin at 900g, at RT, for 5 min
    f. Repeat wash
13. **Perm**
    a. Decant and resuspend the cells in residual volume
    b. Add 1 ml cold Methanol (-20C)
    c. Vortex immediately
    d. Incubate O/N -20C (minimum 10 minutes)

DAY 3:

14. **MeOH Removal**
    a. Wash with 2 ml PBS, 900g, RT, 5 min
    b. Vortex and decant
    c. Repeat wash with PBS+BSA
15. **Intracellular Stain**
    a. Resuspend cells in stain media
    b. Transfer 30ul to new tube
    c. Add 20ul premade stain
    d. Vortex
    e. Incubate at RT for 30 min
    f. Wash with 2ml PBS+BSA, 900g, RT, for 5 min.
16. **Nucleic Acid Stain**
    a. Add 500ul of Ir191/193 (working .25uM / 1:2000 dilution of 500uM stock)
    b. Incubate for 15 min at RT
    c. Wash with 2 ml PBS at 900g, at RT, for 5 min
17. Leave in residual volume until acquisition
18. Wash with 1 ml DDiH20
19. Spin 900g, at RT, for 5 min
20. Bring volume up in 1 X norm beads

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