CHARACTERIZATION OF REGULATORY B CELLS IN PATIENTS WITH ANTI-NEUTROPHIL CYTOPLASMIC AUTOANTIBODY VASCULITIS

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ABSTRACT

Lydia Tatiana Aybar: Characterization of Regulatory B cells in Patients with Anti-Neutrophil Cytoplasmic Autoantibody Vasculitis
(Under the direction of Ronald J. Falk)

ANCA-associated vasculitis (AAV) is B cell dependent; however, which B cell subsets modulate immunopathogenesis remains unknown. Although their phenotype is controversial, regulatory B cells (Bregs) play a critical role in immunological tolerance via secretion of IL-10. In this study, we investigate three B cell phenotypes reported to produce IL-10: $CD5^+$, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cells in patients with AAV. We further analyzed the $CD5^+$ subsets of the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cell populations.

In our first investigation, we discovered a lower percentage of $CD5^+$ B cells in patients with active AAV, whereas the percentage of $CD5^+$ B cells from patients in remission was indistinguishable from healthy controls (HCs). After rituximab, median time to relapse was 31 months in patients maintaining a normalized percentage of $CD5^+$ B cells with or without maintenance immunosuppression. Among patients whose B cells repopulated with a low or sharply declining percentage of CD5, those who were on low or no maintenance immunosuppression relapsed sooner (median 17 months) than patients who were maintained on high levels of oral maintenance immunosuppression (29 months; $p=0.002$).

The second phase of our investigation identified that the $CD5^+$ subset of $CD24^{hi}CD38^{hi}$ B cells was decreased in patients with active disease relative to patients in remission ($p\leq0.001$) and HCs ($p\leq0.0001$). B cells from patients with active disease produced less IL-10 than those from patients in remission ($p=0.005$) and HCs ($p=0.001$). As $IL\text{-}10^+$, $CD5^+CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD38^{hi}$ B cells increased in disease remission within an individual, ANCA titers decreased. The $CD5^+$ subset of $CD24^{hi}CD38^{hi}$ B cells decreased in active disease and

rebounded during remission similarly to IL-10-producing B cells, suggesting that CD5 may identify functional IL-10-producing Bregs. These data indicate that Bregs malfunction during active disease due to reduced IL-10 expression, permitting ANCA production.

$CD5^+$ B cells, specifically the $CD5^+$ subset of $CD24^{hi}CD38^{hi}$, may be useful indicators of disease activity, remission, and future relapse. These Breg subsets may be used to guide remission maintenance therapy after rituximab treatment to balance immunological B cell tolerance, maintain beneficial B cells, reduce pathogenic ANCA production, and sustain remission with minimal therapeutic interventions.

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AAV	ANCA Associated Vasculitis
ACI	Adoptive Cellular Immunotherapy
ANCA	Anti-Neutrophil Cytoplasmic Autoantibody
AZA	Azathioprine
B10	IL-10 producing B cell
B10pro	B10 Progenitor
BCR	B cell Receptor
BM	Bone Marrow
Breg	Regulatory B cell
BVAS	Birmingham Vasculitis Activity Score
CD40	Cluster of Differentiation 40
CD40L	CD40 Ligand
CIA	Collagen-Induced Arthritis
CHS	Contact Hypersensitivity
CpG	Cytosine-phosphate-Guanosine
CYC	Cyclophosphamide
EAE	Experimental Autoimmune Encephalitis
FasL	Fas Ligand
GPA	Granulomatosis with polyangiitis
HC	Healthy Control
HEL	Hen Egg Lysozyme
HLA	Human Leukocyte Antigen
IBD	Inflammatory Bowel Disease
IL-10	Interleukin-10
IFN-γ	Interferon gamma

ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
iTreg	Induced Regulatory T cell
LPS	Lipopolysaccharide
L-TROT	Long-Term Remission Off Therapy
mAb	Monoclonal Antibody
MHCII	Major Histocompatibility Complex II
MMF	Mycophenolate Mofetil
MPA	Microscopic Polyangiitis
MPO	Myeloperoxidase
MS	Multiple Sclerosis
NK	Natural Killer
nTreg	Natural Regulatory T cell
ODN	Oligodeoxynucleotide
PBMC	Peripheral Blood Mononuclear Cell
PIM	PMA, Ionomycin and Monensin
PMA	Phorbol 12-Myristate 13-Acetate
PR3	Proteinase 3
PTPN22	Protein Tyrosine Phosphatase Non-receptor type 22
RA	Rheumatoid Arthritis
RAVE	Rituximab in ANCA-Associated Vasculitis
SLE	Systemic Lupus Erythematosus
TCRα	T cell Receptor Alpha
T1D	Type 1 Diabetes
T2-MZP	Type 2 Marginal Zone Progenitor
TNF-α	Tumor Necrosis Factor alpha
Treg	Regulatory T cell

CHAPTER 1: B CELL IMMUNOLOGY AND REGULATION

Introduction

In the immune system, B cells dynamically participate in the innate, adaptive, cellular, and humoral immune responses that protect the body from infection. B cells play a diverse range of roles in a healthy individual; they can present antigen to T cells, develop into memory B cells, initiate secondary immune responses, differentiate into plasma cells that secrete protective antibodies, and many more (1). To provide protection against infection without causing damage to the host, they must maintain the ability to distinguish foreign antigens from self antigens. Many B cell subsets exist, with different subsets performing effector and/or regulatory functions which can be accomplished through either antibody-dependent or antibody-independent mechanisms (1). An important function of B cells is the maintenance of immunological homeostasis through secretion of anti-inflammatory cytokines, including the potent and pleiotropic cytokine Interleukin-10 (IL-10) (2-5). A specific subset of B cells, called regulatory B cells (Bregs) after their T cell counterparts, have been shown to inhibit inflammation, autoimmunity, and innate and adaptive immune responses through the production of IL-10 (3, 6, 7).

B cells have unique antigen-specific B cell receptors (BCRs). The BCR is a form of membrane-bound immunoglobulin that can be secreted once the B cell has differentiated into a plasma cell, and can then target antigenic epitopes (8). The expression of this antigen-specific BCR distinguishes B cells phenotypically and functionally from other leukocytes. Upon binding of a cognate antigen to its BCR, the B cell is activated and clonally expands to become either an effector B cell that may further differentiate in to an antibody-secreting plasma cell (9), or a memory B cell (10). With each subsequent encounter of antigen, the immune response becomes more rapid and efficacious through an elaborate process of immunoglobulin class

switching and affinity maturation. Secreted antibodies are a soluble form of the BCR that can then neutralize viruses and bacteria, demonstrating the important role of B cells in infection and clearance of non-self antigens (11). Autoantibodies are destructive antibodies that are specific for self-antigens and may incite an immune response against self (12). If this process is not regulated, B cell immunopathogenicity can occur, which results in production of harmful autoantibodies, immune complexes, inflammatory cytokine secretion, ectopic neolymphogenesis, as well as dendritic cell and T cell activation (13).

Self-tolerance is maintained by removing autoreactive B cells that would produce such potentially harmful antibodies at two checkpoints (14). Normally, B cells expressing an IgM molecule that recognizes a self-antigen are eliminated in the bone marrow (BM), the first checkpoint (central tolerance) (15). Here, >90% of high-affinity self-reactive and polyreactive immature B cells are removed by deletion (16), anergy (17), or receptor editing(18).

Low-affinity self-reactive B cells can escape central tolerance and enter the blood and tissues, where they must pass a second checkpoint by complex and non-redundant peripheral tolerance mechanisms before the B cells mature into naive immunocompetent cells (19). Tolerance strategies at the second checkpoint in the periphery are not completely understood, but involve negative regulation by T cells via interaction of the T cell receptor, CD40 ligand (CD40L) and Fas ligand (FasL) with Major Histocompatibility Complex II (MHCII), CD40 and Fas respectively on B cells (20, 21). An additional checkpoint at the plasmablast to plasma cell transition has also been described in mice (22). Failure to remove auto-reactive B cells at either stage may result in circulating self-reactive B cells in the periphery. When auto-reactive B cells escape tolerance checkpoints and are activated by their cognate self-antigen, they may differentiate into autoantibody-secreting plasma cells, thereby increasing susceptibility to autoimmunity (23).

Regulatory cells like regulatory T cells (Treg) and Bregs can contribute to peripheral tolerance through provision of IL-10 (2, 24). In several human autoimmune diseases, regulatory

cells are diminished in frequency or are dysfunctional (3, 25, 26). Tregs are present but lack suppressive ability in type 1 diabetes (T1D) (27, 28), systemic lupus erythematosus (SLE) (29), rheumatoid arthritis (RA) (30), multiple sclerosis (MS) (31), and AAV (32). Breg abnormalities have been reported in human autoimmune diseases such as SLE (3) and MS (33, 34), where they are phenotypically present but are unable to produce IL-10. Subsets of B cells isolated from patients with RA are unable to perform regulatory tasks and may promote disease (35). B cells from these patients are incapable of inducing functionally suppressive Tregs in order to prevent TH17 cell development (25). Patients with RA appear to have normal levels of $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$, and $CD5^{+}$ B cells; however, levels of B10 (B cells that are actively secreting IL-10) cells are lower in RA patients than in controls, particularly in patients with ≤ 5 years disease duration. Further, B10 cells inversely correlate with rheumatoid factor. $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cells from patients with RA are also unable to induce Tregs (compared to $CD24^{lo}$ B cells) (35). In SLE, a $CD24^{hi}CD38^{hi}$ Breg phenotype is reported to be in circulation, but it is unable to secrete IL-10 (3). More impressively, elimination of Bregs exacerbates inflammation and autoimmune disease in mouse models dependent on their production of IL-10 (36-41)

Regulatory B cells, like other regulatory cells such as Type 1 regulatory T cells (Tr1) (42, 43) exert suppressive effects through the secretion of IL-10, a cytokine that can drive a change in immunological response from T-Helper 1 (Th1) (44) to T-Helper 2 (Th2) (45). Fiorentino *et al* demonstrate that when murine splenic and peritoneal antigen presenting cells (APC) are incubated with IL-10 and co-cultured with TH1 clones, their ability to synthesize IFNγ is impaired (46). IL-10 is a protective agent in a spectrum of chronic inflammatory diseases (47, 48). A lack of B cell-derived IL-10 is common to several relapsing and remitting inflammatory autoimmune diseases characterized by pathogenic B cells like MS (49) and SLE (3).

B cell transfer in the experimental autoimmune encephalitis (EAE) mouse model of human inflammatory autoimmune disease and MS results in an IL-10-dependent increase in

Treg Foxp3 expression (50). IL-10 secreting B cells are essential for recovery in arthritis and EAE mice (28, 29). Interestingly, IL-10 expression protects from metabolic syndrome and T1D (51). Several groups, including ours, have begun to take great scientific and clinical interest in examining the impact of Breg phenotypes and their ability to secrete IL-10 in AAV (26, 52-54).

Regulatory B Cells

Immunoregulation and maintenance of peripheral tolerance is an important research topic in immunology and human autoimmune disease. Although we know Bregs are important and have a significant role in autoimmunity, the underlying mechanisms have not been well established, particularly in humans. Bregs are deficient or dysfunctional in several autoimmune diseases including SLE, RA and AAV, as discussed previously. Bregs from healthy individuals maintain immunological homeostasis through effects on a variety of other leukocytes by suppression of $CD4^+$ T cell proliferation. They suppress naïve and memory T cell pro-inflammatory cytokine production thus balancing TH1/TH2 responses, as well as neutrophil, natural killer (NK) cell, and effector T cell activation. Bregs can suppress monocytic inflammatory cytokine production (55) and promote iTreg ($CD4^+CD25^+CD127^{lo}$ Tregs) differentiation. In mice, Bregs suppress auto-antibody production. Flores-Borja *et al* showed that Bregs can prevent naive T cell differentiation into TH1 and TH17 cells (25). As previously discussed, these Breg functions require B cell-derived IL-10.

<u>IL-10 is Required for Regulatory B Cell Suppressive Functions</u>

IL-10 is an anti-inflammatory cytokine produced mainly by monocytes and lymphocytes. This cytokine has pleiotropic effects on immunoregulation and inflammation. IL-10 suppresses inflammation by multiple mechanisms, including down regulation of proinflammatory cytokines such as IFN-γ, IL-17, IL-3, TNFα and GM-CSF. IL-10 also down regulates the expression of Th1 cytokines, MHC II antigens (56), and co-stimulatory molecules and thus decreases antigen presentation. IL-10 production is crucial in the maintenance of Foxp3 expression for Treg function. While IL-10 is required for Breg suppressive functions (3, 7), it is also important to B

cells as a growth factor that promotes B cell survival, proliferation and maturation to antibody- (or autoantibody-) secreting plasma cells (57).

In several relapsing and remitting inflammatory autoimmune diseases characterized by pathogenic B cells, a common problem is lack of B cell-derived IL-10. For example, in patients with SLE $CD24^{hi}CD38^{hi}$ Bregs are present but deficient in IL-10 production and do not suppress inflammatory cytokine secretion as seen in healthy controls (3). Patients with MS have a reduced percentage of IL-10-producing B cells (33). An IL-10 dependent increase in Foxp3 expression, a Treg marker, has been shown in the central nervous system after B cell transfer in the EAE mouse model. IL-10 secreting B cells are essential for recovery in arthritis and EAE murine models of human inflammatory autoimmunity and MS (50). Human genetic research demonstrates that elevated IL-10 protects individuals from metabolic syndrome and diabetes mellitus (51). These data support IL-10 as an important mediator for Breg function and the notion IL-10 dysregulation may lead to autoimmune disease.

In 2009, Hruskova and colleagues showed that patients with AAV in remission who relapsed produced significantly less circulating IL-10 than those without relapse (58). Patients with AAV have an increased frequency of the IL-10 -1082AA genotype that is associated with decreased IL-10 production (59).

Regulatory B Cell Origin and Phenotype

Multiple phenotypes of Bregs have been reported. Each may develop by different means or they all may derive from a common progenitor. Lack of consensus about Breg phenotype and function has led to multiple hypotheses for the ontogeny of Bregs. One hypothesis is that all Breg subsets have a common progenitor that differentiates into various subtypes of Bregs after exposure to environmental factors (60). A second is that they develop from toll-like receptor (TLR) engagement of naive follicular B cells and then are amplified via stimulation of CD40 and the BCR (61). Mauri and collaborators rationalize that multiple markers corresponding to those used to identify Type 2 marginal zone progenitor (T2-MZP) B cells recur amongst published

Breg subsets, and therefore Bregs may derive from a common T2-MZP B cell progenitor (60). Further support for Mauri's hypothesis is that T2-MZP B cells can be autoantigen-specific and immature, and can respond quickly to environmental stimuli (62). Primary activation of a potentially autoreactive T2-MZP B cell by TLR stimulation induces IL-10 is secretion.

Are Regulatory B Cells Antigen-Specific?

BCR signaling is important for Breg IL-10 secretion; thus, IL-10 production may result from antigen-specific BCR activation. Mice lacking CD19, a BCR co-receptor, have fewer IL-10$^+$ B cells (11) whereas hCD19Tg mice, which have increased CD19 expression and therefore amplified BCR signaling, have more IL-10$^+$ B cells than wild-type mice (63). Anti-IgM stimulates the BCR, mimicking antigen stimulation, and causes Breg expansion *in vitro*. One reported phenotype of human Bregs includes CD27, a memory cell marker, suggesting that they are antigen-experienced (12). In adoptive transfer experiments, B10 cells isolated from antigen-experienced mice suppress inflammation or disease more so than those from naive mice. Lastly, murine studies demonstrate B10 cells can differentiate into plasma cells that secrete IgM and IgG and are even enriched for autoantigen specific Ig compared to IgM generated from non-B10 cells (64). These data suggest that BCR drives IL-10 competence upon B cell antigen binding and that Bregs work in an antigen-specific manner.

How Are Regulatory B Cells Produced *ex vivo*?

B10 and B10pro are terms used to identify B cells that are actively synthesizing IL-10 (B10) and those that are capable of Breg differentiation (B10pro). Circulating B10 cells are rare in mice and humans comprising less than 1% of the B cell repertoire. In humans, there is no consensus regarding a surface marker to identify cells capable of secreting IL-10.

To evaluate B10 cells, cells must be cultured for 5 hours with phorbol 12-myristate 13-acetate (PMA), ionomycin and a golgi inhibitor, such as monensin (PIM) to intracellularly sequester synthesized IL-10. They are then analyzed by flow cytometry to identify IL-10-

producing B cells. This process destroys cellular viability rendering B10 cells useless for mechanistic studies.

Multiple stimulants to induce B10pro cells are published giving a variety of ways to study them. To induce B cell IL-10 production, culture systems have included various concentrations of CD40L, agonistic CD40 mAb, LPS, alum, anti-IgM, cytosine-phosphate-guanosine (CpG) oligodeoxynucleotide (ODN) (a TLR9 agonist), PMA, ionomycin, monensin and brefeldin A. Since many research groups' culture systems are unique, comparisons of the resulting Breg data becomes complicated thereby adding confusion to our understanding of Breg phenotype and function.

Two seminal papers in the human Breg field were published in 2010 (3) and 2011(7). Tedder *et al.* have performed a substantial research of human and murine B10 and B10pro cells. This culture system includes enriched B cells, LPS, CpG DNA for 2 days and a final addition of PIM in the final 5 hours of culture. This line of research elucidated that T cell signals and factors may be important for Breg function because mice with B cells deficient in MHC class II or the IL-21 receptor are incapable of IL-10-dependent suppression of autoimmunity (65). Furthermore, they identified the cell surface phenotype of IL-10^{+} B cells as being $CD24^{hi}CD27^{+}$. Mauri and colleagues have also considerably contributed to the Breg literature. Their culture system includes enriched B cells, transgenic murine epithelial cells that express human CD40L, CpG DNA for 2 days and a final addition of PIM in the final 5 hours of culture. Mauri's group showed that Il-10^{+} B cells are enriched in the $CD24^{hi}CD38^{hi}$ B cell population (3).

As mentioned previously, there is no unique marker(s) or transcription factor(s) that exclusively identifies human $IL10^{+}$ Bregs without rendering them inert in mechanistic studies. Most phenotypes overlap with overarching marker sets like those ascribed to T2-MZP B cell ($CD19^{+}CD2^{hi}CD23^{hi}CD24^{hi}IgD^{hi}IgM^{hi}CD1d^{hi}$) phenotypes and have been studied in murine splenocytes, which is an organ difficult to harvest in humans.

Murine Regulatory B Cells

In mice, B10 and B10pro cells are enriched within the $CD1d^{hi}CD5^{+}$ B cell subset and develop an inhibitory function that suppresses IFN-γ and TNF-α expression in T cells (6), neutrophils and natural killer (NK) cells. Murine splenic B10 cells are $IgM^{hi}IgD^{lo}CD19^{hi}$MHC-$II^{hi}CD21^{int/high}CD23^{lo}CD24^{hi}CD43^{+/-}CD93^{-}$ (63). They reside primarily in the spleen (66), and can also be found in gut-associated lymphoid tissues, peripheral blood and lymph nodes (66, 67). A number of studies in mouse models of autoimmunity have shown that IL-10-producing B cells were capable of suppressing disease development. Murine B10 cells are integral for regulating autoimmune diseases in mouse models of EAE (41), inflammatory bowel disease (IBD) (68), contact hypersensitivity (CHS) (69), collagen-induced arthritis (CIA) (37) and SLE (70). Elimination of $IL\text{-}10^{+}$ Bregs intensifies inflammation and autoimmune disease manifestations in diverse mouse models (36-41, 69, 71). In mice, $CD5^{+}CD1d^{hi}$ B cells secrete IL-10 after 48 h of *in vitro* stimulation with either agonistic CD40 mAb or LPS (17). TLR stimulation is necessary for maturation and expansion of B10 cells (66) as evidenced by TLR9-deficient MRL/*lpr* mice that develop an exacerbated SLE-like disease (72). Administration of agonistic TLR9 stimulators suppresses the course of diabetes (73) and arthritis in mice (74). Other Bregs that have been phenotypically identified in murine models are reported to be $TIM1^{+}$ (75), $FASL^{+}$ (76) and $CD19^{hi}Fc\gamma RIIb^{+}$ (77).

Human Regulatory B Cells

IL-10-producing Bregs and their possible contribution to autoimmune responses have also been studied in humans. Several cell surface phenotypes to identify IL-10-producing Bregs in humans have been described, including $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$ and $CD25^{+}$ (3, 7, 52). Incidentally, the majority of Breg phenotypes studied in humans and mice are also $CD5^{+}$. As mentioned earlier, dysregulated frequencies and/or defective function of Bregs have been identified in MS, SLE and RA. Overall, current evidence indicates that Bregs play an important

immunoregulatory role and suggests that alterations in either number or function of Bregs contribute to pathogenic immune responses in autoimmune diseases.

Important Regulatory B Cell Subsets in Humans

Iwata *et al.* show that in humans the majority of, *ex vivo* B10 and B10pro cells reside within the $CD24^{hi}CD27^{+}$ B cell subpopulation and that this subset using IL-10 can negatively regulate monocyte inflammatory cytokine production *in vitro*. This subset was enumerated in patients with several autoimmune diseases finding that circulating B10 cells were present and in some cases expanded in 91 patients with RA, SLE, primary Sjögren syndrome, autoimmune vesiculobullous skin disease, or MS (7). On B cells, CD24 (heat-stable antigen) is expressed at high levels on B cell progenitors and mature resting B cells. Blocking CD24 interaction with T cells inhibits T cell proliferation demonstrating that APC CD24 expression provides costimulation for CD28-independent proliferation of CD4 and CD8 T cells (78). CD27 is a marker for memory B cells, which suggests that they are potentially class-switched and antigen-experienced. CD27 binds to CD70 and induces T-cell co-stimulation and B cell activation. Both markers support the Breg need for T cell engagement.

Mauri's group demonstrated that human B cells with the transitional markers $CD24^{hi}CD38^{hi}$ were able to secrete IL-10. CD38 is a B cell activation marker that monitors intracellular calcium levels. In Breg/T cell co-culture studies this $CD24^{hi}CD38^{hi}$ B cell subset was able to suppress T cell IFN-γ and TNF-α secretion. Lastly they discovered that B cell IL-10 is decreased due to a delay of STAT3 phosphorylation (3). Importantly, this subset was present but unable to produce IL-10 in patients with SLE. Flores-Borja *et al.* studied $CD24^{hi}CD38^{hi}$ Bregs in RA and found that patients with active RA had lower numbers of $CD24^{hi}CD38^{hi}$ Bregs in circulation and were insufficient to induce functionally suppressive Treg cells to inhibit TH17 differentiantion (25).

Since these discoveries, several groups have documented these two phenotypic subsets, $CD24^{hi}CD38^{hi}$ or $CD24^{hi}CD27^{+}$, in several autoimmune diseases including AAV.

Generally, they report both Breg phenotypes are present in patients with AAV and harbor IL-10-competent B cells. However, the ability of B cells to produce IL-10 and their capacity to suppress activation of other immune cells were not compromised in patients in clinical remission, implying that Bregs are functional in AAV patients with quiescent disease (26, 54). Data adding to the understanding of Breg phenotypes and B10pro cells in AAV are reported in chapters two and three. These reports will be analyzed in contrast to other Breg/AAV research in detail in the discussion.

CD5

CD5 is one of the first cell surface markers discovered to identify B10 cells in mice defined as $CD5^{+}CD1d^{hi}$ thereby cementing CD5 as an important molecule in the Breg literature. CD5 is a scavenger-like lymphocyte receptor (also named T1, Tp67 in humans or Lyt-1 in mouse) that associates with the antigen specific receptor complexes on B and T cells to regulate signal transduction (79). CD5 is constitutively expressed on T cells and at higher levels on both natural Tregs (nTreg) (80) and induced Tregs (iTregs) (81). CD5 mutes B cell signaling and maintains immune tolerance via anergy (79, 82, 83). Transgenic $CD5^{-/-}$ mice that have a BCR specific for hen egg lysozyme (HEL) and membrane-bound self-antigen (HEL) mice have elevated levels of anti-HEL IgM, enhanced proliferative responses *in vitro* and increased intracellular Ca^{2+} levels. In the same transgenic mouse model with B cells with CD5, B cells are sequestered in the BM and undergo clonal deletion and are able to maintain tolerance (83). When CD5 levels are increased on both B and T murine lymphocytes, it renders them unresponsive to antigen (83, 84) through immunoreceptor tyrosine-based inhibitory motifs (ITIMs). CD5 is expressed by B-1a B cells that secrete natural polyreactive antibodies, as well as leukemic B cells (85). B cells can use two forms of CD5 by the modulation of exon 1 and can be expressed as a cell-surface form or a truncated cytoplasmic form (86). CD5 expression weakens the responsiveness of effector cell and is a clear marker to analyze for immune system regulation. It is unknown whether CD5 is necessary for B10 cell IL-10 production (79); however,

CD5 is reported to induce IL-10 expression and promote cell survival in human B cells (27), human chronic lymphocytic leukemia B cells (28) and mice (29). Many hospitals utilize CD5 in their repertoire of clinical laboratory analyses as one component to evaluate for disease progression. Preliminary results in patients with AAV demonstrate that repopulation with normal levels of $CD5^+$ B cells portends a longer time to relapse than patients who repopulate with reduced levels of $CD5^+$ B cells potentially making CD5 a feasible biomarker of disease.

CD5 Ligand

Several ligands have been proposed for CD5; however, none has been confirmed and thoroughly investigated. The pan-B cell marker, CD72, was the first ligand reported for CD5. CD5 has been shown to interact with CD72 and this interaction is blocked by anti-CD72 antibodies (87). Anti-CD5 mAbs can be used to stimulate CD5 (88). Another candidate, in rabbits, for the ligand of CD5 is $F(ab')_2$ fragments of antibodies that express V_H a2 framework sequences (IgG V_H framework regions) (89). CD5 has been shown to function as a pathogen recognition receptor (PRR) that recognizes pathogen associated molecular patterns (PAMPs) on fungal but not bacterial surfaces (90). Other groups claim CD5 is its own ligand (91). Homophilic binding by CD5 domain 1 to CD5 has the potential to mediate interactions between cells, in trans or on the same cell, in cis (92).

The Pathogenesis of Anti-Neutrophil Cytoplasmic Autoantibody (ANCA) Vasculitis

AAV is a severe relapsing disease caused by pathogenic autoantibodies directed against myeloperoxidase (MPO) (93) and/or proteinase 3 (PR3) (94), which are called anti-neutrophil cytoplasmic autoantibodies (ANCA). ANCA systemically activate neutrophils, which harbor MPO and PR3. This leads to inflammation and necrosis of small blood vessel walls thereby resulting in decreased organ function of highly vascularized tissues such as the kidney, lungs and skin (95). Clinical manifestations common to both MPO and PR3 serotypes of AAV may include fatigue, arthralgias, vasculitic skin lesions and necrotizing capillaritis leading to pulmonary hemorrhage and glomerulonephritis. AAV-like disease can be induced in mice upon

adoptive transfer of splenocytes, B cells, or ANCA alone (3-5). Therefore, ANCA are pathogenic in AAV and may be the “prime mover” of disease. The effectiveness of rituximab therapy that depletes B cells from circulation in patients with AAV (96, 97) is additional evidence that directly implicates B cells in the immunopathogenesis of AAV.

AAV is associated with a breach in self-tolerance during B cell development leading to autoreactive B cells circulating in peripheral blood of patients. Healthy individuals have B cells that express autoreactive antibodies including some that are specific for the self-proteins MPO and PR3. They are normally IgM, polyreactive antibodies and are likely to be generated by gene rearrangement during early B cell development in the bone marrow. ANCA are predominantly somatically mutated, class-switched IgGs; therefore, they must be produced by antigen-experienced B cells, suggesting a breach in the late stages of B cell tolerance. The checkpoint at which B cell tolerance is first broken in AAV is unknown.

AAV Epidemiology and Genetics

ANCA disease has a reported incidence of 10–20 cases per million per year worldwide that varies racially and geographically (98, 99). The severity of this disease leads to significant morbidity and mortality wherein 15% of patients die within the first year of diagnosis and 35% die within 5 years

A mixture of predisposing genetic and environmental factors contributes to the etiology of AAV. Genetic studies implicate several genes associated with B cells, IL-10, and the immune system that predispose patients to AAV such as human leukocyte antigen (HLA), protein tyrosine phosphatase non-receptor type 22 (PTPN22), and Fcγ receptors. Patients with AAV have an increased frequency of the IL-10 -1082AA genotype that is associated with decreased IL-10 production (59).

Current Treatment Options for Patients with AAV

AAV is a systemic autoimmune disease often characterized by an alternating pattern of active disease (either new-onset or relapse) and remission. In order to induce disease

quiescence, immunosuppressive therapy comprised of corticosteroids and cyclophosphamide, azathioprine and/or mycophenolate mofetil (MMF) must be administered. The aforementioned treatment options are non-specific and have deleterious side effects that immunocompromise patients leaving them susceptible to infection – the leading cause of mortality in patients with AAV (100), and cancer. Therefore there is a need for safer and more specific therapies. Rituximab, a humanized monoclonal anti-CD20 antibody used in many B cell mediated autoimmune diseases and lymphomas to deplete circulating B cells, is the newest therapy approved for patients with AAV. Rituximab is an effective therapy for patients suffering from AAV (6,7) further supporting the integral nature of B cells in AAV pathogenesis (101, 102). Because of its efficacy, some clinicians and researchers propose that rituximab-induced continuous B cell depletion should become the mainstay of therapy for patients with AAV thereby rendering them permanently purged of B cells. One major side effect of this treatment can be increased risk of infection. Importantly, rituximab depletes peripheral B cells indiscriminately; thus, some would argue that rituximab could delay or prevent durable remission due to concomitant eradication of beneficial Bregs that may be necessary to induce and/or maintain immunological homeostasis. We are interested in AAV as a human disease as a model of humoral autoimmune disease in general to understand basic Breg biology and clinical relevance.

Regulatory B cells in AAV

The importance of B cells in the immunopathogenesis of AAV is underscored by the fact that ANCA cause disease in mice (103, 104) and by the therapeutic effectiveness of rituximab, a B cell-depleting therapy (96, 105). The concept that functional Bregs contribute to the maintenance of tolerance by means of IL-10 is robustly supported by results from animal models and human studies described in the previous sections (106). Along with the immunopathogenic role of certain B cells in AAV, Bregs negatively regulate the immune response and may be beneficial because they may play a role in the maintenance of

immunological tolerance. Recently several groups have investigated the role of Bregs in AAV and will be discussed in detail in chapter four. Of note these studies have not addressed the role of $CD5^+$ Bregs or their suppression of autoantibodies. In the two following manuscripts (chapters two and three) we investigate Breg subsets in patients with AAV and modulation of these subsets during active and remission disease states.

In chapter two, we tested the hypothesis that B cell phenotype might be used as an indicator of disease activity, response to treatment or future relapse. We investigate $CD5^+$ B cells in patients during the course of disease activity and with response to rituximab therapy (107). We report a B cell population that partially overlaps with the immunophenotype for regulatory B cells and correlates with disease activity in patients with AAV. To further evaluate the relationship of $CD5^+$ B cells and states of remission and relapse in AAV, we examined peripheral blood samples from patients who received rituximab therapy and underwent B cell depletion. We hypothesized that patients who repopulated with normalized $\%CD5^+$ B cells following rituximab would have a more sustained remission than patients who repopulated with low $\%CD5^+$ B cells.

We show that B cells from patients with active AAV express low levels of CD5, a surface molecule that negatively regulates B cell signaling through the BCR to maintain immunological tolerance (82, 107). In contrast, patients who are in remission have $CD5^+$ B cell levels comparable to those in healthy individuals (107). Moreover, we found that $CD5^+$ B cells are a harbinger of relapse following rituximab therapy when low or in decline.

In chapter three, we explore the role of regulatory B cells in patients with AAV, by (1) measuring the reported phenotypes, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ as well as $CD5^+$ subsets of these populations, (2) determined B cell IL-10 production and (3) correlated these findings with changes in ANCA titer. Herein, we show that the $CD5^+$ subset of $CD24^{hi}CD38^{hi}$ B cells ($CD5^+CD24^{hi}CD38^{hi}$) is reduced in patients with active AAV compared with healthy controls and patients in remission. Moreover, IL-10-producing B cells also decrease during active disease.

As patients go into remission, both $CD5^{+}CD24^{hi}CD38^{hi}$ and IL-10-secreting B cells are present at levels similar to healthy controls. Although not significantly decreased during active disease, the $CD24^{hi}CD38^{hi}$ B cell population expands during disease remission. Longitudinal analysis of paired active and remission samples from patients' B cells reveals that $CD24^{hi}CD38^{hi}$, $CD5^{+}CD24^{hi}CD38^{hi}$ B cells, and $IL\text{-}10^{+}$ B cells all increase upon disease remission. Our data are consistent with the hypothesis that functionally competent regulatory B cells characterized as $CD5^{+}CD24^{hi}CD38^{hi}$ or $IL\text{-}10^{+}$ support long-term clinical remission and that absence of functional regulatory B cells may be associated with disease onset and relapse in patients with AAV.

In conclusion, we demonstrate that a low percentage (≤30%) of circulating $CD5^{+}$ B cells correlates with disease activity and a shorter time to relapse. Patients in remission had a percentage of $CD5^{+}$ B cells similar to HCs, which was significantly higher than patients with active disease. After rituximab therapy, low or declining $\%CD5^{+}$ B cells was associated with a shorter time to disease relapse among patients on no or low dose maintenance therapy with mycophenolate mofetil (MMF). In the second study, CD5 appears again as a potential marker for $IL\text{-}10^{+}$ B cells: specifically, the $CD5^{+}CD24^{hi}CD38^{hi}$ population, which modulates with AAV disease activity. Further, this study demonstrates that as Bregs increase (either $CD24^{hi}CD38^{hi}$, $CD5^{+}CD24^{hi}CD38^{hi}$ or $IL\text{-}10^{+}$ B cells), circulating ANCA titers decrease, indicating an important function for human Bregs in autoimmune disease.

Overall, these insights may contribute to the identification of biomarkers of disease activity and may facilitate the design of safe, targeted, therapeutic agents to improve induction of disease remission with the ultimate goal of ensuring that patients sustain durable remission without additional immunosuppression.

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CHAPTER 2: DECREASED CD5+ B CELLS IN ACTIVE ANTI-NEUTROPHIL CYTOPLASMIC AUTOANTIBODY (ANCA) VASCULITIS AND RELAPSE AFTER RITUXIMAB[1]

Summary

Background and Objectives

B cell significance in antineutrophil cytoplasmic autoantibody (ANCA) disease pathogenesis is underscored by the finding that ANCA alone can cause disease in mouse models and by the effectiveness of rituximab as therapy in ANCA-small vessel vasculitis (ANCA-SVV). To avoid infections and adverse events from therapy, clinicians require improved markers of disease activity and impending relapse to guide immunosuppression strategies post-rituximab.

Design, Setting, Participants, and Measurements

We investigated B cell phenotype in patients with active ANCA-SVV and in remission. From 2003 to 2009, 54 patients were followed longitudinally for 4 to 99 months and compared to 68 healthy controls. In a subset of 19 patients we examined B cell immunophenotype in samples following rituximab.

Results

Patients with active ANCA-SVV had lower %$CD5^+$ B cells, whereas %$CD5^+$ B cells from patients in remission were indistinguishable from healthy controls. After rituximab, median time to relapse was 31 (IQR=25,48; n=7) months in patients maintaining normalized %$CD5^+$ B cells, with or without maintenance immunosuppression. Among patients whose B cells repopulated with low %$CD5^+$ B cells or had a sharply declining %$CD5^+$ B cells, those who were on low or no

[1] This chapter previously appeared as an article in the Clinical Journal of the American Society of Nephrology. The original citation is as follows: Bunch DO, McGregor JAG, Khandoobhai NB, Aybar LT, Burkart ME, Hu Y, et al. Decreased CD5+ B Cells in Active ANCA Vasculitis and Relapse after Rituximab. Clinical Journal of the American Society of Nephrology 8 :382-391, 2013. PMID: 23293123.

maintenance immunosuppression relapsed sooner (median=17 (12,20) months; n=7) than patients who were maintained on high levels of oral maintenance immunosuppression (29 (29,35) months; n=5; p= 0.002).

Conclusions

The %$CD5^+$ B cells, as a component of the human B regulatory cell phenotype, is a useful indicator of disease activity, remission and future relapse, and therefore, may guide remission maintenance therapy following rituximab.

Introduction

Anti-neutrophil cytoplasmic autoantibody-small vessel vasculitis (ANCA-SVV) is a severe relapsing disease wherein B cells produce autoantibodies directed against myeloperoxidase (MPO) (1) or proteinase 3 (PR3) (1,2). These autoantibodies can cause disease in mouse models (3-5). Recently, rituximab (a B cell depleting monoclonal antibody) has been shown to be effective in treating ANCA-SVV, suggesting that B cells play an important role in the pathophysiology of this disease (6,7). We predicted that B cell phenotype might be used as an indicator of disease activity, response to treatment or future relapse. CD5 mutes B cell signaling and maintains immune tolerance via anergy (8-12). Recently, human B regulatory cells characterized as $CD24^{hi}$ and either $CD38^{hi}$ (13) or $CD27^+$ (14) were described. These cells are also noted to be $CD5^+$ (13). We investigated $CD5^+$ B cells in patients during the course of disease activity and with response to rituximab therapy.

We report a B cell population that partially overlaps with the immunophenotype for regulatory B cells and correlates with disease activity in patients with ANCA-SVV. To further evaluate the relationship of $CD5^+$ B cells and states of remission and relapse in ANCA-SVV, we examined peripheral blood samples from patients who received rituximab therapy and underwent B cell depletion. We hypothesized that patients who repopulated with normalized %$CD5^+$ B cells following rituximab would have a more sustained remission than patients who repopulated with low %$CD5^+$ B cells.

Study Population and Methods

Patient and Healthy Control Samples

We performed flow cytometry analysis of lymphocyte samples from 54 patients with ANCA-SVV and 68 healthy controls between the years 2003 and 2009. Informed consent was obtained in accordance with our Institutional Review Board's guidelines for human subjects. Peripheral blood samples were collected from patients positive for MPO-ANCA and/or PR3-ANCA by either indirect immunofluorescence or antigen-specific ELISA. Patients with Churg-Strauss Syndrome, anti-GBM or overlap ANCA/anti-GBM disease were excluded. Forty-nine of 54 patients had biopsy-proven ear, nose and throat, pulmonary, renal or dermatologic small vessel vasculitis. Clinical and serological data were gathered during routine clinic visits at the time of blood draw for B cell analysis. Patients in end-stage kidney disease were excluded from this study unless there were overt extra-renal manifestations of vasculitis.

Patient Groups

Vasculitis disease activity was measured using the Birmingham Vasculitis Activity Score (BVAS) (15). Patients with a BVAS ≥1 were considered to have active disease. When possible, "active" samples were obtained at disease onset; otherwise, the sample corresponding to the highest BVAS score was used in these analyses. Samples were classified as "remission" if patients were in remission for 3 months before and after the collection date. "Active" versus "remission" samples were compared in rituximab-naive patients.

When available, blood samples were evaluated before and after rituximab treatment. We examined the last sample obtained before rituximab treatment and samples obtained after rituximab treatment where the %$CD19^+$ B cells were ≥1%. For post-rituximab evaluation, patients were separated into 3 groups. Patients whose %$CD5^+$ B cells measured at >30% ("normal" based on the mean of healthy controls) at the time of B cell repopulation and in the samples following B cell repopulation were labeled Group 1 regardless of remission maintenance therapy dose. Patients whose %$CD5^+$ B cells measured ≤30% at the time of B cell

repopulation, or decreased to ≤30% within 12 months, were sub-divided based on the dose of mycophenolate mofetil (MMF) received post-rituximab treatment. Patients who had low-dose MMF (≤1 gram/day) were labeled Group 2, whereas those maintained on higher doses of MMF (>1 gram/day) post-rituximab infusion were labeled Group 3. Only two of our patients were on any steroids in addition to the MMF dose stated for maintenance therapy after Rituximab infusion. One of our Group 2 patients was on 100 mg/d cyclosporine and 6 mg/d prednisone instead of MMF. One of our Group 3 patients (on 2 g/d of MMF) was also on 10 mg prednisone every other day following B cell recovery through time of flare. Since there were only 2 patients taking prednisone as part of their maintenance therapy and this dose was quite minimal, we did not consider the prednisone dose in our division of patients with low %$CD5^+$ B cells into low and high immunosuppression sub-groups (Groups 2 and 3).

We performed a sensitivity analysis by regrouping the patients based on $CD5^+$ B cells at the time of B cell repopulation only, without considering the subsequent trend of $CD5^+$ B cells, and then reanalyzing the data as done for the primary analysis.

Cell Preparation and Cell Surface Staining

Peripheral blood mononuclear cells were purified from heparinized peripheral blood samples by centrifugation in cell preparation tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Cells were washed in phosphate-buffered saline, resuspended in Hank's buffered salt solution (2% fetal calf serum, 0.1% sodium azide) and stained with CD19-APC (HIB19) in combination with 2 of the following either FITC- or PE-fluorescently labeled antibodies to: CD21 (B-ly4), CD24 (ML5), CD27 (M-T271), CD38 (HIT2), CD5 (UCHT2), IgM (G20-127) or IgD (IA6-2) (BD Pharmingen, San Diego, California). After fixation with 1% paraformaldehyde, cells were analyzed using a FACSCalibur flow cytometer. B cells were gated based on $CD19^+$ staining. Data was analyzed with Summit (DakoCytomation) or FlowJo (Treestar, Ashland, OR) software.

Statistical Analysis

Mean and standard deviation (SD) or median and interquartile range (IQR) were used to describe demographic and clinical characteristics as appropriate. Wilcoxon rank-sum or Kruskal-Wallis tests were used to compare groups for continuous variables and Fisher's exact tests were used for categorical variables. Paired Wilcoxon signed rank test tested the paired difference of B cell phenotypes in the subgroups. P-values reported with a two-side p value of ≤0.05 indicate a significant difference. Analyses were conducted using SAS 9.1 (SAS Institute, Cary, NC).

Results

The %CD5$^+$ B Cells is Reduced in Patients with Active Disease and Before Relapse

We first examined %CD5$^+$ B cell expression in rituximab-naïve ANCA-SVV patients. Samples were evaluated at the time of either active disease (BVAS≥1, n=24) or remission (BVAS=0, n=19) (Table 2-1). There were no significant differences between active disease patients compared to those in remission with respect to age, sex, ethnicity, ANCA type, disease category, organ involvement or peak creatinine at disease onset (Table 2-1). Patients with active disease had significantly lower %CD5$^+$ B cells (median=17%, IQR=10,28) than those in remission (26%, IQR=21,36, p=0.02) and healthy controls (28%, IQR= 21,35, p<0.001) (Figure 2-1A, Table 2-1). The %CD5$^+$ B cells during remission did not differ significantly from the percentage found in healthy controls. Although patients were significantly older than healthy controls, the %CD5$^+$ B cells did not correlate with age in healthy controls, patients with active disease or patients in remission (data not shown). In patients for whom active and remission samples were available, the %CD5$^+$ B cells increased from a median of 14% (IQR=10,17) in active disease to a median of 25% (IQR=17,45; p=0.008) as patients entered remission (Figure 2-1B). When %CD5$^+$ B cells were compared to disease activity over time, downward trends in CD5 were associated with relapse (representative Figure 2-1C). An example of a patient who maintained >30% of CD5$^+$ B cells and remained in remission without maintenance immunosuppression with a persistently high MPO-ANCA titer for 82 months is shown in Figure 2-1D.

B Cell Phenotypes Following Rituximab Therapy

To further elucidate the relationship between %CD5$^+$ B cells and disease activity, we studied a subset of 19 patients who received rituximab (Table 2-2). The %CD5$^+$ B cells were measured following B cell repopulation after rituximab. Group 1 (patients who repopulated with >30% CD5$^+$ B cells) was diverse with regard to MMF dose; there were 3 patients on no immunosuppression, 2 on low immunosuppression and 2 on high immunosuppression with a

mean dose of 0.75 ±0.8 g/day (Table 2-2). By definition, patients who repopulated with ≤30% $CD5^+$ B cells and were maintained on ≤1g/day MMF (Group 2) were prescribed 75% less MMF (mean 0.43±0.5 g/day) than Group 3 patients who also repopulated with ≤30% $CD5^+$ B cells but were maintained on >1g/day MMF (mean 1.95±0.7 g/day) (p=0.005). On average, Group 2 and Group 1 were similar with regard to immunosuppression dose (p=0.4). All patients treated with oral remission maintenance therapy following rituximab infusion were prescribed MMF with the exception of two patients who received low dose cyclosporine (<1 mg/kg/d) in addition to MMF. Patient characteristics were similar across the 3 groups (Table 2-2).

Group 1 had a significantly higher %$CD5^+$ B cells (median=57%, IQR 48,70) at the time of B cell repopulation than Group 2 (18%, IQR 11,31, p=0.003, Table 2-2). Group 3 had a similarly low %$CD5^+$ B cells (23%, IQR 13,53) but did not reach statistical significance due to the small number of patients. The median %$CD5^+$ B cells at the last sample available prior to flare for Group 1 was 34% $CD5^+$ B cells (IQR 27,41). Median %$CD5^+$ B cells at the time proximal to flare was 16% (IQR 15,18) and 4% (IQR 4,16) for Groups 2 and 3 respectively. Time to relapse following rituximab infusion was significantly shorter when CD5 was ≤30% at the time of B cell repopulation (Group 2) (p=0.002, Table 2-2). In patients who had CD5 levels >30% at the time of B cell repopulation and remained >30% for all subsequent samples evaluated (Group 1), but similarly low levels of oral remission maintenance therapy, time to flare was 18 months longer on average than Group 2 (Table 2-2). Group 3 patients had similarly low CD5 levels to Group 2 patients (p=0.52), but were maintained on significantly higher doses of MMF (p=0.005). Their time to flare after rituximab infusion was on average 20 months longer than Group 2 (p=0.006, Table 2-2). Time to flare from B cell repopulation was also significantly different between Group 2 patients and either patients whose %$CD5^+$ B cells remained >30% following B cell repopulation maintained on similarly low remission maintenance therapy (p=0.002, Group 1) or when oral remission maintenance therapy was maintained at significantly higher doses (Group 3) (p=0.006, Table 2-2).

Sensitivity Analysis

Sensitivity analysis was performed to evaluate whether the association between %$CD5^+$ B cells and time to relapse held up after regrouping patients based strictly on %$CD5^+$ B cells at the time of B cell recovery (Supplemental Table S2-1). Patients having >30% $CD5^+$ B cells at the time of B cell repopulation became Group 1^S (n=10) regardless of whether the %$CD5^+$ decreased below 30% subsequently. Group 2^S (n=6) had ≤30% $CD5^+$ B cells at B cell repopulation and were on ≤1 gram of MMF daily (mean = 0.33±0.5 g/d). Group 3^S (n=3) had ≤30% $CD5^+$ B cells at B cell repopulation and were on >1 gram of MMF daily (mean = 2±1 g/d), (p=0.04 compared to Group 2^S). By definition, Group 1^S repopulated with higher %$CD5^+$ B cells (median = 55%, IQR 48,70) compared to both Group 2^S (17%, IQR 11,30) and Group 3^S (13%, IQR 12,23) (p=0.001) after rituximab. The time to flare post-rituximab therapy for Group 2^S was significantly shorter (median = 16 months, IQR 12,18) compared to both Group 1^S (28 months, IQR 25,34) and Group 3^S (35 months, IQR 29,65) (p=0.002). The %$CD5^+$ B cells at the time of documented flare did not differ for Group 1^S and Group 2^S (median = 26% and 16% respectively, p=0.18), whereas the %$CD5^+$ B cells were lower when relapses occurred in Group 3^S (4%, p=0.05).

To evaluate %$CD5^+$ B cells with respect to clinical disease activity in patients treated with rituximab, %$CD5^+$ B cells were plotted against BVAS and MMF dose. Three examples depict the consistent decline in %$CD5^+$ B cells we observed prior to disease relapse (Figure 2-2, A-C). Time to relapse appears delayed if higher levels of remission maintenance therapy were given when CD5 levels were <30%.

Other B cell populations including naïve, switched and non-switched memory, IgD,CD27-double negative, and pre-germinal center founder (Bm2'3δ) cells, are different in ANCA patients compared to controls but do not correlate with disease activity (Supplemental Table S2-2). CD21 differs between active disease and remission (p<0.001) but is not clearly associated with time to relapse.

CD5 as a Surrogate Marker for Putative B Regulatory Cells

Because of its role as a negative regulator of B cell receptor signaling and its inclusion in the immunophenotype reported for B regulatory cells, we compared $CD5^+$ B cells with other phenotypes reported for B regulatory cells. $CD5^+$ B cells correlate well with the $CD24^{hi}CD38^{hi}$ population of B regulatory cells that has been shown to secrete IL-10 ($R^2 = 0.50$, Figure 2-3A) in all samples for which both stains were available (n=21 HC, 17 Active, 13 Remission). When flow cytometric data was available for all 3 stain sets ($CD24^{hi}CD38^{hi}$, IgM^+CD5^+, and $CD5^+$) these B cell populations correlated well over time (representative patients shown in Figure 2-3B).

Discussion

The last two decades have witnessed a marked improvement in the induction treatment of patients with ANCA vasculitis, with remission rates around 80% (16-18). A major remaining challenge in the long term management of patients pertains to the prevention and treatment of relapses. The risk of relapse is not uniform for all patients with ANCA vasculitis. PR3-ANCA (as compared to MPO-ANCA), lung disease, upper respiratory tract disease, a clinical diagnosis of GPA (as compared to MPA or renal limited disease), cardiovascular involvement and a lack of renal impairment (creatinine <200 µmoles/liter) have been reported as risk factors for relapse (19-21). Nevertheless, no clinical or serologic measure is currently available that allows effective disease monitoring and distinguishes patients in long-term stable remission from those at imminent risk of relapse (22-26). Such a tool would allow physicians to better tailor the duration and intensity of immunosuppressive therapy based on the individual patient's needs. Our goal was to evaluate whether certain B cell subpopulations could be used to assess immunologic disease activity or a patient's risk of relapse. Although limited to a small number of patients, we determined that a low percent (≤30%) of circulating $CD5^+$B cells correlates with disease activity and a shorter time to relapse. Patients in remission had %$CD5^+$ B cells similar to healthy controls and significantly higher than patients with active disease. After rituximab therapy, low or declining %$CD5^+$ B cells was associated with a shorter time to disease relapse among patients

on no or low dose maintenance therapy with MMF. The use of full dose MMF was associated with a longer time to relapse in the setting of a low %$CD5^+$ B cells. Additional data will be required to definitively address the correlation of %$CD5^+$B cells with sustained remission.

If our findings are confirmed in a larger population, then the clinical implications of our results may pertain to the decision to use maintenance immunosuppression following rituximab and its timing. Our data suggest that patients whose %$CD5^+$ B cells remain low or decline after a period of normalization following rituximab therapy would be at higher risk of subsequent relapse and likely benefit from maintenance immunosuppression. Conversely, such immunotherapy could be avoided in patients who maintain a normal %$CD5^+$ B cells.

Our results are consistent with current knowledge of B cell subtypes and function. B regulatory cells, defined by their ability to suppress INF-γ and TNF-α expression in T cells via expression of IL-10, have been described as having a $CD24^{hi}CD38^{hi}$ phenotype (13). B regulatory cells were also reported to be $CD5^+IgM^{+/hi}IgD^{+/hi}CD10^{low/+}CD27^{neg}CD1d^{hi}$, although consensus on their immunophenotype is not yet fully established (14). We propose that the CD5 marker is an acceptable measure of B regulatory (Breg) cells based on our data demonstrating a high correlation with $CD24^{hi}CD38^{hi}$ and IgM^+CD5^+ subpopulations. CD5 is reported to induce IL-10 expression and promote cell survival in human B cells (27), human chronic lymphocytic leukemia B cells (28), and mice (29). In mice, $CD5^+CD1d^+$ B cells secrete IL-10 and have a regulatory function evidenced by their inhibition of INF-γ and TNF-α expression in T cells (30). Our results add to accumulating evidence that a paucity of, or non-functional B regulatory cells are associated with increased disease activity in autoimmune disease (13,14,31). Years ago, when dogma was that $CD5^+$ B cells were increased in autoimmune disease (32), patients with active Kawasaki disease were reported to have a decreased %$CD5^+$ B cells (33). These and our findings raise the possibility that a robust Breg subpopulation could be a goal of immunotherapy, as well as a means of monitoring its efficacy. This hypothesis would best be tested prospectively as part of a clinical trial.

Other B cell populations including naïve, switched and non-switched memory, IgD,CD27-double negative, and pre-germinal center founder (Bm2'3δ) cells have been reported to correlate with response to rituximab therapy in SLE and RA (34-36). Neither these B cell populations nor ANCA titer correlated with disease activity or time to flare after rituximab therapy in our patient cohort.

Patients in our study were treated with rituximab for induction of remission after a clinical relapse (to avoid repeat exposure to cyclophosphamide) or because of persistent disease activity despite cyclophosphamide and corticosteroids. Although B cell phenotype data emanate from rituximab-treated patients, they may not be restricted to this form of therapy. Indeed, treatment with cyclophosphamide results in peripheral B cell depletion albeit more slowly and to a lesser magnitude than with rituximab (6). Studies are ongoing to assess whether similar effects on the $CD5^+$ B cell subpopulation are detectable with cyclophosphamide-based therapies.

The optimal choice and duration of maintenance therapy is the subject of current clinical investigations. In this study, the choice of MMF as maintenance therapy after rituximab was not predetermined by protocol, and antedates the published results on the International Mycophenolate Mofetil Protocol to Reduce Outbreaks of Vasculitides (IMPROVE) study in which azathioprine was associated with a statistically significant decrease in the rate of relapses compared to MMF (24). The demonstrated efficacy of rituximab in treating active ANCA-SVV has raised the question as to its possible role in maintenance therapy, given at regular intervals regardless of clinical signs of disease activity (37). It will be interesting to test the validity of our hypothesis in a setting where a robust $CD5^+$ Breg population may be suppressed by a regimen of prolonged B cell depletion. It is possible that a state of immune tolerance may require the presence of robust Breg and/or Treg populations, which would be prevented by sustained B cell depletion.

There are limitations to our study. The relatively small sample size of patients with longitudinal data limits our ability to evaluate the correlation between %$CD5^+$ B cells and time to relapse while correcting for other risk factors such as PR3-ANCA, organ involvement or disease phenotype. Although we attempted to obtain patient samples every 3 months, the timing of our blood collections was not standardized. Samples were obtained from patients whenever they presented for care.

A future research direction will be to validate our findings in a larger cohort of patients treated with either rituximab or cyclophosphamide-based regimens, while formally assessing the time to relapse from the time of decline in %$CD5^+$ B cells. We aim to study the relationship between CD5 levels, IL-10 expressing B regulatory cells, and disease activity in ANCA-SVV. The expression of an alternatively spliced variant of CD5 resulting in reduced membrane expression of CD5 through methylation changes driven by IL-6 has recently been described (38), which may regulate the function of B regulatory cells. The impact, if any, of the CD5 splice variant on disease activity or response to therapy will be interesting to evaluate.

In summary, we identified a $CD5^+$ B cell subpopulation as a potential immunological marker of sustained remission when robust, or a harbinger of subsequent relapse when low or declining. These findings may offer a clinical tool to monitor disease activity and modulate maintenance immunotherapy.

Disclosures

None

Table 2-1. Demographic Characteristics of Patient Groups and Healthy Controls

	Active N=24	Remission N=19	Healthy Controls N=68	P value*
Age				<0.001
Mean ± SD	55±19 [b]	54±17[b]	35±12 [a]	
Median, IQR	58(48,68)	58(38,66)	34(25.46)	
Sex				0.32
Female	11(46%)	13(68%)	41(60%)	
Ethnicity				0.76
Non-white	5(21%)	5(26%)	20(29%)	
White	19(79%)	14(74%)	48(71%)	
ANCA			N/A	0.07
MPO	8(33%)	12(63%)		
PR3	16(67%)	7(37%)		
Disease			N/A	0.23
GPA	10(42%)	8(42%)		
MPA	8(33%)	10(53%)		
ANCA GN (Renal limited)	6(25%)	1(5%)		
Organ involvement			N/A	
Upper Respiratory	12(50%)	14(74%)		0.13
Pulmonary	14(58%)	11(58%)		>0.99
Renal	N=16	N=15		
	15(94%)	14(93%)		>0.99
Peak serum creatinine at disease onset (mg/dl)	N=22	N=18	N/A	0.97
	3±2	3±3		
	3(1,5)	3(1,5)		
BVAS	N=32	N=19	N/A	<0.001
	12±6	0		
	12(7,16)	0(0,0)		
%CD5$^+$ B Cells	N=32	N=19	N=68	0.003
	21±13	30±14	30±13.	
	17(11,28)[a]	26(21,36)[b]	28(21,35)[b]	
ANCA titer¥ (U/ml)	N=33	N=17	N/A	0.36
	62±63	36±40		
	43(98)	19(67)		
MPO-ANCA titer (U/ml)	N=14	N=12	N/A	0.11
	51±37	26±24		
	50(19,71)	19(8,37)		
PR3-ANCA titer (U/ml)	N=19	N=5	N/A	0.94
	101±64	107±42		
	102(50,162)	117(76,121)		

Data are summarized as mean ± SD and median with IQR. B cell data are reported as a percentage of CD19$^+$ B cells. ANCA titers were determined by the McLendon Clinical Laboratories at the University of North Carolina using ELISA kits specific for either MPO or PR3 (Inova Diagnostics, San Diego, CA). Negative titers are ≤ 20 U/ml.

¥ANCA titer indicates the MPO-ANCA titer for MPO-ANCA patients or the PR3-ANCA titer for PR-3 patients combined together as a group for all patients in either remission or active disease.

*p values were calculated by Kruskal-Wallis test for comparison in three groups and Wilcoxon two sample test for two groups. Different superscript letters indicate a statistically significant difference between groups after a Bonferroni correction ($p < 0.017$).

Table 2-2. Comparison of Patient Groups after Treatment with Rituximab

	Group 1 N=7 Repopulation with normal %CD5$^+$ B cells	Group 2 N=7 Repopulation with low %CD5$^+$ B cells (≤30%), low remission maintenance medication[♯]	Group 3 N=5 Repopulation with low %CD5$^+$ cells (≤30%), high remission maintenance medication[§]	P values*
Age Mean ± SD Median, IQR	 51±14 59(32,61)	 50±16 52(45,59)	 51±16 51(38,58)	0.93
Sex Female N (%)	 1(14%)	 6(86%)	 2(40%)	0.03
Ethnicity Black N (%) Other N (%) White N (%)	 0(0%) 1(14%) 6(86%)	 0(0%) 1(14%) 6(86%)	 1(20%) 0(0%) 4(80)	0.80
ANCA (PR3) MPO N (%) PR3 N (%) PR3&MPO N (%)	 3(43%) 4(57%) 0(0%)	 0(0%) 6(86%) 1(14%)	 2(40%) 3(60%) 0(0%)	0.18
Disease GPA N (%) MPA N (%) ANCA GN (Renal limited) N (%)	 5(71%) 1(14%) 1(14%)	 4(57%) 3(43%) 0(0%)	 4(80%) 1 (20%) 0(0%)	0.70
Organ involvement N (%) Upper Respiratory N (%) Pulmonary N (%) Renal N (%)	 4(57%) 5(71%) 5(83%)	 5(72%) 7(100%) 4(100)	 5(100%) 3(60%) 4(80%)	0.35 0.25 >0.99
Peak serum creatinine at disease onset (mg/dl)	N=7 1.9±1.0 1.7(1.0,2.9)	N=5 2.9±1.9 2.5(1.8,2.9)	N=4 1.5±0.5 1.6(1.2,1.8)	0.28
%CD5$^+$ B cells at time of B cell repopulation	58±16 57(48,70)[a]	22±13 18(11,31)[b]	34±26 23(13,53)[a,b]	0.02
%CD5$^+$ B cells at last sample available prior to flare	N=2 34±10 34(27,41)	N=5 15±5 16(15,18)	N=4 10±11 4(4,16)	0.06
Dose of MMF for remission maintenance (g/day)	0.75±0.78 1.00(0,1.25)[a,b]	0.43±0.53 0(0,1.0)[a]	1.95±0.67 2.0(1.5,2)[b]	0.007
Time to relapse from rituximab (months)	34±11 31(25,48)[a]	16±5 17(12,20)[b]	37±16 29(29,35)[a]	0.002
Time to relapse	25±9	7±4	29±15	

from B cell repopulation (months)	22(17,36)[a]	7(3,11)[b]	22(20,27)[a]	0.002
Total B cell number (x10^4/ml blood)	7.5±6.0 5.7(2.4,15.0)	2.4±2.1 1.7(1.0,3.2)	3.6±2.1 3.9(2.4,4.5)	0.15
ANCA titer[¥] (U/ml)	49±43 39(10,95)	52±39 52(5,71)	16±14 8(6,24)	0.28

Values for variables examined in patient groups after rituximab therapy are reported as Mean ± standard deviation (SD) and Median (IQR). ANCA titers were determined by the McLendon Clinical Laboratories at the University of North Carolina using ELISA kits specific for either MPO or PR3 (Inova Diagnostics, San Diego, CA). Negative titers are ≤ 20 U/ml.

[¥]ANCA titer indicates the MPO-ANCA titer for MPO-ANCA patients or the PR3-ANCA titer for PR3 patients combined together as a group for all patients in either Group 1, 2 or 3.

*P values were calculated by Fisher exact test for categorical variables and Kruskal-Walls Test for continuous variables. Different superscript letters indicate a statistically significant difference between groups after a Bonferroni correction ($p < 0.0167$).[♯]One group 2 patient was on Cyclosporine (50mg, BID) and prednisone (6 mg/day)

[§]One group 3 patient was on MMF (1g, BID) and prednisone (10 mg every other day) following Rituximab therapy concurrent with 7 monthly intravenous doses of cyclophosphamide

Supplemental Table S2-1. Sensitivity Analysis of Patient and Disease Characteristics in Rituximab Treated Population

	Group 1[S] N=10	Group 2[S] N=6	Group 3[S] N=3	P value*
Age				
Mean ± SD	51±13	50±17	54±19	0.98
Median, IQR	55(38,61)	49(45,59)	51(36,74)	
Sex				
Female N (%)	2(20%)	6(100%)	1(33%)	0.005
Ethnicity				0.16
Black N (%)	0(0%)	0(0%)	1(33%)	
Other N (%)	2(20%)	0(0%)	0(0%)	
White N (%)	8(80%)	6(100%)	2(67%)	
ANCA				0.11
MPO N (%)	3(30%)	0(0%)	2(67%)	
PR3 N (%)	7(70%)	5 (83%)	1(33%)	
PR3&MPO N (%)	0(0%)	1(17%)	0(0%)	
Disease				0.57
GPA N (%)	7(70%)	3(50%)	3(100%)	
MPA N (%)	1(10%)	0(0%)	0(0%)	
ANCA GN (Renal limited) N (%)	2(20%)	3 (50%)	0(0%)	
Organ involvement N (%)				
Upper Respiratory N (%)	6(60%)	5(83%)	3(100%)	0.49
Pulmonary N (%)	7(70%)	6(100%)	2(67%)	0.32
Renal N (%)	8(89%)	3(100%)	2(67%)	0.66
Peak serum creatinine	10	4	2	
at disease onset (mg/dl)	1.7±0.9	3.2±2.1	1.6±0.0	
	1.8(1.0,2.1)	2.7(1.8,4.5)	1.6(1.6,1.6)	0.33
%CD5$^+$ B cells at time	58±15	19±10	16±6	
of B cell repopulation	55(48,70)[a]	17(11,30)[b]	13(12,23)[b]	0.001
%CD5$^+$ B cells at last sample available prior to flare	24±14	14±5	4.0±0.2	
	26(18,27)	16(11,17)	4.0(3.9,4.2)	0.05
Dose of MMF for remission maintenance (g/day)	0.90±0.69	0.33±0.51	2.0±1.0	
	1.0(0,1.25)	0(0,1.0)	2.0(1.0,3.0)	0.01
Time to relapse from	31±10	15±5	43±19	
rituximab (months)	28(25,34)[a]	16(12,18)[b]	35(29,65)[a,b]	0.002
Time to relapse from	22±9	6.3±4.2	35±19	
B cell repopulation (months)	20(17,25)[a]	7.0(3.0,8.5)[b]	27(22,56)[a,b]	0.002
Total B cell number (x10^4/ml blood)	6.0±5.5	2.7±2.2	3.8±2.8	
	4.6(2.4,8.3)	1.9(1.4,3.2)	3.9(1.0,6.5)	0.49
ANCA titer[¥] (U/ml)	42±38	53±42	17±18	
	38(10,50)	60(5,71)	8.3(6.0,38)	0.55

ANCA titers were determined by the McLendon Clinical Laboratories at the University of North Carolina using ELISA kits specific for either MPO or PR3 (Inova Diagnostics, San Diego, CA). Negative titers are ≤ 20 U/ml.

¥ANCA titer indicates the MPO-ANCA titer for MPO-ANCA patients or the PR3-ANCA titer for PR-3 patients combined together as a group for all patients in either remission or active disease.

* P values were calculated by Kruskal-Wallis Test for continuous variables and by Fisher Exact Test for categorical variables. Different superscript letters indicate a statistically significant difference between groups after a Bonferroni correction ($p < 0.017$).

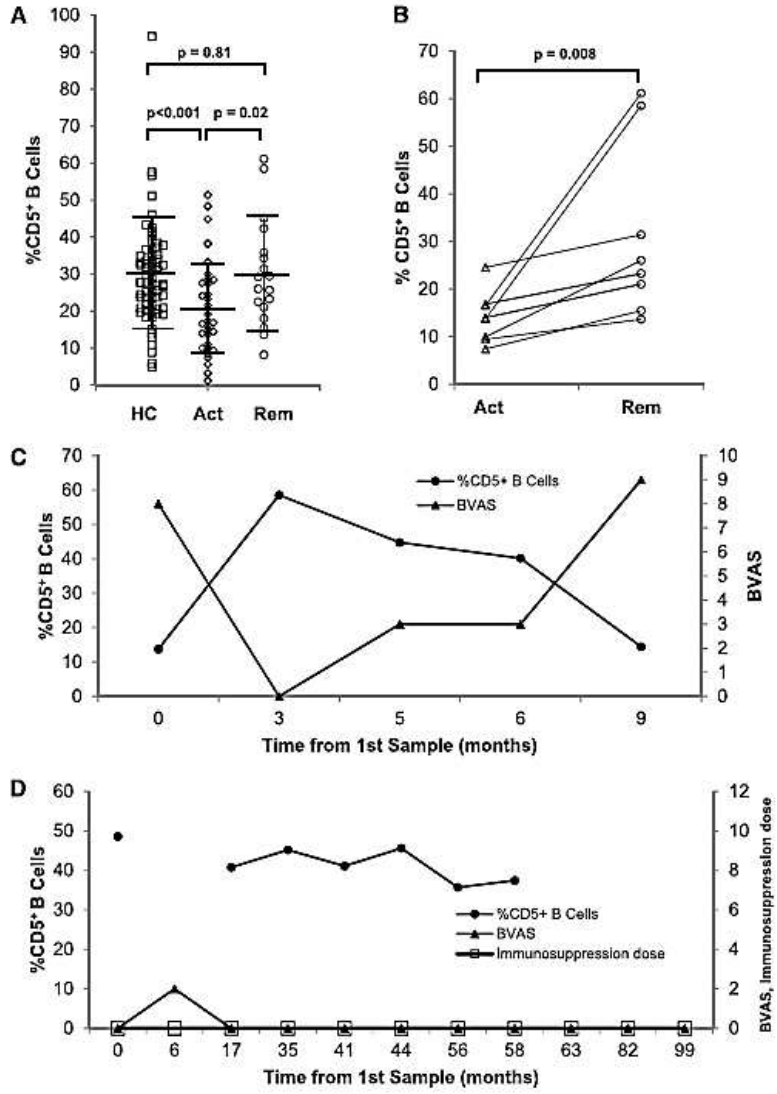
Supplemental Table S2-2. Comparison of Additional B Cell Subsets in Remission and Active Disease

	Active	Remission	Healthy Control	P value*
CD21 median fluorescence intensity (MFI)	116±54 123(58)	220±102 225(136)	244±83 234(102)	<0.001
% Naïve B cells	63±22 69(30)	65±19 68(30)	57±14 57(17)	0.04
%IgD,CD27 Double-negative B cells	10.4±6.9 8.8(7.5)	10.1±8.3 8.0(7.7)	6.8±4.7 5.5(4.6)	0.009
%Switched memory B cells	17.3±12.5 15.2(16.6)	17.0±11.9 14.5(12.4)	22.2±8.8 21.6(13.2)	0.004
%Non-switched memory B cells	8.4±6.3 6.2(7.0)	8.7±8.3 5.7(7.7)	14.1±7.6 13.1(9.3)	<0.001
%IgM^+CD5^+ B cells	15.3±12.8 11.0(13.7)	20.7±9.4 19.5(11.5)	20.8±10.3 20.2(12.4)	0.01
%Bm2'3δ B cells	7.6±12.3 3.6(7.2)	12.7±15.0 8.4(14.0)	8.8±4.7 8.3(4.7)	0.002

B cell data are given as mean ± Standard Deviation (SD) and as median (range) for $CD19^+$ B cells.

*p values were calculated by Kruskal-Wallis test for comparison in three groups and Wilcoxon two sample test for two groups.

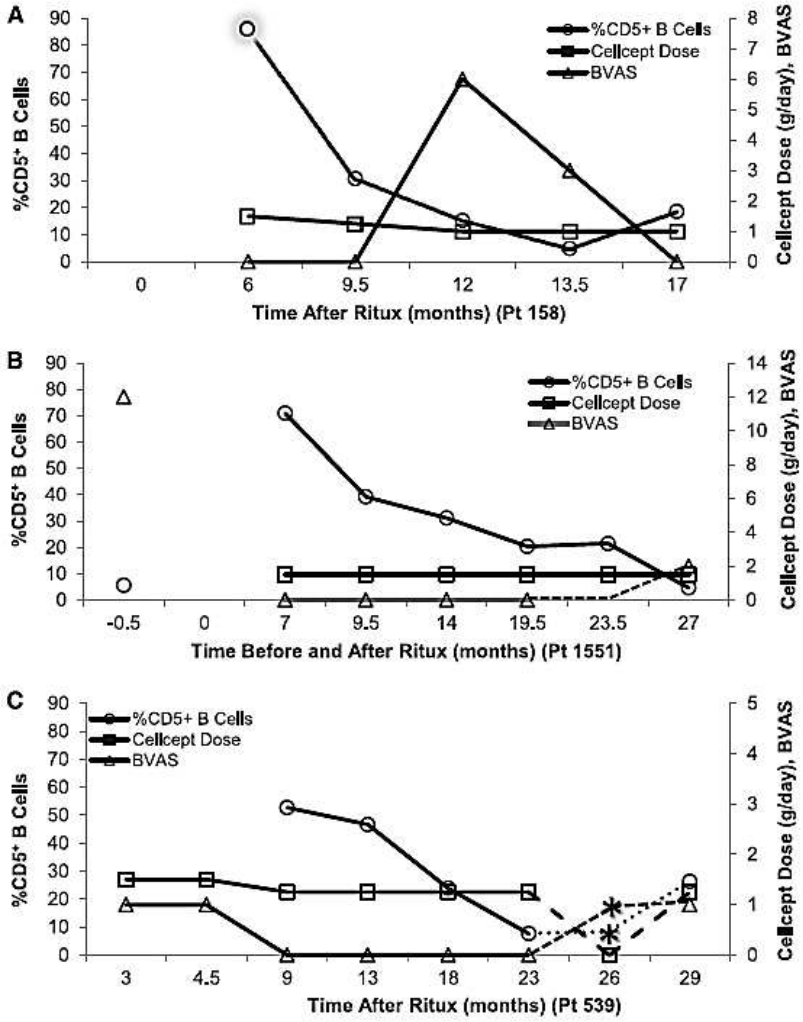
Figure 2-1. The %CD5+ B cells decreases in active ANCA disease and rebounds with remission



Shown (1A) are the %CD5$^+$ B cells in healthy controls (□, HC, n = 68), patients with active disease (◊, n = 24) and patients in remission (○, n = 19). Error bars represent the mean ± SD. The %CD5$^+$ B cells is lower in patients with active disease ($p < 0.001$) and returns to levels similar to healthy controls during remission of disease ($p=0.81$). Paired active and remission samples from 8 patients demonstrate the increase in %CD5$^+$ B cells observed as an individual transitions from active disease to remission ($p=0.008$) (B). The relationship between %CD5$^+$ B cells (●) on the left axis and BVAS (▲) on the right axis over time is depicted (C and D); the

immunosuppression dose (g/day) is indicated on the right axis (D). A reciprocal pattern of %$CD5^+$ B cells and BVAS is observed in a patient (C) who is active at time 0, enters remission at 3 months as %$CD5^+$ B cells reach normal levels and then relapses at 9 months after a steady decline in %$CD5^+$ B cells. A representative example of a patient who maintained a normal %$CD5^+$ B cells over 82 months and remained in remission off therapy with a persistently high MPO ANCA titer during this period is shown in D.

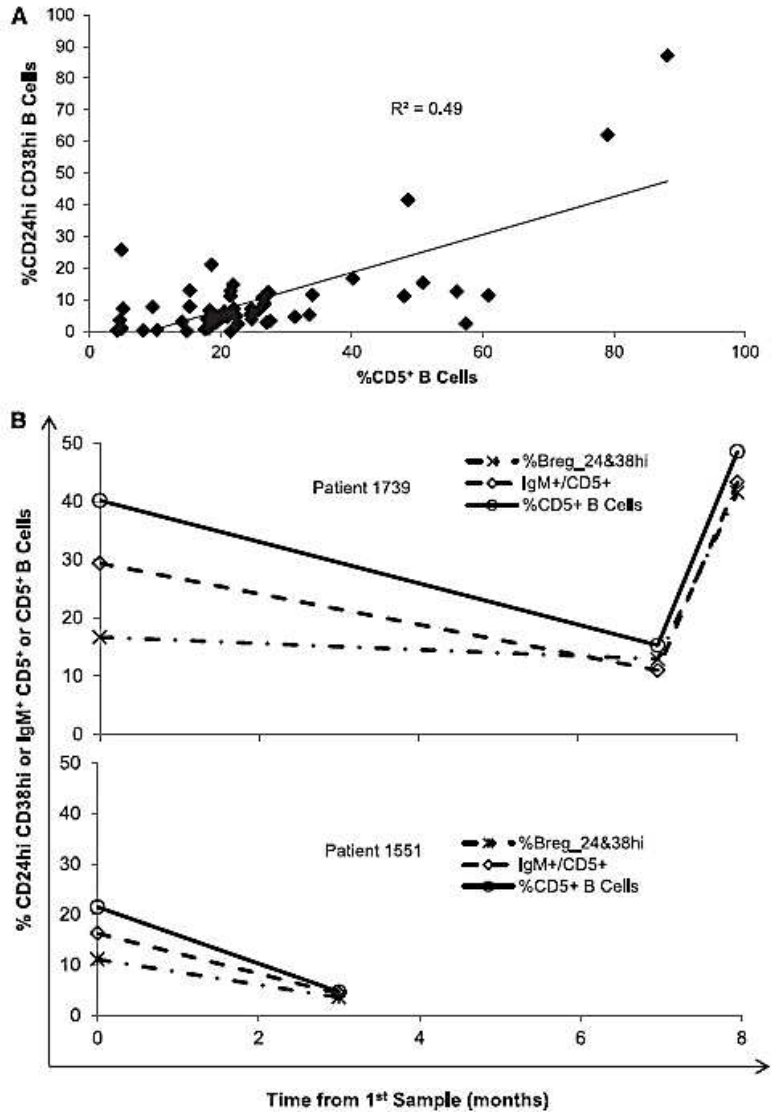
Figure 2-2. Decrease in %CD5+ B cells is associated with an increase in disease activity



Examples of the longitudinal relationship between %CD5⁺ B cells (○) on the left axis compared to BVAS (Δ) and CellCept (MMF) dose (□) on the right axis over time before and/or after rituximab are depicted (A – C). Patient 158 (A, Group 2) who had 85% CD5⁺ B cells prior to full B cell recovery (<1% B cells at 6 m, shadowed circle) showed a precipitous drop in CD5 during the next 3 to 6 months after B cell recovery. As this patient appeared to be in clinical remission, the CellCept dose was decreased during this time period and the patient flared 12 m post-rituximab. Patient 1551 (B, Group 3) had a BVAS of 12 and 5.6% CD5⁺ B cells prior to rituximab treatment. Although the %CD5⁺ cells is initially normal at B cell repopulation, it steadily declines

over the next 2.5 to 20 months without overt clinical activity in the context of high immunosuppression until month 27. Another Group 3 patient 539 (C) had a decrease in %$CD5^+$ cells from 9 to 23 months following rituximab therapy with “no signs of active disease” at months 18 and 23. Upon self-discontinuation of CellCept while %$CD5^+$ B cells were below normal, the patient flared prior to the clinic visit at 29m. The %$CD5^+$ B cells, BVAS and Cellcept dose during the time period between 23 and 29 months are depicted by dashed lines to indicate inferred information. The %$CD5^+$ B cells are assumed to be the same as the previous sample; the BVAS is assumed to be at least equal to the subsequent sample. Asterisks indicate the approximate time of flare gleaned from clinic notes for this time period.

Figure 2-3. The %CD5+ B cells reflect putative B regulatory cells



The %CD5$^+$ B cells correlates with the %B regulatory cells identified as CD24^{hi}CD38^{hi} B cells (R^2 = 0.50) (A). This correlation includes all samples for which both CD19$^+$CD5$^+$ and CD19$^+$CD24^{hi}CD38^{hi} data were available (n=21 HC, 17 Active, 13 Remission). The correlation between percentages of CD24^{hi}CD38^{hi} (dash-dot line), IgM$^+$CD5$^+$ (dash-dash line), and CD5$^+$ B cells (solid line) is shown for 2 representative patients for whom all 3 stain sets were available (B).

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CHAPTER 3: REDUCED $CD5^+CD24^{HI}CD38^{HI}$ AND $IL10^+$ REGULATORY B CELLS IN ACTIVE ANTI-NEUTROPHIL CYTOPLASMIC AUTOANTIBODY ASSOCIATED VASCULITIS PERMIT INCREASED CIRCULATING AUTOANTIBODIES[2]

Summary

Pathogenesis of ANCA-associated vasculitis is B cell dependent yet, how particular B cell subsets modulate immunopathogenesis remains unknown. Although their phenotype remains controversial, regulatory B cells (Bregs), play a role in immunological tolerance via IL-10. Putative $CD19^+CD24^{hi}CD38^{hi}$ and $CD19^+CD24^{hi}CD27^+$ Bregs were evaluated in addition to their $CD5^+$ subsets in 69 patients with AAV. B cell IL-10 was verified by flow cytometry following culture with CD40 ligand and CpG DNA. Patients with active disease had decreased levels of $CD5^+CD24^{hi}CD38^{hi}$ B cells and $IL10^+$ B cells compared to patients in remission and HCs. As $IL-10^+$ and $CD5^+CD24^{hi}CD38^{hi}$ B cells normalized in remission within an individual, ANCA titers decreased. The $CD5^+$ subset of $CD24^{hi}CD38^{hi}$ B cells decreases in active disease and rebounds during remission similarly to IL-10-producing B cells. Moreover, $CD5^+$ B cells are enriched in the ability to produce IL-10 compared to $CD5^{neg}$ B cells. Together these results suggest that CD5 may identify functional IL-10-producing Bregs. The malfunction of Bregs during active disease due to reduced IL-10 expression may thus permit ANCA production.

Introduction

Anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV) is an autoimmune disease where pathogenesis is dependent on autoantibodies that target the self-antigens myeloperoxidase (MPO) and/or proteinase 3 (PR3) (1, 2). The importance of B cells in

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the immunopathogenesis of AAV is underscored by the fact that these autoantibodies cause disease in mice (3, 4) and by the effectiveness of rituximab, a B cell-depleting therapy (5, 6). However, a key subset of B cells, referred to as regulatory B cells (Bregs), also play a role in the maintenance of immunological tolerance. Bregs, which control immunological homeostasis via the hallmark immunosuppressive cytokine interleukin-10 (IL-10) (7-11), have been reported in mice and humans, but are not well characterized in humans.

We recently demonstrated that B cells from patients with active AAV express low levels of CD5, a surface molecule which negatively regulates B cell signaling through the B cell receptor (BCR) to maintain immunological tolerance (12, 13). In contrast, patients who are in remission have $CD5^+$ B cell levels comparable to those in healthy individuals (13). Moreover, we found that $CD5^+$ B cells are a harbinger of relapse following rituximab therapy when low or in decline. In mice, $CD5^+CD1d^{hi}$ B cells secrete IL-10 and have a regulatory function evidenced by their inhibition of INF-γ and TNF-α expression in T cells (14). We surmised that CD5 might serve as a marker of regulatory B cells. Two phenotypes for IL-10-producing regulatory B cells in humans have been described, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ (7, 11).

To explore the role of regulatory B cells in patients with AAV, we (1) measured the reported phenotypes, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ as well as $CD5^+$ subsets of these populations, (2) determined B cell IL-10 production and (3) correlated these B cell populations with changes in ANCA titer. Herein, we show that the $CD5^+$ subset of $CD24^{hi}CD38^{hi}$ B cells ($CD5^+CD24^{hi}CD38^{hi}$) is reduced in patients with active AAV compared with healthy controls (HC) and patients in remission. Moreover, IL-10-producing B cells also decrease during active disease. As patients go into remission, both $CD5^+CD24^{hi}CD38^{hi}$ and IL-10-producing B cells are present at levels similar to HCs. In contrast, the $CD24^{hi}CD38^{hi}$ B cell population does not significantly decrease during active disease, but expands during disease remission. Longitudinal analysis of patients' B cells reveals that $CD5^+CD24^{hi}CD38^{hi}$ B cells and $IL\text{-}10^+$ B cells normalize upon disease remission. Our data are consistent with the hypothesis that functionally competent

regulatory B cells characterized as $CD5^{+}CD24^{hi}CD38^{hi}$ or $IL\text{-}10^{+}$ support long-term clinical remission and that absence of functional regulatory B cells may be associated with disease onset and relapse in patients with AAV.

Study Population and Methods

Patient and Healthy Control Samples

Peripheral blood mononuclear cell (PBMC) samples were collected from 30 HCs and 69 patients with AAV (see Table 3-1) after informed consent was obtained in accordance with the University of North Carolina's Institutional Review Board. Patient inclusion required diagnosis of AAV in accordance with criteria established by the Chapel Hill Consensus Conference (15). Diagnosis of microscopic polyangiitis (MPA) or granulomatosis with polyangiitis and/or crescentic glomerulonephritis without overt signs of systemic vasculitis were based on previously established criteria (16, 17). Individuals with anti-glomerular basement membrane disease, immunoglobulin A nephropathy, eosinophilic granulomatosis with polyangiitis or any other glomerular disease process in addition to AAV were excluded. Patients who had reached end stage renal disease (on dialysis or a renal transplant recipient) were excluded. Clinical and serological data were gathered during routine clinic visits at the time of blood draw for B cell analysis.

Disease activity was classified, in part, based on the Birmingham Vasculitis Activity Score (BVAS) (18). Charts were reviewed extensively to distinguish persistent or recurrent disease from disease quiescence or non-vasculitic symptoms. Patients were classified as being in remission at the time of the sample only if they had a BVAS of 0 and had no clinical evidence of active disease for at least 3 months before and 3 months after the sample date. Patients were classified as having active disease if they had a BVAS greater than 0 and had clear clinical evidence of active disease (eg. hematuria). In those patients for whom BVAS and clinical presentation were discordant (BVAS = 0 with clinical signs and/or symptoms), clear clinical evidence of disease activity superseded BVAS, and these patients were classified as "active."

Any patients designated as having “unclear” disease activity by clinical evaluation, regardless of BVAS, were not used in this study.

The protocol for treatment has been extensively described (19, 20). All patients included in this study received immunosuppressive therapy with corticosteroids and cyclophosphamide or rituximab at disease onset or at time of relapse. Remission maintenance therapy included azathioprine (AZA), mycophenolate mofetil (MMF) or rituximab. Patients with ≥1% B cells at the time of sample collection were included in our analysis. Patients not requiring remission maintenance treatment were followed at close intervals during periods of remission off therapy. ANCA titers were determined by the McLendon Clinical Laboratories at the University of North Carolina using enzyme-linked immunosorbent assay (ELISA) kits specific for either MPO or PR3 (Inova Diagnostics, San Diego, CA).

Blood Collection

Whole blood was collected in Sodium Heparin tubes (BD, Franklin Lakes, New Jersey)**.** To facilitate erythrocyte removal, 1 part HetaSep (STEMCELL Technologies Inc., Vancouver, Canada) was added per 5 parts heparinized whole blood and centrifuged at room temperature at 90 x *g* with the brake off for 6 minutes. The leukocyte-rich supernatant was harvested and layered onto 5 mls of Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged at room temperature at 400 x *g* with no brake for 30 minutes. The buffy coat was washed twice and resuspended in Hank's Balanced Salt Solution (HBSS**,** Life Technologies, Grand Island, NY) supplemented with 2% fetal bovine serum (FBS).

Flow Cytometric Analysis

The expression of cell surface molecules reported to designate Bregs was examined by flow cytometry at the time of blood collection. First, cells were stained with Human TruStain FcX™ Fc Receptor Blocking Solution (Biolegend, San Diego, California) to prevent non-specific antibody binding to Fc receptors. Next, cells were stained with the following fluorochrome-labeled anti-human antibodies: CD19 Pacific Blue (clone HIB19 Biolegend, San Diego,

California), CD38 PerCP-CY5.5 (clone HIT2, Biolegend, San Diego, California), CD24 PE-CY7 (clone ML5 Biolegend, San Diego, California), CD27 Alexa Fluor-647 (clone O323 Biolegend, San Diego, California), and CD5 PE (clone UCHT2 Biolegend, San Diego, California) and then fixed with 1% paraformaldehyde. Cells were analyzed using a LSRII (BD, Franklin Lakes, New Jersey) flow cytometer. Data was analyzed with FlowJo software (Treestar, Ashland, OR). After selection of the lymphocyte population based on forward and side scatter, B cells were gated based on $CD19^+$ staining and categorized according to their expression of CD38 and CD24, CD24 and CD27 and $CD5^+$ subsets of these populations. The gating strategy for each B cell phenotype examined is provided in Supplemental Figure S3-1.

Cell Culture

Human PBMCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco® Life Technologies, Carlsbad, USA) supplemented with 100 U/µg/ml penicillin/streptomycin (Life Technologies, Carlsbad, USA) and 10% FCS (Gibco® Life Technologies, Carlsbad, USA).To ascertain B cell ability to produce IL-10, PBMCs were stimulated with 1 µg/ml recombinant human CD40 ligand (CD40L) (R&D Systems, Inc. Minneapolis, MN) and 1 µg/ml CpG oligodeoxynucleotide (ODN) 2006 (Invivogen, San Diego, CA) for 96 hours. PBMC were cultured for the final 6 hours with 1µl/mL GolgiPlug (BD Biosciences, Franklin Lakes, New Jersey), 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich), St. Louis, Missouri) and 1 µg/mL ionomycin (Sigma-Aldrich, St. Louis, Missouri). $CD19^+IL\text{-}10^+$ B cells were measured by intracellular cytokine staining. To exclude dead cells from our analysis, cells were labeled using the LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Life Technologies, Carlsbad, USA). To prevent non-specific antibody binding, cells were incubated with Human TruStain FcX™ Fc Receptor Blocking Solution (Biolegend, San Diego, California) and stained with CD19 Pacific Blue (clone HIB19 Biolegend, San Diego, California). Post surface staining, cells were fixed and permeablilized using the FIX & PERM® cell fixation and cell permeabilization kit (Life Technologies, Carlsbad, USA). Permeabilized cells were stained with anti-IL-10 antibody (PE,

clone JES3-9D7, Biolegend, San Diego, California). IL-10 expression in $CD19^+$ B cells was assessed relative to a fluorescence minus one (FMO) control where the IL-10 antibody was omitted (21).

Sorting of B Cell Populations

Leukocytes were obtained from healthy controls (Gulf Coast Regional Blood Center; Houston, Texas) and processed as described above to obtain a buffy coat containing lymphocytes. Cells were stained with antibodies to CD19 and CD5 and sorted into $CD19^+CD5^+$ and $CD19^+CD5^{neg}$ populations using a FACSAria II flow cytometer (Becton Dickinson). Cells were collected into Iscove's Modified Dulbecco's Medium (IMDM) containing 50% FBS (unless otherwise specified, all culture reagents from Life Technologies; Grand Island, NY). Sorted populations were washed twice and then cultured in IMDM containing 5% human AB serum, 1µg/ml CpG, 0.1µg/ml CD40L and PenStrep in U-bottom 96-well plates (Falcon, Corning Incorporated; Corning, NY) at 2.5×10^6 cells per ml. After 72-96 hours, cells were processed for IL-10 intracellular staining as described above.

Statistical and Graphical Analysis

Demographic and clinical characteristics were summarized by descriptive statistics. P-values were calculated by Fisher's exact test for categorical variables and Wilcoxon two-sample tests and Kruskal-Wallis test for continuous variables. A paired signed rank test was used to test the paired difference of B cell phenotypes in the subgroups. P-values reported with a two-side p-value of ≤ 0.05 indicate a significant difference. A Bonferroni correction was used in all analyses that compared more than two groups making values ≤ 0.0056 significant. Analyses were conducted using SAS 9.1 (SAS Institute, Cary, NC). Graphs were created using GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA).

Results

Cohort Description

To investigate regulatory B cells in patients with AAV, we analyzed PBMC samples from 69 patients with AAV and 30 HCs by flow cytometry (Table 3-1). Patient samples were evaluated at the time of either active disease or remission. There were no significant differences between active disease patients compared to those in remission with respect to age, sex, ethnicity, ANCA serotype, disease diagnosis, or peak creatinine at disease onset (Table 3-1). A higher proportion of patients with renal limited organ involvement were in remission compared with active disease (22% versus 4%; p=0.04). HCs were significantly younger than patients; however, B cell phenotype and IL-10 production did not correlate with age in patients or healthy individuals (data not shown). Patient therapy is summarized in Table 3-2. Standard induction therapy was high dose prednisolone and cyclophosphamide. Maintenance therapy was comprised of AZA or MMF in combination with low-dose prednisolone. More patients with active disease were treated with cyclophosamide and corticosteroids as well as prednisone than patients in remission (Table 3-2); however, median values of $CD24^{hi}CD38^{hi}$ and $CD5^{+}CD24^{hi}CD38^{hi}$ B cells did not differ between active patients on medication compared to patients prior to initiation of therapy. A greater percentage of patients in remission had been previously treated with rituximab than patients in active disease; however, median values for B cell populations did not differ from those for rituximab-naïve patients with comparable disease activity (data not shown).

$CD24^{hi}CD38^{hi}$ B Cells Do Not Correlate with Disease Activity

For our initial examination of putative regulatory B cells in patients with AAV, we analyzed B cells with the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ phenotypes (Supplemental Figure S3-1). When compared to healthy individuals (median = 11, [IQR = 8,12]), $CD24^{hi}CD38^{hi}$ B cells did not differ in patients with AAV during active disease (8, [3,22]; Figure 3-1A; Table 3-3). Patients in remission, however, had elevated percentages of $CD24^{hi}CD38^{hi}$ B cells (17, [10, 32])

compared to HCs. The trend of increased percentages of $CD24^{hi}CD38^{hi}$ B cells during disease remission when compared to HCs was observed in patients with both MPO and PR3 serotypes, but only reached significance in patients with PR3-AAV (p=0.002, Figure 3-1A). In patients with an MPO-ANCA serotype, $CD24^{hi}CD38^{hi}$ B cells were significantly increased in remission (15 [10, 27] when compared to patients in active disease (5 [2,11]); p=0.002; Figure 3-1A), but not HCs (p=0.03). In patients who were in remission and had previously been treated with rituximab, there was a modest expansion of $CD24^{hi}CD38^{hi}$ B cells (median=22%) compared to patients with no prior rituximab treatment (median=12%). In contrast, there was no difference in the median $CD24^{hi}CD38^{hi}$ Bregs in patients with active disease whether they had been treated with rituximab (median=13%) or not (7%). Regardless of whether patients had prior treatment with rituximab or not, there was no difference in $CD24^{hi}CD38^{hi}$ Bregs during active disease compared to disease remission. No differences were observed in $CD24^{hi}CD27^{+}$ B cells when patients with active or quiescent AAV were compared to HCs or when patients with active disease were compared to patients in remission (Supplemental Figure S3-2A).

<u>$CD5^{+}CD24^{hi}CD38^{hi}$ B Cells are Reduced During Active Disease and Rebound Upon Disease Remission</u>

CD5 is a negative regulator of B cell receptor signaling, and in concert with CD1d (14), CD5 is known to be a marker of B cells that secrete IL-10 in mice. We previously investigated CD5 on total B cells and established its importance as an indicator of AAV disease activity and future relapse when decreasing or low (13). To examine the $CD24^{hi}CD38^{hi}$ B cell subpopulation expressing this inhibitory protein, we analyzed the CD5 marker on $CD24^{hi}CD38^{hi}$ B cells in patients with AAV and healthy individuals. Patients with active disease have significantly lower percentages of the $CD5^{+}$ subset of $CD24^{hi}CD38^{hi}$ B cells ($CD5^{+}CD24^{hi}CD38^{hi}$) (median = 28 [17,40]) than HCs (74 [IQR=50,92], p≤0.0001, Figure 3-1B; Table 3-3). This decrease in $CD5^{+}CD24^{hi}CD38^{hi}$ B cells during active disease was observed in patients of both MPO (24 [0,50]), p≤0.003) and PR3 (29 [20,39]), p≤0.0001) serotypes compared to HCs (Figure 3-1B).

Further, the reduced percentage of $CD5^{+}CD24^{hi}CD38^{hi}$ B cells during active disease increased to an intermediate level not different from HCs during remission for all AAV patients together (54 [31,65], p=0.0064) as well as both MPO (54 [39,59], p=0.05) and PR3 ANCA (48 [30,76], p=0.02) serotypes (Figure 3-1B). The percentage of $CD5^{+}CD24^{hi}CD38^{hi}$ B cells did not differ in patients with active disease whether they had prior treatment with rituximab (median=31%) or not (28%). Likewise, patients in remission had comparable levels of $CD5^{+}CD24^{hi}CD38^{hi}$ B cells whether they had been treated with rituximab (52%) or not (55%). Moreover, the percent $CD5^{+}CD24^{hi}CD38^{hi}$ B cells remains significantly lower (median=28%, p=0.006) in patients with active disease compared to patients in remission (median=55%) when only patients who had not been treated with rituximab are considered. In contrast, examination of the $CD5^{+}$ subset of $CD24^{hi}CD27^{+}$ B cells did not reveal a B cell population that correlated with disease activity. No significant differences were observed in $CD5^{+}CD24^{hi}CD27^{+}$ B cells when patients with AAV were compared to HCs or when patients with active disease were compared to patients in remission (Supplemental Figure S3-2B).

B Cells in Patients With Active Disease Have Reduced Production of IL-10

To determine the competency of B cells to produce IL-10, 33 patients with AAV and 14 HCs were examined for IL-10 producing B cells after 96 hours of stimulation with CD40 ligand and CpG DNA. Percentages of IL-10-producing B cells in patients with AAV during remission (24 [17,34]), of either MPO (24 [23,32]) or PR3 (34 [13,41]) serotype, were similar to healthy individuals (25 [22,34]) with regard to IL-10 producing B cells (Figure 3-1C; Table 3-3). In contrast, B cells from patients with active disease (13 [4,19]) produced significantly less IL-10 than patients in remission (p=0.005) and HCs (p=0.001).

$CD5^{+}CD24^{hi}CD38^{hi}$ and $IL\text{-}10^{+}$ B Cells Normalize as Individual Patients Transition from Active Disease to Remission

To eliminate any inter-patient variation, B cells of individual patients with AAV were analyzed during times of disease activity and remission. Although levels during active disease

did not differ from the baseline level of HCs, paired active disease and remission samples from 9 patients exhibited a significant expansion (p=0.004) of $CD24^{hi}CD38^{hi}$ B cells as individuals transitioned from active disease into remission (Figure 3-2A). Inclusion of CD5 as a marker denoted a B cell population that was significantly decreased in our population studies. This finding was substantiated in our paired analysis. When CD5 was included as a marker, 6 out of 7 paired active and remission disease samples demonstrated an increase of $CD5^{+}CD24^{hi}CD38^{hi}$ B cells as individuals transitioned from active disease to remission (p=0.05, Figure 3-2B). Likewise, paired active and remission disease samples from 6 patients demonstrated a significant increase (p=0.02) in IL-10-producing B cells as individuals transitioned fromina active disease to remission (Figure 3-2C) in all 6 cases.

$CD5^{+}$ B cells are Enriched in B cells Capable of Producing IL-10

Given the similar decrease of $CD5^{+}CD24^{hi}CD38^{hi}$ and $IL\text{-}10^{+}$ B cells during active AAV and similar rebound during disease remission, we tested whether $CD5^{+}$ B cells contained a population of B cells capable of producing IL-10. Enriched B cells, $CD5^{+}$ B cells and $CD5^{neg}$ B cells were evaluated for IL-10 producing B cells after 72-96 hours of stimulation with CD40 ligand and CpG DNA. $CD5^{+}$ B cells are enriched in IL-10 producing B cells (median=19%) when compared to $CD5^{neg}$ B cells (11%) or total B cells (16%, n=2). Representative flow histograms depicting the percentage of $IL\text{-}10^{+}$ B cells in cultured populations of total B cells (Figure 3-3A), $CD5^{neg}$ B cells (3B) and $CD5^{+}$ B cells (3C) demonstrate that $CD5^{+}$ B cells are enriched in IL-10-producing B cells compared to $CD5^{neg}$ and total B cells.

As $CD24^{hi}CD38^{hi}$, $CD5^{+}CD24^{hi}CD38^{hi}$ or $IL\text{-}10^{+}$ Regulatory B Cells Increase, Circulating ANCA Titers Decrease

To determine if the increase in $CD24^{hi}CD38^{hi}$, $CD5^{+}CD24^{hi}CD38^{hi}$ and $IL\text{-}10^{+}$ B cells during disease remission had a suppressive effect, the influence on circulating ANCA titers was calculated. ANCA titers are reported from either MPO-ANCA or PR3-ANCA tests as appropriate. In paired active and remission samples from the same patient, the percentage of

each B cell phenotype during active disease (A) was subtracted from the value during remission (R) to generate the change or delta (Δ; R - A = Δ). Likewise, the ANCA titer during active disease was subtracted from the ANCA titer during remission for each patient. The change in each B cell phenotype is a positive integer when the percent B cells at the remission time point is greater than the percent B cells in active disease. Conversely, the ANCA titer values are negative integers when the ANCA titer is higher in active disease than in disease remission. Figure 4 contains graphical representations of how the B cell phenotype relates to the change in ANCA titer during remission compared to active disease for $CD24^{hi}CD38^{hi}$ (Figure 3-4A), $CD5^{+}CD24^{hi}CD38^{hi}$ (Figure 3-4B), and IL-10^{+} B cells (Figure 3-4C). In all 3 analyses, the trend indicates that as $CD24^{hi}CD38^{hi}$, $CD5^{+}CD24^{hi}CD38^{hi}$ and IL-10^{+} B cells increase, ANCA titer decreases (p=0.02, 0.02 and 0.03 respectively).

Discussion

In this study, we demonstrate that the $CD5^{+}$ subset of $CD24^{hi}CD38^{hi}$ B cells and IL-10^{+} B cells are reduced in patients with active AAV compared with HCs and patients in remission. As patients go into remission, both $CD5^{+}CD24^{hi}CD38^{hi}$ and IL-10-producing B cells are present at levels similar to HCs. These data suggest that $CD5^{+}CD24^{hi}CD38^{hi}$ B cells may infer IL-10-producing B cells. In our population-based examinations, two of the phenotypes reported for human regulatory B cells, $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$, did not correlate with disease activity. Although patients with active disease do not show a significant decrease in $CD24^{hi}CD38^{hi}$ B cells, we did observe an expansion of this population as patients went into remission. In paired, longitudinal analysis of the same patient, $CD5^{+}CD24^{hi}CD38^{hi}$ B cells and IL-10^{+} B cells are similarly decreased during active disease and increased upon disease remission. Moreover, we show that $CD5^{+}$ B cells are enriched in IL-10 producing cells compared to $CD5^{neg}$ B cells. Importantly, for the first time, we demonstrate that an increase in $CD24^{hi}CD38^{hi}$ B cells, $CD5^{+}CD24^{hi}CD38^{hi}$ B cells and IL-10^{+} B cells correlates with a decrease in autoantibody titer, specifically ANCA titer.

In humans, regulatory B cells have not been distinguished from their reported $CD5^{+}CD1d^{hi}$ (14, 22), $CD19^{hi}FcγRIIb^{+}$ (23), $FASL^{+}$ (24), or IL-10-producing counterparts in mice. Human B cells with the phenotype $CD24^{hi}$ and either $CD38^{hi}$ (7) or $CD27^{+}$ (11) have been described as $IL\text{-}10^{+}$ regulatory B cells. These multiple immunophenotypes reported for regulatory B cells may simply indicate multiple Breg subsets with different functions. In a chronic inflammatory environment, a murine antigen-specific $CD1d^{hi}CD5^{+}$ B cell subset has been shown *in vitro* and *in vivo* to differentiate and suppress T cells via IL-10, IL-1β and STAT3 activation and secretion of TGF-α, IFN-γ, and IL-12 (10, 25). CD5 is one of the surface molecules that defines most murine Breg subsets (14). Although not included in the reported definitions of human regulatory B cells, a subset of both of these phenotypes also expressed CD5 in healthy individuals. We have recently shown that CD5 marks B cells that portended active disease when low or decreasing (13), a pattern expected for B cells with a regulatory function. Moreover, CD5 is reported to induce IL-10 expression and promote cell survival in a human Daudi B cell line (26), human chronic lymphocytic leukemia B cells (27), and mice (28). Our own data confirm that $CD5^{+}$ B cells are enriched in IL-10-producing B cells when compared to $CD5^{neg}$ B cells.

In several autoimmune diseases including type 1 diabetes (29), systemic lupus erythematosus (SLE), and AAV (30), regulatory T cells (Treg) are present but lack suppressive ability (7, 31). Regulatory B cells and Type 1 regulatory T cells (Tr1) (32, 33) exert suppressive effects through IL-10, a cytokine which can drive a change in immunological response from T-helper 1 to T-helper 2. A lack of B cell IL-10 is common to several relapsing and remitting inflammatory autoimmune diseases characterized by pathogenic B cells like multiple sclerosis (MS) (34) and SLE (7). IL-10 deficiency infers regulatory B cell malfunction. IL-10-producing B cells have been proven to diminish clinical symptoms in MS (34). An IL-10 dependent increase in Foxp3 expression, a Treg indicator, was shown in the central nervous system after B cell transfer in the experimental autoimmune encephalitis (EAE) mouse model (35). IL-10 secreting

B cells are essential for recovery in arthritis and EAE murine models of human inflammatory autoimmune disease and MS (9, 36). Interestingly, a high capacity to produce IL-10 protects from metabolic syndrome and diabetes mellitus in geriatric adults (37). Much evidence implicates IL-10 as a protective agent in a spectrum of chronic inflammatory diseases. Our findings indicate that IL-10 producing B cells are decreased during active disease and reappear in disease remission at levels similar to healthy individuals. These findings are in line with those of Hruskova and colleagues who showed that patients with AAV in remission who relapsed produced significantly less circulating IL-10 than those without relapse; however, these investigators did not determine the source of IL-10 (38). Patients with AAV have an increased frequency of the IL-10-1082AA genotype that is associated with decreased IL-10 production (39). Our results confirm and extend those of Wilde *et al*. who showed that B cells from 11 patients with active AAV produced less IL-10 (40). In contrast, these investigators also reported a significant decrease in IL-10^{+} B cells during disease remission, whereas we observed an increase in IL-10^{+} B cells during remission to a level that did not differ from that observed in HCs. Todd (41) and Lepse *et al*. (42) also investigated Breg subsets in AAV. Todd *et al*. found that CD19^{+}CD24^{hi}CD38^{hi} cells are more decreased in remission than during active disease; conversely, the 'tolerant' patient population (defined as: 'those with a history of active AAV who subsequently became negative for ANCA by ELISA, remaining free from pathology after withdrawal of treatment for a minimum of 2 years' (41) had the highest values of CD24^{hi}CD38^{hi} B cells that were indistinguishable from the HC population. In their study of patients with PR3-AAV, Lepse *et al*. reported the frequency of CD19^{+}CD24^{hi}CD38^{hi} cells was not different in patients in remission compared with HCs, but was decreased in patients with active disease compared to either HCs or patients in remission. Our findings with regard to CD24^{hi}CD38^{hi} B cells differed from both of these groups which also differed from each other. Our results greatly extend the analysis of this putative Breg phenotype by investigating the CD5^{+} subset of CD24^{hi}CD38^{hi} B cells and demonstrating that CD5^{+}CD24^{hi}CD38^{hi} B cells are decreased during

disease activity and normalize upon remission as expected for a regulatory B cell population. Furthermore, whereas Lepse's group found that $CD24^{hi}CD27^{+}$ B cells were significantly decreased in both remission and active patients when compared with HCs, we found no significant differences in either $CD24^{hi}CD27^{+}$ or $CD5^{+}CD24^{hi}CD27^{+}$ B cell populations (Supplemental Figure S3-2).

The differences in our observations could be due to our strict definition of remission that disallowed inclusion of patients with clinically active disease within 3 months of remission time points or our inclusion of CD40L in B cell cultures for IL-10 stimulation. Although culture conditions were different (2 vs 4 days and slight concentration differences in CpG and CD40L), our findings are in concert with those of Todd *et al.* (41) where $IL\text{-}10^{+}$ B cells in ANCA remission patients do not differ in frequency from HCs. Of note, this is the first demonstration that patients in active disease have fewer $IL\text{-}10^{+}$ B cells that rebound to HC levels when the patient goes into remission.

One strength of this study is the inclusion of patients with both MPO- and PR3-ANCA, as we now realize these are genetically and serologically distinct diseases with different risks for relapse (16, 43). $CD5^{+}CD24^{hi}CD38^{hi}$ B cells and IL-10 producing B cells were decreased in patients with active disease and were similar to healthy individuals when patients were in remission regardless of ANCA serotype. The strongest evidence presented is our analysis of paired samples from the same patient over time demonstrating that a significant increase in $CD24^{hi}CD38^{hi}$, $CD5^{+}CD24^{hi}CD38^{hi}$ and $IL\text{-}10^{+}$ B cells was observed when patients transitioned from active disease to remission. Our results suggest that $CD5^{+}CD24^{hi}CD38^{hi}$ and $IL\text{-}10^{+}$ B cell phenotypes can be used as indicators of disease activity since these B cell populations are decreased during active disease and rebound to levels similar to HCs during remission. Furthermore, it could logically be proposed that therapeutic up-regulation of these regulatory B cells in patients with humoral autoimmune disease could promote disease quiescence.

Rituximab treatment eradicates all peripheral $CD20^+$ B cells indiscriminately and is a prominent, effective therapy for AAV (5, 6). The high values of $CD24^{hi}CD38^{hi}$ B cells (>40% of total B cells) observed in some patients with AAV may be influenced by the repopulation of B cells post rituximab treatment; however, only patients who had repopulated to ≥1% B cells in their lymphocytes were included in our studies. Additionally, there was no correlation between the percentage of total B cells present and the percentages of $CD24^{hi}CD38^{hi}$, $CD5^+CD24^{hi}CD38^{hi}$ or IL-10^+ B cells detected (data not shown). Moreover, prior rituximab therapy did not significantly alter median values for B cell populations relative to those for rituximab-naïve patients with comparable disease activity. Regardless of whether patients had been treated with rituximab or not, there was no difference in $CD24^{hi}CD38^{hi}$ B cells during active disease compared to disease remission. In contrast, the percent $CD5^+CD24^{hi}CD38^{hi}$ B cells remained significantly lower in patients with active disease compared to patients in remission when only rituximab-naïve patients were considered.

Patients with SLE that repopulated with $CD24^{hi}CD38^{hi}$ B cells, which have been described as both transitional and regulatory, had a longer time to relapse post-rituximab therapy, suggesting that the phenotype of repopulating B cells may be important to follow with respect to disease outcome (44). Our own previous findings demonstrate that repopulation with a low percentage of $CD5^+$ B cells portends a shorter time to relapse after B cell depletion with rituximab (13). Addition of CD5 to this $CD24^{hi}CD38^{hi}$ B cell phenotype denotes a crucial B cell subpopulation that not only correlates inversely with active disease but also parallels IL-10 production and suppressive function.

Elucidation of crucial molecules that define and orchestrate the regulatory functions of B cells including the suppression of pathogenic autoantibodies is crucial to the development of more directed and safer therapies for individuals who suffer from AAV and conceivably, autoimmune diseases as a whole. Our data are consistent with the hypothesis that functionally competent regulatory B cells characterized as $CD5^+CD24^{hi}CD38^{hi}$ and IL-10^+ support long-term

clinical remission by inhibiting production of autoantibodies that drive disease pathogenesis. Whether the $CD5^{+}CD24^{hi}CD38^{hi}$ or IL-10 producing B cells can prospectively guide immunosuppressive therapy in patients to prevent unnecessary treatment and ensure treatment when appropriate is an open question that would be best answered in a clinical trial.

Table 3-1. Patient and Healthy Control Demographic Characteristics

Characteristic	Active (n=28)	Remission (n=41)	Healthy Controls (n=30)	P value*
Age				<0.0001
Median (IQR)	60 (42, 69)	59 (54, 72)	32 (26, 48)	
Sex				0.43
Female	16 (57%)	26 (63%)	22 (73%)	
Ethnicity				0.47
Asian	0 (0%)	1 (2%)	2 (7%)	
Black	4 (15%)	8 (20%)	2 (7%)	
Hispanic	1 (4%)	2 (5%)	0 (0%)	
White	21 (81%)	30 (73%)	25 (86%)	
ANCA			NA	0.34
PR3	15 (54%)	17 (41%)		
MPO	13 (46%)	24 (59%)		
Disease			NA	0.18
GPA	9 (39%)	14 (34%)		
MPA	13 (57%)	18 (44%)		
GN	1 (4%)	9 (22%)		
Organ involvement			NA	
Upper Respiratory	11 (48%)	24 (60%)		0.43
Pulmonary	13 (57%)	18 (44%)		0.43
Renal Limited	1 (4%)	9 (22%)		0.04
Peak Creatinine at disease onset			NA	0.12
n/N	20/28	38/41		
Median (IQR)	2.1 (1.4, 3.0)	1.4 (0.9, 2.8)		
BVAS			NA	<0.0001
n/N	26/28	41/41		
Median (IQR)	6.5 (2, 12)	0 (0, 0)		

Data are summarized as number (n) and percent (%) or median with interquartile range (IQR). MPO, myeloperoxidase; PR3, proteinase 3; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; ANCA, antineutrophil cytoplasmic autoantibody; GN, glomerulonephritis; BVAS, Birmingham Vasculitis Activity Score; n, number of observations; N, total number of patients.

*P values were calculated by Fisher Exact Test for categorical variables and Wilcoxon Two-Sample Tests and Kruskal-Wallis Test for continuous variables.

Table 3-2. Medications

	Active N=27*	Remission N=39	P values**
Cyclophosphamide and corticosteroids	12 (44%)	1(3%)	0.000034
Mycophenolate mofetil (MMF)	6 (22%)	8 (21%)	1.0000
Azathioprine	3 (11%)	5 (13%)	1.0000
Prednisone	11 (46%)	6 (15%)	0.0176
Off therapy	6 (22%)	17 (44%)	0.1144
Prior rituximab therapy (>1% B Cells)	9 (33%)	29 (74%)	0.0012

*n=1 active and n=3 remission patients had no medications documented in clinic records

**P values were calculated by Fisher's exact test.

Table 3-3. Regulatory B cell Phenotypes in Healthy Controls and Patients with ANCA Vasculitis

B Cell Population	Healthy Controls	All ANCA Active	All ANCA Remission	MPO Active	MPO Remission	PR3 Active	PR3 Remission
$CD24^{hi}CD38^{hi}$ *(%$CD19^+$ B cells)* *(IQR)* *number*	11 (8, 12) n=30	8 (3, 22) n=29	17[a] (10, 32) n=43	5[c] (2, 11) n=13	15 (10, 27) n=25	14 (4, 35) n=16	23[a] (12, 33) n=18
$CD5^+CD24^{hi}CD38^{hi}$ *(%$CD24^{hi}38^{hi}$ B cells)*	74 (50, 92) n=23	28[a, b] (17, 41) n=24	54 (31, 65) n=35	24[a] (0, 50) n=10	54 (39, 59) n=18	29[a] (20, 39) n=14	48 (30, 76) n=17
IL-10^+ B cells *(%$CD19^+$ B cells)*	25 (22, 34) n=14	13[a, b] (4, 19) n=10	24 (17, 34) n=23	7 (4, 14) n=5	24 (23, 32) n=15	19 (12, 19) n=5	34 (13, 41) n=7

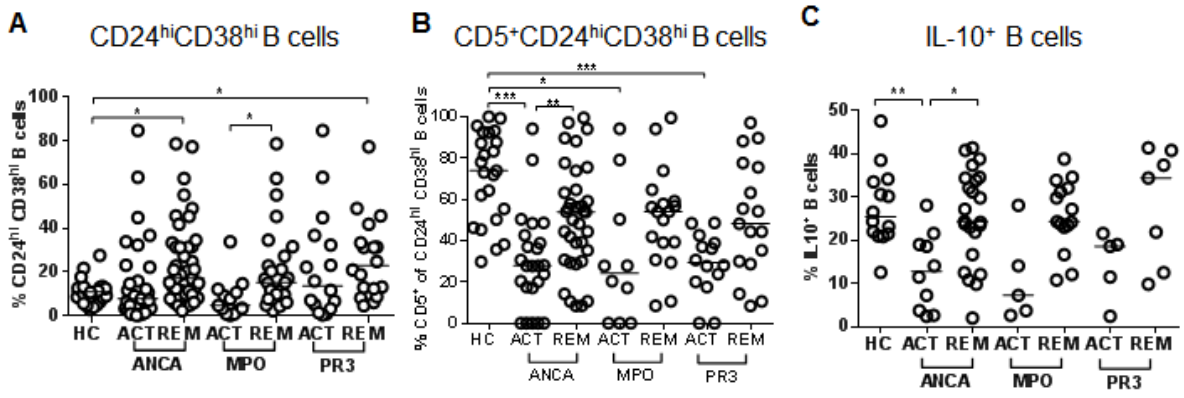
Data are presented as the median and interquartile range (IQR). The number of samples examined (n) is also given. P values were calculated by a Wilcoxon two sample test. Due to multiple comparisons between groups, a Bonferroni correction was applied resulting in a p value ≤ 0.006 being considered statistically significant.

[a]Statistically different from healthy control

[b]Statistically different from all ANCA remission

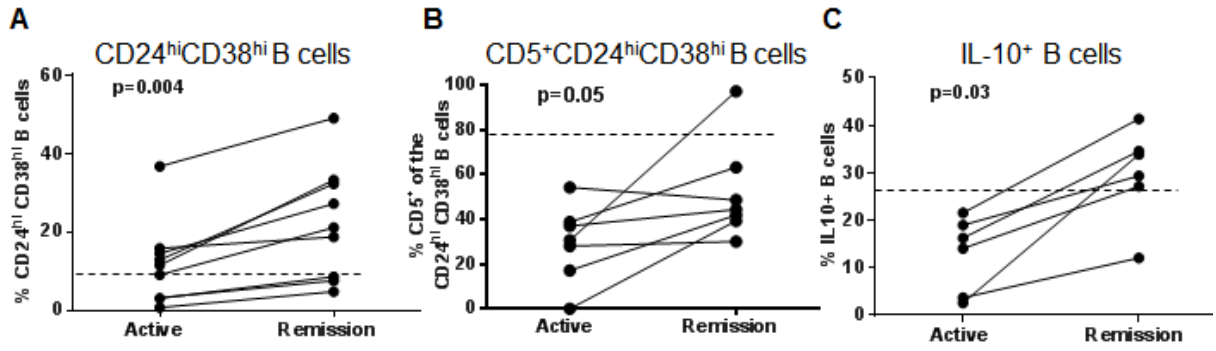
[c]Statistically different from MPO-ANCA remission

Figure 3-1. CD5+CD24^{hi}CD38^{hi} and IL-10+ B cells decrease during active disease and rebound during remission



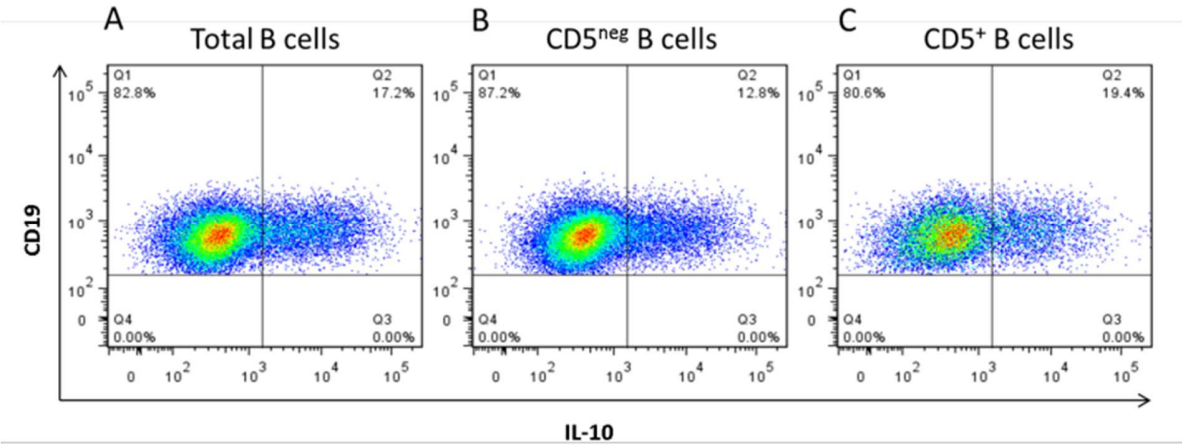
Population analysis of the percentage of CD24^{hi}CD38^{hi} B cells (A), the CD5^{+} subset of CD24^{hi}CD38^{hi} B cells (B) and IL-10^{+} B cells (C) is shown for healthy controls (HC, n=14-30), patients with active AAV (ANCA ACT, n=10-29), remitting AAV (ANCA REM, n=23-43), active MPO-ANCA (MPO ACT, n=5-13), remitting MPO-ANCA (MPO REM, n=15-25), active PR3-ANCA (PR3 ACT, n=5-16), and remitting PR3-ANCA (PR3 REM, n=7-18). IL-10 producing B cells (C) were analyzed after 96 hours of PBMC stimulation with CD40 ligand and CpG DNA. Aggregate data show increased CD24^{hi}CD38^{hi} B cells during disease remission (A) but no significant change during active disease. Percentages of both the CD5^{+} subset of CD24^{hi}CD38^{hi} B cells and IL-10^{+} B cells decrease during active disease and rebound during remission. Lines indicate median values. * indicates p≤0.005; ** indicates p≤0.001 and *** indicates p≤0.0001.

Figure 3-2. CD24^{hi}CD38^{hi}, CD5+CD24^{hi}CD38^{hi} and IL-10+ B cells increase during remission in paired active and remission samples.



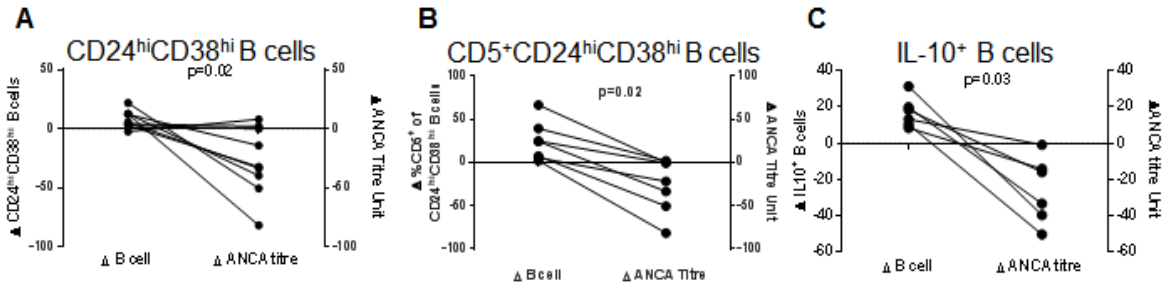
Paired active and remission disease samples from 9 patients demonstrate an increase in CD24^{hi}CD38^{hi} B cells (A) as an individual transitions from active disease to remission. Paired active and remission disease samples from 7 patients demonstrate that the CD5^{+} subset of CD24^{hi}CD38^{hi} B cells (shown in B) decreased in patients with active disease and increased during remission. Paired active and remission disease samples from 6 patients demonstrate a similar increase in CD19^{+}IL-10^{+} B cells (C) as all individuals transition from active disease to remission. The dashed line indicates the median value for healthy controls.

Figure 3-3. CD5+ B cells are enriched in B cells capable of producing IL-10



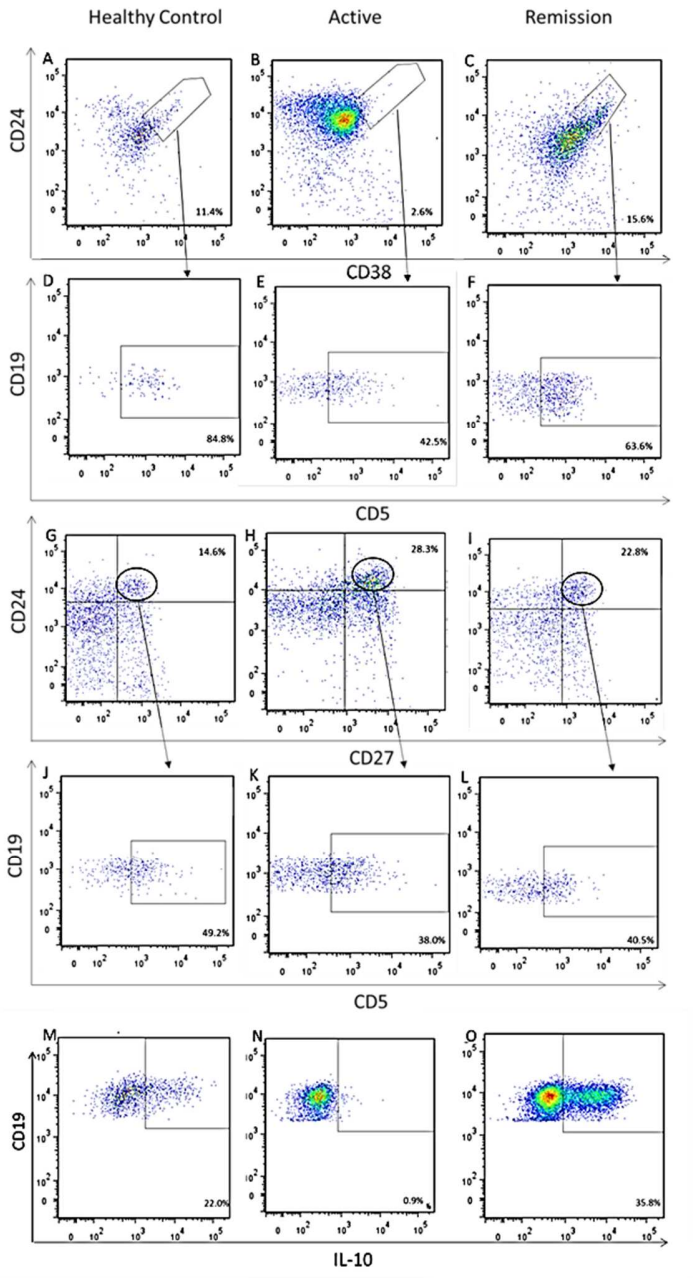
Total B cells, $CD5^{+}$ B cells and $CD5^{neg}$ B cells were evaluated for IL-10 producing B cells after 72-96 hours of stimulation with CD40 ligand and CpG DNA. Shown are representative flow histograms depicting the percentage of $IL\text{-}10^{+}$ B cells in cultured populations of total B cells (A), $CD5^{neg}$ B cells (B) and $CD5^{+}$ B cells (C) demonstrating that $CD5^{+}$ B cells are enriched in IL-10-producing B cells compared to $CD5^{neg}$ and total B cells.

Figure 3-4. As CD24^{hi}CD38^{hi}, CD5^{+} CD24^{hi}CD38^{hi} or IL-10^{+} B cells increase, circulating ANCA titers decrease



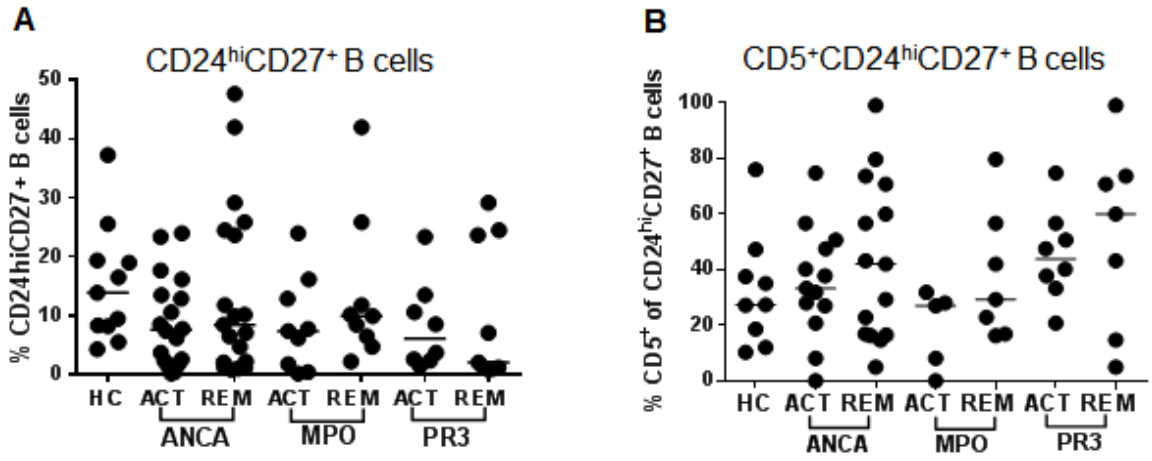
Paired active and remission sample data was used to generate the change (Δ) in each B cell subset CD24^{hi}CD38^{hi} (A) CD5^{+}CD24^{hi}CD38^{hi} (B) or IL-10^{+} (C) by the subtraction of the active value from the remission value (R - A = Δ). A positive value for ΔB cell subset indicates a greater percentage of B cells present during remission than during active disease. The change in ANCA titer was generated by subtracting the ANCA titer during active disease from the ANCA titer at remission utilizing the same time points used for B cell subset data. A negative value for ΔANCA titer indicates a greater titer during active disease than remission. Statistical significance was determined with a signed rank test; p≤0.05 is considered significant.

Supplemental Figure S3-1. Gating strategies for B cell subpopulations



Shown are the gating strategies for $CD24^{hi}CD38^{hi}$ (A-C), $CD5^{+}CD24^{hi}CD38^{hi}$ (D-F), $CD24^{hi}CD27^{+}$ (G-I), $CD5^{+}CD24^{hi}CD27^{+}$ (J-L) and $IL\text{-}10^{+}$ B cells (M-O). Typical examples of these B cell subpopulations observed in HCs (A, D, G, J and M), patients with active AAV (B, E, H, K and N) and patients in remission (C, F, I, L and O) are provided.

Supplemental Figure S3-2. CD24^{hi}CD27^{+} and CD5^{+}CD24^{hi}CD27^{+} B cells do not correlate with disease activity



Percentages of CD24^{hi}CD27^{+} B cells in healthy controls (n=11), patients with active ANCA (n=18), ANCA patients in remission (n=19), active MPO-ANCA (n=9), remission MPO-ANCA (n=9), active PR3-ANCA (n=8) and remission PR3-ANCA (n=9) are shown (A). Percentages of the CD5^{+} subset of CD19^{+}CD24^{hi}CD27^{+} B cells are depicted for healthy controls (HC, n=9), patients with active ANCA (n=13), ANCA patients in remission (n=15), active MPO-ANCA (n=5), remission MPO-ANCA (n=7), active PR3-ANCA (n=8) and remission PR3-ANCA (n=7) are presented (B).

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CHAPTER 4: DISCUSSION

Discussion

In order to better treat patients with AAV, the role of regulatory B cells in this disease needs to be better defined. With this in mind, this work addresses the repertoire of regulatory B cell subsets and how their ability to secrete IL-10 (B10pro) impacts AAV patients. In the first study, we demonstrate that a low percentage (≤30%) of circulating $CD5^+$ B cells correlates with disease activity and a shorter time to relapse. The percent of $CD5^+$ B cells observed in remission patients was similar that of healthy controls (HC), and both were significantly higher than in patients with active disease. After Rituximab therapy, low or declining %$CD5^+$ B cells was associated with a shorter time to disease relapse among patients on either no or low dose maintenance therapy with mycophenolate mofetil (MMF). The results of this study have recently been confirmed in a larger cohort of patients in collaboration with the clinical labs at UNC Hospital (manuscript pending publication in the Annals of Rheumatic Disease). In this study, patients were separated into assessment groups solely based on their $CD5^+$ B cells at repopulation post-rituximab therapy. Patients who repopulated with low $CD5^+$ B cells relapsed sooner than patients who repopulated with high $CD5^+$ B cells (median 16 months (IQR=12, 19) vs. 23 months (18,30); p=0.005) after rituximab treatment (Figure 4-1). This study expands our finding that low %$CD5^+$ B cells at B cell repopulation portends a shorter time to relapse following rituximab therapy regardless of immunosuppression dose. This indicates that monitoring $CD5^+$ B regulatory cells repopulation may serve as an immunological biomarker to follow induction of remission or detect impending flare.

Regulatory B Cells in AAV

To date, three publications [Wilde *et al.* (2), Todd *et al.* (3) and Lepse *et al.* (4)] address Breg cell surface phenotypes, B10 cells and B10pro cells and their function in AAV. All three

publications document unique criteria for their cohorts and experimental design, i.e. inclusion/exclusion guidelines, activity status assessment, Breg subsets evaluated, and culture system timing and components. While these differences limit the ability to compare findings amongst research teams, they broaden the understanding of the role of Bregs in AAV and in human autoimmune disease. Importantly, all three studies have European cohorts and therefore include a predominance of patients with PR3-ANCA- rather than MPO-ANCA-associated disease. Thus, it is conceivable that the results, due to differences in genetic and epigenetic factors, cannot be extrapolated to patients with MPO-ANCA-associated disease.

Our results confirm and add to those of Wilde *et al*., who show that B cells from 11 patients with active AAV produced less IL-10 (2). Notably, these investigators reported a significant decrease in IL-10^{+} B cells in disease remission when compared to HCs, whereas we observed an increase in IL-10^{+} B cells during remission to a level that did not differ from that observed in HCs.

Todd *et al*. (3) found that the frequency of circulating CD24^{hi}CD38^{hi} cells is decreased in AAV patients in remission more so than during active disease; however, the “tolerant” patient population (defined as: “those with a history of active AAV who subsequently became negative for ANCA, remaining free from pathology after withdrawal of treatment for a minimum of 2 years” (3)) had the highest values of this subset of circulating Bregs, indistinguishable from the HC population. Lepse *et al*. (4) reported the frequency of circulating CD24^{hi}CD38^{hi} cells was not different in PR3-AAV patients in remission compared with HCs, but instead it was decreased in patients with active disease compared to either HCs or patients in remission.

Our results greatly increase the phenotypic analyses of Bregs by investigating the CD5^{+} subset of CD24^{hi}CD38^{hi} B cells and demonstrate that modulations in these cells do occur in different disease states: CD5^{+}CD24^{hi}CD38^{hi} B cells are decreased during disease activity and increase upon remission. Furthermore, we found no significant differences in either CD24^{hi}CD27^{+} or CD5^{+}CD24^{hi}CD27^{+} B cell populations (Chapter 3, Supplemental figure S3-2),

whereas Lepse's group found that $CD24^{hi}CD27^{+}$ were significantly decreased in both remission and active patients when compared with HCs (4). This may be an effect due to different protocols of therapeutic interventions, their effects on B cell sub-populations and small cohort sizes. A collaborative study with a larger cohort size would help to better understand the changes in B cell populations. Data from the RAVE trial will help elucidate the effects of medication on B cell population dynamics (the RAVE trial will be discussed in greater detail in a later section).

An important difference between our study and the aforementioned studies is the inclusion of patients treated with rituximab, a $CD20^{+}$ B cell depleting monoclonal antibody that is being used with increasing frequency in patients with AAV for induction therapy both at onset of disease and flare (5, 6), and even to maintain durable remission (7). Neither Wilde *et al*. (2) nor Lepse *et al*. (4) included any rituximab-treated patients. Todd *et al.* (3) included a sub-cohort of 7 patients who had been treated with rituximab between 6 months and 7 years prior to enrollment and analyzed their $CD24^{hi}CD38^{hi}$ Bregs separately from their population studies. Among the non-rituximab-treated cohort, they observed no significant difference between patients with active disease compared to those in remission in percentage of B cell population comprising B10pro cells. However, 4 of the 7 rituximab-treated patients (3 were lost to follow-up) were found to have up to 80% of their total B cell population comprising B10pro cells suggesting that rituximab therapy promotes Bregs, specifically B10 cell proliferation.

The differences in our observations from those of Wilde *et al.* (2), Todd *et al.* (3) and Lepse *et al.* (4) could be a result of our strict definition of remission, which excluded patients with clinically active disease within 3 months of remission time points, or our inclusion of CD40L in B cell cultures. Although culture conditions were different (2-4 days, differences in stimulants like CpG, anti-IgM, and CD40L and in their concentrations), our findings are in concert with those of Todd *et al.* (3) where $IL10^{+}$ B cells in ANCA remission patients do not differ in frequency from HC. Of note, our study used the largest cohort (almost doubling those published

in AAV/Breg literature) and is the first demonstration that patients with active disease, regardless of serotype, have fewer $IL10^{+}$ B cells that then rebound, to levels seen in healthy individuals, as patients transition from active disease to remission.

Another strength in our studies that differs from those previously published is the inclusion of equal amounts of patients with both MPO- and PR3-ANCA. This inclusion is important because we now realize these are genetically and serologically distinct diseases with different risks for relapse (8, 9). $CD5^{+}CD24^{hi}CD38^{hi}$ B cells and IL-10 producing B cells were decreased in patients with active disease and were similar to healthy individuals when patients were in remission regardless of ANCA serotype. The strongest evidence presented is our analysis of paired samples from the same patient over time demonstrating that, in all cases, a significant increase in $CD24^{hi}CD38^{hi}$, $CD5^{+}CD24^{hi}CD38^{hi}$ and $IL\text{-}10^{+}$ B cells was observed when patients transitioned from active disease to remission.

CD5

CD5 is one of the surface molecules that defines most murine Breg subsets (10). CD5 is reported to induce IL-10 expression and promote cell survival in human B cells (11), human chronic lymphocytic leukemia B cells (12), and mice (13). In mice, $CD5^{+}CD1d^{hi}$ B cells are enriched in B10 cells and have a regulatory function evidenced by their inhibition of INF-γ and TNF-α expression in T cells (10). Although not included in the phenotypic definitions of human Bregs (Table 4.1), a subset of both of these phenotypes also expressed CD5 in healthy individuals. The $CD24^{hi}CD38^{hi}$ phenotype of Bregs was also reported to be $CD5^{+}IgM^{+/hi}IgD^{+/hi}CD10^{low/+}CD27^{-}CD1d^{hi}$ (13). We propose that the CD5 marker is an important measure of Bregs based on our data demonstrating a correlation with $CD24^{hi}CD38^{hi}$ and $IgM^{+}CD5^{+}$ subpopulations (shown in Chapter 2). We have recently shown that CD5 identifies B cells that portend active disease when percentages of $CD5^{+}$ B cells are low or decreasing (14). As indicated previously, our second study ties $CD5^{+}$ B cells with the published human Breg phenotypes, $CD24^{hi}CD38^{hi}$ (15) and $CD24^{hi}CD27^{+}$(16) and sub-classifies them further by

analyzing their $CD5^+$ subsets. We added CD5 to our analysis of the reported phenotypes for circulating regulatory B cells to better understand the putative functional role of CD5 in Bregs. None of the aforementioned Breg/AAV studies include CD5 analysis. $CD5^+$ B cells are present in the periphery at a higher frequency and phenotypically overlap with many of the reported Breg phenotypes, including naïve and memory B cells, and therefore are a bridge between the two reported human Breg phenotypes: $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$. A major challenge in connecting $IL10^+$ and $CD5^+$ B cells to confirm that they are the same subset is that, in our culture system, CD5 is down regulated and not detectable at the time that IL-10 is analyzed. Our hypothesis is that CD5 has transformed to an intracellular form. Our future work will investigate this hypothesis by measuring intracellular CD5 using a CD5 antibody that detects the intracellular form exclusively. Alternatively, CD5 may be sloughed from the surface, which could be measured by ELISA of culture supernatants. Both these experiments will yield data to increase our understanding of CD5 activity with relation to B cells. If either of these experiments relate to AAV disease activity, then they could possibly be translated into a non-invasive clinical test.

Limitations

This doctoral work contributes to the body of knowledge of Breg identification; however, until there can be agreement about which B cells to analyze, we are limited in our investigations. The relatively small sample size of patients with longitudinal data limits our ability to evaluate the correlation between the percentage of $CD5^+$ B cells and time to relapse while correcting for other risk factors such as PR3-ANCA, organ involvement or disease phenotype. Although we attempted to obtain patient samples every 3 months, the timing of our blood collections was not standardized, as samples were obtained from patients whenever they presented for care.

Another complication in the investigation of regulatory B cells is that we are limited to studying circulating B cells in humans. The majority of the regulatory B cell research has been done in murine models using spleen and tissue samples (17). As we work with human subjects,

it is difficult to obtain tissue samples from which viable B cells can be harvested for experimental studies. Unfruitful attempts were made in the beginning of this investigation to analyze B cells from urine, nasal lavage, and bronchoalveolar lavage samples from patients with AAV, as the signal to noise ratio compromised the data rendering it unreliable.

Medications also confound the data pertaining to Bregs in the natural state of AAV during active disease and remission. To better understand the role of regulatory B cells, future studies should compare B10 frequency, IL-10 production, plasma cell differentiation, Ig isotype, and glycosylation among healthy individuals and patients with AAV during active disease and remission. These issues are being addressed with the study of new onset patients with ANCA who have not received any treatments and in long-term remission off-therapy (L-TROT).

Future Directions

What is the Cell Surface Phenotype of an IL-10^{+} B Cell?

The first question to address is which B cell subset makes IL-10. To identify B10 and B10pro cells we will sort CD5^{+}CD24^{hi}CD38^{hi} to culture for IL-10 production. Once this is established in healthy individuals, we can then survey patients with AAV to better understand if B cell IL-10 production is compromised. One limitation is cell number; B cells are a minority of even the lymphocyte population, and obtaining a yield that is culturable may be a challenge. This line of investigation may also help with B10 and B10pro biomarker identification that may lead to a consensus among investigators. Finally, we may be able to isolate these cells for better understanding of B10 mechanism, effects on other cells, characterization of other cytokines that may be essential for their suppressive actions, and expansion of regulatory B cells for adoptive cellular immunotherapies to use in patients for recalibration of immunological tolerance.

Can Regulatory B Cells Suppress Autoantibody Production in Humans?

To test the ability of B10pro cells to suppress ANCA production, we will culture B cells from ANCA patients and HCs with a cytokine cocktail including IL-21 to induce plasma cell

differentiation with MPO or PR3 to promote ANCA production (18, 19). Autologous B cells will be cultured separately with CD40L and CpG then added to the plasma cell cultures. Bregs will not be included in the positive control samples. We will then perform ANCA and total immunoglobulin ELISAs of culture supernatants and confirm plasma cell differentiation by flow cytometry. Understanding regulatory B cell functions will aid in the understanding of how immunological tolerance is broken and maintained in AAV immunopathogenesis. This will also allow clinicians to ensure that they are treating patients effectively and potentially change the regimen in which rituximab is used.

How Do Therapeutic Interventions Affect Breg Populations?

Rituximab in ANCA-Associated Vasculitis (RAVE) trial, a multicenter, randomized trial comparing the effects of rituximab compared with cyclophosphamide (CYC) followed by azathioprine (AZA) for the induction of remission generated B cell data for 197 subjects with ANCA-positive patients diagnosed with Granulomatosis with Polyangiitis (GPA) or Microscopic Polyangiitis (MPA) (5). This study has just been completed and all data from this trial are publicly available to the via the ITN TrialShare system at: https://www.itntrialshare.org/ar/figures.html. This will be a clear next step in further confirmation of $CD5^+$ B cell population in AAV remission maintenance and will help glean information about how the medications rituximab, CYC and AZA impact changes in B cell populations.

Can the Analysis of Circulating $CD5^+$ and IL-10^+ Regulatory B Cells Help Guide Clinical Decision-Making to Promote Durable Remission in AAV?

Another future direction will be to test the hypothesis that normalization of IL-10-secreting $CD5^+$ regulatory B cells is protective of future relapse in a proof-of-concept, randomized clinical trial. CD5 is reported to drive IL-10 expression and therefore may identify Bregs poised to produce IL-10, underscoring the importance of this cellular marker (11, 12). To avoid adverse events from therapy (i.e., infections), clinicians require improved markers of disease activity and impending relapse to guide immunosuppression strategies post-rituximab

and induction of remission therapy. We hypothesize that $CD5^+$ B cells and other Bregs studied in this investigation can serve as biomarkers of active disease. Our data suggest that patients whose $\%CD5^+$ B cells remain low or decline after a period of normalization following rituximab therapy would be at higher risk of subsequent relapse and would likely benefit from maintenance immunosuppression. Conversely, such immunotherapy should be avoided in patients who maintain a normal $\%CD5^+$ B cells. Our hypothesis is that numerical and functional changes in circulating $CD5^+$, $CD5^+CD24^{hi}CD38^{hi}$ and B10pro B cell subsets are related to disease activity and predict response to treatment and disease relapse in AAV. To improve disease monitoring and enable prevention of relapse via identification of sensitive diagnostic and more specific therapeutic strategies, we will follow Breg populations in patients with AAV. Clinicians may use this information to make informed decisions concerning use maintenance immunosuppression and its timing.

We propose a proof-of-concept prospective, randomized, open-label clinical trial whereby patients who recover a normal proportion of Bregs at the end of induction therapy will be followed expectantly without immunotherapy. Patients who do not recover regulatory B cells will be randomized to either maintenance immunosuppressive therapy or clinical monitoring with immunotherapy guided by clinical signs of active vasculitis (Figure 4-2).

In the second study, CD5 appears again as a potential marker for $IL\text{-}10^+$ B cells: specifically, the $CD5^+CD24^{hi}CD38^{hi}$ population, which modulates with AAV disease activity and may be a new (more specific $CD5^+$ B cell subset) Breg cell surface marker phenotype to follow in patients. Further, this study demonstrates that as Bregs increase (either $CD24^{hi}CD38^{hi}$, $CD5^+CD24^{hi}CD38^{hi}$ or $IL\text{-}10^+$ B cells), circulating anti-neutrophil cytoplasmic antibody (ANCA) titers decrease, indicating an important and protective role for Bregs in autoimmune disease (1).

<u>Breg Adoptive Cellular Immunotherapy</u>

Clinical trials using adoptive cellular immunotherapies (ACI) with expanded or genetically manipulated autologous cells for treatment of cancer and autoimmune diseases like T1D have

shown to be effective (20). The current immunosuppressive drug regimen for treatment of AAV non-specifically suppresses the immune system and compromises the patent, making them vulnerable to adverse events like infection, the leading cause of mortality for patients with AAV (21). Bregs are believed to work in an antigen-specific mechanism, and systemic administration of a cytokine like IL-10 may be ineffective due to its short half-life. Furthermore, IL-10 may be potentially harmful to the patient due to its pleiotropic action. Expanded autologous Bregs may deliver IL-10 in antigen specific manner directly to the cellular environment where it will be effective in attenuating the pathogenic autoimmune response.

Bregs and Medications in AAV

The last two decades have witnessed a marked improvement in the induction treatment of patients with AAV, with remission rates around 80% (16-18). In the second chapter, we determined that a low percent (≤30%) of circulating $CD5^+$B cells correlates with disease activity and a shorter time to relapse. After rituximab therapy, low or declining %$CD5^+$ B cells was associated with a shorter time to disease relapse among patients on no or low-dose maintenance therapy with MMF. The use of full dose MMF was associated with a longer time to relapse in the setting of a low %$CD5^+$ B cells. Clinicians may monitor patients' B cell CD5, which is available in many hospital rituximab panels, and if this population is ≤30% or declining, they may choose to use MMF. Conversely, if a patient is in remission and their %$CD5^+$ B cells is >30% and rising, they may wean the subject off therapy and monitor them more closely.

Because the risk of relapse is not uniform for all patients with AAV, Bregs may be important in identifying alternative treatment strategies. No clinical or serologic measure is currently available that allows effective disease monitoring and distinguishes patients in long-term stable remission from those at imminent risk of relapse (22-26). Such a tool would allow physicians to better tailor the duration and intensity of immunosuppressive therapy based on the individual patient's needs. Our goal was to evaluate whether certain B cell subpopulations could be used to assess immunologic disease activity or a patient's risk of relapse. Our results

suggest that $CD5^{+}CD24^{hi}CD38^{hi}$ and $IL\text{-}10^{+}$ B cell phenotypes can be used as indicators of treatment responsiveness and disease activity. Furthermore, it could also be proposed that therapeutic up-regulation of regulatory B cells in patients with humoral autoimmune disease could promote disease quiescence.

Rituximab Therapy in AAV

Patients in our study were treated with rituximab for induction of remission after a clinical relapse (to avoid repeat exposure to cyclophosphamide) or because of persistent disease activity despite cyclophosphamide and corticosteroids. Due to the efficacy of rituximab in treating active AAV, some clinicians view rituximab as a possible maintenance therapy to administer to patients at regular intervals regardless of clinical signs of disease activity, thus, permanently depleting them of B cells (37). It is possible that a state of immune tolerance may require the presence of robust Breg and/or Treg populations, which would be prevented by sustained B cell depletion. It will be interesting to test the validity of our hypothesis in a setting where a robust $CD5^{+}$ Breg population may be suppressed by a regimen of prolonged B cell depletion. Patients with SLE that repopulated with $CD24^{hi}CD38^{hi}$ B cells had a longer time to relapse post-rituximab therapy, suggesting that this may be an important B cell population to follow with respect for disease outcome (22). Addition of CD5 to this $CD24^{hi}CD38^{hi}$ B cell phenotype denotes a crucial B cell subpopulation that not only correlates inversely with active disease but also parallels IL-10 production and suppressive function.

Conclusion

The main objectives of this investigation were to gain insight into regulatory (and potentially protective) B cells in patients suffering from AAV and to understand the contribution of regulatory B cells to maintenance of immunological homeostasis in order to harness them for safer, directed immunotherapeutics and ultimately improve patient care. B cells in patients with ANCA are autoantigen-specific and a subset of B cells produce pathogenic ANCA, which implicates them as one of the principal problems in this disease; thus, B cells are an important

target for therapy. Our results add to accumulating evidence that a paucity of Bregs or non-functional Bregs are associated with increased disease activity in autoimmune disorders (13,14, 31). Our findings suggest that a robust Breg subpopulation could be a goal of immunotherapy, as well as a means of monitoring its efficacy. This hypothesis would best be tested prospectively in a clinical trial setting. This study contributes to information pertaining to a B cell subset that may be important for patients to maintain disease remission and suppress pathogenic ANCA production. These data provide an initial window into the idea of tailoring individual therapies, and may lead to B cell-specific therapies that can induce durable remission in AAV.

One of the main problems in harnessing the power of B10 cells is their basic identification. Distinct membrane markers or transcription factors are necessary for a more accurate and standardized identification. We may be analyzing many Breg subsets that obscure our ability to understand their qualities and function. Further, there is disagreement with regard to stimulation strategies *ex vivo* to harness their regulatory properties. This study begins to shed light on the phenotype of an IL-10 producing B cell and suggests that they have multiple functions, including Treg induction, monocyte suppression and control of autoantibody production. Furthermore, understanding how Bregs suppress pathogenic ANCA production is essential to AAV remission strategies and will shed light on basic mechanisms of Breg function.

Despite a lack of consensus on the immunophenotype of Breg subsets, our data are still consistent with the generalized knowledge in this area and extend our understanding of this elusive cell type (14). We identified a $CD5^+$ B cell subpopulation as a potential immunological marker of sustained remission when robust, or a harbinger of subsequent relapse when low or declining. We also demonstrate that the $CD5^+$ subset of $CD24^{hi}CD38^{hi}$ B cells and IL-10^+ B cells are reduced in patients with active AAV compared with healthy controls and patients in remission. As patients go into remission, both $CD5^+CD24^{hi}CD38^{hi}$ and IL-10-secreting B cells are present at levels similar to healthy controls. These data suggest that $CD5^+CD24^{hi}CD38^{hi}$ B

cells may infer IL-10-secreting B cells. These findings may also offer a clinical tool to monitor disease activity and modulate maintenance immunotherapy. Finally, we demonstrate that an increase in B cells with these regulatory phenotypes correlates with a decrease in autoantibody titer, specifically ANCA titer.

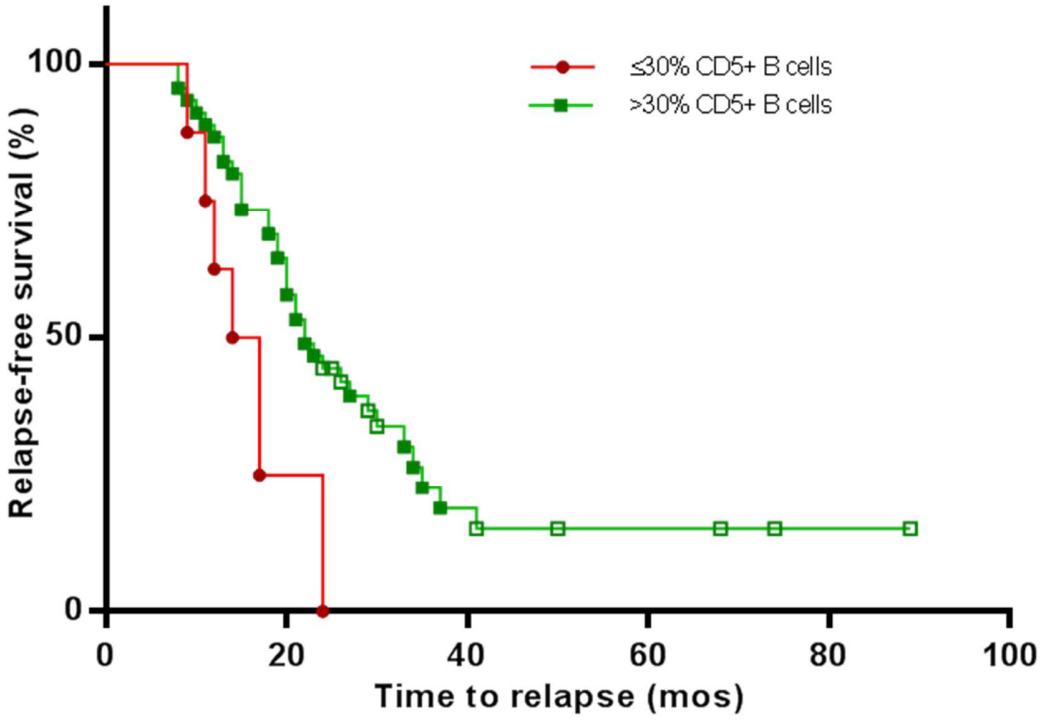
Based on these findings, we predict that CD5+ B cells (more specifically, the CD5+CD24^{hi}CD38^{hi}) are enriched in IL-10+ B cells. Furthermore, we predict that some of these cells are autoantigen-specific in patients with AAV. When Bregs are functioning properly in a healthy individual, we hypothesize that this cell subset can suppress MPO or PR3 inflammatory TH1 cells (usurping them to become iTregs), induce autoantigen-specific iTregs, suppress inflammatory monocytes, and importantly halt production of ANCA. Our data are consistent with the hypothesis that functionally competent regulatory B cells characterized as CD5^{+}CD24^{hi}CD38^{hi} and IL-10^{+} support long-term clinical remission by inhibiting production of autoantibodies that drive disease pathogenesis. Whether the CD5^{+}CD24^{hi}CD38^{hi} or IL-10 producing B cells can prospectively guide immunosuppressive therapy in patients to prevent unnecessary treatment and ensure treatment when appropriate is an open question.

Table 4-1. Regulatory B Cell Phenotypes Reported in Humans

Breg Phenotype	Stimulation	Breg Function Tested	Disease Correlation	Reference
$CD24^{hi}CD38^{hi}$ (enriched in IL-10+ B cells)	CD40L via CD154-CHO cells	IL-10 production ↓Th1 differentiation via IL-10, not TGF-β reversed by anti-CD80 & anti-CD86	(SLE) ↓IL-10 response to CD40 stim	Blair PA, et al., Immunity 2010; 32:129
$CD24^{hi}CD27^{hi}$ (enriched in IL-10+ B cells)	CpG plus CD40L (CD154)	IL-10 production ↓TNF-α secretion in T cells and monocytes Inhibition dependent on IL-10	SLE, RA, SJS, BD, & MS Expanded compared to HC	Iwata Y, et al., Blood 2011;117:530
$CD25^{+}$, $CD5^{+}CD25^{+}$	Not tested	Not tested	AAV Expanded in remission Active not different from HC	Eriksson P, et al., J Rheumatol 2010; 37:2086
$CD5^{+}$	Not tested	Not tested	AAV Decreased in active disease Normalized in remission	DO Bunch, et al., CJASN 2013; 8:382
$IL\text{-}10^{+}$	CpG	IL-10 production Correlation with Tregs Correlation with INF-γ+ Th1 cells	AAV ↓IL-10 in active & remission Pos. correlation w/ Tregs in rem Neg. correlation w/ INF-γ+ T cells	B Wilde, et al., Ann Rheum Dis 2013; 72:1416
$CD24^{hi}CD38^{hi}$	CD40L via CD154-CHO cells	IL-10 production Th1 TNF-α & INF-γ not affected Failed conversion of naïve T cells to Tregs Differentiation of Th17 cells not blocked	RA (in active disease) ↓number & percentage ↓IL-10+ ↓ability to inhibit Th17 diff. ↓ability to promote Tregs	Flores-Borja F, et al., Sci Transl Med 2013;5:173ra23
$CD24^{hi}CD38^{hi}$	CpG ± CD40L	IL-10 production Th1 INFγ and TNFα	AAV ↓number in active & remission ↓IL-10+ Th1 INFγ and TNFα not altered	SK Todd, et al., 2014, Rheumatology, ePub ahead of print
$CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{hi}$	CpG ± CD40L	IL-10 production Monocyte TNF-α suppression	$CD24^{hi}CD38^{hi}$ ↓ in active disease $CD24^{hi}CD27^{hi}$ ↓ in active & rem IL-10 production not altered Monocyte TNF-α not altered	N Lepse, et al., 2014,Rheumatology, ePub ahead of print

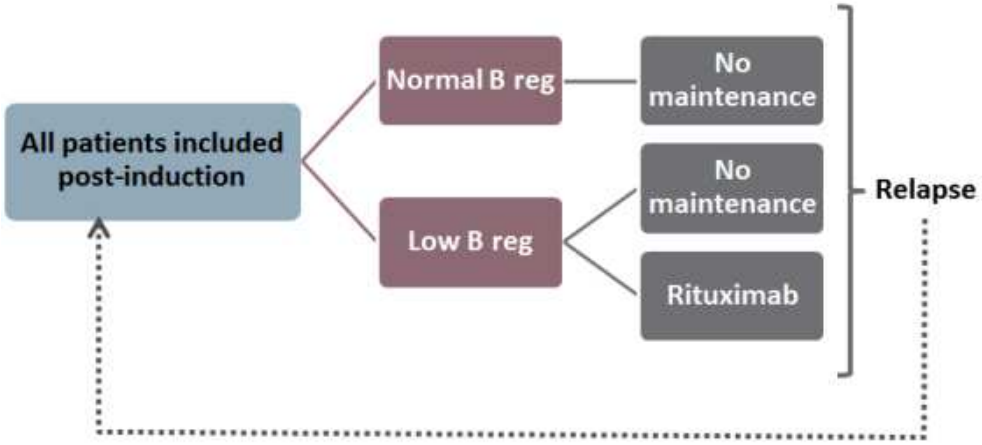
$CD5^+$ $CD24^{hi}CD38^{hi}$, $IL\text{-}10^+$	CpG ± CD40L	IL-10 production Autoantibody production	$CD5^+$ $CD24^{hi}CD38^{hi}$ ↓ active, ↑ rem IL-10 ↓ active disease, rebound in rem Negative correlation with ANCA titer	LA Aybar, et al., 2014, Clinical and Experimental Immunology

Figure 4-1. Relapse-free from time of rituximab



Patients who repopulated with ≤30%CD5+ B cells (●) relapsed sooner than patients who repopulated with >30% CD5+ B cells (■; p=0.005). Open squares denote patients who did not relapse during the time of our study. Adjusting for differences in upper respiratory involvement, the low CD5 group at B cell repopulation remained significantly associated with a shorter time to relapse from time of rituximab (p=0.002) and from time of B cell repopulation (p=0.001).

Figure 4-2. Diagram of Breg clinical trial



Immunosuppression guided by $CD5^+$ B cells to avoid unnecessary treatment when protective $CD5^+$ B cells are present and avoid relapse by proactive treatment when $CD5^+$ B cells are declining could offer immeasurable benefit to patients.

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