ROLE OF CIRCULATING T CELLS IN AUTOIMMUNE KIDNEY DISEASE: IMPLICATIONS FOR ANCA DISEASE AND MINIMAL CHANGE DISEASE

Meghan Elizabeth Free

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Approved by:

Ronald J. Falk, M.D.

J. Charles Jennette, M.D.

Yuri Fedoriw, M.D.

Maureen Su, M.D.

Donald Cook, Ph.D.

ABSTRACT

MEGHAN E FREE: Role of Circulating T Cells in Autoimmune Kidney Disease: Implications for ANCA Disease and Minimal Change Disease
(Under the direction of Dr. Ronald J. Falk, M.D.)

This dissertation is focused on circulating T cells and the role they play in the immunopathogenesis of two autoimmune kidney diseases: anti-neutrophil cytoplasmic autoantibody (ANCA) disease and minimal change disease (MCD). Results demonstrated in the subsequent chapters explain known regulatory T cell defects in ANCA disease, reveal altered effector T cell dynamics in ANCA disease and present novel data of autoantibody/autoantigen interactions in minimal change disease.

Data presented in Chapter 1 addresses the known defect of regulatory T cell suppression in patients with ANCA disease. The lack of T cell suppression by ANCA disease regulatory T cells is confirmed in our patient cohort. However, our data reveal that a splice variant of FOXP3 lacking exon 2 is highly prevalent in ANCA disease patients and expression of exon 2-deficient FOXP3 correlates with a decreased suppressive function of the same regulatory T cells. Yet, the data in Chapter 1 also demonstrates that defective regulatory T cells are not the sole culprit of effector T cell non-suppression in ANCA disease patients.

Additional data in Chapter 1 demonstrates that ANCA disease patients have an expansion of a $CD25^{intermediate}$ T cell which comprises the majority of their peripheral T cell pool. These $CD25^{int}$ T cells produce pro-inflammatory cytokines, are antigen-experienced and are resistant to suppression by regulatory T cells from healthy individuals. As such, T cell dysfunction and aberrant proliferation in ANCA disease stems from both altered regulatory T cells and an expanded $CD25^{int}$ population which is difficult to suppress by conventional means.

Chapter 2 focuses on the discovery of anti-TCR autoantibodies found in patients with minimal change disease. These anti-TCR autoantibodies target a specific subset of circulating T cells found at a higher frequency in MCD patients compared to healthy individuals. Additionally, this autoantibody/autoantigen interaction induces cellular activation leading to cytokine production from targeted cells. We hypothesize that these interactions and downstream effects ultimately lead to the immunopathogenesis of MCD by causing injury to podocytes. In sum, the data presented herein comprise a comprehensive body of work which reveals previously unknown roles of circulating T cells in ANCA disease and minimal change disease.

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LIST OF ABBREVIATIONS

[A]

AAV	anti-neutrophil cytoplasmic autoantibody associated vasculitis
ANCA	anti-neutrophil cytoplasmic autoantibodies

[B]

BVAS	Birmingham vasculitis activity score

[C]

c-ANCA	cytoplasmic-antineutrophil cytoplasmic autoantibodies
CCR5	C-C chemokine receptor type 5
CCR6	C-C chemokine receptor type 6
CCR7	C-C chemokine receptor type 7
CD2	cluster of differentiation 2
CD3	cluster of differentiation 3
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CD25	cluster of differentiation 25
CD28	cluster of differentiation 28
CD45RA	cluster of differentiation 45RA
CD45RO	cluster of differentiation 45RO
CD127	cluster of differentiation 127

CDR	complementarity determining region
CFSE	carboxyfluorescein succinimidyl ester
CNBr	cyanogen bromide
CPT	cell preparation tube
CXCR3	chemokine (C-X-C motif) receptor 3

[D]

DRB1*15	human leukocyte antigen-DRB1*15
DNA	deoxyribonucleic acid

[E]

E-GPA	eosinophilic granulomatosis with polyangiitis
ELISA	enzyme-linked immunosorbent assay

[F]

FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluoresceine-isothiocyanate
FOXP3	forkhead box P3
FPLC	fast protein liquid chromatography
FSGS	focal segmental glomerulosclerosis

[G]

GDCN	Glomerular Disease Collaborative Network
GITR	glucocorticoid-induced TNFR-related protein
GPA	granulomatosis with polyangiitis

[H]

HBSS	Hank's balanced salt solution
HC	healthy control
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	human leukocyte antigen

[I]

IDEAS	Image Data Exploration and Analysis Software
IFNγ	interferon gamma
Ig	immunoglobulin
IgE	immunoglobulin class E
IgG	immunoglobulin class G
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
IL-7Rα	interleukin 7 receptor chain alpha
IL-8	interleukin 8
IL-10	interleukin 10
IL-13	interleukin 13
IL-17	interleukin 17
IL-23	interleukin 23
IV	intravenous

[K]

kD	kilodalton

[M]

MALDI-TOF/TOF	matrix-assisted laser desorption ionization time of flight
MCD	minimal change disease
MHC	major histocompatibility complex
mL	milliliter
MPA	microscopic polyangiitis
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MS	mass spectrometry
mTOR	mammalian target of rapamycin
MyH9	myosin heavy chain 9, non-muscle

[N]

NCBI	National Center for Biotechnology Information
NFAT	nuclear factor of activated T cells

[O]

OD	optical density

[P]

p-ANCA	perinuclear- antineutrophil cytoplasmic autoantibodies
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PI	propidium iodide
PMA	phorbol myristate acetate
PNPP	para-nitrophenyl phosphate
PR3	proteinase 3
pSIVA	polarity sensitive indicator of viability and apoptosis

[R]

RA	rheumatoid arthritis
RORα	retinoic acid receptor-related orphan nuclear receptor alpha
RORγt	retinoic acid receptor-related orphan nuclear receptor gamma t
RPMI	Roswell Park Memorial Institute medium
RT	room temperature

[S]

SLE	systemic lupus erythematosus

[T]

T1DM	type 1 diabetes mellitus
TCR	T cell receptor
TGFβ	transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
Treg	regulatory T cell

[U]

UNC	University of North Carolina at Chapel Hill
UV	ultraviolet

[W]

WBC	white blood cell

[Y]

YAG	yttrium-aluminum gamet

PROLOGUE: CIRCULATING T CELLS IN AUTOIMMUNE DISEASE

The adaptive, antigen-specific immune system relies heavily on T cells with the capacity to adapt to the nuances of each pathogen. Healthy individuals maintain T cell homeostasis with regulatory T cell suppression of inappropriate effector T cell responses. In autoimmune diseases, there is a breakdown of this balance leading to uncontrolled autoreactive T cell responses. Possible T cell disturbances in autoimmune disease are any combination of the following: a lack or overrepresentation of certain T cell subsets, regulatory T cell dysfunction or inappropriate effector responses. However, autoimmune disease without a monogenetic cause cannot result from just one disturbance. It is more likely that multiple aberrations are interrelated in each autoimmune disease.

Autoimmune diseases affecting the kidney are numerous and include anti-glomerular basement membrane disease[1], lupus nephritis[2] and anti-neutrophil cytoplasmic autoantibody (ANCA) disease[3]. Additionally, there exist kidney diseases with suspected autoimmune involvement, though lack direct evidence, as is the case with minimal change disease (MCD) and primary focal segmental glomerulosclerosis (FSGS)[4].

Circulating autoantibodies are found in ANCA disease and these autoantibodies are class-switched and high affinity, indicating previous T cell help. Therefore, CD4+ T cells must play some role in the immunopathogenesis of ANCA disease. While even less is known about the role of T cells in MCD, anecdotal evidence is highly suggestive of some amount of T cell involvement. The function of T cells in the immunopathogenesis and disease progression in the

context of medical intervention is crucial to achieve a better understanding of autoimmune disease.

Effector T cell abnormalities in autoimmune disease

CD4+ T cells comprise a large part of an individual's adaptive immune system. Antigen specificity of T cells provides tailored immunity towards any pathogen with potential for invasion. Additionally, CD4+ T cells can be further subdivided into different "helper" subsets, each with a different "preference" of pathogen and capacity for cytokine secretion. Until the mid-2000s, immunologists had only defined two helper subsets, Th1 and Th2, which were thought to antagonize each other.

Th1 cells were directed against intracellular pathogens such as *Listeria monocytogenes*, and preferentially secreted IFN-gamma. For years, many autoimmune diseases were linked to a preponderance of Th1 cells or Th1-related cytokines. Both the mouse model of multiple sclerosis, experimental allergic encephalomyelitis, and the mouse model of insulin-dependent diabetes were generally accepted to result from a preponderance of Th1 cells[5,6]. However, many autoimmune diseases could not be categorized as Th1 or Th2-driven and presented as a mystery for years.

In contrast to Th1 cells, Th2 cells are critical for the clearance of extracellular pathogens, especially helminths and parasites. Cytokines secreted by Th2 cells, IL-4, IL-5, IL-10 and IL-13 induce downstream effects central to immunoglobulin class-switching. While this cascade is important for the formation of IgE to clear parasites, IgE is also a central mediator of allergic responses. Furthermore, Th2 cells are incriminated in the pathogenesis and progression of inappropriate allergic reactions and asthma.

For decades, Th1 cells were thought to be the major T cell subset responsible for the immunopathogenesis of autoimmune diseases, however experimental research to this end has often returned conflicting results. The discovery of Th17 cells helped to reconcile many discrepancies in the literature surrounding the pathogenic effector T cell in autoimmune disease[7]. Th17 cells are important in the clearance of extracellular bacteria and fungi by the secretion of IL-17A, IL-17F and other related cytokines. Current research in most autoimmune diseases has now demonstrated an increased frequency of Th17 cells in patients. Th17 cells are particularly adept at promoting tissue inflammation and recruitment of neutrophils to sites of inflammation[8], therefore, it is not surprising that Th17 cells are found in targeted organs of various autoimmune diseases.

Further investigation into Th17 cell development revealed numerous similarities between Th17 and regulatory T cell differentiation. Both cell subsets require the presence of TGFβ for differentiation[9]. This finding helped to reconcile an ongoing paradox in the inflammatory literature as some experiments demonstrated the pro-inflammatory nature of TGFβ[10] while others demonstrated an anti-inflammatory effect[11]. The defining feature of T cell differentiation is the microenvironment. If TGFβ is present, a naïve T cell will essentially "default" into an induced Treg phenotype. However, if the pro-inflammatory cytokine(s) IL-6 (in mice) and IL-1β (in humans) are in the same microenvironment, the same naïve T cell would be diverted to a Th17 phenotype[9,12,13].

Th17 and Treg cell differentiation are further linked by common transcription factor induction. Th17 cells are predominantly controlled by RORγt and Treg cells by FOXP3. Interestingly, naïve T cells in the presence of TGFβ express both RORγt and FOXP3 initially[14]. It is only with further downstream commitment to T cell subsets that one transcription factor dominates the other. If TGFβ is present in a non-inflammatory milleu, FOXP3 antagonizes the function of RORγt, allowing a Treg program to initiate[15]. However, when TGFβ exists with IL-6, IL-1β or other pro-inflammatory Th17-inducing cytokine, RORγt will antagonize FOXP3, thus driving towards a Th17 cell[16]. This reciprocity in Th17 and Treg development underlines the potential for the immune system to go awry in autoimmune disease.

Regulatory T cell dysfunction in autoimmune disease

Autoreactive T cells occasionally escape thymic deletion and are released into the circulation. Despite the presence of circulating autoreactive T cells, most individuals do not development autoimmune disease. This is, in part, attributable to regulatory T cells and their ability to dampen inappropriate effector T cell responses. Tregs have been classically defined as CD4+ $CD25^{high}$ and FOXP3+ with the recent addition of $CD127^{low}$ [17,18]. The transcription factor FOXP3+ is the critical component of functional Tregs as evidenced by patients with FOXP3 mutations, resulting in uncontrolled lymphoproliferation and numerous autoimmune diseases[19]. Along this line, scurfy mice with spontaneous mutations in FOXP3, demonstrate a fatal lymphoproliferative disease[20]. However, implementation of the scurfin transgene (analogous to FOXP3) into scurfy mice abrogates the massive lymphoproliferation[20].

Current research provides evidence that Tregs can suppress effector T cell proliferation and cytokine production through both direct and indirect means. Examples of some mechanisms of Treg suppression are secretion of anti-inflammatory cytokines, consumption of cytokine(s) needed for effector proliferation, cell surface molecule engagement and direct killing of effector T cells. Two anti-inflammatory cytokines produced by Tregs that are commonly focused on are TGFβ and IL-10. While controversial, TGFβ produced by regulatory T cells has been shown to regulate effector T cell proliferation[21]. IL-10 is also frequently associated with Treg suppression, although IL-10 produced by Tregs seems to have differential effects depending on the involved organ[22]. Also controversial is the consumption of IL-2 by regulatory T cells, thereby starving effector T cells so proliferation does not proceed[23]. This makes teleological sense as Tregs have high expression of CD25, the receptor for IL-2, but these findings have been disputed over the years[24]. Additionally proposed is the hypothesis that regulatory T cells secrete granzyme A and induce effector T cell death through perforin[25].

The fact that there are numerous pathways of Treg suppression with conflicting literature reports points to a rationale that Treg mediated suppression does not stem from one pathway and is dependent on the type of inflammation as well as the location of inflammation. To this end, some type of Treg dysfunction has been demonstrated in the majority of autoimmune diseases. However, the mechanism(s) behind Treg dysfunction are varied and controversial. Three main mechanisms of impaired Treg function were proposed by Buckner[26] as 1) diminished numbers of regulatory T cells, 2) inability of regulatory T cells to perform suppressive functions, and 3) effector T cells that are resistant to regulatory T cell suppression.

The decreased frequency of peripheral Tregs can be due to diminished induction of Tregs or altered survival of Tregs. However, enumeration of Tregs has been controversial over the years and in most autoimmune diseases, there is no consensus as to if Tregs are decreased, increased or equal to healthy control frequencies. Additionally, recent research has proposed that regulatory T cells may be unstable in chronic inflammatory states and can convert into other T cell phenotypes[27].

Dysfunctional Tregs that are unable to control effector proliferation is another common finding in autoimmune disease. Some studies suggest that this could be due to, in part, the inability of disease Tregs to secrete anti-inflammatory cytokines such as IL-10[28,29]. Treg suppression assays are performed *in vitro* which are intrinsically problematic. Recent hypotheses have questioned whether the defect of suppression *in vitro* can be solely attributable to Tregs. This has led to research to probe the potential of effector T cells that resist Treg suppression.

In some cases, Tregs from a diseased individual may be capable of suppressing allogeneic cells from a healthy individual, but not their own autologous effector cells. Suppression-resistant effector cells have been observed in mouse models of SLE, experimental autoimmune encephalomyelitis and type 1 diabetes[30,31,32]. Effector cell resistance may be intrinsic to the T cell phenotype (Th17 cells are notoriously more difficult to suppress)[33,34], or cytokine secretion from effector T cells may disrupt the suppressive mechanisms of the regulatory T cells[35].

CD4+ T cells in ANCA disease

ANCA disease can manifest with systemic small vessel vasculitis, leading to organ damage of highly vascular organs such as kidneys, lungs and upper respiratory tracts. These vasculitides can be subdivided into granulomatosis with polyangiitis (GPA) formerly Wegener's, microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA) formerly Churg-Strauss syndrome. The common immunologic feature of these diseases is the formation of ANCAs that target either myeloperoxidase (MPO) or proteinase-3 (PR3) found in the granules of neutrophils and monocytes. The known production of autoantibodies in anti-neutrophil cytoplasmic autoantibody (ANCA) disease provides evidence towards T cell involvement in the autoimmune process[3]. Therefore, T cells in ANCA disease are likely to be dysregulated in some manner to permit autoantibody formation.

Pathogenic ANCAs cause activation of neutrophils resulting in degranulation and release of proteases[36]. In turn, this neutrophil activation leads to pauci-immune necrotizing crescentic glomerulonephritis and systemic small vessel vasculitis. Studies to incriminate a role for CD4+ T cells in ANCA disease have yielded results suggesting MPO or PR3 specific T cells exist in the periphery[37,38] which proliferate in response to autoantigen. Other studies have also noted persistent activation of effector T cells in the periphery of ANCA disease patients[39,40]. Th17 cells are involved in a number of autoimmune diseases and are also present in increased frequencies in patients[41]. In corroboration with this, circulating levels of Th17-associated cytokines (IL-1β, IL-6, IL-23) are also increased in the serum of patients[29,42].

Of additional concern are the studies demonstrating a functional deficit of Tregs in patients with ANCA disease[43,44,45]. However, no functional mechanism has been demonstrated for this Treg dysfunction. One hypothesis which may explain the functional deficit of Tregs in ANCA disease is that in the face of chronic inflammation, Tregs have converted into Th17 cells[46,47,48,49]. This phenomenon would not only diminish any suppressive function of the purported Tregs, but could potentiate ongoing inflammation by production of IL-17 and related cytokines.

T cells in Minimal Change disease

MCD accounts for the majority of nephrotic syndrome cases in children and FSGS may derive from an underlying etiology as MCD. Autoimmune involvement, specifically T cells, has been inferred by the efficacy in treating MCD patients with corticosteroids[50] or medications that alter T cells such as cyclosporine[51] or tacrolimus[52]. The pathologic finding in MCD is podocyte foot process effacement wherein slit diaphragms are lost and foot processes become fused. These cellular changes are visible only by electron microscopy[53,54]. For decades, it has been hypothesized that a "permeability factor" in MCD patients induces podocyte effacement, although no factor has been inexplicably implicated[55].

The role of T cells in MCD has perplexed investigators as T cells are rarely found in or around the glomerulus upon kidney biopsy in suspected MCD[56]. Therefore, the investigational focus has been on circulating T cells in patients with MCD. Early studies did note that CD4+ T cells had phenotypic markings of an "activated" phenotype in the periphery of MCD patients[57,58]. However, the majority of studies have focused on secreted proteins from MCD T cells and their potential to be injurious to podocytes. A very early study noted that a "factor" was produced from PBMCs from MCD patients and the "factor" induced proteinuria when injected into rats[59].

The limitations from this study were 1) total PBMCs were used and it is unknown which cell type produced the "factor" and, 2) the "factor" was never identified. Later studies were refined and demonstrated a "factor" was produced by T cell hybridomas made from MCD patients[55]. While this study implicated T cells as the cell type producing an injurious protein, the actual protein was still not identified but was hypothesized to be a "lymphokine." More recently, IL-4 and IL-13 have been implicated as potential candidates as the glomerular permeability factor, and both of these cytokines are secreted by Th2 cells[60]. However, the field continues to rely on hypotheses and anecdotal evidence as to the true etiology of MCD.

Central Hypothesis

In summary, T cell dysfunction is incriminated in the majority of autoimmune diseases and provides insight into the immunopathogenesis and progression of disease. The central hypothesis of this body of work is that T cells are integral to the immunopathogenesis of ANCA disease and MCD. In addition to the central hypothesis, this body of work incorporates two main sub-hypotheses, 1) ANCA disease patients have dysfunctional regulatory T cells and an expanded effector population resistant to Treg suppression 2) MCD patients have circulating autoantibodies reactive to a T cell receptor present on peripheral "thymocyte-like" cells.

The central hypothesis and sub-hypotheses will be addressed in the following two chapters. Chapter 1 details the confirmation that Tregs from ANCA patients are incapable of suppressing effector T cell proliferation. Studies are also presented to demonstrate a contributing factor of Treg dysfunction—a splice variant of FOXP3. Additionally, a population of effector cells that are overrepresented in ANCA patients are functionally and phenotypically characterized. Chapter 2 is focused on the discovery of both a novel autoantibody and autoantigen in MCD and an interesting subset of FSGS patients.

Chapter 1

ANCA-ASSOCIATED VASCULITIS PATIENTS HAVE DEFECTIVE TREG FUNCTION EXACERBATED BY PRESENCE OF A SUPPRESSION-RESISTANT EFFECTOR POPULATION

This chapter consists of material from a manuscript reprinted with permission from *Arthritis and Rheumatism* 2013[1] However, the breakdown in immune tolerance that results in the induction and persistence of ANCAs is not well-understood. We hypothesized that abnormal T cell regulation is central to disease pathogenesis and demonstrate here two separate abnormalities in T cell regulation in ANCA-associated vasculitis patients. Peripheral blood samples were obtained from patients with ANCA-associated vasculitis (n=63) and healthy controls (n=19) for flow cytometric analysis of CD4+ T cell populations. Functional T cell studies were performed with FACS sorted CD4+ T cell populations stimulated with anti-CD3/28. First, we show that the Treg frequency in the peripheral blood of active disease patients is increased, but Tregs from patients with ANCA-associated vasculitis have decreased suppressive function. Tregs from active disease patients disproportionately utilize a FOXP3 isoform lacking exon 2, which may alter Treg function. Second, we identify a CD4+ T cell population with increased frequency that is resistant to Treg suppression, produces pro-inflammatory cytokines, and is antigen-experienced. ANCA-

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This chapter consists of material from a manuscript reprinted with permission from *Arthritis and Rheumatism* 2013[1] [1]Meghan E Free, Donna O Bunch, JulieAnne McGregor, Britta E Jones, Elisabeth A Berg, Susan L Hogan, Yichun Hu, Gloria A Preston, J. Charles Jennette, Ronald J Falk, Maureen A Su

associated vasculitis is associated with disruption of the suppressive Treg network and increased frequency of a distinct pro-inflammatory effector T cell subset which comprises the majority of peripheral CD4+ T cells.

Introduction

T cells, an integral part of adaptive immunity, are additionally incriminated in a number of autoimmune diseases. The role of T cells in autoimmunity is diverse and can be attributed to multiple pathogenic mechanisms, including regulatory T cell (Treg) dysfunction[61], effector hyperactivation[62] and an imbalance of certain subsets[31]. Therefore, the contribution of T cells to the pathogenesis of a particular autoimmune disease is unlikely to stem from a single aberration.

CD4+ regulatory T cells (Tregs) are primary mediators of peripheral tolerance and express the master transcription factor FOXP3[63]. A critical role for Tregs in preventing autoimmunity is demonstrated by the development of fulminant autoimmunity in rare patients lacking Tregs due to FOXP3 mutations[19]. How quantitative changes in Treg numbers and function contribute to the pathogenesis of more common autoimmune diseases that do not involve FOXP3 mutations are less clear[61].

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is an autoimmune disease wherein patients have pathogenic autoantibodies reacting to myeloperoxidase (MPO) or proteinase 3 (PR3) and exhibit necrotizing, crescentic glomerulonephritis and systemic vasculitis[3]. The production of isotype-switched autoantibodies in AAV suggests the involvement of CD4+ helper T cells in the autoimmune process[3]. Therefore, primary dysregulation of T cells in AAV is likely to permit autoantibody formation. Studies on the frequency and function of Tregs in patients with AAV have yielded conflicting results. Treg frequency in AAV patients has been reported to be increased[43], or decreased[44,45]. Additionally, Treg function in AAV patients has been reported as normal[64] and decreased[43,44,45]. Furthermore, little is known about how Treg functional deficits may negatively affect other T cell subsets in AAV.

Although attention has focused on abnormalities intrinsic to Tregs in autoimmunity, recent evidence suggests that effector T cell resistance to Treg suppression may also contribute to the development of autoimmune disease[62]. We report herein the potential contributions of both Treg dysfunction and effector T cell resistance in AAV. Patient Tregs are defective in suppressing effector CD4+ T cells and this dysfunction is correlated with increased usage of a FOXP3 splice variant. Additionally, patient peripheral blood samples demonstrate an increased frequency of a distinct CD4+ T cell population that is resistant to functional Treg suppression and secretes pro-inflammatory cytokines. Taken together, these data delineate two separate and novel mechanisms by which dysregulation of CD4+ T cells contribute to AAV.

Materials and Methods

Patient cohort

Patients with biopsy-proven AAV enrolled in this study gave informed, written consent and participated according to UNC Institutional Review Board guidelines. Patient demographics were similar between healthy controls and AAV patients with the exception of age which was significantly lower in the healthy control cohort and are listed in Table 1.1.

Disease activity was determined by the Birmingham Vasculitis Activity Score (BVAS). BVAS is a clinical assessment of vasculitic activity, the most recent updated version was published by Mukhtyar et al[65]. In this study, patients with a BVAS of 0 were considered to be in remission while a BVAS >0 determined active disease. Diagnosis of microscopic polyangiitis or granulomatosis with polyangiitis was based on previously well established criteria[66,67].

Table 1.1 Patient demographics

	ANCA-associated vasculitis (Total)	Healthy Control (Total)	ANCA-associated vasculitis (Functional studies)	Healthy Control (Functional studies)
Sex				
Male	41.9%	47.2%	37.5%	33.3%
Female	58.1%	52.8%	62.5%	66.7%
Race				
Asian	1.6%	0.0%	0.0%	0.0%
African American	12.9%	10.5%	25.0%	0.0%
Hispanic	1.6%	0.0%	0.0%	0.0%
Caucasian	83.9%	84.2%	75.0%	83.3%
Other	0.0%	5.3%	0.0%	16.7%
Mean Age	52.0	33.8	54.6	45.7
Range	13-86	20-54	24-79	26-54
Serology				
MPO	42.9%	NA	25.0%	NA
PR3	57.1%	NA	75.0%	NA
Diagnosis				
MPA	48.4%	NA	25%	NA
GPA	40.3%	NA	62.5%	NA
Renal limited	11.3%	NA	12.5%	NA
Mean BVAS*	7	NA	0.5	NA
Range	0.5-23	NA	0-4	NA
Mean Disease Duration (days)				
Presentation to most recent follow-up	1957	NA	2136	NA
Range (days)	11-6809	NA	295-4417	NA
Medication use** (within 6 months prior to sample date of functional study				
IV cyclophosphamide	8.1%	NA	0.0%	NA

Oral cyclophosphamide	3.2%	NA	0.0%	NA
Mycophenolate mofetil	33.9%	NA	50%	NA
Glucocorticoid	43.5%	NA	12.5%	NA
Azathioprine	17.7%	NA	12.5%	NA
Rituximab	41.9%	NA	37.5%	NA
TOTAL	**62**	**19**	**8**	**6**

*BVAS average for AAV (total) only includes active patients
**Immunosuppressive medication

Cell isolation and flow cytometry

PBMCs were washed and resuspended in HBSS, 2% FBS, 0.1% sodium azide and stained with: CCR6, CD45RO, CD45RA (BD Biosciences), CCR5, CCR7, CXCR3, CD25, CD4, CD127, CD8, CD3 (BioLegend). For FOXP3 detection, cells were fixed and permeabilized using a FOXP3 Staining Buffer Set (eBioscience). FOXP3 was detected by clones PCH101 (eBioscience) and 150D (BioLegend). These two antibodies have previously been used to identify FOXP3 splice variants by flow cytometry[68]. We additionally confirmed the specificity of the antibodies by western blot (data not shown). Intracellular cytokine staining of IL-4 and -17 was performed after 4 hour stimulation with PMA and ionomycin while incorporating GolgiPlug (BD Biosciences). Cells were then permeabilized with CytoFix/CytoPerm (BD Biosciences) and stained with anti-IL-4 or anti-IL-17 (BioLegend). Cells were acquired on a BD LSRII and data was analyzed by FlowJo software (Tree Star, Inc.).

Suppression assays

Demographics for patients used in functional studies are shown in Table 1. PBMCs were isolated from heparinized peripheral blood by Histopaque-1077 (Sigma-Aldrich) and washed twice in HBSS plus 2% FBS. CD4+ T cells were isolated by EasySep® Human CD4+ T Cell Enrichment Kit (StemCell) and subsequently stained with the following fluorochrome-labeled anti-human antibodies: CD4, CD25 and CD127. Cell sorting was performed on an iCyt Reflection (Sony). Post-sort cell population purity was routinuely >95% for Tregs and $CD25^{neg}$ and >85% for $CD25^{int}$ with occasional $CD25^{neg}$ contamination.

Responder T cells were labeled with 0.1 μM CFSE (Invitrogen), washed twice and resuspended in RPMI, 10% FBS, pen/strep, HEPES. Cells were plated in 96-well plates pre-coated with immobilized anti-CD3 (5 ug/mL) and anti-CD28 (1 ug/mL) (BioLegend), as done previously[69]. Responders were plated at 50,000 cells per well and suppressors were titrated. Cells proliferated for 4 days before analyzing on a BD LSRII.

Suppression assays using allogeneic Tregs were performed as previously described[62], wherein 50,000 CFSE-labled effectors were plated in anti-CD3/28 pre-coated wells and proliferated for 4 days before analyzing on a BD LSRII.

Cytokine array and ELISA

To detect IL-17A or IL-4 in the cell culture supernatants we utilized a Human IL-17A ELISA MAX™ Deluxe and Human IL-4 ELISA MAX™ Deluxe (BioLegend). Cell culture supernatants were analyzed for additional cytokine content using a multiplex assay, Beadlyte Human 22-plex (Upstate).

Statistics

The Wilcoxon rank-sum test was used for analysis of two groups. Kruskal-Wallis test was utilized in analyses involving more than two groups.

Results

Tregs from AAV patients are hypofunctional

To clarify if and how Tregs are abnormal in AAV we characterized Treg population dynamics in patients with active disease, in disease remission, and healthy controls. Tregs, defined as CD4+, $CD25^{high}$, $CD127^{low}$ and FOXP3+[17,18], were quantified as percentage of CD4+ T cells. We obtained remission and most proximal relapse samples from the same patient to determine Treg population dynamics in internally controlled samples. In patients followed longitudinally, active disease was associated with an increased frequency ($p=0.003$) of Tregs compared to remission (Figure 1.1A left panel). We also obtained multiple remission samples on these patients, allowing us to compare the remission sample having the lowest Treg frequency with the active sample (Figure 1.1A right panel). The lowest Treg frequency occurred 6 months after relapse in most cases. Consistent with our previous data, active disease was associated with a higher frequency of Tregs in this analysis.

Additionally, we compared Treg frequencies in our large cohort of patient samples to healthy controls as a composite. We noted significantly increased Treg frequencies ($p=0.03$) in

active disease compared to healthy controls (Figure 1.1B left panel). The Treg frequency was not significantly different between AAV patients in remission and healthy controls (Figure 1.1C). Absolute numbers of Tregs per mL of blood were calculated and were not statistically significant between AAV patients and healthy controls (Figure 1.1D). Additionally, there were no differences in Treg frequency when comparing MPO and PR3 reactive AAV patients (Figure 1.1B right panel). As some reports have demonstrated increased Treg frequencies with increase in patient age[70], we performed a linear regression analysis of Treg frequency and patient age (Figure 1.1E). Age and frequency of Tregs were not correlated in our cohort of AAV patients.

We also sought to determine the functional capacity of Tregs in AAV. CD4+, $CD127^{low}$, $CD25^{high}$ Tregs and $CD25^{neg}$ effector T cells were sorted from healthy controls and patients. Prior studies have demonstrated the suppressive function of $CD127^{low}$, $CD25^{high}$ T cells was equal, if not superior to a gating scheme which identified Tregs as the highest 5% of CD25+ T cells[17]. AAV patients used for functional studies were not exposed to cyclophosphamide in the preceeding six months as cyclophosphamide is known to inhibit Treg function[71]. As expected, healthy Tregs suppressed >50% of effector proliferation at a 1:1 ratio (Figure 1.1F, diamonds; 1.1G left). In contrast, Tregs from AAV suppressed only 20% of effector proliferation on average at a 1:1 ratio (Figure 1.1F, squares; 1.1G, right). The possibility remained that AAV $CD25^{neg}$ effectors are resistant to suppression since syngeneic Tregs and effectors were used in this experiment. To explore this possibility, we mixed healthy control Tregs with AAV $CD25^{neg}$ effectors in an allosuppression assay (Figure 1.1F, triangles; 1.1G, middle). Healthy Tregs (n=4) were able to suppress >50% of AAV $CD25^{neg}$ effector proliferation on average. Therefore, $CD25^{neg}$ T cells from AAV patients are similarly susceptible to suppression by functional Tregs. Together, these results point to a defect in Treg functional capacity and are consistent with those studies demonstrating defective Treg suppressive function in AAV[43–45].

Figure 1.1

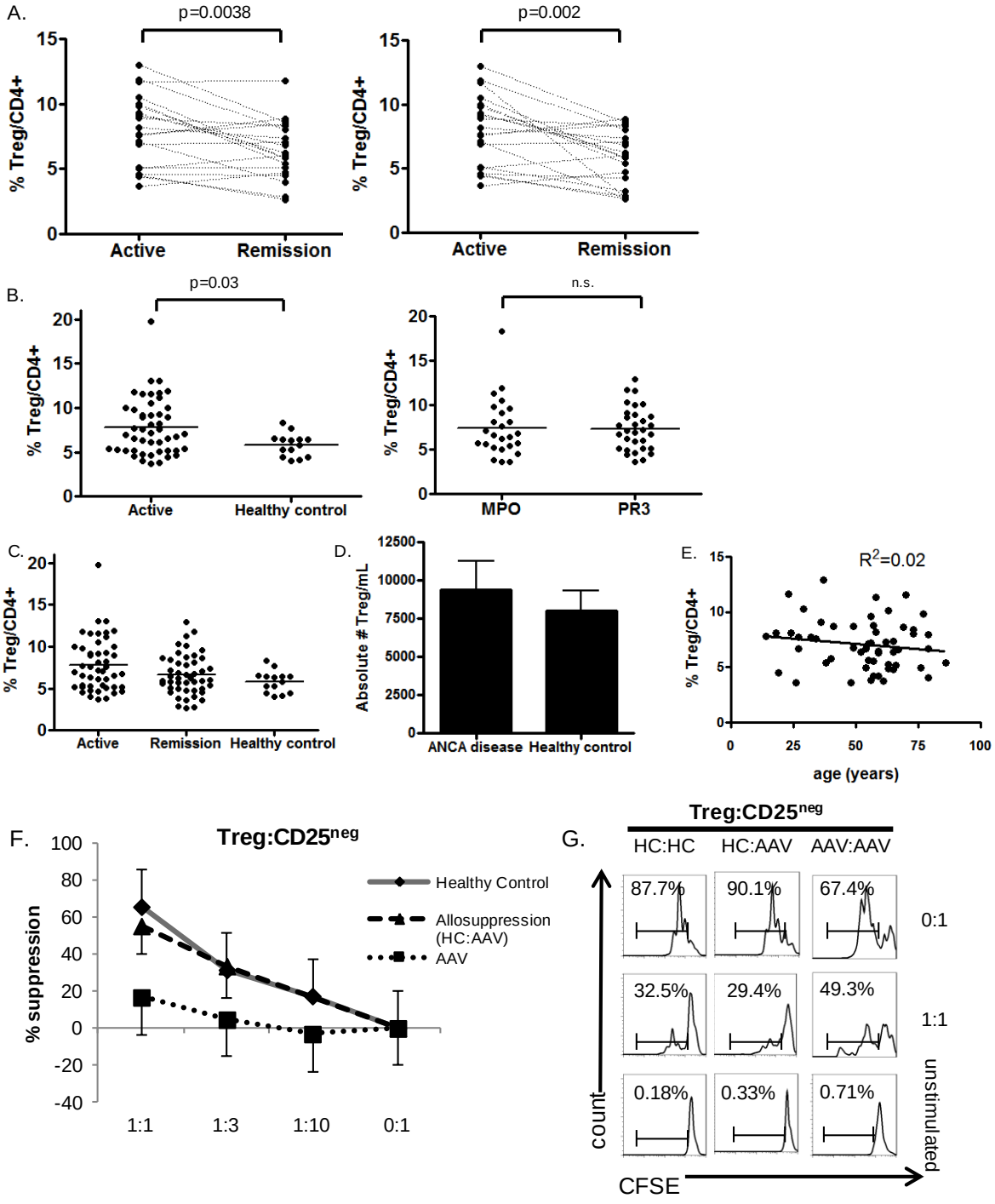

Figure 1.1. Altered Treg function in AAV. (A) Longitudinal studies of individual AAV patients indicated by dashed line. Active disease point compared to most proximal remission point (left panel). Active disease point compared to remission point with lowest Treg frequency (right panel)

(n=20 pairs). (B) Left: Treg frequency with CD4+ T cells in active disease versus HC. Right: Treg frequency in MPO and PR3 reactive AAV patients. (C) Treg frequencies in ANCA patients with active or remitting disease compared to healthy controls. (D) Absolute number of Tregs per mL of blood in patients and healthy controls. (E) Linear regression analysis of Treg frequency and age of AAV patients. (F) In vitro suppression by HC and patient Tregs; HC, healthy control. Sorted, CFSE-labeled $CD25^{neg}$ T cells were stimulated with anti-CD3, -28. Syngeneic or allogeneic Tregs were added to test suppressive ability. Proliferation was assessed on day 4 in HC (n=4), AAV (n=6), allosuppression (n=4). (G) Representative flow cytometry plots of in vitro suppression assay using autologous or allogeneic Tregs with CFSE-labeled $CD25^{neg}$ effector T cells; %=proliferation. Active, BVAS>0; Remission, BVAS=0.

Tregs from AAV patients disproportionately utilize a variant FOXP3 isoform

With confirmation of the functional deficit of Tregs from AAV patients we explored the underlying mechanism behind AAV Treg dysfunction. FOXP3 is the master transcriptional regulator of CD4+ Tregs and drives their suppressive regimen. FOXP3 has two isoforms in humans, full-length and exon 2-deficient, due to alternative splicing at the mRNA level[72]. Exon 2 lies within a repressor domain important in binding pro-inflammatory transcription factors, such as RORγt and RORα, which drive Th17 induction (Figure 1.2A, top)[73,74]. Lack of this domain would be predicted to prevent repression of these Th17 transcription factors and promote Th17 lineage differentiation[74]. We hypothesized that increased expression of exon 2-deficient FOXP3 may contribute to non-suppressive function of AAV Tregs.

We utilized two FOXP3 antibodies recognizing two distinct domains on the FOXP3 protein. The FOXP3 antibody clone PCH101 recognizes the N-terminus and both isoforms of FOXP3 are recognized by this antibody. The FOXP3 antibody clone 150D recognizes the exon 2 portion of FOXP3 and only recognizes full-length FOXP3 containing exon 2. Western blots using the same FOXP3 antibodies confirmed the specificity of these antibodies for the two isoforms (data not shown). Additionally, these antibodies were previously used to distinguish these two FOXP3 isoforms by flow cytometry and have concordance with mRNA analysis of these two FOXP3 isoforms[68]. Consistent with these reports we found two staining patterns among Treg populations by flow cytometry. In some samples, CD4+ $CD127^{neg}$ $CD25^{high}$ cells Tregs stained positively with both antibody clones and therefore predominantly express full-length FOXP3 (Figure 1.2A, bottom left panel). In other samples, Tregs stained positively with only the N-terminus clone and therefore predominantly express exon 2-deficient FOXP3 (Figure 1.2A, bottom right panel).

We utilized this flow cytometric approach to quantify the percentage of CD4+ T cells harboring the FOXP3 splice variant in healthy controls and patients during active disease and remission. AAV patients have an increased frequency ($p=0.001$) of exon 2-deficient cells

compared to healthy individuals (Figure 1.2B left). Strikingly, exon 2-deficient FOXP3 is associated with both active and remitting disease (Figure 1.2B left). We also examined the frequency of cells expressing full-length FOXP3 in patients and healthy individuals. Overall, AAV patients tended to have a lower frequency of cells expressing full-length FOXP3 compared to healthy individuals (Figure 1.2B right). The increased frequency of exon 2-deficient FOXP3 is not solely due to the increased Treg frequency in active AAV because exon 2-deficient FOXP3 often exceeds >90% of total FOXP3+ cells (Figure 1.2C).

We correlated the degree of suppression when Tregs were cultured with syngeneic $CD25^{neg}$ T cells at a 1:1 ratio with the percentage of T cells utilizing exon 2-deficient FOXP3 to corroborate the hypothesis that exon 2-deficient isoform of FOXP3 is associated with Treg dysfunction. These two variables were inversely correlated (Figure 1.2D; R^2=0.72), demonstrating that an increased preponderance of exon 2-deficient FOXP3 is linked to a Treg population with diminished suppressive ability.

Figure 1.2

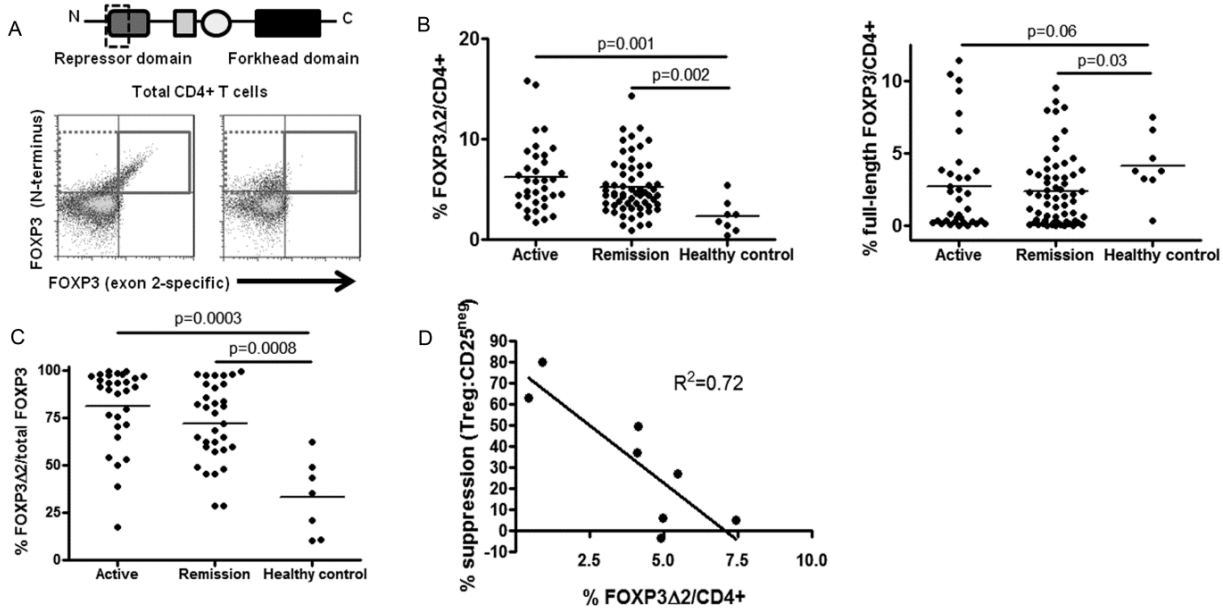

Figure 1.2. Exon 2-deficient FOXP3 splice variant predominates in AAV. (A) Top: Protein structure of FOXP3. Exon 2 (dashed line) encompasses part of the repressor domain; Bottom: Representative flow cytometry plots of CD4+ cells using an antibody recognizing exon 2 (clone 150D) and the N-terminus (clone PCH101) of FOXP3. Dual positive cells contain full-length FOXP3 (solid gray box) while single positive cells contain the splice variant (hatched gray box). (B) Left: Percentage of exon 2-deficient FOXP3 within CD4+ T cells in active AAV patients, remission AAV patients and healthy controls. Right: Percentage of full-length FOXP3 within CD4+ T cells in active AAV patients, remission AAV patients and healthy controls. (C) Exon 2-deficient FOXP3 as a percentage of total FOXP3 positive cells in active AAV patients, remission AAV patients and healthy controls. (D) Correlation of the suppressive ability of Tregs and expression of exon 2-deficient FOXP3 in healthy controls and AAV patients. Active, BVAS>0; Remission, BVAS=0.

Increased frequency of a distinct CD4+ CD25^{intermediate} CD127^{high} T cell population is associated with AAV

We used a recently described gating strategy[17,18] to stratify CD3+ CD4+ T cells with CD127 and CD25 (Fig 1.3A, top panels) to examine effector and suppressor CD4+ T cell populations. T cell populations were dramatically altered between healthy controls and AAV patients (Figure 1.3A, compare two bottom panels). Most CD4+ T cells in healthy controls are CD127^{high} CD25^{neg} while the majority of patient CD4+ T cells are CD127^{high} CD25^{intermediate} (Figure 1.3A, bottom panels). The frequency of the CD25^{int} population in AAV patients was significantly increased ($p<0.0001$) compared to healthy controls (Figure 1.3B). Both MPO and PR3 positive AAV patients demonstrated increased frequency of this population. The absolute number of CD25^{neg} T cells in the peripheral blood of AAV patients is two-fold less than healthy controls, while the absolute number of CD25^{int} T cells is two-fold higher in patients (Figure 1.3C). Therefore, there is an increased frequency of CD25^{int} cells in AAV patients.

To determine if other autoimmune diseases have an increased frequency of this CD25^{int} population we obtained samples from systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and type 1 diabetes mellitus (T1DM) patients. An increased frequency of this population occurred in SLE (data not shown; mean 52.1 ± 17.1%) and RA (data not shown; mean 48.3 ± 9.5%), but not in T1DM (mean 28.2 ± 12.4%) (Figure 1.3B). Thus, a higher frequency of CD25^{int} T cells is not a universal property of all autoimmune diseases, but is associated with a subset of autoimmune diseases.

The CD25^{int} population was not associated with AAV disease activity as there were no significant differences between active and remission disease states (Figure 1.3D). Therefore, it is unlikely that increased frequency of the CD25^{int} population is merely a disease severity marker. In patients followed over time, a stable CD25^{int} T cell population persists for as long as patients have been monitored (Figure 1.4). We also obtained samples at disease onset and the CD25^{int} population was already increased at that time which argues against an artifact of drug therapy.

Figure 1.3

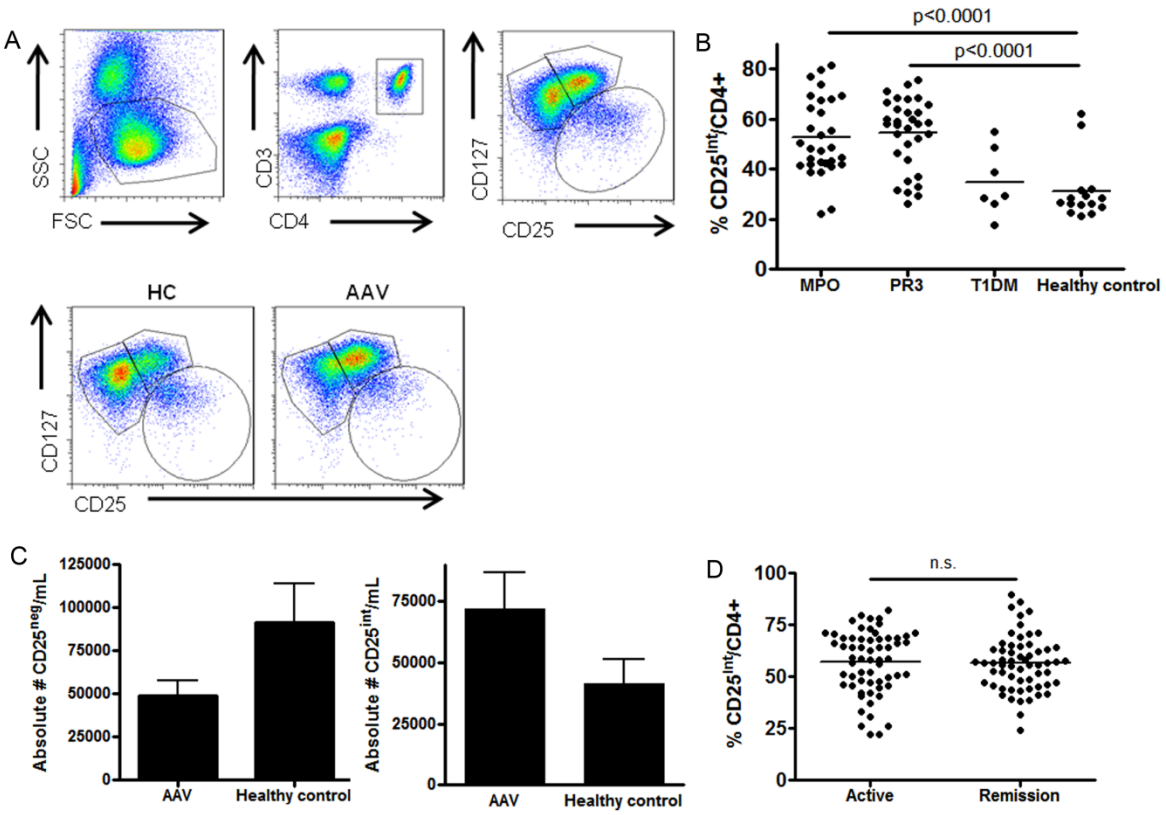

Figure 1.3. Altered T cell dynamics in PBMCs of AAV patients. (A) Gating strategy of CD3+ CD4+ lymphocytes stratified by CD127 and CD25 (top panel). Representative flow cytometry analysis of CD3+ CD4+ T cells in HC and patients (bottom panel). (B) Cumulative frequency of the $CD25^{int}$ T cell population in MPO+ AAV patients, PR3+ AAV patients, type 1 diabetes mellitus (T1DM), and healthy controls. (C) Absolute cell numbers per mL of peripheral blood of the $CD25^{neg}$ and $CD25^{int}$ T cell populations. (D) Cumulative frequency of the $CD25^{int}$ population expansion in active AAV patients or AAV patients in remission. n.s., not significant. Active, BVAS>0; Remission, BVAS=0.

Figure 1.4

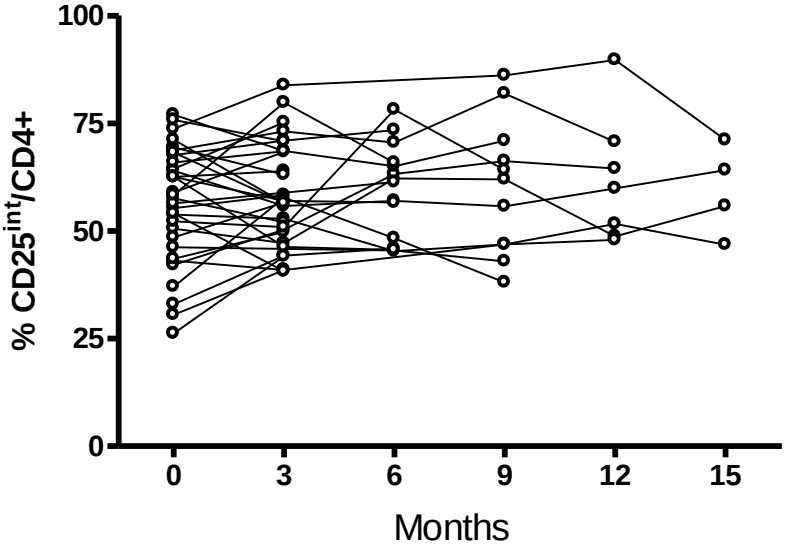

Figure 1.4. Longitudinal studies of $CD25^{int}$ T cells. The $CD25^{int}$ T cell population was monitored in patients every 3 months from time of entry into study. n=32 individual patients.

CD25^{int} T cells are resistant to healthy control Treg suppression

CD25 expression is associated with both effector T cell activation and Tregs[75]. We therefore sought to determine whether the CD25^{int} T cell population had effector or suppressor functions. Three CD4+ T cell populations were sorted by flow cytometry (CD25^{neg}, CD25^{int} and CD25^{high}) as delineated in Figure 1.3A, top panels. Functional studies were performed with cells from healthy controls (n=4) and AAV patients (n=4).

Sorted CD25^{int} T cells from AAV patients and healthy controls were serially diluted and cultured with syngeneic effectors stimulated with anti-CD3/CD28 to test potential suppressive function. As mentioned previously, CD25^{neg} cells from healthy controls or AAV patients are equally susceptible to Treg suppression (Fig 1.1F). CFSE dilution in CD25^{neg} effectors determined the suppressive ability of CD25^{int} T cells. As a positive control, we sorted bonafide CD25^{high} CD127^{neg} Tregs from a healthy control and showed that this population suppresses 70-80% of effector proliferation in this assay (Figure 1.5A, closed triangle). CD25^{int} T cells from healthy controls have moderate suppressive ability as they suppressed 40% of effector proliferation at a 1:1 ratio (Figure 1.5A, closed diamond). In contrast, sorted CD25^{int} T cells from AAV patients were unable to suppress CD25^{neg} effector proliferation (Figure 1.5A, closed squares). Analysis of FOXP3 expression among CD25^{int} T cells revealed that they rarely express FOXP3 and therefore would not be predicted to have substantial suppressive function (Figure 1.6). However, an average of 3-4% of healthy CD25^{int} T cells did express FOXP3 compared to less than 1% FOXP3+ in AAV. It remains possible that an additional suppressive CD127^{high} T cell population, such as recently identified CD25^{low} CD127^{high} GITR+ T cells[76], resides in the CD25^{int} population in healthy controls. .

Sorted CD25^{int} T cells were cultured with syngeneic Tregs in serial dilution to test how CD25^{int} T cells function as responders. CD25^{int} T cells from both healthy controls (n=6) and AAV patients (n=7) were anti-CD3/CD28 stimulated for 4 days. CD25^{int} cells from healthy controls were resistant to suppression by autologous Tregs (Figure 1.5B) when compared to CD25^{neg}

effectors. Only 10% of proliferation on average was suppressed at a 1:1 ratio of Tregs to $CD25^{int}$ T cells, whereas at a 1:1 ratio of Tregs to $CD25^{neg}$ effectors suppressed approximately 70% of proliferation on average (Figure 1.5B, right). $CD25^{int}$ cells from healthy controls are thus resistant to Treg suppression. Tregs were unable to suppress $CD25^{int}$ T cell proliferation (Figure 1.5B) when patient cells were tested. However, as we and others have demonstrated, patient Tregs have a functional deficit. Therefore the exact degree of suppression-resistance of $CD25^{int}$ T cells could not be ascertained from this particular experiment.

We performed allo-suppression assays using functional Tregs from healthy individuals and effector T cells from AAV patients to differentiate between resistance of $CD25^{int}$ T cells to suppression and lack of Treg function. Healthy Tregs were able to suppress 50%, on average, of $CD25^{neg}$ T cells from an AAV patient as mentioned previously (Figure 1.1F). This demonstrates that $CD25^{neg}$ T cells are able to be suppressed in AAV if functional Tregs are present. However, when healthy Tregs were added to proliferating $CD25^{int}$ T cells from an AAV patient, less than 20% of effector cells were suppressed on average (Figure 1.5B). These data demonstrate that even functional, healthy Tregs are incapable of suppressing $CD25^{int}$ T cells in AAV. In sum, $CD25^{int}$ T cells are increased in frequency in AAV and $CD25^{int}$ T cells from both healthy controls and AAV patients are resistant to Treg suppression.

Figure 1.5

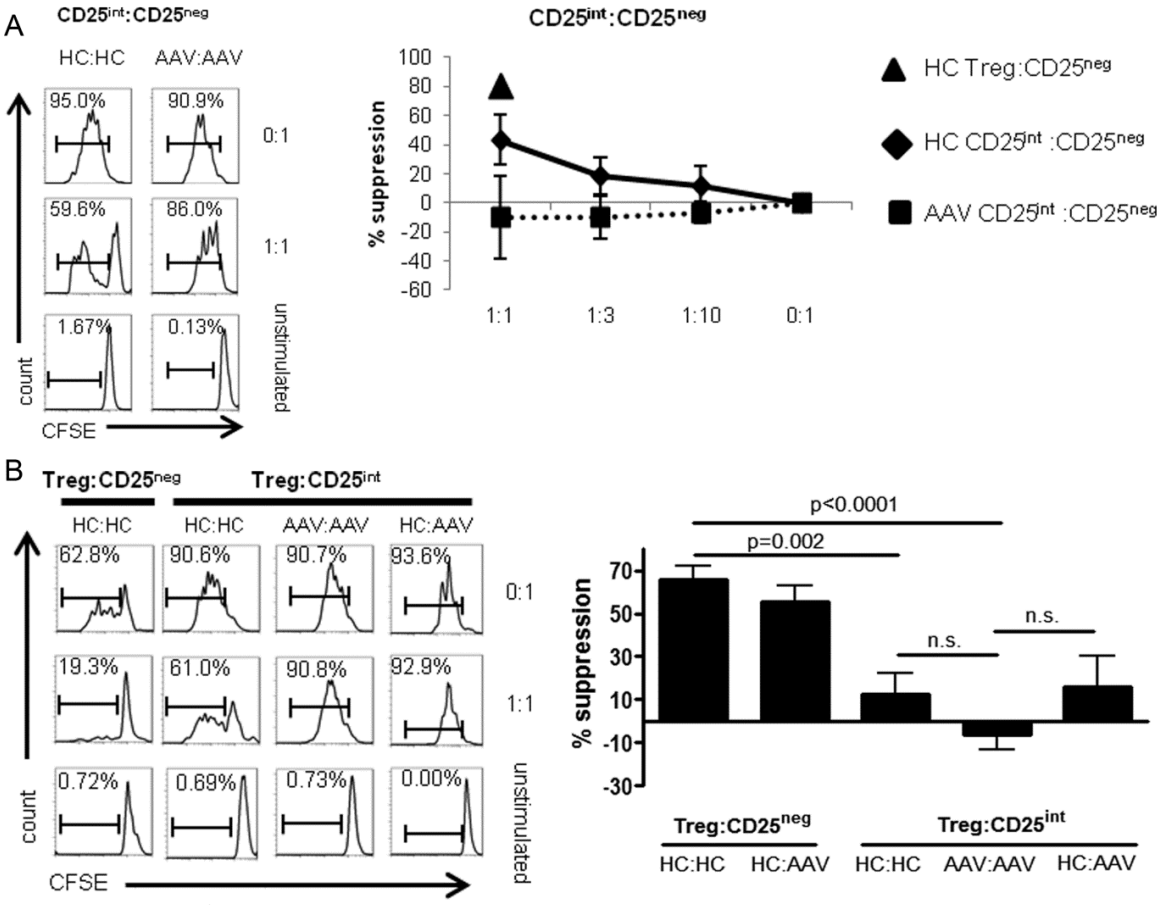

Figure 1.5. CD25^{int} T cells are resistant to suppression compared to CD25^{neg} T cells. (A) Left: Representative flow plots of suppression assay using CD25^{int} as suppressors and CFSE-labeled CD25^{neg} as effectors. Right: Compiled suppression data using CD25^{int} as suppressors and CD25^{neg} as effectors. Data from HC (n=4) and AAV (n=4). (B) Left: Representative flow plots of suppression assay using Tregs as suppressors and CD25^{int} as effectors; % proliferation. Right: Compiled suppression data using Tregs as suppressors and CD25^{int} as effectors. Data from HC (n=6) and AAV (n=7). Allosuppression assays (HC:AAV) were repeated with HC (n=4) and AAV (n=4).

Figure 1.6

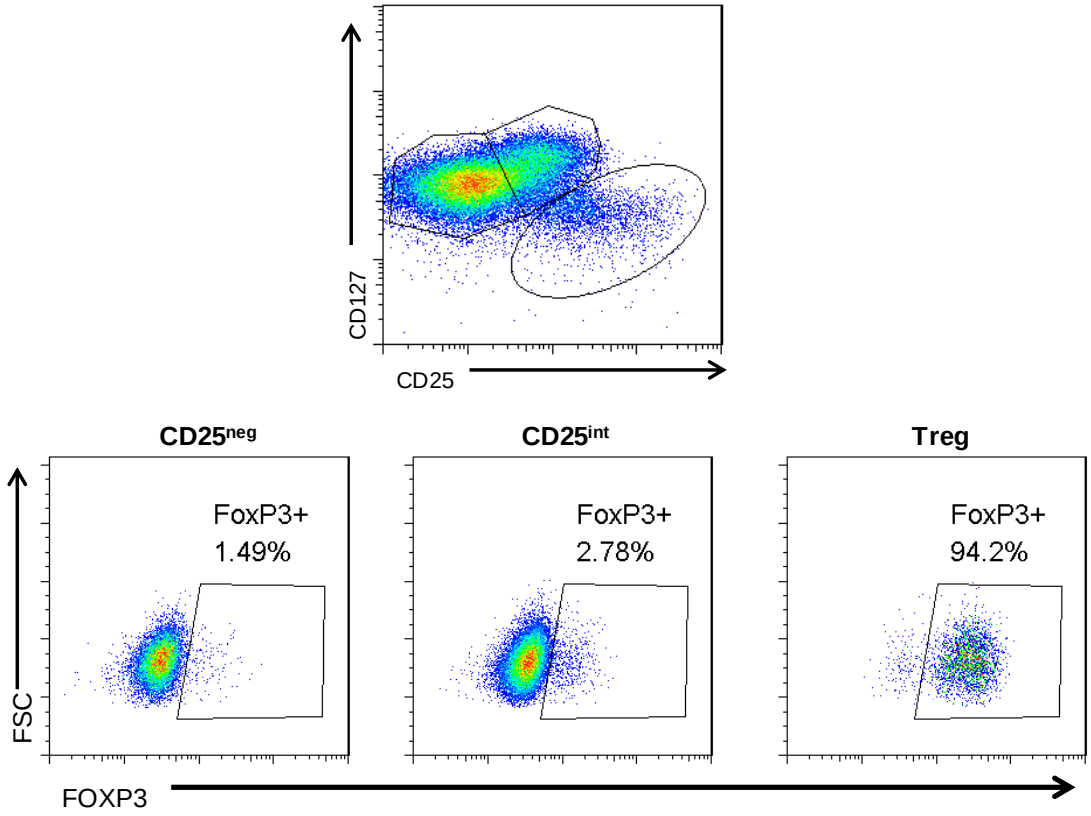

Figure 1.6. FOXP3 expression between T cell subsets. Representative FACS plots depicting FOXP3+ intracellular staining of $CD25^{neg}$, $CD25^{int}$ and regulatory T cell populations.

The CD25^{int} T cell population is phenotypically distinct from the CD25^{neg} population

We hypothesized that CD25^{int} T cells might have pathogenic effector T cell capacity since these cells are resistant to suppression. Thus, we investigated phenotypic differences between CD25^{int} and CD25^{neg} T cells. T cell cytokine profiles are a key determinant of T helper subsets and also indicate the immunomodulatory capacity of T cells. We used capture ELISAs to determine cytokine production in culture supernatants of stimulated, sorted T cell populations from healthy controls and patients. The CD25^{int} population produced increased amounts of IL-17 and IL-4 compared to the CD25^{neg} population; this corroborates our hypothesis that these cells act predominantly as effector cells (Figure 1.7A, left). IL-4 was never detected in the supernatants of stimulated CD25^{neg} T cells from healthy controls or AAV patients (Figure 1.7A, left). There was no significant difference in IL-4 and IL-17A production between CD25^{int} T cells from healthy controls or patients. Following short cultures with PMA and ionomycin, we also detected intracellular IL-4 and IL-17 in T cells and these cytokine-producing cells localized predominantly to the CD25^{int} population (Figure 1.7A, right). Thus, CD25^{int} T cells are similar in cytokine secretion between AAV patients and healthy controls on a per cell basis. However, the absolute number of CD25^{int} T cells is at least two-fold higher in patients and are therefore likely to produce more cytokines than healthy controls.

T cell populations utilize different chemokine receptors which allows for specialized functions such as exiting blood vessels and entering inflamed sites. We compared chemokine receptor expression between CD25^{neg} and CD25^{int} T cells from AAV patients to gain more insight regarding their function. These two populations differentially expressed CCR5 and CCR6. No significant differences in CCR7 and CXCR3 expression were observed. CD25^{neg} T cells expressed CCR5 while CD25^{int} T cells had a statistically significant higher ($p<0.0001$) expression of CCR6 (Figure 1.7B). Therefore, CD25^{neg} and CD25^{int} T cell populations have differential expression of chemokine receptors.

Additional phenotyping of the T cell populations from AAV patients demonstrated that most $CD25^{neg}$ T cells are a relatively equal split of CD45RA+ and CD45RO+ cells; indicating a population with a large percentage of naïve cells (Figures 1.7C and 1.7D). However, the $CD25^{int}$ population preferentially expressed CD45RO+ (Figures 1.7C and 1.7D). CD45RO expression suggests that the $CD25^{int}$ population is enriched for activated or memory T cells.

Figure 1.7

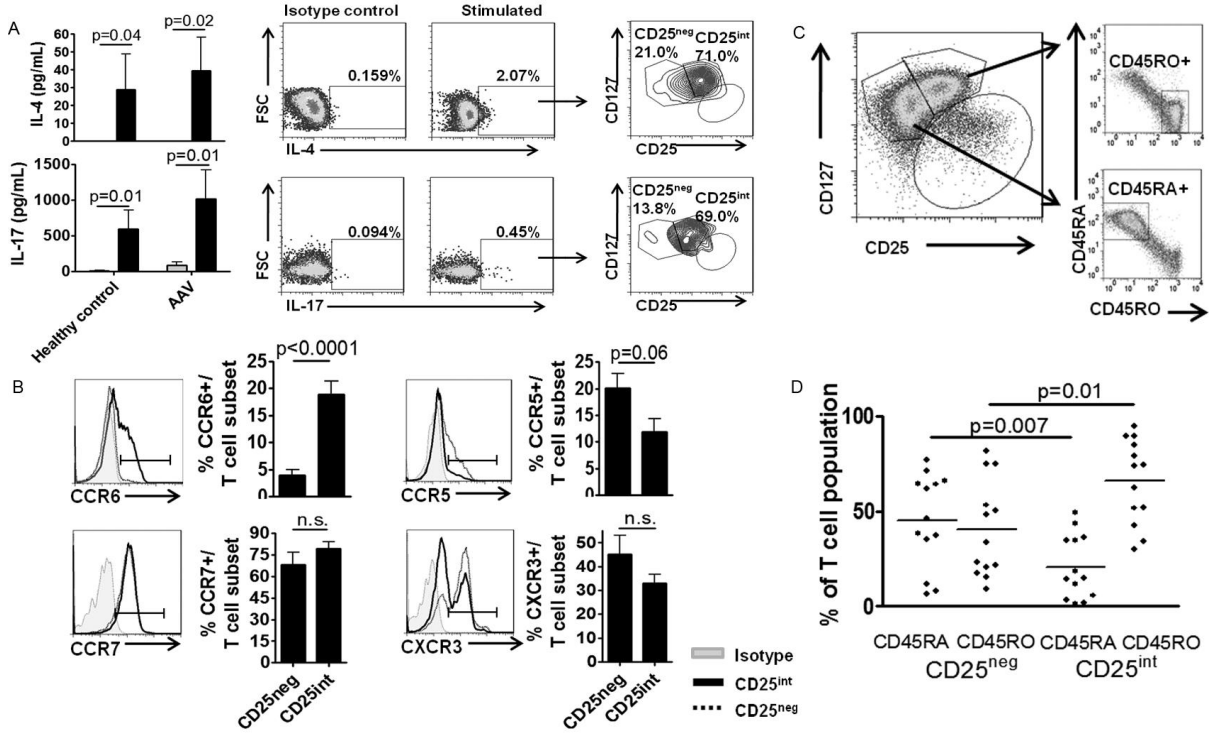

Figure 1.7. Phenotypic analysis of CD25^{int} T cells. (A) Left: Concentration of IL-17A and IL-4 in supernatants of in vitro stimulated CD25^{neg} and CD25^{int} T cells from HC (n=8) and AAV patients (n=9). Right: Representative plots of intracellular detection of IL-17A and IL-4 post-stimulation. Localization of cytokine-producing cells in CD25^{neg} or CD25^{int} T cell populations. Flow sorted T cell populations were stimulated with anti-CD3, -28 for 4 days. (B) Representative flow cytometry plots and cumulative analysis of chemokine receptor expression between AAV T cell subsets. (C) Gating strategy for CD45 isoform analysis of T cell subsets in AAV. (D) Compiled data of CD45 isoform usage between AAV patient T cell subsets (n=13).

Discussion

In conclusion, our data demonstrate two important findings: 1) Defective CD4+ Treg suppressive function in AAV which is associated with increased usage of a FOXP3 splice variant and 2) Increased frequency of a distinct effector CD4+ T cell population is associated with AAV patients which is resistant to Treg suppression. Both findings are critical to the understanding of AAV and autoimmune diseases in general by providing evidence that defects in T cell suppression can stem from both suppressive and responder T cell populations.

Whether Tregs are intrinsically defective in number and function in AAV patients has been controversial[43–45]. Some controversy can be attributed to the fact that other reports examine Treg populations in patients with only remitting disease or only with granulomatosis with polyangiitis (formerly Wegener's)[43,44]. Our studies confirm that comparing Tregs across a large cohort of patients in relapse or remission is not straightforward. However, statistically significant changes in Treg populations are found when studies examine internally controlled active and remission samples, in concordance with Morgan et al[44]. We note that while Treg frequencies significantly change with disease activity, absolute numbers of Tregs are not statistically different from healthy controls. We can partially attribute this discrepancy to altered numbers of effector T cells, specifically decreased numbers of $CD25^{neg}$ T cells in AAV (Figure 1.3C). It remains possible that transient lymphopenia induced during flares of disease could globally affect the T cell pool which may be reflected by increased frequency of Tregs. It is less likely that glucocorticoids could impact Treg frequencies in that 9 of 20 patients in the paired Treg studies had continuous or no glucocorticoid use during sampling. Additional controversy surrounds the markers used to identify Treg populations. We utilize a highly specific method for phenotypic classification of CD4+ FOXP3+ T cells based on surface markers of CD4+ $CD25^{high}$ $CD127^{low}$ [17,18].

The use of immunosuppressives in our patient cohort is an additional confounding factor. However, AAV patients were routinely followed for up to two and a half years with serial sample

collection every three months. During this time many patients' immunosuppressives were changed, yet Treg population changes only occurred with changes in disease activity and the $CD25^{int}$ population remained unchanged despite medication alterations. Additionally, 41.9% of our patient cohort was exposed to Rituximab during sample acquisition (Table 1). While monoclonal anti-CD20 antibodies target the depletion of circulating B cells, there are likely to be unknown off-target effects of Rituximab on peripheral T cells. The effects of Rituximab on T cell populations in ANCA disease have not been directly examined, however studies in other diseases have noted reduced Th17 responses and depletion of a controversial CD20+ T cell population[77,78]. Despite this, our data demonstrates a robust Th17 response from $CD25^{int}$ T cells in our cohort. Yet we do acknowledge that future studies should examine the effects of Rituximab treatment on T cell populations of ANCA disease patients.

Our data demonstrate that AAV Tregs have an increased frequency in active disease that is linked with defective function. We further demonstrate that this dysfunction is associated with increased usage of an exon 2-deficient FOXP3 splice variant. In fact, the majority of FOXP3+ T cells in AAV patients are exon 2-deficient. Healthy controls with minimal exon 2-deficient FOXP3 have a normal suppressive function while AAV patients lose suppressive function as there is an increase in the usage of exon 2-deficient FOXP3. One other study has utilized this method to examine FOXP3 isoforms in the peripheral blood of inflammatory bowel disease patients. Yet that study found no differences in isoform usage between patients and healthy controls[68]. Therefore, our study is the first to identify a patient population that significantly differs in FOXP3 isoform usage compared to healthy controls. We hypothesize that the suppressive regimen of Tregs is lost when exon 2 of FOXP3 is aberrantly spliced. Exon 2 of FOXP3 comprises part of the repressor domain which sequesters other proteins, such as RORγt and NFAT[74,79]. We hypothesize that in the absence of exon 2, RORγt and potentially other unknown proteins, are not sequestered and are able to initiate pro-inflammatory cascades. In line with this speculation, the previously mentioned publication demonstrated cells expressing the exon 2-

deficient FOXP3 were the main producers of IL-17 among FOXP3 expressing cells[68]. Therefore, exon 2-deficiency may alter FOXP3 and Treg function in AAV and increased exon 2-deficiency predicts a lack of suppressive ability.

Our identification of a distinct CD4+ effector T cell ($CD25^{int}$) reveals additional T cell dysregulation in AAV patients. The increased frequency of $CD25^{int}$ T cells, and their CD45RO positivity, leads us to hypothesize that this population may be a clonal expansion of autoreactive cells. A study by Hirota et al suggests that CCR6-expressing Th17 cells were the tissue-infiltrating pathogenic T cell in RA[80]. Therefore, we hypothesize that CCR6-expressing, IL-17-producing $CD25^{int}$ T cells may be able to infiltrate sites of inflammation in AAV and contribute to tissue immunopathogenesis. Consistent with their resistance to Treg suppression, $CD25^{int}$ T cells produce elevated levels of the pro-inflammatory cytokines IL-4 and IL-6 (Figure 1.8), which have been associated with resistance to Treg suppression[31,81]. $CD25^{int}$ T cells also produce robust amounts of IL-17A which has been described to be elevated in AAV patients[41,42]. The data presented herein demonstrate that not only are Tregs implicated in immune dysregulation in AAV, but that a $CD25^{int}$ effector population compounds the dysregulation through suppression resistance and production of pro-inflammatory cytokines. Combination of these events could permit the formation of ANCAs, thereby perpetuating the disease. Several patient samples were obtained at presentation of disease and the $CD25^{int}$ population was already increased in frequency. Therefore, alterations of the $CD25^{int}$ population precede disease presentation and must play a role in disease immunopathogenesis.

Currently, therapies aimed at augmenting Treg function are under development for autoimmune diseases[82]. Increased frequency of a CD4+ T cell population resistant to Treg suppression in AAV highlights the need to consider not only the augmentation of Treg function, but also the ability of effector T cells to be suppressed. Future studies are aimed at deciphering the inciting event(s) leading to changes of the $CD25^{int}$ population and their role in other autoimmune diseases.

Figure 1.8

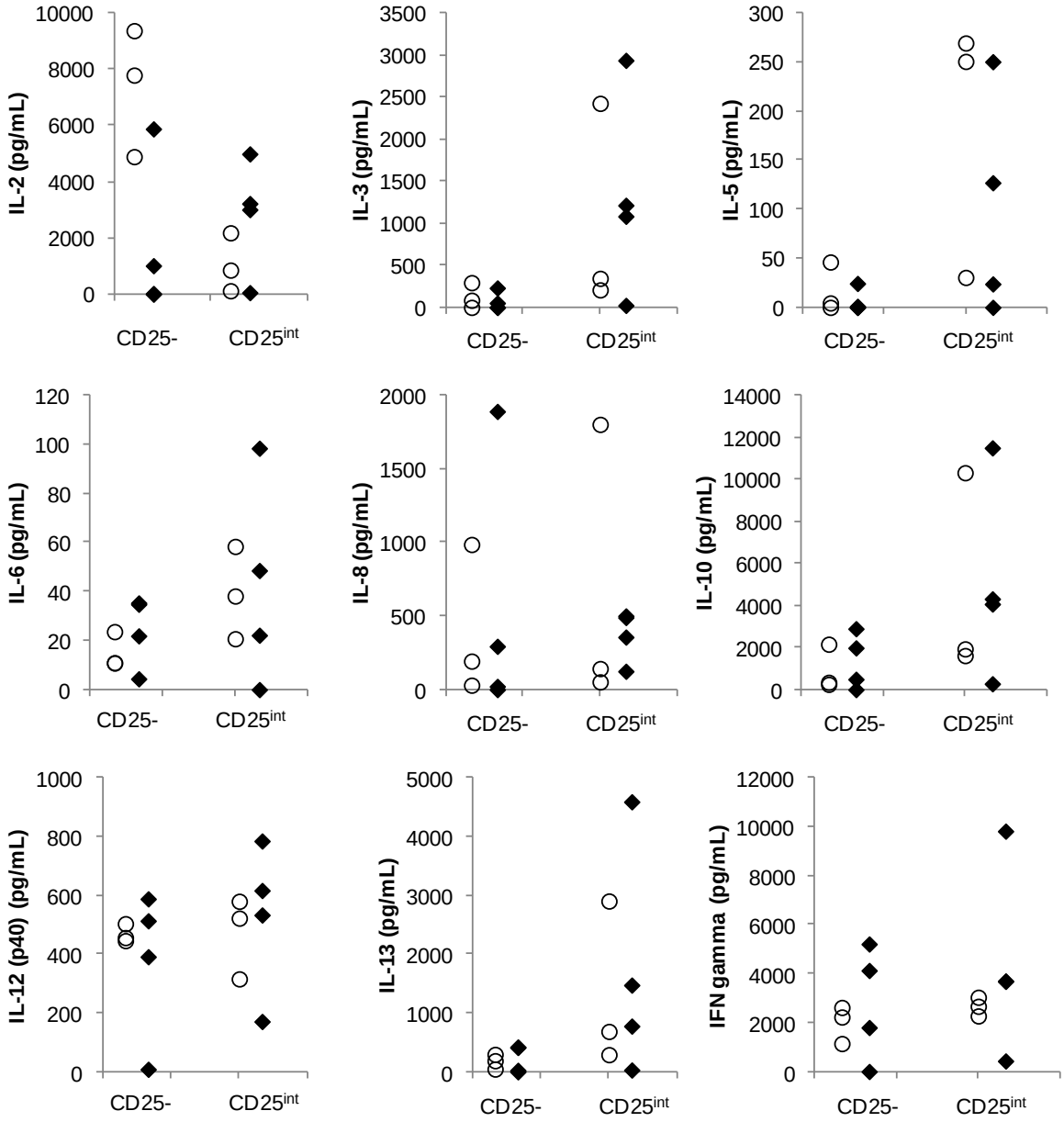

Figure 1.8. Cytokine expression from $CD25^{neg}$ and $CD25^{int}$ T cells. Cytokine profiles of T cell subsets from healthy controls (open circles) and ANCA disease patients (closed diamonds). $CD25^{neg}$ and $CD25^{int}$ T cells from healthy controls and patients with ANCA disease were sorted and subsequently stimulated with anti-CD3/-28 for 4 days. Cell supernatants were then harvested and analyzed by cytokine multiplex.

Chapter 2

DISCOVERY OF NOVEL AUTOANTIBODIES AND AUTOANTIGEN IN IDIOPATHIC NEPHROTIC SYNDROME

The immunopathogenesis of minimal change disease has eluded scientists and clinicians for years. While the hypothesis that T cells are integral to the immunopathogenesis is generally accepted, definitive evidence is lacking. Using a mass spectrometry/epitope excision protocol previously used in our lab to define autoantibody peptide reactivity in ANCA disease, we discovered autoantibodies in MCD patients reactive to a conserved portion of T cell receptors. These anti-TCR autoantibodies reacted with a specific subset of circulating T cells which phenotypically resembled escaped thymocytes. Additionally, these anti-TCR autoantibodies induced changes in targeted T cells which resembled cellular activation. This cellular activation was accompanied by cytokine production from the targeted T cells. Our data demonstrate the presence of autoantibodies present in MCD, a phenomenon not generally hypothesized to occur in MCD patients. Anti-TCR autoantibodies target specific T cells to induce a pro-inflammatory response which may ultimately lead to the pathology and disease manifestation of minimal change disease.

Introduction

Idiopathic nephrotic syndrome, including minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS), affects podocyte architecture and thus the ability of the glomerular filter to prevent urinary protein loss. The initial hypothesis that idiopathic nephrotic syndrome resulted from T-cell dysfunction was put forth in 1974 by Dr. Shalhoub [4]. Shalhoub hypothesized that a clone of T cells produced a "lymphokine" that was toxic to the glomerular basement membrane. Subsequent studies have attempted to identify the "permeability factor" responsible for nephrotic syndrome. IL-8 and IL-13 have been proposed as mediators of minimal change disease by several studies[60,83]. One study has suggested that soluble urokinase receptor (suPAR) causes podocyte injury in FSGS[84], although controversy surrounds this proposal. Another recent hypothesis of the etiology of MCD is that CD80 is upregulated on podocytes in MCD, thereby inducing architectural changes within the podocyte leading to proteinuria[85,86]. The upregulation of CD80 is hypothesized to be a result of cytokine release from T cells or toll-like receptor activation by infectious or environmental agents[86]. Some features of these combined hypotheses are likely to contribute to the immunopathogenesis of MCD. However, there remain a number of unanswered questions.

Traditional hypotheses of the immunopathogenesis of nephrotic syndrome often discount any involvement of B cells. However, the successful use of rituximab in MCD patients questions this paradigm[87–90]. Our group has succeeded at identifying specific peptide sequences recognized by anti-MPO autoantibodies in anti-neutrophil cytoplasmic autoantibody (ANCA) disease by use of a technique called epitope excision utilizing MALDI-TOF/TOF mass spectrometry (Roth et al, JCI 2013 in press). We applied this approach in MCD using total IgG from patients and protein lysates from total peripheral mononuclear blood cells to preliminarily identify proteins targeted by antibodies in MCD. Subsequent peptide ELISAs have confirmed the identification of autoantibodies present in the circulation of MCD. Downstream experiments utilizing affinity-

purified anti-TCR autoantibodies enabled us to determine the targeted T cells, their phenotype and cellular activation induced by autoantibody/autoantigen interactions.

Methods

Patient Cohort/Sample Acquisition

Patients with biopsy-proven or clinically reviewed minimal change glomerulopathy or FSGS, biopsy-proven and chart reviewed patients with ANCA disease, and patients with idiopathic membranous enrolled in this study gave informed, written consent and participated according to UNC Institutional Review Board guidelines. Samples from children with peanut allergies were deidentified. Consent and sample storage procedures have been previously documented[91]. In addition, deidentified serum specimens were obtained from patients concurrent with renal biopsy from UNC-Nephropathology. The institutional review board of the University of North Carolina at Chapel Hill waived the requirement of consent for use of these samples.

Minimal change glomerulopathy, focal segmental glomerulosclerosis, and membranous nephropathy were diagnosed by means of renal biopsy. A subset of pediatric patients (not included in analyses) with clinician diagnosis of minimal change nephrotic syndrome were included after chart review confirmed this to be the most likely diagnosis. All membranous nephropathy patients underwent chart review to identify only idiopathic etiology.

Epitope mapping of immunoglobulin from MCD/FSGS patients against circulating leukocyte proteins

Protein A/G (Santa Cruz) purified immunoglobulin (Ig) was immobilized on CNBr-activated Sepharose 4B (GE Healthcare) in compact reaction columns (CRC, USB Corporation) and incubated with human peripheral leukocyte cell lysate or thymic cell lysate. PBMCs were isolated from healthy donor blood using Vacutainer CPT tubes with sodium heparin (BD). Thymocytes from a healthy thymus were isolated by tissue mincing and straining through 70 µm nylon mesh (BD Falcon). Cell membranes and cytoplasm were then fractionated using a subcellular protein fractionation kit (Pierce). Sepharose-Ig-cell lysate complex was then

sequentially digested with sequencing grade TPCK-treated trypsin (Worthington) for 2h at 37C. TCR-peptides which remain bound to Ig after digestion were eluted with 0.1% Trifluoro acetic acid (ThermoScientific) and sequences were determined by mass spectrometry. Samples were analyzed using a 4800 Plus Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (MADLI TOF/TOF MS/MS) in conjunction with ProteinPilot software (AB SCIEX). Samples were spotted on a stainless-steel target with α-cyano-4-32hydroxycinnamic acid matrix (Sigma). The instrument used has a YAG laser with λ=355nm and the potential difference between the source acceleration voltage and the collision cell was set at 2kV. Calibration was done internally with self-digested TPCK-treated trypsin (same as above). Peak absorbance in MS spectra are not indicative of the abundance of peptide species because of the differences in individual peptides' ability to ionize. All analysis was done with ProteinPilot using an NCBI Mascot search. This protocol is a variation of epitope excision using MS from Roth et al, JCI 2013, in press.

ELISA

Immunoglobulin was isolated from patient plasma or serum using Protein A/G beads (Santa Cruz). ELISA plates (Corning, #3590) were coated with 2-3 ug of peptide diluted in carbonate/bicarbonate buffer (Sigma) and incubated overnight at 4 degrees. Plates were blocked with Superblock-PBS (Thermo Scientific). Patient immunoglobulin (1 ug/well) was added and incubated for 3 hours at 37 degrees. Secondary anti-human antibody with horseradish peroxidase was incubated for 45 min at 37 degrees. Substrate was 1-step PNPP (Thermo Scientific) and absorbance was read at 405 nm at 10 minute intervals.

Immunoprecipitations

Cellular protein lysate from either MCD patients or healthy thymus (as collected in MS protocol) was incubated with immunoprecipitating antibody (anti-myosin, pan-anti-TCR) overnight at 4C. Samples were then washed 6 times before elution by boiling. Eluted samples were run on Mini-Protean TEX Precast Gels 4-15% (BioRad) before transfer to nitrocellulose.

After blocking with Superblock (Thermo Scientific), immunoblotting antibodies (affinity-purified anti-TCR from patients) were incubated overnight at 4C. Appropriate secondary antibodies conjugated to alkaline phosphatase were incubated for 1 hour at room temperature before development with Western Blue stain (Promega).

Purification/Labeling of anti-TCR autoantibodies

TCR peptides identified by mass spectrometry were synthesized and bound to a HiTrap NHS-activated column (GE). Sera from MCD patients were then subjected to FPLC with the TCR peptide column. Affinity-purified anti-TCR autoantibodies were then dialyzed over night prior to fluorescent labeling. All affinity-purified anti-TCR autoantibody samples were concentrated to 1 mg/mL using Amicon Ultra-0.5 mL Centrifugal Filters (Millipore). Samples were then fluorescently labeled with either Alexa Fluor 488 or Alexa Fluor 647 with Alexa Fluor Antibody Labeling Kits (Life Technologies) per manufacturer's protocol.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were washed and resuspended in HBSS, 2% FBS, 0.1% sodium azide, Fc blocked, and stained with the following anti-human fluorescently labeled antibodies: CD2, CD3, CD4, CD8, CD45, TCRαβ, TCRγδ, CD95, CD69, CD154 (all from BioLegend), Myh9 (abcam), and fluorescently labeled anti-TCR autoantibodies from MCD patients. with minimal change disease were affinity purified for peptides found by mass spectrometry. Annexin V and propidium iodide staining was performed according to the FITC Annexin V Apoptosis Detection Kid with PI (BioLegend) manufacturer's instructions. To determine kinetic annexin positivity, the Kinetic Apoptosis Kit (abcam) was followed per manufacturer's instructions. For intracellular cytokine detection, PBMCs were stimulated with either affinity-purified anti-TCR autoantibodies or PMA/ionomycin for 4 hours with GolgiStop incorporated the final 3 hours. Surface staining was performed as above prior to cell fixation and permeabilization with CytoFix/CytoPerm (BD). Intracellular cytokines were detected by the

following anti-human fluorescently labeled antibodies: IL-8 and IL-13 (BioLegend). Cells were acquired on a BD LSRII and data was analyzed by FlowJo software (Tree Star, Inc.).

Images of individual cells during flow cytometry were acquired on an Amnis ImageStream and data analysis was performed using the Amnis Image Data Exploration and Analysis Software (IDEAS™).

Results

Identification of a target protein in peripheral blood leukocyte lysate

Previous studies from our group have utilized mass spectrometry approaches to successfully pinpoint autoantibody targets in ANCA disease. ANCA disease is an "indirect" autoimmune disease as the autoantibodies to not target the organ with pathological manifestations, in this case, the kidneys. Rather, in ANCA disease autoantibodies target an intermediate circulating cell, neutrophils, which spur the kidney pathology. We questioned if a similar phenomenon occurred in MCD. Epitope excision utilizing MALDI-TOF/TOF mass spectrometry was performed using IgG from MCD patients and total leukocyte cell lysate from a healthy control or total protein lysate from normal human thymocytes. Initial MS spectra identified a TCR beta peptide of interest at an observed mass of 1904.7864. The target epitope was identified as the sequence TVTSAQKNPTAFYFCAR at a mass peak at 1904.7864 and protein matched to the human T cell receptor (TCR) beta chain.

Follow up MS experiments also used normal human thymus cell lysate as the source of protein. These experiments also detected TCR peptides bound to IgG from MCD patients; however these peptides matched to the delta chain of the TCR with an amino acid sequence of LEDSAKYFCALGNPLR. Additional MS experiments were performed with several IgG samples from different individual MCD patients. The peptide sequences discovered by all of these experiments are listed in Table 2.1.

After several MS experiments, while the majority of amino acids within the peptide sequence were different from experiment to experiment, we did note a conserved amino acid

sequence which appeared in all MS experiments. This YXCA motif is underlined in Table 2.1. Further investigation into amino acid sequences of TCR beta chains revealed that the majority of TCR variable regions have two highly conserved cysteine residues[92]. These two cysteines should form a disulfide bridge, contributing to the tertiary structure of the TCR. Additionally, as no reducing agents are utilized in the mass spectrometry experiments, it would be expected that both cysteines would be detected by mass spectrometry. However, in all experiments, only one cysteine is detected. This prompts our hypothesis that this disulfide bridge is perturbed in patients with MCD, thereby allowing exposure of an otherwise cryptic epitope and downstream formation of anti-TCR autoantibodies. This "conformeropathy" has been noted in other autoimmune diseases, notably Goodpasture's or anti-glomerular basement membrane (GBM) disease wherein the autoantibody target of the noncollagenous-1 domain of type IV collagen is only exposed after a change in the quaternary structure[93].

Table 2.1

Amino acid sequence	Protein match	Source of protein for MS experiment
TVTSAQKNPTAFYFCAR	TCRβ	Peripheral leukocyte
TAFYLCASS	TCRβ	Peripheral leukocyte
VYFCASSEGTYKYIFGTGTRLK	TCRα	Mouse thymoma
LEDSAKYFCALGNPLR	TCRδ	Normal human thymus
KYFCALGNPLRLGG	TCRδ	Normal human thymus
LLSDSGFYLCAWR	TCRβ	Peripheral lymphocyte
YLCASVVGIPPR	TCRβ	Peripheral lymphocyte
QYLCASSDSFR	TCRβ	Peripheral lymphocyte

Table 2.1. Peptide sequences identified by mass spectrometry/epitope excision. IgG samples from MCD patients incubated with various cell protein lysates were subjected to epitope exicision mass spectrometry experiments to determine the peptide targets of autoantibodies in MCD patients. Table depicts amino acid sequences of identified targeted peptides, protein match and initial source of input protein. Highlighted regions are conserved amino acid motifs found in all mass spectrometry experiments.

Anti-TCR autoantibodies are present in patients with minimal change disease

Peptide ELISAs were performed using the portion of the T cell receptor (TCR) beta and delta found by mass spectrometry (peptides 1 and 4 listed in Table 2.1). IgG samples from patients with MCD, FSGS, idiopathic membranous, anti-neutrophil cytoplasmic autoantibody (ANCA) disease and healthy individuals were tested for reactivity to the TCR peptide (Figure 2.1A). Samples from MCD and FSGS patients were inclusive of all subsets and were acquired at onset of disease in addition to disease remission and relapse of disease. To address the relatively young ages of the MCD patients, we acquired 25 IgG samples from children with peanut allergies. In our MCD cohort, the frequency of positivity for anti-TCR autoantibodies was 36.8%, with the caveat that our cohort was not prospectively collected and includes all patients from a wide range of ages and clinical disease courses. The combined healthy cohort with pediatric peanut allergies had a positivity rate of 3.77%.

To better address the frequency of anti-TCR autoantibodies in the MCD population, we acquired serum and plasma samples from Nationwide Children's Hospital in conjunction with the Midwest Pediatric Nephrology Consortium. Fifty-seven samples acquired at or around onset of disease and fifty-six paired samples at one month after diagnosis were used in a replication study of our peptide ELISA. Samples from the onset of disease had a high reactivity to TCR peptides with a 96.5% positivity rate (Figure 2.1B). At one month post initiation of treatment, most patients had decreased reactivity to TCR peptides but were still positive compared to healthy controls (Figure 2.1B). Therefore, the presence of anti-TCR autoantibodies may be enriched during disease onset or relapse. Figure 2.1A depicts all MCD patients tested on our ELISA (all UNC patients and onset MCD patients from the MWPNC); the positivity rate of this inclusive cohort was 67.8%.

Interestingly, while our cohort of FSGS patients was inclusive of all variants and stages of disease, there was a clear demarcation of FSGS patients positive for anti-TCR autoantibodies (Figure 2.1A). Clinical analysis of FSGS patients who had an IgG reactivity of greater than or

equal to an OD of 1 revealed that this subset of patients was enriched for those with a pathologic diagnosis of tip lesion FSGS. Additionally, clinical review noted that a number of these patients, despite having biopsy-proven FSGS, clinically "acted" like MCD patients. Positivity for anti-TCR autoantibodies may better predict FSGS patients with either tip lesion pathologies or those who may benefit from clinical treatment similar to MCD patients. Additionally, the single idiopathic membranous patient with anti-TCR autoantibody positivity was clinically reviewed and found to have global podocyte activation on kidney biopsy; therefore, this patient may not be truly representative of idiopathic membranous patients and their autoantibody status.

Figure 2.1

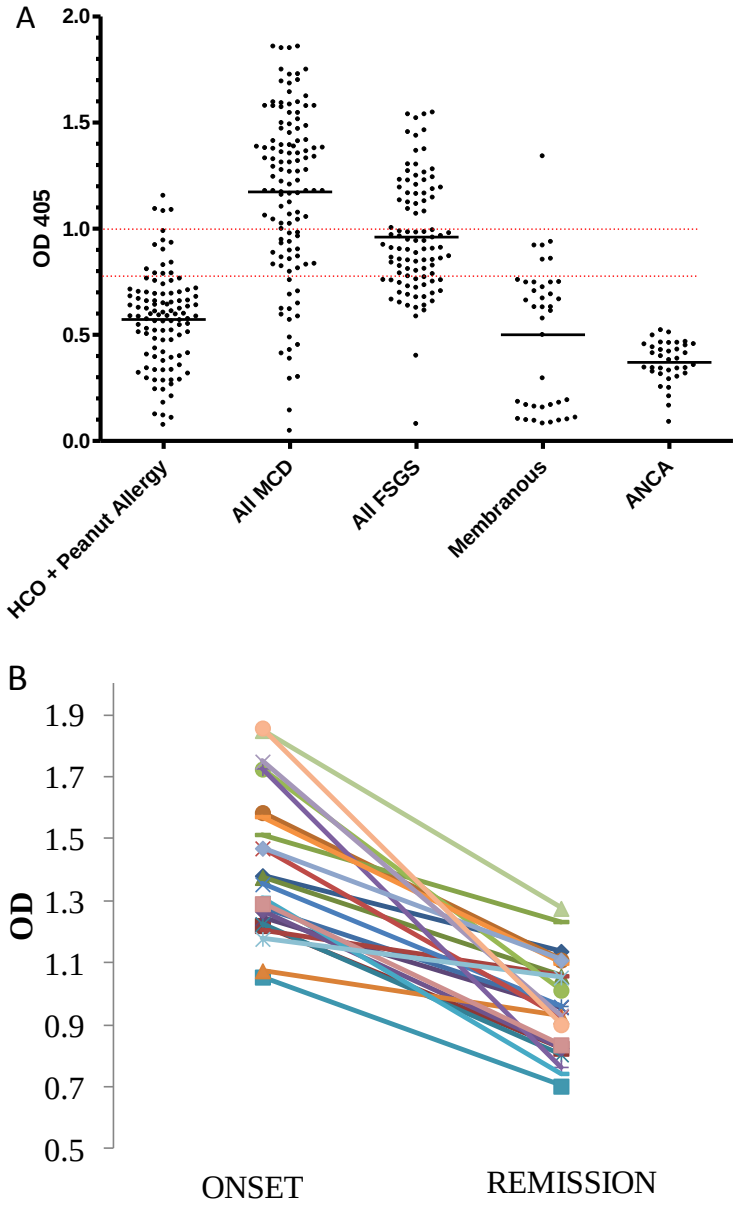

Figure 2.1 Patient IgG reactivity to TCR peptide. (A) IgG isolated from healthy controls, MCD, FSGS, idiopathic membranous and ANCA disease patients were used to determine reactivity to the TCR peptide. Dashed red lines represent one and two standard deviations above the mean of the healthy controls. (B) Paired onset and clinical remission anti-TCR ELISA data from patients in the Midwest Pediatric Nephrology group.

Additionally, anti-TCR autoantibodies were affinity-purified by FPLC with a column to which peptides discovered by mass spectrometry were attached. Relative abundance of anti-TCR autoantibodies was inferred from FPLC peaks when serum from either MCD patients, FSGS patients or healthy controls was used during affinity-purification. Representative FPLC peaks demonstrate that the selected MCD and FSGS patients have anti-TCR autoantibodies (Figure 2.2 top two panels) while the healthy control only exhibited a small amount of anti-TCR autoantibodies, likely representative of natural autoantibodies (Figure 2.? bottom panel). The data from the affinity-purification process strengthened our hypothesis that anti-TCR autoantibodies are enriched in the MCD population and a subset of FSGS patients.

Figure 2.2

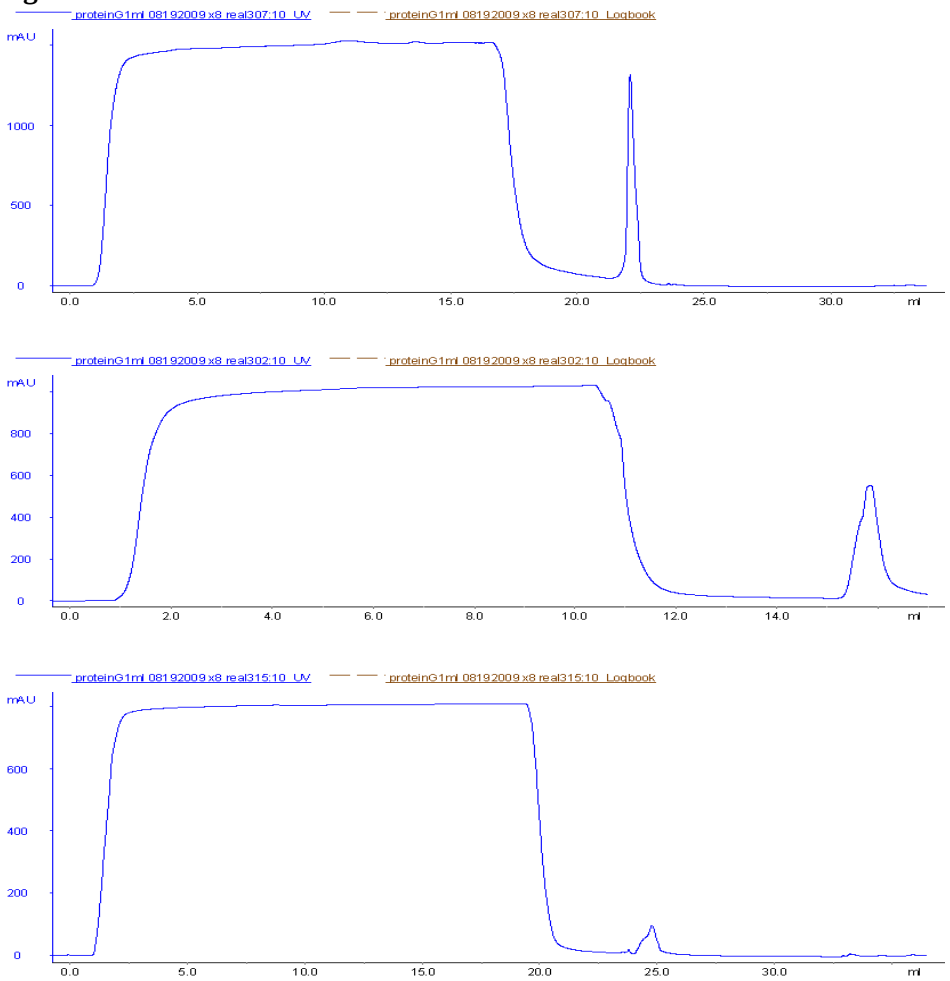

Figure 2.2 Antibody elution peaks from affinity-purification of anti-TCR autoantibodies by FPLC. Serum from MCD patient, FSGS patient or healthy control was subjected to an FPLC column with the TCR peptide. Peaks represent eluted affinity-purified antibodies.

Characterization of T cells targeted by anti-TCR autoantibodies

As the sequence identified by mass spectrometry matched to a portion of the TCR, we sought to determine if these autoantibodies recognize peripheral blood lymphocytes. Anti-TCR autoantibodies from patients with MCD were affinity purified and fluorescently labeled for use in flow cytometry. Initial experiments observed a well-defined population of small lymphocytes recognized by the anti-TCR autoantibody (Figure 2.3A). To control for any artifact or non-specific binding of IgG purified from MCD patients, we also fluorescently labeled total IgG from MCD patients that was depleted of anti-TCR autoantibodies (Figure 2.3A middle panel). These antibodies had minimal background recognition of PBMCs, although they may represent additional autoantigenic targets not currently known.

With confirmation of a readily discernible population of PBMCs recognized by anti-TCR autoantibodies from MCD patients, we sought to further phenotype these cells. As initial mass spectrometry results matched the peptide to a region of a TCR, we hypothesized that cells recognized by these autoantibodies were likely to be T cells. On average, >50% of cells recognized by the anti-TCR autoantibodies expressed surface CD3 (Figure 2.3B left panel). Additionally, >80% of cells recognized by the anti-TCR autoantibodies were CD2+ (Figure 2.3B), which is expressed earlier in T cell development compared to CD3 expression. In further characterization utilizing CD4 and CD8, very few cells were CD4 single positive, a low proportion (~20%) were CD8 single positive, however, the majority of cells were CD4+ CD8+ double positive (Figure 2.3B right panel). The distribution of CD4 and CD8 positivity of cells recognized by the anti-TCR autoantibodies is vastly different when compared to the normal frequency of CD4+, CD8+ or CD4+ CD8+ double positive cells in the periphery (Figure 2.3B far right). As there is a portion of anti-TCR targeted cells that are not CD3+, we were interested to determine if these CD3- cells were still T cells and expressed T cell receptors. Indeed, the majority of anti-TCR targeted cells that are CD3- are expressing a TCR when stained with a pan-TCR antibody (Figure 2.3C). Therefore, the CD3 negativity of some cells could be due to either

steric hindrance of the anti-TCR autoantibody, or these cells are early in development and do not yet express surface CD3. Based on these phenotypic results, we hypothesize that the anti-TCR targeted cells represent a population of "thymocyte-like" T cells.

The anti-TCR autoantibody recognized, on average, 1% of PBMCs from patients with MCD (range 0.1-8%) which was significantly higher than healthy controls which only had an average of 0.25% PBMCs recognized by the anti-TCR autoantibody (Figure 2.3D). These "thymocyte-like" T cells are present in the periphery of healthy individuals, albeit they constitute only a fraction of lymphocytes (0.1-0.5%). A much larger proportion of these cells exist in the periphery of patients with MCD or FSGS, occasionally constituting upwards of 8% of the total lymphocyte population. Glucocorticoids are not known for altering peripheral immunoglobulin levels, therefore the efficacious use of glucocorticoids in MCD must alter some other feature of the immune system. It is widely accepted that T cells and particularly thymocytes are very susceptible to apoptosis induced by glucocorticoids[94,95]. Therefore, disease quiescence in steroid-responsive MCD patients may be due to apoptosis of "thymocyte-like" T cells, thereby decreasing the amount of autoantigen present in the circulation. To this end, with the few MCD patients followed longitudinally, we have documented impressive alterations in the frequency of "thymocyte-like" T cells with changes in disease activity. Perhaps the most impressive data is that from one pediatric case of MCD wherein during relapse the patient's peripheral blood contained "thymocyte-like" T cells at a frequency of 7.88% of total PBMCs. Four months later, after glucocorticoid initiation and remission of disease, "thymocyte-like" T cells only accounted for 2.7% of PBMCs. It will be important to examine the frequency of "thymocyte-like" T cells in a prospective cohort of MCD patients.

Figure 2.3

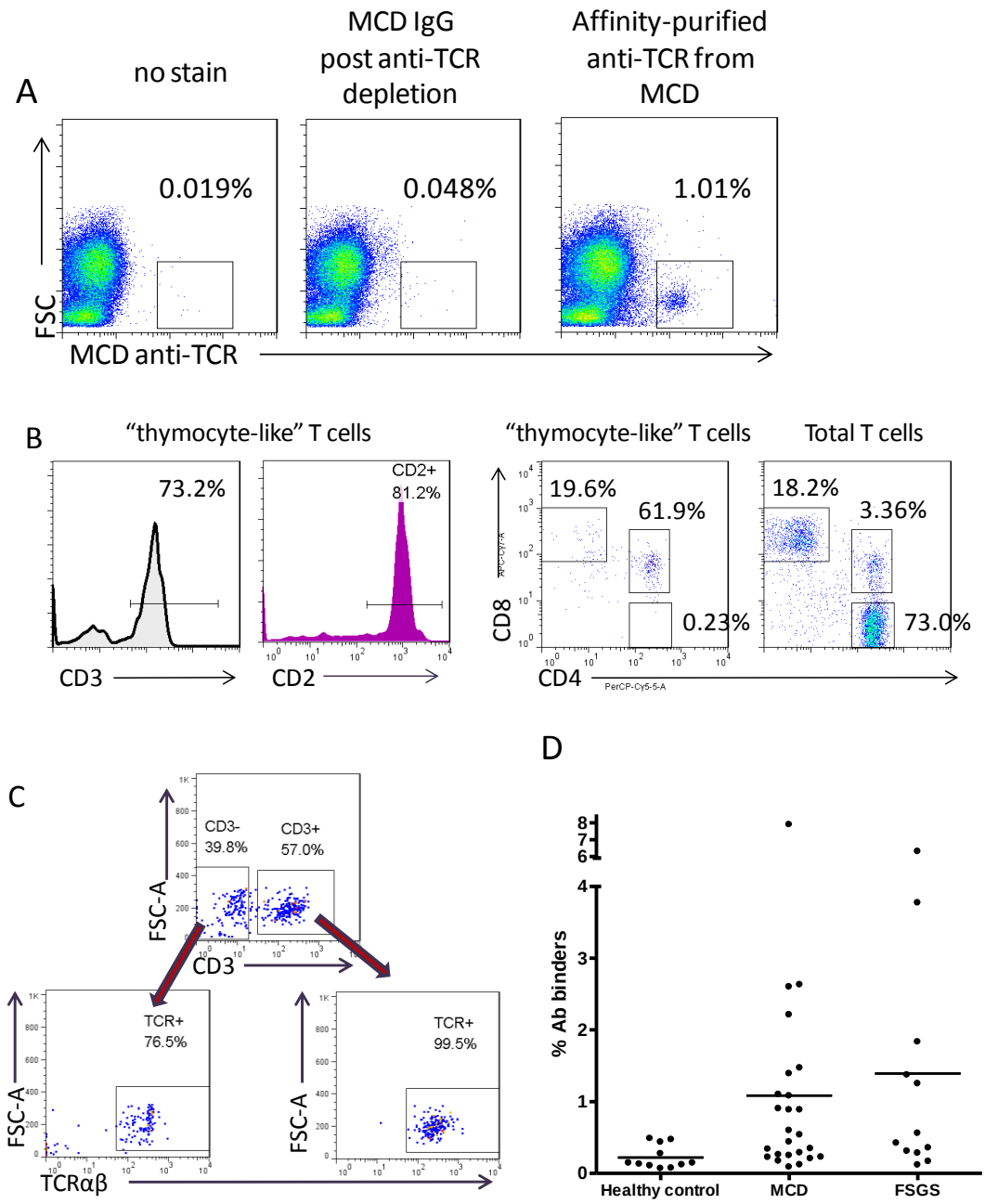

Figure 2.3 Detection of "thymocyte-like" T cells in the periphery of MCD patients. (A) Left panel: unstained PBMCs; Middle panel: Alexa Fluor 647-labeled total IgG from MCD patient depleted of anti-TCR autoantibodies; Right panel: Alexa Fluor 647-labeled affinity purified anti-TCR autoantibodies from MCD patient. (B) Left panel: cells recognized by affinity purified anti-TCR autoantibodies further analyzed for CD3 and CD2 positivity. Right panel: cells recognized

by affinity purified anti-TCR autoantibodies further analyzed for CD4 and CD8 expression in comparison to total peripheral CD3+ T cells. (C) Analysis of pan-TCR positivity on cells recognized by anti-TCR autoantibodies. Targeted cells were sub-analyzed based on CD3 positivity or negativity. (D) Frequency of anti-TCR autoantibody positive cells as a percentage of PBMCs in healthy controls and patients with MCD or FSGS.

To further confirm the cellular phenotype of cells recognized by the anti-TCR autoantibodies, we analyzed fluorescently labeled cells on an Amnis ImageStream which captures images of individual cells through a flow sheath. We were able to visualize individual cells recognized by the anti-TCR autoantibodies and confirmed identical protein expression as was demonstrated by traditional flow cytometry (CD3, CD8 positivity) (Figure 2.4A). Additionally, through the use of the nuclear dye, Hoechst 33342, we confirmed that these cells are indeed nucleated cells despite their relatively small size based on forward scatter observed in flow cytometric analysis (Figure 2.4B).

Figure 2.4

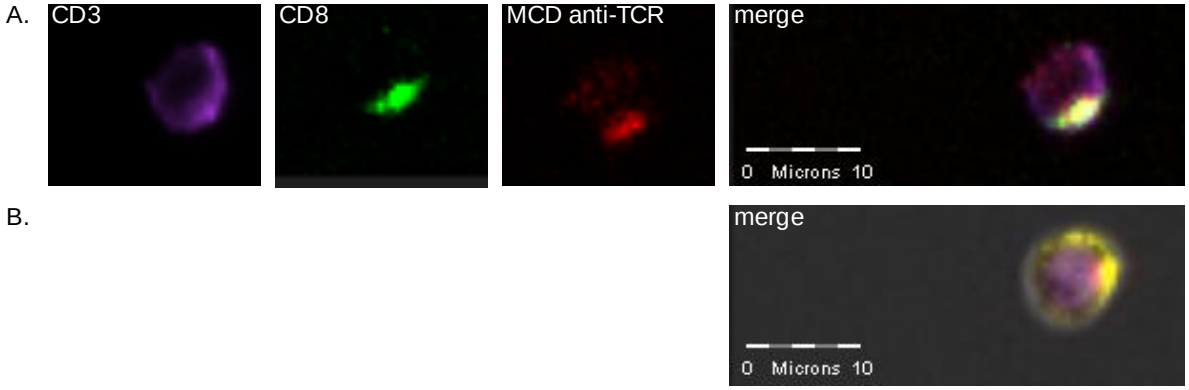

Figure 2.4. Single cell fluorescent phenotype of "thymocyte-like" T cells. (A) FACS images from Amnis Imagestream depicting CD3 and CD8 localization on the surface of cells targeted by anti-TCR autoantibodies. Right panel demonstrates merged pattern of CD3/CD8/anti-TCR positivity. (B) FACS images from Amnis Imagestream depicting nuclear staining (Hoechst 33342) and pan TCR positivity of a cell targeted by the anti-TCR autoantibody. Right panel demonstrates merged pattern of nuclear staining/pan-TCR/anti-TCR positivity.

Anti-TCR autoantibodies activate target T cells

Upon discovery of anti-TCR autoantibodies and their binding to a specific population of "thymocyte-like" T cells, we hypothesized that these autoantibodies could alter the target T cells to become pathogenic. Initial hypotheses about activation of T cells after binding of anti-TCR autoantibodies were formed after visualization of "capping" of CD3/CD8/anti-TCR autoantibody, indicative of activation of the cells[96]. To directly test activation of cells post-autoantibody binding, we assessed surface expression of several activation markers, CD69, CD95 and CD154. CD69 is considered the earliest activation marker expressed after T cell stimulation and activation, and not surprisingly, CD69 expression was the highest expressed activation marker on T cells recognized by the anti-TCR autoantibodies (Figure 2.5A). CD95 had a bimodal expression within the population of cells recognized by the anti-TCR autoantibodies which also confirms activation of these cells. However, CD154 was only modestly expressed after anti-TCR autoantibody binding.

Prior genetic analyses identified a genetic variation of nonmuscle myosin, MyH9, as a risk allele within the FSGS population[97]. While MyH9 is known to localize to podocytes[98], MyH9 is also the dominant isoform of nonmuscle myosin II present in T cells[99,100]. MyH9 plays an integral role in the formation and stabilization of the immunological synapse after T cell activation and aides in the movement of TCR microclusters[101]. Therefore, we assessed intracellular expression of MyH9 in lymphocytes in general. Not surprisingly, lymphocytes had robust intracellular expression of MyH9 (Figure 2.5B left). However, we were interested to examine any potential surface expression of MyH9 on total lymphocytes and also "thymocyte-like" T cells recognized by the anti-TCR autoantibodies. Total lymphocytes rarely expressed MyH9 on the surface (Figure 2.5B middle), but "thymocyte-like" T cells expressed high levels of surface MyH9 (Figure 2.5B right). This finding was further confirmed by captured images on the Amnis ImageStream where surface MyH9 demonstrated a punctate pattern and additionally

localized to a TCR microcluster recognized by the anti-TCR autoantibodies (Figure 2.5C merged).

Figure 2.5

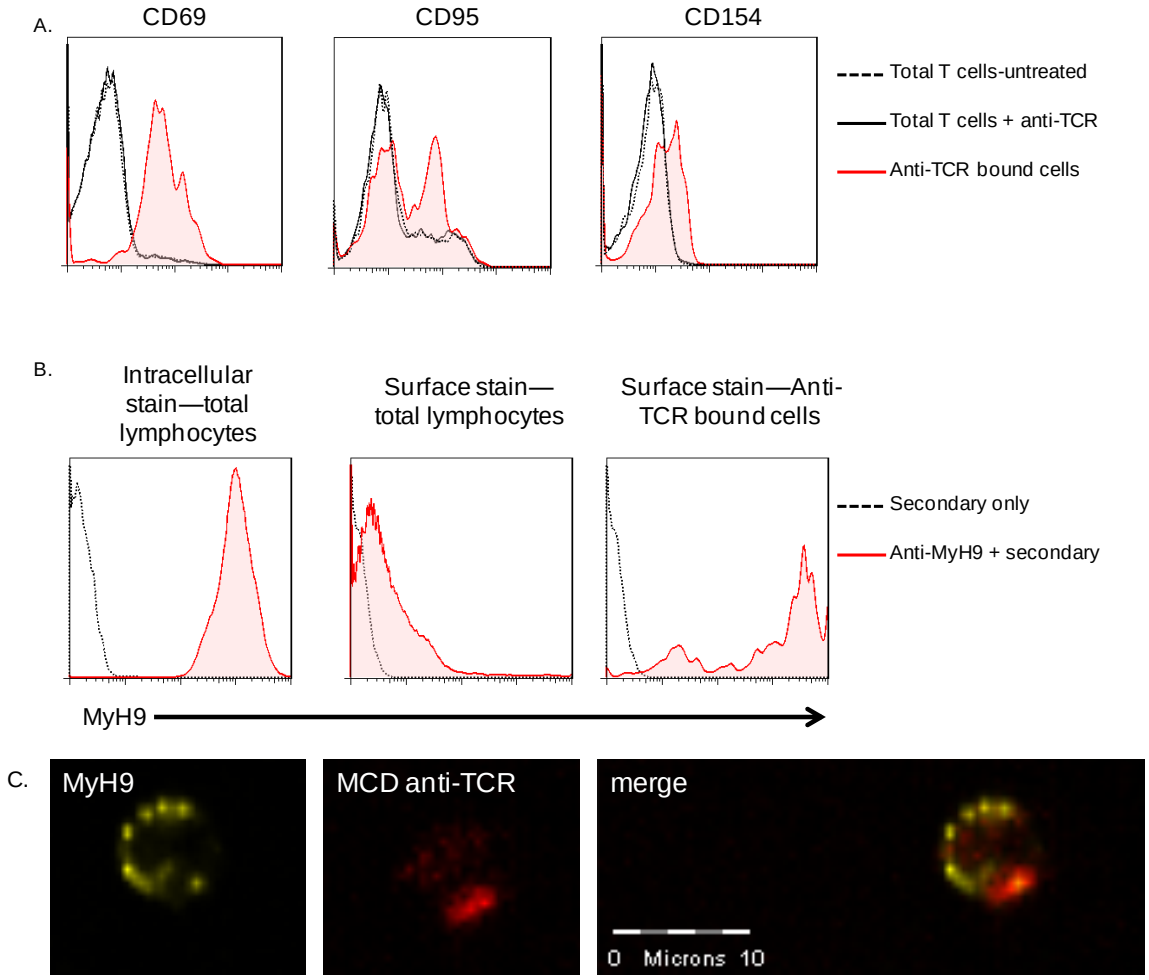

Figure 2.5. Anti-TCR autoantibodies induce cellular activation of target cells. (A) Representative flow plots of experiments in which affinity-purified anti-TCR autoantibodies were added to total PBMCs for 30 minutes and subsequently stained for various markers of cellular activation. Dashed line denotes total T cells not exposed to anti-TCR autoantibodies, solid black line denotes total T cells post anti-TCR autoantibody exposure, and shaded red denotes cells recognized by the anti-TCR autoantibodies. Left panel: surface expression of CD69; Middle panel: CD95 surface expression; Right panel: CD154 expression. (B) Representative flow plots of MyH9 expression on PBMCs. Dashed line denotes cells only stained with secondary antibody, shaded red denotes

cells stained with primary anti-MyH9 and secondary PE antibody. Left panel: intracellular expression of MyH9 in total lymphocytes; Middle panel: surface expression of MyH9 on total lymphocytes; Right panel: surface expression of MyH9 on cells recognized by anti-TCR autoantibodies. (C) FACS images from Amnis Imagestream depicting surface expression of MyH9 and anti-TCR autoantibodies. Right panel demonstrates merged pattern of MyH9 and anti-TCR autoantibodies.

Our initial flow cytometry observations of "thymocyte-like" T cells revealed the cells to be relatively small as demonstrated by forward scatter (Figure 2.3A). Therefore, there was concern that the "thyomcyte-like" T cells could be apoptotic or necrotic. Annexin V staining was performed to delineate if cells were apoptotic. Initial observations revealed that "thymocyte-like" T cells bound extremely high amounts of Annexin V compared to other peripheral blood immune cells (Figure 2.6A). This high binding of Annexin V correlates to high exposure of phosphatidylserine, which occurs during cell apoptosis.

Subsequent experiments examined Annexin V and propidium iodide (PI) simultaneously to assess cell necrosis. While a large proportion of "thymocyte-like" T cells were positive for Annexin V, very few cells were PI positive, indicating that cells were not necrotic (Figure 2.6B). With confirmation that the "thymocyte-like" T cells were still viable, we questioned if the phosphatidylserine exposure truly meant these cells were apoptotic, or instead was yet another marker of cellular activation. To assess the kinetics of phosphatidylserine exposure, we utilized a commercial Annexin XII with polarity sensitive indicator of viability and apoptosis (pSIVA™) technology. This technology allows the discernment of permanent phosphatidylserine exposure (apoptosis) versus reversible, transient exposure (more indicative of cellular activation). The fluorescent signal from Annexin XII is only detected when bound to exposed phosphatidylserine. If the cellular membrane is flipped back to the original orientation, Annexin XII detaches and the fluorescent signal is lost.

In simultaneous experiments, "thymocyte-like" T cells were assessed for Annexin V and XII positivity (Figure 2.6C). Again, we demonstrated that the majority of "thymocyte-like" T cells are Annexin V positive, however none of the "thymocyte-like" T cells stained positively for the polarity-dependent Annexin XII (Figure 2.6C). This indicated that "thymocyte-like" T cells are "flipping" their lipid membranes, but this is a reversible phenomenon. We hypothesize that this phosphatidylserine exposure on "thymocyte-like" T cells is a function of cellular activation

by the anti-TCR autoantibodies as another group has demonstrated that TCR-mediated antigen recognition in CD8+ T cells induces phosphatidylserine exposure on the external membrane[102].

Figure 2.6

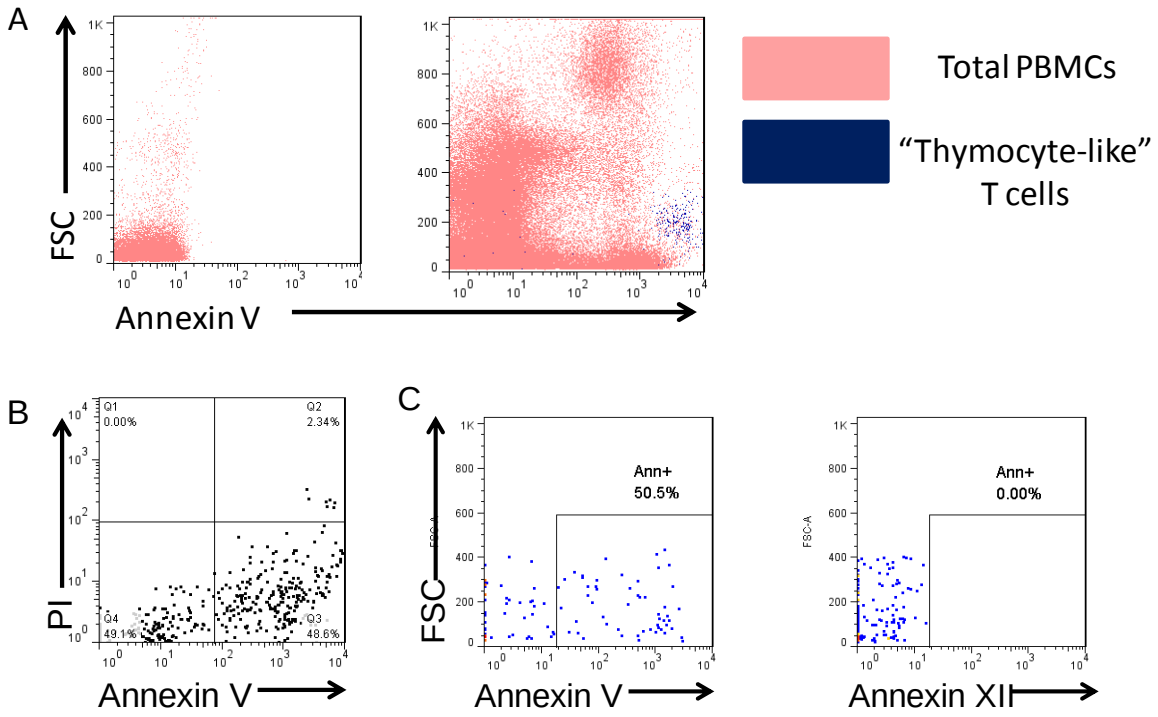

Figure 2.6. Annexin positivity of "thymocyte-like" T cells. (A) Annexin V staining to discern phosphatidylserine exposure on total PBMCs. Left panel: unstained control. Right panel: pink cells are total PBMCs, dark blue cells are "thymocyte-like" T cells recognized by anti-TCR autoantibodies. (B) "Thymocyte-like" T cells analyzed for Annexin V and propidium idodide incorporation simultaneously. (C) Analysis of different Annexin positivity on "thymocyte-like" T cells. Left: flip-independent Annexin V staining. Right: flip-dependent Annexin XII staining.

Presence of co-immunoprecipitants with anti-TCR autoantibodies

Upon demonstration that MCD anti-TCR autoantibodies react to a specific subset of peripheral T cells and the coincidental finding of surface expression of MyH9, we hypothesized that these proteins could be physically linked to one another. Protein lysates from the membrane fraction of healthy thymocytes derived from thymus or protein from peripheral CD3+ cells of a MCD patient were subjected to a series of immunoprecipitations and immunoblots to determine protein proximity or co-involvement.

In one series of experiments involved immunoprecipitation with an anti-pan-TCR antibody and immunoblot with affinity-purifed anti-TCR autoantibodies (Figure 2.7; left panel). In this instance, the prominent band was revealed at ~165 kD from both healthy thymocyte and MCD peripheral CD3+ protein lysate. As even the highest combined molecular weight of the TCR is only 110 kD, there must be another protein co-immunoprecipitating. This could potentially represent the "masking" protein in serum which prevents use of serum on peptide ELISA.

The other set of experiments; cellular proteins were probed for immunoprecipitation with anti-MyH9 antibodies and subsequently immunoblotted with affinity-purified anti-TCR autoantibodies (Figure 2.7; right panel). Both healthy thymocyte and MCD peripheral CD3+ protein lysate revealed a prominent band at approximately 115 kD (Figure 2.7; right panel). The molecular weight of human TCRs (heterodimer) can vary between 40-55 kD per chain[103]. Therefore, the TCR heterodimer could run anywhere between 80-110 kD on Western blot. Considering the initial immunoprecipitation was targeted to Myh9, with a native molecular weight of 226 kD, one would expect a higher molecular weight band on Western blot. However, MyH9 has been reported to denature into a form with a molecular weight of only 54 kD[104]. A complex of MyH9 and TCR heterodimer may be represented by the ~165 kD band visualized from the CD3+ lysate from MCD (Figure 2.7.; right panel lane 2).

Figure 2.7

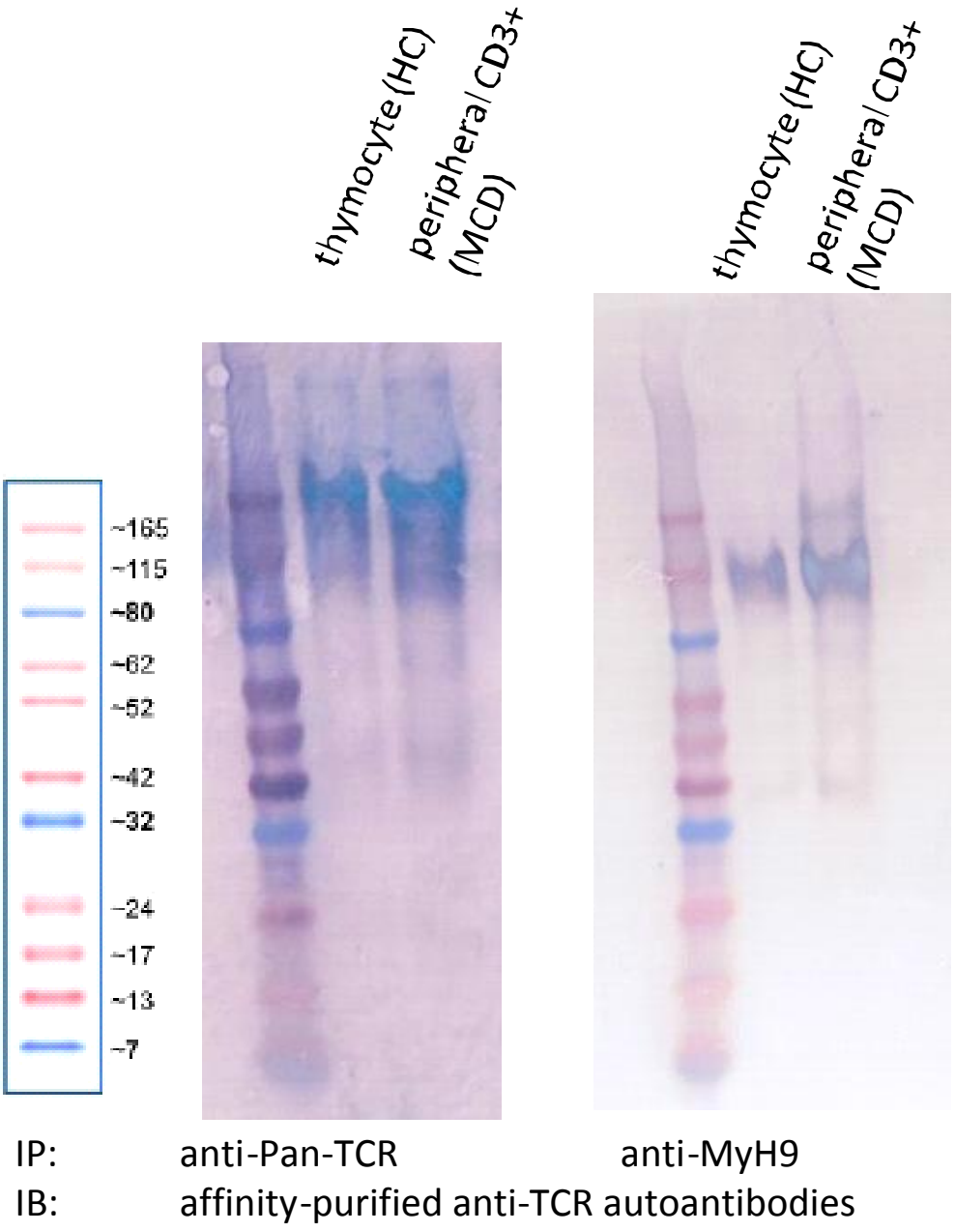

Figure 2.7. Presence of co-immunoprecipitating proteins with anti-TCR autoantibodies. Protein lysate was extracted from the cellular membranes of either healthy thymus-derived thymocytes (lane 1) or peripheral CD3+ cells from MCD patients (lane 2). (Left) Overnight immunoprecipitation was performed with commercial anti-pan-TCR antibodies. Immunoblot was performed with affinity-purified anti-TCR autoantibodies and appropriate secondary antibody to

reveal protein bands. (Right) Overnight immunoprecipitation was performed with commercial anti-MyH9 antibodies. Immunoblot was performed with affinity-purified anti-TCR autoantibodies and appropriate secondary antibody to reveal protein bands.

Anti-TCR autoantibodies induce cytokine production in "thymocyte-like" T cells

With phenotypic confirmation that anti-TCR autoantibodies induce cellular activation of targeted "thymocyte-like" T cells, we sought to determine if any cytokines were produced in response to activation by anti-TCR autoantibodies. Total CD3+ T cells from patients with MCD were incubated overnight with anti-TCR autoantibodies and controls. Cell-free supernatants were then analyzed by cytokine ELISAs. Cytokines previously suspected in the literature were assessed, IL-8, IL-13 and sUPAR in addition to other T cell activation cytokines, IL-2, IL-4 and IL-17A. CD3+ T cells from three separate MCD patients all secreted high levels of IL-8 (range 1500-3000 pg/mL) while CD3+ T cells from a healthy individual or a healthy thymus did not secrete detectable levels of IL-8 in response to anti-TCR autoantibody stimulation (Figure 2.8A). IL-2, IL-4, IL-17A, IL-13 and sUPAR were not detected in any supernatants of stimulated cells.

To further confirm that IL-8 secretion was produced by T cells and specifically, "thymocyte-like" T cells, we also examined intracellular cytokine production by flow cytometry. Four hour stimulation with anti-TCR autoantibodies elicited some IL-8 production in CD3+ T cells (Figure 2.8B middle panel); although this was not as potent as four hour stimulation with PMA and ionomycin (Figure 2.8B right panel). With additional subset gating, we determined that the majority of IL-8 secretion in response to anti-TCR autoantibody stimulation was coming from "thymocyte-like" T cells (Figure 2.8C). While these studies are not inclusive of all potential cytokines, we can confirm that anti-TCR autoantibodies activate "thymocyte-like" T cells and elicit cytokine production.

Figure 2.8

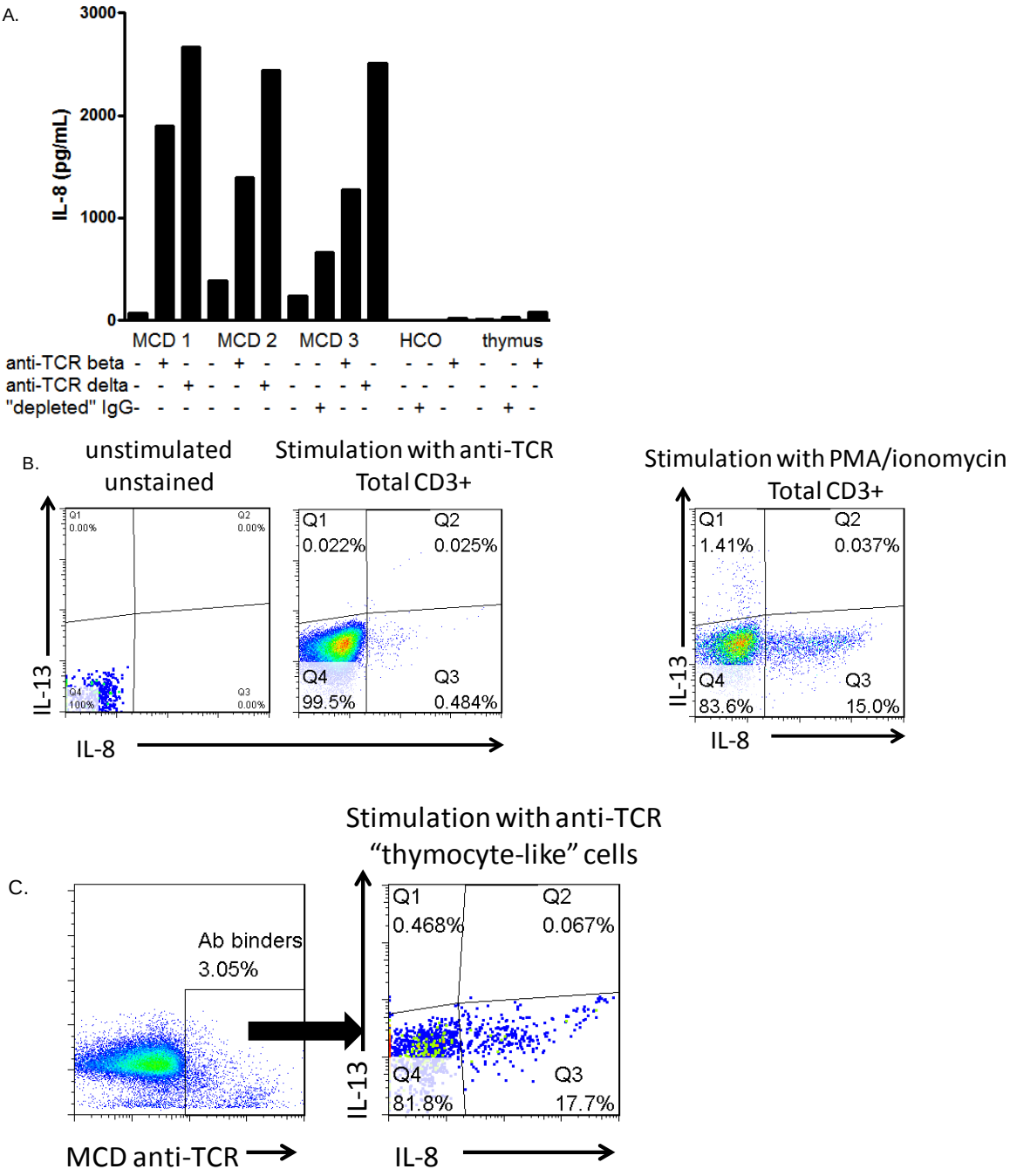

Figure 2.8. Anti-TCR autoantibodies induce cytokine production from "thymocyte-like" T cells in MCD patients. (A) CD3+ T cells were negatively selected for from MCD patients (n=3), healthy control (n=1) or healthy thymus (n=1) and were either unstimulated, stimulated with 5 ug of anti-TCR autoantibodies or stimulated with 5 ug of "depleted" MCD IgG per 2 million cells overnight. Cell-free supernatants were then used for IL-8 cytokine ELISA. (B) Intracellular

cytokine detection of IL-13 and IL-8. Left: unstimulated and unstained PBMCs. Middle: PBMCs stimulated with anti-TCR autoantibodies for 4 hours. Right: PBMCs stimulated with PMA and ionomycin for 4 hours. (C) Gating on “thymocyte-like” T cells targeted by anti-TCR autoantibodies; Right: cytokine production from “thymocyte-like” T cells post 4 hour stimulation with anti-TCR autoantibodies.

Discussion

Perhaps one reason the immunopathogenesis of MCD has eluded scientists and clinicians for years, is the emerging evidence that MCD is another example of an indirect autoimmune disease, akin to ANCA disease. In cases of indirect autoimmune disease, the autoantigenic target is not necessarily part of the organ(s) with the ultimate disease manifestation. Therefore, the autoantigenic target is likely to be elusive. Findings in MCD and ANCA disease now provide evidence that research must cast a wider net to fully understand the immunopathogenesis of autoimmune diseases.

The coincidental finding of surface expression of MyH9 on "thymocyte-like" T cells additionally clarifies the role of MyH9 in MCD and some variants of FSGS. MyH9 was found to be linked to FSGS in a GWAS study[97], but the absolute function and impact on disease pathogenesis was not known. As other investigators have demonstrated the importance of MyH9 in TCR synapse formation[101], we now demonstrate this same MyH9, TCR coalescence also occurs in "thymocyte-like" T cells in response to stimulation by anti-TCR autoantibodies. Since we only detected very robust surface expression of MyH9 on "thymocyte-like" T cells, this may represent a potential biomarker for targeted cells in minimal change disease.

Our findings in MCD also have implications for some patients with FSGS. The pathological diagnosis of FSGS tends to be a "catch all" with many subsets and variants found on kidney biopsy. However, the etiology and pathogenesis of FSGS also remains elusive. Clinicians have often noted that certain variants of FSGS (some not otherwise specified and tip lesion) clinically "behave" more like MCD. It has long been hypothesized that MCD and FSGS may lie on a pathological continuum. Our data provide evidence that a subset of FSGS patients do have anti-TCR autoantibodies and circulating "thymocyte-like" T cells. This confirms that some FSGS patients may immunologically "behave" more as a MCD patient and may benefit from altered therapeutics.

One confounder of our ELISA is the necessity to use patient IgG instead of total serum to demonstrate positive autoantibody titers. Our group demonstrated this same requirement of IgG instead of sera in patients with ANCA disease in recognition of a certain masked epitope of MPO (Roth et al, JCI 2013, in press). With MPO, our group demonstrated that a fragment of ceruloplasmin, the natural inhibitor of MPO, was present in serum and masks a crucial epitope. By using purified IgG, the fragment of ceruloplasmin is no longer present and autoantibody reactivity to a specific peptide was demonstrated. A similar phenomenon must be occurring in MCD as we had to use IgG to demonstrate positive reactivity to the TCR peptide. We currently do not know what serum protein is responsible for the blocking effect in minimal change disease.

One group has previously published the finding that patients with RA or SLE do have antibodies reactive to the lambda chains of immunoglobulin and the beta chain of a TCR, specifically the CDR1 and framework 3 regions of the variable region[105]. These autoantibodies were predominately IgM in patients with RA[105]. This is not surprising as the classically defined rheumatoid factor is IgM directed against the Fc portion of circulating IgG. Immunoglobulins have a number of similarities to the structure of TCRs. Therefore, this may represent rheumatoid factors that have crossreactivity to portions of the TCR. Patients with SLE had both IgM and IgG reactive to the CDR1 and framework 3 regions of the variable region of the TCR. It is unknown from these previous studies the exact peptide to which RA and SLE patients react to the TCR. However, the critical feature in differentiating these previous findings from our current MCD findings is the targeted cell and downstream effects of autoantibody/autoantigen interaction. The prior findings in RA and SLE determine that anti-TCR autoantibodies downmodulated target T cells and the autoantibody/autoantigen interaction was actually a beneficial response to dampen the immune response[106]. Our studies of anti-TCR autoantibodies in MCD patients have demonstrated the activation of targeted “thymocyte-like” T cells inducing a pro-inflammatory response. Therefore, not all anti-TCR autoantibodies have the same function or induce the same downstream effects.

There are certainly limitations to the studies put forth in this chapter. One limitation is the narrow exploration of cytokines produced by "thymocyte-like" T cells in response to anti-TCR autoantibodies. For the purpose of this study, we focused on cytokines either previously demonstrated in the literature, hypothesized in the literature, or were cytokines often produced during T cell activation. Future studies are aimed to further investigate a more robust list of cytokines or proteins produced during this autoantibody-cell interaction and downstream effects on podocyte architecture. Another limitation of these studies is the age discrepancy between healthy controls and patient samples. Many MCD patients are under the age of 5 and acquisition of PBMCs and serum from "healthy" children poses as an obstacle. This was somewhat remedied by the inclusion of children with peanut allergies for the ELISA screen. We have acquired PBMCs from a few children (10 years and younger) to assess for "thymocyte-like" T cells. These preliminary studies suggest that the increased frequency of "thymocyte-like" T cells in MCD patients is not solely attributable to patients' young age as the large population of "thymocyte-like" T cells was not evident in healthy children.

We demonstrate herein that anti-TCR autoantibodies found in MCD target "thymocyte-like" T cells, induce cellular activation and cytokine production. As a result of this cascade, we hypothesize that the activated "thymocyte-like" T cells secrete a protein or cytokine that is ultimately injurious to podocytes. IL-8 may be a contributing factor in this progression of events. Preliminary data using supernatants from anti-TCR autoantibody-activated T cells are suggestive that the "permeability factor" is released from "thymocyte-like T cells." *In vitro* podocyte studies demonstrate architectural changes in podocytes after several hours of exposure to activated supernatants. Future studies are aimed to determine the critical protein responsible for podocyte injury and formal characterization of podocyte injury in MCD.

As with all protein-protein interactions, tertiary conformational structure is equally important as the amino acid sequence which determines reactivity. Repeated mass spectrometry, epitope excision experiments with MCD patients revealed a common amino acid sequence with

the TCR to which patient autoantibodies reacted. We found that the common amino acid motif YXCA appeared in the majority of peptides discovered during mass spectrometry experiments. Interestingly, upon examining the crystal structure of T cell receptors, the cysteine of the common MCD motif should be associated to another cysteine by a disulfide bridge. As no reducing agents are utilized during our mass spectrometry protocols, the inability to detect the associated cysteine initially proved to be conundrum. In further analysis of the TCR crystal structure, the presence of the disulfide bridge invokes a tertiary structure in which the common MCD motif is not readily accessible.

While the YXCA amino acid sequence can be found in a large percentage of T cells, affinity-purified anti-TCR autoantibodies only recognize a small, but distinct population of circulating T cells. From this finding, we hypothesize that the disulfide bridge has only been disrupted in "thymocyte-like" T cells and thus, the epitope is available for autoantibody recognition (Figure 2.9). Conformational changes in proteins eventually allowing exposure of an otherwise cryptic epitope has also been demonstrated with the collagen protein in Goodpasture's disease[93]. We hypothesize that either through a genetic, environmental or infectious aberration, the disulfide bridge either does not exist or has been disrupted in the "thymocyte-like" population of T cells.

In sum, the data presented herein demonstrate that MCD and a subset of FSGS patients have IgG reactive to a conserved amino acid motif present in the majority of T cell receptors. Despite the fact that most circulating T cells contain the YXCA motif in their TCRs, affinity-purified anti-TCR autoantibodies from MCD patients only react with a subset of T cells with defined characteristics. This may represent a "conformeropathy" in which a structural change must occur in specific TCRs to be exposed for autoantibodies to recognize the target peptide. Additionally, this may be the same reason why these "thymocyte-like" T cells are readily detectable in MCD patients, but are found at an exceedingly low frequency in healthy individuals. These anti-TCR autoantibodies not only bind "thymocyte-like" T cells but induce cellular

activation leading to cytokine production which we hypothesize causes podocyte damage as an end result. Changes in podocyte architecture induced by these “thymocyte-like” T cell cytokines likely propel the immunopathogenesis of minimal change disease.

Figure 2.9

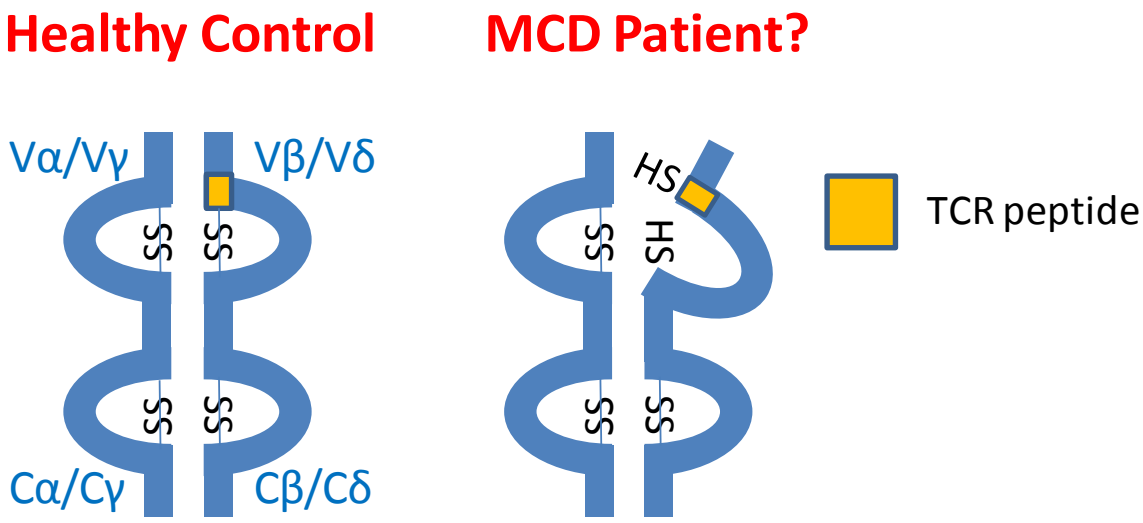

Figure 2.9. Hypothesis of TCR structure in MCD patients. Cartoon structure of TCR chains, left chain could represent TCRα or γ chains and right chain could represent the pairing TCRβ or δ chains. Top half represents the variable portion of the TCR with the bottom half representing the constant region. –SS- represents the disulfide bridges created between conserved cysteine residues. Yellow box represents the putative epitope of anti-TCR autoantibodies found in MCD patients.

SUMMARY

The presented body of work has focused on the role of circulating T cells in the immunopathogenesis of ANCA disease and nephrotic syndrome. Chapter 1 elucidates how both regulatory T cells and effector T cells contribute to the lack of T cell suppression exhibited by ANCA disease patients. The dysfunction of regulatory T cells from ANCA disease has been demonstrated by a number of groups over the years, yet little progress has been made to determine the cause of Treg dysfunction in these patients. With the discovery that Tregs from ANCA disease disproportionately utilize exon 2-deficient FOXP3, we can move forward to better understand Treg dysfunction from a molecular standpoint. However, the data presented in Chapter 1 suggests that Tregs cannot be the sole culprit in T cell aberrancies in ANCA disease. The expanded population of $CD25^{int}$ T cells in ANCA disease, which are resistant to suppression by healthy control Tregs, demonstrates that effector T cells must be equally considered as a culprit in dysfunctional T cell suppression.

Chapter 2 delineates not only the role of circulating T cells in the immunopathogenesis of minimal change disease, but demonstrates a novel autoantibody-autoantigenic target. Autoantibodies to a region of the TCR are present in patients with minimal change disease. But, perhaps more importantly, is the discovery and characterization of cells targeted by these anti-TCR autoantibodies. Data presented in Chapter 2 reveal that anti-TCR autoantibodies bind to a specific subset of T cells that are reminiscent of thymocytes and we have termed these cells "thymocyte-like" T cells. Importantly, these "thymocyte-like" T cells are found infrequently in the peripheral blood of healthy individuals, but have an increased frequency in patients with MCD and some patients with FSGS. Therefore, it is not only the presence of autoantibodies that

dictates disease immunopathogenesis, but rather the additional altered presence of the autoantigenic target.

The discovery of anti-TCR autoantibodies and targeted “thymocyte-like” T cells in MCD and a subset of FSGS patients alters the paradigm of autoimmunity and autoimmune disease by providing another example of “indirect” autoimmunity similar to ANCA disease. Acknowledgement of this phenomenon will enable further advances in the discovery of additional autoantibody-autoantigen interactions in other autoimmune diseases.

EPILOGUE

T CELLS IN AUTOIMMUNE KIDNEY DISEASE: IMMUNOPATHOGENESIS AND TREATMENT

T cell alterations in ANCA disease

Diversity of the CD4+ T cell pool in healthy individuals provides an efficient mechanism to combat an array of potential pathogenic insults. However, this diversity is problematic in autoimmune disease as multiple facets of T cell immunity are altered in order to contribute to disease progression. This is certainly true in ANCA disease. For years, the predominant T cell discovery in ANCA disease was that Tregs were unable to suppress effector proliferation[43–45]. While this finding was reproduced by many labs with different patient cohorts, the underlying cause of the Treg dysfunction remained an enigma. The data presented in Chapter 1 not only recapitulates the dysfunction of Tregs in ANCA, but reveals a contributor to the non-suppressive function of those Tregs. ANCA patients disproportionately utilize a variant of FOXP3 lacking part of the repressor domain found in exon 2. To corroborate this finding, the ability of Tregs to suppress effector proliferation is inversely correlated with the frequency of exon 2-deficient FOXP3 cells. Treg dysfunction in ANCA disease is not likely due to only one aberration; however, this FOXP3 discovery is certainly a contributing factor.

While the Treg defect in ANCA disease is of obvious concern, the CD4+ effector T cell population must also be taken into account. As the data in Chapter 1 demonstrate, $CD25^{int}$ T cells are of additional concern as these cells are "primed" to be pro-inflammatory. The production of IL-4 and IL-17 in addition to CCR6 expression on these cells are evidence that $CD25^{int}$ cells have the capacity to extravasate into peripheral tissues and contribute to inflammatory cascades. While $CD25^{int}$ cells exist in healthy individuals and are also capable of cytokine secretion, the key factor is the impressive expansion of the $CD25^{int}$ population in the periphery of not only ANCA disease patients, but also patients with SLE and RA. The balance of effector T cell subsets is weighed in favor of the pro-inflammatory subsets in ANCA disease.

The predominance of the $CD25^{int}$ population in ANCA disease also contributes to the lack of suppression demonstrated in ANCA disease patients multiple times. Only recently has the hypothesis of suppression-resistant effector cells been proposed[26]. Prior to this hypothesis, any lack of suppression was attributed solely to some defect within the regulatory T cell population. Data presented in Chapter 1 demonstrate that the lack of regulation of T cell responses in ANCA disease stems from two aberrations: exon 2-deficient FOXP3 in regulatory T cells, and $CD25^{int}$ T cells that are resistant to suppression by healthy control Tregs. In moving forward in ANCA disease and other autoimmune diseases, any defect in T cell regulation must be investigated from both a Treg and effector T cell perspective

Insight into the immunopathogenesis of MCD

The immunopathogenesis of minimal change disease has largely been based on speculation, hypotheses and anecdotal evidence. The common belief that T cells participate in some role of disease induction and progression was probably best summarized by Shalhoub[4]. Data presented in Chapter 2 have unlocked a great deal of the mystery in MCD. The data demonstrate that B cells and antibodies cannot be ignored in the immunopathogenesis of minimal change disease and some variants of focal segmental glomerulosclerosis.

Our finding that autoantibodies in MCD target a population of T cells supports the long accepted hypothesis that T cells play a role in the disease pathogenesis. Additionally, the fact that the autoantibody-targeted cells are reminiscent of thymocytes is not surprising considering the anecdotal evidence of MCD "burning out" in some patients at the same time of thymic involution. If fewer aberrant thymocytes are released into the periphery, the autoantigenic target is no longer accessible for continuation of autoantibody-autoantigen interaction. As the targeted T cells are "thymocyte-like," this may also explain why many patients respond favorably to glucocorticoids. It is known that thymocytes are especially sensitive to apoptosis by glucocorticoids[94,95].

Additionally, while the consensus sequence of YXCA is present in the majority of circulating TCRs, only a subset of cells are recognized by anti-TCR autoantibodies. While the conformational change within the TCR is not fully understood, one hypothesis stands that either an environmental or infectious agent could disrupt the nature disulfide bridge at that motif. The hypothesis of an infectious agent causing this fits with the anecdotal evidence that a viral prodrome often precedes the clinical manifestation of minimal change disease.

The data presented in Chapter 2 also lends credence to the hypothesis of a "permeability factor" that causes podocyte injury, ultimately leading to proteinuria. We demonstrate that anti-TCR autoantibodies induce IL-8 secretion from "thymocyte-like" T cells. While we cannot confirm that IL-8 alone induces podocyte injury, it remains possible that it is a contributing factor. IL-8 has already been shown to alter focal adhesion kinases and heparan-sulfate interactions[107,108]. Both of these are important in maintaining podocyte architecture and function.

Altering the paradigm of autoimmunity and autoimmune disease

Historically, antibody-mediated autoimmune diseases manifest with autoantibodies that target the organ directly involved in the disease. Goodpasture disease or anti-glomerular basement membrane disease patients have autoantibodies reactive to a portion of collagen IV and immunoglobulin deposition in the basement membrane is visible on biopsy[109]. Similarly, patients

with type 1 diabetes mellitus have autoantibodies targeting pancreatic islet cells[110]. However, autoantibodies present in ANCA disease did not follow the historical autoantibody precedent. Anti-neutrophil cytoplasmic autoantibodies target either MPO or PR3, cause neutrophil activation and degranulation, which in turn leads to damaged endothelium manifesting as vasculitis. This "indirect" autoimmunity involves a step-wise process utilizing an intermediary circulating cell to ultimately cause disease.

The discovery of a similar autoimmune phenomenon in MCD alters the paradigm of conventional autoimmunity and autoimmune disease. Minimal change disease should also be considered an "indirect" autoimmune disease as patients form autoantibodies which recognize a circulating cell (thymocyte-like T cells) which are activated and in turn produce a factor injurious to podocytes ultimately causing the clinical presentation of MCD. With now at least two autoimmune diseases whose pathogenesis relies on an indirect autoimmune cascade, the scientific field must consider the potential for other autoimmune diseases to be governed by "indirect" autoimmunity; especially those diseases for which no autoantibody has been found to date.

Potential for improvement on current therapies

Drug therapy for ANCA disease was historically focused on non-specific immunosuppressants such as glucocorticoids and Azathioprine. Recently, the use of Rituximab has gained favor for use in ANCA disease[111]. However, the role of T cells in both disease progression and drug therapy are not often considered in ANCA disease. Recently recognized are the effects of Rituximab on T cells in other autoimmune diseases, particularly RA. Currently controversial is a small population of CD3+ T cells which co-express CD20 and have a pro-inflammatory phenotype. These CD20+ T cells are depleted by Rituximab and may play a role in achieving disease remission[78]. To date, these CD20+ T cells have not been investigated in ANCA disease, but it would be interesting if these cells are also $CD25^{int}$ and how various medication regimens may affect their function. Additionally, in RA is has been shown that Rituximab

decreases Th17 responses but does not affect other T cell subsets[77]. If these same findings are consistent in ANCA disease is unclear, but should be investigated.

Minimal change disease and a subset of FSGS patients can therapeutically benefit from the findings in Chapter 2. The current standard of care for minimal change disease is glucocorticoids. A large percentage of patients respond well to glucocorticoids, but there remain a subset of patients who eventually become steroid-resistant. These difficult to treat patients are usually tried on other medications such as cyclosporine. Both glucocorticoids and cyclosporine affect T cells and therefore affect the autoantigenic target. Historically, B cells and antibodies have not been thought to be part of the disease process of MCD and therefore B cell related therapies were not used until recent years. With the current knowledge that B cells and autoantibodies are indeed a part of the immunopathogenesis of MCD, Rituximab, a B cell depleting monoclonal antibody, could have efficacy in patients. In particular, steroid-resistant MCD patients could benefit the most from medications targeting the B cell arm of immunity.

Focal segmental glomerulosclerosis presents as a conundrum to clinicians as the diagnosis can be a "catch-all." Additionally, FSGS can be further subdivided into collapsing, tip lesion variants, 'not otherwise specified,' all of which have varying etiologies and disease outcomes. Data presented in Chapter 2 have could help to better treat different sub-categories of patients with FSGS. The patient population with biopsy-proven FSGS and a positive titer of anti-TCR autoantibodies is enriched for those patients with either tip lesion FSGS or FSGS that is clinically reminiscent of MCD. This presents two clinical possibilities to be addressed in the future, 1) could some FSGS patients be spared a kidney biopsy if they have a positive anti-TCR titer? and 2) those FSGS patients that clinically "act" more like MCD could have a more tailored medication regimen.

Future directions

The data presented in the previous chapters certainly provide groundwork for future studies in ANCA disease, minimal change disease and application to other autoimmune diseases. With the discovery of exon 2-deficient FOXP3 and its prevalent usage in ANCA disease patients, the actual functional implications for this FOXP3 isoform should be investigated. Prior *in vitro* studies hint at the effects of exon 2-deficiency. Studies suggest that without the full repressor domain, certain known (RORγt, RORα, NFAT) and unknown proteins are not sequestered[15,74,79]. However, these studies do not directly address the impact on Tregs in ANCA disease patients. In conjunction with these studies, the potential "fluctuation" between full-length and exon 2-deficient FOXP3 over time is unknown. Can a cell in essence be "reprogrammed" to express full-length FOXP3? A potential therapy to this effect would be the use of rapamycin. Rapamycin has been used in the setting of transplant to inhibit the activation of T and B cells by blocking cellular response to IL-2 through the mTOR pathway. Interestingly, rapamycin has an altered effect on regulatory T cells. Instead of inhibiting cellular proliferation (as in the case of effector cells) rapamycin has been shown to expand the regulatory T cell compartment[112]. A subsequent study demonstrated that in a heterogeneous mixture of FOXP3+ and exon 2-deficient FOXP3+ regulatory T cells subjected to *in vitro* rapamycin, only FOXP3+ regulatory T cells expanded[113]. Thus, it is possible that supplementation with rapamycin could improve the suppressive function of regulatory T cells which could be due to, in part, not allowing exon 2-deficient FOXP3 Tregs to survive.

The impressive frequency of $CD25^{int}$ T cells in ANCA disease combined with CD45RO positivity suggests that autoreactive T cells are likely represented in that population. A proposed experiment to test this theory is the use of MHC class II tetramers for flow cytometric staining of peripheral T cell populations. Until this past year, this experiment was virtually impossible as an investigator must know the HLA of the patient and the peptide sequence of the autoantigen to be loaded into the tetramer. Our research group recently discovered a predominant HLA allele

(DRB1*15) in our ANCA disease patient cohort[114]. While the ANCA disease field has known that patients have seroreactivity to MPO or PR3, the exact peptide sequences of MPO reactivity remained an enigma. Simultaneous, independent studies in mice and humans revealed an epitope of MPO for which reactive antibodies conferred pathogenicity[115] (and Roth et al, JCI 2013, in press). The combined knowledge of predominant HLA and critical peptide sequences of MPO provide an opportunity for tetramer studies in human T cells.

During T cell education in the thymus, thymocytes with strongly autoreactive TCRs are diverted to either apoptosis or become FOXP3+ regulatory T cells[116,117]. Therefore, in the periphery, T cells with high affinity for self proteins are pre-programmed to act in a suppressive, anti-inflammatory manner. In the normal, healthy individual, this arrangement is in place to prevent autoimmune disease from developing. However, under stress conditions such as lymphopenia, infection or inflammation, Tregs have the potential to differentiate into a more effector-like T cell[48,49,118]. The plasticity of regulatory T cells sets up the potential to have high-affinity autoreactive cells now functioning as a pro-inflammatory effector cell. As demonstrated in Chapter 1, patients with systemic autoimmune diseases exhibit an increased frequency of $CD25^{int}$ T cells that are pro-inflammatory and antigen-experienced. These cells have some phenotypic characteristics reminiscent of regulatory T cells. Could the expanded $CD25^{int}$ population be a function of "de-differentiated" regulatory T cells in ANCA disease? Future studies could address this question by sorting $CD25^{int}$ and Treg populations from patients and subsequent TCR sequencing. Fine TCR sequencing of human T cells to reveal T cell subsets has previously been done with success[119]. If clonality existed between the $CD25^{int}$ and Treg populations, but not the $CD25^{neg}$ population, one could postulate that $CD25^{int}$ T cells likely came from a Treg precursor.

A coincidental finding during the initial characterization of the $CD25^{int}$ population in ANCA disease was also finding an increased frequency of these T cells in SLE and RA., but not type 1 diabetes mellitus. We provide two hypotheses to explain this finding: 1) $CD25^{int}$

population expansion occurs in systemic autoimmune diseases, but not organ-specific autoimmune diseases or 2) the expansion occurs in autoimmune diseases that exhibit cycles of relapse and remission but not in autoimmune diseases that have essentially "burned out." To further dissect this phenomenon, studies would need to address the frequency of the $CD25^{int}$ population in several other autoimmune diseases, systemic versus organ-specific alongside diseases with different clinical courses.

Another incidental finding was that patients who received a kidney transplant greater than two years prior to sample, had no clinical suggestion of rejection and their initial kidney disease was not autoimmune in nature also exhibited an increased frequency of $CD25^{int}$ T cells compared to healthy individuals. Vallotton et al. noted the increase of a similar T cell population in the periphery of kidney transplant patients[120]. These activated effector T cells were CD4+ $CD25^{high}$ IL-7Rα^{high} ($CD127^{high}$) and CD45RO+ which share a number of phenotypic characteristics as our $CD25^{int}$ population. The most intriguing finding from the Vallotton study was serial measurements of the T cell populations before transplantation and 3, 6 and 12 months post-transplant. Prior to transplantation, the activated effector T cell population was comparable between healthy controls and future transplant recipients. However, three months post-transplantation, the activated effector T cell population had increased 200-300% over the frequency seen in healthy individuals. The combination of our data and the Vallotton data leads us to hypothesize that the $CD25^{int}$ T cell population expands upon chronic exposure to auto- or alloantigen.

Another preliminary finding from our studies suggest that while the frequency of the $CD25^{int}$ T cell population is equivocal between stable kidney transplant patients and those who are actively rejecting their graft, the function of the $CD25^{int}$ population is vastly different. Our initial studies in ANCA disease revealed that $CD25^{int}$ proliferate rapidly and at a rate that exceeds the proliferation of $CD25^{neg}$ T cells. However, purified $CD25^{int}$ T cells from stable kidney transplant patients did not proliferate despite four day stimulation with anti-CD3/anti-CD28.

Therefore, preliminary evidence suggests that despite the expansion of the $CD25^{int}$ population, these T cells are anergic in a stable setting of transplant. Acquisition of $CD25^{int}$ T cells from a transplant recipient with active rejection revealed that the T cell population now proliferated and were reminiscent of the proliferation demonstrated in ANCA disease patients. Further studies should endeavor to determine the "stop-gap" mechanism of $CD25^{int}$ T cells in stable transplant patients. This could potentially serve as a biomarker for transplant patients who are more prone to rejection.

Our studies in MCD have unveiled a type of T cell unknown to the literature. Preliminary characterization of these "thymocyte-like" T cells leaves many questions unanswered and provokes future studies to better understand these cells. We know these cells are reminiscent of thymocytes by virtue of CD3+ and CD4+ CD8+ double positivity. However, it is unknown as to the exact stage of T cell development of the "thymocyte-like" cells. Future studies to elucidate their maturity and lineage would provide additional insight into the immunopathogenesis of MCD.

The presence of a circulating "permeability factor" has long been heralded as the key to podocyte injury in MCD. Such a factor has also remained elusive over the years. Our current data suggest that the anti-TCR autoantibody in MCD activates the target "thymocyte-like" T cells. We preliminarily know that IL-8 is produced as a result of this interaction. Future studies are directed to further investigate all factors produced during this interaction. Dissection of these factors will be needed to determine the critical factor or factors that ultimately cause podocyte damage leading to the manifestation of MCD.

The most intriguing aspect of the studies presented herein is the known production of autoantibodies reactive to circulating cells in two different autoimmune diseases. Historically, autoimmune diseases caused by antibodies are "direct" in the sense that the autoantibodies target the organ in which the disease manifests. Both ANCA disease and minimal change disease (and some FSGS) are types of "indirect" autoimmune diseases. This compels one to consider that

perhaps other autoimmune diseases which have no known autoantibody to date or perhaps the known clinical autoantibody does not correlate well disease are actually other examples of "indirect" autoimmunity. With this in mind, future studies should employ the epitope excision/mass spectrometry approach to investigate potential unknown autoantibodies in the circulation of other autoimmune diseases.

Conclusion

Circulating T cells comprise a large portion of adaptive immunity with numerous subsets, creating a system of checks and balances of inflammation and tolerance. However, these multiple T cell subsets can also be a source of dysfunction in autoimmune diseases. Be it changes in regulatory T cells or the effector T cells themselves, numerous perturbations of the T cell arm of adaptive immunity must be considered when investigating the source of dysfunction in autoimmune diseases. Of additional interest, there is possibility of immune cells themselves being autoantigenic targets, as in the case of ANCA disease and minimal change disease. In future studies of other autoimmune diseases, this "indirect" pathway of autoimmunity must be recognized.

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