Antigen Drives Regulatory B10 Cell Development and Function

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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 cells mediate immune responses via the secretion of antibody and interactions with other immune cell populations through antigen presentation, costimulation, and cytokine secretion. Although B cells are primarily believed to promote immune responses using the mechanisms described above, some unique regulatory B cell populations that negatively influence inflammation have also been described. Among these is a rare interleukin (IL)-10-producing B lymphocyte subset termed “B10 cells.” B cell-derived IL-10 can inhibit various arms of the immune system, including polarization of Th1/Th2 cell subsets, antigen presentation and cytokine production by monocytes and macrophages, and activation of regulatory T cells. Further studies in numerous autoimmune and inflammatory models of disease have confirmed the ability of B10 cells to negatively regulate inflammation in an IL-10-dependent manner. Although IL-10 is indispensable to the effector functions of B10 cells, how this specialized B cell population is selected in vivo to produce IL-10 is unknown. Some studies have demonstrated a link between B cell receptor (BCR)-derived signals and the acquisition of IL-10 competence. Additionally, whether antigen-BCR interactions are required for B cell IL-10 production during homeostasis as well as active immune responses is a matter of debate. Therefore, the goal of this thesis is to determine the importance of antigen-driven signals during B10 cell development in vivo and during B10 cell-mediated immunosuppression.
Chapter 3 of the dissertation explored the BCR repertoire of spleen and peritoneal cavity B10 cells using single-cell sequencing to lay the foundation for studies to understand the full range of antigens that may be involved in B10 cell selection. In both the spleen and peritoneal cavity B10 cells studied, BCR gene utilization was diverse, and the expressed BCR transcripts were largely unmutated. Thus, B10 cells are likely capable of responding to a wide range of foreign and self-antigens in vivo.

Studies in Chapter 4 determined the predominant antigens that drive B cell IL-10 secretion during homeostasis. A novel in vitro B cell expansion system was used to isolate B cells actively expressing IL-10 in vivo and probe the reactivities of their secreted monoclonal antibodies. B10 cells were found to produce polyreactive antibodies that bound multiple self-antigens. Therefore, in the absence of overarching active immune responses, B cell IL-10 is secreted following interactions with self-antigens.

Chapter 5 of this dissertation investigated whether foreign antigens are capable of driving B10 cell expansion and effector activity during an active immune response. In a model of contact-induced hypersensitivity, in vitro B cell expansion was again used to isolate antigen-specific B10 clones, which were required for optimal immunosuppression.

The studies described in this dissertation shed light on the relative contributions of BCR-derived signals during B10 cell development and effector function. Furthermore, these investigations demonstrate that B10 cells respond to both foreign and self-
antigens, which has important implications for the potential manipulation of B10 cells for human therapy. Therefore, B10 cells represent a polyreactive B cell population that provides antigen-specific regulation of immune responses via the production of IL-10.
Dedication

This thesis is dedicated to my grandfather, Philip A. Candando, who had a deep curiosity about the natural world and who would have been pleased to know that I became a scientist.
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1. Introduction

Portions of this text have been adapted from the original manuscripts “B10 Cell Regulation of Health and Disease” published in *Immunological Reviews* in 2014 and “Regulatory B10 Cell Development and Function” published in *International Immunology* in 2015.

1.1 The Immune System

The human immune system is the primary defense against invading pathogens, including viruses, bacteria, fungi, and anything recognized by immune cells as foreign or not “self.” Cells of the immune system can be broadly divided into the innate and adaptive arms. Innate immune cells provide a first line of defense against threats and respond to pathogens rapidly in a non-specific manner. The adaptive immune cells, known as T cells and B cells, require more time than do cells of the innate immune system to mobilize an anti-pathogen response. This is due to the exquisite specificity of the adaptive immune system. T and B cells express unique cell-surface receptors that recognize specific chemical moieties present on disease-related organisms or molecules. Once a cell of the adaptive immune system recognizes its molecule of interest, or antigen, it can rapidly proliferate and traffic to sites of inflammation to combat pathogens and neutralize threats. The innate and adaptive immune systems often work in concert to remove pathogens or foreign invaders (1).
Proper immune system function is dependent on the ability of immune cells to discriminate between “self” and “non-self.” This ability to leave the organism’s own cells in tact is known as tolerance. A break-down in tolerance sometimes occurs and can lead to autoimmune disease wherein immune cells recognize self-derived molecules as foreign and mount inappropriate immune responses that can lead to significant tissue damage and in some instances, death (1).

1.1.1 B cells

B cells are part of the adaptive immune system and are principally known for their ability to secrete antigen-specific antibody. Antibodies are Y-shaped proteins that bind to pathogens and act as signals for immune system clearance. In this way, B cells promote inflammation and drive the immune response forward (1).

1.2 Regulatory B10 cells

B cells are defined by their humoral effector function through the secretion of antibodies and are also known to play prominent roles in the activation of CD4+ T cells and the development of lymphoid tissue architecture (2). However, it has long been suggested that B cells are also critical negative regulators of both normal and aberrant immune responses. Nearly 40 years ago, Katz, Parker, and Turk (3) observed an increase in the severity and duration of contact hypersensitivity responses in guinea pigs following selective B-cell depletion and concluded that B cells were capable of inhibiting T-cell activation. Further studies characterized a similar effect of B-cell suppression on
anti-tumor T-cell responses and suggested that in addition to regulatory T cells (Tregs), immunosuppressive B cells are also important for maintaining immune homeostasis (4). Despite these findings, the identification of bona fide regulatory B cells and the mechanisms by which these cells function remained elusive in the years to follow.

The past decade has seen tremendous advances in our understanding of B-cell immunoregulation. Mizoguchi et al. (5, 6) first used the term “regulatory B cells” to describe a subset of gut-associated CD1d^+ B cells that suppressed inflammatory cytokine production during colitis progression in mice by secreting the anti-inflammatory cytokine IL-10. Shortly thereafter, Fillatreau et al. (7) observed that recovery from experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), was dependent on the presence of splenic IL-10-producing B cells. Since these seminal observations, multiple additional studies have conclusively demonstrated the significance of IL-10-producing regulatory B cells in divergent models of autoimmunity, infection, and hematologic malignancy, as summarized in numerous reviews of regulatory B-cell function (8-17). Despite a number of studies that focused on identifying the numerous cell surface markers expressed by regulatory B cells, the specific identification of the individual B cells that were producing IL-10 remained confusing and difficult to untangle. To transcend this complexity, we focused specifically on the characterization of individual B cells uniformly defined by their
characteristic production of IL-10 although it is appreciated that other subsets of regulatory B cells function in an IL-10-independent manner.

1.2.1 B10 cell characterization and identification

B10 cells were first characterized in a mouse model of contact hypersensitivity (CHS) in which dendritic cells serve as the predominant if not exclusive antigen-presenting cells and T cells mediate inflammation upon rechallenge with a sensitizing antigen (18). In this model, mice are immunized with oxazolone and resultant inflammation is observed following secondary exposure. Relative to wild-type mice, CD19−/− mice, which have reduced B-cell activation due to the lack of this key B-cell signaling molecule, experienced significantly greater levels of ear swelling up to 96 h post-oxazolone challenge. By contrast, mice overexpressing the human CD19 transgene (hCD19Tg) exhibited far less inflammation than did their wild-type counterparts. This inverse relationship between B-cell function and oxazolone-induced T-cell inflammation was due to a subset of IL-10-producing spleen B cells that were absent in CD19−/− mice, yet represented 1–2% of spleen B cells in wild-type mice and approximately 10% of spleen B cells in hCD19Tg mice. Adoptive transfer experiments of these cells demonstrated that their immunosuppressive effects were selectively dependent on IL-10 secretion, and thus the term B10 cell was introduced to functionally define this unique regulatory subset on the basis of their production of the potent anti-inflammatory cytokine IL-10.
Following the initial characterization of B10 cells in wild-type and hCD19Tg mice, further studies were undertaken to define the activity and understand the in vivo development of this unique regulatory population. However, the identification of IL-10-producing immune cells in vivo is hardly a straightforward task and remains challenging in the field of regulatory B-cell biology (19). This is because individual spleen B cells isolated from naive wild-type mice do not constitutively express or secrete measurable IL-10 protein without ex vivo activation. Given the inability to observe B10 cells directly ex vivo, well-described in vitro assays to detect cytokine production in T cells were modified to identify B cells that were “competent” to produce IL-10 ex vivo (18, 20). Stimulation of purified B cells using the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin along with monensin added to block protein secretion (together, PIM) resulted in accumulation of cytoplasmic IL-10 at sufficient levels to allow detection of rare IL-10-competent spleen B cells by immunofluorescence staining. The addition of lipopolysaccharide (LPS) to these cultures along with PIM (L+PIM) results in marginally greater frequencies of spleen B10 cells among total B cells (1–3%), thus making a short-term 5-h culture with L+PIM the ideal assay to identify mouse B cells capable of producing IL-10 directly ex vivo. Additionally, incubation of splenic B cells with LPS, CpG, or apoptotic cells induces significantly elevated IL-10 secretion versus PMA and ionomycin stimulation alone, indicating that
B10 cells are primed to respond to mitogenic stimuli and are therefore likely antigen-experienced cells (21-23).

Although short-term stimulation of spleen B cells resulted in the identification of 1–3% IL-10-competent B cells within the spleen, 48-h stimulation of B cells by agonistic CD40 monoclonal antibody (mAb) with L+PIM added during the final 5 h results in approximately 12% IL-10+ B cells within the total splenic B-cell pool of wild-type C57BL/6 mice (21). This significant increase in IL-10+ B cells relative to those obtained from the 5 h L+PIM assay revealed that some splenic B cells required additional stimulation to acquire IL-10 competence, which is in accordance with studies showing the expansion of regulatory B10 cells in vivo following chronic CD40 signaling (24). These cells were termed “B10 progenitor” (B10pro) cells and are considered to be a functionally immature precursor population relative to B10 cells. While CD40 signals mature B10pro cells to B10 cells, BCR cross-linking inhibits this process (21). Thus, although visualization of immune cells actively producing IL-10 in vivo remains a difficult task, these ex vivo assays to characterize IL-10 competence have shed light on the small subset of B cells that has turned on the IL-10 functional program and are capable of producing this potent regulatory cytokine.

1.2.2 B10 cell distribution in vivo

The spleen is the predominant reservoir of B10 cells in mice (21). However, mouse B10 cells have also been identified in the gut-associated lymphoid tissues, such as
the mesenteric lymph nodes and peritoneal cavity, as well as in the peripheral blood and lymph nodes (5, 21, 25). The peritoneal cavity also contains substantial frequencies of B10 and B10pro cells. The greatest frequencies of B10 cells in the peritoneal cavity were identified within the CD5+ CD11b+ B1a cell subset (38%), followed by the CD5− CD11b+ B1b (18%) and the CD5− CD11b− B2 (4%) subsets. Peritoneal cavity B cells also contain substantial proportions of B10pro cells, as 48-h CD40 mAb with 5 h L+PIM stimulation increases IL-10+ B-cell frequencies within the B1a, B1b, and B2 subsets to 41%, 22%, and 6%, respectively. Although the overall peritoneal cavity B-cell compartment contains the greatest frequency of B10 cells, the significantly reduced number of total B cells in the peritoneal cavity as compared to that of the spleen results in a greater overall number of B10 cells in the spleen. Within other mucosal tissues, B10 cells represent approximately 1% of mesenteric lymph nodes, 4% of lamina propia, and 3% of Peyer’s patch B cells. Additionally, few, if any, B10 cells are present in the peripheral blood and lymph nodes, though 3–8% of peripheral blood and lymph node B cells are B10pro cells (21). Thus, B10 and B10pro cells are rare within the blood, peripheral lymph nodes, and gut-associated lymphoid tissues, with the exception of the peritoneal cavity, where IL-10+ cells represent a substantial portion of the local B-cell pool.

1.2.3 B10 cell phenotype

L+PIM stimulation for 5 h does not change the expression of most cell surface molecules, particularly in the presence of monensin, which allows for the
characterization of the *in vivo* B10 cell phenotype (21). An extensive cell surface phenotyping study revealed that mouse spleen B10 cells are IgM\(^{hi}\)IgD\(^{lo}\)CD19\(^{hi}\)MHC-II\(^{hi}\)CD21\(^{int/high}\)CD23\(^{lo}\)CD24\(^{hi}\)CD43\(^{+/−}\)CD93\(^{−}\). Additionally, spleen B10 cells are predominantly enriched (15–20%) within the CD1d\(^{hi}\)CD5\(^{+}\)subset, as are B10pro cells. However, this designation should not be interpreted as a definitive phenotype for B10 cells, but rather as a feasible means to enrich these cells for functional studies without having to stimulate the cells with L+PIM to induce IL-10 expression (18).

Spleen and peritoneal cavity B10 cells have similar phenotypic profiles with notable exceptions. As in the spleen, peritoneal cavity B10 cells express high levels of IgM, CD5, CD19, CD24, CD43, and MHC class II (MHC-II) and low levels of IgD and CD23 relative to their non-B10 cell counterparts (25). However, the CD1d\(^{hi}\)CD5\(^{+}\)phenotype cannot be used to enrich peritoneal cavity B cells for B10 cells because high-level CD1d expression is not induced within the peritoneal cavity (18). Furthermore, CD5 expression in this compartment is typically associated with the delineation of B1 and B2 cells, both of which are known to contain B10 cells, as discussed above. Thus, B10 cells are present within multiple phenotypically-defined B cell subsets in both the spleen and peritoneal cavity, demonstrating that cell surface phenotype does not necessarily delineate B-cell functional homogeneity. The demonstrated capacity to produce IL-10 thereby remains the best way to identify pure B10 cell populations for study.
1.2.4 B10 cell development

The identification of B10pro cells after in vitro stimulation led to the hypothesis that some B cells are selected in vivo for the unique capacity to produce IL-10 but nonetheless require additional signals to become IL-10 competent. The current developmental scheme for B10 cells posits that this in vivo selection is mediated by appropriate BCR-derived signals, which are essential for B10 cell development and function (Fig. 1). Evidence for the role of BCR signaling in B10 cell development is provided by hCD19Tg and CD19−/− mice, which have amplified and reduced BCR signaling and greater and lesser frequencies of B10 cells, respectively, than do wild-type mice (18). This positive correlation between BCR signaling and the ability to produce IL-10 indicates that the BCR drives the acquisition of IL-10 competence in a given B cell following antigen receptor selection and the binding of antigens in vivo. Further, transgenic mice with fixed BCR specificities have a definitive lack of IL-10-competent spleen B cells (21), thus proving that appropriate BCR specificities are required for B10 cell development in vivo. Lastly, BCR signals also play a role in B10 cell effector function, as evidenced by the fact that B10 cells isolated from mice with prior antigen exposure are more effective at suppressing inflammation or disease in adoptive transfer experiments than their counterparts isolated from naïve littermates (26). For example, B10 cell negative regulation of oxazolone-induced CHS is more pronounced when the cells are isolated from mice sensitized with the eliciting hapten. Whether this is due
solely to enhanced BCR signaling in the appropriate antigen-specific B10 cells or to the clonal expansion of antigen-specific B10 cells in donor mice is unknown. Nonetheless, these observations demonstrate that BCR-derived signals play a role in early B10 cell development in vivo and may confer enhanced B10 cell immunosuppressive activity upon adoptive transfer.

BCR signals select B cells into the B10 cell compartment, but are not the only molecular events required for IL-10 secretion. B10pro cells likely reflect the subpopulation of B cells in vivo that have already received appropriate BCR signals to begin opening the \textit{il10} locus, but require additional molecular events to make the locus fully accessible to \textit{il10} gene transcription and/or protein translation. B10 cells thereby represent a further stage of functional maturation following the B10pro phase and have received the correct combination of signals in vivo to remodel the \textit{il10} locus such that B10 cells remain poised for IL-10 production (Figure 1). As such, B10 cells are readily identified after appropriate short-term in vitro stimulation, as the provision of L+PIM acts as a potent stimulus to drive intracellular signaling pathways that result in the transcription and translation of genes in an open configuration.

Although B10 cells are poised for IL-10 production, a final set of T-cell-derived signals is indispensable for the actual secretion of IL-10 and B10 cell regulation of immunity in vivo (27). For example, B cells deficient in MHC class II or the IL-21 receptor (IL-21R) are incapable of exerting IL-10-dependent suppression of autoimmunity. These
observations, coupled with normal numbers of B10 cells in T-cell-deficient mice as assayed by 5-h L+PIM (21), indicate that while B10 cells do not require T cells to acquire IL-10 competence, they do need cognate interactions with IL-21-producing T cells to become B10 effector cells (B10eff) and secrete IL-10 in vivo (Fig. 2). These checkpoints further explain the antigen-specific regulatory function of B10 cells in vivo.

Given that B10 cells require additional signals in vivo to actually secrete IL-10, the current model for understanding B10 cell development now includes the recently characterized B10 effector cells described above (Figure 1). B10 effector cells are those B cells that are actively secreting IL-10 in vivo and are modulating immune responses via antigen-specific interactions with T cells. Thus, B10pro cells represent a maturing population of B10 cells, with B10 cells poised to secrete IL-10 but lacking the T-cell-derived signals to do so. B10 effector cells, however, are those B cells that have activated the correct molecular program to transcribe il10 and have received appropriate input in vivo to secrete IL-10 protein and suppress immune responses following cognate interactions with antigen-specific T cells. Antigen-specific B10 effector cells are thereby controlled by multiple levels of regulation, leading to rare numbers of these cells in vivo and partially explaining why it has been so difficult to identify B cells producing IL-10 in situ above the background levels inherent in the assays used for IL-10 detection.

Consistent with this, it has been possible to observe rare but measurable numbers of B cells expressing IL-10 reporter transgenes in vivo after polyclonal B cell stimulation with
LPS, bacteria, or other mitogens (28), but this is facilitated by the more durable half-lives of the reporter molecules in comparison with IL-10 (19).

The discovery of B10 effector cells was aided in part by the development of a 9-day *in vitro* culture system that massively expands B10 effector cells from mouse spleen B cells (27). In this system, mouse B cells are cultured on highly selected NIH-3T3 cells expressing BLyS and CD154 (CD40L) in the presence of IL-4 for 4 days and IL-21 for an additional 5 days. By the end of the 9-day culture period, B10 effector cells are expanded 4,000,000-fold and provide potent IL-10-dependent suppression of autoimmune inflammation before or during the course of EAE. These cells are therefore referred to as induced B10 (iB10) effector cells to distinguish them from B10eff cells generated in vivo. The development of this culture system will allow a more comprehensive investigation of B10 cell biology, as the acquisition of this normally rare population for molecular and biochemical studies is greatly facilitated.
BCR signals select B cells into the B10 cell compartment, in vivo that have already received appropriate BCR signals that result in the transcription and translation of genes in an open configuration. Stimulation of B10 cells with either PMA and ionomycin or LPS induces the acute production of IL-10 transcripts and cytoplasmic IL-10 protein that accumulates to measurable levels within 5 hours when monensin is present. Once B10 cells begin to secrete IL-10, they are functionally defined as B10 effector (B10eff) cells, which are rarely visualized in vivo. Based on their cell surface phenotypes, B10pro and B10 cells are likely to be chronically stimulated through their BCRs but require additional signals to become B10 effector cells that produce IL-10, which can include LPS or CpG. Following transient IL-10 production in vivo, some B10 effector cells acquire cell surface markers found on plasmablasts and differentiate into plasma cells (PCs) that secrete polyreactive, autoreactive antibodies depending on their BCR specificity. Alternatively, the culture of spleen or blood B cells with NIH-3T3 cells expressing cell surface CD154 and BLyS in the presence of IL-4 for 4 days can induce B10pro cell expansion and the development of proliferating B10 cells when stimulated with IL-21 for five days, resulting in the generation of iB10 effector cells.

Figure 1. Model for mouse B10 cell development. After B cells encounter the correct self or foreign antigen in vivo, appropriate levels of B cell antigen receptor (BCR) signaling induce a small subset of B cells to progress down a developmental program that leads to IL-10 production. B10 progenitor (B10pro) cells do not express IL-10 but can become competent to do so after culture with agonistic CD40 monoclonal antibody (mAb) or lipopolysaccharide (LPS). Once B10pro cells become IL-10-competent, they are functionally defined as B10 cells. B10 cells do not express measurable IL-10 but are primed for transcription of the il10 locus. Stimulation of B10 cells with either PMA and ionomycin or LPS induces the acute production of IL-10 transcripts and cytoplasmic IL-10 protein that accumulates to measurable levels within 5 hours when monensin is present. Once B10 cells begin to secrete IL-10, they are functionally defined as B10 effector (B10eff) cells, which are rarely visualized in vivo. Based on their cell surface phenotypes, B10pro and B10 cells are likely to be chronically stimulated through their BCRs but require additional signals to become B10 effector cells that produce IL-10, which can include LPS or CpG. Following transient IL-10 production in vivo, some B10 effector cells acquire cell surface markers found on plasmablasts and differentiate into plasma cells (PCs) that secrete polyreactive, autoreactive antibodies depending on their BCR specificity. Alternatively, the culture of spleen or blood B cells with NIH-3T3 cells expressing cell surface CD154 and BLyS in the presence of IL-4 for 4 days can induce B10pro cell expansion and the development of proliferating B10 cells when stimulated with IL-21 for five days, resulting in the generation of iB10 effector cells.
**Figure 2. Model for B10 cell regulation of in vivo innate and adaptive immune responses following antigen-specific cognate interactions with T cells.** To become B10 effector cells and secrete IL-10 in vivo, B10pro cells must (1) receive appropriate signals through BCR interactions with antigen. These B cells are presumed to display antigenic peptides through their cell-surface MHC class II molecules (2), which facilitates their cognate interactions with peptide-specific CD4+ T cells (3). The antigen-specific T cells become activated and express CD154, which binds CD40 on the cognate B cells and induces B10 cell maturation and IL-10 competence. T cell receptor (TCR) and CD154 engagement induces T cell IL-21 production (3), which then acts locally on B10 cell IL-21 receptors (IL-21R) to promote IL-10-secreting B10 effector (B10eff) cell generation (4). IL-10 production by B10 effector cells in the local microenvironment negatively regulates adaptive immune responses by inhibiting antigen-specific T cells (5) and innate macrophage function (Mac, 6) during immune responses.

### 1.2.5 Molecular regulation of IL-10 production and the fate of B10 cells

Although several studies have described crucial in vivo signals that confer IL-10 competence to B cells, the transcriptional network and upstream signaling molecules controlling B-cell IL-10 production remain incompletely understood. Although molecular programs regulating IL-10 expression in T cells and macrophages have been described, B-cell IL-10 regulation is largely unexplored, most likely due to the rarity of
these cells (29). Previous studies have shown that some transcription factor genes related to plasma cell differentiation are upregulated in B10 cells, which is consistent with IL-21 induction of B10 cells (27, 28). A more complete picture of the regulatory transcription factor landscape in B10 cells is needed to further understand basic B10 cell biology and reveal molecular targets that can be exploited to manipulate B10 cells for therapeutic purposes.

Whether B10 cells retain their capacity to express IL-10 in vivo or adopt other fates following the induction of IL-10 expression has been characterized using the following two strains of IL-10 reporter mice: Tiger knockin mice, which contain an IRES-GFP element following the endogenous il10 locus, and transgenic 10BiT mice that contain an ectopic Thy1.1 gene under control of the IL-10 promoter (28). In both of these IL-10 reporter strains, B-cell reporter protein expression was predominantly observable only after in vitro stimulation with L+PIM. Reporter-positive B10 effector cells also appear to only produce measurable IL-10 transiently in vivo for 24–48 h before some of the cells progress toward plasma cell differentiation and antibody production. This is consistent with transcriptional data indicating an upregulation of known plasma cell genes such as irf4, prdm1, and xbp1 in reporter-positive B cells. Neither B10 nor B10 effector cells express cell surface markers commonly associated with plasmablasts and plasma cell populations. However, the IL-10 reporter Thy1.1 remains on the cell surface for a period of time after IL-10 expression has ended; thus, this durable reporter allows
the tracking of B10 effector cells after they have ceased to express measurable IL-10. Remarkably, \textit{in vivo} LPS administration in 10BiT mice revealed that a significant proportion of IL-10 reporter-positive B cells subsequently adopt a plasmablast phenotype following IL-10 production (28), which also occurs with non-B10 cells following antigen or mitogen encounter and activation.

Whether all or only a fraction of B10 cells become plasma cells following IL-10 induction is unknown, but it is quite clear that the majority of plasmablasts do not derive from B10 cells. The long-term contribution of B10 effector cell antibody production to their regulatory function has yet to be determined, but the vast majority of B10 cell function results from acute IL-10 production. Additional fates of B10 effector cells following IL-10 production, such as memory cell commitment or the secretion of other regulatory molecules, also remain active areas of investigation (Fig. 2).

\textbf{1.2.6 B10 cell BCR specificity and antibody production}

As mentioned above, some B10 cells are known to progress toward the plasma cell differentiation program following IL-10 secretion. Terminal plasma cell differentiation and antibody secretion by B10 cells has been confirmed \textit{in vivo} by the adoptive transfer of B10 cells into lymphocyte-deficient RAG2−/− mice (28), whereby LPS-stimulated B10 cells were able to reconstitute serum IgG and IgM antibody levels within 10 d. Subsequent \textit{in vitro} studies of antibodies derived from these recipient mice showed that B10 cell-derived serum contains antigen-specific IgM and IgG, as well as IgM that is
significantly enriched for autoreactivity when compared to IgM derived from non-B10 cells. This is consistent with the observation that B10 cells represent a population of B cells with diverse, predominantly germline BCRs, including those with reactivity for self-antigens. Thus, B10 cells secrete both foreign antigen-specific and autoreactive antibodies.

Despite the demonstrated importance of BCR signals in B10 cell activity, B10 cells are not measurably restricted in their usage of BCR genes. Single-cell repertoire analyses in spleen and peritoneal cavity B10 cell populations have revealed diverse heavy and light chain gene usage with a remarkable absence of somatic mutations in B10 cell immunoglobulin genes (25, 28). Even in the peritoneal cavity, where some restricted gene usage has been reported in B1a and B1b cells, IL-10+ B cells contain diverse BCR genes associated with both the B1 and the B2 subsets. These studies bolster the hypothesis that B10 cells are a functionally-defined population and are not a restricted lineage with a single cell type of origin.

1.3 B10 cell regulation of immune responses

The observation that B-cell-derived IL-10 could suppress T-cell-mediated inflammation in a diverse array of mouse models led us to study the role of B10 cells in autoimmunity, hematologic malignancy, and infection. In the context of autoimmunity and transplantation, B-cell-derived IL-10 acts on a local, antigen-specific level to combat T-cell autoimmune responses and dampen inflammation (27). During infection,
however, B10 cells have significant regulatory effects on the innate immune system that can negatively affect the clearance of certain pathogens (30). In a separate mouse model of hematologic malignancy, B10 cells were enriched during chronic lymphocytic leukemia (31). Thus, B10 cell interactions with the immune system are context-dependent, and additional studies have expanded upon those described above to conclusively demonstrate a potent and important function for B10 cells in immune system homeostasis as well as the suppression of both appropriate and deleterious immune responses (32-37).

1.3.1 B10 cell regulation of autoimmunity

A role for B10 cells in the regulation of autoimmunity was first observed by characterizations of B10 cell numbers in several autoimmune-prone mouse strains. Both CD1d$^{hi}$CD5$^+$ and IL-10$^+$ spleen B cells are significantly increased in non-obese diabetic mice as well as in NZB/W F1 and MRL.lprfas mice, both of which are prone to lupus like disease (21). Subsequent studies to understand how both endogenous and adoptively transferred B10 cells regulate autoimmunity have revealed that B-cell-derived IL-10 is a potent inhibitor of autoimmune inflammation with great promise for translation into human therapies.

1.3.1.1 EAE

The role of B10 cells during autoimmunity has been investigated most thoroughly in the antigen-specific model of MS, EAE. In this experimental system, mice
are immunized with myelin oligodendrocyte (MOG) peptides, and autoimmune disease development is typically observed over a 28-day time course. Hallmarks of EAE-induced autoimmunity include MOG-specific T-cell infiltration into central nervous system tissues, which is accompanied by increased levels of Th17-derived pro-inflammatory cytokines (38, 39).

B cells exhibit both pro- and anti-inflammatory roles during the course of EAE. B-cell autoantibodies specific for MOG can contribute to increased levels of inflammation and worsened disease (40, 41). For this reason, CD20 mAb-induced depletion of mature B cells once disease is established (at least 14 days post-MOG immunization) can lower disease scores, reduce demyelination of the CNS tissue, and limit MOG-specific T-cell infiltration into the CNS (42). However, B-cell depletion 7 days prior to MOG immunization results in the opposite phenomenon, with B-cell-depleted mice experiencing heightened disease as compared to mice treated with control mAb. Increased inflammation when B cells are depleted before disease initiation implicated a regulatory role for B cells during EAE. This B cell-mediated regulation was attributed to B10 cells themselves because the adoptive transfer of B10 cell-enriched CD1dhiCD5+ splenic B cells normalized disease in B-cell-depleted, MOG-immunized mice (42). Similar results were obtained when µMT mice that congenitally lack B cells were immunized with MOG peptide and experienced worsened disease and enhanced Th1-derived autoimmunity compared to wild-type mice (7). Further studies of EAE severity
in different mouse strains revealed that CD19−/− mice had worse EAE than wild-type controls, most likely due to markedly decreased numbers of endogenous B10 cells. By contrast, hCD19Tg mice had elevated B10 cell numbers and milder EAE disease severity than did wild-type mice, which confirmed an inverse relationship between the abundance of B10 cells and EAE severity (26). Thus, B10 cells play a definitive role in the amelioration of antigen-specific T cell-mediated autoimmunity.

The EAE model not only provides a physiologic means for assessing the role of B10 cells during autoimmunity but also allows for the investigation of B10 cell kinetics in the context of other immunoregulatory populations. Interestingly, B-cell depletion late in the EAE disease course lessened disease, but late ablation of Tregs significantly worsened disease (26). Similarly, B10 cells were evident in the CNS tissue before disease initiation and increased only slightly in the 28 days following MOG immunization. Tregs, however, significantly increased in the CNS during EAE and peaked at 21 days after MOG immunization. Collectively, these studies highlight an early role for B10 cells during EAE wherein B-cell-derived IL-10 prevents disease initiation. At later stages of disease, B-cell antigen presentation and autoantibody production may dominate the antiinflammatory effects of B10 cells.

Although adoptive transfers of B10 cells confirmed the role these cells play in blocking early autoimmune inflammation, B10 effector cells are also capable of blunting established disease (27). Adoptive transfer of 1 × 10^6 iB10 effector cells at days −1, 7, or
14 relative to MOG immunization significantly reduced EAE disease scores in recipient mice when compared to those mice that received an equivalent amount of *ex vivo*-expanded non-B10 cells. These studies demonstrated the *in vivo* potency of B10 effector cells, as they were able to significantly reduce disease at a time-point when previous studies showed that the absence of B cells is beneficial for combating autoimmunity. Furthermore, the transfer of $1 \times 10^6$ CD1d$^{hi}$CD5$^+$ splenic B cells at 14 days post-MOG immunization was without effect in previous studies (26). This appears to be attributable to the fact that B10 effector cells represent a more pure population of B10 cells than any freshly isolated splenic B-cell population as well as the potentially increased anti-inflammatory potency of iB10 effector cells.

### 1.3.1.2 Systemic lupus erythematosus

As in many other autoimmune diseases, B cells are able to promote the progression of systemic lupus erythematosus (SLE), a multi-organ autoimmune disease characterized by autoantibody production. The autoimmune-prone mouse strains NZB/W F1 and MRL.lprfas are the models of choice for investigating SLE due to their spontaneous lupus-like disease that can be accelerated by treatment with pristane. B cells produce anti-nuclear antibodies and other autoantibodies in both of these models and contribute to renal disease caused by IgG deposition (43). As such, mature B-cell depletion at 12–32 weeks of age in NZB/W F1 mice increases overall survival as compared to mice treated with control mAb (44).
As in EAE, however, CD20 mAb-mediated depletion of B cells prior to disease development in the NZB/W F1 SLE model (4 weeks of age) leads to decreased survival, and this effect is attributable to the loss of B10 cells (44). NZB/W F1 mice have approximately four-fold more B10 cells at 10 weeks of age than do age-matched C57BL/6 mice that are not prone to autoimmune disease. The observation that B10 cells play a protective role during lupus-like disease was further highlighted by the generation of a CD19−/− mouse on the NZB/W background. CD19−/− NZB/W F1 mice experienced worse disease than did their wild-type counterparts, with increased proteinuria and glomerulonephritis and decreased overall survival (45). As in CD19−/− C57BL/6 mice, CD19−/− NZB/W mice had a distinct lack of B10 cells with a barely detectable CD1dhiCD5+ compartment and less il10 transcription in the overall splenic B-cell population when compared with B cells from wild-type NZB/W F1 mice.

The increased severity of lupus-like disease in CD19−/− NZB/W F1 mice was especially striking considering that CD19 deficiency impaired the production of characteristic autoantibodies thought to drive the lupus-like disease phenotype (45). Therefore, even with the decrease in autoantibodies, CD19 deficiency and the subsequent lack of B10 cells drove more aggressive disease. Furthermore, the adoptive transfer of CD1dhiCD5+ spleen B cells from wild-type NZB/W F1 mice ameliorated disease in CD19−/−NZB/W F1 mice and increased Treg frequencies. Thus, as in other
models of autoimmunity, B cells play both pathogenic and protective roles during pristane-accelerated lupus.

The notion that B10 cells were capable of blunting autoimmunity during lupus was questioned in a study in MRL.lprfas mice with a B-cell-specific deficiency in IL-10. In this system, CD19\textsuperscript{Cre}\textsuperscript{IL-10\textsuperscript{fl/fl}} MRL.lprfas mice did not exhibit worse lupus symptoms as compared to wild-type MRL.lprfas mice, implying that endogenous B10 cells do not limit spontaneous autoimmunity (46). These observations are difficult to interpret because not all CD19\textsuperscript{+} B cells express sufficient levels of CD19-driven Cre recombinase (47), leading to the possibility that residual B10 cells may remain that inhibit disease severity. Additionally, a congenital defect in B10 cell development may be compensated for by the increased availability of a separate IL-10-producing population. The same compensatory mechanisms are not evident in mice depleted of mature B cells, as discussed above, which may provide a more comprehensive assessment of the role of B10 cell function during lupus.

1.3.1.3 Inflammatory bowel disease

Early studies of B cell immunoregulation relied on colitis as a model of autoimmune inflammation. A role for IL-10-mediated suppression of colitis was demonstrated by the generation of IL-10\textsuperscript{−/−} mice, which develop spontaneous, chronic colitis by 7–11 weeks of age (48). Mizoguchi et al. (5, 49) demonstrated that B cells are capable of suppressing colitis in a separate model using TCR\textalpha\textsuperscript{−/−} mice in which
mesenteric lymph node B cells upregulated CD1d and provided the IL-10 necessary for dampening disease.

While studies by Mizoguchi and others have shown that B cells can mitigate gut-associated inflammation, a definitive role for B10 cell-mediated suppression by cells from both the spleen and the peritoneal cavity was demonstrated in three separate inflammatory bowel disease (IBD) models. Dextran sulfate sodium (DSS)-induced colitis, which is achieved by short-term administration of DSS in rodent drinking water and causes acute intestinal injury throughout the length of the colon, is significantly worse in B10 cell-deficient CD19−/− mice than in wild-type mice (50). The adoptive transfer of splenic CD1dhiCD5+ cells ameliorates disease by slowing weight loss and intestinal bleeding in an IL-10-dependent manner. Additional studies of DSS-induced colitis in IL-10 reporter Tiger mice have also shown that B10 cells in the spleen, mesenteric lymph nodes, and peritoneal cavity are capable of expressing IL-10 during the acute inflammation caused by DSS treatment (25). Thus, B10 cells play an active role in reducing gut-associated inflammation.

B10 cells also suppress the spontaneous, chronic IBD modeled in IL-10−/− mice. Peritoneal cavity-derived B10 cells significantly delayed the onset of IBD in IL-10−/− recipients when transferred at 10–12 weeks of age and decreased the frequency of activated and cytokine-producing CD4+ T cells in the peritoneal cavity, mesenteric lymph nodes, and inguinal lymph nodes of recipient mice. The anti-inflammatory effects
of peritoneal cavity B10 cells were confirmed in a separate colitis model where the
transfer of CD25\(^-\)CD45RB\(^{hi}\)CD4\(^+\) T cells into RAG2\(^{-/+}\) mice caused an IBD-like disease
(51). As in spontaneous IBD, the transfer of peritoneal cavity B10 cells suppressed T-cell-
induced colitis in an IL-10-dependent manner and decreased levels of IFN-\(\gamma\)- and IL-17-
producing T cells in the peritoneal cavity, mesenteric lymph nodes, and spleen (25).
Therefore, B10 cells from both the spleen and peritoneal cavity are capable of inhibiting
gut-associated inflammation in both spontaneous and induced models of IBD.

1.3.1.4 Collagen-induced arthritis

Collagen-induced arthritis (CIA) serves as a model for human rheumatoid
arthritis (RA) and is characterized by joint destruction and infiltration of antigen-specific
T cells to sites of inflammation following collagen immunization in the DBA/1 strain of
mice (52). B-cell depletion prior to collagen immunization results in reduced disease, as
pathogenic autoantibodies can no longer drive autoimmune inflammation (53). Despite
evidence that B cells are inflammatory mediators of autoimmunity during CIA, some B
cell subsets have also been shown to negatively regulate antigen-specific immune
responses during arthritic disease. Mauri et al. (54) have described that in vitro activation
of B cells with agonistic CD40 mAb induces production of IL-10 and to a much lesser
extent, IFN-\(\gamma\). Transfer of these in vitro-activated B cells into collagen-immunized DBA/1
with transgenic T-cell receptors specific for collagen significantly delayed disease onset
and ameliorated established disease. The observed effects were attributed to the ability
of the transferred B cells to produce IL-10, as B cells derived from IL-10−/− mice were without effect.

An immunosuppressive role for B cells during CIA was further described in studies where the CD21hiCD23+IgM+ transitional 2 marginal zone precursor (T2-MZP) cell population was isolated and transferred to collagen-immunized DBA/1 mice (55). TZ-MZP cell adoptive transfers delayed disease onset and treated established disease via inhibition of Th1-type immune responses in an IL-10-dependent manner. Moreover, adoptive transfer of total splenic B cells from apoptotic cell-treated mice also suppressed autoimmune pathogenesis in an IL-10-dependent manner that encouraged CD4+ T cell IL-10 production, thus verifying the ability of particular B-cell subsets to instigate tolerance and inhibit arthritic inflammation in CIA (23).

A role for B10 cells specifically during CIA was investigated by Yang et al. using B cells cultured in vitro with BAFF for 72 h, which is reported to induce IL-10 production in approximately 32% of CD1dhiCD5+B cells (56). The B10 cell-enriched B cells were then transferred to collagen-immunized DBA/1 mice where they delayed the onset of arthritis and specifically downregulated Th17 responses. Taken together, these studies confirmed the role of B cells during CIA and documented that while some pathogenic B-cell populations certainly contribute to the progression of disease, regulatory B-cell subsets, especially B10 cells, are capable of inhibiting effector T-cell responses during the course of CIA.
2. Materials and Methods

2.1 Mice

Tiger (B6.129S6-Il10tm1Flv/J)(57), Il10−/− (B6.129P2-Il10tm1Cge/J), ROSA-26TdTomato (B6.Cg-Gt(Rosa)26Sor^{tm9(CAG-tdTome)Hze/J}), and C57BL/6 mice were from The Jackson Laboratory. MHC-II−/− (B6.129-H2-Ab1^{tm1}B2m^{tm1}GtmN17) mice were from Taconic Farms. All mice were bred in a specific pathogen–free barrier facility and were used at 8–20 wk of age. The Duke University Animal Care and Use Committee approved all studies.

2.2 Cell isolation and preparation

Peritoneal cavity leukocytes were isolated as described (25). Single-cell splenocyte suspensions were generated by gentle dissection with cells passed through 70-µm cell strainers (BD Biosciences). Viable cells were counted by hemocytometer, with relative lymphocyte percentages determined by flow cytometry analysis. B cells were purified from splenocyte preparations by negative selection. Briefly, splenocytes were incubated with a cocktail of biotinylated mAbs specific for CD4 (GK1.5; eBioscience), TER-119 (TER-119; eBioscience), and CD43 (S7; BD Pharmingen) for 15 minutes at 4 °C before washing and centrifugation at 300 x g for 5 min. Labeled cells were then incubated with strepavidin-coated magnetic microbeads (Streptavidin Particles Plus-DM, BD Biosciences) and washed before cell separation using the IMag separation system (BD Biosciences) according to the manufacturer’s instructions. Where indicated,
subsets of CD19+ B cells were purified following immunofluorescence analysis using a FACSDiva flow cytometer (BD Biosciences) or a BC Astrios flow cytometer (Beckman Coulter) with ≥98% purities.

2.3 Immunofluorescence analysis

FITC-, PE-, or APC-conjugated CD19 (6D5), CD5 (53-7.3), CD1d (1B1), CD38 (90), B220 (RA3-6B2), and CD23 (B3B4) antibodies were from BioLegend. FITC-, PE-Cy7-, or APC-conjugated CD138 (281-2), CD21 (7G6), CD95 (Jo2), and IgG1 (A85-1) antibodies were from BD Biosciences. PE-conjugated CD93 (AA4.1) and GL7 (GL7) antibodies were from eBioscience. Biotinylated oxazolone reagents were generated using the EZ Link Sulfo-NHS-Biotinylation Kit (Thermo Scientific) according to the manufacturer’s instructions. IL-10 antibody (JES5-16E3; eBioscience) was used for intracellular staining. Background staining was assessed using non-reactive, isotype-matched mAbs. For three- to six-color immunofluorescence analysis, single-cell suspensions (10^6 cells) were stained at 4 °C using predetermined optimal antibody concentrations for 20 min as described (27).

B cell intracellular IL-10 expression was visualized by immunofluorescence staining and analyzed by flow cytometry as described (20, 27). Briefly, isolated spleen or peritoneal cavity cells or iB10pro cells were resuspended (2 × 10^6 cells/ml) in complete medium (RPMI 1640 containing 10% FCS, 200 µg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine and 50 µM 2-mercaptoethanol, all from Gibco) with LPS (10 µg/ml,
Escherichia coli serotype 0111: B4; Sigma), PMA (50 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma) and monensin (2 µM; eBioscience) for 5 h in 48-well flat-bottom plates. In some experiments, the cells were incubated for 48 h with agonistic anti-mouse CD40 mAb (1 µg/ml; HM40-3; BD Pharmingen) as described (18). Fc receptors were blocked with mouse CD16/32 mAb (2.4G2; BioLegend), with dead cells detected using a LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen-Molecular Probes) before cell surface staining. For IL-10 detection, stained cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions and stained with PE-conjugated mouse anti-IL-10 mAb (BioLegend). Splenocytes from Il10−/− mice served as negative controls to demonstrate specificity and were used to establish background IL-10 staining levels. Viable cells were analyzed using a BD FACSCanto II (BD Biosciences).

2.4 Immunoglobulin sequence analysis

Purified spleen B cells from three mice were stimulated with LPS (10 mg/ml), PMA (50 ng/ml), and ionomycin (1 mg/ml) for 5 h. IL-10−secreting cells were identified using the Mouse IL-10 Secretion Assay Kit (Miltenyi Biotec). Individual Lambda IL-10+CD19+ cells were sorted into single wells of 96-well PCR plates using a FACSARia II cell sorter (BD Biosciences). cDNA was synthesized with Ig H and L chain transcripts amplified using nested PCR primers, as described (58). PCR products were purified (QIAquick PCR Purification Kit; Qiagen, Valencia, CA) and cloned (StrataClone PCR
Cloning Kit, Agilent Technologies, La Jolla, CA) before sequencing (Duke University DNA Analysis Facility). Productive Ig 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 (59) to determine V(D)J gene family usage. Mutation frequencies were determined using germline V, D, and J sequences from Ig Basic Local Alignment Search Tool. When L chain sequences obtained from adjacent wells were identical, only one sequence was reported. $V_{H}\rightarrow D\rightarrow J_{H}$ and $V_{K}\rightarrow J_{K}$ transcript alignments and phylogenetic trees based on average percent identity were constructed using ClustalW2 (60).

2.5 In vitro B cell expansion and adoptive transfer experiments

B10 effector cells were generated from spleen B cells as described (27). Briefly, purified spleen B cells were cultured with NIH-3T3 cells expressing CD154 and BLyS (NIH-3T3-CD154TK) with exogenous recombinant IL-4 (2 ng/ml; eBioscience) or IL-21 (10 ng/ml; eBioscience) added to the cultures (24, 27, 61, 62). After culture, B10 effector cells were purified by cell sorting based on selective CD5 expression. Bulk iB10pro cells were generated as above using NIH-3T3-CD154EAT cells. For the single-cell iB10pro culture system, single spleen B cells were mechanically sorted into 96-well plates containing NIH-3T3-CD154EAT cells (1,000 cells/well). IL-4 (2 ng/ml) was added on day 0,
and IL-21 (10 ng/ml) was added at days 0, 2, 4, 6, and 8 before harvesting the cells and culture supernatant fluid on day 9.

For adoptive transfer experiments, unfractionated iB10pro cells or CD5+ B10 effector cells (1-10 x 10^6) were transferred after culture to the indicated recipient mice by tail vein injections. In some experiments, iB10pro cells generated using the single-cell culture system were pooled prior to transfer. For transfers of ≤0.2 x 10^6 iB10pro cells, 2 x 10^6 total splenocytes from wild-type mice were added to the cell preparations to act as carrier cells. For experiments using TdTomato+ iB10pro cells, 20 x 10^6 cells were transferred to recipient mice by intraperitoneal injection.

**2.6 Immunohistochemistry**

HEp-2 cells obtained from the American Type Culture Collection were cultured in eight-chamber tissue culture slides with complete medium for 48 h. At room temperature, the adherent cells were washed with phosphate buffered saline (PBS), fixed in 4% formaldehyde for 20 min, washed, and permeabilized with 0.15% Triton X-100 for 4 min. After washing, the slides were blocked using 10% fetal bovine serum in PBS for 1 h. Supernatant fluid (200 µl) was then added to each slide well for 1 h. After washing, PE-conjugated anti-IgG1 antibody (Southern Biotech) was added to each well for 1 h in the dark. Washed slides were then mounted with coverslips using Fluoromount-G (Southern Biotech), with the stained cells visualized by fluorescence microscopy.
For immunohistochemistry analyses in Figure 3H, approximately 0.1 x 10⁶ cells were cytocentrifuged onto microscope slides. Cells were stained with APC-conjugated CD138 and FITC-conjugated IgG₁ antibodies as well as 4’, 6-diamidino-2-phenylindole (Sigma). Slides were visualized by fluorescence microscopy, with identical exposures between photomicrographs within each picture set.

2.7 Enzyme-linked immunosorbent assays (ELISAs) and enzyme-linked immunospot (ELISpot) assays

IL-10 secretion was measured using the OptEIA Mouse IL-10 ELISA set (BD Biosciences) according to the manufacturer’s instructions. Antibody isotypes were quantified by ELISA (63). ELISAs for dsDNA, ssDNA, histone proteins, and MAA-BSA were as described (24, 64). Background OD values (≤10%) were subtracted from ELISA values. BSA-oxazolone conjugate was produced as described (65). Briefly, 75 mg of oxazolone was added to 20 ml of 50 mg/ml BSA (Sigma) in 5% (w/v) NaHCO₃ and shaken gently for 24 h at 4 °C. After centrifugation for 30 min at 30,000 x g, the supernatant fluid was dialyzed against 2 L of 0.15 M NaCl at 4 °C with gentle stirring for 12 h. Dialysis was repeated twice. Oxazolone ELISAs were completed by coating 96-well plates with oxazolone-BSA conjugate (10 µg/ml). Plates were blocked with tris-buffered saline (TBS) containing 1% BSA before cell culture supernatant fluid (diluted 1:10 from bulk B cell cultures or undiluted if from single B cell cultures) was added to the plates. Alkaline-phosphatase-conjugated IgG₁ antibody (Southern Biotech) was used to detect bound antibody, and 1 M diethanolamine/0.5 M MgCl₂ with 4-nitrophenyl phosphate
disodium salt hexahydrate used as the detection reagent. Absorbance was read at 405 nm. Control plates were coated with PBS containing 1% BSA and blocked as above before the addition of culture supernatant fluid and detection reagents. Wells were considered positive when their mean optical density (OD) values were greater than three standard deviations above the mean OD values of control wells. ELISpot assays were as described (28).

**2.8 DNAse-seq and ATAC-seq analyses**

Purified gDNA from CD19+ B cells directly *ex vivo* or iB10pro cells at the end of culture was used to construct libraries for high-throughput sequencing of Dnase I-hypersensitive sites (DNase-seq) as described (66). IL-10+ B cells were purified using the Mouse IL-10 Secretion Assay Kit (Miltenyi) according to the manufacturer’s instructions. Purified gDNA from IL-10+ B cells was used to construct libraries for high-throughput sequencing of transposase-accessible chromatin sites (ATAC-seq) as described (67). DNA-seq and ATAC-seq libraries were assembled and sequenced at the Genome Sequencing Center at the Duke Center for Genomic and Computational Biology. Samples were sequenced using 50-bp single reads in single lanes on the Illumina HiSeq 2000 platform. All reads were aligned to the mm9 mouse genome assembly.
2.9 CHS induction

Mice were sensitized with an acetone/olive oil (4:1 v:v) mixture containing 100 mg/ml oxazolone (Sigma) as described (18). Mice were shaved on the upper right abdomen, and 25 µl of the oxazolone mixture was evenly applied to exposed skin on days 0 and 1. On day 5, sensitized mice were challenged by the application of 10 µl of acetone/olive oil (4:1 v:v) with oxazolone (10 mg/ml) to the right ear (5 µl on the dorsal side and 5 µl on the ventral side). In certain experiments, 25 µl of 0.5% DNFB (Sigma) in acetone/olive oil (4:1 v:v) was used as the sensitization agent. In either oxazolone or DNFB challenge, 10 µl acetone/olive oil (4:1 v:v) was administered to the left ear as a control for inflammation. In oxazolone hypersensitization experiments, mice were repeatedly sensitized with oxazolone on days 0, 1, 2, 3, 4, 7 and 8 (68), with tissues collected at days 5 and 14.

After oxazolone or DNFB challenge, ear thickness was measured using a calibrated digital thickness gauge (Mitsutoyo Corp., Tokyo, Japan), with the thickness of the control ear subtracted from that of the challenged ear to determine the extent of inflammation. Whole ears (day 7 post-oxazolone sensitization) were fixed in 10% formalin and stained with Hematoxylin and Eosin (Duke University Pathology Research Histology and Immunohistochemistry Laboratory).
2.10 Statistical analyses

All data are shown as means + s.e.m. Significant differences between sample means were determined using an unpaired, two-tailed Student’s t-test.
3. B10 cells express a diverse, predominantly unmutated BCR repertoire

Portions of the following text were adapted from the original manuscripts, “Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo” published in The Journal of Immunology in 2011 and “Peritoneal Cavity Regulatory B Cells (B10 Cells) Modulate IFN-γ⁺CD4⁺ T Cell Numbers During Colitis Development in Mice” published in The Journal of Immunology in 2013.

3.1 Introduction

The ability to re-arrange antigen receptor genes from immunoglobulin (Ig) loci to form either a BCR or a TCR is a distinguishing feature of adaptive lymphocytes. In B cells, the BCR is composed of a heavy chain containing re-arranged variable (V), diversity (D), and joining (J) gene segments linked to a constant region gene that determines the B cell antibody isotype. The BCR also contains a light chain, which includes re-arranged V and J gene segments that are linked to either Lambda or Kappa constant region genes (1). The large number of possible gene combinations along with stochastic nucleotide additions by terminal deoxynucleotidyl transferase can lead to a vastly diverse antigen receptor repertoire that renders B cells capable of responding to a varied pool of antigens. Some B cell populations have restricted BCR repertoires that exhibit skewed or preferential usage of particular Ig gene segments. For instance, certain
populations of B cells found in the peritoneal cavity in mice preferentially bind self-antigens, such as phosphatidylcholine (69). Therefore, understanding the full range of the BCR repertoire for a given B cell population can provide clues as to B cell origin and receptor reactivity.

The current developmental scheme for B10 cells posits that B cells are selected for the unique ability to produce IL-10 by the provision of the appropriate BCR-derived signals (70). Evidence for the role of BCR signaling in B10 cell development is provided by human CD19 transgenic and CD19−/− mice, which have amplified and reduced BCR signaling and greater and lesser frequencies of B10 cells, respectively, than do wild-type mice (18). This positive correlation between BCR signaling and the ability to produce IL-10 indicates that the BCR drives the acquisition of IL-10 competence in a given B cell following antigen receptor selection and the binding of antigens in vivo. Further, transgenic mice with fixed BCR specificities have a definitive lack of IL-10-competent spleen B cells, thus proving that appropriate BCR specificities are required for B10 cell development (21). Lastly, BCR signals also play a role in B10 cell effector function, as evidenced by the fact that B10 cells isolated from mice with prior antigen exposure are more effective at suppressing inflammation or disease in adoptive transfer experiments than their counterparts isolated from naive littermates (26, 42). Thus, understanding the full scope and diversity of BCR genes expressed by B10 cells will aid in understanding
how B cells are selected for regulatory function in vivo and what antigens may drive this process.

Understanding the composition of the B10 cell BCR repertoire is also crucial to determining the importance of B10 cell-derived antibodies. After IL-10 production, some B10 cells can differentiate into antibody-secreting cells that predominatly secrete IgM+ antibodies that are enriched for reactivity with self-antigens (28). As antibody secretion is a major effector function of all B cells, delineating the B10 cell BCR repertoire may aid in further studies to evaluate the potentially protective role of B10 cell antibodies.

Not only does study of the B10 cell BCR repertoire help to determine the antigenic pool with which B10 cells may react, it also provides clues as to what processes B10 cells undergo during active immune responses in vivo. For instance, the extent of somatic hypermutation present in B10 cell BCR genes can indicate whether B10 cells enter germinal centers and undergo affinity maturation during response to a particular antigen. Furthermore, other studies have suggested that usage of certain V\textsubscript{H} genes, particularly those proximal to the D\textsubscript{H} segment of the locus, are indicative of multiple rounds of B cell re-arrangement during development, and repeated re-arrangements may be due to increased reactivity between the nascent BCR and self-antigens (71). Thus, although BCR re-expression and antibody binding characterization remains the gold standard for identifying B cell cognate antigens, sequencing of the B10 cell BCR
repertoire establishes a foundation for understanding the antigenic reactivity of this population and provides a genetic context for further receptor reactivity studies.

This chapter presents data regarding the BCR repertoire for B10 cells isolated from the spleen and peritoneal cavity, both of which represent anatomical locations in which B10 cells are known to be preferentially expanded. IL-10+ B cells are found within each of the phenotypically-defined peritoneal cavity B1a, B1b, and B2 subsets. Single-cell sequencing data from both spleen and peritoneal cavity B10 cells was used to determine the relative restriction or diversity of BCR gene utilization by IL-10-competent B cells. In addition, BCR gene sequencing information revealed any mutations present in framework regions (FWRs) or complementarity-determining regions (CDRs). Collectively, these data provide information as to whether B10 cells have a distinct ontogeny in vivo or whether they resemble the overall B cell population from a genetic perspective.

3.2 Results

3.2.1 Spleen B10 cells express a diverse, predominantly unmutated BCR repertoire

PCR methods were used to obtain an unbiased representation of the heavy and light chain repertoires of single IL-10+λCD19+ cells from wild-type C57BL/6 mice. Only λ-B10 cells were isolated for this study because λ+ B cells represent approximately 5% of the overall B cell population in wild-type C57BL/6 mice (72). B10 cell heavy chain transcripts were largely encoded by members of the VH1 family (~40%), which was not
unexpected given that the V_{H}1 gene family contains the greatest number of V gene segments within the Ig locus (Figure 3A-B). Additionally, when compared with the BCR repertoire of conventional spleen B cells, gene utilization by spleen B10 cells was similar to that of conventional spleen B2 cells (73). Light chain transcripts from spleen B10 cells also exhibited diverse genetic composition, and most spleen B10 cells utilized light chain V genes from the V_{K}1 gene family. As with heavy chain B10 cell transcripts, the light chain repertoire resembled that of conventional spleen B2 cells.

Not only was gene usage diverse among the B10 cells isolated, but the transcripts sequenced were also typically germline-encoded (Figure 3A-B). Only 16% of heavy chain transcripts and 9% of light chain transcripts contained any nucleotide mutations. Therefore, B10 cell BCR transcripts were typically devoid of somatic hypermutation-induced mutations.

The diversity of the B10 cell BCR repertoire was further examined by phylogenetic analysis of B10 cell heavy and light chain amino acid sequences (Figure 3C-D). Although some B10 cell BCR sequences were similar in composition, no obvious clustering or restriction within the repertoire was observed when considering distances among the branches of the displayed phylogenetic trees. Thus, spleen B10 cells expressed a diverse, predominantly unmutated BCR repertoire.

The mutations that were observed within the spleen B10 cell transcripts were distributed among FWRs and CDRs, and did not appear to localize to certain regions of
the BCR transcripts (Tables 1 and 2). Additionally, as observed within conventional spleen B2 cells, B10 cells contained N and P nucleotide additions and had CDR3s of normal lengths (73). Thus, the gene repertoire and BCR sequences of spleen B10 cells largely resemble those of conventional spleen B2 cells.
IL-10 is not required for B10 cell ASC differentiation. IL-10 induces human plasma cell differentiation in vitro (34–36). To determine whether autocrine IL-10 drives mouse B10 cell development or differentiation, the 10BiT transgene was bred into an IL-10<sup>-/-</sup> background to create 10BiT.IL-10<sup>-/-</sup> mice. Spleen Thy1.1<sup>+</sup> B cell frequencies were identical in both 10BiT and 10BiT.IL-10<sup>-/-</sup> mice after in vitro stimulation with agonistic CD40 mAb or LPS for 48 h (Fig. 5A). Identical frequencies of IgM ASCs were also found within the spleen Thy1.1<sup>+</sup> subsets of 10BiT and 10BiT.IL-10<sup>-/-</sup> mice following in vivo LPS treatment (Fig. 5B). ASC frequencies within the spleen CD1d<sup>hi</sup>CD5<sup>+</sup> subset were also equivalent in LPS-treated IL-10<sup>-/-</sup> and wild-type mice, with the B10 cell-enriched CD1d<sup>hi</sup>CD5<sup>+</sup> B cells containing a higher frequency of ASCs compared with CD1d<sup>lo</sup>CD5<sup>-</sup> B cells. Thus, autocrine IL-10 was not required for either B10 cell development or ASC differentiation.

Figure 3. Spleen B10 cells use diverse V genes that are largely unmutated.
Figure 3. Spleen B10 cells use diverse V genes that are largely unmutated. A, \( V_H \) family gene usage by 50 representative IL-10^+ B cells from three individual mice. Mutation frequencies within the \( V_H-D-J_H \) gene sequences are shown on the right. B, \( V_K \) gene family usage by 69 representative IL-10^+ B cells. \( V_K-J_K \) mutation frequencies are shown on the right. C, Phylogenetic tree showing relationships between the \( V_H-D-J_H \) amino acid sequences of individual B cells from mice named A through C, with numbers indicating different B cells. Branches indicate the average distance between two sequences based on percent identity. D, Phylogenetic tree showing the relationship between the \( V_K-J_K \) amino acid sequences of individual B cells.
Table 1. B10 Cell Heavy Chain V-D-J Sequences.

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Table I. (Continued)

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Table 1. Spleen B10 Cell Heavy Chain V-D-J Sequences. V_{H-D_{H-J}} genes from single IL-10+ B cells were identified as in Figure 3. Italicized text in “V End” column denotes the V gene codon sequence immediately prior to the CDR3 region. In the “Mutation Locations” column, italicized text indicates a synonymous mutation. FWR, framework region; N, N nucleotide addition; P, P nucleotide addition.
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**Table 2. Spleen B10 Cell Light Chain V-J Sequences**
Table 2. Spleen B10 Cell Light Chain V-J Sequences. V<sub>k</sub>-J<sub>k</sub> genes from single IL-10<sup>+</sup> B cells were identified as in Figure 3. Italicized text in “V End” column denotes the V gene codon sequence immediately prior to the CDR3 region. In the “Mutation Locations” column, italicized text indicates a synonymous mutation. FWR, framework region; N, N nucleotide addition; P, P nucleotide addition.
3.2.2 Peritoneal cavity B10 cells express a diverse, predominantly unmutated BCR repertoire

As in the case of spleen B10 cells, PCR methods were also used to obtain an unbiased representation of the BCR repertoire for B10 cells isolated from the peritoneal cavity. In these studies, total peritoneal cavity B cells with no division into B1a, B1b, or B2 subsets were isolated for sequencing. Heavy chain transcripts from peritoneal cavity B10 cells exhibited diverse V gene usage, with genes from V_{H}3 and V_{H}12 families being the most prominent (Figure 4A-B). This gene usage was consistent with previous observations that some B1 cells preferentially use V genes from these families due to their reactivity with self-antigens and their abilities to produce so-called “natural” antibodies (74). Similarly, light chain transcripts from peritoneal cavity B10 cells contained frequent usage of V_{K}12 genes, associated with the B1 repertoire, and V_{K}1, the mouse Vkappa family with the greatest number of V gene segments. Although more peritoneal cavity B10 cells contained mutations that did spleen B10 cells, more than 75% of both heavy and light chain transcripts from peritoneal cavity B10 cells were germline-encoded. Phylogenetic analyses underscored the diversity of the peritoneal cavity B10 cell BCR repertoire (Figure 4C). Thus, peritoneal cavity B10 cells also expressed a diverse, predominantly unmutated BCR repertoire.

The mutations observed in peritoneal cavity B10 cell BCR transcripts were distributed among FWRs and CDRs, as in spleen B10 cells (Tables 3 and 4). Additionally, peritoneal cavity B10 cells contained N and P nucleotide additions and CDR3s of typical
lengths. Therefore, the BCR repertoire of peritoneal cavity B10 cells resembled that of peritoneal cavity B1a, B1b, and B2 cells (73).

Figure 4. Peritoneal cavity B10 cells use diverse V genes that are largely unmutated
Figure 4. Peritoneal cavity B10 cells express diverse V genes that are largely unmutated. **A**, $V_H$ family gene usage by 36 representative IL-10$^+$ B cells and $V_K$ family gene usage by 81 IL-10$^+$ B cells from two mice. **B**, Mutation frequencies within the $V_H$-$D$-$J_H$ and $V_K$-$J_K$ gene sequences. **C**, Phylogenetic trees showing relationships between the $V_H$-$D$-$J_H$ (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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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, N nucleotide addition; P, P nucleotide addition.
Table 3. Peritoneal cavity B10 cell heavy chain V-D-J sequences. $V_{H}$-$D_{H}$-$J_{H}$ genes from single IL-10$^+$ cells were identified as in Figure 4. 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 re-arrangement. FWR, Framework region; N, N nucleotide addition; P, P nucleotide addition.
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3.3 Discussion

The BCR repertoires of spleen and peritoneal cavity B10 cells were remarkably diverse, involving a wide spectrum of V, D, and J elements, normal frequencies of non-coded nucleotide insertions, and considerable CDR3 diversity (Figures 3 and 4, Tables 1-4). Thereby, spleen and peritoneal cavity B10 cell BCR gene usage was similar to that observed for conventional spleen B cells or peritoneal cavity B1 and B2 cells. These results indicate that different selective and/or developmental forces may ultimately shape the B10 cell BCR repertoire, leaving B10 cells capable of responding to a diverse array of foreign and self-antigens. The diverse gene usage observed combined with previous studies in which B10 cells were shown to contribute to the “natural” IgM antibody pool indicate that B10 cells are poised to produce polyreactive, potentially self-
reactive IgM antibodies that may augment the protective effector functions of B10 cells during instances of autoimmunity and inflammation.

B10 cells also exhibited a distinct lack of somatic hypermutations in the transcripts isolated in this study. Thus, the B10 cells examined and sequenced here did not appear to have undergone any degree of affinity maturation or significant germinal center activity in vivo. These results are not unexpected given that these B10 cell clones were isolated from naïve mice not actively inflamed or undergoing significant immune activation. However, this predominance of germline-encoded transcripts sets the stage for further inquiries into B10 cell dynamics in vivo and studies of whether they undergo somatic hypermutation and affinity maturation.

A remaining question and logical extension of the studies presented here is how the B10 cell BCR repertoire may be shaped and focused during cases of antigen exposure. As B10 cells are known to actively participate in mitigating autoimmune inflammation, it would be of interest to determine the same B10 cell repertoire in models of autoimmune disease. However, as B10 cells are known to act in a local, cell-to-cell manner, isolating the B10 cells of interest that are actively delivering tissue-specific anti-inflammatory IL-10 remains a challenge. Therefore, understanding the complex scope of the B10 cell BCR repertoire during different disease states and within various anatomical locations will build upon these studies to further knowledge of B10 cell BCR-antigen interactions.
4. Self-antigens drive regulatory B cell IL-10 production under homeostatic conditions

Portions of this text have been adapted from the original manuscript, “Self-antigens Drive Regulatory B Cell IL-10 Production Under Homeostatic Conditions,” which is currently in submission for publication.

4.1 Introduction

IL-10 is a potent cytokine that negatively regulates inflammation and autoimmunity as well as innate and adaptive immune responses, particularly within local microenvironments. Regulatory B10 cells with the capacity to express IL-10 are dependent on IL-10 for both their identification and regulatory function (70). B10 cells are relatively rare in mice and humans, generally representing only 1-3% of mouse spleen B cells and 0.6% of human blood B cells, but they can increase in number during inflammation and autoimmune disease (18, 75).

The capacity of B10 cells to express IL-10 can be visualized by immunofluorescence staining for cytoplasmic IL-10 after short-term ex vivo stimulation, which drives il10 gene transcription and IL-10 protein production (18, 20, 21). B10pro cells do not express IL-10 following short-term stimulation, but become functionally competent to express IL-10 after agonistic CD40 stimulation in vitro (21, 70). Lastly, B10 effector cells are those regulatory B cells that are actively producing IL-10 in vivo without
the need for external stimulation. Although B10 and B10pro cells are readily quantified 
*ex vivo*, IL-10-producing B10 effector cells have been difficult to enumerate *in situ* or *ex 
vivo* as their presence is obscured by the intrinsic instability of *il10* mRNA, transient IL-
10 expression, or low-level IL-10 protein expression (19). Furthermore, B10 effector cells 
have been difficult to enumerate relative to the low-level background staining of 
autofluorescent dead or dying cells and debris that is observed in both wild-type and IL-
10-deficient mice (20). IL-10 reporter mice have also facilitated the study of IL-10 
production by immune cells, in part due to the longer intracellular half-life of reporter 
molecules after IL-10 expression is terminated (19, 28, 76, 77). Although B cells 
expressing IL-10 or reporter molecules are not measurable in naïve mice, B10 cells 
frequencies increase *in vivo* after stimulation and during inflammation or *Salmonella* 
infection (25, 78). Nonetheless, B10 effector cells have been difficult to identify in the 
absence of inflammation or disease.

Whether B10 effector cells contribute to the maintenance of homeostasis *in vivo* is 
unknown due to their scarcity (70). Alternatively, B10 effector cells may only be 
generated *in vivo* during responses to foreign antigens, infection, and autoimmune 
disease, or may only produce IL-10 at measurable levels following *in vitro* stimulation. 
To clarify these issues, novel methods were developed to expand single B10 effector cells 
*ex vivo* in order to determine B10 effector cell frequencies and to identify antigens that 
drive their IL-10 production under homeostatic conditions *in vivo*.  

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4.2 Results

4.2.1 B10 effector cells are rare \textit{in vivo}

To quantify B10 effector cells, B cells were examined directly \textit{ex vivo} or after culture with monensin for 5 h to enhance cytoplasmic IL-10 accumulation. Under these conditions, cytoplasmic IL-10 staining was not discernable in spleen B cells from naïve wild-type mice relative to normal low-level background fluorescent events that are normally observed in wild-type and \textit{il10}−/− B cells (≤0.2%, Figure 5). B10 effector cells were thereby not measurable, even though the B cell preparations were cultured with monensin to enhance IL-10 retention and were gated for live cell staining. Despite this, B10 and B10+B10pro cells were found at normal levels in wild-type and Tiger mice (GFP reporter mice for IL-10). While B10 and B10+B10pro cells were present at similar levels in Tiger and Vert-X reporter mice, GFP+ B10 effector cell frequencies in naïve Tiger and Vert-X mice were not distinguishable from background autofluorescent events that were observed in wild-type control mice. Thus, B10 effector cell numbers were not measurable in wild-type, Tiger, or Vert-X mice under homeostatic conditions.
Figure 5. **B10 effector cells are rare in vivo.** Representative B10 effector, B10 and B10+B10pro cell identification and isolation from control wild-type or IL-10 reporter Tiger and Vert-X mice. Numbers indicate mean frequencies of fluorescent events within the individual gates. Results are representative of 3-10 individual mice.

### 4.2.2 Single B cells can be expanded ex vivo

As spleen B10 effector cell numbers were below the frequencies of background fluorescent events in naïve mice, a culture system that promotes single B cell survival and expansion was developed using described methods (24, 27, 62). Mouse fibroblasts were modified to express B lymphocyte stimulator (BLyS) and CD154, with IL-21 stably expressed or added to cultures along with exogenous IL-4 at the beginning of culture (Figure 6A). Single fluorescence-positive or –negative cells/events were isolated from
wild-type and Tiger mice by fluorescence-based cell sorting into individual culture wells containing modified feeder fibroblasts as described above. Fluorescence-positive B cells/events sorted from Tiger mice expanded in approximately 10% of the culture wells, whereas <1% of the “control” fluorescence-positive cells/events sorted from wild-type B cells proliferated in this culture system (Figure 6B). By contrast, GFP B cells sorted from wild-type mice, and GFP+ B10 cells or GFP+ B10+B10pro cells from Tiger mice proliferated in approximately 60% of the wells. Single B cells from wild-type and Tiger mice expanded 10,000-fold on average under these culture conditions, and clonal expansion was not dependent on the ability of the B cells to express IL-10 (Figure 6C). Thereby, reporter-positive B10 effector cells were infrequent within the ≤0.2% background of fluorescence-positive events normally observed by flow cytometry, but could be isolated and quantified from naïve Tiger mice under homeostatic conditions.
Figure 6. Single B cell expansion ex vivo. A, Representative single B cell expansion during in vitro cultures. Single B cells were cultured on either 3T3-CD154/BLyS^BEAT^ or 3T3-CD154/BLyS^EAT^ cells with the addition of IL-4 and/or IL-21 before harvesting the cells and culture supernatant fluid on day nine. Representative microscopy images show single B cell cultures at the indicated days and magnifications. B, Isolation and expansion efficiencies of single B cells cultured as in (A). Individual fluorescence-positive (GFP^+^) and fluorescence-negative (GFP^-^) events from wild-type or Tiger mice were identified using the gating strategy shown in Figure X and cultured in single wells containing 3T3-CD154/BLyS^BEAT^ cells for 9 days. Bars indicate mean frequencies of wells containing expanded B cells (n=264 wells/group; three experiments pooled; **P<0.01). C, B cell expansion after culture. Dots indicate the number of B cells within individual culture wells after expanding single B cells from wild-type, Tiger, and il10^-/-^ mice for 9 days as in (A). Horizontal bars show means. Numbers of B cell clones quantified in each group are indicated.
4.2.3 *Ex vivo*-expanded B cells class switch and secrete mAbs during culture

As a result of IL-4 exposure during their expansion, the B cells within each clone were predominantly surface IgM negative, but IgG1 and CD19 bright, confirming their B cell origin (Figure 7A). Similar results were obtained with wild-type B cells, and GFP⁺ B10 effector cells or GFP⁻ B cells cultured from Tiger mice. Most of the B cells within each clone also expressed the CD5 activation marker or were CD38⁻CD138⁺ cells. Some B cells expressed high levels of CD138 (<1%, n=3) after stimulation, in agreement with observations that some B10 cells can differentiate into antibody-secreting cells *in vivo* and *in vitro* following transient IL-10 production (28). Consistent with this, 17±3% of the expanded B cells within each clone secreted detectable mAb in ELISpot assays, whereas 0.03±0.01% of *ex vivo* spleen B cells had this capacity (Figure 7B). All B cell clones secreted significant IgG1 (range 2-12 μg/ml, mean 4.0 μg/ml) into the culture supernatant fluid, but significant amounts of IgM, IgG2a, IgG2b, IgG3, or IgE were not detected (Figure 7C). GFP⁺ B10 effector cells and GFP⁻ B cell clones from Tiger mice secreted similar amount of IgG (Figure 7D). Thereby, the B cells switch antibody isotype during clonal expansion, with some stochastically differentiating into antibody-secreting cells.
Figure 7. Antibody secretion by ex vivo-expanded B cells. A, Cell surface phenotype of B cell clones from wild-type mice. B cell clones expanded for 9 days as in Figure X were stained for cell surface CD19, IgM, IgG1, CD5, CD38, and CD138 expression relative to isotype control antibody. Representative immunofluorescence and frequencies of cells within quadrants are shown for ≥4 experiments. B, Antibody-secreting cell frequencies among B cell clones and spleen B cells. Representative ELISpot wells from ≥3 experiments are shown. C, IgG1 mAb is secreted by B cell clones after 9 days of culture as measured by isotype-specific ELISAs. D, IgG1 mAb secretion by expanded B cells on
day 9 of culture from single B cells. C-D, Dots represent single B cell clones. Bars indicate mean culture supernatant fluid mAb concentrations.

4.2.4 Self-antigens drive B10 effector cells in vivo

The ability to isolate single B cell clones that secrete mAb enabled the characterization of B10 effector, B10, and B10+B10pro cell antigen receptor specificities. Apoptotic cells induce regulatory B cells (23), and antibodies secreted by B10 cells include autoreactive specificities (28), suggesting that some B10 cells are reactive with self-antigens. Therefore, single B10 effector cells and GFP B cells were purified from Tiger mice. Individual B10 and B10+B10pro cells were also isolated after ex vivo stimulation to induce GFP expression. After their in vitro expansion, culture supernatant fluid from each B cell clone was used to characterize its BCR autoreactivity. At least 59% of B10 effector cell clones produced mAbs that were reactive with self-antigens (Figure 8A), revealing remarkably high levels of self-reactivity among B10 effector cells. B10 effector cell mAbs (~11%) also bound malondialdehyde acetylaldehyde (MAA), an oxidation-specific adduct generated during atherosclerosis (64, 79). By contrast, <3% of mAbs from GFP non-B10 effector cells were self-reactive, despite the fact that these clones produced antibody to the same extent that B10 effector cell clones did (Figure 8B). Thus, the self-reactivity evident in B10 effector cells was a unique property of this population and not a general trend among all B cells isolated.
Over half of the self-reactive B10 effector cell mAbs were polyreactive (59%) and bound multiple self-antigens (Figure 8C). Only 10% of B10 cell and 5% of B10+B10pro cell clones expressed DNA- or histone-reactive BCRs (Figure 8B). None of the B cell populations studied reacted with the foreign antigens oxazolone (Oxa) or bovine serum albumin (BSA), which served as negative controls. Thus, B10 effector cell selection and IL-10 secretion appear to be predominantly driven by autoreactive and polyreactive BCRs under homeostatic conditions as a likely means to combat the development of autoimmunity and atherosclerosis-associated inflammation.
Figure 8. Self-antigens drive B10 effector cells in vivo
Figure 8. Self-antigens drive B10 effector cells in vivo. A, Culture supernatant fluid mAbs from B10 effector cell clones generated from naïve Tiger mice as in Figure X were tested for autoreactivity using HEp-2 cell monolayers. Representative images of HEp-2+ and HEp-2- cell staining are shown for mAbs that were not reactive with dsDNA, ssDNA, histones or malondialdehyde acetylaldehyde (MAA) by ELISA. B, Self-antigen specificities of the mAbs produced by expanded single B10 effector, B10, B10+B10pro cell, or non-B10 clones were determined by ELISA using the indicated antigens. Oxazolone (Oxa) and bovine serum albumin (BSA) reactivities were evaluated as negative controls. Bars indicate mean frequencies of the reactive mAbs (top graph). Mean supernatant fluid mAb concentrations are shown in the lower graphs with numbers of clones tested indicated. Significant differences between means are indicated; **p<0.01. C, Antigen specificities of single B10 effector clones as determined in Figure X. The polyreactivity of mAbs with self-antigens is shown in the Venn diagram, which represents the B10 effector cell clones contained in the black and grey sections of the pie chart.

4.3 Discussion

These studies specifically provide the first direct proof that B10 effector cells exist under homeostatic conditions, where they represent at least 0.02% of spleen B cells (Figures 5-7). The majority of spleen B10 effector cells were autoreactive, with most binding to at least two self- or inflammation-associated antigens (Figure 8). That B10 effector cell BCRs were significantly more reactive with ssDNA, dsDNA, and histones than were B10, B10pro or B cell BCRs (p<0.05) also indicates that the generation of B10 effector cells was preferentially driven by self-antigens under homeostatic conditions rather than resulting from foreign antigens or stochastic mechanisms. Polyreactive B10 and B10pro cell BCRs were also common (Figure 8), thereby empowering the relatively small numbers of B10 and B10 effector cells to counterbalance immune responses to a more diverse population of antigens. Thus, B10 effector cells provide an auto-antigen-
specific mechanism for delivering IL-10 within appropriate tissue microenvironments before and during inflammation and autoimmune disease.

The single B cell ex vivo expansion system used in this study revealed that most B10 effector cells acquire their functional capacity to secrete IL-10 in response to self-antigens under homeostatic conditions (Figure 8). B10 cell regulation of inflammation and autoimmunity under physiologic conditions also appears to be antigen-specific (7, 18, 23, 27, 42). In this way, autoreactive B10 effector cells may provide one of the first lines of defense against autoimmunity by negatively regulating self-reactive T cells (27). Consistent with their self-reactivity and polyreactivity, B10 cell-derived antibodies are also predominantly, if not exclusively, germline-encoded and of the IgM isotype (Chapter 3, refs.25, 28). The preferential capacity of B10 effector cell BCRs to bind DNA and DNA-associated auto-antigens suggests that these and similar multivalent auto-antigens may have a unique capacity to induce the IL-10 functional program. However, not all self-reactive B cells acquire the capacity to express IL-10 (Figure 8). In these cases, more powerful DNA and DNA-associated signals such as those that activate autoreactive B cells via sequential BCR engagement and Toll-like receptor 9 may circumvent the acquisition of IL-10 competence and induce pathogenic autoimmunity (80, 81). Vigorous BCR-crosslinking by anti-IgM antibodies also down-regulates B10 pro-cell IL-10 competence in vitro, arguing that strong BCR signals can induce alternative functional programs (21, 25, 75). Thereby, B10 cells likely provide an antigen-specific
mechanism for sensing autoreactive versus pathogenic immune responses as a way to help maintain tolerance to self-antigens while not suppressing protective immunity. Thus, antigen specificity, autoreactivity, and the modest numbers of B10 effector cells in vivo may limit the production of IL-10, a potent immunosuppressive cytokine, so as to also maintain normal immune system function in response to foreign antigens.

Although autoreactive B cells are historically thought to promote pathology and autoimmunity, these studies extend the concept that self-reactive B cells, and B10 cells in particular, have protective functions (23, 28, 82). Chronic B cell stimulation in vivo due to self reactivity helps explain why B10 cells expand quickly during immune responses to self-antigens (21, 42) and mount rapid proliferative responses in vitro relative to those of follicular B cells (21, 25, 75). Chronic stimulation of B10 cells by self-antigens also explains why the functional effects of B10 cell depletion are most obvious during acute immune responses, whereas regulatory T cell depletion has more dramatic effects during the later phases of autoimmune responses (26, 42). Consistent with the concept of prior antigen experience in vivo, human B10 and B10pro cells are found within the CD24hi and CD27+ memory compartments (75). Furthermore, after terminating transient IL-10 expression, some B10 effector cells differentiate into antibody-secreting cells that produce polyreactive natural antibodies and antigen-specific antibodies that may neutralize immune responses to self or foreign antigens. Thereby, the high degree of
autoreactivity among B10 effector cells supports the emerging evidence that regulatory B10 cell production of IL-10 contributes to tolerance regulation.

The precise role of antigen-BCR interactions in B cell acquisition of IL-10 competence and subsequent regulatory activity has been a matter of speculation among studies of regulatory B cells (70). These questions have remained unanswered largely due to the technical challenges of isolating primary B cells and expanding them on a clonal basis to generate sufficient material to investigate their antigen specificity. Herein, we demonstrate an ex vivo culture system in which single B cells can be expanded approximately 10,000-fold in a short period of time (Figure 6). In addition, a fraction of the expanded B cells in each clone differentiate into antibody-secreting cells (Figure 7) that generate sufficient quantities of mAb for antigen screening assays (Figure 8). This culture system also enable the separation and isolation of labeled B10 effector cells, which represented 10% of the inherent background fluorescence signals that normally obscure their enumeration by flow cytometry (Figure 6). That <1% of the ≤0.02% background fluorescence signals sorted from wild-type B cells gave rise to B cell clones confirmed that these sporadic background signals are predominantly fluorescent debris and autofluorescent dead or dying cells (Figure 5). Thus, in addition to being useful for the identification and characterization of rare antigen-specific B10 effector cells, B10 cells, and B10pro cells, this culture system may be useful for the identification and characterization of rare antigen-specific B cells and their BCRs before, during, and after
active immune responses. Moreover, the ability to identify and expand antigen-specific regulatory B10 cells exponentially \textit{ex vivo} may eventually allow their manipulation for the treatment of inflammation and induction of tolerance in diverse human diseases.
5. Antigen-specific B10 cells suppress inflammation

Portions of this text have been adapted from the original manuscript, “Antigen-specific B10 Cells Suppress Inflammation,” which is currently in submission for publication.

5.1 Introduction

As discussed in Chapters 3 and 4, antigen-derived BCR signals from self and foreign antigens are essential to B10 cell development and may be the crucial first step in selecting B cells to become IL-10-competent. How BCR-related signals drive B10 cell effector function and the tissue-specific delivery of IL-10 during active immune responses remains less well understood.

Several previous studies have established that antigen-BCR interactions are indispensable for B10 cell effector function. In studies where B10 cell deficient in MHC class II were transferred into mice with EAE, the MHC-II⁻ B10 cells were incapable of suppressing autoimmune inflammation. This observation, coupled with the fact that B10 cells also require IL-21-derived signals, likely supplied by nearby T cells, to secrete IL-10 led credence to the hypothesis that B10 cells interact with T cells in a local, intimate fashion that relies on cognate antigen specificity (27).

This model in which B10 cells recognize their antigen of interest via MHC class II-T cell receptor interactions was bolstered by studies in which antigen-experienced B10 cells and other regulatory B cells exhibited enhanced immunosuppression upon
adoptive transfer when compared with naïve B cells. In models of EAE, B cells derived from mice that had already experienced active disease were more effective at suppressing EAE-induced inflammation that were their B cell counterparts derived from naïve mice (7, 26). Similarly, in collagen-induced arthritis, B cells that were pre-incubated with apoptotic cells prior to adoptive transfer provided superior immunosuppression of autoimmune inflammation, thus underscoring the augmentative effect that prior antigen exposure has on regulatory B cell populations (23). Whether this occurs because antigen exposure expands a population of antigen-specific regulatory B cells or because BCR-derived signals differentially focus the biology of a given B cell population remains unknown.

To address this question and determine the importance of antigen specificity in B10 cell-mediated effector functions, we used novel methods to show that foreign antigens can promote antigen-specific B10 effector cell expansion in vivo and that these antigen-specific B10 effector cells are required for optimal suppression of inflammation.

5.2 Results

5.2.1 Ex vivo-expanded B cells (iB10pro cells) acquire IL-10 competence after receiving appropriate molecular signals

One inherent challenge of studying the dynamics of antigen-specific lymphocytes is the difficulty in generating enough antigen-specific cells for rigorous evaluation. To this end, a bulk B cell expansion system similar to that described for single B cells in Chapter 4 was designed in which mouse spleen B cells were cultured on
NIH-3T3 cells stably expressing BLyS and CD154 (NIH-3T3-CD154-BLyS<sup>tk</sup> cells) to support B cell growth and survival. With the addition of IL-4 for 4 days and IL-21 for 5 days, B cells expanded 30,000-fold, and approximately 39% of B cells expressed cytoplasmic IL-10 at the end of culture (27). A second generation version of this culture system using NIH-3T3-CD154-BLyS<sup>EAT</sup> cells was established in which spleen B cells again expanded approximately 30,000-fold but did not produce cytoplasmic IL-10 at the end of the nine-day culture period (Figure 9A). However, the expanded B cells were capable of secreting IL-10 after 24-h stimulation with LPI (Figure 9B). In addition, the expanded B cells has a demonstrably accessible <i>il10</i> gene locus when analyzed by DNase-seq at the end of culture (Figure 9C). The propensity of these cells to open the <i>il10</i> locus but not readily produce IL-10 protein suggests that they may function in a manner similar to that of B10pro cells, which also require longer term stimulation to produce IL-10. Thus, B cells cultured on NIH-3T3-CD154/BLyS<sup>EAT</sup> cells are hereafter referred to as induced B10pro-like (iB10pro) cells.

After culture, iB10pro cells expressed CD19, MHC class II, IgG1, and the activation markers CD5 and CD38 (Figure 9D). The majority of iB10pro cells expressed CD138, which is associated with plasmablast development and has also been correlated with regulatory B cell activity in previous studies (83, 84). However, as occurs with B10 cells <i>in vivo</i>, iB10pro cells terminated IL-10 expression as they differentiated into antibody-secreting cells (Figure 9E). This was demonstrated by fractionation of the
cultured B cells on the basis of CD138 expression after culture where a clear inverse correlation between IL-10 production and intracellular IgG1 could be observed. Thus, iB10pro cells were IL-10-competent at the end of culture, and they ceased IL-10 expression as they differentiated into plasmablasts and less frequently, plasma cells.

**Figure 9. iB10pro cell culture system.** A, iB10pro cell generation and expansion during culture. Purified spleen B cells were cultured on NIH-3T3-CD154/BLyS*EAT cell
monolayers with exogenous IL-4 for four days and then IL-21 for five days before harvest. Bar graphs indicate mean (+ s.e.m.) cell numbers at indicated days of culture from three independent experiments. B, iB10pro cells resemble B10pro cells and secrete IL-10 following 24-h stimulation in vitro. iB10pro cells generated from wild-type (WT) or Il10−/− mice were stained for cell surface CD19 and intracellular IL-10 following 5-h LPIM stimulation. The bar graph shows mean (+ s.e.m.) secreted IL-10 concentrations for iB10pro cells cultured with media alone, LPS, PI, or LPI (2 x 10⁶ cells/ml) for 24 h in three independent experiments. C, il10 locus accessibility for B cells, B10 cells, and iB10pro cells. Regions of the il10 locus in accessible configurations were evaluated by DNase-seq (ex vivo B cells and iB10pro cells at day nine of culture as in [A]) or ATAC-seq (B10 cells). Solid blocks indicate il10 gene exons. D, Cell surface phenotype of iB10pro cells. Spleen B cells or iB10pro cells generated as in (A) were stained for cell surface CD19, CD5, CD38, CD138, IgG₁ and MHC-II expression. Representative histograms are shown with black lines representing B cells or iB10pro cells and dashed lines representing isotype control staining. E, iB10pro cells were sorted according to cell surface CD138 immunofluorescence staining, permeabilized and stained for cytoplasmic IgG₁ expression with representative images shown. In separate experiments, CD138-sorted populations (1 x 10⁶ cells/ml) were stimulated for 24 h with LPI. Bars indicate mean concentrations of supernatant IL-10.

5.2.2 iB10pro cells are selected to become B10 cells in vivo

Further characterization of iB10pro cells revealed that mouse B cell subsets proliferated 10,000-30,000-fold and produced IL-10 at the end of culture, with the exception of germinal center B cells (Figure 10A). Strikingly, when this culture system was adapted to single B cell culture, every B cell clone that was capable of expanding during culture also produced IL-10 after 24-h stimulation. The amount of IL-10 produced by the expanded B cell clones was comparable to that produced by B10 cell-enriched CD1dhiCD5+ B cells isolated directly ex vivo (Figure 10B). Thus, nearly all spleen B cells were capable of expanding in the iB10pro culture system, and all cells that were able to expand acquired IL-10 competence during culture.
Whether iB10pro cells are capable of producing IL-10 in vivo was assessed by adoptively transferring iB10pro cells constitutively expressing tandem dimer Tomato fluorescent protein into the peritoneal cavities of wild-type recipients. When the transferred cells were recovered from the peritoneal cavity two days after transfer and stimulated for five hours with LPIM, approximately 14% of the transferred cells produced IL-10 (Figure 10C). Therefore, although all B cells cultured acquired IL-10 competence, only some iB10pro cells were selected to produce IL-10 in vivo.

Figure 10. iB10pro cells are selected to become B10 cells in vivo. A, B cell subset expansion and IL-10 secretion during iB10pro culture. Spleen or peritoneal cavity B cells were isolated based the following phenotype: transitional 1 (T1, B220⁺CD93⁺CD21 CD23⁻), transitional 2 (T2, B220⁺CD93⁺CD21⁺CD23⁺), follicular (FO, B220⁺CD93⁻CD21⁺CD23⁻),
marginal zone (MZ, B220^CD93^CD21^-^CD23^), germinal center (GC, B220^GL-7^-^CD95^), CD1d^{hi}CD5^+ and peritoneal cavity-derived B cells (PC) and expanded as in (Figure 9A). The bar graphs show expansion fold and IL-10 secretion following 24-h LPI stimulation (2 x 10^6 cells/ml) of cells at the end of culture for each subset indicated. B, Expanded single B cells produce IL-10 at the end of culture. Plate images show representative IL-10 ELISA results for single B cell clones (5,000-15,000 cells/well) expanded from wild-type or Il10^-/- B cells as in Fig. 1C and then stimulated for 24 h with LPI. Serially-diluted control IL-10 standards (St) are displayed in the left two columns of each plate. The dot graph shows relative IL-10 concentrations for iB10pro clones in comparison with supernatant fluid from ex vivo purified spleen CD1d^{hi}CD5^+ and CD1d^{lo}CD5^- B cells (10,000 cells/well) stimulated for 24 h with LPI. Horizontal bars indicate means. C, iB10pro cells become B10 cells in vivo. TdTomato^+ iB10pro cells were transferred into wild-type mice, with peritoneal cavity B cells isolated two days later. Some TdTomato^+ iB10pro cells acquire IL-10 competence and express IL-10 following 5-h LPIM stimulation. B cells from Il10^-/- mice are shown as a control. Bar graph indicates mean (+ s.e.m.) IL-10^+ B cell frequencies from three mice.

5.2.3 iB10pro cells suppress contact hypersensitivity in an antigen-specific manner

Whether iB10pro cells would also be selected to produce IL-10 and regulate immune responses during active inflammation was determined using a model of oxazolone-induced CHS. When iB10pro cells generated from naïve B cells were transferred into oxazolone-sensitized mice before antigen challenge, CHS-induced inflammation was significantly reduced (Figure 11A) and was comparable to that observed when B10 effector cells generated using NIH-3T3-CD154/BLyS^{TK} cultures are transferred (Figure 11B). Suppression of inflammation by iB10pro cells was dependent upon the presence of MHC-II and IL-10.

Immunosuppression mediated by iB10pro cells was dose-dependent, with maximal disease inhibition was observed at a dose of 5 x 10^6 cells (Figure 11C).
However, iB10pro cells provided durable suppression of inflammation and mitigated disease when mice were re-challenged with oxazolone 28 days after iB10pro cell adoptive transfer (Figure 11D). Moreover, iB10pro cells derived from oxazolone-sensitized mice reduced inflammation to a significantly greater extent than did iB10pro cells from naïve or DNFB-sensitized mice. Reciprocally, iB10pro cells from DNFB-sensitized mice reduced disease to a greater extent than did iB10pro cells from naïve or oxazolone-sensitized mice during DNFB-induced CHS (Figure 11E). Therefore, iB10pro cells are likely to be selected by antigen to become B10 and B10 effector cells, which suppress disease through IL-10- and MHC-II-dependent mechanisms following antigen recognition.
Figure 11. iB10pro cells suppress CHS in an antigen-specific manner. A-B, iB10pro and B10 effector cells inhibit CHS through IL-10- and MHC-II-dependent pathways. Oxazolone-sensitized mice were given iB10pro cells or B10 effector cells (2 x 10^6) generated from the indicated mice one day before ear challenge. Representative ear histologies from untreated control or oxazolone-sensitized mice that received PBS only or iB10pro cells before oxazolone challenge are shown. Line graphs show mean (+ s.e.m.) increases in ear thickness following challenge (n=5 mice/group). C, Dose-dependent iB10pro cell suppression of CHS. Oxazolone-sensitized mice were given iB10pro cells from a naïve mouse at the indicated doses one day before ear challenge. Line graphs
indicate mean (+ s.e.m.) increases in ear thickness (n=5 mice/group). D, iB10pro cell suppression of CHS is durable. Oxazolone-sensitized mice were given iB10pro cells generated from naïve mice one day before ear challenge on day five (n=5 mice/group). Recipient mice were challenged with oxazolone at day 5, and re-challenged on days 28 and 56. Line graphs indicate mean (+ s.e.m.) decreases in ear thickness. E, iB10pro cells generated from antigen-sensitized mice optimally suppress antigen-specific CHS. Line graphs show mean (+ s.e.m.) increases in ear thickness of oxazolone- or DNFB-sensitized mice that received iB10pro cells (1 x 10⁶) from the indicated oxazolone- or DNFB-sensitized mice before ear challenge (n=5 mice/group).

5.2.4 Foreign antigens drive the expansion of antigen-specific B10 effector cells in vivo

Whether foreign antigens drive B10 effector regulation during active immune responses was assessed using a model of oxazolone hypersensitization. Oxazolone-specific B cells measured by flow cytometry were rare in naïve mice and increased significantly following sensitization, but remained <1% of B cells except in Il10⁻/⁻ mice where inflammatory responses are augmented (Figure 12A). Oxazolone-reactive B cells were confirmed by ex vivo culture and oxazolone ELISA with equivalent cloning efficiencies of single B cells observed throughout oxazolone hypersensitization. Thus, oxazolone-reactive B cells could be readily isolated and expanded.

Oxazolone-specific B10 effector cells were also apparent following oxazolone hypersensitization. In naïve Tiger mice, only background levels of B cells were reactive with oxazolone, but after sensitization, 0.5% of B cells stained with oxazolone of which ~40% expressed IL-10 in vivo (Figure 12B). At the single-cell level, GFP⁺ B10 effector cells reactive with oxazolone were not detected in naïve Tiger mice (Figure 12C). However, almost 40% of GFP⁺ B10 effector cells from sensitized Tiger mice produced oxazolone-
specific mAbs, whereas only 2% of GFP B cell clones reacted with oxazolone (Figure 12C). Most (~60%) oxazolone-specific mAbs from B10 effector cells did not bind autoantigens. Thus, oxazolone sensitization can drive antigen-specific B10 effector cell expansion in vivo, and most of these cells are reactive for the foreign antigen eliciting the immune response, not self-derived proteins.
Figure 12. Foreign antigens drive the expansion of antigen-specific B10 effector cells in vivo
Figure 12. Foreign antigens drive the expansion of antigen-specific B10 effector cells in vivo. A, Oxazolone-specific B cells are expanded in vivo during CHS. Schematic for oxazolone hypersensitization to induce CHS. Wild-type (WT) and il10−/− mice were hypersensitized with oxazolone as indicated. Oxazolone-specific spleen B cells were visualized by immunofluorescence staining using biotinylated oxazolone (Oxa) and streptavidin-APC (St-APC). Single Oxa+ B cells were used to generate oxazolone-specific iB10pro cell clones, with representative plate images showing supernatant fluid oxazolone-specific ELISA results (day 14 mouse). Bar graphs show mean (+ s.e.m., n=3 experiments) frequencies of cloning efficiencies and clones reactive with oxazolone as determined by ELISA, with Oxa+ iB10 cell clones (day 14 mouse) shown as controls (Ctrl). B, Oxazolone-specific B10 effector cells expand in vivo. GFP+ B10 effector cell frequencies were quantified in naïve and oxazolone-sensitized (day 5) Tiger mice. Wild-type B cells treated with streptavidin-APC alone were used as controls. Bar graph shows mean (+ s.e.m.) frequencies of IL-10/GFP+ cells among oxazolone- and non-oxazolone-specific B cells from three experiments. C, B10 effector cell antigen specificities. IL-10/GFP+ B10 effector cell frequencies were assessed in Tiger and control wild-type mice before or five days following oxazolone hypersensitization as in (A) with single cell expansion as in Fig. 1B. Antigen specificities of individual B cell clones from three Tiger mice were determined by ELISA (bar graph). Some oxazolone-specific B10 effector cell clones were also reactive with self-antigens (Venn diagram of grey pie chart section). A-C, Significant differences between means are indicated: *p<0.05; **p<0.01.

5.2.5 Antigen-specific B10 effector cells are required for optimal immunosuppression

Whether immunosuppression requires antigen-specific B10 cells was examined using B cells from oxazolone-hypersensitized Tiger mice. After iB10pro cell culture, B cells enriched for oxazolone reactivity contained ~25% oxazolone-specific cells, whereas oxazolone-reactive cells were rare in oxazolone-depleted or naïve iB10pro cell cultures (Figure 6A). Oxazolone-specific antibody was only produced in oxazolone-enriched cultures and to a small extent naïve iB10pro cultures, whereas all three iB10pro cell preparations produced equivalent IL-10 after stimulation. iB10pro cells enriched for
oxazolone reactivity suppressed CHS to a significantly greater extent than did iB10pro cells from naïve mice, whereas iB10pro cells depleted of oxazolone-reactive clones were without significant therapeutic effect (Figure 13A). Identical results were obtained when single oxazolone-specific iB10pro clones were generated, pooled, and transferred into sensitized mice, while non-oxazolone-reactive iB10pro clones did not suppress inflammation (Figure 13B). Pooled oxazolone-reactive iB10pro clones from Il10⁻/⁻ mice did not reduce inflammation, thereby eliminating a measurable role for antibody or other iB10pro cell-derived products besides IL-10 in immune suppression. As with iB10pro cells from naïve mice (Figure 13A), oxazolone-specific iB10pro cells suppressed CHS responses in a dose-dependent manner (Figure 13C). Thus, antigen-derived signals and IL-10 were indispensable for B10 cell regulatory function.
Figure 13. Antigen-specific B10 effector cells are required for optimal immunosuppression. A, Oxazolone-specific iB10pro cells are required for CHS suppression. Oxa\(^+\) and Oxa\(^-\) enriched B cell populations were isolated from oxazolone hypersensitized mice (day 14). Naïve, Oxa\(^+\), and Oxa\(^-\) B cells were expanded as in Fig. 3B, with Oxa\(^+\) iB10pro cell frequencies shown after culture. Culture supernatant fluid anti-oxazolone antibody levels and iB10pro cell activation-induced IL-10 secretion were determined by ELISA. Naïve, Oxa\(^+\), and Oxa\(^-\) iB10pro cells (1 x 10\(^6\) cells, n=5 mice/group) were transferred into oxazolone-sensitized mice before challenge with subsequent mean.
(± s.e.m.) increases in ear thickness shown (line graph). B, Antigen-specific iB10pro clones suppress CHS through IL-10. Pooled Oxa-specific or pooled non-antigen-specific (Oxa') iB10pro cell clones generated from wild-type or Il10−/− oxazolone-hypersensitized mice (day 14, ≥100 clones each group) as in Fig. 1C were transferred into mice (0.2 x 10^6 cells, n=5 mice/group) before ear challenge, with mean (± s.e.m.) increases in ear thickness shown. C, Dose-dependent antigen-specific iB10pro cell suppression of CHS. Oxazolone-sensitized mice were given pooled Oxa' iB10pro clones as in B at the indicated doses. A-C, Significant differences relative to control mice are indicated: *p<0.05; **p<0.01.

5.3 Discussion

Antigen-specific B10 cells elicited during foreign antigen challenge also limited inflammatory immune responses within tissues (Figures 11-13). Polyreactive B10 cell BCRs were common, thereby allowing small numbers of B10 cells to counterbalance diverse immune responses. Thus, self and foreign antigens not only elicit B cell pro-inflammatory immune responses, but may also generate regulatory B10 cells that prevent autoimmunity and promote tolerance depending on the balance of signals and the nature, concentration, and longevity of the eliciting antigen in vivo (Figures 12-13).

Not only are B10 effector cells reactive with self-antigens during homeostasis, but they are also capable of expanding in response to foreign antigens. Previous studies investigating the role of antigenic stimulation and B10 effector function have demonstrated that antigen-experienced B10 cells provide superior regulation of immune responses when compared with B10 cells from naïve mice (7, 26). Here we show for the first time that not only are antigen-experienced B10 cells better at suppressing disease than their naïve counterparts, they are in fact required for optimal immunosuppression.
upon adoptive transfer. These findings underscore the fact that antigen-specific B10 cells, although rare, are the primary population that responds to suppress immune responses. Indeed, although large numbers of B10 cells are often used for adoptive transfer, the population that is likely responding to combat disease is the exceedingly small number of antigen-specific cells contained in the adoptive transfer.

Thereby, the adoptive transfer of iB10pro cells is followed by the in vivo selection of regulatory B10 cells by BCR specificity to provide an antigen-specific delivery mechanism for IL-10 within tissue microenvironments. As numerous regulatory immune cell-based immunotherapies are in development, the antigen specificity of regulatory B10 cells and their capacity for exponential ex vivo expansion may allow their manipulation for the treatment of inflammation and diverse human diseases.
6. Discussion

6.1 Regulatory B cells are selected by self-antigens in vivo

The studies presented here demonstrate the importance of antigen-BCR interactions during B10 cell development and function. B10 cells suppress immune responses through the provision of IL-10 and are likely selected to do so via BCR signals derived from a diverse pool of self-antigens. B10 cells from both the spleen and peritoneal cavity expressed diverse BCR gene utilization with a distinct lack of BCR mutations (Chapter 3). These studies are consistent with other observations that B10 cells secrete weakly reactive, polyreactive antibodies enriched for reactivity with self-antigens (28). Thus, the wide variability within the B10 cell BCR repertoire poises B10 cells to respond to a diverse pool of antigens in vivo.

That self-antigens play a crucial role in driving B cell IL-10 secretion was confirmed by studies of B10 effector cells in vivo. B10 effector cells expressed BCRs that were reactive with multiple self-antigens (Chapter 4). Furthermore, non-B10 effector cells, representing more than 99% of the general spleen B cell pool, were demonstrably lacking in self-reactivity. Therefore, although B10 cells express a varied BCR repertoire, the antigenic population that selects B cells for IL-10 production is unique and this reactivity is not reflected in the overall B cell pool.

Studies of the B10 cell BCR repertoire are especially useful for understanding the fact that B10 cells are a functionally-defined immune cell subset uniquely identified by
their ability to secrete IL-10. Current paradigms for classifying the myriad of adaptive lymphocyte subpopulations often rely on expression of certain cell-surface markers or a narrowed antigen receptor repertoire, as in the case of peritoneal cavity B1a and B1b cells (73). Although these phenotypic definitions are useful for charting the immune cell landscape, they often lead to models of different immune cell lineages and underestimate the complexity and plasticity of immune cell subsets. Despite numerous attempts to identify unique phenotypic markers for B10 cells, IL-10-competent B cells continue to appear in multiple phenotypically distinct B cell subsets and anatomic locations. This is reflected in the BCR repertoire studies presented in this dissertation where both spleen and peritoneal cavity B10 cells exhibited BCR repertoires remarkably similar to that of the overall B cell pools from which they were isolated. That B10 cells continue to defy a traditional lineage classification makes them a versatile population of regulatory cells able to exert their effects in a variety of contexts. Therefore, B10 cells express a diverse BCR repertoire and phenotype, and expression of IL-10 remains the best way to identify this unique subset.

Despite the fact that B10 cells can be found in multiple locations, they remain a rare population in vivo, especially the active IL-10 producers, B10 effector cells. The single B cell expansion methods described in Chapter 4 represent an unprecedented opportunity to study rare B cells on a clonal level. Additionally, the rarity of B10 effector cells points to the potency of their immunosuppressive effects. It is likely that they are so
limited in number and capable of only modest expansion during active immune responses (Chapter 5) to keep IL-10 levels in check and maintain the delicate balance between pro- and anti-inflammatory immune cell activities.

The extensive self-reactivity of B10 effector cells is perhaps also linked to their exceedingly small numbers in vivo. The finding that such a beneficial B cell population capable of providing protection against autoimmunity is also significantly reactive with self-antigens is surprising and inverts the classically-held view that self-reactivity is undesirable within the immune system. However, as in the case of B cells that produce natural antibody and have been proposed as a protective B cell subset (74), B10 effector cells may be driven by self-antigens in vivo to modulate potentially destructive autoimmune responses raised against self-antigens resulting from typically occurring cell death and maintenance. Thus, the self-reactivity of B10 effector cells may aid in B10 cell effector functions and B10 cell-mediated protection from disease.

6.2 B10 cell regulation of inflammation is antigen-specific

Foreign antigen exposure has long been thought to augment B10 cell regulatory activity upon adoptive transfer in models of autoimmunity (70). This hypothesis was confirmed by studies of iB10pro cells in CHS. iB10pro-mediated suppression of inflammation was definitively antigen-specific (Chapter 5). Indeed, the additive effects of prior antigen exposure on iB10pro cells were limited to the sensitizing antigen of interest and could not be replicated by non-specific activation with an unrelated antigen.
Further studies demonstrated that antigen-specific B10 cells are necessary for optimal immunosuppression and that potent inhibition of inflammation can be achieved by the transfer of verifiably antigen-specific iB10pro cells. Thus, the greatest extent of B10 cell effector activity is likely accomplished by a scarce but influential population of antigen-specific B10 cells.

The requirement for antigen specificity during B10 cell regulation of homeostasis and disease could act as a control mechanism for the tissue-specific delivery of IL-10. As discussed above, linking BCR reactivity with the secretion of IL-10 prevents an influx of anti-inflammatory cytokine into the cellular milieu, thus helping to maintain equilibrium within the immune system. Furthermore, these studies create a model of B cell responses in which both pro- and anti-inflammatory B cell populations are expanded during inflammation. In the CHS model described here, antigen-specific B10 cells were a dynamic population that expanded along with the course of antigen exposure. These cells likely act as a counter to the pro-inflammatory B cells and limit potentially dangerous excessive immune activation. B10 cells therefore represent a potential rheostat for B cell responses.

**6.3 Implications for B10 cell therapies in humans**

B10 cells represent a potential source of anti-inflammatory cells that could be used to treat autoimmune diseases in humans. However, these studies underscore the necessity of understanding whether the antigen-specific phenomena observed during
mouse B10 cell activity can be extended to human B10 cells. Understanding the requirement for antigen specificity in humans would help to direct potential human therapies as certain populations of B10 cells could be isolated and expanded for treatment. Although challenging, it is also necessary to determine whether a B10 effector cell counterpart exists in humans and what antigens drive their IL-10 production. These proposed studies would expand on current knowledge of human B10 cells and provide direction for how to optimally harvest the potential of this unique population.

6.4 Remaining Questions

The B10 cell BCR studies presented here provide a foundation for further investigations into antigen-driven B10 cell dynamics. Of particular interest is how B10 effector cell BCR repertoires shift or mutate in antigen-specific populations. Given the single B cell culture system and methods for isolating antigen-specific B cells described here, it would be feasible to obtain antigen-specific B10 effector cells and sequence their BCRs using the clonally-expanded B cells. Determining whether these clones are mutated in vivo would provide insight into B10 cell responses and whether these cells are capable of undergoing affinity maturation, which would further understanding of their antigen-driven biology.

Identifying that self-antigens drive B cell IL-10 production under homeostatic conditions is a step forward in understanding the role of B10 cell-derived antibodies in vivo. Although many studies have highlighted the importance of regulatory B cells
antibodies (83, 84), the results shown here have demonstrated that B10 cell effects on the immune system are dependent upon IL-10 and as yet, no other effector molecules. Studies using antibody-deficient mice may help elucidate the contributions of B10 cell-derived antibodies.

Remaining questions surrounding the antigen-specific effector of B10 cells are largely related to other disease models in which B10 cells are known to play relevant roles. Do B10 cells also act in an antigen-specific manner during suppression of autoimmune disease? Additionally, how do antigen-specific B10 cell populations change during the course of diseases known to have significant epitope spreading, such as EAE or CIA? Defining the full extent of the antigen-specific B10 cell regulation network will provide valuable clues as to how B10 cells can be exploited for the treatment of human disease.
References


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Biography

I was born in Abington, Pennsylvania in 1985. I graduated from Juniata College in 2007 with a Bachelor’s of Science in Biology and completed an Intramural Research Training Award Fellowship at the National Institutes of Health from 2007-2009 prior to enrolling in the Graduate School of Arts and Sciences at Duke University. I have co-authored the following peer-reviewed articles:


4. Maseda, D., Smith, S.H., DiLillo, D.J., Bryant, J.M., Candando, K.M., Weaver, C.T., and


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