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ANCA-associated Vasculitis Patients Have Defective Treg Function Exacerbated by Presence of a Suppression-Resistant Effector Population

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^{*,†}, and Maureen A Su[‡]

^{*}Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[†]UNC Kidney Center, Department of Medicine, Division of Nephrology and Hypertension, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[‡]Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract

Objective—The development of pathogenic anti-neutrophil cytoplasmic autoantibodies (ANCA) can result in systemic small vessel vasculitis. However, the breakdown in immune tolerance that results in the induction and persistence of ANCA 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.

Methods—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.

Results—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.

Conclusion—ANCA-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 [1], effector hyperactivation [2] and an imbalance of certain subsets [3]. 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 [4]. 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 [5]. 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 [1].

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 [6]. The production of isotype-switched autoantibodies in AAV suggests the involvement of CD4⁺ helper T cells in the autoimmune process [6]. 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 [7], or decreased [8, 9]. Additionally, Treg function in AAV patients has been reported as normal [10] and decreased [7–9]. 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 [2]. 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.

Disease activity was determined by the Birmingham Vasculitis Activity Score (BVAS). 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 [11, 12].

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 [13]. 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 routinely >95% for Tregs and CD25^{neg} and >85% for CD25^{int} with occasional CD25^{neg} contamination.

Responder T cells were labeled with 0.1 μ M CFSE (Invitrogen), washed twice and resuspended in RPMI, 10% FBS, pen/strep, HEPES. Cells were plated in 96-well plates precoated with immobilized anti-CD3 (5 μ g/mL) and anti-CD28 (1 μ g/mL) (BioLegend), as done previously [14]. 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 [2] wherein 50,000 CFSE-labeled 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 MAXTM Deluxe and Human IL-4 ELISA MAXTM Deluxe (BioLegend).

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⁺ [15, 16], 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 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 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 1B left panel). The Treg frequency was not significantly different between AAV patients in remission and healthy controls (data not shown). Absolute numbers of Tregs per mL of blood were calculated and were not statistically significant between AAV patients and healthy controls (data not shown). Additionally, there were no differences in Treg frequency when comparing MPO and PR3 reactive AAV patients (Figure 1B right panel). As some reports have demonstrated increased Treg frequencies with increase in patient age [17], we performed a linear regression analysis of Treg frequency and patient age (Figure 1C). 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 [15]. AAV patients used for functional studies were not exposed to cyclophosphamide in the preceeding six months as cyclophosphamide is known to inhibit Treg function [18]. As expected, healthy Tregs suppressed >50% of effector proliferation at a 1:1 ratio (Figure 1D, diamonds; 1E left). In contrast, Tregs from AAV suppressed only 20% of effector proliferation on average at a 1:1 ratio (Figure 1D, squares; 1E, 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 1D, triangles; 1E, 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 [7–9].

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 [19]. Exon 2 lies within a repressor domain important in binding pro-inflammatory transcription factors, such as ROR γ t and ROR γ , which drive Th17 induction (Figure 2A, top) [20, 21]. Lack of this domain would be predicted to prevent repression of these Th17 transcription factors and promote Th17 lineage differentiation [21]. 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 [13]. 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 fulllength FOXP3 (Figure 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 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 2B left). Strikingly, exon 2-deficient FOXP3 is associated with both active and remitting disease (Figure 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 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 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 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.

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

We used a recently described gating strategy [15, 16] to stratify CD3⁺ CD4⁺ T cells with CD127 and CD25 (Fig 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 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 3A, bottom panels). The frequency of the CD25^{int} population in AAV patients was significantly increased ($p<0.0001$) compared to healthy controls (Figure 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 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 $52.1 \pm 17.1\%$) and RA (data not shown; mean $48.3 \pm 9.5\%$), but not in T1DM (mean $28.2 \pm 12.4\%$) (Figure 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 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 (data not shown). 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 (data not shown).

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

CD25 expression is associated with both effector T cell activation and Tregs [22]. 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 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 1D). 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 4A, 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 4A, closed diamond). In contrast, sorted CD25^{int} T cells from AAV patients were unable to suppress CD25^{neg} effector proliferation (Figure 4A, 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 (data not shown). 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} GI TR⁺ T cells [23], 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 4B) 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 4B, right). CD25^{int} cells from healthy controls are thus resistant to Treg suppression. Tregs were unable to suppress CD25^{int} T cell proliferation (Figure 4B) 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 1D). 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 4B). 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.

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, which corroborates our hypothesis that these cells act predominantly as effector cells (Figure 5A, left). IL-4 was never detected in the supernatants of stimulated CD25^{neg} T cells from healthy controls or AAV patients (Figure 5A, 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 5A, 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 5B). 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 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 5C and 5D). However, the CD25^{int} population preferentially expressed CD45RO⁺ (Figures 5C and 5D). CD45RO expression suggests that the CD25^{int} population is enriched for activated or memory T cells.

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 [7–9]. 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) [7, 8]. 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 [8]. 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 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} [15, 16].

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, 42% 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 [24, 25]. 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 AAV 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 [13]. 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 ROR γ and NFAT [21, 26]. We hypothesize that in the absence of exon 2, ROR γ 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 [13]. 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 tissueinfiltrating pathogenic T cell in RA [27]. As such, 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 (data not shown), which have been associated with resistance to Treg suppression [3, 28]. CD25^{int} T cells also produce robust amounts of IL-17A which has been described to be elevated in AAV patients [29, 30]. The data presented herein demonstrates 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 [31]. 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.

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Figure 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) Linear regression analysis of Treg frequency and age of AAV patients. (D) 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). (E) 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.

Figure 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.

Figure 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 in active AAV patients or AAV patients in remission. n.s., not significant. Active, BVAS>0; Remission, BVAS=0.

Figure 4. $CD25^{int}$ 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 5.

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). Flow sorted T cell populations were stimulated with anti-CD3, -28 for 4 days. 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. (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).

Table 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	21 36	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
Mycophenolatemofetil	33.9%	NA	50 %	NA
Glucocorticoid	43.5%	NA	12 .5%	NA
Azathioprine	17.7%	NA	12 .5%	NA
Rituxi mab	41.9%	NA	37 .5%	NA
TOTAL	62	19	8	6

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

** Immunosuppressive medication