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Innate Pathways to B cell Activation and Tolerance

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Abstract

B cells represent an important link between the adaptive and innate immune systems, as they express both antigen-specific B cell receptors (BCRs) as well as various toll-like receptors (TLRs). Several checkpoints in B cell development ensure that self-specific cells are eliminated from the mature B cell repertoire to avoid harmful autoreactive responses. These checkpoints are controlled by BCR-mediated events, but are also influenced by TLR-dependent signals from the innate immune system. Additionally, B cell-intrinsic and extrinsic TLR signaling are critical for inflammatory events required for the clearance of microbial infections. Factors secreted by TLR-activated macrophages or dendritic cells directly influence the fate of protective and autoreactive B cells. Additionally, naïve and memory B cells respond differentially to TLR ligands, as do different B cell subsets. We review here recent literature describing intrinsic and extrinsic effects of TLR stimulation on the fate of B cells, with particular attention to autoimmune diseases.

Keywords

B cells; Toll-like receptors; TLRs; autoimmunity; autoreactive; tolerance; innate; SLE; Lupus; BAFF; IFN-I

Introduction

The generation of a diverse antibody repertoire occurs in a stochastic fashion and requires checkpoints to either eliminate or tolerize B cells with anti-self specificity [1]. Autoreactive B cells that escape these checkpoints have the potential to initiate or contribute to the development of autoimmune diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis [2, 3]. In contrast, effective activation and clonal expansion of antigen-specific B cells is required to quickly neutralize and eradicate both viral and bacterial infections.

It is well established that B cell receptor-triggering events regulate both tolerance to self-derived antigens in the bone marrow and periphery, as well as activation in response to foreign antigens [4]. Central tolerance mechanisms occur in the bone marrow and involve clonal deletion to eliminate B cells with self-reactive BCRs [5-7]. Additionally, receptor editing of autoreactive BCRs via alternative light chain usage can alter B cell-specificity and thus, eliminate self-reactivity [8]. In the event that an autoreactive B cell escapes central tolerance in the bone marrow, it is subject to a number of peripheral tolerance mechanisms. First, anergy, or functional unresponsiveness, can prevent peripheral autoreactive B cells from becoming activated and differentiating into plasma cells[1]. In addition, self-reactive B cells can be deleted from the peripheral lymphocyte pool [9, 10]. Mature autoreactive B cells in

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the germinal center reaction can undergo V(D)J recombination to change otherwise harmful self-specific BCRs [11-14]. Antigen-dependent exclusion of autoreactive B cells from follicles also prevents overt self-specific responses [15, 16]. Finally, in some cases, fully functional B cells with self-specificity were found to be auto-antigen ignorant [17-19]. All of the above mentioned mechanisms are thought to require recognition of the BCR with soluble or membrane-bound self-antigen. However, it is becoming more appreciated that signals from the innate immune system can also help determine the fate of auto-reactive, as well as foreign-antigen-specific B cells [20, 21]. Herein, we review the most recent literature describing methods the innate immune system uses to drive as well as dampen B cell responses.

Intrinsic effects of TLR ligands on naïve and memory B cells

Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs), which recognize conserved microbial products and activate the innate immune system[22]. TLR-1, -2, -4 and -5 are expressed on the cell surface and recognize non-nucleic acid components of microbes [23]. TLR2, in conjunction with TLR1 or TLR6, recognize lipopeptides while TLR4 and TLR5 recognize bacterial lipopolysaccharide (LPS) or flagellin, respectively [24]. Other TLRs localize to endocytic compartments such as TLR-3, -7, -8 and -9. These TLRs recognize dsRNA (TLR3), ssRNA(TLR7/8) and CpG-containing DNA(TLR9) [23]. Proximal signaling events downstream of all TLRs except TLR3 involve the adaptor molecule myeloid differentiation primary response gene 88 (MyD88) and interleukin-1 receptor associated kinase (IRAK) [24]. Distal signaling events lead to interferon regulatory factor (IRF) and/or NFκB-dependent induction of type one interferon (IFN-I) as well as pro-inflammatory cytokines such as IL-6, TNF-α, and IL12 [22]. B cell activation, proliferation, and class-switch recombination are all influenced by the TLR pathways [25-31], but exactly which B cell population is most sensitive and which additional factors are required for full B cell activation are still open questions. Among recent studies focused on this issue, we will summarize, on one side, reports of activation of various B cell populations with TLR ligands, and on the other side, reports of B cell suppressor activity as a consequence of TLR activation.

TLR-mediated activation of B cells

The immune system uses a hierarchy of B cell subtypes to respond to TLR ligands[32]. Thus, marginal zone B cells respond to a greater extent in response to TLR3 and TLR4 ligands than transitional or follicular B cells with regard to plasma cell differentiation and IgM secretion [32, 33]. This is consistent with the characterization of marginal zone B cells being more “innate-like” B cells[34]. Also, due to the functional differences between naïve and memory B cell subsets, there has been interest in delineating the differential effects of TLR agonists on each. For instance, human memory B cells were shown to have increased levels of CD180, a TLR-related molecule that cooperates with TLR4 to recognize LPS, compared to naïve cells [35]. This correlated with increased proliferation of memory B cells in response to CD40L and anti-CD180. Other studies have demonstrated higher protein levels of TLRs on memory versus naïve B cells [36]. Resiquimod, a TLR7 agonist, was shown to induce IgM and IgG secretion without T cell help in both naïve and memory human B cells *in vitro*, but naïve B cells required boosting with IL-2 + IL-10 for an efficient response (Fig. 1) [37]. These data indicate that TLR ligands alone may be sufficient to lead to preferential activation of memory B cells and differentiation into IgM and IgG-secreting plasma cells in the absence of T cell help. But the *in vitro* data on B cell differentiation upon TLR stimulation might not be easily translated to *in vivo* findings. For example, it was found that TLR4, 7 & 9 ligands could stimulate B cells from NP-immunized mice to secrete high affinity NP-specific IgM and IgG *in vitro*. Purified NP-specific memory B cells had the greatest capacity to differentiate into high-affinity NP-specific IgG^+ cells in response to TLR4 or TLR9 ligands,

compared to plasma cells and non-switched NP-specific cells. The situation was very different *in vivo*, however, as TLR4 and 9 ligands were unable to boost NP-specific antibody titers in the serum of immunized mice [36]. Thus, it appears that the real consequence of activating the TLR pathway in B cells *in vivo* in the absence of BCR engagement or other co-stimulators is still uncertain.

It is interesting to note that B cells with high affinity autoreactive BCRs are less responsive to ligands for TLR4 and 9, with respect to differentiation into antibody-secreting cells, despite having a higher proliferation index, than B cells with lower affinity autoreactive BCRs [32]. This suggests that high affinity autoreactive B cells may be intrinsically programmed to respond poorly to endogenous TLR ligands. The concentration of TLR ligand and duration of stimulation strictly regulate how many cell divisions a B cell will undergo [38]. Under optimal stimulation conditions, however, a maximum number of B cell divisions, termed population division destiny, is reached and will not be exceeded [38]. Since cell proliferation is tightly linked to plasma cell formation, B cells most likely use this to limit the extent of TLR effects during a normal inflammatory response. Additional mechanisms may exist in B cells to limit TLR signaling. For instance, in HEK293 cells stimulation of TLR2, -4 or -9 leads to downregulation of the critical IL1R/TLR adaptor IRAK4 [39, 40]. Receptors with regulatory function on TLR signaling such as the inhibitory TIR8/SIGRR could also limit B cell activation. Indeed, TIR8 has been shown to be a factor in maintaining B cell tolerance *in vivo* [41] as will be discussed below in the section describing mouse models of autoimmune disease.

Since B cell-intrinsic TLR signaling can promote B cell activation, proliferation, and class-switch recombination, its role in the production of pathogenic antibodies in autoimmune diseases is currently being investigated [26, 30]. In the AM14 transgenic mouse model, in which B cells are expressing a BCR specific for IgG2a, it was shown that administration of IgG2a anti-chromatin antibodies lead to extrafollicular plasma cell expansion, class-switch recombination, affinity maturation and antibody secretion [42, 43]. This process could proceed at least partially in the absence of T cell help and required B cell-intrinsic expression of MyD88, a critical adaptor molecule for TLR signaling. Furthermore, the appearance of antibody-forming cells (AFC's) required the expression of TLR7 and TLR9, as combined deficiency completely eliminated these antigen-specific AFCs [42]. These results seem to indicate that chromatin-containing immune complexes activate B cells *in vivo* by engaging endogenous TLRs and thus induce their differentiation into plasmablasts [29, 42, 44, 45].

Role of TLRs in B cell suppressive functions

New reports suggest that B cell-intrinsic TLR signaling can lead to tolerance in T cell-mediated responses. Using the mouse model of autoimmune disease experimental autoimmune encephalomyelitis (EAE), B cells were shown to play a suppressive role in the recovery phase of disease and they required expression of MyD88 or TLR2/4 [46]. Furthermore, IL-10 from TLR-activated B cells prevented CpG-stimulated DCs from promoting T cell activation *in vitro*, thus providing a possible molecular mechanism whereby B cells suppress EAE[46, 47]. In separate experiments where B cells present the encephalitogenic peptide MOG 35-55 on MHC class II molecules, T cells become tolerized and do not initiate MOG-induced EAE [48]. This process did not require IL10 expression. Human B cells have also been reported capable of suppressing T cell responses in certain conditions. It was shown that TLR9-activated human $CD25^+$ B cells could suppress T cell proliferation and IFN-γ production through a contact-dependent mechanism [49]. B cells appeared to require IL2 for the suppressive effect, as blocking the IL2R on B cells partially reversed the repression. Thus, these studies indicate that B cells can suppress T cell function through TLR-dependent and independent mechanisms.

Extrinsic effects of innate immune pathways on B cell activation and tolerance

In this section we will discuss how TLR signaling and subsequent innate responses in cell types other than B cells influence the outcome of B cells during infection or autoimmune responses.

Effects of IFN-I on B cells

Dendritic cells (DCs) and macrophages express a variety of TLRs important for microbe recognition [50]. Inflammatory cytokines secreted by DCs and macrophages stimulated with TLR ligands can ultimately influence the fate of B cells during normal inflammatory responses or during autoreactive ones [22, 51, 52]. For example, IFN-I, secreted at high levels by plasmacytoid DCs (pDCs) upon viral infection or TLR stimulation, increased the expression of TLR7 in naïve B cells [53-55]. Others have shown that pDC-derived IFN-I increased the sensitivity of memory B cells to TLR7 ligand-induced plasma cell formation [56]. This was limited to TLR7, as TLR3 and TLR9 ligands had little combined effect in the presence of pDCs or IFN-I in inducing plasma cell formation. Additionally, pDCs, versus myeloid DCs (mDCs), were able to support better memory plasma B cell differentiation and proliferation.

Elevated levels of IFN-I and interferon stimulated gene expression have been found in association with human lupus as well as mouse lupus models [57-62]. Immune complexes containing DNA antigens can be internalized by pDCs, which leads to the induction of IFN-I [63, 64]. This process, downstream of the initiation and expansion of autoreactive B cells, is thought to amplify lupus [65]. In the pristane-induced lupus mouse model, IFN-I receptor deficiency eliminated autoantibody production and glomerulonephritis [60]. TLR7 expression was also required in this model for IFN-I production as well as the increase in anti-nuclear antibodies [66, 67]. Interestingly, FcγRI and FcγRIII, which are required for uptake of IgG-antibody containing immune complexes in pDCs, were dispensable for IFN-I production in response to pristane. Taken together, these results suggest that in addition to being a result of immune complex-mediated stimulation of pDCs, IFN-I may also have effects upstream of the initial break in tolerance against nuclear antigens by B cells. In support of this idea, patients treated with IFN-I for cancer or Hepatitis C viral infection have an increase in circulating anti-nuclear antibodies [68, 69].

Suppressive effects of innate immune cells and cytokines on B cells

TLR-activated macrophages and DCs can also have suppressive effects on autoreactive B cells. In co-culture experiments, LPS-stimulated mDCs and macrophages suppressed the differentiation of autoreactive B cells into antibody-secreting cells [70, 71]. The suppressive effect of mDCs and macrophages was dependent on IL-6 and sCD40L secretion, respectively [70, 71]. Importantly, the suppressive effects of these soluble factors seem to be limited to chronically activated autoreactive B cells, as naïve B cell differentiation into antibody-secreting cells was unaffected. Follicular B cells were also more sensitive to the inhibitory effects of DCs and macrophages than marginal zone B cells[70]. Plasmacytoid dendritic cells have also been shown to play a suppressive role in a mouse model of autoimmune arthritis [72]. In this model, Th1-polarized T cells specific for OVA were adoptively transferred into BALB/c recipients and one day later challenged with a subcutaneous injection of OVA in complete Freund's adjuvant (CFA). 10 days later, the mice were challenged with a subcutaneous periarticular injection of heat-aggregated OVA into both ankles [73]. Inflammatory arthritis was established, characterized by ankle joint swelling, and the breakdown of tolerance to collagen antigens [73]. Anti-collagen antibodies are detected in the serum of these animals and increase in the absence of pDCs, indicating a

suppressive role on autoreactive B cells. Anti-OVA antibody levels were similar between pDC-depleted and non-depleted animals, suggesting that the suppression may be specific to autoreactive B cells [72].

Effects of BAFF on the fate of B cells

B-cell-activating factor (BAFF) is a member of the tumor-necrosis-factor family and induces B cell proliferation and differentiation both *in vitro* and *in vivo*[74]. BAFF is an essential component of B cell homeostasis, as it is critical for B cell survival and effector function [75, 76]. Transgenic overexpression of BAFF results in a severe autoimmune disorder characterized by the production of autoantibodies, proteinuria, splenomegaly, immunoglobulin deposition in kidneys, spontaneous germinal center formation and expanded marginal zone cells [75, 76]. Increased serum levels of BAFF have also been detected in patients with SLE [77].

Some phenotypic features seen in BAFF transgenic mice are similar to autoimmune regulator gene (AIRE)-deficient mice [76]. *Aire*$^{-/-}$ mice contain elevated levels of BAFF in their serum and expanded, BAFF-expressing antigen presenting cells, such as dendritic cells, which persist in the absence of T cells [76, 78]. Patients with loss-of-function mutations in Aire frequently develop Autoimmune Polyendocrine Syndrome type I [78, 79]. Elevated levels of circulating BAFF were detected in these patients, which was shown to be dendritic cell-intrinsic. These results suggest the intriguing possibility that BAFF regulates tolerance in the absence of functional AIRE.

Early activation of B lymphocytes in the target organs of some autoimmune diseases may be the consequence of increased levels of BAFF, secreted by activated innate immune cells[80]. For instance, viral infections can induce secretion of BAFF in human salivary gland epithelial cells, which are primary targets of Sjögren's syndrome. Viral-induced BAFF upregulation was shown to involve TLRs, IFN-Is, as well as other unidentified factors. This study demonstrates how viral infection could link the innate immune system with BAFF-dependent activation of B cells.

Blockade of BAFF is a promising therapy in several autoimmune diseases. BAFF antagonists have been shown to be effective in the prevention and treatment of the lupus-prone mice [81]. Several BAFF antagonists, including soluble forms of the receptor and neutralizing monoclonal antibodies are currently in human clinical trials for SLE treatment [81-83]. More experiments are needed to determine the optimal way to neutralize BAFF without compromising adaptive immunity to microbes.

In addition to an inflammatory role for BAFF in promoting B cell activation, it has also been demonstrated to play an anti-inflammatory role by supporting the expansion of T regulatory cells [84]. Deregulated BAFF expression alters T cell-dependent alloimmune responses. BAFF transgenic mice with the H-2^{b} haplotype failed to reject transplanted islet allografts from BALB/c donors with the H-2^{d} haplotype. Furthermore, skin allografts were also tolerated in BAFF Transgenic MHC-mismatched recipients. Tolerance was due to BAFF-dependent expansion of CD4^{+}CD25^{+}Foxp3^{+} T regulatory cells (T regs) in the periphery, which suppressed T cell-mediated effector function. Finally, the mechanism of BAFF-mediated expansion of T regs was shown to be B cell-intrinsic [84].

Mouse models of B cell autoimmunity and tolerance

Recently, several mouse models of B-cell associated autoimmune diseases induced by dysregulation of innate immune responses have been described. Using *in vivo* transgenic expression, it was recently shown that TNF-receptor associated factor 3 (TRAF3) plays a

critical role in the control of tolerance and the development of autoimmunity [85]. TRAF3 overexpression produced a phenotype similar to SLE, characterized by systemic inflammation and elevated levels of inflammatory cytokines such as TNF-α, MCP-1, IL-6 and IFNγ. TRAF3 transgenic mice also contained an elevated frequency of plasma cells, correlating with hypergammaglobulinemia and increased serum levels of IgG2a and IgG2b. Transgenic expression of TRAF3 also alters TLR-mediated B-cell differentiation, as LPS or CpG enhanced humoral responses without affecting proliferation, compared to wild type mice.

Another example, which underscores the importance of TLR regulation in the prevention of autoimmune disease, is the mouse deficient in the inhibitory TIR8/SIGIRR receptor. The TLR-related molecule TIR8 negatively regulates TLR and IL-1R signaling through the inhibition of TRAF-6 and IRAK [86-88]. Mice deficient in this molecule on the lupus-prone B6$^{lpr/lpr}$ background develop a lymphoproliferative disease characterized by splenomegaly, lymph follicle hyperplasia and the presence of both anti-dsDNA and anti-snRNP antibodies[41]. In addition to the known role of TIR8 in DCs and epithelial cells, Lech and co-workers demonstrated that B6.$^{lpr/lpr}$*Tir8*$^{-/-}$ B cells are hyperresponsive to ligands for TLRs -4, -7 and -9 compared to B6.$^{lpr/lpr}$*Tir8*$^{+/+}$[41, 86, 89]. These data suggest that TIR8 could operate in B cells to restrict the production of anti-nuclear and anti-nucleolar plasmablasts.

A mouse with a hypomorphic allele of the inhibitory protein-tyrosine phosphatase SHP-1 is another example of spontaneous multi-organ autoimmune disease that depends on TLR/IL-1 activity [90]. Disease in this new mouse model was shown to be dependent on MyD88, IRAK-4 and the IL-1 receptor, but not on STAT1, suggesting that IFN-I production might not be critical in this case [90].

Innate immune regulation of human B cells and autoantibodies

Recently, B cell depletion therapies have proven effective for the treatment of human autoimmune diseases such as rheumatoid arthritis and lupus[91-93]. These observations underscore the importance in understanding the genetic and environmental basis for how human B cells contribute to the break in tolerance to self-antigens. As described above, TLR signaling contributes to both B cell activation and tolerance to auto-antigens (e.g., antigens of nuclear origin). But paradoxically, it was recently discovered that patients who have loss-of-function mutations in IRAK4, UNC-93B or MyD88, all of which are molecules critical for TLR signaling, have an emergence of autoreactive B cells in their peripheral blood [94]. Using a single-cell BCR cloning and expression system, Isnardi, et al. determined that new emigrant B cells isolated from IRAK4-, MyD88- and UNC-93B-deficient patients contain between a 5-10 fold higher frequency of multi auto-antigen specific B cells than healthy controls. This suggests that IL-1R/TLR signaling events are required early in B cell development to effectively eliminate autoreactive cells. Although the removal of new emigrant B cells specific for anti-nuclear antigens required IRAK4 and MyD88, UNC-93B was dispensable for this process [94]. In contrast, all three proteins were required for the removal of multi auto-antigen and nuclear-specific mature naïve B cells in the periphery. Interestingly, despite the elevated frequency of B cells with nuclear specificity in their blood, serum from these patients did not contain anti-nuclear antibodies. This observation is in agreement with mouse studies demonstrating the importance of MyD88 and TLR7 for systemic autoimmunity [28, 95-98]. It would be interesting to examine the B cell repertoire of mice deficient for IRAK4 or MyD88 to determine if, similar to humans, they have increased frequencies of B cells with self-specificities. Crossing these mice to various lupus-prone strains would help to elucidate the mechanisms by which these B cells are silenced in the periphery.

Others have recently shown that B cells with anti-nuclear specificity are part of the normal human peripheral repertoire [99]. These cells are functionally anergic and phenotypically identical to naïve B cells with the exception of the reduced expression of surface IgM. Anergy in these B cells could be reversed when they were rested overnight in the absence of self-antigen. Autoreactive IgG^+ B cells have also been demonstrated in the memory pool of normal individuals [100]. It is plausible that perturbations in TLR signaling (such as a viral infection) may give these autoreactive pools of B cells a selective advantage and lead to a break in peripheral tolerance and eventually autoimmunity.

Blockade of TNF-α has proven to be a very effective treatment for patients with rheumatoid arthritis and Crohns disease [101]. Recently, however, it was discovered that patients treated with anti-TNF-α have an emergence of anti-dsDNA and anti-nucleosome antibodies in their serum [102, 103]. Interestingly, the presence of anti-nucleosome antibodies in the serum of patients treated with TNF-α blockers positively correlated with circulating nucleosomes [104]. This suggests that availability of antigen drives *de novo* production of autoreactive B cells in the presence of the TNF-α-blocking antibody, rather than a change in the inflammatory environment favoring the expansion of low frequency precursor cells. Nucleosomes from late apoptotic cells can also stimulate cytokine release and the upregulation of costimulatory markers in macrophages and DCs, respectively [105]. This effect appears to be dependent on high mobility group box protein 1 (HMGB1), contained within the nucleosomes, and TLR2 expression on responding cells. This process, in combination with the TLR9-dependent activation of DCs by chromatin-containing immune complexes, may contribute to the overall amplification of inflammation seen in SLE [29, 63, 64].

Conclusions

B cell fate during an inflammatory response must be tightly regulated by signals from the adaptive and innate immune systems. Balance between activating and inhibitory signals can help prevent overt autoreactive responses while maintaining immunity against harmful infectious agents. We reviewed here the most recent progress in understanding how innate immune signals originating from TLRs control the fate of B cells (Fig. 1). Interestingly, in addition to a role for TLR signaling in promoting humoral immunity and autoimmunity, it seems that under certain conditions TLRs contribute to B cell-mediated suppression of autoreactive responses. These effects appear to be both B cell intrinsic and extrinsic. Understanding the innate mechanisms balancing activation and suppression of B cells will help in strategies for treating autoimmune diseases or for designing better vaccination protocols.

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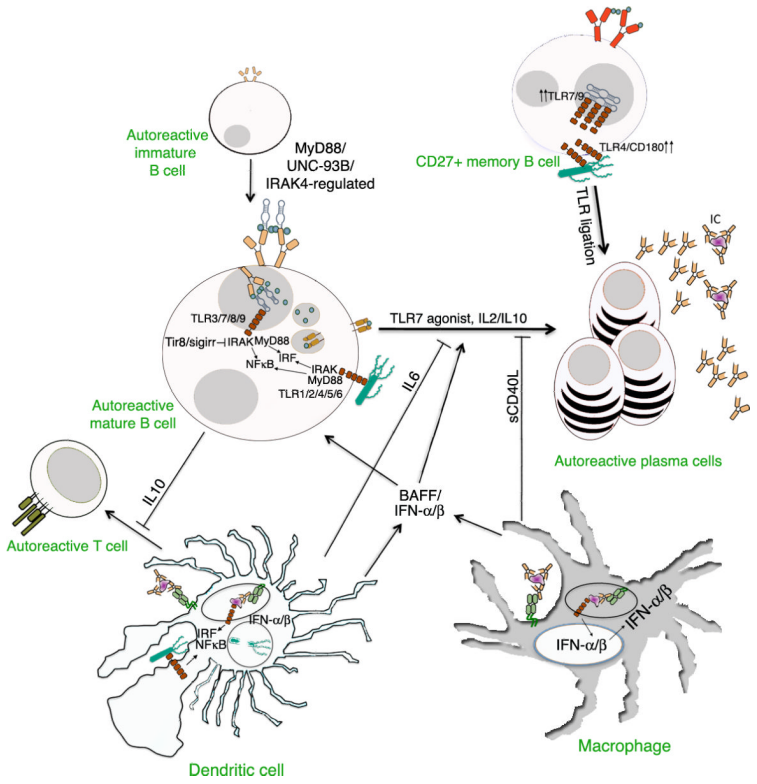

Figure 1. Innate pathways to B cell activation and tolerance

Intrinsic TLR signaling in naïve and memory B cells can lead to IgM and IgG-secreting plasma cell formation through IRAK and MyD88. Memory B cells express increased levels of TLRs and have a greater capacity to differentiate into plasma cells via TLR stimulation than naïve B cells. TLR-activated B cells can suppress T cell responses indirectly through an IL10-dependent mechanism. Type one IFN production from macrophages and dendritic cells stimulated with immune complexes or TLR agonists can promote the differentiation of B cells into plasma cells. TLR-stimulated dendritic cells and macrophages can suppress autoreactive B cell differentiation into plasma cells through IL6 and sCD40L, respectively. BAFF, produced by APCs, can also influence B cell homeostasis and differentiation into plasma cells. MyD88, UNC-93B and IRAK4 each decrease the level of autoreactive B cells in the periphery.