IL-10-producing Regulatory B Cell Development in Human Autoimmune Disease

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Immunology in the Graduate School of Duke University

2016
ABSTRACT

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Abstract

B cell abnormalities contribute to the development and progress of autoimmune disease. Traditionally, the role of B cells in autoimmune disease was thought to be predominantly limited to the production of autoantibodies. Nevertheless, in addition to autoantibody production, B cells have other functions potentially relevant to autoimmunity. Such functions include antigen presentation to and activation of T cells, expression of costimulatory molecules and cytokine production. Recently, the ability of B cells to negatively regulate cellular immune responses and inflammation has been described and the concept of “regulatory B cells” has emerged. A variety of cytokines produced by regulatory B cell subsets have been reported with interleukin-10 (IL-10) being the most studied. IL-10-producing regulatory B cells predominantly localize within a rare CD1dhiCD5+ B cell subset in mice and the CD24hiCD27+ B cell subset in adult humans. This specific IL-10-producing subset of regulatory B cells has been named “B10 cells” to highlight that the regulatory function of these rare B cells is primarily mediated by IL-10, and to distinguish them from other regulatory B cell subsets that regulate immune responses through different mechanisms. B10 cells have been studies in a variety of animal models with autoimmune disease and clinical settings of human autoimmunity. There are many unsolved questions related to B10 cells including their surface phenotype, their origin and development in vivo, and their role in autoimmunity.
In Chapter 3 of this dissertation, the role of the B cell receptor (BCR) in B10 cell development is highlighted. First, the BCR repertoire of κ+ mouse peritoneal cavity B10 cells is examined by single cell PCR sequencing; peritoneal cavity B10 cells have clonally diverse germline BCRs that are predominantly unmutated. Second, mouse B10 cells are shown to have higher frequencies of λ+ BCRs compared to non-B10 cells which may indicate the involvement of BCR light chain editing early in the process of B10 cell development in vivo. Third, human peripheral blood B10 cells are examined and are also found to express higher frequency of λ chains compared to non-b10 cells. Therefore, B10 cell BCRs are clonally diverse and enriched for unmutated germline sequences and λ light chains.

In Chapter 4 of this dissertation, B10 cells are examined in the healthy developing human across the entire age range of infancy, childhood and adolescence, and in a large cohort of children with autoimmunity. The study of B10 cells in the developing human documents a massive transient expansion during middle childhood when up to 30% of blood B cells were competent to produce IL-10. The surface phenotype of pediatric B10 cells was variable and reflective of overall B cell development. B10 cells down-regulated CD4+ T cell interferon-gamma (IFN-γ) production through IL-10-dependent pathways and IFN-γ inhibited whereas interleukin-21 (IL-21) promoted B cell IL-10 competency in vitro. Children with autoimmunity had a contracted B10 cell compartment, along with increased IFN-γ and
decreased IL-21 serum levels compared to age-matched healthy controls. The decreased B10 cell frequencies and numbers in children with autoimmunity may be partially explained by the differential regulation of B10 cell development by IFN-γ and IL-21 and alterations in serum cytokine levels. The age-related changes of the B10 cell compartment during normal human development provide new insights into immune tolerance mechanisms involved in inflammation and autoimmunity.

These studies collectively demonstrate that BCR signals are the most important early determinant of B10 cell development in vivo, that human B10 cells are not a surface phenotype defined developmental B cell subset but a functionally defined regulatory B cell subset that regulates CD4+ T IFN-γ production through IL-10-dependent pathways and that human B10 cell development can be regulated by soluble factors in vivo such as the cytokine milieu. The findings of these studies provide new insights into immune tolerance mechanisms involved in human autoimmunity and the potent effects of IL-21 on human B cell IL-10 competence in vitro open new horizons in the development of autologous B10 cell-based therapies as an approach to treat human autoimmune disease in the future.
Dedication

I would like to dedicate this dissertation to myself, all those that truly supported me during the course of my studies and especially to those that opposed my choices. My path has been uniquely self-driven and it provided me the opportunity to explore myself and the world both intellectually and philosophically. The six year period I spent at the Duke Immunology Graduate School marked the beginning of my formal and informal intellectual endeavors for the rest of my life and loaded me with an unlimited source of motivation to make genuine authentic difference in the world.
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List of Abbreviations

APC, antigen-presenting cell
B10pro, B10 progenitor cell
BCR, B cell receptor
BFA, Brefeldin A
CD, cluster differentiation antigen
CDR, complementarity determining region
CIA, collagen-induced arthritis
CLL, chronic lymphocytic leukemia
CpG, CpG oligodeoxynucleotide
EAE, experimental autoimmune encephalomyelitis
EDTA, ethylenediaminetetraacetic acid
FMO, fluorescence minus one
GM-CSF, granulocyte-monocyte colony stimulating factor
IBD, Inflammatory Bowel Disease
IFN, interferon
IL-, interleukin-
IRB, Institutional Review Board
JDM, juvenile dermatomyositis
JIA, juvenile idiopathic arthritis
LPS, lipopolysaccharide
mAb, monoclonal antibody
MCTD, mixed connective tissue disease
MFI, mean fluorescence intensity
MHC-I, major histocompatibility complex class I
MHC-II, major histocompatibility complex class II
MMF, mycophenolate mofetil
PBMC, peripheral blood mononuclear cells
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PMA, phorbol 12-myristate 13-acetate
RA, rheumatoid arthritis
SLE, systemic lupus erythematosus
T1D, type 1 diabetes
TCR, T cell receptor
TGF, transforming growth factor
TLR, toll-like receptor
T_{REG}, regulatory T cell
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1. Introduction

Traditionally, B cells have been thought to contribute to the pathogenesis of autoimmune disease through antigen-specific autoantibody production (1). Nonetheless, the role of B cells in autoimmunity extends beyond the production of autoantibodies. It is now well established that B cells can have both positive and negative regulatory effects during immune responses through the secretion of antibody or soluble factors such as pro-inflammatory or anti-inflammatory cytokines.

B cells can positively regulate immune responses by producing antigen-specific antibody and inducing optimal T-cell activation (2, 3). B cells can serve as professional antigen-presenting cells (APCs), capable to present antigen up to 10^4-fold more efficiently than non-professional APCs (4). B cell Ag presentation is required for optimal antigen-specific CD4^+ T cell expansion, memory formation, and cytokine production (5-7). B cells may also positively regulate CD8^+ T cell responses in mouse models of autoimmune disease (8, 9). Furthermore, costimulatory molecules (such as CD80, CD86, and OX40L) expressed on the surface of B cells are required for optimal T cell activation (10, 11). The positive regulatory roles of B cells extend to multiple immune system components; the absence of B cells during mouse development results in significant quantitative and qualitative abnormalities within the immune system including a remarkable decrease in thymocyte numbers and diversity (12), significant defects within spleen DC and T cell compartments (13-15), absence of Peyer’s patch organogenesis and
follicular DC networks (16, 17), and absence of marginal zone and metallophilic macrophages with decreased chemokine expression (15, 17). B cells also positively regulate lymphoid tissue organization (18, 19). Finally, dendritic cell (DC), macrophage, and T helper (Th) cell development may all be influenced by B cells (20).

B cells can negatively regulate cellular immune responses through the production of cytokines. B cell negative regulation of immune responses has been demonstrated in various mouse models of autoimmunity and inflammation (21-29). The important negative regulatory effects of B cell subsets are now broadly recognized and well described (30-33). IL-10-producing regulatory B cells (B10 cells) have been the most widely studied (33-35). B10 cells have been phenotypically characterized and predominantly localize within the rare CD1dhiCD5+ B cell subset in mice (34) and the CD24hiCD27+ B cell subset in humans (36). This specific subset of regulatory B cells was labeled as “B10 cells” to highlight that the regulatory function of B10 cells is primarily mediated by IL-10, and to distinguish them from other B cell subsets that regulate immune responses through different mechanisms (37). The regulatory functions of B10 cells depend on their competence to produce and secrete IL-10. This functional subset of B cells is defined solely by its IL-10-dependent regulatory properties and extends beyond the concept of transcription factor-defined cell lineages (30, 31).
1.1. Biology of B10 cells

One of the most fundamental basic biology questions about B10 cells relates to the stimuli driving their development. Antigen and B cell receptor (BCR) signaling are critical in early development although additional stimuli appear to be involved in the developmental process such as CD40 ligation and TLR ligands. Figure 1 illustrates our current understanding of B10 cell development in vivo both in mice and humans, where their development shows similarities.

B10 cells are a functionally defined subset. There are no unique phenotypic markers for B10 as these cells are currently defined only by their competence to produce and secrete IL-10 following appropriate stimulation. B10 cells share surface markers with other previously defined B cell subsets but no one marker or set of markers is unique to B10 cells. For identification of B10 cells, intracellular cytoplasmic IL-10 staining is used, following ex vivo stimulation with lipopolysaccharide (LPS) or CpG oligonucleotides, phorbol esters (PMA) and ionomycin for 5 hours (38). B10 cells originate from a progenitor population (“B10PRO cells”). B10PRO cells develop into B10 cells following maturation through CD40 ligation and exposure to LPS or CpG. B10PRO cells can be identified indirectly following ex vivo stimulation with LPS or CpG in the presence of CD40 ligation for 48 hours with the addition of PMA and ionomycin for the
Figure 1. Linear differentiation model of B10 cell development in vivo.
Figure 1. Linear differentiation model of B10 cell development in vivo. B10 cells originate from a progenitor population (“B10\text{PRO}”). In mice, B10\text{PRO} cells are found in the CD1dCD5− adult blood and lymph node B-cell subsets and within the CD1dCD5+ neonatal spleen and adult peritoneal cavity B-cell subsets. CD40 stimulation induces B10\text{PRO} cells to become competent for IL-10 expression, while LPS induces B10\text{PRO} cells to become competent for IL-10 expression and induces B10 cells to produce and secrete IL-10. IL-10-competent B cells are induced to express IL-10 following ex vivo stimulation with lipopolysaccharide (LPS) plus phorbol esters (PMA) and ionomycin for 5 hours. Following a transient period of IL-10 expression, a small subset of B10 cells can differentiate into antibody-secreting plasma cells (“PC”). B10 development in humans appears to follow the differentiation scheme observed in mice. B10 and B10\text{PRO} cells have been identified in human newborn and adult blood. B10+B10\text{PRO} cells in adult human blood express CD27 and CD24. Whether human B10 cells further differentiate into plasma cells (PC) remains to be determined. Solid arrows indicate known associations, while dashed arrows represent speculation.
last 5 hours. The IL-10+ B cells measured following this 48 hour stimulation include cells that would have been IL-10+ even with the shorter 5 hour stimulation (“B10 cells”) and thereby represent the sum of B10 plus B10PRO cells (“B10+B10PRO”).

1.1.1. Mouse B10 cell development

BCR specificity, affinity and signaling are the most important currently identified factors in B10 cell development. B10 cell regulation of inflammation and autoimmunity is antigen-specific (23, 34). Signaling through the B cell receptor (BCR) appears critical during early development in vivo. The importance of BCR diversity is demonstrated by the fact that B10+B10PRO cells are reduced by approximately 90% in transgenic mice with a fixed BCR (39). CD19-deficient mice (where BCR signaling is decreased) have a 70–80% decrease in B10+B10PRO cells (34). In contrast, B10 cells are expanded in human CD19 transgenic mice (where the over-expression of CD19 augments BCR signaling). The absence of CD22, which normally dampens CD19 and BCR signaling (40), also results in increased B10 cell numbers. Ectopic B cell expression of CD40L (CD154) in transgenic mice, which induces increased CD40 signaling (41), also increases B10 cell numbers. CD22−/− mice that also ectopically express CD40L show dramatically enhanced numbers of CD1dhiCD5+ B cells and B10 cells (42). The induction of IL-10+ B cells with regulatory activity by T cell immunoglobulin domain and mucin domain protein 1 (TIM-1) ligation (43) further highlights the importance of BCR signaling in B10 cell development. BCR signaling and TIM-1 are closely related. BCR ligation induces TIM-1 expression on B
cells (43, 44), and TIM-1 ligation appears to enhance BCR signaling since it increases antibody production both in vitro and in vivo (45). Additional evidence supporting the importance of BCR signaling in B10 cell development is the requirement of the stromal interaction molecules 1 (STIM1) and 2 (STIM2) for B cell IL-10 production. Remarkably, B cells lacking both STIM proteins failed to produce IL-10 after BCR stimulation in the presence of PMA and ionomycin for 5 hours (46). All of the above indicate that BCR-related signals are particularly important in B10 cell development.

Despite the requirement for BCR expression and function during B10 cell development, B cell stimulation with mitogenic anti-IgM antibody alone does not induce cytoplasmic IL-10 expression. The combination of anti-IgM stimulation with CD40 ligation and LPS or CpG significantly reduces IL-10 competence (47). Thus, BCR-generated signals inhibit the abilities of LPS or CpG and CD40 ligation to induce cytoplasmic IL-10 production. Whether BCR stimulation inhibits the induction of IL-10 competence by inducing B cells to mature or differentiate down a divergent pathway or diverts intracellular signaling is unknown. Another possibility is that the signals generated by mitogenic anti-IgM BCR cross-linking are too intense and low-affinity Ag-BCR interactions are driving B10 early development in vivo.

B10 cells can be driven to produce IL-10 by TLR4 (lipopolysaccharide, LPS) or TLR9 (CpG oligonucleotides) ligands. B10PRO cells acquire the ability to function like B10 cells after in vitro “maturation” following stimulation with LPS or CpG in the presence of
agonistic CD40 monoclonal antibody (mAb) (6, 32). Although myeloid differentiation primary response gene 88 (MyD88) is necessary for TLR signaling, it is not required for normal B10 cell development and/or expansion in vivo (47). When MyD88−/− B cells are stimulated with PMA and ionomycin, equivalent B10 cell numbers are detected when compared with similarly treated wild type cells. Nevertheless, MyD88 expression is required for LPS to induce optimal IL-10 expression and secretion in vitro and may also be important for optimal LPS-induced IL-10 production by B10 cells in vivo. Therefore, although TLR ligands and signaling may be involved in optimal B10 cell function, they are not required for B10 cell development.

The cytokine milieu can also influence the development of B cell IL-10 competence. A variety of cytokines have been reported to be involved in regulatory B cell development including IL-21 (48), IL-33 (49), IL-35 (50, 51), granulocyte-monocyte colony stimulating factor (GM-CSF) (52, 53), IL-6 and IL-1β (54).

Following a transient period of IL-10 transcription characterized by increased expression of the blimp1 and irf4 transcription factors and decreased expression of pax5 and bcl6, a significant but small fraction of B10 cells can differentiate into antibody-secreting cells (ASCs) producing IgM and IgG polyreactive antibodies that are enriched for autoreactivity to single- or double-stranded DNA and histones (55). It is important to stress that, although certain transcription factors are involved at some point in B10 cell
development, there is no transcription factor “signature” unique to B10 cells. Whether B10 cells can produce and secrete IL-10 repeatedly remains to be determined.

T cells do not appear to be required for B10 cell development. B10 cells are present in T-cell-deficient nude mice and their frequencies and numbers are approximately five-fold higher when compared with wild-type mice. This observation is strengthened by the fact that major histocompatibility complex (MHC) class I and class II molecules and CD1d expression are not required for B10 cell development (47). Therefore, the role of T cells in B10 cell development in vivo is currently unknown.

1.1.2. Human B10 cell development

Most of what is known about B10 cell development stems from mouse studies. However, B10<sub>PRO</sub> and B10 cells have been recently identified in humans (36) and their responses to LPS, CpG and CD40 ligation appear to follow the general scheme of mouse B10 cell development (Figure 1). One notable difference in mouse versus human B10 cell development is the lack of response of mouse B10<sub>PRO</sub> cells to CpG compared to their human counterparts. Human B10<sub>PRO</sub> and B10 cells are predominantly IgM<sup>+</sup> IgD<sup>-</sup>. B10<sub>PRO</sub> can be driven to develop ex vivo to B10 cells with LPS or CpG stimulation in the presence of CD40 ligation. Whether human B10 cells develop to antibody-secreting plasma cells in vivo remains to be determined.
1.1.3. Critical unsolved questions on B10 cell development

The most critical unsolved issue relates to the nature of antigenic stimuli driving their development. The identification of B10 cell BCR specificity is imperative since it will provide new insights in their early development. The autoreactive nature of B10 cell BCRs (55) suggests that auto-antigens may be driving early B10 cell development and B10 cells may represent one of the ways enabling the immune system to peripherally “tolerate” auto-antigens. B cells responding to auto-antigens in an IL-10-dependent regulatory way can potentially limit inflammatory responses and limit autoimmune phenomena (see later section on B10 cell mechanisms of action and Figure 2). It is likely that additional unidentified antigenic and non-antigenic stimuli are involved in B10 cell development. The identification of such stimuli will provide additional insights in B10 cell development which may prove invaluable for their potential future manipulation as a novel therapeutic approach in treating autoimmune disease. Another important question is whether B10 cells enter the B cell memory pool during their development. This is suggested by human studies demonstrating that B10\textsubscript{PRO} and B10 cells share phenotypic features with memory B cells (see section on human B10 cell phenotype).

1.1.4. Mouse B10 cell phenotype

Although a variety of cell surface markers have been proposed (32, 33), there is no known surface phenotype unique to B10 cells and, currently, the only way to identify these cells is functionally by means intracellular IL-10 staining (38). Only a small portion
of B cells (i.e. ~1-3% of splenic B cells in wild type C57BL/6 mice) produce IL-10 following PMA and ionomycin stimulation implying that not all B cells are “competent” to produce IL-10. Intracellular cytokine staining combined with flow cytometric phenotyping shows that mouse spleen B10 cells are enriched (~20% in C57BL/6 mice) within the small CD1dhiCD5+ B cell subset. This phenotypically unique CD1dhiCD5+ subset shares overlapping cell surface markers with a variety of phenotypically-defined B cell subsets (23, 26, 34, 56-58). Mouse B10 cells are predominantly IgD-IgM+. Recently, IL-10+ B cells were shown to be enriched in the TIM-1+ compartment and TIM-1+ B cells are enriched in the CD1dhiCD5+ compartment (43). However, IL-10+ B cells are also present in the TIM-1- compartment and TIM-1+ B cells are present in the non-CD1dhiCD5+ compartment.

1.1.5. Human B10 cell phenotype

The IL-10-producing B cell subset characterized in humans represents ~1% of peripheral blood B cells (36). In humans, a diverse variety of surface phenotype-defined developmental B cell subsets have been reported to contain B10 cells such as transitional B cells (59-62), memory B cells (36, 60, 62, 63), germinal center B cells (64) and plasmablasts/plasma cells (63). Peripheral blood B10 and B10PRO cells are highly enriched in the CD24hiCD27+ B cell subset, with approximately 60% also expressing CD38. Similar total numbers of IL-10+ B cells have been described in the CD24hiCD38hi and CD24intCD38int B cell subsets (59). Our studies indicate that human B10 cells highly
Figure 2. B10 cell regulatory effects in autoimmune disease. In this model, unidentified autoantigens drive early development of B10\textsubscript{PRO} cells. Following exposure to CD40 ligation and/or TLR ligands (LPS, CpG), B10\textsubscript{PRO} cells “mature” into B10 cells that can actively secrete IL-10 and regulate both innate and adaptive immune responses. B10 cells regulate macrophage function by decreasing their activation, phagocytosis and cytokine and nitric oxide (NO) production. In antigen-presenting cells (APC), B10 cell negative regulation of antigen presentation, expression of costimulatory molecules (such as CD86) and proinflammatory cytokines limits T cell activation. In CD4\textsuperscript{+} T helper (T\textsubscript{H}) cells, B10 cells skew responses towards T\textsubscript{H2} and away from T\textsubscript{H1} and T\textsubscript{H17}. 
express CD48 and CD148. CD48 is a B cell activation marker (65) and CD148 is considered a marker for human memory B cells (66). CD27 expression is another well-characterized marker for memory B cells, although some memory B cells may be CD27− (67-69). The CD27− B cell subset can also expand during the course of autoimmunity and has been proposed as a marker for disease activity (67, 69). The CD24hiCD148+ phenotype of B10 and B10PRO cells may indicate their selection into the memory B cell pool during development, or they may represent a distinct B cell subset that shares common cell surface markers with memory B cells. Consistent with a memory phenotype, the proliferative capacity of human blood B10 cells in response to mitogen stimulation is higher than that for other B cells, as is seen for mouse B10 cells (47).

Human transitional B cells are rare (2%-3% of B cells) in adult human blood and are generally CD10+CD24hiCD38hi cells that are also CD27 negative (68, 69); since CD10 expression is a well-accepted marker for most cells within the transitional B-cell pool (39), its absence on B10 cells suggests that these cells are not recent emigrants from the bone marrow. In conclusion, human B10 cells share phenotypic characteristics with other previously defined B cell subsets, and, currently, there is no known surface phenotype unique to B10 cells.

1.2. B10 cell regulatory effects in autoimmune disease

B10 cells exert a variety of IL-10-dependent regulatory effects potentially involved in autoimmune disease. The anti-inflammatory effects of IL-10 are mediated by multiple
mechanisms involving both the innate and adaptive arms of the immune system. In innate cells, this includes the downregulation of proinflammatory cytokine production (TNF-α, IL-1, IL-6, IL-8, and IL-12) (70) and decreased expression of MHC class II and costimulatory molecules (such as CD86) (71) resulting in decreased T cell activation. In CD4+ T cells, IL-10 suppresses TH1 (56) and enhances TH2 polarization (43, 71). Coculture of CD1dhiCD5+ B cells with CFSE-labeled naive CD4+ T cells suppresses TH17 cell differentiation (72) and IL-10 is known to suppress TH17 responses (73). A number of studies suggest that IL-10-producing B cells are important for the generation and/or maintenance of the regulatory T cell (TREG) pool (74-84). However, a recent study (85) and our previously published data (23) do not support this view. IL-10 production by human B10 cells regulates monocyte activation and cytokine production in vitro; it is thereby likely that human B10 cell IL-10 production will also have pleiotropic regulatory effects on the immune system, as occurs in mice (86). The potential regulatory effects of B10 cells in autoimmune disease limiting inflammatory responses and subsequent tissue damage are summarized in Figure 2.

1.3. B10 cells in human autoimmune disease

Studies on B10 cells and human autoimmune disease are limited but of outmost importance since they provide valuable insights relevant to the potential future therapeutic application of B10 cells in humans. Peripheral blood B10 and B10PRO cells are present in patients with autoimmune diseases, including rheumatoid arthritis (RA),
systemic lupus erythematosus (SLE), primary Sjögren’s syndrome, autoimmune bullous diseases, and multiple sclerosis (MS). Interestingly, B10+B10PRO cell frequencies are expanded in some but not all cases, and mean B10+B10PRO cell frequencies are significantly higher in patients with autoimmune disease compared to age-matched healthy controls (36). A different study examined cytoplasmic IL-10 production by B cells from SLE patients and normal controls by flow cytometry (86). Blood mononuclear cells were cultured for 24 hours in the presence or absence of PMA, ionomycin, or LPS and significantly more SLE B cells spontaneously produced cytoplasmic IL-10 than controls. Both unstimulated and LPS-stimulated CD5+ B cells from SLE patients were enriched for cells producing high levels of IL-10. Spontaneous IL-10 production by blood B cells and monocytes cells is higher in untreated RA, systemic sclerosis, and SLE patients than in controls, as measured using RT-PCR and ELISA assays (87). A recent study introduced the concept of functional impairment of B10 cells in autoimmune disease by demonstrating functional impairment of CD24hiCD38hi regulatory B Cells in human SLE (59). These CD24hiCD38hi regulatory B cells were able to suppress the differentiation of T\(_{\text{H1}}\) cells, partially via IL-10, when derived from healthy individuals. However, when isolated from the peripheral blood of SLE patients they were refractory to CD40 ligation, produced less IL-10, and lacked the suppressive capacity of their healthy counterparts. The results of this study are rather intriguing but these findings need to be validated using B cell subpopulations that are more uniform in regard to their
IL-10 regulatory properties. In conclusion, B10 cells are present in the peripheral blood of patients with autoimmune diseases where they appear to be expanded.

1.4. B10 cells in mouse models of autoimmune disease

The important regulatory effects of B10 cells in vivo and their therapeutic potential have been demonstrated in a variety of mouse models of human autoimmune disease.

1.4.1. Experimental Autoimmune Encephalomyelitis (EAE)

EAE is an established model of MS induced by immunization with myelin peptides (such as myelin oligodendrocyte glycoprotein, MOG) leading to demyelination mediated by auto-Ag-specific CD4+ T cells (88, 89). B cells were shown over a decade ago to have regulatory properties during the induction of EAE with genetically B cell deficient mice developing a severe non-remitting form of the disease (21). However, it was recently shown that these B cell regulatory effects are not IL-10-dependent (37). Nonetheless, other studies highlight the importance of B cell-derived IL-10 in EAE. Specifically, EAE severity during the late phase of disease increases in B cell-deficient µMT mice that do not fully recover from their disease compared to wild type mice and the adoptive transfer of wild-type B cells but not IL-10+/− B cells normalizes EAE severity in µMT mice (22). Disease recovery is dependent on the presence of autoAg-reactive B cells, and B cells isolated from mice with disease produced IL-10 in response to autoAg
stimulation. In the absence of Ag-specific B cell IL-10 production, the pro-inflammatory Th1-mediated immune responses persist and mice do not recover from the disease.

The EAE model demonstrates the complexity of regulatory mechanisms which are mediated by different cell subsets during different stages of the disease. When B cells from wild-type mice are depleted by CD20 mAb treatment 7 days before EAE induction, there is an increased influx or expansion of encephalitogenic T cells within the CNS and exacerbation of disease symptoms (23). This is related to B10 cell depletion since similar effects are observed with selective B10 depletion by means of CD22 mAb. The protective effect is IL-10-dependent since the adoptive transfer of CD1dhiCD5+ B cells purified from IL-10−/− mice does not affect EAE severity. Thus, IL-10 production by B10 cells negatively regulates EAE initiation. However, once disease is established, adoptive transfer of B10 cells does not suppress ongoing EAE. Thereby, B10 cells appear to normally counterbalance the positive effector roles of B cells during acute autoimmune responses in EAE. The adoptive transfer of MOG-sensitized B10 cells into wild-type mice also reduces EAE initiation dramatically (90). During disease initiation, the preferential depletion of B10 cells in vivo enhanced EAE pathogenesis, whereas TREG cell depletion enhances late-phase disease. B10 cells did not regulate T cell proliferation during in vitro assays, but significantly altered CD4+ T cell IFN-γ and TNF-α production. Furthermore, B10 cells downregulated the ability of DCs to act as APCs and thereby indirectly modulated T cell proliferation. Thus, in EAE, depending on the stage
of the disease, different regulatory mechanisms are involved in limiting inflammatory responses with B10 cells regulating disease initiation and T$_{REG}$ cells are involved in the regulation of late-phase disease.

1.4.2. Inflammatory Bowel Disease (IBD)

IL-10-producing B cells regulate intestinal inflammation during IBD (26). B cells and their autoantibody products suppress colitis in T cell Ag receptor $\alpha$ chain-deficient mice that spontaneously develop chronic colitis, while B cells are not required for disease initiation (91). It was subsequently demonstrated that B cells are the regulatory mediators and a B cell subset with upregulated CD1d expression was identified that is induced in the gut-associated lymphoid tissues of mice with intestinal inflammation (25). This IL-10-producing B cell subset appears during chronic inflammation and suppresses the progression of intestinal inflammation by downregulating inflammatory cascades associated with IL-1 upregulation and signal transducer and activator of transcription 3 (stat3) activation rather than by altering polarized T$_H$ cell responses. In addition, the adoptive transfer of mesenteric lymph node B cells also suppresses inflammatory bowel disease through a mechanism that correlated with an increase in regulatory T cell subsets (79). Oral administration of dextran sulfate sodium (DSS) solution to mice is widely used as a model of human ulcerative colitis (UC) and, it was recently shown that DSS-induced intestinal injury is more severe in CD19$^{-/}$ mice than in wild type mice (92). These inflammatory responses are negatively regulated by
CD1d<sup>hi</sup>CD5<sup>-</sup> B cells and adoptive transfer of CD1d<sup>hi</sup>CD5<sup>-</sup> B cells from wild type mice reduces inflammation in CD19<sup>-/-</sup> mice in an IL-10–dependent manner. Therefore, B10 cells emerge during chronic inflammation in mouse models of IBD where they suppress the progression of inflammatory responses and ameliorate disease manifestations.

1.4.3. Collagen-Induced Arthritis (CIA)

CIA is a model for human RA that develops in susceptible mouse strains immunized with heterologous type II collagen emulsified in complete Freund’s adjuvant (93, 94). CIA and RA share in common an association with a limited number of major histocompatibility complex class II haplotypes that determine disease susceptibility (95, 96). B cells are important for initiating inflammation and arthritis since mature B cell depletion significantly reduces disease severity prior to CIA induction, but does not inhibit established disease (97). Activation of arthritogenic splenocytes with Ag and agonistic anti-CD40 induced a B cell population that produced high levels of IL-10 and low levels of IFN-γ, and adoptive transfer of these B cells into DBA/1-TcR-β-Tg mice, immunized with bovine collagen (CII) emulsified in complete Freund’s adjuvant, inhibited Th1 responses, prevented arthritis development, and was also effective in ameliorating established disease (98). A different study demonstrated that adoptive transfer of CD21<sup>hi</sup>CD23<sup>-/hi</sup>IgM<sup>+</sup> B cells from arthritogenic mice (remission phase) prevents CIA incidence and reduces disease severity, and that the majority of IL-10 generated is produced by the CD1d<sup>hi</sup>CD21<sup>hi</sup>CD23<sup>-/hi</sup>IgM<sup>-</sup> B cell subset (99). The adoptive transfer of
CD21<sup>hi</sup>CD23<sup>+</sup>IgM<sup>+</sup> B cells from naive mice also has a significant but less dramatic effect on CIA progression. Administration of apoptotic thymocytes to mice up to 1 month before the clinical onset of CIA is protective for severe joint inflammation and bone destruction (75). Activated spleen B cells respond directly to apoptotic cell treatment by increasing secretion of IL-10, and inhibition of IL-10 in vivo reverses the beneficial effects of apoptotic cell treatment. A recent study demonstrated that adoptive transfer of ex vivo expanded CD1d<sup>hi</sup>CD5<sup>+</sup> B cells in collagen-immunized mice delays arthritis onset and reduces the severity of clinical symptoms and joint damage, accompanied by a substantial reduction in the number of T<sub>H17</sub> cells (72). Coculture of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells with CFSE-labeled naive CD4<sup>+</sup> T cells suppresses T<sub>H17</sub> cell differentiation in vitro via the reduction of stat3 phosphorylation and RORγt expression. Coculture of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells with T<sub>H17</sub> cells results in decreased proliferation responses in vitro and adoptive transfer of T<sub>H17</sub> cells triggers CIA in IL-17<sup>-/-</sup> DBA mice. However, when T<sub>H17</sub> cells are co-transferred with CD1d<sup>hi</sup>CD5<sup>+</sup> B cells the onset of CIA is significantly delayed. Thereby, several studies on mouse models of RA demonstrate the negative regulatory effects and therapeutic potential of B10 cells.

1.4.4. Systemic Lupus Erythematosus (SLE)

B cell depletion initiated in 4-week-old NZB/W F1 mice hastens disease onset, which parallels depletion of B10 cells (100). B10 cells are phenotypically similar in NZB/W F1 and C57BL/6 mice, but are expanded significantly in young NZB/W F1 mice (100). In
wild type NZB/W mice, the CD1d^{hi}CD5^{+} B cell subset, which is enriched in B10 cells, is increased by 2.5-fold during the disease course, whereas CD19^{+} NZB/W mice lack this CD1d^{hi}CD5^{+} regulatory B cell subset (101). The transfer of CD1d^{hi}CD5^{+} B cells from wild type NZB/W mice into CD19^{+} NZB/W recipients significantly prolongs their survival (101). A recent study suggests that B cell-derived IL-10 does not regulate spontaneous autoimmunity in the MRL.Fas(lpr) mouse lupus model (102). By lineage-specific deletion of IL-10 from B cells, it was shown that B cell-derived IL-10 is ineffective in suppressing the spontaneous activation of self-reactive B and T cells during lupus. Severity of organ disease and survival rates in mice harboring IL-10-deficient B cells were unaltered. In contrast to previously published studies from our lab and elsewhere, putative regulatory B cell phenotypic subsets, such as CD1d^{hi}CD5^{+} and CD21^{hi}CD23^{hi} B cells, were not enriched in IL-10 transcription. This suggests fundamental differences in the pathogenesis and immune dysregulation in the NZB/W lupus model compared to the MRL.Fas(lpr) model.

1.4.5. Type 1 Diabetes (T1D)

Based on studies using the non-obese diabetic (NOD) mouse, a spontaneous model of T1D, autoimmune destruction of the pancreatic β cells is primarily T-cell mediated (103). B cells activated in vitro can maintain tolerance and transfer protection from T1D in NOD mice (104, 105), despite the pathogenic role that B cells play in disease initiation (106). The repeated transfusion of 1.2x10^7 BCR-stimulated NOD spleen B cells into NOD
mice starting at 5-6 weeks of age both delays the onset and reduces the incidence of T1D, while treatment at 9 weeks of age before disease onset only delays disease onset. Protection from T1D requires B cell IL-10 production since the transfusion of activated NOD-IL-10−/− B cells does not confer protection from T1D or the severe insulitis observed in NOD recipients. The therapeutic effect of transfusing activated NOD B cells correlates with T_{H2} polarization. LPS-activated B cells upregulate Fas ligand (FasL) and secrete transforming growth factor beta (TGF_{β}), which may contribute to their ability to inhibit autoimmunity when transferred to pre-diabetic NOD mice (105). However, whether these B cells also secrete IL-10 was not assessed. The limited data above suggest that B10 cells may be protective in preventing establishment of T1D in NOD mice.

1.5. Therapeutic potential of B10 cells

Harvesting the anti-inflammatory properties of B10 cells can provide a new approach in the treatment of autoimmunity. Manipulation of this subset for treating autoimmune disease may occur either by selective depletion of mature B cells while sparing B10/B10_{PRO} cells or by the selective expansion of B10_{PRO} cells. Selective depletion of mature B cells while sparing B10/B10_{PRO} cells is not possible with the agents currently available and highlights the need for discovery of new drugs that specifically deplete non-B10/B10_{PRO} cells. This approach appears quite difficult since there are no identified surface molecules specific for non-B10/B10_{PRO} cells that could be utilized in a depletion strategy.
Expansion of B10\textsubscript{pro} cells appears to be a more viable approach since some of the stimuli driving their development have been identified. Expanding B10 cells \textit{in vivo} carries additional risks since the currently identified stimuli driving B10 cell development are rather non-specific and, if administered systemically, may trigger multiple responses in a variety of immune cells with rather unpredictable results. For example, systemic administration of agonistic CD40 antibodies has been associated with serious adverse effects such as the cytokine release syndrome \cite{107}. Nevertheless, expansion of B10 cells \textit{in vivo} by means of agonistic CD40 antibody has shown benefit in CIA, a mouse model of human RA \cite{98}. On the other hand, expanding B10 cells \textit{ex vivo} does not carry the risk of non-specific immune effects and offers a potentially highly effective therapy without “off-target effects”. Expanding B10 cells \textit{ex vivo} introduces new challenges related to the method of expansion, the magnitude of expansion and the time it takes to reach B10 numbers that will be sufficient for therapeutic use. The most common approach of generating regulatory B cells is by means of genetically manipulating immature B cells through lentiviral transfection \cite{108}. Although this approach can efficiently generate large numbers of regulatory B cells \textit{ex vivo}, concerns about administering infusions of lentivirus-infected B cells to humans (with retroviral and infectious potential) remain, and this may render this approach inappropriate for use in human studies. Therefore, a new approach not utilizing infectious agents is needed. Another challenge to B10 cell \textit{ex vivo} manipulation relates to the magnitude of
expansion since the number of cells infused during adoptive transfer experiments is critical. In humans, the most convenient potential source of B10/B10<sub>PRO</sub> prior to <i>ex vivo</i> expansion is obviously peripheral blood. Since B10/B10<sub>PRO</sub> cells are rare in peripheral blood and there are limitations on the volume that can be drawn at any given time, a method of expanding B10 cells by several million fold is needed. Furthermore, since this will be used for treatment of active disease, the time that will take to expand these cells <i>ex vivo</i> is also of great significance; ideally, this process should not take more than 1-2 weeks. In conclusion, a new approach that expands B10 cells <i>ex vivo</i> effectively, quickly and without the use of viral vectors will provide a promising novel approach in treating human autoimmune disease potentially without undesirable “off-target effects”.

1.6. Conclusions

It is likely that numerous functionally defined subsets of regulatory B cells will be identified in the near future. The phenotypic and functional characterization of B10 cells is an important advance for the field. B10 cells share phenotypic markers with a variety of previously defined subsets but the only unique phenotypic marker is their intracellular IL-10 production. B10 cells are a functionally defined subset with regulatory IL-10-dependent effects and their definition extends beyond transcription factor-committed lineages. Although certain transcription factors are involved at different points in B10 cell development, there is no transcription factor “signature” unique to B10 cells. BCR-related signals are most critical in B10 cell development. Our recent findings
of B10 cell BCR autoreactivity raise intriguing questions about the nature of signals driving their early development and suggest that autoantigens may be of particular importance. The identification of the specificity of antigenic and non-antigenic stimuli driving B10 development is imperative since it will provide invaluable clues in the design of \textit{ex vivo} expansion methods for future therapeutic applications.
2. Methods

2.1 Study Design

These studies consist of controlled laboratory experiments using mice and human peripheral blood mononuclear cells (PBMC). All data, including outliers, were included in the presented experiments. Unless otherwise noted, all experiments were performed at least three times with multiple samples to ensure reproducibility.

2.2 Mouse Studies

2.2.1. Mice

C57BL/6 and BALB/c mice were from The Jackson Laboratory. All mice were bred in a specific pathogen–free barrier facility and were used at 8–20 weeks of age, unless indicated otherwise. Peritoneal cavity cells were obtained from 12–14-week-old mice. The Duke University Animal Care and Use Committee approved all studies.

2.2.2. Cell isolation and culture

Single-cell suspensions were generated by gentle dissection of spleen, lymph nodes and bone marrow. Mouse PBMC were isolated by a discontinuous gradient-based separation similar to the method used for human PBMC (see below). To isolate peritoneal cavity leukocytes, 5 mL ice-cold RPMI 1640 medium (Cellgro) containing 5% FBS was injected into the peritoneal cavity of euthanized mice followed by gentle massage of the
abdomen and recovery of the fluid with a large-gauge needle. Viable cells were counted using a hemocytometer, with relative lymphocyte percentages determined by flow cytometry analysis. B cells were enriched by positive selection using CD19 mAb-coupled MicroBeads (Miltenyi), respectively, according to the manufacturer’s instructions, with obtained purities >95%. Where indicated, subsets of CD19+ B cells were further purified following immunofluorescence staining using a FACS DIVA flow cytometer (BD Biosciences) with 98% purities.

2.2.3. Immunofluorescence

Single-cell leukocyte suspensions (1 x 10^6 cells) were stained on ice for 20–30 min using predetermined optimal concentrations of mAbs, as described (109). Washed cells were resuspended in PBS containing 1.5% paraformaldehyde and kept at 4°C in the dark until final analysis. Cells with light scatter properties of singlet lymphocytes were analyzed for four- or five-color immunofluorescence staining using a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA). Background staining was determined using unreactive isotype-matched control mAbs (Caltag Laboratories, San Francisco, CA). Flow cytometry data analysis by FlowJo (version X). Mouse κ chains were stained with goat polyclonal antibody (Southern Biotech) whereas λ chains were stained with a monoclonal antibody (clone R26-46, BD Pharmingen).
2.2.4. Single cell BCR sequencing

In two individual experiments, enriched peritoneal cavity CD19⁺ B cells from three individual wild-type C57BL/6 mice were stimulated with LPS (10 mg/ml), PMA (50 ng/ml), and ionomycin (1 mg/ml) for 5 hours. Individual IL-10⁺CD19⁺ cells were identified using the Mouse IL-10 Secretion Assay Kit (Miltenyi Biotec), according to the manufacturer’s instructions, and sorted into single wells of 96-well PCR plates using a FACSaria II cell sorter (BD Biosciences). cDNA was synthesized, and transcripts were amplified using nested PCR primers, as described (110). PCR products were purified (QIAquick PCR Purification Kit; QIAGEN) and cloned (StrataClone PCR Cloning Kit; Agilent Technologies) before sequencing (Duke University DNA Analysis Facility). Productive rearrangements were compared against germline Ig sequences, according to the Ig Basic Local Alignment Search Tool database (National Center for Biotechnology Information, Bethesda, MD), and analyzed using the Immunogenetics V-query and Standardization tool (111) to determine V(D)J gene family usage. Mutation frequencies were determined using germline V, D, and J sequences from the Ig Basic Local Alignment Search Tool. VH-DH-JH and VK-JK transcript alignments and phylogenetic trees based on average percent identity were constructed using ClustalW2 (112). In one case, identical sequences were obtained from adjacent wells, so only one sequence is reported.
2.3. Human studies

2.3.1. Human subjects

The study protocol was approved by the Duke University Institutional Review Board (IRB) in compliance with the Helsinki Declaration. Participants were recruited from the Research Triangle Area (North Carolina, USA) between February 2012 and July 2015. Following written informed consent, samples were obtained from neonates (umbilical cord samples, n=4), healthy children (n=20), and healthy adults (n=16). An 11-month-old infant was recruited from the University of South Florida under their IRB-approved protocol. Children with autoimmune diseases (n=52) included juvenile idiopathic arthritis (JIA; n=25), juvenile dermatomyositis (JDM; n=13), systemic lupus erythematosus (SLE; n=13) and mixed connective tissue disease (MCTD; n=1). All children with SLE satisfied the American College of Rheumatology criteria (113). 12 of 13 children with JDM had diagnostic muscle biopsy and/or electromyography and satisfied the Bohan and Peter criteria (114). The child with MCTD had myositis, lymphopenia, Raynaud’s phenomenon, periungual telangiectasias, polyarthritis, parotitis, and positive serum autoantibodies (rheumatoid factor, anti-Smith, anti-ribonucleoprotein). All 24 children with JIA satisfied the International League of Associations for Rheumatology criteria (115). Due to sample limitations, there were only 5 white blood cell (WBC) measurements, 14 B10 cell measurements and 11 B10+B10<sub>PRO</sub> cell measurements in adults, and only 24 B10 cell measurements and 23 B10+B10<sub>PRO</sub> cell
measurements in children with JIA. Exclusion criteria included systemic-onset JIA, intercurrent illness, surgical procedures or vaccination within 4 weeks, and treatment with rituximab, belimumab or cyclophosphamide in the last 12 months. The demographics and study characteristics of healthy children are summarized in Table III, and the clinical data of children with autoimmune diseases are summarized in Table IV.

Since JIA, JDM, SLE and MCTD lack a common disease activity assessment tool, physician global assessment as a continuous visual analogue scale score (0-10 cm) was used to assess disease activity and minimize bias due to the use of multiple disease activity assessment tools. 1 of 8 pediatric rheumatologists at the Duke Children’s Health Center scored each participant following their ambulatory clinic visit and prior to any laboratory testing. Scores < 1 were categorized as “inactive disease” and scores ≥1 as “active disease,” respectively.

2.3.2. Cell isolation and culture

Fresh human peripheral blood collected in EDTA (ethylenediaminetetraacetic acid)-coated Vacutainer tubes (BD Biosciences) was processed within 12 hours. WBC counts were measured with a Beckman Coulter automated counter. Serum was obtained after whole blood centrifugation and stored at -80 °C. PBMC were isolated by centrifugation over a discontinuous gradient (Lymphoprep, Axis-Shield PoC As) and quantified manually using a hemocytometer. PBMCs were resuspended in culture medium (RPMI 1640 with 10% fetal calf serum, 200 U/mL penicillin, 200 μg/mL streptomycin, and 2 mM
L-glutamine) and seeded in 48-well flat-bottom tissue culture plates (Genesee Scientific) at a final volume of 1 mL (2x10⁶ PBMCs/mL). Cultures were stimulated with CpG (ODN 2006, 10 μg/mL; InvivoGen), human recombinant CD40L (1 μg/mL; Insight Genomics), phorbol esters (PMA, 50 ng/mL; Sigma-Aldrich), ionomycin (1 μg/mL; Sigma-Aldrich) and Brefeldin A (BFA; BioLegend) as indicated. Alternatively, commercially available magnetic separation-based negative selection kits were utilized to purify B cells (EasySep, StemCell Technologies) and CD4⁺ T cells (Human CD4⁺ T Cell Isolation Kit, Miltenyi Biotec). The following human recombinant proteins (100 ng/mL) were used to stimulate B cell IL-10 production in vitro: interleukin-12 (IL-12), IL-21, interleukin-27 (IL-27) and interleukin-35 Fc fusion protein (IL-35 Fc FP) from BioLegend; IFN-γ from PeproTech.

2.3.3. Immunofluorescence

PBMC were stained using pre-determined optimal concentrations of antibodies for cell surface staining, permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained for intracellular IL-10, IFN-γ, GM-CSF or IL-1β expression as described (36). Fixable cell viability dyes (Fixable Violet Dead Cell Stain, Invitrogen; Zombie Aqua, BioLegend) were used to exclude dead cells. The following anti-human fluorochrome-conjugated monoclonal antibodies (mAbs) were used (clones in parentheses): IgM (MHM-88), IgD (IA6-2), CD3 (OKT3), CD4 (OKT4), CD5 (UCHT2), CD10 (HI10a), CD19 (HIB19), CD20 (2H7), CD21 (Bu32), CD22 (HIB22), CD24 (ML5), CD38 (HIT2), CD44 (IM7), CD48 (BJ40),
CD73 (AD2), CD360 (2G1-K12), granulocyte-monocyte colony stimulating factor (GM-CSF) (BVD2-21C11), interleukin-1 beta (IL-1β) (H1b-98) and IL-10 (JES3-9D7) from BioLegend; IgG (G18-145), CD27 (M-T271) and IFN-γ (B27) from BD Pharmingen; interleukin-12 receptor beta 2 subunit (IL-12Rβ2) (305719) from R&D Systems; CD1d (51.1) and CD9 (eBioSN4) from eBiosciences. Surface IgA and immunoglobulin light chains (κ and λ) were visualized using goat polyclonal antibodies (Southern Biotech). Immunofluorescence was quantified using a FACSCanto II cytometer (BD Biosciences) and FlowJo (version X) analysis.

2.3.4. In vitro functional assays

Purified CD4+ T cells (1x10^6 cells/mL) were cultured for 72 hours with soluble CD3 mAb (clone HIT3a, 1 μg/mL, BD Pharmingen), CD40L (1 μg/mL; Insight Genomics) and CpG (ODN 2006, 10 μg/mL; InvivoGen) in the presence or absence of purified B cells (1x10^6 cells/mL) (1:1 ratio) in 48-well flat-bottom plates at a final volume of 1 mL. IL-10 receptor (IL-10R)-blocking mAb (5 μg/mL, clone 3F9, Biolegend) or control rat IgG2a (5 μg/mL, clone KLH/G2a-1-1, Southern Biotech) was added to the B-T cell cocultures as indicated. During the last 5 hours, cultures were stimulated with PMA, ionomycin and BFA. Cells were then stained with fluorophore-conjugated mAbs against CD3, CD4, CD19 and IFN-γ, followed by flow cytometry analysis as above.
2.3.5. Serum cytokine analysis

Serum IFN-γ, IL-10 and IL-21 concentrations in duplicate samples were quantified by ELISA MAX kits (Biolegend) according to the manufacturer’s protocols with results quantified using a Molecular Devices microplate reader (model Emax). Four-parameter regression analysis was used to fit standard curves and calculate serum cytokine values.

2.3.6. Statistical analysis

For all comparisons, two-sided tests were used; alpha was set at 0.05. All differences between groups were tested with the independent sample Mann-Whitney U test (unpaired) unless otherwise indicated. All statistical testing was performed on the IBM SPSS platform (version 22). Graphics were created with GraphPad PRISM (version 7) and IBM SPSS statistics.
3. B10 cell B cell receptor (BCR) studies

3.1. Introduction

BCR specificity, affinity and signaling are the most important currently identified factors in B10 cell development. B10 cell regulation of inflammation and autoimmunity is antigen-specific (23, 34). Signaling through the B cell receptor (BCR) appears critical during early development in vivo. The importance of BCR diversity is demonstrated by the fact that B10+B10PRO cells are reduced by approximately 90% in transgenic mice with a fixed BCR (39). CD19-deficient mice (where BCR signaling is decreased) have a 70–80% decrease in B10+B10PRO cells (34). In contrast, B10 cells are expanded in human CD19 transgenic mice (where the over-expression of CD19 augments BCR signaling). The absence of CD22, which normally dampens CD19 and BCR signaling (40), also results in increased B10 cell numbers. Ectopic B cell expression of CD40L (CD154) in transgenic mice, which induces increased CD40 signaling (41), also increases B10 cell numbers. CD22−/− mice that also ectopically express CD40L show dramatically enhanced numbers of CD1dhiCD5+ B cells and B10 cells (42). Therefore, the study of B10 cell development closely relates to the study of their BCRs since it provides information about the signals involved in their selection in vivo.
3.2. Results

3.2.1. Peritoneal cavity B10 cells express germline-encoded unmutated BCRs

The BCRs of individual peritoneal cavity IL-10^+CD19^+ B cells of wild-type mice were sequenced to obtain an unbiased representation of their heavy and light chain repertoires. Both heavy (H) and light (L) chain transcripts from single cells revealed the use of diverse V_H and V_K family members (Fig. 3A, Tables I, II). V_H3 and V_H12 were the most frequently observed V_H families, consistent with the predominance of these families within the B1 cell repertoire (116, 117). Germline sequences without somatic mutations encoded 76% of 39 representative V_H-D_H-J_H sequences and 84% of 77 representative V_K-J_K sequences (Fig. 3B). The BCR repertoire of peritoneal cavity B10 cells was remarkably diverse, involving a wide spectrum of V_H, D_H, and J_H elements, normal frequencies of non-coded nucleotide (N) insertions, as well as considerable CDR3 diversity. Peritoneal cavity B10 cell V_H use was similar to that observed for spleen B10 cells (55) and conventional B cells (116), but it also included sequences commonly associated with peritoneal cavity B1 cells (118, 119). Whether the B10 cell BCR repertoire in other anatomic locations is similar to the peritoneal cavity B10 cell BCR remains to be determined.
Figure 3. Peritoneal cavity B10 cells use largely unmutated diverse V genes.
Figure 3. Peritoneal cavity B10 cells use largely unmutated diverse V genes. (A) $V_H$ family gene usage by 36 IL-10$^+$ B cells and $V_K$ family gene usage by 81 IL-10$^+$ B cells from two mice. (B) Mutation frequencies within the $V_H$-$D_H$-$J_H$ and $V_K$-$J_K$ gene sequences. (C) Phylogenetic trees showing relationships between the $V_H$-$D_H$-$J_H$ (n = 36) or $V_K$-$J_K$ (n = 50) amino acid sequences of individual B cells from individual mice named A or B, with numbers indicating different B cells. Branches indicate the average distance between two sequences based on percent identity.
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\(V_α-D_β-J_β\) genes from single IL-10+ cells were identified as in Fig. 5A-C. Italicized text in the "V End" column denotes the codon sequence immediately prior to the CDR3. In the "Mutations" column, italicized text indicates a synonymous mutation. Parentheses indicate nucleotides that may have been deleted during Ig gene rearrangement.

FWR, Framework region; N, Nucleotide addition; P, P nucleotide addition.
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Table II. B10 cell V<sub>K</sub>-J<sub>K</sub> sequences (continued)

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V<sub>K</sub>-J<sub>K</sub> genes from single IL-10<sup>+</sup> cells were identified as in Fig. 5A-C. Hatched text in the “V End” column denotes the codon sequence immediately prior to the CDR3. In the “Mutations” column, hatched text indicates a synonymous mutation. Parentheses indicate nucleotides that may have been deleted during Ig gene rearrangement. FWR: Framework region; N, N nucleotide addition; P, P nucleotide addition.

3.2.2. B10 cells are enriched among λ<sup>+</sup> B cells

The surface expression of B10 and B10+B10<sub>pro</sub> cell light chains in C57BL/6 and BALB/c mice was examined by flow cytometry. Single cell suspensions were stained for live/dead, CD19, κ and λ light chains and IL-10 following 5 hours of culture with LPS and PMA/ionomycin for B10 cells or 48 hours of culture with CD40L (CD154) and LPS (last 5 hours cells were stimulated with PMA/ionomycin) for B10+B10<sub>pro</sub> cells. B10 cells were enriched in the λ<sup>+</sup>κ<sup>−</sup> B cell population in all tissues examined including spleen, lymph nodes, blood, peritoneal cavity and bone marrow both in C57BL/6 and BALB/c mice (Figure 4A-E). B10+B10<sub>pro</sub> cells were also enriched in the λ<sup>+</sup>κ<sup>−</sup> B cell population in the spleen and lymph nodes (Figure 4F-G). Positivity for light chain staining was determined using isotype control matched mAbs and fluorescence minus one (FMO) controls; in addition, different clones of mAbs with a variety of fluorophores were utilized to exclude staining artifacts.
Figure 4. Surface expression of λ lights chains by B10 and B10<sub>PRO</sub> cells. The surface expression of B10 and B10+B10<sub>PRO</sub> cell light chains in C57BL/6 and BALB/c mice was examined by flow cytometry. Cells were stained for live/dead, CD19, κ and λ light chains and IL-10 following 5 hours of culture with LPS for B10 cells or 48 hours of culture with CD40L and LPS for B10+B10<sub>PRO</sub> cells; during the last 5 hours of culture, cells were stimulated with PMA/ionomycin. The percentages of λ<sup>+</sup>κ<sup>-</sup> B cells between IL-10<sup>-</sup> and IL-10<sup>+</sup> B cells are shown in different anatomic locations; (A) spleen B10 cells, (B) lymph node B10 cells, (C) blood B10 cells, (D) bone marrow B10 cells (E) peritoneal cavity B10 cells, (F) spleen B10+B10<sub>PRO</sub> cells, (G) lymph node B10+B10<sub>PRO</sub> cells, and (H) bone marrow B10+B10<sub>PRO</sub> cells.
3.2.3. The κ/λ ratio is inverted in human B10 cells

Following the observation in mice that B10 cells are enriched among λ⁺ B cells, I examined the λ chain surface expression of human B10 cells. Human PBMC from healthy adults (n=2) and healthy children (n=4) were cultured with CpG and PMA/ionomycin in the presence of Brefeldin A and stained for intracellular IL-10, surface CD19 and λ. Positivity for light chain staining was determined using isotype control matched mAbs and fluorescence minus one (FMO) controls; in addition, different clones of mAbs with a variety of fluorophores were utilized to exclude staining artifacts. The normal κ/λ ratio for conventional B cells is inverted in B10 cells (Figure 5). Specifically, whereas ~40% of conventional B cells are λ⁺, ~70% of B10 cells are λ⁺; the κ/λ ratio for conventional B cells is ~1.5, whereas the κ/λ ratio for B10 cell is ~0.4. The reversal of the κ/λ ratio in B10 cells is another piece of evidence pointing at the importance of BCR signals during early B10 cell development and their selection through antigen in vivo.
Figure 5. Human B10 cells have inverted κ/λ ratio.
Figure 5. Human B10 cells have inverted $\kappa/\lambda$ ratio. Human PBMC were cultured with CpG and PMA/ionomycin in the presence of Brefeldin A and stained for intracellular IL-10, surface CD19 and $\lambda$. **(A)** Modal histograms of IL-10$^+$ (thick line) and IL-10$^-$ (interrupted line) B cells showing $\lambda$ chain expression; isotype-matched monoclonal antibody (mAb) staining is shown as a gray shaded area. The two leftmost panels are from healthy adults (n=2) and the rest from healthy children (n=4). **(B)** Cumulative comparison of $\lambda$ chain expression between B10 (IL-10$^+$) and non-B10 (IL-10$^-$) cells from healthy children and healthy adults. **(C)** Cumulative comparison of the $\kappa/\lambda$ ratio between B10 (IL-10$^+$) and non-B10 (IL-10$^-$) cells from healthy children and healthy adults. P values were calculated using the paired-sample $t$-test.
3.3. Conclusions

Peritoneal cavity B10 cells expressed clonally diverse BCRs (Fig. 3C) that were predominantly germline encoded with no somatic mutations in most clones (Fig. 3; Tables I and II), as it has been previously observed for splenic B10 cells (55). Thus, the B10 cell BCR repertoire appears to be generated in response to diverse foreign and/or self-antigens, which may explain B10 cell enrichment within the peritoneal cavity relative to other tissues.

The enrichment of B10 cells in the λ+ B cell compartment is another piece of evidence pointing at the importance of BCR signals during early B10 cell development and their selection through antigen *in vivo*. B10 cells were enriched in the λ+κ− B cell population in all tissues examined including spleen, lymph nodes, blood, peritoneal cavity and bone marrow both in C57BL/6 and BALB/c mice (Figure 4A-E). The normal κ/λ ratio for conventional B cells was inversed in human B10 cells (Figure 5). Specifically, whereas ~40% of conventional B cells were λ+, ~70% of B10 cells were λ+; the κ/λ ratio for conventional B cells was ~1.5, whereas the κ/λ ratio for B10 cell was ~0.4. The preferential expression of λ light chains by both mouse and human B cells may indicate extensive light chain rearrangements and the involvement of receptor editing during their early development *in vivo* (120-123).
4. IL-10-producing regulatory B cells in the developing human and in pediatric autoimmunity

4.1. Introduction

Regulatory B cells that inhibit immune responses through the secretion of cytokines have been described in a variety of mouse models of autoimmunity and inflammation (30, 31). A subset of B cells that are competent to express the negative regulatory cytokine IL-10 (B10 cells) is among those best studied. Because phenotypic markers unique to B10 cells have not been identified, these cells are defined by their IL-10 expression following appropriate in vitro stimulation (36, 38). As in mice (34, 47), B10 cells are found at low frequencies in humans (36, 124). Human regulatory B cells have been reported within B cell subsets expressing phenotypic markers associated with transitional B cells (59-62, 125), memory B cells (36, 60, 62, 63), germinal center B cells (64), and plasmablasts (63). Although the functional importance of B10 cells is well described in mice, their role in human autoimmunity remains unclear. In adult humans, B10 cell frequencies are increased or maintained with autoimmunity (36). However, defects in the size and/or functionality of various regulatory B cell compartments have also been reported (59, 61, 62, 126-131).
B10 cell frequencies and numbers are increased in newborn and aged mice (47). Two studies have examined B10 cells in healthy children, one in cryopreserved cord blood samples (3) and one in the context of Wiskott-Aldrich syndrome (132). There are no studies examining B10 cells across the entire age range of normal human development. Common low-grade inflammatory conditions (such as hypertension or obesity) that potentially confound the assessment of immunologic parameters in adults are rare in children (133, 134). Furthermore, the incidence of autoimmunity is lower in children compared to adults (135-141), in contrast with the extensive autoantigen exposure associated with tissue remodeling during normal growth. Additionally, the surface phenotype of blood B cells changes with age during childhood and reflects overall changes in B cell development (142-144). Thereby, studies of B10 cells in the developing human offer unique opportunities to examine this regulatory subset during normal growth and in the context of autoimmunity, and to better define the relationship between B10 cells and surface phenotype-defined developmental B cell subsets.

4.2. Results

4.2.1. The B10 cell compartment transiently expands in childhood

The size of the “B10 cell compartment” (B10 cells and B10 progenitor cells) was assessed (Figure 6) as described (36). B10 cells are defined by their capacity to express IL-10 after
Figure 6. Gating approach for the identification of B10 and B10+B10PRO cells.

Peripheral blood mononuclear cells (PBMCs) from a healthy 7-year-old child were cultured with either CpG for 5 hours (B10 cells, top row) or CD40L+CpG for 48 hours (B10+B10PRO cells, bottom row); the cultures were stimulated the last 5 hours with PMA/ionomycin in the presence of Brefeldin A (“PIB”). Flow cytometry dot plots following immunofluorescence staining are shown. Prior to the identification of B10 and B10+B10PRO cells, the gating approach (left to right) consists of singlets and lymphocytes based on size distribution (forward scatter area and height, FSC-A and FSC-H) and granularity (side scatter area, SSC-A), live cells based on the exclusion of a fixable live/dead dye, and B cells based on CD19 positivity.
short-term (5 hours) *ex vivo* stimulation with PMA, ionomycin and Brefeldin A (BFA) ("PIB"; Figure 7A). B10 progenitor (B10*PRO*) cells do not express IL-10 following short-term *ex vivo* stimulation (23) but acquire IL-10-competence following 48 hours of agonistic CD40 stimulation (CD40L) *in vitro* (Figure 7A); the addition of CpG further enhances the acquisition of IL-10 competence (36). Adding BFA alone during the last 5 hours serves as a negative control for the 0.1-0.2% background staining, similar to the frequencies observed in IL-10-deficient mice (38, 47).

Healthy children (n=20, age range: 3-16 years, Table III) had significantly increased frequencies and numbers of B10 and B10+B10*PRO* cells (Figure 7B) when compared to healthy adults (age range: 27-52 years). Mean frequencies of B10 and B10+B10*PRO* cells in children were increased by 1.7-fold and 2.4-fold, and mean numbers of B10 and B10+B10*PRO* cells increased by 3.9-fold and 5.7-fold, respectively. Adult B10 (n=14) and B10+B10*PRO* (n=11) cell measurements were representative of those from >100 healthy adults (36, 124). Children had higher absolute lymphocyte counts (ALC), B cell frequencies and B cell numbers compared to adults (Figure 8A). The B10 cell compartment size was similar between males and females (Figure 8B). Expansion of the B10+B10*PRO* cell compartment was transient and peaked in middle childhood (5-11 years); a less prominent relationship with age was observed for B10 cells (Figure 7C).
Figure 7. The B10 cell compartment transiently expands in childhood.
Figure 7. The B10 cell compartment transiently expands in childhood. (A) PBMCs from healthy individuals were cultured with CpG for 5 hours to visualize B10 cells, or with CD40L+CpG for 48 hours to visualize B10+B10\textsuperscript{PRO} cells; BFA or PMA, ionomycin and BFA (“PIB”) added to cultures during the last 5 hours. IL-10\textsuperscript{+} B cell frequencies were measured by flow cytometry. To position the IL-10\textsuperscript{+} gate, only BFA (instead of PIB) was added to the cultures during the last 5 hours. Average background staining for B cell IL-10 positivity was 0.1% and 0.2% for B10 and B10+B10\textsuperscript{PRO} cell cultures, respectively; numbers represent the frequencies (% of all CD19\textsuperscript{+}) within the IL-10\textsuperscript{+} gate. (B) Healthy children have increased frequencies and numbers of B10 and B10+B10\textsuperscript{PRO} cells compared to healthy adults. (C) The relationship between age and B10 (left panel) or B10+B10\textsuperscript{PRO} (right panel) cell frequencies. Interrupted lines represent the upper and lower limits of the 99% confidence interval of fitted polynomial regression curves, with adults grouped as a single age point (20+). P values were calculated using the independent sample Mann-Whitney U test (unpaired); crosses within boxplots represent means.
### Table III. Demographics and study characteristics of healthy children

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Table III. Demographics and study characteristics of healthy children. From left to right, the identification of each child is provided along with male or female sex, age (estimated gestational age is provided for the 4 cord blood samples), race and ethnicity, and whether surface phenotyping data (Figures 9-12), intracellular phenotyping (Figure 13), functional assays (Figure 14) and ex vivo cytokine effect assays were performed. Abbreviations: identification (ID), cord blood (CB), infant (INF), healthy child (HC), male (M), female (F), non-Hispanic (NH).
Figure 8. Basic immunologic parameters of study participants, and comparisons of the B10 cell compartment between male and female healthy children.
Figure 8. Basic immunologic parameters of study participants, and comparisons of the B10 cell compartment between male and female healthy children. (A) Comparisons of white blood cell (WBC) counts, absolute lymphocyte counts (ALC), B cell frequencies and B cell numbers between healthy adults and healthy children. (B) Comparisons of B10 and B10+B10 PRO cell frequencies and numbers between female and male healthy children. (C) Comparisons of WBC counts, ALC, B cell frequencies and B cell numbers in healthy children (HC), and children with JIA and JDM/SLE/MCTD. (D) Comparisons of WBC counts, ALC, B cell frequencies and B cell numbers in children with JIA and JDM/SLE/MCTD in relation to disease activity (“-” for inactive disease and “+” for active disease). Disease activity was measured using global physician assessment on a visual analogue scale. P values were calculated using the independent sample Mann-Whitney U test (unpaired); crosses in the boxplots represent the mean.
4.2.2. The B10 cell surface phenotype reflects overall B cell development

The surface phenotype of B10 cells in children was variable. B10 cells were enriched within the non-naïve (IgD), class-switched (IgG⁺ or IgA⁺) B cell compartments, indicating their prior exposure to antigen in vivo (Figure 9A). Nonetheless, the majority of B10 cells in children were IgD⁺⁺IgM⁺⁺. The frequency and density of B10 cell surface IgM, IgD, IgG, IgA, CD19, CD20, CD21, and CD22 expression were similar between subjects, whereas the expression and density of surface markers that dynamically change during B cell development and maturation such as CD5, CD9, CD10, CD24, CD27, CD38, CD44, CD48 and CD73 were more varied between subjects. The 5-hour stimulation with PMA/ionomycin and CpG did not alter the cell surface expression of any of the B cell phenotypic markers presented, although this treatment did induce significant changes in CD360 (IL-21 receptor) and IL-12Rβ2 expression (Figure 10). The surface phenotype of B10+B10PRO cells was not examined since it reflects the effects of CD40 and CpG signals during the 48-hour cultures (36).

Regulatory B cells in adults have been “localized” previously to either the memory CD24⁺CD27⁺ or the transitional CD24⁺⁺CD38⁺⁺ B cell compartments (36, 59-63, 125). While most adult B10 cells were CD27⁺ and ~50% were CD24⁺⁺CD27⁺⁺, only 12% of B10 cells in children were CD24⁺⁺CD27⁺⁺ (Figures 9B and 11A). In contrast, 32% of B10 cells in children were CD24⁺⁺CD38⁺⁺, while only 8% of adult B10 cells were CD24⁺⁺CD38⁺⁺
(Figures 9C and 11B). The surface phenotype of B10+B10Pro cells is not presented since it reflects the effects of CD40L and CpG signals during the 48-hour cultures. B10 or B10+B10Pro cell frequencies in children did not correlate with the size of either the memory CD24hiCD27+ or transitional CD24hiCD38hi B cell compartments (Figure 12). Compared to older children, neonates had low B10 and B10+B10Pro cell frequencies (Figure 7C) despite their higher frequencies of CD24hiCD38hi and lower frequencies of CD27+ B cells (142-144). Neonates and adults had similar B10 and B10+B10Pro cell frequencies, despite the dramatically different surface phenotypes of neonatal B cells (high immature/naive and transitional B cell frequencies) compared to high frequencies of memory B cells in adult blood (144). As in adults (36), CD5 was a poor marker for B10 cells in children and CD1d was generally not expressed by blood B cells (Figure 9A). In addition, the low frequencies of B10 and B10Pro cells in cord blood (Figure 7C) contrasts with the abundance of CD5+ B cells in neonates (145). Thus, although there are no cell surface markers specific for B10 cells, these cells were more prevalent within certain surface phenotype-defined B cell subsets depending on the age of the individual.
Figure 9. The surface phenotype of B10 cells varies during B cell development.
Figure 9. The surface phenotype of B10 cells varies during B cell development. PBMCs from healthy children and/or healthy adults were cultured with CpG and stimulated with PIB for 5 hours. The surface phenotype of B10 cells was examined by flow cytometry. All flow cytometry plots are gated on CD19+ live lymphocytes. (A) Modal histograms of IL-10+ (thick line) and IL-10– (interrupted line) B cells; isotype-matched monoclonal antibody (mAb) staining is shown as a gray shaded area. Representative data from at least 3 different samples from middle childhood are shown. (B-C) Contour plots comparing IL-10+ (B10 cells, top row) with IL-10– (bottom row) B cells from a healthy 7-year-old child (left column) and a healthy adult (right column) in regards to the surface expression of (B) CD24 and CD27 and (C) CD24 and CD38. Numbers represent the cell frequencies (% of total) within each gate, with mean (±SEM) values for children (n=5, age range: 3-11 years) and adults (n=5, age range: 27-52 years) shown in italics.
Figure 10. Effects of CpG and PMA/ionomycin on B cell surface phenotype. PBMCs from healthy children were cultured with either CpG and PIB (solid line) or Brefeldin A alone (BFA, interrupted line) for 5 hours. The surface phenotype of total B cells (defined as CD19+ lymphocytes) was examined by flow cytometry. The surface expression of IgM, IgD, IgG, IgA, CD1d, CD5, CD9, CD10, CD19, CD20, CD21, CD22, CD24, CD27, CD38, CD44, CD48 and CD73 did not change. The surface expression of CD360 (IL-21 receptor) decreased. The surface expression of IL-12Rβ2 increased. All data are representative of at least 3 separate experiments.
Figure 11. B10 cell phenotypic comparisons among healthy children and healthy adults. B cells from healthy adults (n=5, age range 27-52 years) and healthy children (n=5, age range: 3-11 years) were cultured with PMA, ionomycin and BFA for 5 hours, followed by immunofluorescence staining for IL-10, CD24, CD27 and CD38, and flow cytometry analysis. The frequencies of CD24$^{hi}$CD27$^+$ and CD24$^{hi}$CD38$^{hi}$ cells among adult and pediatric B10 cells are shown. (A) Adult B10 cells have higher frequencies of CD24$^{hi}$CD27$^+$ cells than pediatric B10 cells. (B) Pediatric B10 cells have higher frequencies of CD24$^{hi}$CD38$^{hi}$ cells than adult B10 cells. P values were calculated using the independent sample Mann-Whitney U test (unpaired); crosses in the boxplots represent the mean.
Figure 12. The size of the B10 cell compartment does not correlate with the frequencies of CD24hiCD27+ or CD24hiCD38hi B cells in healthy children.
Figure 12. The size of the B10 cell compartment does not correlate with the frequencies of CD24^hiCD27^hi or CD24^hiCD38^hi B cells in healthy children. (A) Scatter plots showing no correlation between B10 cell frequencies and CD24^hiCD27^hi B cells frequencies in healthy children (n=17, age range: 3-16 years). (B) Scatter plots showing no correlation between B10+B10^{PRO} cell frequencies and CD24^hiCD27^hi B cells frequencies in healthy children (n=17, age range: 3-16 years). (C) Scatter plots showing no correlation between B10 cell frequencies and CD24^hiCD38^hi B cell frequencies in healthy children (n=15, age range: 3-16 years). (D) Scatter plots showing no correlation between B10+B10^{PRO} cell frequencies and CD24^hiCD38^hi B cell frequencies in healthy children (n=15, age range: 3-16 years).
4.2.3. Most IL-10+ B cells do not express GM-CSF, IL-1β, or IFN-γ

Since B10 and B10+B10\textsubscript{PRO} cells cannot be visualized without PMA/ionomycin stimulation, we examined the possibility that these cells represent activated B cells expressing multiple cytokines in response to non-specific \textit{in vitro} stimulation. The B10 and B10+B10\textsubscript{PRO} cell expression profile of three proinflammatory cytokines was examined using purified B cells from three healthy children (Figure 13, Table III). In all three subjects, B10 cells did not express GM-CSF, IL-1β, or IFN-γ, while less than 15% of B10+B10\textsubscript{PRO} cells expressed GM-CSF and less than 5% expressed IL-1β or IFN-γ. Therefore, the B10 cell compartment in children has a specific cytokine expression profile, characterized by the limited or no expression of proinflammatory cytokines.

4.2.4. B10 cells negatively regulate CD4\textsuperscript{+} T cell IFN-γ expression \textit{in vitro}

The high B10+B10\textsubscript{PRO} cell frequencies in children provided the unique opportunity to examine the regulatory effects of the entire B10 cell compartment, eliminating the need to fractionate B cell subpopulations based on cell surface marker expression. The effects of regulatory B cell-derived IL-10 on activated CD4\textsuperscript{+} T cell IFN-γ expression was assessed in three healthy children (Figure 14, Table III). Purified CD4\textsuperscript{+} T cells stimulated through CD3 were cultured for 72 hours with purified B cells (ratio 1:1) in the presence of CD40L and CpG. The presence of B cells reduced the frequency of CD4\textsuperscript{+} T cells expressing IFN-γ, while CD4\textsuperscript{+} T cell IFN-γ expression was normalized by the addition of IL-10 receptor-
Figure 13. Relationship between IL-10-producing and proinflammatory cytokine-producing B cells.
**Figure 13. Relationship between IL-10-producing and proinflammatory cytokine-producing B cells.** B cells purified from a healthy 12-year-old child (HC17, Table III) were cultured with CpG for 5 hours (B10 cells) or CD40L+CpG for 48 hours (B10+B10<sub>PRO</sub> cells), with BFA or PIB added to the cultures during the last 5 hours. B cells were stained intracellularly for IL-10 and either GM-CSF (A), IL-1β (B) or IFN-γ (C). The position of all gates was determined using isotype-matched control mAb staining and fluorescence minus one (FMO) controls. These data are representative of those obtained in three separate experiments (n=3; HC3, HC7 and HC17 in Table III). All flow cytometry dot plots were gated on CD19<sup>+</sup> live lymphocytes, with numbers representing their frequencies (% of total) within each gate; mean (±SEM; n=3) values are shown within parentheses in italics.
Figure 14. B cells negatively regulate CD4⁺ T cell IFN-γ production in vitro via IL-10-dependent mechanisms.
Figure 14. B cells negatively regulate CD4+ T cell IFN-γ production in vitro via IL-10-dependent mechanisms. (A) Purified CD4+ T cells were cultured either alone (1st panel) or with purified B cells (1:1 ratio) (2nd-4th panels) in the presence of soluble anti-CD3, CD40L and CpG for 72 hours. In the 3rd and 4th panels, the cultures contained either rat IgG2a (control) or IL-10 receptor blocking antibody (anti-IL-10R). PMA, ionomycin and Brefeldin A were added during the final 5 hours of culture. CD4+ T cells are shown as CD19- cells because CD3 and CD4 are down-regulated during the 72 hour stimulation with anti-CD3 mAb and the 5 hour PMA/ionomycin stimulation, respectively. Numbers represent the frequencies (% of all CD19-) within the IFN-γ gate; the mean fluorescent intensity (MFI) value for IFN-γ staining is displayed in the right lower corner of each dot plot. (B) Cumulative comparisons of IFN-γ CD4+ T cell frequencies between the 3 different culture conditions (left to right), CD4+ T cells cultured alone, CD4+ T cells cultured with B cells and CD4+ T cells, cultured with B cells in the presence of anti-IL-10R mAb, respectively, from three separate experiments (n=3; HC14, HC16 and HC20 in Table III). P values were calculated using the paired-sample t-test.
-blocking monoclonal antibody to the B-T cell cocultures (Figure 14). Thus, B10 cells from children had significant IL-10-dependent negative regulatory effects on CD4+ T cell IFN-γ expression, as occurs in mice (90).

### 4.2.5. The B10 cell compartment contracts in children with autoimmunity

The size of the B10 cell compartment was compared between age-matched healthy children and children with autoimmunity (Figure 15A, Tables III and IV). Most children with autoimmunity had significantly lower B10 cell frequencies and numbers when compared to healthy children (Figure 16A). Children with autoimmunity were further categorized based on data clustering into two groups: 1) children with JIA excluding systemic-onset JIA (“JIA group”), and 2) children with JDM, SLE and MCTD (“JDM/SLE/MCTD group”). B10+B10PRO cell frequencies and numbers were decreased only in the JDM/SLE/MCTD group (Figure 16A). Children with JDM/SLE/MCTD had lower ALC compared to healthy children, but there were no significant differences in B cell frequencies and numbers or WBC counts among disease groups (Figure 8C).

### 4.2.6. The B10 cell compartment expands with active disease

Since JIA, JDM, SLE and MCTD do not share a disease activity measure, physician global assessment was used to assess disease activity after age-matching (Figure 15B). Both B10 and B10+B10PRO cell frequencies and numbers increased during active disease
Figure 15. Age-matching in children with autoimmunity and healthy controls. (A) Comparisons of the age distributions of HC, children with LA and SA. (B) Comparisons of the age distributions within children with LA and SA in relation to disease activity groups (“-” for inactive disease and “+” for active disease); disease activity was measured using global physician assessment on a visual analogue scale. (C) Comparisons of the age distributions of children not treated with MMF (MMF⁻, n=7) and children treated with MMF (MMF⁺, n=7). P values were calculated using the independent sample Mann-Whitney U test (unpaired); crosses in the boxplots represent the mean.
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Table IV. Clinical characteristics of children with autoimmunity. From left to right, the diagnosis of each child with autoimmunity during their initial study visit is provided along with whether longitudinal data were collected, sex, age, race and ethnicity, disease activity scores (visual analogue scale, VAS), disease duration, and medication exposures at the time of blood sampling. Where longitudinal data are available, the time between the two assessments is provided in parentheses in the longitudinal data column and the disease activity score at the second assessment in parentheses in the disease activity score column. Abbreviations: juvenile idiopathic arthritis (JIA), systemic lupus erythematosus (SLE), juvenile dermatomyositis (JDM), male (M), female (F), Hispanic (H), non-Hispanic (NH), methotrexate (MTX), leflunomide (LFL), sulfasalazine (SLFZ), etanercept (ETA), abatacept (ABA), adalimumab (ADA), prednisone (PRED, daily dose in mg in parentheses), mycophenolate mofetil (MMF), tacrolimus (TAC), intravenous immunoglobulin (IVIG), hydroxychloroquine (HCQ), MPRED (intravenous methylprednisolone), chloroquine (CLQ), non-steroidal anti-inflammatory drug (NSAID), and acetylsalicylic acid (ASA).
Figure 16. Children with autoimmunity have a contracted B10 cell compartment compared to age-matched healthy children.
Figure 16. Children with autoimmunity have a contracted B10 cell compartment compared to age-matched healthy children. (A) B10 and B10+B10\textsuperscript{PRO} cell frequencies and numbers are decreased in children with JIA (n=23-24) or JDM/SLE/MCTD (n=27) autoimmunity compared to age-matched healthy children (HC, n=18). Children with JDM, SLE and MCTD are shown as solid triangles, open triangles and open diamonds respectively. (B) B10 cell and B10+B10\textsuperscript{PRO} cell frequencies and numbers in children with JIA (n=23) or JDM/SLE/MCTD (n=27) in relation to disease activity (“-” for inactive disease and “+” for active disease); the B10 cell compartment shows a limited expansion with disease activity only in children with JDM/SLE/MCTD. P values were calculated using the independent sample Mann-Whitney U test (unpaired); crosses in the boxplots represent means. (C) Correlation of B10+B10\textsuperscript{PRO} cell frequencies with disease activity scores in children with JDM/SLE/MCTD. The interrupted lines represent the upper and lower limits of the 99% confidence interval of the fitted line. (D) Longitudinal measurements of B10+B10\textsuperscript{PRO} cell frequencies and disease activity scores in 6 children with JDM/SLE/MCTD (2 with SLE and 4 with JDM). The two time points are connected with a line ending with an arrowhead pointing at the second sampling time point.
in the JDM/SLE/MCTD group but not in the JIA group (Figure 16B). Despite the limited B10+B10\textsuperscript{PRO} cell expansion during active disease in the JDM/SLE/MCTD group, the frequencies and numbers of B10+B10\textsuperscript{PRO} cells remained below those of healthy children (Fig. 16B). B10+B10\textsuperscript{PRO} cell frequencies correlated linearly with VAS scores (Figure 16C). A similar trend was observed when B10+B10\textsuperscript{PRO} cell frequencies were followed longitudinally in 4 children with JDM and 2 with SLE (Figure 16D, Table IV). There were no significant differences between disease activity groups in WBC counts, ALC, and B cell frequencies or numbers (Figure 8D). Thereby, although the B10 cell compartment is contracted in children with autoimmunity, B10 cells retain their ability to expand during active disease in children with JDM and SLE.

### 4.2.7. Medication effects on the B10 cell compartment

Each group of children with autoimmunity (JIA and JDM/SLE/MCTD) was classified into two “medication subgroups” for each medication (Table IV) based on whether or not the child was on the medication at the time of blood sampling. No significant differences in B10 or B10+B10\textsuperscript{PRO} cell frequencies between medication subgroups were noted with the exception of mycophenolate mofetil (MMF) in children with SLE. MMF-treated children had higher B10 cell frequencies compared to children not treated with MMF (Figure 17A). B10+B10\textsuperscript{PRO} cell frequencies were not significantly higher in MMF-treated children compared to children not treated with MMF (p=0.097, Figure 17B).
Figure 17. Effects of mycophenolate mofetil (MMF) on the B10 cell compartment and basic immunologic parameters in children with SLE. (A) MMF-treated children (MMF+, n=7) have higher frequencies (left panel) but not numbers (right panel) of B10 cells (left panel) compared to children not treated with MMF (MMF-, n=7). (B) The frequencies (left panel) and numbers (right panel) of B10+B10PRO cells are no different between MMF- and MMF+ children. (C) Comparisons of B cell frequencies and B cell numbers between MMF- and MMF+ children. (D) Comparisons of WBC counts and ALC between MMF- and MMF+ children. P values were calculated using the independent sample Mann-Whitney U test (unpaired); crosses in the boxplots represent the mean.
MMF-treated children had lower B cell numbers compared to children not treated with MMF (Figure 17C), but there were no significant differences in the age distribution (Figure 15C) or WBC counts, ALC and B cell frequencies (Figure 17C-D) between MMF-treated children and children not treated with MMF. Therefore, MMF therapy or other treatments did not explain the contracted B10 cell compartment found in children with autoimmunity.

4.2.8. Children with autoimmunity have high IFN-γ and low IL-21 serum levels

Although inherently low in most subjects, serum IL-10 concentrations were highest in the JDM/SLE/MCTD group and healthy adults (Figure 18A). Higher levels of IL-10 among adults and among children with systemic autoimmunity contrasts with their lower frequencies of blood B10 cells, but multiple cell types produce IL-10 and undoubtedly contribute to the serum IL-10 pool. By contrast, healthy children had significantly lower serum IFN-γ and higher IL-21 levels compared to children with autoimmunity or healthy adults (Figure 18A). However, serum cytokine levels did not correlate with the size of the B10 cell compartment between individuals in any group and regardless of disease activity. Nonetheless, IL-21 promotes whereas IFN-γ inhibits mouse B cell IL-10 expression in vitro (48). Therefore, the differences in serum cytokines could provide a partial explanation for the differences of the B10 cell compartment among the study populations.
Figure 18. Serum cytokine levels and differential regulation of B cell IL-10 expression by IFN-γ and IL-21.
Figure 18. Serum cytokine levels and differential regulation of B cell IL-10 expression by IFN-γ and IL-21. (A) Serum IL-10, IFN-γ and IL-21 levels in HC (n=18), age-matched children with JIA (n=25) or JDM/SLE/MCTD (n=27), and healthy adults (n=16). Serum cytokines were measured by ELISA. P values were calculated using the independent sample Mann-Whitney U test (unpaired); crosses in the boxplots represent means. Children with JDM, SLE and MCTD are shown as solid triangles, open triangles and open diamonds respectively. (B) Representative effects of IL-21 and IFN-γ on B cell IL-10 competence. Purified B cells from a healthy 12-year-old child were cultured for 48 hours with CD40L+CpG in the presence or absence of IFN-γ or IL-21 and stimulated with either Brefeldin A (BFA) or PMA, ionomycin and BFA (“PIB”) during the last 5 hours. IL-10+ B cell frequencies were measured by immunofluorescence staining with flow cytometry analysis. Numbers represent the frequencies (% of all CD19+) within the IL-10+ gate. (C) Cumulative results of IL-10+ B cell frequencies following culture of purified B cells with CD40L+CpG (“control”) alone or with IFN-γ, IL-21 or IL-35 Fc FP. Paired samples (from the same individual) are connected with an interrupted line. P values were calculated using the paired-sample t-test.
4.2.9. IL-21 promotes whereas IFN-γ inhibits human B cell IL-10 competence

In view of the observed differences in serum cytokine levels, along with the reported effects of IFN-γ (48), IL-21 (48), and interleukin-35 (IL-35) family members (50, 51) on B cell IL-10 expression, the effects of these cytokines on B cell IL-10 competence was examined. Purified B cells from healthy children were cultured for 48 hours with CD40L and CpG in the presence or absence of IL-21, IFN-γ, IL-12, IL-27, or IL-35 Fc FP. IL-10 expression was not detectable in any of these cultures without the addition of PIB during the last 5 hours. IL-21 enhanced whereas IFN-γ inhibited B cell IL-10 expression (Figure 18B-C), while IL-35 Fc FP, IL-12 or IL-27 had no effect (Figures 18C and 19A-B). When both IL-21 and IFN-γ were added to cultures, the effects of IL-21 on B cell IL-10 expression predominated (Figures 18C and 19A). Similar results were obtained with B cells from healthy adults (Figure 19C). Thus, B cell IL-10 production was predominantly and differentially regulated by IL-21 and IFN-γ, which may also influence the size of the B10 cell compartment in vivo.
Figure 19. *In vitro* effects of cytokines on human B cell IL-10 competence.
Figure 19. *In vitro* effects of cytokines on human B cell IL-10 competence. (A) Purified B cells from a healthy 12-year-old child were cultured for 48 hours with CD40L+CpG in the presence or absence of either IFN-γ+IL-21, IL-12, IL-27, or IL-35-Fc FP and stimulated with either Brefeldin A (BFA) alone or with PMA, ionomycin and BFA (“PIB”) during the last 5 hours. The two leftmost on the first row panel are duplicate from Figure 6B. (B) Cumulative measurements of IL-10+ B cell frequencies following stimulation with either CD40L+CpG (“control”) alone or with IL-12 (n=5) or IL-27 (n=6); p values were calculated using the paired-sample *t*-test; paired samples (from the same individual) are connected with an interrupted line. (C) Purified B cells from a healthy adult were cultured for 48 hours with CD40L+CpG in the presence or absence of either IFN-γ, IL-21, IFN-γ +IL-21, IL-12, IL-27, or IL-35-Fc FP and stimulated with either BFA or PIB during the last 5 hours. The frequencies of IL-10+ B cells were measured by flow cytometry. Numbers in flow cytometry dot plots represent the frequencies (% of all CD19+) within the IL-10+ gate.
4.3. Conclusions

The study of B10 cells in the developing human revealed that the B10 cell compartment transiently expands in childhood. The expansion peaks in middle childhood when up to ~30% of B cells are competent to produce IL-10 under in vitro polarizing conditions. The phenotype of B10 cells from children was variable and reflective of overall B cell development. The B10 cell compartment has a specific cytokine-expressing profile that does not include proinflammatory cytokines such as GM-CSF, IL-1β, or IFN-γ. Pediatric B10 cells were able to negatively regulate CD4+ T cell IFN-γ expression in vitro. Children with autoimmunity had a contracted B10 cell compartment. The B10 cell compartment was more profoundly contracted in children with JDM/SLE/MCTD compared to children with JIA. In children with JDM/SLE, The B10 cell compartment showed a limited expansion with disease activity. Medication effects were not able to explain the observed deficiency of the B10 cell compartment in children with autoimmunity. Children with autoimmunity had higher IFN-γ and lower IL-21 serum levels compared to age-matched healthy controls and IL-21 promotes whereas IFN-γ inhibits human B cell IL-10 competence in vitro. Therefore, the observed deficiency of the B10 cell compartment in children with autoimmunity may be explained by the differences in the cytokine milieu that B cells have been exposed to in vivo and does not necessarily indicate an intrinsic B cell defect.
5. Discussion

BCR specificity, affinity and signaling are the most important currently identified factors in B10 cell development. B10 cell regulation of inflammation and autoimmunity is antigen-specific (23, 34). BCR specificity dramatically influences spleen B10 cell development and antigen-specific regulatory function (34, 47, 48). Receptors or pathways that positively or negatively regulate BCR signaling, such as CD19, CD22, and CD40, can also significantly modulate B10 cell numbers (42, 46, 47, 146, 147). In contrast, potent BCR cross-linking by anti-IgM Abs significantly reduces the number of peritoneal B cells that expressed or secreted IL-10 (148), as also occurs with spleen B cells (47). Thereby, appropriate BCR signals are thought to induce a select subset of B cells to become B10\textsuperscript{pro} and then IL-10–competent B10 cells, whereas strong BCR signals may divert B cells into a different functional program (32). Consistent with their \textit{in vivo} antigen stimulation, spleen B10 cells are hyper-responsive to mitogens (36, 47) and can give rise to antigen-specific, self-reactive, or natural antibodies (55). Peritoneal cavity B10 cells expressed clonally diverse BCRs (Fig. 3C) that were predominantly germline encoded with no somatic mutations in most clones (Fig. 3; Tables I and II), as it has been previously observed for splenic B10 cells (55). Thus, the B10 cell BCR repertoire appears to be generated in response to diverse foreign and/or self-antigens, which may explain B10 cell enrichment within the peritoneal cavity relative to other tissues.
The enrichment of B10 cells in the λ⁺ B cell compartment is another piece of evidence pointing at the importance of BCR signals during early B10 cell development and their selection through antigen in vivo. B10 cells were enriched in the λ⁺κ⁻ B cell population in all tissues examined including spleen, lymph nodes, blood, peritoneal cavity and bone marrow both in C57BL/6 and BALB/c mice (Figure 4A-E). The normal κ/λ ratio for conventional B cells was inverted in human B10 cells (Figure 5). Specifically, whereas ~40% of conventional B cells were λ⁺, ~70% of B10 cells were λ⁺; the κ/λ ratio for conventional B cells was ~1.5, whereas the κ/λ ratio for B10 cell was ~0.4. The preferential expression of λ light chains by both mouse and human B cells may indicate extensive light chain rearrangements and the involvement of receptor editing during their early development in vivo (120-123).

The studies in the developing human demonstrate that the blood B10 cell compartment is uniquely expanded in children relative to newborns and adults. B10+B10^PRO cell frequencies peaked at ~30% in middle childhood (Figure 7C), while the B10 cell compartment normally represents <10% of newborn and adult blood B cells (36). This increase in children did not correlate with the expansion of a specific phenotypically-defined B cell subset (Figures 9-12). Despite differences between the B10 cell compartments of children and adults, B10 cells from children retained their regulatory capacity and down-regulated CD4⁺ T cell IFN-γ production through IL-10-
dependent pathways in vitro (Figure 14). There were also significant reductions in B10 cell and B10+B10^{pro} cell frequencies and numbers in children with autoimmune disease, but their frequencies nonetheless increased significantly with disease severity in children with JDM and SLE (Figure 16). Dynamic changes in the size of the B10 cell compartment are likely to be functionally important as small increases in B10 cell and B10^{pro} cell numbers in mice during adoptive transfer experiments can reduce inflammation and autoimmune disease (23, 34, 48, 50, 149), while reducing B10 cell numbers enhances subsequent innate and adaptive immune responses (42, 90, 150). Thus, alterations in the size of the B10 cell compartment in children may differentially affect tolerance regulation and autoimmune disease.

The size of the IL-10-producing regulatory B cell compartment varies during normal human development, at least in peripheral blood, and expands transiently, peaking in middle childhood. Whether this phenomenon is limited to circulating B cells, indicating their trafficking to tissues during a specific time period of human development, or it extends to B cells in other tissues remains unknown. When LPS is used instead of CpG for the B10 and the B10+B10^{pro} assays (Figure 20), healthy children have increased frequencies of B10 cells, but not of B10+B10^{pro} cells, compared to adults or children with autoimmunity. This suggests that TLR9-specific signals are more
Figure 20. B10 and B10+B10PRO cell assays using LPS instead of CpG.
Figure 20. B10 and B10+B10\textsubscript{PRO} cell assays using LPS instead of CpG. PBMCs from healthy adults, healthy children (“HC”) and children with JIA or JDM/SLE/MCTD autoimmunity were cultured with CpG for 5 hours (“B10 cells”) or CD40L+LPS (“B10+B10\textsubscript{PRO} cells”) for 48 hours. Brefeldin A (“BFA”) or PMA/ionomycin/BFA (“PIB”) was added to the cultures during the last 5 hours. The frequencies of IL-10+ B cells were measured by immunofluorescence staining and flow cytometry. To determine the position of the IL-10+ gate, only BFA (instead of PIB) is added to the cultures during the last 5 hours; the background staining for B cell IL-10 positivity is 0.1% and 0.2% for the (A) B10 and (B) B10+B10\textsubscript{PRO} cell assays respectively. (C) Healthy children (n=19) have increased frequencies/numbers of B10 cells compared to healthy adults (n=3). (D) Healthy children (n=20) have increased numbers but not increased frequencies of B10+B10\textsubscript{PRO} cells compared to healthy adults (n=4). (E) Children with JIA (n=24) or JDM/SLE/MCTD (n=27) have decreased B10 cell frequencies/numbers compared to HC (n=17). (F) Children with JDM/SLE/MCTD (n=27) have decreased B10+B10\textsubscript{PRO} cell frequencies/numbers compared to HC (n=18) or children with JIA (n=23). P values were calculated using the independent sample Mann-Whitney U test; crosses in the boxplots represent the mean.
important than TLR4-specific signals during the earlier stages of B10 cell development. Alternatively, CpG may mediate its effects through the BCR (151-153).

Human regulatory B cells have been reported within a diverse variety of surface phenotype-defined developmental B cell subsets (36, 59-64, 125). While most adult B10 and B10pro cells express a CD24hiCD27+ memory phenotype (36, 124), the surface phenotype of B10 cells in children was variable, and expansion of the B10 cell compartment in children did not correlate with the normal age-related shifts among B cell subsets (Figures 9-12). B10 cells from children were highly represented within the transitional CD10+CD24hiCD38hiCD44low B cell subset and relatively few pediatric B10 cells expressed CD27 and high levels of CD24. A lack of correlation between phenotypically-defined developmental B cell subsets and IL-10-competent B cells is well documented in adults (142-144). The phenotypic variability of B10 cells during normal human development and their lack of correlation with surface phenotype-defined developmental B cell subsets highlights the concept that B10 cells are a unique B cell subset that is functionally programmed to produce IL-10 in response to appropriate B cell antigen receptor (BCR) signals rather than representing a cell lineage or a specific stage of B cell development (31). The transient capacity B10 cells to express IL-10 following appropriate activation in mice is highlighted by their termination of IL-10 production prior to plasmablast and plasma cell differentiation (55). Furthermore, both
B10 and B10+PRO cells display a specific cytokine-expression profile with limited or no expression of inflammatory cytokines such as GM-CSF (154), IL-1β and IFN-γ (Figure 13). Thus, their capacity to express IL-10 remains the most comprehensive marker for B10 and B10PRO cell identification and function.

Blood B10 cell frequencies increased during childhood, but the most dramatic increase was observed in B10PRO cells (Figure 7C). Although B10 cells are inherently competent to produce IL-10 following PMA/ionomycin stimulation, B10PRO cell IL-10 competence is acquired during prolonged CD40 ligation along with CpG or LPS stimulation in vitro prior to PMA/ionomycin treatment (36). Like B10 cells in adults, B10PRO cells predominantly localize within the blood CD27+ memory B cell subset, but CD40 plus CpG or LPS stimulation does not induce significant numbers of purified CD27-negative B cells to acquire IL-10 competence. Thereby, B10pro cells are presumed to have encountered the appropriate BCR and transmembrane signals in vivo that initiate IL-10 competence, but they have not acquired the ability to transcribe the il10 gene (31). Consistent with this, BCR crosslinking with anti-IgM antibody in vitro terminates B10pro cell acquisition of IL-10 competence in humans and mice (31, 36), arguing that non-specific high intensity BCR signals induce a different functional program in B cells. The acquisition of IL-10 competence relates more to the selection of B cells by previous antigen exposure and the activation of specific signaling thresholds that elicit this
functional program rather than the simple differentiation of B cells into plasmablasts or plasma cells (31). Moreover, the vast majority of B cells can be activated with CD40 ligand, CpG or LPS, but only a subset of B cells can be induced to express IL-10. Thus, B10pro cells are likely to represent a precursor pool for the selection of functionally mature B10 cells during antigen-specific cognate interactions with T cells that induce some B10 cells to become B10 effector cells in vivo (48).

Human B10pro cell maturation into B10 cells was negatively regulated by IFN-γ and positively regulated by IL-21 in vitro (Figure 6) as occurs in mice (48). Remarkably, healthy children had lower serum IFN-γ concentrations as previously documented (155, 156), and higher serum IL-21 when compared to either children with autoimmunity or healthy adults (Figure 18). Thereby, contraction of the B10pro cell compartment in children with autoimmunity would be expected due to differences in the in vivo cytokine milieu that B cells are exposed to, which would predictably lead to reduced B10 cell numbers. Thus, rather than representing inherent functional defects within the B10 cell compartment, increased serum IFN-γ concentrations during autoimmune disease may reduce the size of the B10pro cell pool in children. Alterations in the cytokine milieu may similarly account for alterations within regulatory B cell compartments that have been reported to underlie autoimmune disease in adults (59, 61, 129, 130). Consistent with this hypothesis, human IL-21 deficiency results in autoimmune manifestations (157). Given
this and the higher levels of IL-21 in healthy children (Figure 18A), our hypothesis is that when stimulated with appropriate antigens plus cognate T cell help, B cells in children are more prone to acquiring IL-10 competence than adult B cells.

B10 cells are developmentally and functionally distinct from regulatory T cells, but they work synergistically with regulatory T cells to control autoimmune disease manifestations in mice (90). High frequencies of regulatory T cells from early and middle childhood were recently documented for healthy children in comparison with adolescents and adults (158). Thus, regulatory B10 cells and regulatory T cell expansion during middle childhood may represent a mechanism for promoting tolerance during this unique stage of human development. Thereafter, blood B10 cell frequencies do not change remarkably within adults until they decrease substantially (<0.2%) with old age (124). By contrast, the mouse spleen B10 cell compartment is expanded in neonatal mice, contracts quickly thereafter and remains stable through adulthood, but frequencies expand in aged mice (47). Thereby, immune tolerance mechanisms may be more prominent in children than in adults, especially prior to adolescence.

The B10 cell compartment can expand with autoimmune disease in both adult humans and mice (36, 47), and in individuals prone to developing chronic lymphocytic leukemia (124). In fact, expansion of the B10 cell compartment in some adults with autoimmune disease is similar to the expansion reported herein for children (36). By
contrast, however, the B10 cell compartment was significantly contracted in children with autoimmune disease (Figure 16A), and the frequencies of B10 cells in children with autoimmune disease resembled those normally observed in healthy adults (Figure 7). Nonetheless, the B10 cell compartment remained dynamic as B10+B10\textsuperscript{PRO} cell frequencies showed a limited increase with disease activity in children with JDM and SLE, but returned to low levels as disease activity decreased (Figure 16B). B10 and B10\textsuperscript{PRO} cell frequencies are also significantly reduced in children with Wiskott-Aldrich syndrome relative to healthy children (132). While defects in the size and/or the functional capacity of adult regulatory B cell compartments may contribute to the development of autoimmunity (59, 61, 62, 126-131), the current results suggest the opposite possibility that autoimmunity and inflammation may contribute to the reduced size of the B10 cell compartment. It thereby remains premature to draw conclusions regarding the dynamic function of the B10 cell compartment until there is a better understanding of the factors regulating their numbers and function \textit{in vivo} during autoimmunity.

The IL-35 family of cytokines has been recently shown to be involved in the development of B cell IL-10 competence (50, 51). However, in our study, the IL-35 family cytokines (IL-12, IL-27, and IL-35 Fc FP) did not promote B cell IL-10 competence (Figures 18-19). It is important to note that our approach for evaluating the involvement of IL-35 in the acquisition of human B cell IL-10 competence is different than the
approach previously taken (51) where purified B cells were stimulated for 3 days with PMA and ionomycin prior to immunofluorescence IL-10 staining. The exposure of human B cells to PMA and ionomycin for more than 6 hours results in extensive B cell death (159) and in all our assays only a brief (5 hours) stimulation with PMA and ionomycin was used to maintain cell viability at the time of immunofluorescence IL-10 staining. Furthermore, in both approaches, IL-35 Fc FP was used, a fusion protein that may not have the same properties as native human IL-35. Therefore, the effects of IL-35 on human B cell IL-10 competence in vitro remain to be determined. Nevertheless, the potent effects of IL-21 on human B cell IL-10 competence in vitro open new horizons in the development of autologous B10 cell-based therapies (48).

There are several inherent limitations and challenges encountered when using human samples and particularly in studies that involve sampling from children. Our studies in children with autoimmunity and age-matched healthy controls are limited to fresh peripheral blood samples, as opposed to lymphoid tissues or tissues affected by the disease process, which is an ongoing constraint in studies like ours. In addition, whether the conclusions of our study can be interpolated to autoimmunity in adults remains a challenge, since pediatric autoimmune diseases are distinct from adult autoimmune diseases in regards to both pathogenesis and treatment. Although our findings suggest that the contracted B10 cell compartment may be involved in the
development of autoimmunity, it remains unclear whether this is a primary defect or a secondary effect of alterations in the cytokine milieu that B cells have been exposed to in vivo. Furthermore, it remains unclear whether the reduced size of the B10 cell compartment or a functional defect of B10 cells contributes to the development of autoimmunity. Even if we had compared the regulatory effects of B cells from healthy children and children with autoimmunity side by side and observed a difference, we would be unable to conclude whether the difference is because of the differences of the B10 cell compartment size or due to an inherent functional defect of B cells in children with autoimmunity. Furthermore, the observed differences in serum cytokines are limited by the fact that several subjects had undetectable serum levels of IL-21 and IFN-γ, as well as by the lack of validated normal serum IL-21 reference values in healthy children and adults. Finally, the use of global physician assessment in the form of a VAS score has its own inherent limitations as a disease activity assessment tool (160); therefore, it would be of interest in the future to formally assess the value of B10+B10PRO cell frequency measurements as a disease activity tool, especially in JDM where comprehensive disease activity assessment tools are lacking.

In conclusion, this first study of B10 cells across the entire age range of developing humans documents that the size of the B10 cell compartment varies during normal human development, and expands transiently in childhood. The peak of the B10
cell compartment expansion occurred during middle childhood, when linear growth rates are lowest (161), suggesting that B10 cells are particularly important in immune tolerance regulation prior to the pubertal growth spurt. The B10 cell compartment responds dynamically to inflammation in both children with JDM/SLE (Figure 16), adults (36, 124) and mice (23, 124), indicating that B10 cell development in vivo may be regulated by antigen exposure and cytokines. Despite the expanded B10 cell compartment in healthy children, serum IL-10 levels remained low in comparison with adults and children with JDM/SLE/MCTD (Figure 18A), indicating that other cells preferentially contribute to serum IL-10 levels and consistent with B10 cells regulating immune responses within local microenvironments rather than through the provision of systemic IL-10 (48). The identification of therapies that restore or augment the B10 cell compartment will clarify these issues and may provide attractive strategies for modulating immune responses and autoimmunity in the future.
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7. Biography

I was born at the island of Chios in Hellas on March 23rd 1975 to Ekaterini and Dimitrios Kalampokis, and I have a younger sister named Charikleia. I went to medical school at and graduated from Charles University in Prague in July 2000. Following completion of the mandatory military service in my home country, I moved to New York where I stayed for 5 years (2004-2009) and completed my residency in Pediatrics and a Master’s degree in public health (MPH). In 2009, I moved to Durham and Duke University Medical Center where I joined the pediatric rheumatology fellowship program which I graduated from in 2012. In 2010, I joined the Duke Immunology graduate program and I have spent the last 6 years studying B cell biology with Dr. Thomas F. Tedder.

I have published or submitted for publication the following manuscripts during my Duke Immunology graduate studies:


I received the following awards and academic honors while at Duke University:

1) In May 2011, I received the Arthritis Foundation Clinical to Research Transition Award for a project named “IL-10-producing regulatory B cells in pediatric autoimmune diseases”.
2) In June 2012, I was nominated for the 2012 American College of Rheumatology Fellow Distinction Award by my mentors at the Duke Pediatric Rheumatology Fellowship Training Program.

3) In November 2013, I was awarded the Rheumatology Research Foundation Scientist Development Award for a project on IL-10-producing regulatory B cells and autoimmunity in children.

I defended my dissertation on June 22nd 2016 with the faculty of the Duke University Department of Immunology.

Following graduation, I will be joining the faculty of the University of New Mexico (UNM) to build a pediatric rheumatology division at the UNM Children’s Hospital and start an independent research program in pediatric autoimmunity.