

Review

Multiple Mechanisms of Immune Suppression by B Lymphocytes

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Abstract

Suppression of the immune system following the resolution of infection or inflammation is an important process that limits immune-mediated pathogenesis and autoimmunity. Several mechanisms of immune suppression have received a great deal of attention in the past three decades. These include mechanisms related to suppressive cytokines, IL-10 and TGF β , produced by regulatory cells, and mechanisms related to apoptosis mediated by death ligands, FasL and TRAIL, expressed by killer or cytotoxic cells. Despite many lines of evidence supporting an important role for B lymphocytes as both regulatory and killer cells in many inflammatory settings, relatively little attention has been given to understanding the biology of these cells, their relative importance, or their usefulness as therapeutic targets. This review is intended to give an overview of the major mechanisms of immunosuppression employed by B lymphocytes in the course of both normal and inflammatory contexts. The more recent discoveries of expression of granzyme B, PD-L2, and regulatory antibody production by B cells as well as the interactions of regulatory and killer B cells with regulatory T cells, NKT cells, and other cell populations are discussed. In addition, new evidence based on independent characterizations of regulatory and killer CD5⁺ B cells point toward the concept of a multi-potent suppressor B cell with seemingly high therapeutic potential.

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Introduction

The immune system contains a vast array of cell types and effector molecules specialized to detect and destroy pathogenic microorganisms and the cells and tissues that harbor them. With this highly toxic protective function comes the need for tight regulation so that once the danger of infection has passed, the system can return to relative calm and further damage can be avoided. This regulation is mediated by subsets of cells and immunosuppressive molecules that are specialized to actively suppress the immune response.(1-7) Ideally, immune suppression balances the destructive forces of inflammation while allowing clearance of pathogenic infectious agents [Fig. 1A].(8) Failures of immune suppression may manifest themselves clinically in the form of severe acute inflammation and cytokine storms that if improperly controlled ultimately result in death [Fig. 1B].(9) In other cases, poor immune regulation may contribute to chronic or aberrant inflammation including allergies, asthma and autoimmune diseases.(10, 11) Reciprocally, over suppression of the immune system may lead to poor clearance of pathogens leading to persistent infections [Fig. 1C],(12) or ineffective tumor surveillance leading to cancer.(13) Many pathogenic strains of viruses and bacteria have adopted immunosuppressive mechanisms to circumvent detection and destruction by the immune system.(14) This delicate balance between inflammation and immune suppression is dependent on all of the components of the immune response working in concert and requires complex mechanisms of communication between all of the effector and regulatory cell populations.(15) Therefore, a thorough understanding of the specialized processes and cell types that control immune suppression is essential to our understanding of many human diseases and in developing effective therapeutic approaches.

Immune suppression can be classified in two broad categories: passive and active suppression. Passive immune suppression is generally a result of the removal of immune stimuli. This can come in the form of clearance of the pathogenic microorganism, sequestration of antigens, or

decreased antigen presentation resulting in decreased production of proinflammatory cytokines and reduced inflammation. In contrast, active immune suppression is mediated by specialized cells and their molecular products that direct the effector cells of the immune system to either decrease their activity or undergo programmed cell death [apoptosis]. Active immune suppression can occur despite the presence of antigens and its mediators are often detectable during acute or chronic inflammatory processes. Cooperation between passive and active immune suppressive mechanisms is critical to controlling inflammation and maintaining homeostasis.

Immune suppression occurs through multiple mechanisms and is mediated by a wide variety of cell populations. While the antibody-producing functions of B cells have been intensely studied, their immunosuppressive functions have received relatively less attention in comparison to those of other regulatory cell populations. Thus, despite sporadic reports of B cell-mediated immune suppression over the last three decades, the regulatory functions of B cells have only been rigorously examined in the last ten years.(16-19) As will be discussed further below, most of the immune suppressive mechanisms previously attributed to regulatory T cells and cytotoxic cells have also been demonstrated in B cells [Fig. 2]. Some immunosuppressive mechanisms are unique to B cells, such as the production of regulatory antibodies that contribute to immune regulation. In this article we aim to survey the demonstrated mechanisms of immunosuppression employed by B cells and discuss the importance of these mechanisms in normal immune responses and in maintaining immunological tolerance. Taken together, the reviewed studies demonstrate that B cells are critical for the effective regulation of the immune system and employ a wide array of immunosuppressive mechanisms. We will also discuss which B cell subsets are known to utilize these suppressive mechanisms, and present the evidence for the existence of a multi-potent suppressor B cell population.

B Cell Secretion of Interleukin-10

The most widely-studied and best understood immunosuppressive mechanism of B lymphocytes is the secretion of IL-10,(1) which is one of the most important anti-inflammatory cytokines regulating inflammation.(8) While IL-10 has multifaceted effects on nearly all hematopoietic cells, its most important roles appear to be in the direct regulation of myeloid-derived antigen presenting cells and T lymphocytes.(20) In antigen-presenting cells, IL-10 inhibits the secretion of pro-inflammatory cytokines such as $\text{TNF}\alpha$ and $\text{IL-1}\beta$ and reduces surface expression of the antigen presentation machinery [MHC Class II, costimulatory molecules, adhesion molecules].(21-25) IL-10 directly affects differentiated CD4^+ T cells as demonstrated in studies in which both $\text{T}_{\text{H}1}$ and $\text{T}_{\text{H}2}$ cells exhibited reduced proliferation and cytokine secretion in the presence of IL-10.(26, 27) It should be noted that both $\text{T}_{\text{H}17}$ and CD8^+ cytotoxic T cells appear to be relatively unaffected by IL-10, suggesting that other anti-inflammatory mediators are necessary for the regulation of these T cell subsets.(28)

Although B cells had been shown previously to express IL-10 under various conditions, the importance of B cell-derived IL-10 in immunosuppression was not definitively demonstrated until the last decade.(1, 18, 29, 30) In 2002, Mizoguchi and colleagues identified a subset of IL-10-producing B cells in the gut-associated lymphoid tissue [GALT] that up-regulated the MHC Class I-like molecule CD1d over the course of chronic intestinal inflammation. These CD1d-expressing B cells mediated IL-10-dependent suppression of intestinal inflammation in recipient animals upon transfer.(30) In the same year, Fillatreau and colleagues showed a role for IL-10-producing splenic B cells in autoimmunity.(29) In these experiments, the authors showed that splenic B cells could produce IL-10 in response to stimulation through the BCR and CD40, and that B cell-specific IL-10 production correlated with recovery in a mouse model of multiple sclerosis. Importantly, they found that IL-10-competent B cells could reduce disease severity upon adoptive transfer, but B cells from $\text{IL-10}^{-/-}$ mice were unable to do so. In the intervening

years, others have demonstrated that B cell-derived IL-10 regulates autoimmunity in various other mouse models of autoimmune conditions including chronic intestinal inflammation,(30) collagen-induced arthritis,(31) type 1 diabetes,(32) and systemic lupus erythematosus [SLE].(33, 34)

A great deal of attention has been paid to the factors that generate or expand IL-10-producing B cells. Stimuli known to increase the number of IL-10-producing B cells *in vitro* include signaling through TLRs,(35) the BCR,(36) CD40,(37) or combinations thereof. Recently it has been shown that the TNF-family cytokine, B cell-activating factor [BAFF, BlyS], increased the frequency of IL-10-producing B cells *in vitro* and *in vivo*, and BAFF-treated B cells were able to suppress inflammation upon adoptive transfer.(38) Genetic studies have revealed that splenic IL-10-producing B cells are found at nearly normal levels in mice deficient in T cells, CD21, MHC Class I and Class II, and CD40.(30, 35) In contrast, loss of CD1d, CD19, or MyD88 expression essentially eliminates IL-10-producing B cells, suggesting these molecules are required for IL-10-producing B cell development or function.(30, 35) Mice that express higher levels of CD19, ectopically express CD40L in B cells, or are deficient in CD22 or NFATc1 have greatly increased numbers of IL-10-producing B cells.(35, 39) BCR-mediated Ca²⁺ flux appears to be required for IL-10 production in B cells, as B cells deficient in the calcium sensors STIM1 and STIM2 have a profound defect in IL-10 secretion and abrogated suppression abilities *in vivo*.(40) Remarkably, antibody responses and B cell development are largely intact in these animals, suggesting that BCR-mediated calcium flux is crucially important for B cell-mediated immune suppression but is dispensable for effector functions. Mice prone to spontaneous autoimmunity [NZB/W, NOD, and MRL/lpr mice] have increased numbers of IL-10-producing B cells relative to normal strains, whereas mice susceptible to induced autoimmunity [DBA/1, SJL] have lower basal numbers of IL-10-producing cells.(35) The mechanisms and consequences of these differences in IL-10-producing B cell frequency have yet to be elucidated.

Splenic IL-10-producing B cells were initially reported to have a surface phenotype consistent with transitional 2-marginal zone precursor [T2-MZP] B cells, namely high expression of CD21, CD23, and IgM.(41) Additionally, transfer of T2-MZP B cells reduced the severity of collagen-induced arthritis in recipients to a greater extent than other B cell subsets, suggesting the presence of regulatory B cells within this subset. Later, a portion of splenic IL-10-producing B cells were shown to co-express CD5 and CD1d in addition to the previously described T2-MZP B cell markers, and CD19⁺CD5⁺CD1d^{high} B cells were found to have IL-10-dependent immunosuppressive properties upon transfer into recipient animals.(36) Using a contact hypersensitivity model, the authors showed that the suppression mediated by CD5⁺CD1d^{high} B cells was antigen specific, as CD5⁺CD1d^{high} B cells from naïve animals or those sensitized with an irrelevant antigen did not suppress the immune response to subsequent antigenic challenge. More recently, the protein TIM-1 (T cell Ig domain and mucin domain protein 1) was reported to identify 70% of IL-10-producing B cells, making this marker the most specific yet identified for IL-10-producing B cells.(42) Treatment of mice with an anti-TIM-1 antibody led to an increase in IL-10-producing TIM-1⁺ B cells and improved tolerance of an allogeneic tissue graft, suggesting that TIM-1 is of functional significance for IL-10-producing B cells rather than simply an identification marker.(42) These results suggest that targeting TIM-1 signaling may provide a novel therapeutic strategy for increasing the number of IL-10-producing B cells in transplant recipients or patients with autoimmunity. It should be noted that the same anti-TIM-1 antibody that mediated tolerance in wild type mice actually hastened allograft rejection in B cell-deficient mice, suggesting that TIM-1 signaling has different effects in non-B cells.

Much controversy still exists regarding the relationship of IL-10-producing B cells to known B cell subsets. While IL-10-producing B cells are enriched in the T2-MZP and CD5⁺CD1d^{high} subsets, not all cells in these subsets express IL-10, suggesting that there is not an absolute correspondence between these B cell subsets and IL-10-producing B cells. The fact that many

IL-10-producing B cells express CD5 suggests that they may also be related to the peritoneal B-1a cell population, which is in accord with the known ability of B-1a cells to secrete large amounts of IL-10.(18) Additionally, splenic IL-10-producing B cells are present at much higher frequencies in neonates than in older mice as is also true of B-1a cells.(35) Although it has been suggested that IL-10-producing B cells represent a distinct lineage of B cell [termed “B10” cells],(43) more study will be required to demonstrate that IL-10-producing B cells are indeed distinct from T2-MZP B and B-1a cells. Finally, while the CD5⁺CD1d^{high} B cell population is enriched for IL-10-producing B cells, only ~25% of IL-10-producing B cells are found in this population. Indeed, IL-10-producing B cells can be found among most major subsets of splenic B cells, albeit at smaller frequencies than that seen among CD5⁺CD1d^{high} B cells.(42) Given that no set of surface markers identifies all IL-10-producing B cells, it is also possible that IL-10 production occurs in several B cell subsets depending upon differentiation or activation state.

While murine IL-10-producing B cells have been well-studied, only recently has evidence for an orthologous human cell emerged. Blair and colleagues compared the capacity of different peripheral blood B cell subsets to suppress T cell cytokine production *in vitro*, and found that the CD24^{high}CD38^{high} subset inhibited IFN γ and TNF α production in autologous T cells.(44) This B cell subset was found to be enriched for IL-10-producing B cells, and blockade of IL-10 largely abrogated T cell suppression mediated by these B cells. The authors also compared the suppressive effects of CD24^{high}CD38^{high} B cells from SLE patients to those of healthy controls, and found that those cells derived from SLE patients had both a reduced ability to produce IL-10 and reduced suppression of T cell cytokine production, although it is not clear if this defect is a cause or a consequence of SLE.(44) In work from another group, human IL-10-producing B cells were found to suppress monocytes in an IL-10-dependent manner, and were present in greater numbers in patients with autoimmune diseases.(45) In this study, IL-10-producing B cells were identified as CD24^{hi}CD27⁺ B cells, a phenotype similar to that recently reported for

human B-1 cells.(46) As in mice, the relationship of IL-10-producing B cells to other B cell subsets is still controversial, and further work will be needed to clarify the identity of IL-10-producing human B cells.

While it is clear from these studies that IL-10 plays a major role in B cell-mediated immune suppression, it is not currently known if B cell-derived IL-10 acts exclusively on target cells or if the loss of IL-10 in B cells also leads to defects in other immunosuppressive functions. This is an important area of study, as IL-10 is a known growth factor for B cells and other suppressive mechanisms employed by B cells may work in synergy with IL-10.(47) Further studies are required to fully understand the role B cell-derived IL-10 has in immunosuppression and autoimmunity.

Granzyme B Expression by B Cells

Granzyme B is an enzymatic component of the cytotoxic granules produced by CTL and NK cells that mediates cleavage of caspases and initiation of apoptosis in virus-infected cells.(48) B lymphocyte-associated granzyme B expression was first detected in a panel of human B-chronic lymphocytic leukemia cells that underwent apoptosis following stimulation with the TLR9 ligand, CpG-ODN, and the cytokine, IL-21.(49) The same group also demonstrated IL-21-inducible expression of granzyme B by EBV-transformed and non-transformed human B cells, as well as B cells isolated from patients with SLE, psoriasis and rheumatoid arthritis.(49-51) In SLE patients, the predominant B cell population that produced granzyme B was the CD5⁺ B cell subset, and treatment of umbilical cord blood-derived B cells with IL-21 led to *de novo* granzyme B expression by CD5⁺ B cells.(51) While these studies clearly show that B cells can express functional granzyme B, the *in vivo* significance of this phenomenon is still unknown.

FasL and TRAIL Expression and Killer B Cell Function

A major mechanism for maintaining peripheral tolerance, establishing sites of immune privilege, and controlling the duration and magnitude of immune responses is the expression of death-inducing ligands by lymphoid and non-lymphoid cells. The best characterized death-inducing ligands in mice and humans are Fas ligand [FasL, CD178](2) and TRAIL [CD253],(52) molecules which activate programmed cell death in target cells through ligation of cell surface death receptors. Defective expression of FasL or its receptor in mice results in increased lymphocyte proliferation, splenomegaly, and high production of self-reactive antibodies.(53) In humans, autoimmune lymphoproliferative syndrome [ALPS] has been linked to defective Fas-mediated apoptosis.(54) TRAIL has also been identified as an important regulator of autoimmunity in mice.(55)

B lymphocyte expression of FasL was first demonstrated in human peripheral blood B cells following activation by the mitogens LPS or PMA and ionomycin.(56) Since this report, several groups have demonstrated expression of FasL by transformed and non-transformed B cells from humans and mice.(57) Expression of FasL by malignant human B cells, most notably in multiple myeloma, diffuse large B cell lymphoma, and B-cell chronic lymphocytic leukemia, has been associated with induction of apoptosis of T cells and other cells and increased tumor pathogenesis.(58-61) FasL-expressing B cells were also detected following several types of viral infections including Epstein-Barr virus,(62) HIV (63) and murine leukemia virus,(64) where it was proposed that death ligand expression led to T cell apoptosis and potentially the persistence of the infections.

Our studies of FasL expression by B lymphocytes began with the observation that FasL was highly expressed by CD19⁺ splenocytes in schistosome-infected mice.(65) In the schistosome infection model, CD19⁺ B cells were the predominant FasL⁺ cell type in the spleen and depletion

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of CD19⁺ cells led to decreased CD4⁺ T cell apoptosis in response to antigenic stimulation. The number of FasL⁺ CD19⁺ 'killer' B cells increased during infection following a time course that closely matched the increase in granulomatous inflammation around schistosome eggs in the liver. In a subsequent study, it was found that the splenic CD5⁺ B cell subset expressed higher levels of FasL than CD5-negative B cells at every time point tested during schistosome infection.(66) Perhaps the most important observations from this latter study were that: 1) splenic CD5⁺ B cells from uninfected mice expressed FasL; 2) that purified CD5⁺ B cells from infected and uninfected mice were potent killers of CD4⁺ T cells in the presence of antigen; and 3) that FasL expression could be enhanced on CD5⁺ B cells by addition of schistosome antigens, IL-4 and IL-10 to cell cultures.(66) These data led to the hypothesis that splenic CD5⁺ B cells are a naturally-occurring and inducible killer cell population with potent apoptosis-inducing capabilities against antigen-specific CD4⁺ T cells.(57)

Additional studies have shown that FasL⁺ B cells may play a role in autoimmune diseases and induction of T cell tolerance. Activated B cells that expressed FasL and TGFβ were able to suppress the onset of autoimmune diabetes in NOD mice.(67) FasL⁺ B cells with cytotoxic activity against T cells were elevated in the lupus susceptible MRL/lpr (Fas-deficient) strain of mice.(68) An inverse correlation between the number of splenic FasL⁺ CD5⁺ B cells and severity of collagen-induced arthritis was also demonstrated.(69) FasL expression increased on lung CD5⁺ B cells during chronic exposure to cockroach antigens in an asthma model, and lung T cell apoptosis was reduced in CD5⁺ B cell deficient Xid mice during asthma.(70) The power of FasL⁺ killer B cells to mediate immune tolerance was best demonstrated in a male-to-female skin graft model in which purified splenic B cells transferred from control male mice were able to tolerize female graft recipients, but purified B cells from FasL-defective [gld/gld] mice could not tolerize.(71) Thus, FasL⁺ killer B cells have been demonstrated in many disease situations as well as in healthy individuals, and they have been shown to have therapeutic potential.

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In comparison to what is known about FasL expression and its role in immune suppression and tolerance, the role of TRAIL is less well characterized. The importance of TRAIL has mainly been studied in the context of cancer apoptosis, and initial studies in TRAIL-deficient mice did not indicate an altered immune phenotype.(72, 73) However, a recent study in TRAIL and FasL double-deficient mice indicated that FasL and TRAIL may have somewhat redundant yet cooperative roles in regulating immune homeostasis.(74) TRAIL/FasL double-deficient mice had significantly increased splenomegaly, lymphadenopathy, and autoantibody production compared to FasL-defective mice. TRAIL/FasL double deficiency also resulted in decreased breeding capacity and increased early mortality indicating the importance of these molecules in maternal/fetal immune tolerance and regulation of inflammation. TRAIL expression by B lymphocytes was first reported on human and mouse B lymphoma cell lines.(75) TRAIL has also been detected on B-chronic lymphocytic leukemia cells and multiple myeloma cells, as well as on non-transformed mouse and human B cells.(76-78) Primary human B cells could be induced to express TRAIL upon treatment with TLR9 ligand or with the cytokine IFN α .(79) The unique role of TRAIL-expressing B cells as compared to FasL-expressing B cells to immune suppression, if one exists, has yet to be determined.

PD-L1 and PD-L2 Expressing B Lymphocytes

The programmed death-1 [PD-1] receptor has been shown to be a critical regulator of immune activation and maintenance of peripheral tolerance.(6) PD-1 expression by T and B cells increases following stimulation and was first detected in T cells undergoing activation-induced cell death.(80, 81) Although the name “programmed death-1” implies that this molecule is directly involved in cell death, its immune suppressive functions are not thought to depend on induction of apoptosis.(82) Two ligands for PD-1, PD-L1 and PD-L2, have been identified that have distinct patterns of cell expression and unique roles in immune suppression.(83-85) PD-L1 is constitutively expressed by T and B lymphocytes, dendritic cells and monocytes, and its

expression is induced by ligation of cell surface receptors and/or stimulation with the T_H1 -associated cytokine IFN γ .(83) In contrast, expression of PD-L2 was originally described as being restricted to activated dendritic cells and monocytes.(86, 87) PD-L2 expression in these cells was inducible by treatment with the T_H2 -associated cytokines IL-4 and IL-13.(84) PD-L1 plays a dominant role in regulating T_H1 -mediated immune responses, while PD-L2 appears to be more important in regulating mucosal and T_H2 -associated responses such as oral tolerance and asthma.(88, 89)

In 2007, Zhong and colleagues first described the expression of PD-L2 on 50-70% of mouse peritoneal CD5⁺ B cells.(90) This subset, which the authors named L2pB1 cells, was enriched for cells that produce anti-phosphatidylcholine antibodies, a specificity that has also been associated with binding to self antigens. These L2pB1 cells are also major producers of anti-double stranded DNA binding antibodies and are more abundant in lupus-prone BXSB mice than in BALB/c control mice.(91) Although the latter study demonstrated that L2pB1 cells were potent APC that favored the activation of IL-17-producing T_H17 cells, the mechanisms behind this ability were not dependent on PD-L2 expression. The immunosuppressive capacity of PD-L2 expressed by B cells has not been directly demonstrated and requires further study.

Inhibitory Effects of Sialylated IgG

Over the course of a productive immune response, high-affinity IgG antibodies are developed that recognize epitopes on the invading pathogen or its toxic products. The Fc region of IgG binds to a family of Fc γ receptors expressed on cells of both the innate and adaptive immune system altering their phagocytic activity and responses to other stimuli.(5) Both activating and inhibitory receptors exist within the Fc γ receptor family, and their relative levels of expression determine the activation threshold for a given cell. The production of IgG is vital for pathogen

clearance, as individuals with hypogammaglobulinemia are extremely susceptible to infection. These patients are often treated with intravenous injection of polyclonal IgG pooled from thousands of blood donors, a method known as IVIG therapy. Despite its clear role in pathogen clearance, it has been known for decades that very high doses of IVIG have an anti-inflammatory effect, and high-dose IVIG is now commonly administered to patients with the autoimmune disease idiopathic thrombocytopenic purpura.(92, 93) As the anti-inflammatory function of IVIG is only evident at doses much higher than that needed for passive IVIG antibody replacement therapy, it was hypothesized that only a minor fraction of IgG molecules possessed anti-inflammatory activity.

The Fc portion of IgG contains a single biantennary N-linked glycan required for the binding IgG to its cognate Fc γ receptors. While all IgG molecules appear to have a core heptasaccharide moiety comprised of N-acetylglucosamine (GlcNAc) and mannose at this site, more than 30 alternatively-modified glycoforms have been identified on naturally-occurring IgG molecules.(94) Given that changes in this glycan had been reported to affect IgG receptor binding and cytotoxicity, it was hypothesized that the immunosuppressive IgG could be distinguished from pro-inflammatory IgG as a unique glycoform. Indeed, IVIG fractions enriched for a glycoform of IgG possessing sialic acid terminal additions displayed an anti-inflammatory activity at much reduced doses relative to un-fractionated IVIG in mouse models.(95) Conversely, the removal of sialic acid residues from IVIG preparations severely abrogated immunosuppressive function.

The mechanism through which sialylated IgG suppresses inflammation involves a novel innate immunosuppressive pathway utilizing T_H2-related cytokines. The addition of sialic acids to the IgG glycan alters its receptor-binding profile, severely reducing its affinity for classical Fc γ receptors while increasing its ability to bind to an alternate C-type lectin receptor, SIGN-R1.(96) Macrophages and dendritic cells from mice expressing the human orthologue of SIGN-R1 (hDC-SIGN) are thought to respond to sialylated IgG by secreting Interleukin-33 (IL-33), which in turn

increases the number of circulating IL-4-producing basophils.(97) IL-4 produced by these activated basophils then acts on effector macrophages in the periphery, causing them to up-regulate the inhibitory Fc γ RIIB receptor thus increasing the activation threshold of these cells and down-regulating their pro-inflammatory capacity.(98) In accord with this model, IVIG treatment shows little benefit in mice lacking SIGN-R1, Fc γ RIIB, or IL-4, and mice treated with exogenous IL-33 have increased levels of IL-4-producing basophils and reduced susceptibility to antibody-mediated arthritis.(96, 97) Additionally, IVIG treatment is ineffective in mice depleted of basophils, strongly suggesting a central role for these cells in IVIG-mediated immunosuppression.(97)

IgG sialylation status may prove to be an important factor in the pathogenesis or disease course of autoimmunity. Rheumatoid arthritis is associated with a decrease in galactosylated and sialylated serum IgG.(94) It has been shown that autoantigen-specific serum IgG displays reduced sialylation, and this reduction was even more pronounced at the site of active inflammation.(99) Remarkably, little is known about what factors act on B cells to influence the glycosylation status of secreted antibodies. It appears that an active inflammatory response reduces the relative amount of sialylated IgG, although no mechanism for this reduction is currently known.(95) A recent study characterized the glycan profile of IgG secreted from human primary B cells in response to various pro-inflammatory cytokines and other stimuli (100). Somewhat surprisingly, it was found that many stimuli tested [including IL-4, IL-6, IL-17, TNF α , TGF β , and LT α] had no measurable effect on the sialylation status of secreted IgG. Even more intriguingly, those pro-inflammatory stimuli that did have an effect [CpG ODN, IL-21, IFN γ , and all-trans retinoic acid] actually increased levels of sialylation and galactosylation rather than decreasing them. As this is only a single *in vitro* study, and the B cells in question were stimulated with other factors in addition to the experimental conditions, further study will be

required to better understand what causes B cells to change the sialylation status of secreted IgG *in vivo*.

While the identity of the B cells that secrete sialylated IgG is currently not known, marginal zone B [MZB] cells may play a role in regulating the expression of SIGN-R1 on macrophages in the marginal zone.(101) In mice genetically lacking MZB cells, macrophages in the marginal zone do not express SIGN-R1. In addition, loss of SIGN-R1⁺ macrophages occurs transiently when B cells are stimulated to exit the marginal zone.

Regulatory Natural Antibodies

Both humans and mice are born with a significant level of circulating IgM antibodies generated by neonatal B cells. As these antibodies are present in mice raised in germ-free conditions, they are often referred to as “natural” or “non-immune” antibodies. Natural antibodies possess a limited and recurring repertoire of specificities and are generated by B-1 cells, a subset of B cells found mostly in the peritoneal and pleural cavities but also in smaller quantities in the spleen.(102) In contrast to B-2 cells, B-1 cells exist as a self-renewing pool with little addition from bone marrow precursors after infancy. Given their limited repertoire and ability to respond to T cell-independent signals, they are thought to represent a primitive “innate-like” lymphocyte population. IgM antibodies made by B-1 cells are unique in that they display lower affinity interactions with their cognate antigen and display more cross-reactivity than antibodies derived from B-2 cells.

Natural antibodies are thought to play a role in the elimination of apoptotic cells, a key function of the immune system.(103) Under steady state, phagocytic cells such as macrophages and dendritic cells efficiently clear apoptotic cells with little induction of inflammation in a process known as efferocytosis. If this process is inhibited or overwhelmed, however, apoptotic cells are not removed before their plasma membrane loses integrity, resulting in the release of self-

antigens and inflammatory signals into the interstitial space. This in turn can lead to increased tissue destruction and induction or exacerbation of autoimmunity.

The surface phenotype of cells undergoing apoptosis is altered from that of healthy cells by the presence of apoptosis-specific neoepitopes, sometimes referred to as “eat-me” signals.(104) These apoptotic surface determinants bind to surface receptors on phagocytic cells and allow for the selective identification and elimination of apoptotic cells. Among these neoepitopes is an alteration in the lipid profile of the outer leaflet of the plasma membrane, such as an enrichment of lipids containing phosphorylcholine [PC] or malondialdehyde [MDA]. The antibody repertoire of B-1 cells is highly enriched for antibodies which bind these apoptosis-induced determinants.

Natural antibodies appear to have a potent immunosuppressive function both *in vitro* and *in vivo*. Upon binding to the surface of apoptotic cells, natural antibodies recruit the complement components C1q and mannose-binding lectin.(105) The deposition of these molecules makes the apoptotic cell more appetizing to macrophages and dendritic cells resulting in the increased phagocytosis of opsonized apoptotic cells. In addition to increasing the rate of phagocytosis, natural antibody-coated apoptotic cells negatively regulate the inflammatory capacity of APC in a variety of ways. Notably, the addition of natural antibodies to cultured dendritic cells inhibits their maturation, blunts their response to a variety of TLR ligands, and reduces their expression of molecules involved in antigen presentation. Similar effects were seen in APC from animals receiving a model monoclonal natural antibody, T15.(106) The administration of T15 has a general immunosuppressive effect, as mice receiving the antibody were protected from both collagen-induced arthritis and a passive transfer model of arthritis induced by anti-collagen IgG antibodies.

In addition to apoptotic cells, the lipid epitopes bound by natural antibodies are also found on the surface of bacteria such as *S. pneumoniae* (104) and oxidized LDL deposits found in

atherosclerotic lesions. In mouse models of atherosclerosis, natural antibodies reduce the severity of disease, and immunization of atherosclerosis-susceptible mice with *S. pneumonia*, which increases the levels of anti-PC natural antibodies, leads to a decrease in atherosclerotic lesion formation.(107) These results suggest that increasing the level of natural antibodies, either by immunization or other means, could be therapeutically beneficial in patients with atherosclerosis.

The production of natural antibodies is critically dependent upon the presence of B-1 cells and occurs independently of T cells. Circulating levels of natural antibodies are increased in mice that have received either large doses of apoptotic cells or have been immunized with pneumococcal extracts, suggesting that the presence of cognate antigen determines the level of natural antibody secretion. In some cases natural antibody responses to different antigens appear to be coordinately regulated, as immunization with one B-1 cell-specific antigen, MDA-LDL, resulted in an increase in natural antibodies recognizing another B-1 cell-specific antigen, PC.(104) Finally, T cell responses in mice immunized with natural antibody-binding antigens are dominated by the T_H2-type response even if the antigen is administered with T_H1-skewing adjuvant, suggesting that the selective expansion of T_H2 cells may be another regulatory mechanism of natural antibodies.(108)

B Cell Interactions With Regulatory T Cell Populations

Although B cells can directly suppress immune responses, there is also strong evidence that part of their immunosuppressive function is mediated by interactions with other regulatory cell populations. B cells constitutively express both MHC Class I and Class II molecules, making them capable of antigen-specific interactions with CD8⁺ and CD4⁺ T cells, respectively. In addition, regulatory B cells may have direct interactions with NKT cells through surface expression of CD1d molecules.

Most studies of regulatory T cells [Treg] have focused on CD4⁺ T cells that exert immune suppressive functions mainly through production of IL-10 and/or TGFβ, or through direct interactions with antigen presenting cells.(109) Although the importance of Treg cells in immune regulation and their potential clinical relevance is well-supported and has been reviewed extensively, the relative importance of B cells as antigen presenting cells for Treg differentiation and activation has only recently become a focus of research. An early study demonstrated that both CD8⁺ and CD4⁺ T cells with immunosuppressive properties were generated by co-culture with purified B cells derived from antigen-tolerized mice in a corneal transplant model.(110) Subsequently, the accumulation of Treg cells in the central nervous system in a murine model of multiple sclerosis was shown to be dependent on B cells, but the direct regulatory properties of these B cells were not determined.(111) However, a recent study demonstrated that IL-10 production by regulatory B cells directly affected the accumulation of Treg cells and suppression of inflammation in an arthritis model.(112) Arthritic mice with IL-10^{-/-} B cells had decreased Treg cells, increased T_H1 and T_H17 cells, and increased joint inflammation, all of which were reversed by adoptive transfer of IL-10-producing B cells.

B cells have also been shown to be critical to the expansion of CD4⁺FoxP3⁺ Treg cells at mucosal sites. Sublingual antigen challenge, a strategy for inducing oral tolerance, was partially impaired in B cell- deficient mice through a mechanism that was dependent on induction of FoxP3⁺ Treg cells.(113) In a separate study, repeated nasal antigenic challenge of mice induced a regulatory B cell population in the local draining lymph node that suppressed lung inflammation and asthma in recipient OVA-sensitized mice following adoptive transfer.(114) The effectiveness of these regulatory B cells was linked to B cell expression of TGFβ but not IL-10, and the accumulation of FoxP3⁺ Treg cells in the lung mucosa and local lymph nodes. Similarly, B cells isolated from the spleens of mice infected with schistosome worms were able to induce lung accumulation of FoxP3⁺ Treg cells, and to suppress allergic asthma following adoptive

transfer.(115) In contrast to the previous study, IL-10 rather than TGF β was shown to have a dominant effect in the function of regulatory B cells from schistosome-infected mice.(114, 115) These discrepancies may have been the result of the different types of stimuli, the different anatomical sources of the regulatory B cells, or possibly because the B cells represented separate subsets. The CD5⁺ and CD5-negative B cell subsets have been shown to differ in their capacity to drive differentiation of CD4⁺ T cells toward effector or regulatory phenotypes.(116) Whether specific cell-to-cell molecular interactions are critically involved in the crosstalk between B cells and subsets of regulatory T cells has yet to be determined.

The ability of B cells to drive the differentiation of Treg cells has sparked some interest in the manipulation of this interaction as a therapeutic strategy. Experiments that induced over-expression of the type 1 diabetes-associated autoantigens GAD65 or insulin specifically in B lymphocytes resulted in immune tolerance that was attributed to an increase in Treg differentiation.(117) Similarly, retroviral transduction of B cells with a construct containing the allergen DerP2 followed by adoptive transfer of the transduced B cells into allergen-sensitized mice resulted in immune tolerance and expansion of a subset of Treg cells.(118) A series of studies using B cells that were not genetically modified in a mixed lymphocyte reaction demonstrated that both CD4⁺ and CD8⁺ alloantigen-specific Treg cells with suppressive abilities can be generated by MHC mismatched B cells.(119, 120)

A unique feature that has been described for IL-10-producing B cells compared to other B cell subsets is high expression of the CD1d cell surface molecule.(30, 36, 41) CD1d is required for the presentation of glycolipid and phospholipid antigens to natural killer T (NKT) cells, a subset of NK1.1⁺ and TCR positive cells that was originally shown to have immune suppressive function in a mouse model of SLE.(121) Immunosuppressive NKT cells have also been described in mouse models of autoimmune diabetes,(122) arthritis(123) and multiple sclerosis.(124, 125) A critically important role for CD1d^{hi} expressing B cells in promoting

immune tolerance through activation of NKT cells has been demonstrated in several mouse models.(115, 126, 127) However, NKT cells have been shown to have immune stimulatory functions as well, and it is now clear that the function of NKT cells varies depending on how they are activated.(85, 128) We have recently determined that FasL⁺ CD5⁺ killer B cells also have relatively high expression of CD1d (manuscript in preparation), suggesting that these cells can interact with NKT cells as well. Further study is required to determine what role B cell-derived FasL and apoptosis play in regulating NKT cell functions. Reciprocally, it is also possible that NKT cells play a significant role in the generation and/or maintenance of FasL⁺ killer B cells.

Resistance to Apoptosis Among T Cell Subsets

In circumstances where immune suppression is mediated by death-ligand expressing cells, the relative susceptibility of T cell subsets to death ligand-induced apoptosis can result in a skewing of the response toward the apoptosis-resistant cell type.(129, 130) Response to death ligands is dependent on the activation status of the target cell and expression of cell intrinsic anti- and pro-apoptotic molecules such as cellular FLIP and members of the Bcl family.(131, 132) While most activated T cells are susceptible to death ligand-mediated apoptosis and eliminated following an immune response, a small percentage of CD8⁺ and CD4⁺ T cells resist apoptosis and can persist in the antigen-specific memory pool.(133, 134) Many studies have attempted to determine whether T cell resistance to apoptosis is related to cytokine expression patterns. Comparisons between T_H1 and T_H2 cell types have mostly concluded that T_H1 cells are more susceptible than T_H2 cells to Fas-mediated apoptosis, however one group determined that there was no difference.(129, 135-137) These results may be partially explained by the presence of T_H1 and T_H2-type cytokines in the cultures, since these have been reported to have pro- and anti-apoptotic effects, respectively.(138, 139) Another factor that regulates the susceptibility of T cells to death receptor signals is costimulation, particularly through the CD28 molecule.(140, 141)

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The identification and functional characterization of other important subsets of T cells, namely Treg, T_H17 and NKT cells has renewed interest in susceptibility to apoptosis as a mechanism of skewing of the immune response. Mouse Treg cells have been shown to be highly resistant to FasL-mediated apoptosis, while freshly isolated human Treg cells were susceptible but became resistant upon stimulation.(142-145) The data on T_H17 cell resistance to apoptosis is relatively sparse. One study in which hen-egg lysozyme [HEL] was specifically expressed in the anterior chamber of the eye, followed by adoptive transfer of HEL-specific T cells, clearly showed that T_H17 cells are more resistant than T_H1 cells to apoptosis.(146) The eye is a site of immune privilege that is known to be protected by the local expression of Fas ligand and TRAIL along with other immune suppressive mediators. Interestingly, ocular tolerance has previously been shown to be dependent on the presence of splenic B cells, although the mechanism has been attributed to induction of Treg cells rather than linked directly to B cell-mediated apoptosis.(147) The generation of Treg cells in ocular immune privilege is also under the control of IL-10-expressing NKT cells, which as has been discussed above may be under the control of CD1d^{hi} expressing regulatory and/or killer B lymphocytes.(126) Although NKT cells have been shown to be sensitive to Fas-mediated apoptosis *in vitro* and *in vivo*, several lines of evidence have also suggested that their susceptibility may be limited by down-modulation of their TCR following activation.(148, 149) Taken together, these data suggest that T cell subsets and even individual T cells may differ in their sensitivity to apoptosis based on their differentiation, activation status, and microenvironment. B lymphocytes which: 1) act directly as APC to T cells and NKT cells; 2) provide various forms of costimulation, cytokines and chemokines; and 3) simultaneously express death ligands, may play an important role in providing these contexts, and are likely to favor some T cell subsets while eliminating or suppressing others.

Are There Multi-Potent Suppressor B Cells?

The studies we have reviewed above clearly demonstrate that individual B cells employ a variety of anti-inflammatory mechanisms and that B cell-mediated immunosuppression plays a critical role in many normal and disease contexts. A question that remains about the nature of these regulatory B cells is whether a single regulatory B cell population can simultaneously employ more than one immunosuppressive mechanism. While there is no direct evidence for the co-expression of multiple immunosuppressive molecules in B cells, circumstantial evidence based on a shared surface phenotype suggests that such a B cell population may exist. Notably, expression of IL-10, FasL, granzyme B, and PD-L2 have all been attributed to B cells that express the surface molecule CD5.(18, 36, 51, 66, 90) CD5⁺ B cells are a minor subset of B cells with unique antigen specificities, developmental patterns, anatomical distributions, and sensitivities to stimuli.(102, 150) Additionally, we have recently found that FasL⁺ B cells share other phenotypic markers with IL-10-producing B cells (manuscript in preparation), effectively localizing both IL-10-producing B cells and FasL⁺ B cells to the same B cell subset. These data support the possibility of a multi-potent suppressor B cell subset residing within the CD5⁺ B cell population (Fig. 2 and Table 1). Further work is required, however, to definitively show that the B cells employing these suppressive mechanisms are indeed the same cells.

The relationship of immunosuppressive CD5⁺ B cells to the other established B cell subsets is still incompletely understood. Based on surface phenotype, regulatory IL-10-producing CD5⁺ B cells appear most similar to either T2-MZP B cells or B-1 cells. Both MZB cells, which differentiate from T2-MZP cells, and B-1 cells appear to arise from a single precursor population distinct from that of B-2 cells.(151, 152) B-1 and MZB cells have been described as having functional similarities, particularly in the production of natural antibodies.(153) Given the known propensity for autoreactivity in these subsets, it makes sense that immunosuppressive B cells would arise from this compartment, as these cells would then be apt to suppress immune

reactions to self antigens rather than inducing or accelerating them. However, it is also possible that these cells may not retain immunosuppressive function at all times and could actually contribute to autoimmune pathogenesis under some circumstances. This makes understanding the biology of these cells even more interesting in the context of treating autoimmunity.

As mentioned above, there was not an absolute correspondence between B-1 or T2-MZP B cell phenotype and IL-10 production. Thus, the immunosuppressive activity of B cells may be transient and dependent on the presence or absence of stimulatory signals. It is also possible that the surface marker phenotype of the B cells is secondary to their functional properties, or that the immunosuppressive B cells may represent a completely distinct population from any of the currently recognized subsets. Much further study is required to define the relationship of immunosuppressive B cells with B-1, T2-MZP, and MZB cells and to confirm their multi-potency.

Conclusion

There are likely to be many clinical implications to the findings cited in this review. The elegant set of studies determining the role of sialylated immunoglobulins in the efficacy of IVIG treatment is just one example of how immune suppressive mechanisms mediated by B lymphocytes have been a part of clinical practice without being fully understood.(95, 97) That the identity of B cell subsets that might preferentially produce sialylated IgG have not been determined, and that the biochemical pathways involved in this process are not fully understood, emphasizes the point that immune suppressive functions of B cells still need further study. Recent progress in classifying B cell subsets and renewed interest in understanding the diverse functions of these subsets should aid in developing new strategies to manipulate the activities of defined populations of regulatory as well as effector B cells.(154)

In the field of oncology, it should be noted that malignancies arising from B cells with natural immune suppressive qualities may pose a greater risk to patients because the transformed cells

may directly inhibit the immune response against them. Although it remains to be formally proven, this could underlie the difference between the aggressive and non-aggressive forms of B chronic lymphocytic leukemia (B-CLL). B-CLL cells have been independently reported to express Fas ligand, TRAIL and granzyme B.(49, 61, 76, 155) Other B cell malignancies such as multiple myeloma and non-Hodgkin's lymphoma have also been reported to express death ligands.(58, 60, 156) The Epstein-Barr virus not only carries a form of human IL-10 in the viral genome, but also drives FasL expression in transformed B cells.(14, 62) A better understanding of the phenotypic properties of human killer and regulatory B cells could therefore lead to improved cancer diagnostics. Yet to be developed pharmacological methods of inhibiting expression of immune suppressive molecules specifically in B lymphocytes might reverse tumor-mediated regulation and significantly improve treatment of B cell lymphoma and leukemia.

Conversely, immune suppressive B cells could prove useful as a tool for therapeutic induction of tolerance in solid organ transplantation. Direct evidence of the efficacy of adoptively transferred FasL⁺ B cells toward inducing immune tolerance has been demonstrated in a male to female skin graft model in mice.(71) Much work is still needed to determine how effective this strategy could be in other models such as organ allograft, in which tolerance to a greater number of antigenic determinants is required. Promising evidence of efficacy was recently reported using TIM-1 stimulated B cells that produce IL-10 to tolerize mice toward an allograft of pancreatic islet tissue.(42) If proven safe and effective, pre-graft tolerization by adoptive transfer of donor B cells would be expected to target a broad spectrum of graft-specific T cells, and yet should not harm the other T cells that are involved in host defense. Developing new and improved methods to identify, purify and grow killer and regulatory B cell subsets from humans should help to advance the possibilities of translating this research into clinical practice.

Many of the studies that we have referenced above are focused on the role of regulatory and killer B cells in T cell-mediated diseases such as asthma and autoimmunity. There should be little doubt that immune suppression directed by B lymphocytes is an important factor in regulating T cell functions, and therefore, in modulating allergic and autoimmune pathogenesis. Impairments in B cell-mediated immune regulation or resistance to their suppressive functions may turn out to be critical contributing factors to T cell-mediated diseases. It will be of interest to determine whether any of the known genetic and environmental risk factors that have been identified in these diseases play a role in the survival and function of suppressive B cells. Finding pharmacological or biological methods for reversing defects in B cell-mediated immune suppression may lead to novel therapeutics for many diseases. It is also possible that functional immune suppressive B cells may contribute to autoimmune pathogenesis in some cases. Elevated IL-10 levels have been linked to increased pathogenesis in systemic lupus erythematosus in patients and mouse models, yet B cell-specific IL-10 production and T cell suppression was impaired in a cohort of SLE patients.(44, 157-159) These seemingly conflicting results highlight the need for a deeper understanding not only of the direct functions of suppressive B cells but also their interactions with other effector and regulatory cell populations. It may matter whether immune suppression is dominated by T cells or by B cells in certain clinical situations and this may influence the patient's response to treatment.

Immune suppression, like other important biological processes involves an integrated network of cells and molecules that work together to balance the potentially deleterious effects of uncontrolled inflammation. B lymphocytes are part of this integrated network and can mediate their immune suppressive functions through many of the known pathways that regulate inflammation. B cells also play important non-redundant roles in immunosuppression through production of regulatory antibodies and unique interactions with other cells. While B cells are clearly not the only regulatory cell population involved in immunosuppression, a growing body of

evidence suggests that B cell-mediated immune suppression is important in many inflammatory contexts. In the current era of increasing usage of clinical therapeutics based on blockade of cytokines and depletion of cell populations, the balance of regulatory and pro-inflammatory immune mechanisms is of critical importance. Ignoring the many immune suppressive functions contributed by B lymphocytes during inflammatory processes would be a mistake. Instead, therapeutic strategies that consider the full range of functions of B cells and their interactions with other cells, and incorporate methods to protect desirable B cell subsets or mechanisms of action will likely result in higher clinical success.

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Table 1: Summary of Immunosuppressive Mediators Employed by B Lymphocytes

Suppressive Mediator*	B Cell Subset Phenotype(s)	Inducers	Main Target Cell(s)	References
Interleukin-10	CD5 ⁺ CD1d ^{high} or CD21 ^{high} CD23 ^{hi} CD1d ^{high} (Mouse) TIM-1 ⁺ (Mouse) CD24 ^{high} CD38 ^{high} or CD24 ^{high} CD27 ⁺ (Human)	TLR ligands, CD40L, BCR ligation, overexpression of CD19, BAFF/BlyS, anti-TIM-1	APC, T Cells	(35)(36)(38) (41-45)
Granzyme B	CD5 ⁺ (Human)	CpG-ODN, IL-21	Unknown	(49)(51)
Fas Ligand	CD5 ⁺ (Mouse)	LPS, schistosome infection, viral infection	CD4 ⁺ T Cells	(56) (62-67) (69)
PD-L2	CD5 ⁺ (Mouse)	IL-4, IL-13	Th2 cells	(84) (89)(90)
Sialylated IgG	Unknown	Unknown	Macrophages, DC	(95-97)
Regulatory Natural Antibodies	CD5 ⁺ CD11b ⁺ (Mouse)	Immunization with <i>S. pneumonia</i> , apoptotic cells, or the lipid antigens PC or MDA	Macrophages, DC	(102-108)

* TRAIL, PD-L1, and TGF β have also been reported from B cells, but much less is known about specific subset phenotypes and target cells of these mediators relating to B cell production.

Figure Legends**Figure 1: Normal Immune Responses Depend on Appropriate Levels of Immune Suppression.**

This schematic depicts three potential outcomes of an infection and the resulting inflammatory responses based on varying the level and dynamics of immune suppression. **A:** In a normal immune response, the infectious agent stimulates an early inflammatory response that begins to control the infection before immune suppression begins. As the infection is cleared, inflammation decreases while immune suppression continues to increase resulting in the resolution of inflammation and return to homeostasis. **B:** An immune response with delayed or weakened immune suppression leads to increased inflammation and impaired resolution despite the absence of the eliciting infectious agent. If unresolved this would lead to severe acute inflammation and death. **C:** Early and/or increased suppression may inappropriately lower inflammation and prevent clearance of the infectious agent. Depending on the nature of the infection and the balance between inflammation and suppression, this could lead to morbidity caused by the infectious agent, a chronic state of inflammation, and/or higher baseline suppression toward the next inflammatory stimulus.

Figure 2: B Cell Subsets Can Mediate Immune Suppression Through Multiple Effector Mechanisms

The major pathways involved in immune suppression have been shown to be expressed by various subsets of B lymphocytes. T2-MZP and B10 cells have been independently described as important B cell sources of IL-10, but IL-10 might also be produced by any B cell given the proper stimulus. In some cases, TGF β has also been shown to be produced by B cells but the phenotype of these cells is undetermined. Human but not mouse B cells have been shown to

express granzyme B, the enzymatic component of cytotoxic granules. B cells expressing PD-L2 on the cell surface (L2pB1 cells) can send either stimulatory or regulatory signals through interactions of PD-L2 with the receptor PD-1 on target cells. Killer B lymphocytes are defined by expression of the death ligands FasL and/or TRAIL. The CD5⁺ B cell subset has been independently reported to express IL-10, PD-L2, granzyme B (in humans), and FasL, suggesting that CD5⁺ B lymphocytes may function as multi-potent suppressor B cells. CD4⁺ T cells are documented targets of immune suppression by B cells, but many of the mechanisms described also have effects on NKT cells, CD8⁺ T cells, antigen presenting cells, and other lymphoid and non-lymphoid cells.

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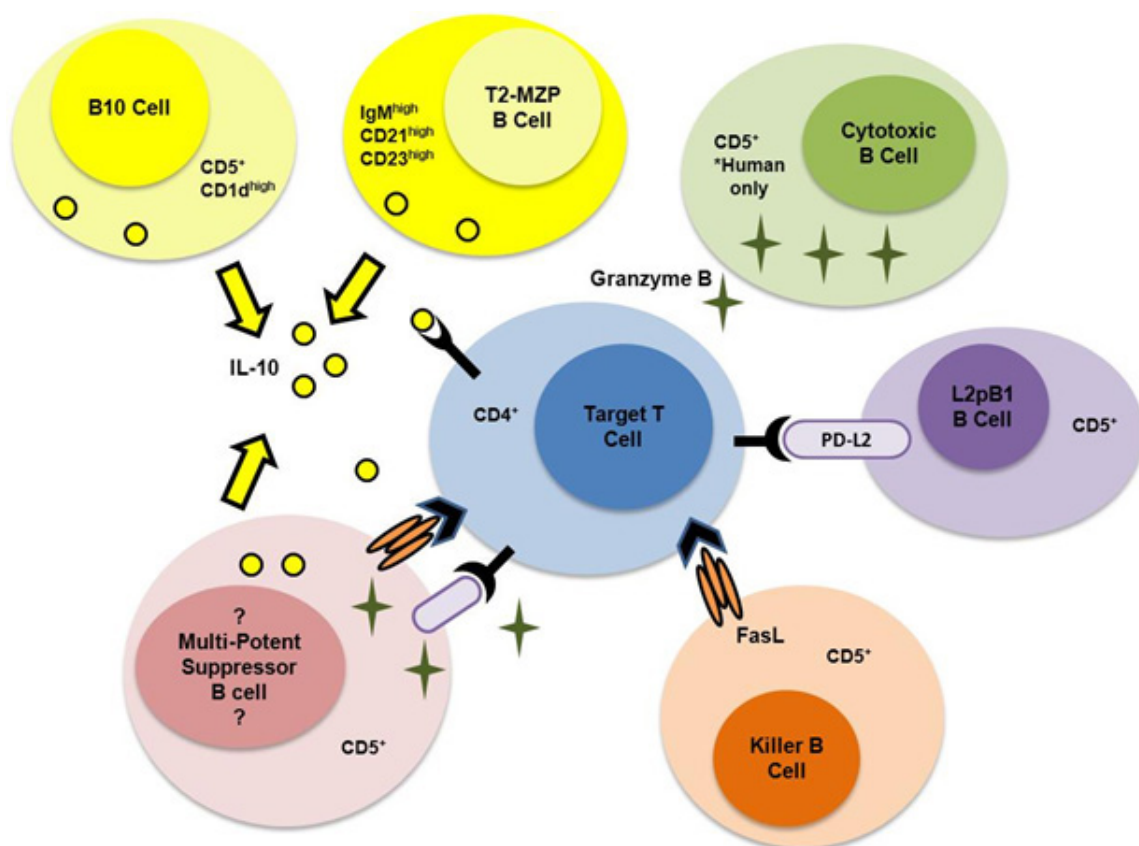
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