MARGINAL ZONE B-CELL PRECURSORS AS CELLULAR AGENTS FOR TYPE I IFN PROMOTED ANTIGEN TRANSPORT

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A DISSERTATION

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Type I IFN is a known pro-inflammatory cytokine that is associated with the development of systemic lupus erythematosus (SLE). At the level of B-cell regulation, current literature primarily attributes the formation of autoantibodies at the post-germinal center stage when type I IFN is known to promote plasmablast differentiation. However, our findings here point to a type I IFN effect at the pre-germinal stage. Type I IFN promotes T-dependent antibody response, activation-induced cytidine deaminase (AID) expression, germinal center formation, all of which are processes before the onset of plasmablast differentiation. We have determined a subset of splenic transitional B-cells, that is known as the precursors to the development of marginal zone (MZ) B-cell population, and are termed marginal zone B-cell precursors (MZPs), whose trafficking from the MZ border to the inner follicle (FO) is promoted by type I IFN. MZPs are more efficient antigen-presenting cells than MZ or FO B cells. Type I IFN up-regulates expression of CD69 and down-regulates expression of S1P1 to promote migration of MZPs into the inner follicle to interact with CD4 T-cells. Together, these observations suggest a novel pathway by which type I IFN can promote the presentation of antigen to CD4 T-cells by MZPs to initiate germinal center reaction prior to type I IFN induction of plasmablast differentiation.
DEDICATIONS

I dedicate this thesis to my parents who worked hard to raise me, my wife for being my companion in tough times, and two daughters for the joys of my life throughout this exciting journey.
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**TYPE I IFN DIRECTLY SIGNALS ON B CELLS TO DRIVE GERMINAL CENTER FORMATION IN BXD2 MICE**

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SUMMARY

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by increased titers of serum autoantibodies, arthritis, dermatological rashes, and glomerulonephritis in affected patients [1-4]. A hallmark of SLE is the presence of autoantibodies to nuclear antigens, including histone and DNA [2]. Clinically, patients may display the characteristic malar rash after sunlight exposure [3]. Current estimates that 1 in 2000 persons is affected by SLE, with a strong gender bias for females (9:1 female to male ratio) and a racial bias for nonwhite populations [3-5]. African Americans and Hispanics develop SLE 2 to 4 times more frequent than Caucasians [4, 5]. Disease onset usually occurs in the third to fourth decade of life [5]. Current life expectancy for SLE patients in the United States, Canada, and Europe is approximately 95% at 5 years, 90% at 10 years, and 78% at 20 years [6].

A variety of murine models of lupus has been generated over the last three decades [4]. These include the New Zealand hybrids, their recombinant inbred strains, and mice carrying single-gene mutations and the so-called disease accelerator genes [4]. All of the murine models develop splenomegaly, the presence of anti-nuclear antibodies, and glomerulonephritis, an array of symptoms mimicking human SLE [4]. Both the New Zealand Black (NZB) and New Zealand White (NZW) were independently generated in New Zealand in the 1950s [7, 8]. Both mice develop glomerulonephritis, although the
NZW strain developed a more severe form albeit with a later onset in life. In 1963, the F1 generation of a cross between the NZB and NZW mice was reported to develop renal lesions with significant resemblance to those found in humans with SLE [7, 9]. By the 1980s, after fortuitous crosses amongst the NZB and NZW strains, a series of 27 New Zealand Mixed (NZM) strains were generated, varying in terms of severity for anti-nuclear antibody production and glomerulonephritis [7, 10]. Of these, the NZM2410 displayed the most severe disease, with a 85% penetrance for glomerulonephritis and 50% mortality by about 6-months of age [7]. The NZB, NZW, (NZBxNZW) F1, and NZW strains, have contributed greatly to identifying the chromosomes upon which lupus-associated loci is located on [4].

Other murine models of lupus include the single-gene or gene segment mutations which accelerate disease progression. The lpr and lpr\textsuperscript{rg} mice which express very little Fas and mutant Fas, respectively, were found to develop lymphadenopathy, splenomegaly, accumulation of CD4\textsuperscript{+}CD8\textsuperscript{−} TCR\textalpha\textbeta lymphocytes, elevated autoantibody titers, glomerulonephritis, vasculitis, and arthritis [11-13]. Disease manifestations vary, depending upon the background strain upon which the lpr gene is crossed onto [14]. On the MRL background, the lpr mice develop severe disease [15-17]. On the other hand, on the C3H/HeJ, C57BL/6J, and AKR/J mice, the lpr gene only induced limited glomerular lesions [15, 16].

Of all the murine models of lupus, the BXSB mouse is the only model that has a male predominance for lupus disease development [4]. Genetic analysis has found that the Y chromosome in the BXSB strain contains at least 17 genes, and is associated with
the accelerated progression of lupus disease, thus named Y chromosome-linked autoimmune accelerator (Yaa) [18]. Recent studies of Yaa found that it is a duplication and translocation of several megabases of DNA from the X chromosome, one of which is the gene which codes for the toll-like receptor 7 (TLR7) [19, 20]. The Tlr7 gene is duplicated in the BXS3B mice and therefore plays a critical role in lupus progression [19, 20].

The BXD2 Murine Model

In 2005, our laboratory identified the BXD2 mouse to spontaneously develop autoimmune diseases resembling lupus-like symptoms, including the elevated serum titers of autoantibodies, autoantinuclear autoantibodies, arthritis, and glomerulonephritis [21]. BXD2 is generated from the intercross of more than 20 generations of brother and sister mating of F1 mice derived from C57BL/6J and DBA/2J mice by Dr. Benjamin Taylor at the Jackson Laboratory [21]. Since the first inbreeding, now more than 100 strains have been generated. Genetic mapping have determined that the BXD2 strain consists of 52% genes from C57BL/6J (B6) and 48% consisting from DBA/2J, both non-autoimmune mice. However, all the major histocompatibility antigens (chromosome 17, 19 CM) and immunoglobulin (chromosome 12, 59 CM) genetic information originate from the B6 mouse, making B6 a sufficient wild-type control for all our experiments. Our laboratory has found that the BXD2 strain developed spontaneous glomerulonephritis and erosive arthritis [21]. BXD2 generated a series of auto-antibodies found commonly in lupus patients, including anti-Ro/SSA, anti-heat shock proteins, anti-DNA and
rheumatoid factor [21, 22]. Significant serum titers of these autoantibodies arise and peak at different ages [22]. Anti-Ro/SSA titers peak at 2 months of age, anti-heat-shock proteins peak at 8 months of age, and anti-DNA titers rising significantly only after 6 months of age [22]. Clinical disease, including significant proteinuria, glomerulonephritis and arthritic severity present themselves after 4 months of age [22]. BXD2 mice, on average have half the lifespan of a normal healthy B6 [21, 22]. The presenting symptoms and the presence of auto-antibodies commonly found in SLE human patients make BXD2 a good model for lupus.

Unlike the BXSB and lpr mice, no single gene or gene segment is responsible for disease development in BXD2 mice. Resembling the New Zealand hybrids and recombinant inbreds, lupus disease in BXD2 mice is polygenic [21]. One of the earliest understanding about lupus with the advent of the NZB as a murine model for lupus is that marginal zone and B-1 B cells may play an essential role in the disease [23]. Both the NZB and the (NZBxNZW)F1 lupus-prone mice exhibit an expanded population of marginal zone B cell and B-1 cells in the spleen and peritoneum, respectively [24-30]. Accordingly, a T-independent process in autantibody production may be primary for lupus induction. NZB mice generate increased titers of serum IgM, increased IgM plasmablasts [31-34], and polyclonal activation of B cells [35-38]. Nevertheless, it was also recognized that NZB and (NZBxNZW)F1 mice generate IgG anti-DNA, suggestive of antibody isotype class-switch [39, 40]. The NZW further reinforced the idea that antibody isotype class-switch may play a prominent role, as exemplified by the presence of high titers of IgG autoantibodies in these mice [41]. Transfer of NZB B cells into T cell-deficient SCID mice resulted in IgM hypergammaglobunemia, and low titer of
IgG2a, whereas the opposite results were obtained when NZW B cells were transferred [42]. These results suggest that in spite of the presence of IgG isotypes, antibody class-switch in these mice occurred in the absence of CD4 T cells, suggestive that intrinsic defects in the B cells are at play.

Nevertheless, the importance of T-dependent antibody responses in lupus-prone murine model was demonstrated when a monoclonal anti-L3T4 was administered into (NZBxNZW)F1 mice, resulting in inhibition of the anti-DNA autoantibody-forming cells [43]. Also, purified splenic T cells in these mice were found to augment both the IgM and IgG anti-DNA autoantibody-forming B cells [43]. Although CD4-deletion in NZB mice only delayed the onset of IgG anti-erythrocyte autoantibody, autoantibody to single-stranded DNA of IgG isotypes were permanently suppressed, suggestive of a significant role for CD4 T cell help in disease induction [44].

Blocking the T cell co-stimulatory pathway by in vivo treatment of BXD2 mice with adenoviral vector-expressed CTLA4 Ig has obviated the onset of autoimmune disease, including the suppression of arthritis, proteinuria, glomerulonephritis, over a period of 6-month follow-up [43]. Serum levels of autoantibodies, including anti-DNA, and anti-histone, were suppressed. In contrast, Ad-CTLA4-Ig injection into BXD2 older than 2 months of age (3 months and 13 months) showed reduced or no effect on autoimmune suppression [43]. Our findings indicate that at <2 months of age, a key immune tolerance defect occurs in the BXD2, leading to long-lasting refractory autoimmune disease after that age period. CTLA-4 is a glycoprotein primarily expressed on the surface of activated T cells and shares with CD28 the ability to ligate to the B7
family of molecules (CD80/86) expressed on antigen-presenting cells [45, 46]. In contrast to CD28, which promotes the T cell co-stimulatory pathway, CTLA-4 is a negative regulator of T cell activity [45, 46]. Consistent with earlier findings in the (NZBxNZW)F1 mice, our findings that CTLA4 Ig treatment in BXD2 mice blocked autoantibody production and obviated the development of autoimmune disease substantiates the idea that T cells do play a significant role in lupus [22].

Further substantiating the predominant role of T cell mediated autoantibody production in BXD2 mice is the observation that these autoimmune mice spontaneously develop large numbers of germinal centers in the follicles [47]. It is well established that germinal center formation requires the interaction between CD4 T cells and B cells in the reactive follicle. In contrast, T-independent antibody responses do not require germinal centers, and these antibodies are primarily generated in the marginal zone in the perimeter of the follicle.

A distinguishing feature of BXD2 mice from the New Zealand strains is that these mice do not have enlarged marginal zone B cell population, suggestive that different mechanisms for disease progression are at play. BXD2 mice surprisingly displayed high levels of serum IL-17 and splenic IL-17 transcripts, suggestive that IL-17 may play a prominent role in the induction of lupus-like disease. CD4 T cell Th17 polarization drove the expansion of a significant population of IL-17 producing CD4 T cells in BXD2 mice compared to B6 mice. My current finding that BXD2 mice also exhibited significant presence of interferon-producers and type I IFN expression is consistent with the current literature which claims that type I IFNs are associated and cause lupus disease. However,
it is also generally recognized that type I IFNs and interferons, in general, suppress Th17 development. The concomitant expression of both high levels of type I IFNs and IL-17 in BXD2 mice led us to determine and propose a scheme by which both cytokines may either antagonize or collaborate to effect similar functions. As the following report will state, both type I IFNs, as well as what was previously reported, IL-17 both drive the formation of autoreactive germinal centers. Herein, lies a new model by which type I IFN and IL-17 can collaborate to induce germinal center formation by a “push-pull” mechanism on a subset of immature B cells, currently known, as the marginal zone B cell precursor.

Plasmacytoid Dendritic Cells (pDCs)

History of pDCs

Lennert and Remmele first identified in 1958 a curious cell population in the T cell rich regions of the human lymphoid tissue [48, 49]. This cell population was named T-associated plasma cells, plasmacytoid T cells, or plasmacytoid monocytes because of their resemblance to plasma B cells, the expression of T cell marker CD4, or myeloid cell markers MHCII, CD36, and CD68 [50].

Separately, well established in the literature, was the observation that human peripheral blood is efficient producer to type I IFN upon viral infection [51]. However, there were no known association between this mysterious interferon-producer in the human peripheral blood and Lennert and Remmele’s curious cell population until well
into the ending decade of the 20th century [51]. This mysterious cell population which
was known to produce most of the type I IFNs in blood, especially in response to viruses,
was termed “natural IFN producing cells” because the phenomenon was associated with
the innate immunity, and this field was then known as “natural immunity” [52, 53]. In
1978, Trinchieri et. al. showed that type I IFN can potently boost the cytotoxic activity of
natural-killer cells [54]. The research group also showed that only a very small
percentage (<0.1%) of blood cells was responsible for the production of type I IFN
during exposure to virus infection and virus-infected cells [54]. This distinctive
population was identified as non-T, non-B, non-natural killer (non-NK), non-monocytic
cells expressing low-affinity Fcγ receptors and MHCII antigen [54-57].

Though expressing MHCII, these interferon-producers were not the usual
conventional dendritic cells, expressing low levels of CD11c, MHCII, and have weak T
cell stimulatory functions [58, 59]. In 1994, O’Doherty et. al. identified what was
apparently a subset of CD11c-negative immature dendritic cells expressing only low
levels of MHCII, and displaying weak antigen-presenting capability [60]. Three years
later, Grouard et. al. published the finding of a CD11c−CD4+CD45RA+ cell type located
in the tonsil with a phenotype almost identical to those of the plasmacytoid T cells
characterized earlier [61]. In spite of phenotypic differences from conventional dendritic
cells, both O’ Doherty and Grouard et. al. found that after stimulation with either
monocyte conditioned medium or IL-3 and CD40L, this cell population differentiated
into the typical dendritic cell morphology with interdigitating protrusions [60, 61].
Initially, this cell population, as identified by the aforementioned research groups, was shown to have the functional capacity to induce Th2 polarization through a IL-4 independent mechanism [62]. Because conventional dendritic cells were known to polarize Th1 and were named type I dendritic cell precursors (pDC1), this CD11\(^{-}\)CD4^+CD3^- plasmacytoid cell population was termed type II dendritic cell (pDC2) [62, 63]. However, other research groups reported that human pDC2 can just as well as polarize Th1 [64], making the distinction between pDC1 and pDC2 moot.

Not until 1999, did two research groups observe that the so-called plasmacytoid T cells, monocytes, or pDC2 were one and the same as interferon-producers long observed in peripheral blood [63]. Siegal et. al. isolated pDC2 cells from human peripheral blood and found that upon herpes simplex virus infection, pDC2s secreted large amounts of type I IFN [65]. In the same year, Cella et. al. have made the observation that so-called plasmacytoid monocytes in inflamed lymph nodes generated large amounts of type I IFN [66]. Because of the greater amounts of type I IFN produced by pDC2s compared to other cell types, including conventional dendritic cells and monocytes, the findings have finally linked type I IFN producers to the subset of dendritic cells first identified by Lennert and Remmele almost four decades previously.

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**Phenotype of Human pDCs**

Currently, pDC2s are termed plasmacytoid dendritic cells (pDCs) because of their plasmacytoid morphology, consisting of a large network of endoplasmic reticulum and
Golgi complex. These human pDCs display the following surface expression markers CD4⁺CD45RA⁺IL-3Rα⁺ILT3⁺ILT1⁻CD11c⁻lineage⁻ [63]. More specific markers for human pDCs are the so-called blood dendritic cell antigens, BDCA-2 and BDCA-4, whose expression is restricted to pDCs in the blood and bone marrow. BDCA-2, a C-type lectin transmembrane glycoprotein, internalizes antigens for antigen-presentation, and is a negative regulator of type I IFN production when bound to its respective antibody [67]. BDCA-4 is a neuronal receptor of the class 3 semaphorin subfamily and is also a coreceptor for vascular growth factor A on endothelial and tumor cells. Because BDCA-4 had little to no effect on pDC function, antibody to BDCA-4 can be used to positively select for pDCs for isolation [68].

**Murine pDCs Identified and Phenotype**

In 2001, three different research groups have identified the pDC counterpart in mice [69-71]. Murine pDCs have most of the same plasmacytoid morphological features, phenotypic, and functional characteristics found with their human counterparts. L-selectin is an important glycoprotein for pDC extravasation across the high-endothelial venules in lymph nodes. Nakano et. al observed that among the CD11c⁺DCs, there was a reduced percentage (=85%) of CD11c⁺CD11b⁻Gr⁻1⁻B220⁻CD19⁻ cell population. These cells displayed the typical morphological features found in the human pDCs, including an extensive endoplasmic reticulum made for cytokine secretion and the development of dendritic cell-like protrusions upon stimulation (Fig. 1). They were found to be efficient
Figure 1. Morphology of pDCs.
(a) By electron microscopy, pDCs appear as lymphoblasts with a well developed rough endoplasmic reticulum. (b) Resting pDCs have a spherical shape (c), whereas CD40L-activated pDCs have a dendritic cell-like morphology.

Grouard, G. et al. J. Exp. Med. 185, 1101–1111
type I IFN-producers, therefore concluding among the first reports of the existence of murine pDCs [69].

As mentioned above, murine pDCs express a different set of surface antigens than do their human counterparts. Murine pDCs express surface antigens B220 and Ly6C [69]. The RB6-8C5 (GR1) antibody, which has specificity for Ly6G, is used to label for pDCs because it cross-reacts with Ly6C [69-71]. Like their human counterparts, murine pDCs express low levels of CD11c compared to conventional DCs. Upon stimulation in culture by antigens, activated pDCs can up-regulate expression MHCII and costimulatory molecules [70].

Three antibodies are currently utilized to identify and deplete/enrich for murine pDCs. The 120G8 antibody specifically binds to a surface antigen on pDCs, whose expression is increased upon type I IFN stimulation [72]. The 120G8 antibody has been shown to effectively deplete pDCs in vivo. The 440c antibody binds to antigen on pDCs that does induce cell death, but does down-regulate type I IFN production [73]. The murine plasmacytoid dendritic cell antigen (mPDCA-1) antibody can specifically identify pDCs and deplete pDCs in vivo by binding to a surface antigen [74, 75].

The Myeloid and Lymphoid Origins of DCs

DCs are a heterogeneous population of cells with efficient antigen-presentation as a defining feature. Conventional DC (cDC) can be divided into subsets according to tissue distribution: Langerhan cells in the epidermis, dermal DCs in the dermis, mucosal
Figure 2. A Developmental scheme of mouse DC development from bone marrow hemopoietic stem cells and lineage-committed progenitors. Flt3 expressing early myeloid or lymphoid progenitor cells can give rise to both cDC and pDCs. Abbreviations: HSC, hemopoietic stem cell; MPP, multipotent progenitors; CMP, common myeloid precursors; CLP, common lymphoid precursors; GMP, granulocyte and macrophage precursors; MEP, megakaryocyte and erythrocyte precursors; M-DC, macrophage and DC precursors; Flt3L, Flt3 ligand. 
tissue-associated DCs, lymphoid tissue-associated DCs, including those in the splenic marginal zone, T cell zone in the follicle, germinal center, and thymus.

The cDCs were thought to have derived from a common myeloid precursor. Culturing mouse bone marrow myeloid precursors in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) produced macrophages, granulocytes, and DCs [76]. Also, monocytes were able to differentiate into DCs in the presence of GM-CSF and IL-4 [77-83]. More definitive conclusions about the myeloid-origins of DCs were confirmed when murine bone marrow common myeloid progenitors reconstituted conventional DCs in the spleen and thymus of irradiated recipients [84, 85] (Fig. 2).

While the myeloid origin of DCs is firmly established, other studies have observed that cDCs can also derive from common lymphoid precursors (Fig. 2). Thymic conventional DCs and populations of lymphoid organ-resident DCs in the spleens and lymph nodes express markers commonly found on lymphocytes, such as CD4, CD8, and CD25 [86]. Transferring intrathymic lymphoid precursors into the thymus of irradiated recipients, research groups have observed thymic development of T cells and conventional DCs [87, 88]. When intravenously injected, lymphoid precursors gave rise to both CD8+ and CD8- cDCs in the spleen [89, 90]. Other studies have firmly confirmed that cDCs, those in the spleen and thymus, can very well also arose from bone marrow common lymphoid precursors [91, 92] (Fig. 2).
The Lymphoid Origin of pDCs?

Human pDCs express many markers found in cells of lymphoid origin, such as germline IgK and pre-T cell receptor [61, 62]. Other research groups have identified the expression of the Rag gene in murine pDCs and can initiate IgH D-J rearrangements [93-95]. Further confirmation of the lymphoid origin of pDCs was shown when overexpression of the dominant-negative transcription factors Id2 or Id3 in human CD34+ hematopoietic progenitor cells blocked the development of pDC, T cells, and B cells, but not cells of myeloid DCs [96]. Human CD34+ hematopoietic progenitor cells with Spi-B deletion, a known transcription factor that is expressed in lymphoid cells, had definitively suppressed pDC development [97]. Together, these studies point to a lymphoid origin for pDCs (Fig. 2).

However, several studies have confounded the neat classification of pDCs as strictly derived from bone marrow common lymphoid precursors. Concurrent with the studies substantiating the lymphoid origin of pDCs, research groups have identified that the transplantation of mouse bone marrow common myeloid precursors into irradiated recipients reconstituted cDCs as well as pDCs [84, 85, 91]. Also, D’Amico and Wu et. al. have found that common myeloid precursors consist of variable expressions of Flt3. The Flt3+ myeloid precursors can develop into cDCs, pDCs as well as B cells [98]. Speculation arises as to the existence of a common Flt3+ precursor that give rise to both pDCs and B cells [99].

Therefore, given the above findings, the origin of pDCs, like their conventional counterparts, cannot be easily defined as either myeloid or lymphoid. Rather, the current
understanding is that the developmental pathways remains fairly plastic, and the myeloid-lymphoid dichotomy is not applicable to pDC lineage development (Fig. 2).

Toll-like Receptors

The discovery that viruses and viral particles can trigger the production and secretion of type I IFNs by specialized cells, which were termed natural-interferon producers (NIPC) before the name “plasmacytoid dendritic cell” was given to them, must have bolstered the begging question as to how viral antigens can interact with surface receptors or the internal cellular machinery to initiate type I IFN expression. Toll-like receptors formed one crucial component of the front-line interface between the cellular signaling pathway for type I IFN signaling induction and the external stimuli which triggered it.

Toll-like receptors are in effect homologues of the toll receptors identified in Drosophila as key molecules in developmental patterning and immunity against fungal and bacterial infections [126-128]. Homologues of the toll receptors, named the Toll-like receptors (TLRs), were identified in mammals. A set of TLRs were discovered, including TLR1, 2, 3, 4, 5, 6, 7, 8, and 9. In humans, TLR1, 2, 3, and 6 map to chromosome 4 [129-131]. TLR4 and 5 map to chromosome 9 and 1, respectively [130]. TLR7 and 8 are located in chromosome X, and TLR9 maps to chromosome 3 [132]. Toll-like receptors are characterized by their leucine-rich repeats in the extracellular domain, and a Toll/IL-1 Receptor (TIR) intracellular domain [129].
Figure 3. Toll-like receptors, adaptors, and PAMPs

TLRs contain TIR domains, which binds to TIR domains-containing adaptors, including MyD88, TIRAP (TIR-containing adaptor protein), TRIF (TIR-containing adaptor-inducing IFN-β), or TRAM (TRIF-related adaptor molecule). TIRAP is utilized by TLR1, TLR2, TLR4, TLR6 to link the TLR to MyD88. TLR3 only utilizes TRIF. TLR4 uses both TRIF and TRAM, and TRAM is essential to linking TLR4 to TRIF. MyD88 contains a TIR and a death domain (DD). TLR5, TLR7, TLR9 uses MyD88-dependent signal transduction.

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TLRs interact with a vast array of microbial and viral antigens by recognizing commonly-expressed molecular patterns on the external antigens, which are termed pattern-associated molecular patterns (PAMPs) (Fig.5). Because this class of receptors does not adapt themselves to any specific antigen by mutation or gene rearrangements, such as is found for antibodies, and T-cell receptors, but rather recognize evolutionarily conserved molecular patterns on viral and other microbial agents, it is deemed part of the innate immune system. Yet, TLRs are also the bridge to the adaptive immune system because various TLRs can signal the expression of cytokines, such as IL-12 and IL-23, both of which are intimately involved in the adaptive response. In both humans and mice, TLR 2, 3, 4, 5, 6, and 8 are selectively expressed on cDCs and monocytes, while TLR 7 and 9 are selectively expressed on pDCs [129, 133-138]. With the exception of TLR 3, 7, and 9, which are localized in endosomes, the other TLRs expressed on conventional DCs and monocytes are plasma transmembrane receptors [129]. Each of the TLRs has specificity for the different motifs presented by bacterial or viral protein and nucleic acids. TLR2 dimerizing with TLR6 interacts with glycolipids and lipoproteins from a vast array of bacterial and fungal invaders [129]. TLR4 interacts with the gram-negative bacterial product lipopolyssacharide (LPS), and TLR 5 binds to flagellin [129]. In constrast, TLR3, 7, 8, and 9 are the intracellularly located endosomal nucleic acid-sensing TLRs [129]. Fc-receptor mediated phagocytosis of nucleic antigens, usually of viral origins, leads to antigen compartmentalization into endosomes containing the intracellular TLRs. Subsequently, the endosomal TLRs then bind to the nucleic acid antigens and initiates their signaling. Nucleic acid-sensing receptors TLR3, 7, 8, and 9 are part of the innate immune repertoire against viruses. TLR3 binds to double-stranded
RNA (dsRNA). TLR7 and 8 binds to single-stranded RNA (ssRNA) in humans. In mice, TLR8 is not functionally expressed and remains a pseudogene. TLR9 recognizes hypomethylated double-stranded DNA (dsDNA) containing the CpG motif. Because single or double-stranded RNA, and unmethylated DNA are commonly found components in viruses, the intracellular TLRs 3, 7, and 9, can transducer signals to initiate an a rapid immune response against viral assault on the host.

*Endosomal TLRs*

TLRs are important receptors to signal for type I IFN expression. TLR3, 7, 8, and 9 are endosomal TLRs. They are transmembrane receptors with their ligand-binding domains in the endosomal space, and signal transduction domains facing outwards into the cellular cytoplasm. Fc-receptors on the surface of dendritic cells or macrophages bind to antibody-bound antigens, containing nucleic acids, such as single- or double-stranded RNA or DNA. The Fc-receptor bound antigen immune complex is brought inside the cell by endosomal engulfment. Inside the endosome, the nucleic acid-containing antigen (e.g. ribonucleic proteins) is brought to close proximity to the endosomal TLRs. As mentioned, double-stranded RNAs are recognized by TLR3, single-stranded RNAs by TLR7, and DNA by TLR9.

All TLRs contain a TIR domain, which recruits a host of factors and kinases. TLR3 initiates its signaling by recruiting a TIR-domain-containing adaptor inducing IFN-β (TRIF) [139, 140]. TRIF then recruits TRAF3 and TRAF6 by its TRAF-binding motifs in its N-terminus and receptor interacting protein (RIP)1 and RIP3 by its RIP homotypic interaction motif (RHIM) [141, 142]. TRAF6 and RIP1 activate NF-κB. TRAF3 activates
TRAF family member-associated NK-κB activator (TANK)-binding kinase 1 (TBK1) and inducible IκB kinase (IKK-i), by which IRF-3 is activated [143]. Activated IRF-3 can translocate in the nucleus and initiate IFN-β expression.

Both TLR7 and -9 utilize their own TIR-containing domain adaptor, myeloid differentiation factor 88 (MyD88), which consists of a TIR domain and a death domain (DD). Upon binding with its ligand, MyD88 binds to interleukin-1-receptor (IL1R)-associated kinase-4 (IRAK-4), IRAK-1, tumor necrosis factor receptor associated factor 3 (TRAF3), TRAF6, Ikκα, and IRF-7 [144-146]. This MyD88-IRAK1/4-TRAF3/6 complex activates IRF-7, thereby starting transcription of IFN-α gene [143]. In non-pDCs, which we will discuss below, the expression of IRF-7 is absent. Thereby, in non-pDCs, TLR3 signaling pathway-initiated IRF-3 dependent IFN-β expression serves as the first step in a biphasic mechanism to drive a IFNAR-initiated JAK-STAT dependent ISGF3-mediated type I IFN and IRF-7 expression.

Type I Interferon (IFN)

The key term “interferon” was first coined in 1957 when Issacs and Lindenmann published their findings on the widely-known phenomenon of “viral interference” [100]. After exposure to one virus in culture, resistance is conferred upon the cell culture upon exposure to a second virus by a hypothesized mechanism in which component of the first virus mediated resistance to the second virus. This was at least the hypothesis of the two investigators. Their conclusion in their 1957 study, however, was that a soluble factor
Table 1. The Interferons (IFNs) and IFN-like proteins

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Alternate names</th>
<th>Human gene locus</th>
<th>Receptor chain 1</th>
<th>Receptor chain 2</th>
</tr>
</thead>
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<tr>
<td>Type I IFN</td>
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</tr>
<tr>
<td>IFN-α</td>
<td>IFN-α</td>
<td>9p21+3 (T)</td>
<td>IFN-αR1</td>
<td>IFN-αR2</td>
</tr>
<tr>
<td>IFN-β</td>
<td></td>
<td>9p21+3 (T)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-δ</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-ε</td>
<td></td>
<td>9p21+3 (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-κ</td>
<td></td>
<td>9p21+1 (T)</td>
<td></td>
<td></td>
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<tr>
<td>IFN-τ</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IFN-ω</td>
<td></td>
<td>9p21+3 (T)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II IFN</td>
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<td></td>
<td></td>
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<tr>
<td>IFN-γ</td>
<td>IFN-γ</td>
<td>12q14+3 (C)</td>
<td>IFN-γR1</td>
<td>IFN-γR2</td>
</tr>
<tr>
<td>IL-28A</td>
<td>IFN-λ2</td>
<td>19q13+2 (T)</td>
<td>IL-28R1</td>
<td>IL-10R2</td>
</tr>
<tr>
<td>IL-28B</td>
<td>IFN-λ3</td>
<td>19q13+2 (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-29</td>
<td>IFN-λ1</td>
<td>19q13+2 (T)</td>
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</tbody>
</table>

The Type I IFN members are clustered together on chromosome 9, type II on chromosome 12, and IL-28A, IL-28B, and IL-29 are clustered together on chromosome 19. The columns from left to right indicate standard names for the ligand or ligand family, alternate names for the ligand (or major subgroups of the Type I IFN family), the location of its gene in the human genome, the components of its receptor complex, and signal transduction pathways. Locations of only the human genes are indicated. Mouse and rat homologs of the human genes are generally found on the respective syntenic chromosome locations in the mouse and rat genomes. Chicken IFN I and IFN II are found on the male sex chromosome. The designation (C) indicates transcription occurs toward the centromere, whereas (T) indicates transcription occurs away from the centromere. Although most IFN-α genes are transcribed toward the telomere, some are transcribed toward the centromere.

Data were obtained from the following web site:
produced by the cell culture after exposure to the first virus conferred resistance to the second virus. This resistance was transferable because the soluble factor was able to confer viral infection in uninfected cell cultures.

*Classes and Isoforms in the Type I IFN Family*

There are seven classes of type I IFN: IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ [101]. IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω are expressed in humans, whereas IFN-δ and IFN-τ are trophoblast interferons expressed in porcine and ovine, or bovine conceptus, respectively, for implantation during gestation [102, 103]. There are thirteen isoforms of IFN-α Table 1. The interferons (IFNs) and IFN-like proteins in humans, with all their genes clustered on the short arm of chromosome 9 (9p21) [104-106]. There are 12 different IFN-α with IFNA1 and IFNA13 genes encoding the identical protein. The IFN-α cytokines shares 76 to 99% of amino acid sequence. There is one functional human isoform of IFN-β and IFN-ω [101]. Similar to other type I IFNs, human IFN-κ display antiviral activity, but is expressed in keratinocytes [107]. In mice, the type I IFN family includes IFN-α, IFN-β, and IFN-ε [108]. The type I IFN genes are localized on chromosome 4. There are fourteen functional Ifna genes. Antibodies specific to each of the isoforms of IFN-α has not been generated, therefore at least at the protein level, the relative abundance of these isoforms in pDCs have not been well determined [109]. However, there are likely functional differences amongst the IFN-α isoforms. For instance, IFN-α1 displays only weak antiviral activity. Unlike the other isoforms, which strongly activate natural killer cell cytotoxic activity, IFN-α7 does not activate natural
killer cells, but instead, even exhibit antagonist function by blocking other IFN-α isoforms from activating natural killer cells [110]. IFN-ε is not well characterized, however, may be involved primarily in reproductive function because its human and murine promoters regulate endocrine expression function and it is constitutively expressed in the murine placental and ovarian tissues [108]. In sum, mice do not express IFN-ω and IFN-κ. IFN-τ is found in only ungulate ruminants and involved in maternal reproduction [111].

Type II IFN family only includes IFN-γ, which primarily mediates Th1 and intracellular cytotoxic immunity. Recently, a type III IFN class of cytokines has been identified in humans, whose genes are clustered on chromosome 19 [112]. IL-28A (IFN-λ2), IL-28B (IFN-λ3), and IL-29 (IFN-λ1), exhibit properties displayed by type I IFNs, including induction by viral infections to initiate antiviral activities [101, 113]. However, like type II IFNs, type III IFNs signal through their own distinct set of receptors [101, 113].

Receptors and Signal Transductions for Type I IFN

Type I, II, and III IFNs share the same JAK-STAT transduction pathway. However, they differ in the sets of receptors. Type I IFNs requires two receptor subunits,
IFN-α is a monomer that binds to the type I IFN receptor complex, which consists of IFN-αR1 and IFN-αR2. Upon binding to complex, IFN-α initiates Stat2 binding to the IFN-αR2 chain and recruits Stat1. Stat1 and Stat2 are phosphorylated, released, associates with p48, and translocates to the nucleus to induce gene expression. The type III IFNs have a similar mechanism for signal transduction except the interleukin-28 (IL-28)/IL-29 receptor complex consists of IL-28R1 and IL-10R2 chains instead of the IFN-αR1 and IFN-αR2c chains, respectively.

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**Figure 5. Interferon-γ (IFN-γ) receptor complex signaling.**

IFN-γ is a dimer that binds to the type II IFN receptor complex by binding directly to the IFN-γR1 chains but not directly to the IFN-γR2. In the absence of the IFN-γR1 chains, IFN-γ does not bind to the receptor. The two receptor chains are pre-assembled prior to ligand binding. After ligand binding, the Jak kinases cross-phosphorylate each other (solid circles), which in turn phosphorylate tyrosine 457 of each IFN-γR1 chain. This phosphorylation becomes the recruitment site for Stat1α, which in turn attaches to phosphotyrosine Tyr457 of IFN-γR1, moves the receptor chains apart and is phosphorylated by the Jak kinases. The phosphorylated Stat1α proteins detach from each IFN-γR1 chain, forming the transcription factor that is translocated to the nucleus to activate IFN-γ regulated genes.

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IFNAR1 and IFNAR2c [101, 114-119]. Type II IFN receptor has the IFNGR1 and IFNGR2 subunits [101, 115, 120-122], and type III IFN receptors requires IL-10R2 and IL-28R1 subunits [101]. IFN-α monomer recruits single subunits IFNAR1 and IFNAR2 with pre-bound tyrosine kinases Tyk2 and JAK1, which now in close proximity, cross-phosphorylates, and becomes active kinases. Active Tyk2 and JAK1 phosphorylates Y466 of IFNAR1, generating docking site for the SH2 domain of STAT2. Phosphorylation of STAT2 in turn recruits STAT1. The STAT21-STAT2 heterodimer is then released into the nucleus, and in conjunction with p48, forms the interferon-stimulated gene factor 3 (ISGF3). This protein complex then binds to interferon-stimulated regulator elements (ISREs) in initiate type I IFN gene transcription [101, 123] (Fig. 3).

Type II IFN, IFNy, are homodimers, which bind to homodimers IFNGR1 and IFNGR2, to form a 4-subunit receptor complex [101] (Fig. 4). The recruitment of IFNGR1 and IFNGR2 brings JAK1 and JAK2 pre-bound kinases to together for trans-phosphorylation and activation, a process which creates a docking site for STAT1 recruitment. Homodimer STAT1 is then released into the nucleus for initiation of transcription. In the case of type III IFN signaling, STAT3 and STAT5 had been reported to be involved in addition to JAK1, Tyk2, STAT1, and STAT2 [101, 124, 125].
Interferons are key initiators of the antiviral response, and so whose response is carefully regulated by upstream regulatory factors. Appropriately named interferon regulatory factor (IRF), IRF1 was first discovered for its capacity to induce IFN-β expression [147]. Subsequently, structurally similar IRF-2 was identified with repressor functions [148]. In all, nine IRFs were identified. IRF-1 is an activator of IFN-β transcription, and has a half-life of approximately only 30 minutes [149]. In contrast, IRF-2, has a half-life of at least 8 hours, indicative of its repressor function [149]. IRF-2 knock-out in primary murine embryonic fibroblasts significantly increased IFN-α/β expression [150]. Both IRF-1 and -2 expression are increased in response to viral infections, and/or IFN stimulation [148, 151], as evidenced by the absence of IRF-1 expression in STAT-deleted cells, and a ISGF3 bind site in the IRF-2 promoter region [152-154]. IRF-3 and -7 are critically important in type I IFN expression, which will be discussed further below. IRF-4 is expressed in T and B cells and not induced by type I IFNs. Deletion of IRF-4 in murine hosts significantly impaired lymphocyte development, such as B cell light chain expression and rearrangements [155]. The functions of IRF-5 and -6 apparently are not well defined, although IRF-5 is induced by type I IFNs, suggesting its association with the regulation of type I IFN system [155]. IRF-8, unlike the other IRFs is expressed only in response to IFN-γ, but not type I IFNs [155]. IRF-8 deletion in macrophage impairs IFN-γ responsive genes, such as iNOS and FcyRI [151]. IRF-9 (p48), as mentioned previously, is an essential component of the trimolecular complex ISGF3 to initiate transcription of type I IFN and type II IFN-inducible genes.
Figure 6. Operation of a positive feedback mechanism

Early Phase: Viral replication intermediate dsRNA is bound by helicase domain of RIG-1 (retinoic acid-inducible gene) and MDA5 (melanoma differentiation-associated gene 5), two cytosolic pattern recognition receptors, unwinding the dsRNA with concomitant conformational change for RIG-1 and MDA5. This conformational change promotes the interaction between RIG-1 and MDA5 and mitochondrial IPS-1. TBK1 is activated via TRAF3 and TRAF6. Activated TBK1 induces phosphorylation of IRF3 and IRF7, resulting in their homo- and heterodimerization, leading to nuclear translocation and IFN-β expression. Late Phase: Secreted IFN-β signals via type I IFN receptor in autocrine and paracrine manner, leading to activation of ISGF3 (heterotrimer of STAT1, STAT2, IRF9) and the IRF7 gene transcription. Activation of IRF7 leads to further amplification of IFN-β and IFN-α expression.

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IRF-9 serves as the anchor which draws STAT1 and STAT 2 together to form the transcription factor ISGF3 [155].

IRF-3 and IRF-7 are two well-known factors involved in type I IFN expression. Both factors are ubiquitously expressed in all tissues and cells. However, IRF-3 is constitutively expressed and its expression is not affected by viral infection or IFN treatment [156]. IRF-7 is not constitutively expressed in most cells and is only induced in response to type I IFN [155]. IRF-3 was found to locate in the cytoplasm, but upon viral exposure, the factor translocates into the nucleus [157-160]. IRF-3 is phosphorylated in the nucleus and binds to co-activator CBP/p300, initiating IFN-β transcription. Over-expression of IRF-3 leads significant increase in IFN-β mRNA levels [157]. Ectopic expression of IRF-7 upregulated the expression of both IFN-α and IFN-β, however, IRF-3 over-expression primarily increased IFN-β expression [161]. Mice with null mutation in IRF-3 alleles did not resists viral infections very well, and IFN-α/β mRNA levels were significantly attenuated [162]. IRF-3 and IRF-9 knock-outs in murine embryonic fibroblasts abrogated IRF-7 and IFN-α/β levels [162]. Restoring the type I IFN system did not occur until both IRF-3 and IRF-7 expressions were reconstituted [155]. Together these findings point to a biphasic program by which type I IFN is expressed in response to viral exposure. The first and rapid phase initiated by a viral antigen assault involves the rapid translocation of IRF-3 into the nucleus. IFN-β levels, on both the message and protein levels, rise quickly. IFN-β mediated IFNAR signaling by JAK-STAT pathway to initiate ISGF3-dependent IRF-7 transcription, which then further maintains and amplifies the type I IFN expression by induction IFN-α expression [155] (Fig 6).
Intracellular Signaling Pathway for Type I IFN Production in pDCs

Type I IFNs are synthesized de novo, as evidenced by the finding that while protein synthesis inhibitor cycloheximide can block the appearance of IFN-α and IFN-β mRNAs, adding the protein synthesis inhibitor 4 hours after type I IFN induction did not block type I IFN message levels [163]. The molecular pathways by which pDCs directly synthesize such interferon proteins de novo is outlined below.

In both humans and mice, pDCs selectively express TLR7 and 9. TLR 9 is also expressed on conventional DCs and B cells in mice [129]. However, only pDCs in both humans and mice possess the unique ability to generate massive amounts of type I IFN in response to TLR7 and 9 signaling [129]. Compared to other cell types, pDCs secrete 10-1000-fold more type I IFN, including IFN-α and –β [63]. As mentioned above, in non-pDCs, type I IFN production appears to stem from a biphasic mechanism [164]. TLR 3 and 4-signaling activates IRF3 via a MyD88-independent pathway [165, 166]. MyD88 is a critical adaptor protein that is recruited to all the TLRs except TLR3 upon ligand binding to the receptor [165]. MyD88 knock-out mice abrogates all TLR signaling except TLR3 and 4, which have a MyD88-independent pathway. Upon phosphorylation, IRF3 dimerizes, relocates into the nucleus and recruits a multi-protein complex consisting of co-activator CBP and p300 [164]. IFN-β and IFN-α4 transcription is initiated [164]. These early type I IFN cytokines signal via their receptor in an autocrine loop, activating the JAK-STAT pathway and leading to IRF-7 expression. IRF-7 establishes a positive feedback loop which then induces the bulk of the type I IFN cytokines secreted [161,
Figure 7. TLR-mediated type I IFN induction pathways

(A) The receptor complex TLR4-CD14-MD-2 binding to LPS recruits the adaptor proteins Trif and TRAM, leading to activation TBK1, which in turn induces IRF-3 phosphorylation and translocation in the nucleus to induce IFN-β. (B) TLR3 binding to dsRNA leads to a similar Trif-TBK1 mediated IRF3-dependent up-regulation of IFN-β, though PI3K is also recruited to enhance signaling (C) Upon TLR7 or TLR9 stimulation, MyD88 is activated by the IRAK4-IRAK1-Ikkα kinase cascade, leading IRF7 upregulation, which in turn by a type I IFN-dependent positive feedback loop induces augmented type I IFN expression. (D) Type II IFN upregulate IRF1 expression by STAT1-dependent mechanisms to activate MyD88-dependent IRF1 transcription of interferons and other cytokines; ?: are unknown mechanisms and factors involved in pathway; 1: IRF1, 3: IRF3, 7: IRF7

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Fig. 7
This biphasic kinetics appears not to present itself in pDCs. Since pDCs lack TLR3 or 4, and selectively express TLR 7 and 9, we expect to see a different mechanism in inducing type I IFN expression [166]. Unlike other cell types, type I IFN production in pDCs is not IRF-3 dependent because IRF-3 knock-out pDCs do not show any significant change in type I IFN production from wild-type. In contrast, IRF-7 knock-out pDCs are almost completely incapable of generating type I IFN [169]. Confirming the importance of IRF-7 in pDCs is the observation that IRF-7 is constitutively expressed in pDCs, whereas in non-pDCs, IRF-7 is not always present in significant amounts [170-172]. The availability of direct IRF-7 mediated type I IFN expression in pDCs, thereby circumventing the biphasic process in non-pDCs, may offer an explanation for the more efficient production of type I IFNs. Deleting IFNAR in pDCs did reduce type I IFN production in response to viral exposure, but did not completely suppress it, indicating that a biphasic-independent mechanism is still at play in these cells [173, 174].

However, IFNAR deletion in pDCs produced only very small amounts of type I IFNs in vivo in response to TLR9 ligand CpG, suggesting that biphasic mechanism may still be important for type I IFN production. In addition, a number of observations found that cells that normally do not constitutively express IRF-7 were able to express IRF-7 to levels even higher than that observed in pDCs in response to viral infections [166]. Yet, these cells do not express type I IFNs at levels observed for pDCs [166]. The identification of type I IFNs as priming agents that further boost type I IFN production supports a biphasic model for type I IFN expression [175].
Aside from constitutive expression of IRF-7 as a possible mechanism for high type I IFN expression in pDCs, pDCs also have the ability to retain hypomethylated CpG oligonucleotides in their endosomes for prolonged periods [176]. Using Cy5-labelled unmethylated type A CpG (also named type D; CpG ODN 2216), containing poly-G motifs at the 5’ and 3’ ends with CpG motif at the oligonucleotide center, the study demonstrated that pDCs were able to retain the CpG ODN 2216, a known TLR9 ligand, in the endosome longer than conventional DCs [176]. In contrast to the pDCs, conventional DCs rapidly sent the TLR9 ligand to the lysosome for degradation [176]. Prolonged retention in the endosome allows for stronger affinity between the TLR9 and its ligand, thereby inducing a stronger MyD88-dependent signal to induce type I IFN expression and secretion [176]. Through this mechanism, researchers speculate this is the true reason for stronger type I IFN expression in pDCs [63, 166, 176]. The various pathways for type I IFN expression is illustrated (Fig. 7)

Type I IFN in SLE

Type I IFNs is associated with lupus pathogenesis. Serum IFN-α correlated positively with SLE severity [177, 178], and peripheral blood mononuclear cells from SLE patients when stimulated in vitro produced elevated levels of IFN-α compared to healthy subjects (36-38). Microarray analysis of SLE patients, exhibit a distinct “type I IFN signature” because type I IFN-inducible genes are up-regulated in their peripheral blood mononuclear cells [179, 180]. Bennett et. al. found that out of 30 pediatric patients with SLE tested, 29 displayed the type I IFN signature [179]. This conclusion is in
contradistinction to the 10 out of 20 SLE patients whose serum was detected with high levels of IFN-α [181]. The authors attributed this apparent contradiction to the limited specificities and sensitivities of the IFN-α ELISA antibodies utilized for detection of the cytokine [179]. Furthermore, SLE patients are known to have anti-IFN antibodies in their serum that may interfere with ELISA measurements [182]. The apparently more sensitive microarray to measure type I IFN-inducible genes offers a more conclusive evidence that type I IFNs are prominent cytokines elevated in SLE patients. Similary, this conclusion was reached by other research groups. Significant positive correlation was found between the levels of type I IFN-inducible genes and the severity of SLE in patients [183, 184].

Though there are elevated levels of serum IFN-α and greater amounts of increased type I IFN-inducible genes in the peripheral blood of SLE patients, in patients with SLE disease, there were reduced counts of pDCs [181, 185]. One study has even found that the residual pDC remaining in the blood produced only normal levels of 5-10 pg of IFN-α per cell after herpes simplex virus stimulation in vitro [185]. The research literature currently hypothesizes that this seemingly contradictory finding in the peripheral blood in SLE patients may be explained by pDC migration into the secondary lymphoid organs and inflamed skin lesions [175]. Evidence for this in SLE patients include the finding that pDCs and cells actively producing IFN-α are identified in the cutaneous lupus erythematosus lesions [186, 187].

IFN therapy against viral infections (Hepatitis C) and for cancer patients had shown that treatment, especially long-term, can induce autoantibody formation in 4-19% of patients, and drive the onset of lupus-like symptoms, including the facial rash typically
found in SLE patients [188]. Conversely, steroid treatment, such as glucocorticoids, a tried and routine treatment method for lupus flares, suppressed the type I IFN signature [179]. Steroid administration is known to decrease the counts of pDCs in human blood and their ability to produce IFN-α [189, 190].

In sum, the the bulk of clinical data and observations supports the important association between type I IFNs and SLE patients. The fact that type I IFN therapy can induce the onset of lupus-like symptoms and autoantibodies, such as anti-DNA, indicate that type I IFNs are the causative factors for lupus disease.

*The Role of Type IFNs in Murine Models of Lupus*

A number of murine models of lupus have demonstrated the importance of type I IFNs in disease onset and maintenance. The New Zealand Black (NZB) mouse, a well-known lupus model, was found to have high levels of IFN-α after poly I:C or CpG treatment [191, 192]. In accordance to the human data, two important mouse studies confirm IFN-α enhances lupus. Deletion of IFNAR in NZB mice by backcrossing with Ifnar-/- Sv129 mice obviated the spontaneous development of serum autoantibodies, immune complex deposition in the glomeruli, glomerulonephritis, prolonged host survival [191]. The injection of poly I:C, a potent TLR7 ligand which induces IFN-α production, into lupus-prone B6.lpr, exacerbated glomerulonephritis and increased serum autoantibodies [193]. In contrast, after introducing Ifnar null mutation into B6 lpr, there
were a dramatic reduction in immune complex deposition in the kidneys and reduced lymphadenopathy [193].

Genetic-predisposing background may be important in IFN-α induced lupus. By injecting adenoviral delivery vector for IFN-α into preautoimmune (NZBXNZW)F1, sera dsDNA autoantibodies appeared as soon as 10 days after cytokine administration [194]. The mice developed proteinuria and death caused by glomerunephritis within 9 and 18 weeks, respectively [194]. In contrast, identical cytokine administration into non-lupus prone mice did not elicit disease [194].

However, murine models studying the role of type I IFNs in lupus do give mixed results. While studies have confirmed the importance of type I IFN in eliciting lupus, other studies arrive at opposite conclusions. In B6.sle2 congenic mice, antibody-mediated IFN-α blockade increased sera autoantibody levels and B-cell hyperactivity, while IFN-α administration protected the mouse from autoimmunity [195]. IFNAR-/- lupus-prone congenic MRL/lpr mouse developed severe lymphoproliferation, lymphadenopathy, renal disease, and elevated sera autoantibodies [196]. The apparent contradiction may because of the distinctive pathogenic mechanisms found in the lpr mice and/or the narrow functional specificity of the sle 2 gene.

**IFN-α-Inducing Factor (II-F) in SLE Patient Serum**

The findings that type I IFN is highly associated with human SLE and may serve as a causative factor for disease induction have led researchers to determine the cellular
and molecular pathways by which type I IFN may induce immune tolerance loss. Earlier studies focused on finding the elusive factor in the sera of SLE patients that induce IFN-α production. Co-cultures of human SLE serum with healthy peripheral blood cells have led to some interesting conclusions. As much as 20% of sera from SLE patients were found to induce IFN-α from healthy peripheral blood mononuclear cells [197]. However, none of the sera from healthy patients could induce IFN-α from healthy peripheral blood mononuclear cells [197]. Supplementation with IFN-α2 and GM-CSF induced IFN-α levels in all co-cultures [197]. However, IFN-α production by SLE sera was enhanced by 5-100 times, with about 3-fold higher levels compared to healthy sera [197].

The mystery factor, termed IFN-α-inducing factor (II-F), was identified to be immune IgG complexes containing unmethylated nucleic acids. Study has found that the presence of anti-DNA antibody correlated positively with SLE-II-F [198]. Purified anti-DNA antibody, SLE-IgG, or plasmid DNA did not induce IFN-α production from the peripheral blood mononuclear cells of SLE patients [198]. However, when DNA plasmid is complexed to either anti-DNA antibody or SLE-IgG, the coupling was able to induce IFN-α to levels comparable with that observed in sera containing SLE-II-F [198]. IFN-α production was boosted by supplementation with IFN-α2b, IFN-β, and GM-CSF [198].

Ribonucleic acids bound to IgG immune complexes may also induce IFN-α production from SLE peripheral blood mononuclear cells. Using apoptotic cellular materials from four cell lines and complexing each to SLE anti-ribonucleoprotein IgG antibody, IFN-α production was stimulated from peripheral blood mononuclear cells [199].
Type I IFN-Induced Immune Tolerance Loss

A variety of mechanisms have been put forth to explain the role of type I IFN in autoimmune disease. Most of these purported mechanisms, unfortunately, to date, have been circumstantial, and are derived from in vitro or non-autoimmune conditions. In vitro studies, have found that pDCs can differentiate into conventional DCs [61]. Type I IFN can expand memory cytotoxic CD8 T cells in response to certain antigens [200]. In humans, pDCs can promote IL-4 independent Th2 differentiation [62]. In mice, pDCs are known to promote IL-12 and IFN-α dependent Th1 differentiation [135, 201]). However, in humans, IFN-α is not sufficient to promote Th1 polarization due to absent stable T-bet expression [202]. In vitro experiments, have also shown that there is an increased frequency of monocyte-derived dendritic cells, and that IFN-α can promote the differentiation of monocytes into dendritic cells [181].

A boost in antibody response to antigen-immunization and antibody isotype class-switch was observed after administration of IFN-α administration [203]. By targeted knock-out of IFNAR on B-cells using a Cre-lox approach, germinal centers and serum antibodies were suppressed even after antigenic stimulation with IFN-α [204]. Using T cell bone marrow chimeras generating IFNAR-/- T cells, sera antibody levels were similarly suppressed, suggesting that IFN-α enhances CD4+ T cell help in mediating the humoral response [204]. Other studies found that type I IFNs promote plasma cell differentiation and germinal center formation, again, under non-autoimmune conditions. In vitro culture of pDCs was observed to promote IFN-α- and IL-6-dependent plasma cell differentiation [205]. Viral infections of type I IFN receptor-deleted non-autoimmune
murine models were found to induce fewer germinal center reactions, although whether type I IFNs directly drove germinal center formation was not elucidated [206, 207].

In summary, given the available data from a variety of quite disparate sources, an emerging picture of type I IFN in lupus is proposed. Type I IFN production can generate and enhance Th1 responses, thereby generating increased cellular debris that can serve as a useful reservoir of self-antigens. Type I IFN can enhance monocyte differentiation into dendritic cells, thereby enhancing antigen-presentation of self-antigens. Type I IFN signaling B cells can drive the formation of plasma cells. In our study, we have some evidence, as will be presented, in which direct type I IFN signaling on B cells can push B cells into germinal centers in an autoimmune context.

*Type I IFN and T-Dependent Humoral Responses*

Studies from Le Bon et al. have demonstrated that type I IFN can boost antibody production and antibody isotype class-switch by generating IgG1, IgG2a and IgG2b subclasses, in addition to IgM and IgG3 T-independent isotypes, in response to immunization with chicken gamma globulin [203, 204]. Type I IFN signaling in dendritic cells was found to induce antibody isotype class-switch, significantly driving the production of higher titers of IgG1, IgG2a, and IgGb subclasses, while type I IFN-deletion suppressed most of the IgG subclasses, with remaining IgM antibody but at substantially reduced serum titers [203]. Defective type I IFN signaling in either B or T cells substantially attenuates the production of antibodies of all isotypes, including IgM
and IgG subclasses [204]. IFNAR-deletion on B cells especially abrogated antibody isotype class-switch, significantly suppressing IgG subclass titers relative to IgM titers [204].

Two research groups has attempted to dissect the cellular pathways for the induction of antibody production and antibody isotype class-switch by type I IFN. Findings have hinted that type I IFN can promote the differentiation of plasma cells from naïve B cells independent of T cell help [205, 208]. The co-culture of pDCs with naïve B cells can promote the differentiation of plasma cells in the presence of antigen and CpG [208]. More specifically, co-culture of CD40L-activated B cells in the presence of intact type I IFN signaling and/or pDCs is required to promote plasma cell differentiation [205]. Although CD40/CD40L signaling pathway in B cells can be mediated by CD4 T cells, Jego et. al. concede that in vivo provision of CD40L can also be furnished by activated endothelial cells, mast cells, platelets, as well as B cells [205]. Although pDCs can promote plasma cell differentiation, Poeck et. al. have found no evidence that pDCs, in spite of IFN-α induction, promote antibody isotype class-switch [208]. Though finding that activated B cells by pDCs predominately favors the production of IgG over IgM titers, Jego et. al. cannot conclusively argue rule out that the pDCs did not specifically target memory B cells to generate the titers of IgG subclasses [205].

Therefore, very possibly, type I IFN signaling initiated by pDCs can promote the development of plasma cells independent of T cell help. In the context of autoimmune murine model, such as in the NZB mice, in which there is an enlarged marginal zone, pDCs in the region may promote the formation of autoreactive antibody-producing cells.
However, the role of type I IFN in promoting antibody isotype class-switch cannot be dismissed. The deletion of intact type I IFN signaling in T cells significantly weakened antibody production in response to CGG immunization [204]. In response to vesicular stomatitis virus infection, IFNAR-deleted murine hosts displayed impaired capacity to class switch from IgM to IgG [206]. In response to adenovirus infection, intact type I IFN signaling in B and T cells is required to produce antibody and class-switch from IgM to IgG2a and IgG2b subclasses [207]. Because antibody isotype class-switch is induced by T-cell dependent processes, and that type I IFN signaling deletion in CD4 T cells significantly attenuated antibody isotype class-switch [204, 207], the role of type I IFN in promoting T cell dependent humoral processes is undeniable. The two findings by Bach and Zhu et. al. also showed that viral infections in IFNAR-deleted murine models displayed weakened germinal center formation [206, 207], indicating that type I IFN does very well play a significant role in T cell dependent responses. Although CD40L is expressed by many cell types, the finding that CD40L-activated B cells can form plasmablasts in the presence of type I IFN supports the possibility that CD4 T cells is involved [205]. Jego et. al. offers the possibility that the localization of pDCs in the T cell zone may enhance the co-ordination between pDCs and CD4 T cells to induce B cell differentiation into plasma cells [205]. However, Asselin-Paturel et. al. have made the observation that in response to murine cytomegalovirus and inactivate herpes simplex virus infections, pDCs migrate from their natural habitat in the T cell zone and into the marginal zone in the perimeter of the follicles geographically located away from the bulk of CD4 T cells [174]. Under certain conditions of chronic inflammation, we may presume that pDCs may have the propensity to migrate away from the T cell zone in the follicles
and towards the marginal zone, in which case, pDC-derived type I IFN may adopt an alternative pathway to the model proposed by Jego et al. to drive a T cell dependent humoral response.

*Geography of Type I IFN Producers*

Although type I IFN has been demonstrated to promote plasmablast differentiation, the process of plasmablast differentiation is widely known to occur in germinal centers located in the follicles. In uninfected hosts, pDCs have been demonstrated to localize in the T cell area and in the red pulp [174]. Under inflammatory conditions, such as viral infections, or simple TLR ligand 7 and 9 injections, pDCs were observed to migrate out of the T cell zone and red pulp, forming clusters in the marginal zone [174]. Thus, some researchers speculate that pDC-derived type I IFN generates plasmablasts from marginal zone B cells, independent of T cell help [208]. Because pDCs are primary type I IFN producers, their distant locations from the germinal centers under inflammatory conditions hint at an inefficient T-dependent pathway by which type I IFN signals on developing plasmablasts.

Our findings in the BXD2 mice have indicated an apparent clustering of pDCs in the marginal zone. This is consistent with literature which states that under inflammatory conditions, pDCs cluster in the marginal zone [174]. As will be mentioned in the subject of our investigation in the following pages, type I IFN does promote T-dependent antibody responses, germinal center formation, and AID expression. Therefore, together,
these data suggest that the pDCs located in the marginal zone may have an association with immune events occurring in the follicle.

Marginal Zone (MZ) B-cells

The humoral arm of the immune system is mediated by B cells. Mature B cells are roughly divided into two groups, marginal zone and follicular. Both names are adjectives for the geographic location of the two B cell populations. Surface markers are utilized to distinguish between follicular and marginal zone (MZ) B cells. The surface antigen combination $\text{IgM}^{\text{low}}\text{IgD}^{\text{high}}\text{CD21}^{\text{int}}\text{CD23}^{\text{high}}$ is used to identify follicular (FO) and $\text{IgM}^{\text{high}}\text{IgD}^{\text{low}}\text{CD21}^{\text{high}}\text{CD23}^{\text{low}}$ for MZ B cells [209].

As the name implies, MZ B cells are located in the marginal periphery beyond the marginal sinus which forms the perimeter of the B cell follicle, and constitute the second largest B cell population in the secondary lymphoid organs [210]. Consisting of 5-10\% of splenic B cells [210], MZ B cells serve as important players in the T-independent response and recent evidence indicate possibly a role in T-dependent responses. The spleen is a blood-trapping organ roughly divided into the lymphocyte-containing white and erythrocyte-containing red pulp [211]. Blood enters the spleen through the splenic artery and traverses into the red and white pulp through a number of central and penicillar arterioles [211]. The central arterioles are surrounded concentrically by a ring of T cells (periarteriolar lymphoid sheath), B cell follicles, and the MZ [211]. Blood enters through the central arterioles, and leaves the spleen through the splenic marginal sinus, which
separates the white pulp from the MZ [212-214], in which blood circulation slows down substantially, maximizing MZ exposure to antigens. Within 30 minutes after introduction into circulation, antigens were found to localize in the MZ [215].

B cells mediate the antibody expression and secretion in the host in response to antigen invasion. Roughly, this humoral arm of the immune response is divided into T-dependent and T-independent responses. Follicular B cells are recognized to initiate and maintain T-dependent responses by germinal center reactions. Marginal zone B cells, on the other hand, are recognized to be plasma effector cells that generate T-independent antibody responses. Antibody isotype subclasses for the T-dependent arm include all IgG subclasses, including IgG1, IgG2a, IgG2b, and IgG2c. Hence, the typical association between antibody isotype class-switch recombination with the “help” provided by CD4 T cells. However, even in T-independent processes, isotype class-switch is known to occur. Aside from the production of pre-switched isotyped antibody subclass IgM, the antibody isotype IgG3 is also produced in T-independent antibody responses. Independent of T cell help, CpG and LPS are know toll-like receptor ligands that mediate the expression of activation-induced cytidine deaminase (AID), and so present a possible mechanism by which T-dependent mechanisms can drive antibody class-switch.

*T-independent Antibody Responses*

The marginal zone consists of a heterogeneous population of cells. Macrophages, the most prominent population in the region, can capture antigens through macrophage scavenger receptors. Plasmacytoid dendritic cells, as mentioned above, also reside in the location. B cells in the marginal zone itself consist of a heterogeneous multi-clonal
population. Some studies indicate the presence of memory B cells generated from T-dependent germinal center reactions by mutational analysis of immunoglobulin heavy chain variable regions [216]. The rapid response by which T-independent antibody response is generated in the marginal zone suggest that memory B cells may accumulate and localize in the marginal zone. The lower threshold for activation, and high capacity to present antigens typically found in memory B cells resemble the phenotypic features present in marginal zone B cells [209].

Even the IgM antibodies commonly found in the peripheral blood circulation before exposure to pathogen and/or immunized antigens serve as rapid and indispensable requirement for effective protective immunity. Thus, these natural serum antibodies do have a protective role against bacterial and viral inoculations [217, 218]. The presence of such antibodies led one question whether MZ B cells are key players in generating these antibodies. Researchers have not found the presence of activated MZ B cells as long-lived plasma cells in the bone marrow [210]. Mice depleted of MZ B cells do not show significant effects on natural serum antibodies, whose production apparently is heavily dependent on the presence of B-1 B cells [31, 219, 220]. In Lsc, RBP-J, and MINT-deficient mice, in which MZ B cells are not present, there still co-exist natural serum antibodies and B-1 B cells [221-223]. Researchers, however, do concede that these studies do not completely eliminate the role of MZ B cells in producing these pre-immunized antibodies. Intense selective pressures and requirement for the host to have normal serum antibodies may lead to expansion of rare B cell clones after the B cell pool is reduced [224-230]. Thereby, even with MZ B cell depletion, it is very well possible
that other B cells can expand and fill in the void left by the depleted cell population [210].

As mentioned previously, in vitro experiments have demonstrated that type I IFN can promote the production of antibodies, including IgM and IgG subclasses, independent of the presence of CD4 T cells [205]. In fact, with only CD40L co-stimulation, type I IFN and IL-6 were able to drive plasma cell differentiation [205]. Although CD40L is provided by CD4 T cells, other cell populations were also just as easily capable of providing such needed co-stimulation [205]. Also, dendritic cell-mediated upregulation of BAFF and APRIL can promote immunoglobulin isotype class-switch in B cells, independent of CD4 T cells [231]. The co-localization of B cells and dendritic cells, including pDC, in the MZ, lead to the interesting conclusion that a T-independent antibody response can easily and frequently occur from the region.

T-dependent Responses

Sequencing the genes from human and rat immunoglobulins support claims that memory B cells reside in the MZ. There is evidence of somatic hypermutation in MZ B cells from human spleens, lymph nodes, and Peyer’s Patches [216, 232, 233]. High-affinity B cells colonizing the MZ and their adopting an MZ B cell phenotype after a T-dependent antigenic exposure further supports the idea that memory B cells do congregate in the MZ [234]. Another study has found that after a T-dependent response to hapten-polysaccharide conjugates, B cells from germinal center reactions, colonize the
MZ and adopt the characteristic MZ B cell phenotype of producing a T-independent antibody response to polysaccharides [235].

Complement receptor CR2 (CD21)-expressing MZ B cells is essential to capture of IgM-immune complexes and transport to the follicular dendritic cells [236-239]. Curiously, T-dependent, as well as T-independent antigens, are transported and deposited onto follicular dendritic cells in this manner. Alternatively, other interesting studies have found that MZ B cells play a more direct and pro-active role in directing T-dependent antibody responses. MZ B cells typically exhibit a pre-activated phenotype, expressing higher levels of CD80, CD86, CD40, CD44, and lower levels of CD62L than their follicular counterparts [240]. Stimulation with LPS, anti-IgM antibodies, and CD40 ligands, can induce robust MZ B cell proliferation for a more prolonged time span than FO B cells [209, 240]. In vitro co-culture of naïve CD4 T cells with antigen-pulsed B cell subpopulations, MZ B cells were found to induce the stronger CD4 T cell proliferation and IFN-γ secretion than FO B cells [240]. Though 2-4 fold less efficient than dendritic cells in priming CD4 T cell help, MZ B cell are potential antigen presenting cells that can contribute to T-dependent humoral response [240].

Histological analyses have evidenced that MZ B cells may very well participate in T-dependent responses. 15-30 minutes after immunization with antigen, labeled antigen concentrate in the MZ, with little remaining in the red pulp [215]. Macrophages can capture antigens and present them to MZ B cells. At 4 hours post-immunization, MZ B cells are found to migrate from the MZ into the follicle and to the T cell zone [215]. LPS and Gram-positive bacterial can also induce MZ B cell migration into the follicle [241].
The in vitro studies, finding that MZ B cells are potent antigen-presenting cells [240], suggest that in the in vivo setting, MZ B cells can act to transport antigens from the MZ to the T cells in the follicles. By 10 hours after immunization, antigen has been transported from the MZ to the T and B cell zones of the white pulp [215]. Antigen-specific B cells have also been shown to migrate from the MZ to the T-B border after immunizing with T-dependent antigen [215, 242-246]. A recent study has found that MZ B cells could induce germinal center formation, though with a delayed kinetics compared to FO B cells [247]

Marginal Zone B cells and Autoimmunity

The MZ has been hypothesized as the site of autoreactive B cells. Anti-DNA B cells are found to localize in the MZ [34, 248], and that these can become precursors to autoreactive plasma cells in murine lupus [32]. In the NZB mice, as mentioned above, increased MZ B cell population precedes the onset of pathogenic T cells [249] Consistent with the T-independent role commonly attributed to MZ B cells, NZB mice displayed elevated serum IgM, increased counts of IgM-producing cells, and increased expression of surface co-stimulatory molecules [26, 36-38, 250-252]. An abnormally increased MZ B cell population and CD5+ B-1 B cell population is associated with this phenotype in NZB mice [26, 253].

While elevated levels of IgM autoantibodies to erythrocytes and single-stranded DNA in NZB mice has hinted at the important role of T-independent pathways, there is
the presence of elevated levels of IgG autoantibodies, especially to single-stranded DNA, in the serum of these mice. Deletion of CD4 T cells in NZB mice has significantly delayed the onset of both IgM and IgG autoantibodies to erythrocytes [44]. However, by eight months of age, 50% of NZB.CD4-/− mice exhibited titers of both IgM and IgG autoantibodies [44]. Interestingly, IgG autoantibody to single-stranded DNA was completely abrogated in these CD4-deficient mice, which the IgM isotype remained present [44]. Thus, the possibility of a prominent role for T-dependent processes may be at work in autoimmune hosts.

A number of studies have pointed to germinal center reactions as responsible for the presence of somatically hypermutated antibodies in lupus-prone mice [254, 255]. However, it is entirely possible that somatically hypermutated antibodies can be the product of extrafollicular reactions, as evidenced by the presence of rheumatoid factor reactive B cells in the bridging channels at the red pulp T cell zone border of MRL/lpr and B6/lpr mice [256].

The Mature B cell Subset Populations

Early classification tended to group B cells into two distinct lineages. Termed B-1 and B-2, the former was argued to have developed during the early stages of host embryonic development. CD5⁺ B1 B cells were found to principally derive from fetal hematopoietic stem cells [257]. Further studies on these cells found that unlike other B cells, these B-1 B cells only express a limited repertoire of immunoglobulin variable region genes, and
which have a preference for binding to T-independent antigens [258]. Generated and secreted prior to antigen exposure, these natural serum antibodies produced by B-1 B cells, whose natural niche is the peritoneal and pleural cavity, serve to act as a rapid and efficient method of protection against invading pathogens [217, 218].

In contrast, the B-2 population was so-named to distinguish it from the T-independent antibody responses characteristically identified with the B-1 population. The bulk of B cells found in the spleen and lymph nodes came under the heading B-2 for B cells involved in the conventional methods of humoral response, which required T cell help. These B-2 cells are the vast majority (>90%) of B cells found in the follicles of spleens and lymph nodes and so are also named follicular (FO). Upon exposure to antigen and activation, FO B cells receive CD40-CD40L and CD80/86-CD28 co-stimulation from CD4 T cells, and initiate germinal center formation and B-cell clonal selection. The ultimate result is the generation of plasma cells producing isotype class-switched and somatically hypermutated antibodies.

In the periphery of the B cell follicle are the marginal zone (MZ) B cells. Like B-1 B cells, MZ B cells initiate and conduct T-independent antibody responses, providing a rapid response to invading pathogens before the onset of the slower T-dependent antibody response started by the FO B cells.
B cell Clonal Selection and Subset Development

Like T cells in the thymus, B cells in the bone marrow undergo negative selection for autoreactive clones. Such negative selection methods include clonal deletion [259-262], and receptor editing [263-268]. If self-reactive clones escape the bone marrow and entered the peripheral circulation, clonal anergy [260, 269] may serve as a “last-resort” alternative to silence self-reactivity. While negative selection has been well-characterized, positive selection were also described in a number of studies, by which mechanism, the various B cell subpopulations are derived.

B cells generated in the bone marrow exhibit random variable heavy chain gene rearrangements, only a subset of these rearranged genes is expressed in the B cell receptors of mature splenic B cells [258]. The B cell receptor composition and density expression on immature B cells was shown by a series of experiments involving knock-in or conventional transgenic mice to directly determine differentiation into B-1 or B-2 subpopulations (Kearney et. al. 14-20) [270-272]. More recent studies have found that certain B cell receptor clones, the phosphorylcholine-binding idiotype M167 and the fetal-associated idiotype 81X, when expressed by B cells, drive their development into the marginal zone [273]. This and other evidence point to a B cell receptor repertoire-driven B cell developmental program once the newly made B cells emerge from the bone marrow [258].

B cell receptor signaling is obviously important in driving B cell development and compartmentalization into different subsets. The ligands, however, has not been easily identified. However, a number of studies hint at the possible candidates, including self-
antigens, commensal organisms, and apoptotic debris. Self-antigens, such as Thy-1, and as-of-yet unidentified determinants on mouse erythrocytes, can drive B cell development to enrich for the presence CD5+ B-1 B cells in the peritoneal cavity [274-277]. In contrast to wild-type mice, Thy-1 deficient mice were not able to enrich for the presence of Thy-1 binding CD5+ B-1 B cells [274]. Other research group has found that in response to transgene-encoded self-antigens, CD45-deficient B cells can enter the mature B cell pool [278].

Other findings have hinted that bacterial antigens may function to positively select for B cell compartmentalization into the different mature B cell subsets. M167 idiotype, another clone for enrichment of marginal zone B cells, was found to only react to self-antigens, but also to phosphorylcholine, a bacterial component, that may just as well originate from the many species of bacteria that live commensally with the host [273]. Other findings indicating the requirement of bacteria, include evidence from the absence of anti-red blood cell transgenic B cells in the B-1 cell population if the murine hosts are kept in a germ-free environment [279].

Another class of compounds that may serve to positively select for B cell development into different subsets are non-protein self-antigens produced from oxidative stress and apoptosis-related processes [215]. The T15 Id clone was been identified to not only bind to phosphorylcholine but also oxidized low-density lipoprotein (LDL) and surface antigens on apoptotic cells [215].
Immature Peripheral B cells

In the early 1990s, there was the growing recognition that there are “transitional” or intermediates in the B cell developmental pathway that takes place some time when newly emergent B cells are produced from the bone marrow and when mature B cells have filled their niches in the secondary lymphoid organs. Newly formed B cells in the bone marrow are defined as immature by their absent expression of IgD and their susceptibility to negative selection [306]. In the periphery, however, most of B cells are mature, a fact that maturation takes place as soon as B cells migrate out of the bone marrow and into the periphery. The obstacle at the time to identifying these intermediate B cell forms was hindered by the lack of an easily identifiable marker [306].

The identification of surface expression of heat-shock antigen (HSA) as a marker for maturing B cells in the periphery has led to the discovery of the so-called transitional B cells [306]. HSA$^+$ B cells comprise about 10% of the adult splenic B cells, are IgM$^+$ and express variable levels of IgD [307]. Four days after birth, murine spleens displayed virtually 100% HSA$^{hi}$ B cells [306]. Gradually, HSA$^{hi}$ B cells declined, while HSA$^{lo}$ B cells increased until 6 weeks of age, by which time, the B cell populations achieve steady-states found in adult mice [306]. Also, IgD expression, a long recognized marker of B cell maturity, increased [306].

The transfer of sIg$^-$ BALB/c B cell-depleted bone marrow to lethally irradiated congenic mice further demonstrated that HSA$^{hi}$ B cells served as precursors to mature B cells. IgM$^+$IgD$^{hi}$HSA$^{hi}$ B cells remained exclusively in the bone marrow throughout the reconstitution period of the peripheral B cell pool until 18 to 26 days after the transfer,
when IgM⁺IgD⁺ became present [306]. By 12 day, all splenic B cells were HSA⁺hi and only 50% were IgD⁺. HSA⁺lo B cells did not appear until day 15 post-transfer [306]. The distribution of HSA profile was not achieved until 26 days after transfer. Together, these observations support the idea that HSA⁺hi splenic B cells are derived from the bone marrow and because IgD⁺ B cells appeared in the spleen prior to in the bone marrow, these observations indicate that there are pivotal intermediate stages in B cell development which occurred in the periphery just shortly after immature B cells had emerged from the bone marrow [306]. B cell reconstitution of SCID mice by splenic HSA⁺hi B cells, in conjunction with antigen-primed T cells, were able to generate both primary and memory antibody responses after immunizations [306].

The Transitional B cells

Since the identification of intermediately matured B cells in the periphery, two classification schemes have been forth to diagram the developmental stages which B cells undergo from the bone marrow to the lymphoid organ in the periphery. In the scheme proposed by Loder et. al., B cells undergo two transitional intermediates, that are termed T1 and T2, or transition B cells, that express surface antigen combination IgM⁺HSA⁺hi with increasing levels of CD23 (FceRII) and complement receptor CD21/35 [308]. The early immature T1 cells are IgM⁺hiHSA⁺hiCD23⁻CD21/35⁺, and the later immature T2 cells are IgM⁺hiHSA⁺loCD23⁺CD21/35⁻ [308]. Loder et. al. found that the T2 population in his scheme proliferate rapidly, and appear to undergo a “proliferative burst” prior to positive selection to become FO B cells. The T2 transition into FO B cells is questioned
Figure 8. Two schemes for B cell development in the periphery.
(A) Loder et. al. proposed that HSA^high sIgM^high immature peripheral B cells be divided into populations that are CD23^− CD21/35^low (T1) and CD23^+ CD21/35^high (T2), from which FO and MZ B cells develop. The proliferative burst that T2 population undergoes is indicated by a curly arrow. (B) Allman et. al. found that all immature transitional B cells are AA4^+ and divided into three populations based on CD23 and IgM expression.

*Allman et. al. Immunological Reviews 2007; 197(1), 147-160*
Table 2. Different nomenclatures, cell-surface phenotypes, and turnover rates for subpopulations of immature peripheral B cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Nomenclature</th>
<th>sIgM</th>
<th>CD21/3</th>
<th>CD23</th>
<th>Turnover Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loder et al.</td>
<td>T1</td>
<td>High</td>
<td>Low</td>
<td>–</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>High</td>
<td>High</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>Allman et al.</td>
<td>T1</td>
<td>High</td>
<td>Low</td>
<td>–</td>
<td>Rapid</td>
</tr>
<tr>
<td></td>
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<td>High</td>
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<td>Rapid</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>Low</td>
<td>Low</td>
<td>+</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

sIgM, surface immunoglobulin M.

*Cellular turnover rates were determined by continuous in vivo BrdU labeling.
by studies in Aiolos-deficient mice which did develop a normal sized FO B cell pool without generating the T2 B cell population as put forth by Loder et. al. [302]. Allman et. al. put forth another B cell developmental scheme by which B cells from the bone marrow undergo three transitional stages in the periphery before becoming mature B cells. The T1, T2, and T3 subsets are defined by expression of C1q receptor homology AA4 [309], variable expression of IgM, CD23, and absent cell proliferation in vivo [294]. In contrast to mature MZ or FO B cells, which do not express AA4, upon B cell receptor cross-linking, T1-T3 cells do not proliferate and undergo apoptosis [294]. (Table 2)

Marginal zone B cell precursor

As cited above, newly emergent B cells from the bone marrow undergo several transitional forms before becoming mature B cells in the periphery. MZ and FO B cells express the following surface antigens IgM^{high} IgD^{low} CD23^{low} CD21/35^{high} AA4^{-} and IgM^{int/low} IgD^{high} CD23^{+} CD21/35^{int} AA4^{-} respectively. Loder et. al. has identified a T2 B cell population that is IgM^{CD23^{+} CD21/35^{high}}, and had postulated that this cell population can generate both MZ and FO B cells [258, 308].

However, studies have shown that this T2 population may not be the precursor for FO B cells. Mice deficient for the expression of the transcription factor Aiolos oblate the development of MZ B cells and the CD21/CD35^{high} CD23^{+} T2 population, whereas only an enlarged FO B cell population is retained [302, 310]. In another study, mice with a conditional deletion of the transmembrane receptor Notch2 did not exhibit the presence of MZ B cells and CD21/CD35^{high} CD23^{+} B cells, and yet, have a normal sized FO B cell
Figure 9. Characterization of marginal zone B cell precursors.
The scheme shown here is a modified form of the scheme put forth by Allman et. al. The AA4\(^+\)/AA4\(^{low}\)IgM\(^{high}\)CD21\(^{high}\)CD23\(^+\) identified by Loder et. al. is now identified as the marginal zone B cell precursor population (MZPs)
Srivastat et. al. JEM 2005; 202(9), 1225-34
population [311]. Both of these studies indicate that CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} B cells are not likely developmental precursors to the FO B cell population. Rather, they suggest critical association between the MZ and CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} B cell populations because these two populations seem to appear either present or absent together.

In 2005, Srivastava et. al. concluded that the CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} B cell population is the precursor to the MZ B cell population [312]. Generating chimeras by reconstituting irradiated C57BL/6 (Ly5\textsuperscript{B6}) hosts with congenic B6.Ly5.2 (Ly5\textsuperscript{SJL}) B220-depleted bone marrow, the chimeras were followed over a period of 25 days. While by day 15, all transitional B cells and FO B cells were already present in significant percentages, CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} B cells became a significant population only day 17. MZ B cells did not become significantly present until on day 21. By day 25, both FO and MZ and CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} B cell populations were present. The observation that CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} B cells became present well after FO B cells had developed indicated that these cells are not the precursors to the FO B cells. Adoptive transfers of CD21/CD35\textsuperscript{high} CD23\textsuperscript{+}, T2 and FO B cells into B6.RAG\textendash/- mice support the thesis that CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} B cells develop into MZ B cells. B cells must first develop into CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} before MZ B cells are developed.

CD21/CD35\textsuperscript{high} CD23\textsuperscript{+} MZ B cell precursors (MZP) share one distinctive feature with MZ B cells, and that is their higher capacity to capture T-independent antigen TNP-Ficoll than do FO B cells [312]. Perhaps not surprisingly, compared to MZ B cells, MZPs do not express high levels of integrin $\alpha_4\beta_7$, which is essential for binding to VCAM and retaining MZ B cells in their niche [312, 313]. This differential in integrin expression may also account for the finding that these MZPs localize into the follicle instead of to
the MZ [308, 314]. Second, MZPs robustly respond to both BCR cross-linking and LPS stimulation, whereas MZ B cells only respond to LPS [312, 315]. Third, MZP development is independent of CD19 because while CD19 \( ^{cre/cre} \) mice do not have MZ B cells, normal frequencies of CD21/CD35\(^{high}\) CD23\(^+\) B cell population is present [312].

\[
\text{S1P}/\text{S1P}_1
\]

A variety of selectins, chemokine receptors, and integrins govern lymphocyte entrance into secondary lymphoid organs [316]. However, currently, there is only one class of factors discovered that governs the rate at which lymphocytes traffic out of the lymphoid organs [317]. Expression of a catalytic subunit of pertussis toxin in thymocytes inhibited mature T cell egress from the thymus, suggestive that a \( \Gamma_a i \)-coupled receptor is involved [318]. The discovery of a fungal metabolite myriocin was found to suppress mixed-lymphocyte reactions. Chemically modified myriocin FTY720 was found to deplete blood and lymph lymphocytes and thus prevent allograft skin rejections [319]. Initially, FTY720 was thought to induce lymphopenia by accelerating lymphocyte homing to lymphoid organs [320]. However, blocking lymphocyte egress was more consistent with the observation that the lymphocyte depletion occurred in the lymph, to which lymphocytes normally enter by exiting from the lymph nodes [321].

Subsequent studies have found that FTY720 is structurally similar to lipid sphingosine and phosphorylated \textit{in vivo}, making it a potent agonist of four out of the five \( \Gamma \) protein-coupled receptors specific to the lysophospholipid S1P, S1P\(_1\), S1P\(_3\), S1P\(_4\), and S1P\(_5\) [321, 322]. The role of S1P in regulating lymphocyte egress was demonstrated by observing that S1P\(_1\)-deficient T cells developed normally in the thymus but failed to exit
the thymus, resulting in the accumulation of mature thymocyte [323, 324]. When these mature S1P₁ deficient T cells are transferred intravenously into wild-type mice, these cells can enter but not exit the secondary lymphoid organs. B cells do not require S1P₁ to leave the bone marrow, but like T cells, require S1P₁ to leave secondary lymphoid organs. [324]

**S1P₁-Regulation of Lymphocyte Egress**

The only known physiologic ligand of S1P₁ is S1P [325]. S1P is generated as a by-product of normal sphingolipid turn-over in many cells [326-328]. Plasma contains micromolar concentrations of S1P, while lymph contains one-sixth of the concentration in plasma, in the nanomolar range [329, 330]. The concentrations of S1P in the secondary lymphoid organs are more difficult to determine, considering that perturbations of the microenvironment can easily induce inflammatory reactions. One method to determine S1P concentrations is to assay for surface expression of S1P₁ on naïve T cells [329]. There is an inverse relationship between the presence of S1P and its receptor S1P₁. Within 20 minutes, thymocytes internalize S1P₁ in response to incubation with S1P [329]. Thus, surface expression of S1P₁ is very sensitive to the presence of S1P. While surface expression of S1P₁ on T cells in lymph and blood was undetectable, surface expression of S1P₁ on T cells in spleen and lymph nodes was detectable, indicating that S1P concentrations inside the lymphoid organs were lower than those in blood and lymph [329, 330]. Relatively high concentrations of S1P in plasma are found to be maintained by red blood cells, which have lacked S1P-degrading enzymes and so generate sufficient S1P to keep steady plasma concentrations [331]. In contrast, the S1P-degrading S1P lyase
inside the secondary lymphoid organs keeps the concentrations of S1P low [329]. Inhibitors of lyase drive up the concentrations of S1P in lymphoid organs [329].

While S1P gradients are a postulated pathway by which to regulate lymphocyte egress, an alternative explanation for S1P’s role on lymphocyte egress could not be ruled out. S1P–deficient embryos die of hemorrhage midgestation because of defects in vascular maturation [332]. S1P induces the assembly of vascular adherens junctions between endothelial cells [333, 334]. Treatment with a S1P antagonist causes pulmonary vascular leakage in mice [334]. The alternative theory to the role of S1P on lymphocyte egress out of lymphoid organs proposes that S1P act on S1P \(_1\) to tighten endothelial cell-cell junctions, blocking cellular egress. Therefore, in this model, lymphocytes simply engage in “random walk” inside the lymphoid organs and are simply blocked from exiting if S1P induces the formation of endothelial cell-cell junctions [335, 336].

Several evidences, however, prove that S1P \(_1\) expressed on lymphocytes intrinsically regulate lymphocyte egress. First, S1P \(_1\)-deficient lymphocytes do not egress from lymph nodes and spleens [337]. Second, in mice deficient in producing functional S1P in the blood and lymph, lymphocytes do not egress from the secondary lymphoid organs [337]. The failure to egress is not because S1P failed to act on egress structures by a failure to form cell junctions. When S1P concentrations were replenished in these mice, lymphocyte egress from lymphoid organs resumed while S1P \(_1\)-deficient cells do not [330].

Immature T cells undergo both positive and negative selection pressures to generate a repertoire of T cell clones that will respond to antigens and not react to self-antigens, thus it is critical that immature T cells complete these processes in the thymus.
before their exit. S1P may regulate this critical trafficking. Mature T cell thymocytes respond to S1P only at the single-positive CD4⁺CD8⁻ and CD4⁻CD8⁺ cell stages [337]. S1P transcripts increase 50-fold between the CD4⁺CD8⁺ double-positive and the single-positive stages, and another 30-fold between the immature and mature single-positive stage [324]. Increased S1P expression at the single-positive stage allows only matured T cells to exit the thymus [337].

S1P also regulate mature lymphocyte trafficking between the circulation and the lymphoid organs. T cells disappear from the lymph one day after antigen-stimulation, concomitant with their ability to recognize S1P and absent surface expression of S1P₁ [324, 338]. Three days, later, S1P₁ expression is restored and T cells reappear in circulation [324]. Likewise, B cells utilize S1P gradient to regulate their homing to the bone marrow after becoming plasma cells in secondary lymphoid organs in response to antigen stimulation. IgG-secreting plasma cells express S1P₁ in order to egress from the spleen and lymph nodes to home onto the bone marrow [339].

*Early Activation Marker CD69*

CD69 is a type II C-type lectin ascribed to the family of natural killer receptors [340]. CD69 is an early activation molecule that is expressed on all cells derived from the bone marrow except erythrocytes [341]. CD69 is commonly found at sites of inflammation and is associated with chronic states of inflammation [342]. A series of in vitro experiments have bolstered the contention that CD69 promotes a pro-inflammatory condition. For instance, anti-CD69 monoclonal antibody stimulate T cell production of
IL-2 and proliferation [343]. Anti-CD69 also significantly blocked direct T cell and macrophage contact to activate the expression of pro-inflammatory cytokines [344].

However, CD69-transgenic mice did not exhibit inflammation [345, 346]. Instead, CD69 was found an immuno-regulatory molecule because CD69-deficient mice displayed exacerbated collagen-induced arthritis (CIA) with reduced TGF-β levels [347]. CD69 cross-linking \textit{in vitro} promotes the synthesis of TGF-β, a known cytokine involved in suppressing the immune response and polarizing the formation of T-regulatory cells [347, 348]. Both CD4 and CD8 T cells, as well as macrophages and natural killer cells were found to express TGF-β after CD69 cross-linking [347, 348]. Therefore, CD69 is postulated to down-regulate an immune response by promoting TGF-β synthesis. The apparent dual role of CD69 as both potential pro-inflammatory and anti-inflammatory molecule clouds any conclusive claim that the molecule may play in autoimmunity. There is significant presence of IgG anti-CD69 autoantibodies in the serum of a subset of rheumatoid arthritis patients, which correlates with disease activity [349]. CD69 cross-linking can stimulate expression of pro-inflammatory cytokines to induce disease, or alternatively, the anti-CD69 autoantibody could block CD69 from its putative ligand and decrease expression of TGF-β [350]. A more recent proposal for the role of CD69 in immune responses involves its role in regulating lymphocyte trafficking.

\textit{Type I IFN, CD69, S1P_{1}, and Lymphocyte Egress}

Poly I:C is a viral mimetic that induces type I IFN production and secretion by dendritic cells. Within six hours of administration of poly I:C, lymphocytes in the lymph were depleted, suggestive of a block in their egress from lymphoid organs [337].
Consistent with this finding were that lymphoid organ lymphocytes had decreased surface expression of S1P1 and did not migrate in response to S1P [337]. In contrast, in CD69-deficient mice, surface S1P1 expression persisted, and lymphocytes remained in the lymph [337]. CD69 decreases surface expression of S1P1 by post-transcriptional mechanisms because very minimal amounts of S1P1 transcripts were found in lymphocytes within moments of antigenic stimulation [337]. A likely mechanism for CD69-dependent regulation of S1P1 is via protein-protein-interactions because the two surface protein receptors co-immunoprecipitate together [351]. CD69 suppression of S1P1 to limit lymphocyte egress is demonstrated in transgenic CD69 mice in which thymocytes failed to exit after maturation [345, 346]. Co-transfection of S1P1 and CD69 demonstrated that the expression of the two proteins has an antagonistic relationship, with the higher expressing protein suppressing the expression of the other [351, 352]. A model for CD69 and S1P1 surface expression proposes that both proteins are retained intracellularly. Naïve lymphocytes consist of substantial transcripts of CD69 and intracellularly store significant quantities of CD69, that is readily released onto the plasma membrane when S1P1 expression is deficient [351, 353].

Type I IFN upregulate surface expression of CD69, which down-regulates surface expression of S1P1, leading to a block in lymphocyte egress from secondary lymphoid organs [351]. The role of type I IFN in promoting a block in lymphocyte egress is consistent with the fact that type I IFN promotes the immune response in a host subjected to an antigen assault, such as a viral infection. Blocking the exit of lymphocytes from the spleens and lymph nodes within hours of infection, type I IFN promotes the accumulation of B and T cells in the lymphoid organs [351]. In this way, accumulated lymphocytes
Figure 10. S1P₁ regulation of T cell trafficking.
S1P₁ permits egress of double-positive T cells, which traffic through the secondary lymphoid organs, until infection-induced type I IFN mediated upregulation of S1P₁ by CD69 up-regulation. T cells are retained within lymphoid organs until S1P₁ is once again down-regulated, by which time, the T cells have become effector T cells that are enabled to exit the organs.
Schwab et. al. Nature Immunology 2007; 8(12), 1295-301
have an enhanced probability of becoming stimulated by antigens presented by macrophages, dendritic cells, and B cells.

**S1P₁-Dependent Marginal Zone B cell Development**

S1P₁ can regulate the exiting of lymphocytes from lymphoid organs, but an important role for S1P₁ in retaining B cells in MZ is also observed. Treatments with S1P antagonists have been shown to rapidly displace MZ B cells and promote their migration into the B cell follicles [354]. S1P₁ message levels are significantly higher in MZ B cells than are present in FO B cells [354]. This S1P₁ differential between the FO and MZ supports B cell residence in the latter. Over-expression of S1P₁ in FO B cells retains the B cells in the MZ. S1P₁-deficient B cells were found to fail to lodge in MZ in a series of experiments using mouse chimeras [354]. B cell residence in the MZ facilitates antigen capture. Displacement of splenic B cells from the MZ to the follicles by FTY720 treatment attenuated antigen capture when treated mice were immunized with TNP-Ficoll [355]. This is consistent with MZ B cells in proximity to the marginal sinus, which drains blood from the peripheral circulation. This is also consistent with MZ B cells as potent antigen presenters [240].

MZ B cells also facilitate antigen transport to the follicles and carry the antigen to the follicular dendritic cells [236, 239], demonstrating their potential as antigen-presenting cells in initiating germinal center formations. Both FTY720 treatment and S1P₁-deficiency diminished antigen deposits on follicular dendritic cells relative to controls [355]. CXCL13 is highly expressed in the follicles and its transcripts extend to
the marginal sinus, but not to the MZ [356, 357]. The receptor of CXCL13 is CXCR5, which is expressed on B cells. Mixed chimeras generated by mixing CD19−/− with either CXCR5+/+ or CXCR5−/− bone marrow cells demonstrated that the shuttling of MZ B cells is critical to antigen capture and delivery to the FDCs in the follicles [355]. CD19−/− B-cells do not position in the MZ, but rather, only in the follicles. CXCR5−/− B cells are retained in the MZ and do not shuttle to the follicles. Mouse chimeras consisting of CXCR5−/− B cells do not deposit antigen to the FDCs after immunization, demonstrating that MZ B cells are required to deliver antigen to the follicles. However, CXCR5+/+ B cells can deliver antigens to the follicular dendritic cells. This CXCR5 ‘pull’ atB cells into the follicles is counter-balanced by high S1P1 expression and S1P signaling in the MZ.

CENTRAL HYPOTHESIS

Lupus-prone BXD2 mice develop significant titers of high-affinity IgG autoantibodies [21]. Spontaneously, BXD2 mice develop germinal center and significant AID transcripts to initiate somatic hypermutation and antibody isotype class-switch [47]. Ad CTLA4 Ig administration blocked autoantibody production, signifying that autoantibody production in BXD2 mice received CD4 T cell help [47]. Together, these findings indicate that autoantibody production in BXD2 mice is initiated through a T-dependent mechanism, likely involving FO B cells. Furthermore, our laboratory has earlier observed increased expression of IL-17 in BXD2 mice that promotes germinal center formation.
In the present study, we have found that type I IFNs generated primarily by pDCs in the MZ can also promote germinal center formation. We hypothesize that type I IFN signaling is important in germinal center formation and autoimmune disease induction in BXD2 mice. Therefore, our first task is to show that type I IFNs do play a role in mediating the T-dependent induction of autoimmunity in BXD2 mice. Second, because the current literature states that type I IFN antagonizes the development of IL-17-producing CD4 T cells, we seek to determine a mechanism by which both type I IFN and IL-17 could conceivably collaborate to induce germinal center formation.

In the present study, we have found a special population of B cells that are known to be precursors to the MZ B cells. These BXD2 MZP B cells are potent antigen presenters because not only do they express higher levels of co-stimulatory molecules but also our in vitro studies found that MZP B cells can induce significant proliferation in CD4 T cells, even more so than either FO or MZ B cells. High levels of type I IFN expression by increased counts of pDCs in BXD2 mice up-regulate CD69 and suppress S1P₁ surface expression in MZ and MZP B cells. The location of pDCs in the MZ facilitates type I IFN signaling on the MZ and MZP B cells, which are found to locate in the outer FO region near the MZ-FO border (unpublished correspondence, Dr. Shiv Pillai). However, S1P₁ suppression is most pronounced in MZP B cells. Therefore, our hypothesis is that high levels of type I IFN in BXD2 mice promote CD69 surface expression in MZP B cells which in turn suppresses S1P₁ surface expression, thereby releasing MZPs from the MZ-FO border to the FO. Once the MZP is inside the FO, they are then trapped by IL-17 producing CD4 T cells that up-regulation of RGS13/16
expression. These MZPs then initiate a cascade of B-T cell conjugate interactions that ultimately forms a germinal center reaction.

First, contained herein in the following report is evidence showing that type I IFN, like IL-17, is crucial to induction of germinal center reactions. Second, evidence demonstrating the importance of MZP in germinal center induction and type I IFN-regulated positional migration from the MZ-FO border will be demonstrated.

SIGNIFICANCE OF STUDY

Type I IFN is associated with the occurrence of human SLE [177, 179, 180, 183, 184, 186]. Lupus-prone murine models with type I IFN receptor deletion abrogate autoantibody production and obviate the occurrence of glomerulonephritis, a hallmark disease in lupus [191, 194]. However, the precise pathway or mechanism that type I IFN induces autoimmunity is not clearly defined. Aside from differentiating monocytes into efficient antigen-presenting dendritic cells, the current SLE literature argues that type I IFN primarily acts on post-germinal center reactions by promoting plasmablast and plasma cell formation [53, 63, 175]. Curiously, such conclusion was derived on in vitro studies involving blood samples from non-autoimmune human subjects [205]. While these studies provide plausible pathways by which type I IFN can induce autoimmunity, they did not address the issue using mouse or conditions that simulate autoimmunity. Also, while in vitro studies have shown that IFN-α can differentiate naïve B cells into plasma cells, we are not certain whether this is the precise pathway by which type I IFN function generate autoantibodies in vivo, and in the context of autoimmunity. Our current study has found an alternative pathway by which type I IFN is involved in pre-germinal
center and active germinal center reactions. In the first part of our study, we substantiate that type I IFN is crucial for direct signaling on B cells to drive germinal center formation and AID expression. This is in contradistinction to the current lupus literature which argues that type I IFN promotes a post-germinal center reaction such as plasma cell differentiation. In the second part of our study, we identify a subset of B cells, the marginal zone B cell precursors (MZP), that is driven by the type I IFN signaling from the MZ-FO border to the FO-T cell border to induce germinal center reactions. If our hypothesis and finding is true, then we have discovered a major route by which type I IFN can induce a pre-germinal center reaction to initiate the formation of a full-fledged repertoire of autoreactive plasma cells.
TYPE I IFN DIRECTLY SIGNALS ON B-CELLS TO DRIVE GERMINAL
CENTER FORMATION IN BXD2 MICE

by

JOHN WANG, HUI-CHEN HSU, QI WU, JOHN D. MOUNTZ
Abstract

Although type I IFN has been demonstrated to promote antibody class-switch and plasma cell differentiation under non-autoimmune conditions, the mechanism for the generation of T-cell dependent class-switched IgG autoantibodies in lupus is not well-defined. In our present study, a lupus-prone BXD2 murine model is utilized to resolve the role of type I IFN in producing pathogenic isotype class-switched IgG autoantibodies, glomerulo-nephritis, and arthritis. Increased frequency and counts of plasmacytoid DCs (PDCs) in BXD2 mice produced significantly elevated expression of type I IFN. Type I IFN signaling was crucial to increasing the T-cell dependent antibody response as demonstrated by immunizing BXD2 and type I IFN receptor(IFNAR)-deleted BXD2 (BXD2.IFNAR-/-) mice with NP-CGG. Type A CpG-induced type IFN expression boosted this T-cell dependent response, and IFNAR-deletion suppressed this response. BXD2.IFNAR-/- mice exhibited the absence of IgG-producing plasmablasts that are normally found in wild-type BXD2 mice. Serum auto-antibodies, normally present in wild-type BXD2 mice, were also significantly suppressed in BXD2.IFNAR-/- mice. Because IFNAR-deletion in BXD2 mice abrogated the development of T-cell dependent antibody responses and the production of IgG class-switched autoantibodies and plasmablasts, we sought to determine the role of type I IFN in germinal center formation and AID expression. BXD2.IFNAR-/- mice displayed significantly decreased counts of germinal centers and decreased frequencies of germinal center B-cells, when compared to BXD2 mice. Consistent with this, AID transcripts were also down-regulated in BXD2.IFNAR-/- mice. Adoptive transfer of IFNAR-competent GFP+/+ B-cells into IFNAR-deficient GFP-/- recipient mice that were subsequently treated with either
phosphate-buffered saline or IFN-α demonstrated a dramatic increase in GFP+/+ germinal center B-cells, while IFNAR-deficient GFP-/- B-cells did not. This result demonstrated that type I IFN signaling drives germinal center formation. Consistent with the absence of isotype class-switched IgG autoantibodies in BXD2.IFNAR-/- mice, these mice, unlike their wild-type counterparts, did not exhibit glomerulo-nephritis and erosive arthritis. Together, our finding indicates an additional pathway to generate T-cell dependent isotype class-switched IgG autoantibodies in lupus.

**Introduction**

Type I interferon (IFN) are a class of cytokines that have been recognized to be associated with several human autoimmune diseases. This includes not only systemic lupus erythematosus (SLE) [1], but also rheumatoid arthritis [2, 3], and psoriasis [4, 5, 6]. While other autoimmune diseases and their associations with type I IFN is important, the role of type I IFN in SLE pathogenesis is most well studied. Intravenous injection of type I IFN to treat viral infected patients have resulted in the development of lupus-like syndrome, including the development of serum autoantibodies, and the characteristic malar rash of SLE [7]. Although this occurred in only a small fraction of patients receiving type I IFN therapy, most patients with active SLE exhibit the presence of the “type I IFN signature”, with significantly increased expression of type I IFN-induced genes [8, 9, 10]. Also, SLE patients exhibited elevated levels of serum type I IFN levels that positively correlated with disease severity [11].

Lupus-prone mouse models have exhibited increased serum levels of type I IFN when compared to their non-autoimmune counterparts [12, 13]. Because BXD2 mice
displayed a lupus phenotype, including the development of serum autoantibodies and glomerulonephritis [14], we attempted to study the role of type I IFN in BXD2 mice. BXD2 mice were generated by Dr. Benjamin A. Taylor at the Jackson Laboratory by inbreeding C57Bl/6 and DBA2/J mice for more than 20 generations. Our laboratory was the first to find that BXD2 mice develop erosive arthritis and glomerulonephritis [14], pathologies resembling those found in rheumatoid arthritis and lupus. BXD2 mice spontaneously develop autoreactive germinal centers that generate high-affinity IgG autoantibodies to various antigens, including those commonly found in SLE, such as histone and DNA [14, 15]. High levels of IL-17 induced increased Rgs13 and Rgs16 expression enabling a stronger interaction between B and T cells [16]. High levels of the message for activation-induced cytidine deaminase (AID) are present in BXD2 mice. AID is an RNA editing enzyme required for somatic hypermutation and antibody isotype class-switch [15]. Blocking CD86/80-CD28 co-stimulation, Ad CTLA4 Ig administration abrogated the production of high-affinity IgG autoantibodies that have undergone AID-mediated somatic hypermutation and isotype class-switch, indicating that the autoimmune process in BXD2 mice is primarily T-cell dependent [15].

The role of type I IFN in T-cell dependent germinal center formation to induce the production of AID-mediated antibody isotype class-switched IgG autoantibodies is not clearly defined. Type I IFN signaling has been demonstrated to induce antibody isotype class-switch. Injections of IFN-α into antigen-immunized mice have enhanced antibody titers and antibody isotype class-switch [17]. Murine models have demonstrated the important role of type I IFN in the spontaneous development of autoantibodies and immune complex deposition in the glomeruli [12, 13, 19]. Although these studies have
indicated the important role of type I IFN to antibody isotype class-switch and the humoral response, the precise mechanisms by which type I IFN effect these processes remain elusive. One study has demonstrated that type I IFN, in conjunction of IL-6, can promote plasma cell differentiation in CD40-stimulated B-cells in the context of non-autoimmunity [20]. Researchers have translated this finding as a mechanism by which type I IFN induce autoantibody production in lupus [20, 21]. While type I IFN can promote plasma cell differentiation, this mechanism alone does not fully explain the observed role that type I IFN can promote antibody isotype class-switch from IgM to IgG. Our present findings here indicate that type I IFN signaling drives germinal center formation, AID expression, and thereby promote the production of T-cell dependent isotype class-switched IgG autoantibodies.

Results

Increased type I IFN expression in BXD2 mice.

We sought to determine whether there is an increased type I IFN expression in BXD2 mice by intravenously injecting mice with type A CpG, a toll-like receptor (TLR) 9 ligand. Type A CpG, once engaged to TLR9 in the PDCs, generate significant amounts of type I IFNs. BXD2 mice produced greater than 2-3-fold higher levels of IFN-α than that produced by C57Bl/6 mice as measured in the serum by ELISA at 2, 4, 8, 12, 24 hours after treatment (Fig. 1A). To determine how this response was affected by age, different age groups (young, middle, old) of BXD2 and C57Bl/6 mice were intravenously injected with type A CpG and serum IFN-α was determined by ELISA at 4 hours after
FIGURE 1. pDCs produced-type I IFN increased in BXD2 mice. (A) CpG was i.v. injected into BXD2 (black circle) and C57Bl/6 (white circle) mice as described in Materials and Methods. Sera were collected at 2, 4, 8, and 12 hours after injections. Serum IFN-α levels were assayed by ELISA. (B) CpG was i.v. injected into BXD2 (black bar) and C57Bl/6 (white bar) mice of different age groups as described in Materials and Methods. Sera were collected at 4 hours after injections. Serum IFN-α levels were assayed by ELISA. (C) RNA was extracted from peripheral blood cells. Transcripts of IFN-α1, -α4, α11, and β isoforms from BXD2 and C57Bl/6 mice at ages 2, 6, 9, and 12 months of age were quantitated relative to copy counts of actin, as described in Materials and Methods.
Relative to the serum levels in C57Bl/6 mice, increased serum IFN-α levels were observed in BXD2 mice, which was most prominent at 3-6 months of age (Fig. 1B).

Quantitative PCR determined that unstimulated BXD2 mice produced increased transcripts of IFN-α1, IFN-α4, IFN-α11, and IFN-β when compared to that produced by C57Bl/6 (Fig. 1C). As our laboratory has previously reported that BXD2 mice exhibited significantly increased titers of pathogenic autoantibodies at the same age range [22], these results suggest that type I IFN may play a significant role in contributing to autoantibody production.

Activated pDCs in proximity to autoreactive germinal centers in BXD2 mice.

In order to determine the role of pDCs and type I IFN in autoimmune pathogenesis in BXD2 mice, we sought to determine the presence and location of pDCs at the major sites of inflammatory reaction in the spleens of BXD2 mice, where spontaneously-formed germinal centers are present. Histological analysis of immunofluorescently stained frozen tissue sections of spleens from BXD2 and C57Bl/6 mice revealed that pDCs are located in the marginal zones surrounding the germinal centers in the spleens of BXD2 mice (Fig. 2A). In the spleens of C57Bl/6 mice, there are fewer pDCs in the marginal zones surrounding non-reactive follicles (Fig. 2A). Flow cytometry analysis of immunofluorescently labeled bone marrow cells polarized to PDCs using Flt3-L indicated that BXD2 mice displayed a more activated phenotype than those from C57Bl/6 mice. Gating on PDCA1+, a specific marker of PDCs, we
FIGURE 2. Increased frequencies of pDCs and activated pDCs in BXD2 mice. (A) Images of spleen sections taken from BXD2 and C57Bl/6 mice that were stained with anti-IgM, anti-CD35 (FDC), anti-PNA, and anti-PDCA1 antibodies as described in Materials and Methods. (B) Flow cytometry was performed on Flt3 ligand (Flt3L) polarized-BXD2 and C57Bl/6 bone marrow cells labeled with anti-PDCA1, anti-MHCII, anti-CD86, and anti-CD80 antibodies. Gating on PDCA1 populations, histograms detailed the counts of MHCII+, CD86+, and CD80+ populations.
determined that PDCs in BXD2 mice expressed increased MCHII, CD86, and CD80 activation/maturation markers compared to those from C57Bl/6 mice (Fig. 2B).

*Type I IFN promotes IgG-producing plasma cells in BXD2 mice.*

We next wanted to determine whether type I IFN signaling is crucial to forming IgG-producing antibody plasmablasts in BXD2 mice. Histological analysis was performed on spleen sections from BXD2, BXD2.IFNAR-/-, C57Bl/6, and C57Bl/6.IFNAR-/- mice for the presence of IgM- and IgG-antibody producing plasmablasts (PB) (Fig. 3). Spleen sections are shown at two different magnifications (Fig. 3A,B). All mice displayed the presence of IgM-producing cells (Fig. 3). However, only the BXD2 spleens exhibited the significant presence of IgG-producing plasma cells around the follicles (FO), indicating that BXD2 mice undergo significant antibody isotype class-switch to generate IgG antibodies (Fig. 3). However, like C57Bl/6, BXD2.IFNAR-/- spleens did not exhibit the presence of IgG-producing plasma cells (Fig. 3). These results indicate that type I IFN is critical to antibody isotype class-switch and the formation of IgG antibody-producing plasma cells in autoimmune BXD2 mice.

*Type I IFN promotes isotype class-switch to generate IgG autoantibodies.*

In order to confirm the role of type I IFN in promoting isotype class-switch for endogenous autoantibodies, we assayed for serum levels of autoantibodies against heat-shock protein 47 (hsp 47), Bip, histone, DNA, and rheumatoid factor (RF) in BXD2, BXD2.IFNAR-/-, and C57Bl/6 mice. For all autoantibodies surveyed, BXD2 mice produced increased IgM and IgG titers compared to C57Bl/6 mice. BXD2.IFNAR-/-
FIGURE 3. Type I IFN receptor deletion abrogates formation of IgG-producing plasmablasts in BXD2 spleens. (A-B) Spleen sections of BXD2, BXD2.IFNAR-/-, C57Bl/6, and C57Bl/6.IFNAR-/ mice were stained with anti-IgM and anti-IgG antibodies as described in Materials and Methods. (A) 10x magnification of images of spleen sections, denoted with respect to the presence of a follicle (Fo) and the surrounding plasmablasts (PB) (B) 40x magnification of images of spleen sections, denoted with respect to the presence of a follicle (Fo) and plasmablasts (PB).
FIGURE 4. Type I IFN receptor deletion abrogates endogenous autoantibody isotype class-switch in BXD2 mice. Sera were collected from BXD2, BXD2.IFNR-/-, and C57Bl/6 mice. Serum autoantibodies against Bip, hsp47, histone, DNA, and RF were assayed by ELISA.
mice produced either similar or increased serum IgM titers of autoantibodies, but produced significantly decreased serum levels of IgG titers (Fig. 4). Serum levels of IgM titers of autoantibodies against hsp27 and Bip were increased greater than 2-fold in BXD2.IFNAR-/- mice than those in BXD2 mice (Fig. 4). For IgG2b isotypes, BXD2 mice produced greater than 2-fold serum levels of anti-hsp47 antibody, greater than 11-fold levels of anti-histone antibody, and greater than 9-fold levels of RF than BXD2.IFNAR-/- mice (Fig. 4). For IgG2c isotypes, BXD2 mice also produced greater than 2-fold serum levels of anti-Bip antibody, greater than 14-fold levels of anti-histone antibody, and about 2-fold higher levels of anti-DNA antibody (Fig. 4). However, we did not observe significant titer differences for IgG1 (Fig. 4). Together, these results indicate that type I IFN signaling is required for endogenous autoantibody isotype class-switch.

Type I IFN signaling drives germinal center formation.

Having determined that type I IFN signaling enhanced germinal center formation, we sought to determine whether type I IFN directly promotes germinal center formation. Enriched B-cells were sorted from the whole spleens of IFNAR-competent BXD2.GFP+/+ mice and adoptively transferred into BXD2.IFNAR-/- mice. IFN-α or placebo was intravenously injected into the recipient mice twice for the first week. Thereafter, recipient mice received one dose per week for another two weeks before the mice were sacrificed. Flow cytometry was performed on the cells extracted from the spleens of recipient BXD2.IFNAR-/- mice. Two populations were observed, one consisting of GFP+/+ CD19+ B-cells, and the other consisting of GFP-/- CD19+ B-cells (Fig. 5A).
FIGURE 5. Type I IFN signaling in B cells directly drives germinal center formation. (A-B) CD19+ B-cells were enriched from GFP+/+.BXD2 spleen cells and adoptively transferred into BXD2.IFNAR-/- mice. The recipient BXD2.IFNAR-/- mice received two doses of IFN-α for the first week, and one dose of IFN-α per week for the next two weeks by i.v. injections. After a total of three weeks after the adoptive transfer, the recipients were sacrificed. Spleen cells were labeled with anti-CD19, anti-Fas, and anti-PNA antibodies. After flow cytometry was performed, (A) the frequencies of GFP+CD19+ and GFP-CD19+ B-cells were determined for recipients BXD2.IFNAR-/- treated with either IFN-α or PBS, and BXD2 mouse. (B) Gating on GFP+CD19+ and GFP-CD19+ B-cells, the frequencies of Fas+PNA+ germinal center cells were determined for BXD2.IFNAR-/- recipients treated with either IFN-α or PBS, and BXD2 mouse.
Fig. 5

IFN-α injected

PBS injected

BXD2 control

GFP-CD19+

GFP+CD19+

Fas

CD19

PNA
Only one population of GFP-/- CD19+ B-cells was observed in a BXD2 mouse control (Fig. 5A). Gating on the GFP-/- B-cells, significantly decreased Fas+PNA+ germinal center B-cells in the recipient BXD2.IFNAR-/- compared to the wild-type BXD2 mouse control was observed (Fig. 5B). However, when comparing the gated GFP-/- B-cell with the GFP+/+ B-cell populations, dramatically increased populations of Fas+PNA+ germinal center B-cells was observed in the latter relative to the former (Fig. 5B). In the placebo treated mouse, we observed 20% of GFP+/+ B-cells were germinal center B-cells. IFN-α treatment further enhanced this population of germinal center B-cells to 32% (Fig. 5B, left). In contrast, the frequency of GFP-/- B-cells that are germinal center B-cells remained relatively constant, in the range of 2 to 4% (Fig. 5B, right).

Discussion

BXD2 mice were found to exhibit pathologies resembling human SLE. The development of glomerulonephritis and elevated autoantibodies, including those against DNA and histone, in BXD2 mice allows us to recognize that these mice are lupus-prone disease [14]. Because type I IFN and pDCs are highly associated with the incidence of SLE in humans, we sought to determine the role of pDCs and type I IFN in autoimmune induction in BXD2 mice. Under normal physiological conditions, pDCs are commonly found in the peripheral blood circulation [23, 24]. In lupus, decreased counts of pDCs are found in the blood stream [25], while pDCs are found to accumulate in the dermatological rashes and inflamed kidneys of SLE patients [26, 27, 28]. In murine models of autoimmunity, such as the New Zealand Black (NZB) mouse, increased counts of pDCs were found to reside in the bone marrow [12]. This accumulated pDC
population is attributed to a defective chemokine expression that incapacitated pDC migration from the bone marrow to other organs[12]. In response to viral infections, pDCs are known to migrate to secondary lymphoid organs where sites of inflammatory reactions take place [369, 370]. We consider the BXD2 spleen as an inflamed organ because BXD2 mice exhibited splenomegaly, lymphoproliferation, and germinal centers in the spleen that contributes to the production of high-affinity multi-reactive pathogenic autoantibodies [14, 15]. Therefore, we determined whether the pDCs are increased in the spleens of BXD2 mice. Unlike NZB mice, BXD2 mice exhibited increased counts of pDCs in the spleen, and were found to reside predominantly in the marginal periphery around the developing germinal centers in the spleen. Flt3 L-derived bone marrow pDCs in the BXD2 mouse were more activated than their counterparts in the non-autoimmune C57Bl/6 mouse. These results suggest that increased numbers of pDCs that reside in the spleen of BXD2 mice may act in the spleen to promote the development of autoantibodies.

Having determined that there are increased pDC counts in the spleens of BXD2 mice, we next sought to determine the levels of type I IFN. Under normal physiological conditions, type I IFN is produced in significant quantities only in response to viral infections to induce the expression of anti-viral proteins that interfere with viral replication [31]. Under autoimmune conditions, such as SLE, patients exhibited increased serum levels of IFN-α [11]. The up-regulation of IFN-inducible gene in the peripheral blood mononuclear cells, known as the “type I IFN signature”, is commonly found in SLE patients [8, 10]. IFN-α therapy for patients with hepatitis and tumors have occasionally induced the presence of serum autoantibodies and symptoms, such as malar
rashes, that are commonly associated with lupus [7]. Lupus-prone mouse models have exhibited increased serum levels of IFN-α [12, 13]. Deletion of the type I IFN receptor (IFNAR) in NZB and lpr mice has abrogated the development of autoantibodies and the onset of glomerulonephritis [13]. Consistent with this finding, IFN-α expression by delivery of an adenoviral vector carrying the IFN-α gene, into (NZBxNZW)F1 mice have accelerated autoantibody production and the earlier onset of fatal glomerulo-nephritis [19]. Because pDCs are known to efficiently produce type I IFNs [32, 33] and type I IFNs are associated with lupus, we determined type I IFN levels in the lupus-prone BXD2 mice. Using a type A CpG, TLR 9 ligand, to induce pDC production of type I IFN, we determined that BXD2 mice exhibited significantly elevated levels of IFN-α for all ages compared to non-auto-immune C57B/6 mice. The highest levels of IFN-α were inducible in vitro and in vivo at 4-6 months of age, which correlates with the age of onset of rapid development of autoantibodies and autoimmune disease. Similarly, we found increased type I IFN message levels in the peripheral blood mononuclear cells of unstimulated BXD2 mice, and this was also highest at 4-6 months of age. The increased levels of type I IFN, at both the protein and transcript levels, began to precipitously rise at the ages 4-6 months, corresponding to the observed increase in autoantibody titers as reported previously [14], suggestive that type I IFN has a causal role in autoantibody production in BXD2 mice.

Several studies have been completed to define the role of type I IFN in promoting antibody responses. In the context of lupus, sera containing high levels of type I IFN from SLE patients have been shown to differentiate monocytes to dendritic cells [34]. In a non-autoimmune context, type I IFN, in conjunction with IL-6, has been shown to
promote the differentiation of CD40 ligand treated naïve B-cells to plasmablasts and plasma cells [20]. Intravenous injections of IFN-α into CGG-immunized mice enhanced antibody titers and promoted antibody isotype class-switch [17]. This study found that CGG-loaded IFNAR-competent dendritic cells promoted antibody isotype class-switch in IFN-α treated mice [17]. The evidence from these studies indicate that type I IFN modulate a T-cell dependent process to either promote CD40-dependent plasma cell differentiation or to initiate antibody isotype class-switch. However, the authors remained skeptical that type I IFN-mediated humoral response must necessarily require T-cell help [17, 20]. In the case of plasma cell differentiation, CD40 ligand can be provided by activated endothelial cells [20], mast cells [36], platelets [37], B-cells [38], as well as activated T-cells, indicating that PDC-driven plasma cell development does not necessarily require T-cell help. Adoptive transfer of antigen-loaded IFNAR-competent dendritic cells into IFN-α treated mice induced antibody isotype class-switch, suggestive that the dendritic cells brought about the antibody response by polarizing T-helper cells [17]. However, virally-infected CD40-/- mice were able to class-switch, indicating that antibody isotype class-switch do not necessarily require T-cell help [39]. While these studies point to a functional role for type I IFN in promoting antibody responses, such as to enhance antigen presentation by differentiating monocytes to dendritic cells, or to enhance antibody production and secretion by differentiating naïve B-cells to plasmablasts, the issue of precisely how and by what mechanism does type I IFN direct antibody isotype class switch remained a mystery. While IFNAR-competent dendritic cells could promote antibody isotype class-switch, the authors of the study concede that the mechanism by which this is completed is unknown [17]. Another study by the same
authors has concluded that type I IFN signaling is required in both B- and T-cells to generate maximal antibody responses [43]. IgG antibody titers were significantly decreased once IFNAR was deleted in either B- or T-cells, or both [43]. However, while ascertaining the importance of both T- and B-cells to type I IFN-mediated antibody response, the pathway for antibody isotype class switch remained relatively undefined.

In the present study, we sought to determine the role of type I IFNs in promoting the spontaneous development of germinal center formation and antibody isotype class-switch. Previous reports from our laboratory have found that Ad CTLA4 Ig administration into BXD2 mice abrogated the development of autoantibodies, indicating that a T-cell mediated process is primarily responsible for the observed autoantibody titers [15]. Because pDCs were located primarily in the marginal zones surrounding the follicles that encapsulate germinal centers in BXD2 mice, our first inclination was to hypothesize that type I IFN promoted a T-cell independent humoral response in these mice. One report has found that pDCs, in response to CpG, can induce plasma cell differentiation in the absence of T-cells [41]. Of note, this same report did not find that pDCs could induce antibody isotype class-switch [41]. Examining for autoantibodies to hsp47 and Bip in IFNAR-deleted BXD2 mice, we found IgM titers were significantly increased, while IgG titers significantly decreased. This titer pattern indicated that IFNAR-deletion blocked isotype class-switch from IgM to IgG. Histological analysis of paraffin-embedded tissues confirmed the absence of IgG-antibody producing cells in IFNAR-/- BXD2 mice, whereas wild-type BXD2 mice displayed the typically high counts of IgG-producing plasmablasts, while IgM-producers remained the same for both mice. Because previously we have demonstrated that autoantibody production in BXD2
mice required T-cell help, we can conclude that type I IFN is intimately involved in promoting the T-cell dependent autoantibody response in BXD2 mice.

As mentioned above, type I IFN can promote antibody isotype class-switch. However, the pathway for this process remained elusive. Studies have found that type I IFN signaling is required for germinal center formation in response to viral infections in murine models [41, 42]. However, none of the studies to date have investigated the role of type I IFN in T-cell dependent germinal center formation in a lupus context, nor have they demonstrated a direct relationship between type I IFN signaling on B-cells and germinal center formation. While IFNAR deletion in the NZB mice have effectively abrogated the development of autoantibodies [13], type I IFN modulation of autoantibody production at the plasma cell formation stage [20] and T-cell independent mechanisms for antibody class-switch remained the predominant mechanisms to explain autoantibody production in lupus. In contrast to the NZB model, in which the mouse has a prominent marginal zone suggestive of autoantibody formation by a T-cell independent pathway, BXD2 mice do not have a prominent marginal zone (unpublished data). Ad-CTLA4 administration effectively disrupted T-B cell interaction and suppressed autoantibody development in the BXD2 mice, indicating that a T-cell dependent process is the predominant pathway. To demonstrate that type I IFN signal transduction directly drives germinal center formation, we transferred B-cells from IFNAR+/+.GFP+.BXD2 mice into recipient IFNAR-/-. GFP-.BXD2 mice and treated the mice with IFN-α for three weeks before sacrifice. By flow cytometry, we found a dramatically increased count of germinal center B-cells in the wild-type GFP+ population compared, while the recipient IFNAR-deleted GFP- B-cells exhibited low counts of germinal center B-cells. There was
a greater than 15-fold increase in the frequencies of germinal center B-cells amongst the adoptively-transferred GFP+/+ B-cells relative to the frequencies of germinal center B-cell normally found in BXD2 mice. This finding indicated that type I IFN signaling drives germinal center formation in the BXD2 mice.

Through the direct signaling on B-cells, type I IFN directly drives germinal center formation, generating isotype class-switched IgG isotype-producing plasma cells. The question as what predominant type of B-cells was driven into germinal center cell formation remains valuable questions for future studies. Candidates include follicular and marginal zone B-cells, both of which have been shown to mediate germinal center formation by migration into follicles. That type I IFN can drive migration of lymphocytes into lymphoid organs hint that likewise similar processes may be at work within the lymphoid organs. Type I IFN-driven migration of particular B-cell subsets into the follicles and their subsequent germinal center differentiation serves as a novel immunological process that opens a new avenue of cellular pathways for autoimmune disease induction.

References


Follicular Entry of MZ-P B cells Induced by Interferon-Alpha

Promotes Spontaneous Germinal Centers

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Running title: IFNα promotes GC formation via influx of MZ-P B cells

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Abstract

Understanding the early event triggering the formation of a spontaneous germinal center (GC) is important to prevent the development of autoantibodies in autoimmune diseases. Here, we show that there was a significantly larger population of marginal zone precursor (MZ-P) B cells, defined as being $\text{IgM}^{\text{hi}}\text{IgD}^{\text{hi}}\text{CD21}^{\text{hi}}$ and $\text{CD23}^{\text{hi}}$ in the spleens of autoimmune BXD2 mice compared to B6 mice. MZ-P B cells expressed high levels of costimulatory signals and promoted a greater Ag specific CD4 T cell activation compared to follicular and conventional $\text{CD23}^{\text{lo}}$ MZ B cells. IFN$\alpha$ produced by plasmacytoid dendritic cells localized in the marginal sinus plays an important role in promoting the migration of MZ-P B cells into the follicle. Knockout of IFN$\alpha$R in BXD2 mice abrogated this response and suppressed the development of GCs and autoantibodies. Our results suggest that IFN$\alpha$-induced entry of MZ-P B cells into the follicle is an early event for autoreactive GC formation in BXD2 mice.

Introduction

Although autoreactive B cells are normally deleted, in autoimmune murine models, B cells can develop and act as antigen presenting cells (APCs) in the development of spontaneous autoimmune germinal centers (GCs) [1-4]. Treatment of autoimmune disease with B cell depletion therapy depletes B cells that interact with CD4 T cells and act as APCs [5-7]. In spontaneous autoimmune thyroiditis, anti-CD20 depleted most
transitional (T)2 and follicular (FO) B cells, but not marginal zone (MZ) B cells [8]. Depletion of these B cells is associated with a reduced disease severity [8], suggesting that transitional T2 and FO B cells are critical for the pathogenesis of autoimmune B-cell responses.

The localization of B cells in appropriate microenvironments of secondary lymphoid organs plays an important role in determining the fate of B-cell immunity. Specifically, the B cell migration pattern into the follicular regions of the spleen can alter the tempo and frequency of T-B cell contact and thus has been proposed as one mechanism to regulate the development of autoreactive B cells [9, 10]. Early studies by Cyster et al [11] showed that under conditions of clonal selection, anergic B cells failed to enter the follicular areas, instead arresting at the T-B cell boundary in a process known as follicular exclusion. Follicular exclusion has been proposed as one B cell checkpoint mechanism to prevent B cell tolerance loss [12]. Although chemokines and chemokine receptors have been paramount in shaping B cell migration patterns, we and other have shown that local cytokine production can provide additional signals to regulate the migration and retention of B cells in the follicles [13, 14, 15].

The BXD2 mouse strain is one of the approximately 20 BXD recombinant inbred strains we have analyzed for development of autoimmune disease, including the development of features resembling human SLE and RA [16]. Development of highly mutated multi-reactive autoantibodies that can induce kidney and joint disease is one of the important pathogenic features of the BXD2 mice [16, 17]. We have found that the BXD2 mice spontaneously form GCs in the spleens and that the expression of the gene encoding activation-induced cytidine deaminase (Aicda) in the GC B cells can be
stimulated by activated CD4+ T cells from BXD2 mice [18]. There is an abundance of T\(_{H-17}\) cells in the follicles and GC area. The high levels of IL-17 produced by these cells is associated with upregulation of regulator of G-protein signaling (Rgs)\(_{13}\) and Rgs\(_{16}\), and the migration arrest of B cells in the follicles, enabling them to form stable T-B cell conjugates and facilitate GC formation in the spleens of BXD2 mice [13].

The importance of IFN\(\alpha\) in the spontaneous development of autoantibodies and immune complex deposition in the glomeruli has been demonstrated using murine models of lupus [20, 21, 46]. Clues to the possible role of IFN\(\alpha\) in the production of high-affinity autoantibodies can be derived from their normal physiologic mechanisms of action. IFN\(\alpha\) has been shown to promote antibody class-switch recombination (CSR) and plasma cell differentiation under non-autoimmune conditions [22, 23]. Strikingly, although the type I IFN signature is associated with lupus in humans, IFN\(\alpha\) by itself has been found to suppress T\(_{H-17}\) development [24]. Here, we demonstrated that high counts of IFN\(\alpha\)-producing plasmacytoid dendritic cells (pDCs) could be detected in the spleens of autoimmune BXD2 mice concurrent with the presence of large numbers of T\(_{H-17}\) cells [13]. To determine the reason for the coexistence of high levels of IFN\(\alpha\) and IL-17 in the spleens of BXD2 mice, we first observed that there was a significantly increased CD21\(^{hi}\)IgM\(^{hi}\)CD23\(^{hi}\)IgD\(^{hi}\)CD93\(^{-}\lo\) B cell population in the spleens of BXD2 mice. B cells with this phenotype are referred to as MZ-precursor (MZ-P) B cells in mice [25, 26]. However, B cells with a similar phenotype have been described as naïve BM2 pre-GC B cells that transport antigen to stimulate CD4 T cells [27]. In this report, we provided evidence to show that these MZ-P B cells are important for the development of spontaneous GC in the spleens of BXD2 mice based on their physical location, their high
levels of expression of costimulatory molecules and expression of IL-21R. Compared to FO B cells, MZ-P B cells exhibit a higher proliferative response to IgM and CD40 crosslinking. Also, MZ-P B cells exhibit strong co-stimulatory activity on CD4 T cells. We have further identified that the entry of MZ-P into the FO region is promoted by IFNα, which is primarily produced by pDCs that are localized in the marginal sinus (MS). Abrogation of IFNαR in BXD2 mice suppressed the development of spontaneous GCs and autoimmune disease. MZ-P B cells were more susceptible to IL-17-induced migration arrest, compared to MZ B cells. Our results therefore suggest that spatial compartmentalization of IFNα producing pDCs and IL-17 producing CD4 T cells can act collaboratively to promote the development of spontaneous GCs through their effects to promote the entry and retention of potent antigen-presenting B cells in the follicles.

**Materials and Methods**

*Mice*

Female homozygous C57BL/6, BXD2 recombinant inbred, and the MOG specific C57BL/6-Tg (Tcrα2D2,Tcrβ2D2)1Kuch/J (2D2 TCR Tg) were obtained from The Jackson Laboratory; B6-Ifnar<sup>−/−</sup> mice were obtained from Dr. Jocelyn Demengeot at the Instituto Gulbenkian de Ciência in Oeiras, Portugal). B6-Il17r<sup>−/−</sup> mouse is a generous gift from Amgen Inc (Thousand Oaks, CA). B6-Ifnar<sup>−/−</sup> mice and B6-2D2 TCR Tg mice were backcrossed with BXD2 mice for six, seven, and nine generations, respectively, using a marker-assisted speed congenic approach with 146 markers. BXD2-Il17r<sup>−/−</sup> mice were generated as we described previously [13]. All mice were housed in the University of Alabama at Birmingham (UAB) Mouse Facility under specific pathogen-free.
conditions in a room equipped with an air-filtering system. The cages, bedding, water and food were sterilized. All mouse procedures were approved by The UAB Institutional Animal Care and Use Committee.

*Analysis of a high affinity anti-NP response*

A subset of mice was treated with type A CpG 24 h after NP-CGG immunization. Mouse was *i.v.* tail injected with 5 µg CpG (ODN 2216, Cell Sciences) complexed to 30 µL DOTAP (Roche) in a total volume of 115 µL PBS. Sera were collected 17 days after immunization as previously described [13].

*Flow cytometry analysis and cell sorting*

Flow cytometry was performed on fluorescently-labeled single cell suspensions derived from spleens using the method we described previously [18]. For the detection of various cell types, including pDCs and B cells, fluorescent –conjugated anti-PDCA1, anti-CD11c, anti-B220, anti-Gr-1, anti-CD19, anti-IgM, anti-IgD, anti-CD21/35, anti-CD23, and anti-CD93/AA4 antibodies were employed. For the detection of activation and other markers on cells, fluorescent-conjugated anti-CD40, anti-CD80, anti-CD86, anti-CD21R, anti-ICOS, anti-ICOSL and anti-CD69 antibodies were employed in addition to the antibodies described above. For the labeling and isolation of pDCs, all spleens were cut and treated with collagenase D (2 mg/mL) for 30 minutes at 37°C/5%CO₂ before obtaining single cell suspensions for antibody labeling. For intracellular detection of IFNα, cells were first permeabilized (BD Biosciences PharMingen Cytofix/cytoperm), washed (BD Biosciences PharMingen Perm/Wash), then incubated
with a 1-to-1 mixture of anti-IFNα antibodies (clones RMMA-1 and F18). Cells (100,000/sample) were washed twice with FACS buffer and fixed in 1% paraformaldehyde/FACS® solution before analysis by flow cytometry using a BD-LSR-II Flow Cytometer ® (Becton Dickenson). The analysis was performed using FlowJo Software (Tree Star, Inc, Ashland, OR). Forward-angle light scatter (FSC) was used to exclude dead and aggregated cells. Some results are presented as fluorescence histograms with the relative number of cells on a linear scale versus the relative fluorescence intensity on a log scale. Acquisition and gating of FO, MZ, and MZ-P B cells was carried out based on the method described by Allman and Pillai [26]. FO B cells were defined as CD23\(^+\) sIgM\(^{low+high}\) sIgD\(^{high}\) CD21/35\(^{int}\) CD19\(^+\) cells; MZ B cells were defined as CD23\(^−\) sIgM\(^{high}\) sIgD\(^{low}\) CD21/35\(^{high}\) CD19\(^+\) cells; MZ-P B cells were defined as CD23\(^+\) sIgM\(^{high}\) sIgD\(^{high}\) CD21/35\(^{high}\) CD19\(^+\) cells. All gatings were set up to ensure that the percent of each sub-population of B cells obtained from normal B6 mice was equivalent to the previously reported results [26].

For cell sorting to isolate the different B-cell populations, single-spleen cell preparations were labeled with fluorescent conjugated antibodies as described above, and were sorted into FO, MZ, and MZP B cells based on the expression of CD21/35, CD23, IgM, IgD as described above. Sorting was carried out using a BD FACS Aria Cell Sorter. All sorting for pDCs were completed by positive selection using anti-PDCA1 microbeads (Miltenyi Biotech).
Immunofluorescent staining and confocal image analysis

Spleens from mice were collected, embedded in Frozen Tissue Media (Fisher Scientific) and snap-frozen in 2-methylbutane. Frozen sections (8 μm thick) were dried in air for 30 minutes and fixed in acetone for 15 minutes. The sections were blocked with 5% horse serum for 30 minutes at room temperature and then stained for 30 minutes at room temperature with various antibodies. The following primary antibodies conjugated to the Alexa flour dyes were used to stain spleen tissues for confocal image analysis: biotin-PNA (Vector Laboratory) to Alexa 350–streptavidin (Invitrogen), anti–mouse CD35 (clone 8C12, BD Bioscience) to Alexa 488; anti-IgM to Alexa 555; anti-CD4 (RM4-5, Invitrogen) to Alexa 488 or 647, anti-CD23 (B3B4) to Alex488, and anti-PDCA1 to Alexa 647. The sections were mounted in Fluormount G (Southern Biotechnology) and viewed with a Leica DM IRBE inverted Nomarski/epifluorescence microscope outfitted with LeicaTCS NT laser confocal optics. In situ quantitation of the PNA⁺ GC response was carried out using the Olympus DP2-BSW software (Olympus America, Center Velley, CA) according to the method we previously described [13].

In vitro stimulation and cell cultures

Cells, including pDCs, B cell subpopulations, or B-T cell mixtures, were cultured with RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 5.5 x 10⁻⁵ M 2-mercaptoethanol and 10% FCS, for all in vitro experiments.

For CD69 expression on different B cell subpopulations, single spleen cells were cultured in 96-well (Costar) tissue-culture plate at 37°C/5% CO₂ in triplicate wells
(1X10^4 cells per well) and stimulated with IFNα (20 ng/mL) as indicated. To measure IFNα levels produced by pDCs, single-cell suspensions were prepared by collagenase D (2 mg/mL) digestion of spleen tissues for 30 minutes at 37°C/5% CO2 before sorting for pDCs. Enriched pDCs were subsequently cultured in 96-well (Costar) tissue-culture plate at 37°C/5% CO2 in triplicate wells (1X10^4 cells per well) and stimulated with medium only, CpG (3 μM), or CpG (3 μM):DOTAP-complex (Roche). After 24 hours, culture supernatants were collected and IFNα levels were measured by ELISA (PBL Biomedical Sciences). CpG:DOTAP preparation for in vitro culture was formulated by mixing 19.2 μg (3 nmole) of CpG in 120 μl of PBS with 30 μl of DOTAP, incubating for 15 min, and then diluting with 850 μl of medium to make 3 μM CpG:DOTAP solution.

MOG-specific CD4 T-cell proliferation assay

Single-cell suspensions were prepared from the spleens of 8 to 10 wk-old mice. B cells from B6 and BxD2 mice were FACS-sorted into FO, MZ, and MZ-P populations based on the expression of cell surface markers and on the gating method described by Allman and Pillai [26]. Sorted B cells were subsequently irradiated (3,300 rad), co-cultured with MACS beads purified 2D2 T cell receptor transgenic BXD2 CD4+ T cells (4x10^5/mL) in a 2-to-1 ratio of B-T cell mixture. The B-CD4 T cell mixtures were cultured in the presence of 50 μg/ml of MOG35-55 peptide (CPC Scientific, San Jose, CA). Cells were cultured in triplicate wells (5 x 10^5 cells/well) of a Costar® 96-well tissue culture plate (Corning, Inc.) for 72 h in the culture medium described above. The proliferative response was measured using a standard [3H]-thymidine incorporation assay.
in which 1 μCi of [3H]-thymidine (Amersham Biosciences) was added to each well at the 72 h time point. Cells were collected 12 h later using a Filtermate harvester (Packard-Perkin Elmer Co) and radioactivity measured using a Top Count liquid scintillation counter (Packard-Perkin Elmer Co.).

Cell migration assay

Single-cell suspensions of anti-CD19 MACS column (Miltenyi Biotech)-purified spleen B cells from B6, BXD2 or BXD2-Ifnar−/− mice were stimulated with either medium only control or mouse recombinant IFNa (20 ng/ml, R&D Systems). The cell migration assay was carried out using the protocol described by Moratz, et al. [29] The stimulated cells were loaded (2 x 10^6) into the upper well insert (5-µm pore size) of a Transwell system (5 µm pore size, Costar), and S1P was added into the bottom chamber at a final concentration of 20 nM. After incubation for 2 h at 37°C in a 5% CO2 incubator, the migrated cells were harvested and resuspended in 300 µl FACS buffer. The cells that remained in the inserts or migrated to the lower chamber were counted using a flow cytometer, and the distribution of the subsets of CD19^+ B cells into migrated versus non-migrated cells was determined. The chemotaxis index was calculated by dividing the number of cells that migrated in response to chemokine by the number of cells that migrated in the absence of chemokine.

Isolation and quality control of RNA

RNA was isolated from 0.2 to 10 x 10^6 cells using the Trizol reagent (Invitrogen) or <0.2 x 10^6 using the PicoPure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA). The quality of the isolated RNA was determined using an Experion Automated
Electrophoresis System (Bio-Rad, Hercules, CA). RNAs that exhibited an 18S/28S ratio >1.8 were then converted to cDNA using the First Strand cDNA Synthesis Kit (Fermentas, Inc).

**qRT-PCR**

Measurements of Aicda and Slp1 transcripts in B cells isolated from the spleens were quantitated by quantitative polymerase chain reaction using the BioRad iQ5 Multicolor Real-Time PCR Detection System. The expression of Aicda, Slp1, Slp3, Cxcr4, and Cxcr5 transcripts was determined quantitatively by real-time PCR using the SYBR Green PCR Master Mix (Bio-Rad) with the following primers: Aicda forward: GCCACCTTCGCAACAAGTCT; Aicda reverse: CCGGGCACAGTCATAGCAC (137 bp); Slp1 forward: TGCTGTAACTGAAGGCTCAC; Slp1 reverse GGATGCTAGTGGACACCCTAG (108 bp); Slp3 forward: CCGTAGTGTAGTGGTGAGTG; Slp3 reverse: GGACAGCCAGCATGATGAAC; Cxcr4 forward: TCCAACAAGGAACCCTGCTTC; Cxcr4 reverse: TTGCCGACTATGCCAGTCAAG; Cxcr5 forward: GAGATTCCCCTACAGGACAGT; Cxcr5 reverse: CCAGCACCGAGATTCCC; Gapdh forward: AGGTCGGTGTAACCGATTTTG; Gapdh reverse: TGTAGACCATGTAGTTGAGGATCTCA (136 bp). A final volume of 25 µl was used for quantitative PCR in an IQ5™ thermocycler (Bio-Rad). Amplification conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and annealing for 30 s. The annealing temperature used was 56°C for Gapdh, 57°C for Slp3, 58°C for Aicda, and 60°C for Slp, Cxcr4 and Cxcr5.
qRT-PCR products were normalized relative to that of Gapdh to correct for potential differences in template input in order to determine relative mRNA levels. Results are expressed as fold differences in expression of the indicated gene relative to the expression of Gapdh. Standard curves were generated for every target using six 4-fold serial dilutions.

Results

Localization of pDCs in the marginal sinus (MS) of the spleens of BXD2 mice

IFNα has been implicated in several human autoimmune diseases [30, 31, 20]. The cell type that most efficiently produces IFNα has been determined to be pDCs [32]. We have previously showed that increased development of T\textsubscript{H}-17 cells is one important pathogenic mechanism associated with the development of spontaneous GCs in the spleens of BXD2 mice. However, because of the unique lupus and arthritis features developed by these mice, we questioned whether high numbers of IFNα-producing pDCs can also be observed in BXD2 mice. Flow cytometry analysis indicated that there was a significantly higher percentage of IFNα-producing pDCs in the spleens of BXD2 mice compared to the spleens of B6 mice (Fig. 1A, left panels, and Fig. S1A). On gating on the IFNα producing cells, we found that the pDCs are the primary IFNα-producing cells in the spleens of BXD2 mice (Fig. 1A, right panel), which is consistent with the reports that pDCs are efficient producers of type I IFN [32-36]. The production of IFNα by pDCs can be stimulated by the addition of type A CpG, which engages the Toll-like receptor-9 [32, 33]. We found that pDCs isolated from the spleens of BXD2 mice were more responsive to in vitro stimulation with either type A CpG or type A CpG:DOTAP.
**FIGURE 1.** Increased type I IFN-producing pDCs in the spleens of BXD2 mice.  

**A**, Flow cytometry was performed on B6 and BXD2 spleen cells labeled with anti-IFNα and anti-PDCA1 antibodies (left panels). Gating on IFNα⁺ cells, the frequencies of BXD2 and B6 PDCA1⁺ cells were determined (right panel). Means ± SEM. (n=6); *P < 0.05 compared with B6 mice.  

**B**, ELISA analysis of the levels of IFNα produced by unstimulated, CpG, or DOTAP complexed to CpG (DOTAP:CpG) stimulated pDCs isolated from the spleens of B6 (black bars) and BXD2 mice (white bars). pDCs were enriched and cultured *in vitro* for 24 hours with the indicated stimulations. Means ± SEM (n=6); *P < 0.05 compared with BXD2 mice.  

**C**, Confocal imaging analysis of spleen sections taken from B6 and BXD2 mice that were stained with fluorescent conjugated anti-PDCA1, anti-IgM, anti-CD35 (FDC), and PNA-biotin followed by an Alexa350-conjugated secondary antibody as described in the Materials and Methods. Data are representative of three independent experiments.
Fig 1
Supplementary Figure S1.

Increased frequency of pDCs in the spleens of BXD2 mice that exhibit increased activation markers and a pattern of chemokine receptor expression that can promote location to MZ. A, Flow cytometry was performed on single-cell suspensions from the spleens of B6 and BXD2 mice labeled with anti-MHC-II, anti-CD11c, anti-B220, and anti-Gr-1 antibodies. MHC-II+ cells were gated to display the CD11clo populations (left panel). Gating on CD11clo and CD11chigh populations, the percentages of B220−Gr-1+ populations were further determined (right panels). Means ± SEM (n=6); * P < 0.05 or ** P < 0.01 compared with BXD2 mice. B, Quantitative RT-PCR analysis of Cxcr4, Cxcr5, S1p1, and S1p3 transcripts in MACS column purified pDCs in the spleens of B6 and BXD2 mice. The levels of mRNAs relative to Gapdh are shown. Means ± SEM (n=6); * P < 0.05 compared with BXD2 mice.
and produced significantly greater amounts of IFNα in response to these agents than equivalent numbers of pDCs isolated from the spleens of B6 mice (Fig. 1B). Immunofluorescent imaging of spleen sections of B6 mice show that there were low numbers of pDCs (green) that form a single layer surrounding non-reactive follicles (Fig. 1C). In the spleens of wild-type naïve BXD2 mice, there were large follicles with active GCs (blue). Surrounding these active GCs, there were increased numbers of pDCs (green) that were located away from the GCs but were primarily in the marginal periphery of the follicles in proximity to the marginal sinus (MS) (Fig. 1C). One factor that influences lymphocyte localization to the MZ is upregulation of S1P1 [354]. Consistent with the localization of pDCs to the marginal zone, *S1p1* transcripts were higher and *Cxcr4* transcripts were lower in the pDCs isolated from BXD2 mice than in the pDCs from B6 mice (Fig. S1B).

**IFNα enhances T-dependent humoral responses in BXD2 mice**

IFNα inhibits TH-17 development [24], which is thought to play a role in GC formation [366, 398, 399]. We therefore asked whether IFNα is associated with either an increase or a decrease in GC formation and antibody production in BXD2 mice. One possibility is that since pDCs are located in the MS away from the T cell zone, production of IFNα at that site may not interfere, and may even promote GC formation in the follicle. To test the effect of decreased type I IFN receptor (IFNαR) signaling on the antibody response, we generated BXD2-*Ifnar*<sup>−/−</sup> mice by backcrossing BXD2 mice with B6-*Ifnar*<sup>−/−</sup> mice. The B6, wild-type BXD2, and BXD2-*Ifnar*<sup>−/−</sup> mice were immunized with either NP-CGG, a T-dependent antigen, or NP-Ficoll, a T-independent antigen and
the titers of IgM or IgG high-affinity anti-NP7 antibody responses in sera were measured 17 days after immunization. The levels of NP-CGG-induced high-affinity anti-NP7 IgG2b and IgG2c antibodies were significantly lower in the BXD2-\(\text{Ifnar}^{-/-}\) mice than in the wild-type BXD2 mice (Fig. 2A and 2B).

To test the effect of increased IFN\(\alpha\)R signaling on the antibody response, mice were further treated with type A CpG which can promote type I IFN through the induction of the TLR9 [400]. Intravenous injection of type A CpG 24 h after immunization resulted in higher titers of anti-NP7 antibody in the wild-type BXD2 mice (Fig. 2A and 2B) and in B6 mice than their untreated counterparts. Consistent with the observation that BXD2 mice exhibited a higher capacity to produce IFN\(\alpha\) than B6 mice, CpG-treated B6 mice did not generate significantly higher antibody titers than similarly treated BXD2 mice. Notably, both IgG2b and IgG2c antibody titers remained significantly low in BXD2-\(\text{Ifnar}^{-/-}\) mice, regardless of whether they were treated with CpG or not (Fig. 2A and 2B). In contrast, the intravenous injection of type A CpG did not enhance the T-independent antigen response, as indicated by a small insignificant increase in the levels of IgG2b and IgG2c anti-NP antibody production in response to NP-Ficoll immunization in the BXD2 mice. NP-Ficoll immunization could induce an IgM response but did not induce IgG2b and IgG2c anti-NP antibody production (Fig. 2C).

To determine the role of IFN\(\alpha\) in GC formation, the presence of GCs in WT BXD2 and age matched BXD2-\(\text{Ifnar}^{-/-}\) mice was compared. Confocal microscopy analysis of frozen spleen sections confirmed the presence of well-formed PNA\(^{+}\) GCs (PNA\(^{+}\); blue) located close to the central areas of follicles in 3-mo-old wild-type BXD2
FIGURE 2. Type I IFN receptor deletion diminishes GC responses in BXD2 mice. A, Confocal imaging analysis of spleen sections from 3-mo-old BXD2 and BXD2-Ifnar−/− mice stained with anti-IgM (red), anti-CD4 (green) antibodies, and PNA (blue) at 10x magnification. B, The quantitative counts of PNA+ GCs per visual field for wild-type BXD2 (WT) and BXD2-Ifnar−/− mice. Means ± SEM (n=5); *P < 0.05 compared with wild-type BXD2 mice. C, Flow cytometry analysis of the percentage of PNA+Fas+ B220+ B cells in the spleens of 6-mo-old BXD2 and BXD2-Ifnar−/− mice. Representative FACS pseudocolor plots are shown in left panels, and quantitative bar graph for wild-type BXD2 (WT) and BXD2-Ifnar−/− is shown in the right panel. Means ± SEM (n=6); **P < 0.01 compared with wild-type BXD2 mice. D, qRT-PCR analysis for transcript levels of Aicda in enriched B cells from the spleens of 3-mo-old wild-type BXD2 (WT) and BXD2-Ifnar−/− mice. Means ± SEM (n=6); *P < 0.05 compared with wild-type BXD2 mice.
Supplementary Figure S2.

Type I IFNαR-deletion attenuates autoimmune disease in BXD2 mice. A, B, ELISA analysis of sera levels of IgM, IgG1, IgG2b, IgG2c isotypes for (A) anti-histone and (B) RF in B6, BXD2, and BXD2-Ifnar<sup>-/-</sup> mice. C, Immunohistochemical images of antibody (IgG) containing immune complexes in the kidneys of B6, B6-Ifnar<sup>-/-</sup>, BXD2, and BXD2-Ifnar<sup>-/-</sup> mice at 10x magnifications. BXD2 and BXD2-Ifnar<sup>-/-</sup> kidneys are displayed at 40x magnifications. D, Quantitation of glomeruli that exhibit IgG-containing deposits as a percentage of total glomeruli per visual field. E, ELISA analysis of urinary protein as determined by the ratio of urinary albumin/creatinine (mg/mg). F, ELISA analysis of C1q containing circulating immune complexes (CIC) in the sera of the indicated strain of mice. For all experiments above, means ± SEM (n=6-10); * P < 0.05 or ** P < 0.01 compared with wild-type BXD2 mice.
mice. In age-matched BXD2-Ifnar<sup>−/−</sup> mice there were fewer PNA<sup>+</sup> GCs and those PNA<sup>+</sup> GCs that were present were smaller (Fig. 2D). Quantification of the number of PNA<sup>+</sup> follicles confirmed that there was a lower number of PNA<sup>+</sup> GCs per section of area in the spleens of BXD2-Ifnar<sup>−/−</sup> mice compared to wild-type BXD2 mice (Fig. 2E) and flow cytometry indicated that BXD2 mice exhibited a higher frequency of PNA<sup>+</sup>Fas<sup>+</sup> GC B cells than BXD2-Ifnar<sup>−/−</sup> mice (Fig. 2F). This decreased formation of GCs in BXD2-Ifnar<sup>−/−</sup> mice was associated with significantly reduced levels of Aicda transcripts, which encode the activation-induced cytidine deaminase (AID) protein required for class-switch recombination and somatic hypermutation, in B cells enriched from the spleens of BXD2 mice (Fig. 2G).

ELISA analysis indicated that the titers of IgG2b and IgG2c anti-histone (Fig. S2A) and rheumatoid factor (RF) (Fig. S2B) autoantibodies in the sera of BXD2-Ifnar<sup>−/−</sup> were significantly lower than the titers in the sera of wild-type BXD2 mice. In contrast, the titers of the IgM autoantibodies were less affected by the deficiency in IFNα (Fig. S2A and S2B). One sign of autoimmune disease in wild-type BXD2 mice is significant IgG immune-complex deposition in the glomeruli, which is not observed in the kidneys of control B6 mice (Fig. S2C). Notably, only minimal IgG immune-complex deposition was observed in the glomeruli of age-matched BXD2-Ifnar<sup>−/−</sup> mice (Fig. S2D). Consistent with this, there was a considerable reduction in the severity of proteinuria, as indicated by a significantly lower ratio of albumin/creatinine (mg/mg) in the urine (Fig. S2E) as well as significantly lower levels of circulating C1q containing IgG immune complexes (CIC) in the sera (Fig. S2F) in the BXD2-Ifnar<sup>−/−</sup> mice compared to age-
matched wild-type BXD2 mice. Collectively, the above results suggest that IFNα promoted T-dependent humoral autoimmune responses in the BXD2 mice.

*Increased IgM*<sup>hi</sup>CD21<sup>hi</sup>CD23<sup>hi</sup> B cells in BXD2 mice*

The presence of pDCs in the marginal periphery of follicles suggests that IFNα may signal on B cells that traffic near the border region between the MZ and FO. We have shown previously that the percentage of MZ B cells, defined by their anatomic location in the MZ, and as exhibiting the CD21<sup>high</sup>CD23<sup>−</sup> phenotype, is lower in the spleens of BXD2 mice than age-matched B6 mice whereas the percentage of FO B cells is higher [13]. To determine whether this reduction in mature MZ in BXD2 mice is due to a lack of precursor or altered B cell development under the influence of pDCs in the MS, we analyzed the phenotype of B cells in B6, BXD2 and BXD2-*Ifnar<sup>−/−</sup>* mice. The relative expression of CD23 on the MZ B cells subdivides these cells into two functionally different populations [25, 26, 41]. The CD23<sup>lo</sup>IgM<sup>hi</sup>CD21<sup>hi</sup> B cells are considered to be mature MZ B cells whereas the CD23<sup>hi</sup>IgM<sup>hi</sup>CD21<sup>hi</sup> B cells are thought to be MZ-precursor (MZ-P) or FO-precursor B cells [25, 26, 41].

As defined by IgM<sup>hi</sup>CD21<sup>hi</sup>, the highest percentage of MZ B cells was seen in B6 mice, with lower percentages in BXD2 and in BXD2-*Ifnar<sup>−/−</sup>* mice (*Fig. 3A*). However, the majority of the IgM<sup>hi</sup>CD21<sup>hi</sup> MZ B cells from the spleens of BXD2 mice exhibited the CD23<sup>hi</sup> phenotype (*Fig. 3B, middle panel*), which was significantly greater compared to B6 mice, in which the majority of IgM<sup>hi</sup>CD21<sup>hi</sup> MZ B cells exhibited the CD23<sup>lo</sup> MZ phenotype (*Fig. 3B, upper panel*). Although exhibiting a higher frequency of IgM<sup>hi</sup>CD21<sup>hi</sup>CD23<sup>hi</sup> MZ B cells than in B6 mice, BXD2-*Ifnar<sup>−/−</sup>* mice displayed
FIGURE 3. Increased MZ-P B cells in BXD2 mice. Single cell suspensions were prepared from the spleens of 3-mo-old B6, BXD2, and BXD2-Ifnar\textsuperscript{+/-} mice. \textit{A}, Flow cytometry analysis of the percent of FO (oval) and total MZ (triangle) B (CD19\textsuperscript{+}) cells in the indicated mouse strain. Means ± SEM (n=6); * \(P < 0.05\) compared with wild-type BXD2 mice. \textit{B}, Gated within the total IgM\textsuperscript{hi}CD21\textsuperscript{hi} MZ B cells, cells were further gated as CD23\textsuperscript{null+lo} MZ or CD23\textsuperscript{hi} MZ-P B cells (rectangle gated cells). Means ± SEM (n=6); ** \(P < 0.01\) or *** \(P < 0.005\) compared with wild-type BXD2 mice. \textit{C}, The counts of total MZ, CD23\textsuperscript{lo} MZ, and CD23\textsuperscript{hi} MZ-P in the spleens of 3-mo-old B6, BXD2, and BXD2-Ifnar\textsuperscript{+/-} mice. Means ± SEM (n=6); * \(P < 0.05\), ** \(P < 0.01\) or *** \(P < 0.005\) compared with wild-type BXD2.
Fig 3
significantly decreased percentages than their wild-type counterparts for this particular cell population (Fig. 3B, lower panel).

The total counts of \( \text{IgM}^{\text{hi}} \text{CD21}^{\text{hi}} \) MZ B cells in the spleens were higher in the wild-type BXD2 mice and in BXD2-Ifnar\(^{-/-}\) mice than in the B6 mice due to the significantly enlarged spleen sizes of these BXD2 mice [23] (Fig. 3C). There were fewer conventional CD23\(^{lo}\)IgM\(^{hi}\)CD21\(^{hi}\) MZ B cells, but greatly elevated CD23\(^{hi}\)IgM\(^{hi}\)CD21\(^{hi}\) B cells, in the spleens of BXD2 mice compared to the spleens of B6 mice (Fig. 3C). Although the total spleen cell count is equivalent between WT BXD2 and BXD2-Ifnar\(^{-/-}\) mice, there was a significantly lower percentage and lower total counts of MZ-P B cells in BXD2-Ifnar\(^{-/-}\) mice compared to BXD2 mice (Fig. 3B and 3C). Together, the above indicate that the increased percentage of splenic MZ-P B cells in BXD2 mice may play a role in the autoimmune features in this model.

\textit{IFNa promotes CD21\(^{hi}\)IgM\(^{hi}\)CD23\(^{hi}\) B cell migration near the GC}

MZ B cells can be distinguished as CD21\(^{hi}\)IgM\(^{hi}\) B cells positioned outside of the MS which enables them to encounter blood-borne IgM-immune complexes [42]. In contrast, the location of MZ-P B cells is less well established. In BXD2 mice, there were lower numbers of IgM\(^{hi}\) B that were physically located in the MZ [13], but there were significantly increased number of MZ-P B cells. We therefore determined the physical location of the CD21\(^{hi}\)IgM\(^{hi}\)CD23\(^{hi}\) MZ-P B cells in the spleens of BXD2 mice. In the spleens of BXD2 mice, we have previously characterized spontaneous GCs as a PNA\(^{+}\) region adjacent to a region of CD4 T cells (Fig. 4A). This is localized beneath a layer of CD23\(^{hi}\)IgM\(^{lo}\) follicular B cells (green). On higher-power magnification (Fig. 4B), there
FIGURE 4. IFNα promotes localization of IgM$^{++}$ CD23$^{+}$ B cells near the GC. Confocal imaging analysis of frozen spleen sections from 3-mo-old BXD2 (A-C) and BXD2-Ifnar$^{-/-}$ mice (D-F) and poly(I:C) (100 μg, iv and analyzed 6 hrs later)-treated 3-mo-old BXD2 (G-I) and BXD2-Ifnar$^{-/-}$ mice (J-L). Histological sections were stained with anti-IgM (red), anti-CD4 (white), and anti-CD23 (green) antibodies, and PNA (blue) at 20x magnifications (A, D, G, J). A higher power view of the gated region (rectangular box) for each panel is also shown (B, E, H, K). Bottom panels (C, F, I, L) show anti-CD4 staining only (white). The location of IgM$^{++}$ CD23$^{+}$ B cells is shown with an arrow.
was an area of IgM<sup>hi</sup> B cells that were also CD23<sup>hi</sup> near the GC (yellow). These IgM<sup>hi</sup>CD23<sup>hi</sup> B cells were found to be situated adjacent to a GC and opposite to the CD4 T cell area, suggesting that these MZ-P B cells are located in the dark zone area of a GC. Nonetheless, CD4 T cells were found to be located in the same areas as were MZ-P B cells (Fig. 4C and Fig. S3). In some GCs of BXD2 spleens, IgM<sup>hi</sup>CD23<sup>hi</sup> B cells were found to overlap with PNA<sup>+</sup> B cells that are in the outer zone of a GC (Fig. S3). However, the inner PNA<sup>+</sup> GC B cells expressed lower levels of CD23 (reddish) (Fig. 4B and Fig. S3). In contrast, in BXD2-Ifnar<sup>−/−</sup> mice, the CD23<sup>hi</sup>IgM<sup>hi</sup> B cells were primarily localized close to the MS in the border region between the MZ and FO (Fig. 4D and 4E). Only sparse number of CD4 T cells could be identified in this area (Fig. 4F).

To further demonstrate the role of IFNα in promoting the follicular oriented migration of MZ-P B cells towards the GC in BXD2 mice, BXD2 mice and BXD2-Ifnar<sup>−/−</sup> mice were administered poly(I:C) (100 μg per mouse), a TLR3 agonist that stimulate type I IFN [43]. Six hours after poly (I:C) treatment, CD23<sup>lo</sup>IgM<sup>hi</sup> MZ B cells (red) were found to migrate from the MZ area into the follicle, as demonstrated by a complete absence of this cell population in the MZ with a concurrent influx of such cells in the follicles (Fig. 4G). There was a mixture of IgM<sup>hi</sup>CD23<sup>hi</sup> MZ-P (yellow) and IgM<sup>hi</sup>CD23<sup>lo</sup> (red) MZ B cells adjacent to the GC (Fig. 4H). These B cells were in close proximity to the CD4<sup>+</sup> T cells
Supplementary Figure S3.

MZ-P B cell population near GCs and CD4 T cells in BXD2 mice. Confocal images of a representative BXD2 spleen section stained with anti-IgM (red), anti-CD23 (green), anti-CD4 (white) antibodies, and PNA (blue). CD23$^{hi}$ IgM$^{hi}$ MZ-P B cells are yellow-colored. Bottom row display higher magnifications of boxed areas in top row.
in the GC of BXD2 mice (Fig. 4I). In BXD2-Ifnar<sup>−/−</sup> mice, there was diminished influx of IgM<sup>hi</sup>CD23<sup>lo</sup> (red) MZ B cells into the follicles after poly-IC administration (Fig. 4J). IgM<sup>hi</sup>CD23<sup>hi</sup> MZ-P (yellow) B cells remained primarily in the MZ-FO border region even after poly (I:C) treatment (Fig. 4K). There were few CD4 T cells that were localized adjacent to MZ-P or MZ B cells in BXD2-Ifnar<sup>−/−</sup> mice even after poly(I:C) injection (Fig. 4I and 4L). Together, these results bolster our observation that endogenous production of IFNα by pDCs promoted the migration of IgM<sup>hi</sup>CD23<sup>hi</sup> MZ-P B cells into the follicles in the spleens of BXD2 mice. When pDCs were stimulated by an exogenous TLR3 agonist, local high induction of IFNα further enhanced the migration of IgM<sup>hi</sup>CD23<sup>lo</sup> MZ B cells into the follicles and promoted close association with CD4 T cells.

**Low threshold for CD69 upregulation on MZ-P B cells**

IFNα can inhibit S1P-induced egress of lymphocytes from the lymphoid organs and their re-entry into the circulation by upregulating the expression of CD69, whose expression in turn down-regulates the expression of the S1P<sub>1</sub> receptor for S1P [351]. Moreover, S1P<sub>1</sub> has been shown to promote recruitment from the FO and the retention of lymphocytes in the MZ [37, 45]. Because of the differential threshold between MZ-P and MZ B cell in their capacity to migrate into the FO in response to IFNα stimulation, this raised the possibility that MZ-P B cells are more sensitive to CD69 upregulation by IFNα stimulation, compared to MZ B cells. We found that there was a higher percentage of CD69<sup>+</sup> MZ-P B cells in the spleens BXD2 mice compared to BXD2-Ifnar<sup>−/−</sup> mice (Fig. 4I and 4L).
FIGURE 5. Increased regulation of CD69/S1P<sub>1</sub> signaling axis in MZ-P B cells by IFNα.  

A, Flow cytometry analysis of the percent of CD69<sup>+</sup> cells within the FO, MZ, and MZ-P subpopulations of B cells in the spleens of 2-mo-old BXD2 and BXD2-Ifnar<sup>−/−</sup> mice. Single cell suspensions from the spleens of mice were stimulated with either cell culture medium only or with IFNα (20 ng/ml). Cells were cultured overnight, and the expression of CD69 on the indicated populations of B cells was determined. One representative experiment of a total of four samples per group is shown.  

B, Bar graph showing the expression of CD69 on each population of B cells stimulated with control medium or with IFNα. Means ± SEM (n=4); * P <0.05 or *** P <0.005 compared with wild-type BXD2 mice or compared with non-MZP B cell population.  

C, qRT-PCR analysis of S1p1 transcripts in FACS sorted MZ and MZ-P B cells from the spleens of the indicated strain of mice. Means ± SEM (n=3); *** P <0.005 compared with wild-type BXD2 mice, or compared with CD23<sup>lo</sup> MZ B cell population.  

D, Chemotactic response on B cells from 3-mo-old BXD2 and BXD2-Ifnar<sup>−/−</sup> mice was analyzed using a Transwell migration chamber with S1P (20 nM) or culture medium loaded in the bottom chamber. Cells were either not stimulated or stimulated with IFNα for 4 hrs prior to carrying out the migration analysis. FACS analysis was carried out to determine the migration response to S1P for the indicated populations of B cells. Means ± SEM (n=6); * P <0.05 or ** P <0.01 compared with wild-type BXD2 mice, or compared with CD23<sup>lo</sup> MZ B cells from BXD2 mice.
5A, upper panels and Fig. 5B). The percentages of CD69⁺ FO and CD69⁺ CD23lo MZ B cells were equivalent between BXD2 and BXD2-Ifnar⁻/⁻ mice. Stimulation of B cells with IFNα resulted in the upregulation of CD69 on FO, MZ, and especially on the CD23hi MZ-P B cell population of BXD2 mice, and this response was greatly decreased in B cells isolated from BXD2-Ifnar⁻/⁻ mice (Fig. 5A, lower panels and Fig. 5B). Consistent with the higher percentage of MZ-P B cells from BXD2 mice that expressed CD69, the MZ-P B cells expressed lower levels of S1p1 transcripts when compared to the CD23lo MZ B cells from these mice (Fig. 5C). Moreover, the relatively low expression of CD69 in the MZ-P B cells from the BXD2-Ifnar⁻/⁻ mice was consistent with our finding for higher S1p1 transcript levels in MZ-P B cells from these mice than their wild-type counterparts (Fig. 5C). Analysis of the chemotactic response towards S1P in vitro indicated that the migration of the MZ B cell in response to S1P was equivalent between BXD2 and BXD2- Ifnar⁻/⁻ mice. However, the S1P-induced chemoattractive effect on MZ-P B cells from the BXD2 mice was lower than that of MZ-P cells from BXD2-Ifnar⁻/⁻ mice (Fig. 5D). Consistent with the elevated expression of CD69 on the BXD2 MZ-P cells, in vitro IFNα stimulation of B cells from BXD2 mice led to further reduction of the chemotactic response toward S1P, especially in the CD23hi MZ-P B cell population, and this decreased was not observed in B cells isolated from BXD2-Ifnar⁻/⁻ mice (Fig. 5D). These results suggest a model by which production of IFNα by the pDCs in the MS of BXD2 spleens “pushes” the entry of CD23hi MZ-P B cells away from the MZ and towards the FO, thereby heightening the probability that they will encounter CD4⁺ T cells in the follicles.
Supplementary Figure S4.

MZ-P B cells from BXD2 mice exhibited highest proliferation by anti-CD40 and anti-IgM stimulation. Single-cell suspensions prepared from the spleens of 8 to 12-wk-old mice were sorted into FO, MZ, and MZ-P B cells based on the expression of CD21/35, CD23, IgM, and IgD. Sorted cells were cultured in triplicate wells (5 x 10^5 cells/well). A, B, Stimulators included (A) 10 µg/ml anti-CD40 (HM40-3; Biolegend) plus 4 µg/ml rabbit-anti-mouse Ig (clone 187.1, BD-PharMingen), and (B) 10 µg/ml goat anti-mouse IgM (F(ab')2 (Jackson ImmunoResearch Laboratories, West Grove, PA). The proliferative response was measured by a standard [³H]-thymidine incorporation assay at
MZ-P B cells exhibited increased ability to stimulate CD4 T cells

The close proximity of MZ-P B cells with CD4 T cells near the dark zone area of spontaneously developing GCs in the spleens of BXD2 mice indicates that MZ-P B cells may be important in contributing to GC formation by either promoting the activation of CD4 T cells or becoming GC B cells themselves. A number of our findings below indicate that MZ-P B cells are potent APCs and can serve as initiators of the GC response.

Compared to FO and CD23lo MZ B cells, MZ-P B cells from BXD2 mice exhibited the highest proliferative response after anti-CD40 and anti-IgM stimulation (Fig. S4). To directly confirm that MZ-P B cells exhibited the highest costimulatory effect to stimulate CD4+ T cells, B cells from BXD2 and BXD2-Ifnar−/− mice were sorted into FO, CD23lo MZ and CD23hi MZ-P B cell subsets. The sorted B cells were irradiated (3,300 rad) and co-cultured with MOG peptide-specific CD4+ T cells (isolated from 2D2 BXD2 Tg mice) in the presence of MOG peptide. The CD23hi MZ-P B cells from BXD2 mice exhibited the greatest stimulatory effect on T cell proliferation than either FO or CD23lo MZ B cells (Fig. 6A). Deficiency of IFNαR on B cells did not alter their effects on Ag-specific CD4 T cell proliferative response, demonstrating that type I IFN signaling on B cells primarily regulates their migratory patterns, as observed above.

Flow cytometry analysis indicated that, in both wild-type BXD2 and BXD2-Ifnar−/− mice, the percentages of cells expressing CD80, CD86, ICOS ligand (ICOSL or B7RP-1) were higher for both the CD23hi MZ-P and CD23lo MZ B cell populations than for the FO B cell population (Fig. 6B-6E). The percentages of cells expressing CD86+ (Fig. 6D), ICOSL (Fig. 6E), and IL-21R (Fig. 6G) was higher for the CD23hi MZ-P B cell
FIGURE 6. MZ-Ps are potent APCs. A, Thymidine incorporation analysis of B cell-promoted proliferative effects on MOG-specific CD4 T cells in response to MOG stimulation. Single cell suspensions prepared from the spleens of 2-mo-old BXD2, and BXD2-Ifnar<sup>−/−</sup> mice were sorted by FACS into FO, CD23<sup>lo</sup> MZ B-cell, and CD23<sup>high</sup> MZ-P subsets. The MOG<sub>35-55</sub> peptide-induced proliferative effects of CD4 T cells isolated from 2D2 TCR Tg BXD2 mice in the presence of the indicated population of B cells (3,300 rad irradiated) was determined. For each experimental group of two independent experiments, cells of the same B cell subset from at least three mice were pooled. Means ± SEM; * P < 0.05 or ** P < 0.01 compared with MZ-P B cells. B, Flow cytometry analysis of single-spleen cell suspensions for FO, MZ, and MZ-P surface expression of CD80, CD86, ICOSL, ICOS, and IL-21R in BXD2 and BXD2-Ifnar<sup>−/−</sup> mice. One representative histogram plot for each marker is shown. (C-G) Bar-graphs of percentages of FO, MZ, and MZ-P expression of (C) CD80, (D) CD86, (E) ICOSL, (F) ICOS, and (G) IL-21R in BXD2 and BXD2-Ifnar<sup>−/−</sup> mice. Means ± SEM (n = 6); ** P < 0.01 or *** P < 0.005 compared with MZ-P B cells.
Fig 6
population than for the CD23\textsuperscript{lo} MZ B cell or FO B cell populations. However, the expression of ICOS is lower on MZP B cells than on FO or MZ B cells (Fig. 6F). Deficiency of IFN R in BXD2 mice had no effects on the expression of these costimulatory molecules on B cells.

**Discussion**

We have established previously that BXD2 mice spontaneously develop high levels of circulating high-affinity nephritogenic and arthrogenic pathogenic autoantibodies and that the spontaneous formation of GCs in the spleen is critical to the production of high-affinity pathogenic autoantibodies [16-18]. Here, we show that there are high numbers of pDCs in MS of the spleens of these mice and that these pDCs exhibit significantly elevated expression of IFN\(\alpha\). We further show that the enhanced levels of IFN\(\alpha\) play a role in the development of lupus in the BXD2 mice by demonstrating that a deficiency of the IFN\(\alpha\)R in these mice leads to a reduction in the spontaneous formation of the GCs and the production of T-dependent, but not T-independent, humoral immune responses. Moreover, the exogenous induction of type I IFNs led to an enhanced T-dependent, but not T-independent, humoral immune response.

We found that there were high numbers of pDCs which were redistributed in the spleens of the BXD2 mice, with the pDCs being preferentially located in the MZ. In the New Zealand Black (NZB) murine model of autoimmunity, increased counts of pDCs are found, but in contrast to the BXD2 mice, these pDCs reside in the bone marrow [46]. In the spleens of normal naïve mice, pDCs are scattered mainly in the T cell area and in the red pulp, and are only rarely found in the MZ; however, upon infection with virus, or
after CpG-ODN stimulation, the pDCs migrate to the MZ area and produce IFNα within the first 36 hours of infection, suggesting that relocation of pDCs into the MZ area and production of IFNα in this area is part of an early host defense mechanism [47]. Our results suggest that the location of IFNα-producing pDCs into the MS provides at least two advantages to promote GC development in BXD2 mice: one is to promote the T-dependent response by directing high numbers of costimulatory B cells to migrate into the follicle; and the other is to prevent the release of IFNα in the follicle to suppress the production of cytokines such as IL-17 that is important to sustaining T-dependent responses [39].

The dynamic motion of B cells is recently verified as being a key process in shaping T-B cell interactions [49, 51]. Our present results have found that in autoimmune BXD2 mice, the IFNα produced locally by increased numbers of pDCs in the MS did not affect the costimulatory function of MZ-P or MZ B cells. Consistent with the role of S1P in retaining B cells in the MZ [45], IFNα-induced upregulation of CD69 leading to down-modulation of Slp1 in BXD2 mice may represent one mechanism that enables the release of MZ-P into the FO. Compared to MZ, MZ-P B cells from BXD2 mice exhibited a lower threshold for the IFNα-induced expression of CD69 to down-regulate levels of Slp1 transcripts. Deficiency of IFNαR in BXD2 mice resulted in the retention of the majority of these MZ-P B cells in the MS, in the area of the MZ-FO border. These results suggest that this increased number of IFNα-producing pDCs in MS provided an important initial step to promote the entry of MZ-Ps into the follicle. Although MZ-P B cells in BXD2 respond well to IFNα-induced CD69 signaling, the majority of MZ-P B cells in BXD2 mice express relatively low levels of CD69 and the
majority of these cells was found to be localized in the inner FO region close to the dark zone area of spontaneous GCs, indicating that once these cells do enter into the FO, down-regulation of CD69 occur in the MZ-P B cells as a result of the lack of IFNα-producing pDCs in the inner FO region. Thus, the major effect of IFNα is to provide the initial force to push the influx of MZ-P B cells into the FO. Additional mechanisms must exist to help retain MZ-P B cells inside a follicle in the spleens of BXD2 mice. Indeed, we have found that, compared to conventional CD23lo MZ B cells, MZ-P B cells expressed higher levels of Rgs13 and Rgs16 (Fig. S5A), which are important for the retention of B cells in the follicle for a GC response [13]. MZ-P B cells also were more susceptible to IL-17 induced migration arrest in response to CXCL12 than were MZ B cells (Fig. S5B). This is consistent with our previous results that upregulation of Rgs13 and Rgs16 inhibits the response of B cells to CXCL12 and CXCL13, leading to B cell localization in the GCs and likely stabilizes their interactions with other cells [13]. Because BXD2-Il17r−/− exhibited a significantly lower percent of MZ-P B cells (Fig. S5C), the results suggest that close contact of CD4 T cells with MZ-P may have provided the necessary signal to promote the survival and proliferation of these highly costimulatory B cells. Alternatively, because there was a significantly increased percentage of MZ B cells in the spleens of BXD2-Il17r−/− mice, compared to WT BXD2 mice (Fig. S5C), the results suggest that due to the absence of IL-17-induced retention of MZ-P B cells in the follicular region, a great percentage of these MZ-P B cells have migrated to the MZ and developed into MZ B cells in the BXD2-Il17r−/− mice.

As observed in our studies, migration of a large number of MZ B cells into the follicles requires a strong signal to stimulate type I IFN production, and this provides an
Supplementary Figure S5.

IL-17 promoted up-regulation of Rgs13/16, inhibited CXCL12 migratory response, and suppressed MZ-P expansion. A, qRT-PCR analysis for transcript levels of Rgs13 and Rgs16 in sorted MZ and MZ-P B cells from the spleens of BXD2 and BXD2-Il17r^{−/−} mice. Means ± SEM (n=6); *P < 0.05 or **P<0.01 compared with BXD2-Il17r^{−/−} mice; ND indicates not detectable. B, Chemotactic response of MZ and MZ-P B cells from BXD2, or BXD2-Il17r^{−/−} mice was analyzed using a Transwell migration chamber with CXCL12 (100 ng/ml) or culture medium loaded in the bottom chamber. Cells were either not stimulated or stimulated with IL-17 for 4 hrs prior to carrying out the migration analysis. FACS analysis was carried out to determine the migration response to CXCL12 for the indicated populations of B cells. Means ± SEM (n=6); *P<0.05 compared with unstimulated B cell populations. C, Left panel: flow cytometry analysis of the percent of FO (oval) and total MZ (triangle) B (CD19^{+}) cells in the indicated mouse strain. Right panel: Within the total IgM^{hi}CD21^{hi} B cells, cells were further gated as CD23^{null/lo} MZ or CD23^{hi} MZ-P B cells (rectangle gated cells). Means ± SEM (n=6); * P < 0.05 or *** P < 0.005 compared with wild-type BXD2 mice.
Fig S5
additional stimulation to lead to further augmentation of a T-dependent response. MZ-P B cells in the spleens of BXD2 mice, on the other hand, are already present in the follicles in proximity to CD4 T cells and developing GCs without exogenous IFNα induction. However, endogenous IFNα signaling is required to keep the MZ-Ps in the follicles near the developing GC border, as demonstrated by the localization of the MZ-Ps at the MZ-FO border when IFNAR is deleted. The proximity of MZ-Ps to GCs thus suggest that migration of MZ-P B cells into the follicle can provide a more rapid and stationary mechanism to promote a T-dependent response. Consistent with this finding, MZ-P B cells expressed higher levels of costimulatory molecules and provided a better signal to promote an Ag specific T-cell proliferative response, compared to both MZ and FO B cells. Because self-antigen stimulation of pDCs is associated with a weak and chronic stimulation of IFNα, spontaneous GC responses in autoimmune disease may be primarily promoted by migration of MZ-P B cells, but not MZ B cells, into follicles where they encounter CD4 T cells and provoke autoreactive CD4 T-cell responses.

One interesting question that remains to be answered is the fate of MZ-P B cells after their contact with CD4 T cells for the induction of a spontaneous GC response. Compared to normal B6 MZ-P B cells, MZ-P B cells from BXD2 mice exhibited dramatically elevated proliferative response by anti-IgM stimulation. Furthermore, MZ-P B cells expressed high levels of IL-21R and also increased anti-CD40 crosslink induced proliferative response. These features further suggest that MZ-P B cells are very susceptible to stimulation provided by follicular T helper cells that produce high levels of IL-21 [39]. Studies in humans suggest that B cells with a similar phenotype (IgM^hiIgD^hiCD23^hi) exhibit a unique role as the BM2 naïve precursor B cells for GC
development [27]. Increased percentage of BM2 B cells has been detected in peripheral blood of Sjögren’s Syndrome patients [51]. High expression of CD80 and CD86 also has been found in the same population of B cells from PBMCs in lupus patients [52]. Interestingly, the anatomic location of IgM^{hi}IgD^{hi}CD23^{hi} BM2 B cells have been previously localized right outside of a GC where B cells encounter CD4 T cells and follicular dendritic cells (FDCs) [27]. The expression of high levels of CD23 on these B cells further makes it possible for these B cells to stimulate CD21^{hi} FDCs that are in the dark zone area of a GC. Our results indicate that MZ-P B cells exhibit many phenotypes that resemble human BM2 naïve B cells. Intriguingly, in human ectopic GCs, it was found that down-regulation of CD23 occurs once the B cells start to undergo a GC response and become the GC founder B cells [27]. This is consistent with our present finding that CD23^{hi}IgM^{hi} B cells are located adjacent to the dark zone PNA^{+} GC B cells that do not express CD23. These results suggest that MZ-P B cells play an important role in either initiating a GC response or transforming into GC B cells during a GC response. We have shown previously that BXD2 mice exhibited high cell counts of T_{H}-17. These IL-17^{+} producing CD4^{+} T cells are located primarily inside the FO area and are crucial in providing the migration arrest signal that leads to the prolonged and close contact between CD4^{+} T cells and B cells, facilitating the formation and stabilization of spontaneous GC development [366]. Deficiency of IL-17R in BXD2 mice leads to a reduction in the spontaneous formation of the GCs and the production of autoantibodies [13]. Our present study suggests that this process is assisted by the production of IFN_{α} by the pDCs in the MS, which promotes migration of MZ-P B cells into the follicle where they help stimulate the CD4^{+} T cells. Thus, the formation of spontaneous GCs in
the spleens of BXD2 mice is due to a localized expression of particular cytokines that are normally invoked during immune responses. In the case of IFNα, its localized expression in the MS acts to enhance migration of MZ-P B cells into the FO to enable contact with CD4 T cells. This is followed by retention of these activated MZ-P B-cells in the FO area by IL-17 to facilitate a GC formation response [13]. Such coupled effects of cytokines on different cells at different anatomic locations within a lymphoid organ may result in responses that are not predicted by the analysis of isolated cytokines on stationary cells.
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Author contributions

J.W., J.D.M, and H.-C.H. contributed to all studies; J.L. contributed to all qRT-PCR experiments; Q.W. and P.A.Y. were involved in all animal experiments, FACS staining, and chemotactic experiments; R.P. contributed to urinary protein and immune complexes analyses; S.X. contributed to analysis of MZ-P B cells and chemotaxis; ; L.T. contributed to identification and analysis of pDCs; CR contributed to the studies of MOG-specific CD4$^+$ T-cell response; D.D.C. contributed to the studies of SHM analysis; L.L. contributed to generation of BXD2-Ifnar$^{-/-}$mice
Footnotes

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3 Abbreviations used in this paper: IFN, interferon; IFNαR, type I IFN receptor; PDC, plasmacytoid dendritic cell; APCs, antigen-presenting cells; GC, germinal center; FO, follicle or follicular; MZ, marginal zone; MZ-P, marginal zone precursor; MS, marginal sinus
References


SUMMARY

Type I IFNs are intricately linked to the development of human SLE [177, 179, 180, 183, 184], and other autoimmune diseases [63, 183, 188]. A solution to the pathogenesis of SLE very likely demands a solution to the “type I IFN signature” found in the vast majority (~100%) of lupus patients [179, 180, 183, 184]. Although certain murine models of lupus have shown type I IFNs can be protective against disease [195, 196], in humans at least, based upon the clinical data and in vitro experiments on human blood cells, type I IFNs appears to have pro-inflammatory potential role in disease induction and maintenance [177-180, 183, 184, 186, 197, 198]. A litany of plausible type I IFN-mediated mechanisms for immune tolerance break-down has been proposed. These include type I IFN-mediated monocyte differentiation into potent antigen-presenting dendritic cells [83], cytotoxic CD8 T cell expansion [200], and the promotion of naïve B cells to become antibody-producing plasmablasts and plasma cells [205]. However, with the exception of type I IFN-induced dendritic cell differentiation [83], all of these proposed pathways were observed in the context of non-autoimmune murine and human hosts. Plasma cell differentiation by pDCs and type I IFNs were observed in a series of in vitro experiments carried on human peripheral blood [83]. Work is still needed to demonstrate that these pathways are indeed major signaling circuits in lupus induction.

Autoantibodies to nuclear antigens are a prominent feature of lupus. Murine models have demonstrated that T-independent pathways to autoantibody production are a
dominant means by which antibodies are produced. Namely, the expansion of the MZ B cell population found in the NZB and (NZBxNZW)F₁ mice prior to disease and the increased titers of polyclonal IgM autoantibodies are testaments to the role of T-independent humoral system. However, the presence of anti-nuclear antibodies to DNA that are of IgG isotype indicate that class-switch recombination do take place in lupus patients and murine models. Furthermore, the finding that autoantibodies do somatically hypermutate suggests that these are products of post-germinal center reactions. Although the presence of somatically mutated class-switched IgG autoantibodies suggest T-dependent pathways for antibody production, it is also very well possible that such antibodies are generated in extrafollicular reactions that do not require T cell help. Namely, the finding of rheumatoid factor-specific B cell clones in the extrafollicular areas that express somatically hypermutated antibody indicate that T-independent mechanisms for somatic hypermutation do exist [406]. Further, MZ B cells are capable of IgG3 production, and B cells are known to express AID when stimulated by LPS [407, 408]. Therefore, any form of antibody-class-switch and somatic hypermutation present must be looked upon with some sort of discrimination as to whether the end-product required T cell help.

In the BXD2 mice, the presence of elevated titers of somatically hypermutated class-switched IgG autoantibodies to nuclear antigens begs the question to whether a primarily T-dependent or T-independent pathway is at play. Unlike the lupus-prone New Zealand mice strains, BXD2 mice do not have expanded MZ B cell populations. In fact, compared to B6 mice, there was a significantly reduced population of MZ B cells in BXD2 mice [47]. While NZB mice are just as capable of spontaneously producing intra-
follicular germinal centers, in our hands, BXD2 mice produced significantly larger germinal centers (unpublished observation). Intravenous administration of CTLA4 Ig at a young age significantly abrogated the production of many IgG autoantibodies in BXD2 mice [47]. Consistent with this is reduced AID expression and germinal center formation in the follicles [47]. Together, these findings in the BXD2 mice bolster the significant role of T-dependent humoral system in lupus induction. Reports of CD4 deletion can also affect antibody production in the New Zealand mice strains also support a role for T-dependent antibody response in these mice [43, 44], although the relative contribution of this response is not gauged. Future studies may be needed to compare the relative contributions of T-dependent and T-independent antibody responses between the New Zealand strains and the BXD2 mouse.

Towards a New Paradigm

In the present study, we made use of the BXD2 mouse model to investigate the role of type I IFN in contributing to the T-dependent antibody response in this mouse. First, we proceeded to substantiate that type I IFN signaling is required to drive germinal center reactions, AID expression, IgG-producing plasmablasts, and class-switched autoantibodies. Second, we proceeded to define a pathway by which this T-dependent antibody response is brought about. Namely, the identification of a precursor cell population to the MZ B cells that are located in the follicles and migrate from the follicle-MZ border to the germinal centers.

Intravenous injection of T-dependent antigen into BXD2 mice gave rise to antibody titers within two weeks, while IFNAR−/−.BXD2 mice exhibited abrogated
antibody response. Type I IFNs by type A CpG further boosted T-dependent response, but IFNAR-deletion obviated such antibody development. It is very well possible that plasmablast formation was suppressed in IFNAR-deleted mice, however, reduced germinal center formation, AID expression, and the severely reduced presence of IgG-producing plasmablasts, indicate that type I IFNs play a significant role in pre-germinal center and germinal center reactions. Our stunning finding that while IgG-producing plasmablasts were completely abrogated in IFNAR-deleted BXD2 mice, there was still an abundance of IgM-producing plasmablasts. This finding argue that type I IFNs are not indiscriminately suppressing the development of plasma cells, for if this were the reason, both IgM and IgG-producing plasmablasts would be absent in IFNAR-deleted mice. Rather, IFNAR-deletion significantly interfered with antibody isotype class-switch, and thereby abrogated the development of IgG-producing plasmablasts. Together, these observations argue a new paradigm in which type I IFNs potentially interfere in either pre-germinal center or germinal center reactions. Consistent with this conclusion is the reduction in AID expression, and germinal center formation. This is in contradistinction to the current lupus paradigm by which type I IFNs interfere in post-germinal center reactions by promoting plasmablast and plasma cell differentiation.

**MZ-P B cells at the Center of Type I IFN-promoted T-dependent Antibody Response**

The observation that type I IFNs can promote a T-dependent antibody response and interfere in pre-germinal center and germinal center reactions led us to determine by what pathway/mechanism this is initiated. One of our early observations about the type I IFN-producers, the pDCs, is that they are oft to be found in the follicular perimeter at the
marginal sinus. Inflammation can lead to a type I IFN-dependent migration of pDCs to the MZ. Consistent with this previous report, we do indeed find many pDCs located in the MZ. The finding that pDCs are co-localized with MZ B cells had led some researchers to speculate that these type I IFN-producers might promote plasmablast differentiation in the MZ, independent of CD4 T cell help. While such a mechanism is plausible and may actually operate for certain autoantibody specificities, in our murine model, a T-independent antibody response is not prominent.

We did observe that upon either CpG or poly I:C treatment, MZ B cells move most conspicuously from the periphery and into the follicle, while in IFNAR-deleted mice, the MZ B cells remained stationary. Type I IFN-dependent migration of MZ B cell has never been reported in the literature, and therefore our discovery further fomented our determination that a similar mechanism governs the migrational capacity of the precursors to the MZ B cells. This population of IgM⁺CD21⁺CD23^high was only recently identified as precursors to the MZ B cells [312], and so are termed MZPs. We have determined that these MZPs are potent antigen-presenting cells and express high levels of co-stimulatory molecules, making them ready candidates for mediating T-dependent antibody responses. Confocal microscopy has located MZPs in the B cell follicles, intermixed with FO B cells. Wild-type BXD2 mice display a slight displacement of MZ B cells into the follicles compared to IFNAR-deleted mice. However, what was most noticeable is that while MZPs are in the follicles adjacent to developing germinal center, MZPs are predominantly at the FO-MZ border in IFNAR-deleted mice. This apparent displacement in the absence of intact type I IFN signaling hint at the significant strength of interferon-mediated migration on MZPs, even more so than its effects on MZ B cells.
MZ-P B cells as Germinal Center Inducers

A well-known mantra is that MZ B cells primarily mediate a T-independent antibody response, while FO B cells require T cell help. However, a number of studies have found that MZ B cells are critical agents that transport antigens to the follicular dendritic cells, which are essential players in germinal center reactions [236, 241]. Furthermore, albeit with delayed kinetics compared to FO B cells, MZ B cells can also initiate germinal centers [247]. Consistent with these reports, is the higher expression levels of co-stimulatory molecules on MZ B cells, and these cells were found to provide greater antigen-presenting capability than FO B cells [240].

It is very plausible that MZ B cells, by virtue of their greater antigen-presenting capabilities than their FO B cell counterparts, that they can play a distinctive role in antigen-transport and germinal center induction in BXD2 mice. However, we did not observe as great as a locational displacement as we did with MZPs when comparing wild-type and IFNAR-deleted BXD2 mice. Further study is needed to examine for the role of other cytokines that may contribute to greater B cell displacement from the MZ. Another possibility is that since type I IFNs are acute disease cytokines generated only in response to acute stress, we may not observe as strong an effect on MZ B cell migration under steady-state conditions in the BXD2 mice. Even though chronic inflammation is a definitive feature of BXD2 mice, it is difficult and likely not biologically feasible for chronic stimulation of type I IFNs. Even in humans, type I IFNs are not readily detectable in serum [178, 181], indicating that these class of interferons is not constitutively expressed. The presence of a distinct “type I IFN signature”, however, in almost 100% of
patients with SLE discounts the notion that type I IFNs is not important for disease [179]. Rather, it is more biologically feasible that normal host physiology, punctuated by disease flares, or periodic disease exacerbation, characterized the steady-state conditions found in humans and murine lupus models. It is only in these periods of disease exacerbation that type I IFNs may become significantly elevated to induce MZ B cell migration in the follicles. Thereafter once the disease flare subsides, type I IFN levels also subsides, leading to a restoration of B cells back into the MZ. After all, the cycling of B cells between the MZ and FO is needed so that B cells can return to the MZ to capture antigens for the next round of transport into the follicles.

Our observation that exogenous induction of type I IFNs by poly I:C can indeed promote an apparent migration of MZ B cells into the follicles in wild-type BXD2 mice does support the mechanism that type I IFNs can move MZ B cells. Interestingly, IFNAR-deletion completely abrogated all MZ B cell migration in response to poly I:C. Without exogenous inductions, in contradistinction to the MZ B cells, MZPs were found to primarily locate in the follicles close to the germinal centers. In the IFNAR-deleted BXD2 mice, MZPs were found at the MZ-FO border. The greater sensitivity upon which MZPs migrate in response to type I IFN signals than the MZ B cells led us to conclude that MZPs may be even better antigen-presenting and antigen-transport cells than the MZ B cells. As shown, transfer of BXD2 MZPs into SCID mice recipients generated the most germinal centers in response to antigen immunization, compared to the transfer of other B cell populations.
**Type I IFN and Th17/IL-17**

Our previous report found that BXD2 mice displayed elevated serum levels of IL-17 and the presence of CD4 T cells that readily polarize to Th17. Although IL-17 and Th17 is usually associated with non-lupus like diseases, such as multiple sclerosis and rheumatoid arthritis, the significant presence of IL-17 in BXD2 mice led to conclude that the cytokine may also be important for lupus disease induction. IL17 receptor deletion in BXD2 mice significantly attenuated germinal center formation and autoantibody production.

One my early findings is that direct type I IFN signaling on B cells is crucial to driving B cell differentiation into germinal centers. This was evidenced when wild-type GFP+ B cells was transferred into IFNAR-/- BXD2 mice and examined for germinal center B cell differentiation after three weeks. Only IFNAR-competent B cells were found to transform substantially into (>20%) germinal center B cells. Interestingly, a similar phenomenon was observed with IL-17R-competent B cells [366]. What is surprising about this observation is that both IL-17 and type I IFNs induce germinal center formation in BXD2 mice. Several reports, however, had found that type I IFNs antagonize the development of IL-17 producing CD4 T cells. Although IL-17 can very well originate from CD4 T cells in BXD2 mice, it is also very possible that bulk IL-17 production in these mice originate from other cellular sources whose development are not blocked by type I IFNs.

IL-17 production and secretion by CD4 T cells in the follicle is proposed as a mechanism by which IL-17 signals on B cells to stabilize their positions inside the follicle by up-regulation of RGS13/16 expression to initiate germinal center formation.
Figure 11. Proposed model in BXD2 mice.
MZPs are potent antigen-presenting cells and capture self-antigens. pDC-expressed IFN-α signals on CD69/S1P1 expression axis to drive MZP migration from the MZ towards the FO. This inward follicular migration obviates MZP development into MZ B cells, and pushes MZPs to contact and stimulate CD4 T cells. IL-17 signaling on MZPs induce migration arrest and stabilizes the position of MZPs inside the follicle. Continued MZP-T cell contact amplify the T cell response and drives more T-B interactions, including those involving FO B cells, to ultimately induce a germinal center reaction.
The finding that type I IFN can also promote germinal center formation prompt us to propose a model by which these two major autoimmune cytokines can act collaboratively to bring about germinal center induction. First, our laboratory propose that pDC expressed type I IFN in response to an endogenous stimulant (i.e. self nucleic antigens) signals potent antigen-presenting MZPs, which have already captured endogenous antigens, by up-regulation of CD69 surface expression and down-regulation of S1P1 surface expression, thereby creating a “push” force that pushes the MZPs away from the MZ and inwards towards the follicular center. Second, once pushed into the follicle near the T cell zone, MZPs come into contact with CD4 T cells, of which, potentially, some are IL-17-producing. IL17 increases the expression of RGS13/16, inhibiting chemotactic response to CXCL12, and thereby stabilizing the position of MZPs inside the follicle, and providing the “pull” force which keeps the MZPs inside the follicle. Persistent contact with CD4 T cells can enable MZPs to present antigens to CD4 T cells, and thereby amplify the T-dependent immune response. With the involvement of regular FO B cell, this initial MZP-T cell contact can trigger an all-out germinal center reaction.

The finding that both type I IFN and IL-17 can promote germinal center formation and T-dependent antibody responses in BXD2 mice presents an interesting combination of having both cytokines promote a common function. The finding that MZPs can potently present antigens and whose geographic position is under the regulation of type I IFNs, and potentially also under the regulation of IL-17, makes for a feasible mechanism by which both type I IFNs and IL-17 can collaboratively promote a common goal. Interestingly, IL-17R-deletion in BXD2 mice significantly depressed the
percentage of MZPs, even more so than the IFNAR-deleted mice, suggestive that IL-17 is a potent survival factor for MZPs. Whether IL-17 can actually regulate MZP and MZ B cell migration remains to be determined for future studies.

Conclusion

The role of type I IFN in promoting a T-dependent humoral response in BXD2 mice acts at the pre-germinal center and active germinal center stage prior to the development of plasma cells. Contrary to conventional wisdom, type I IFN can act more than just to differentiate plasma cell formation in post-germinal center reactions. Our findings have indicated a profound type I IFN-dependent effect on germinal center formation, AID expression, and development of class-switched IgG plasmablasts. All of these processes are pre-germinal center or active germinter reactions by which type I IFN signaling is critical. In response to type I IFNs, MZPs serve as ideal antigen-presenting cells that transport antigens from the MZ-FO border to the developing germinal centers. Consistent with their potent antigen-presenting fuction and high co-stimulatory functions, MZPs were found to induce the greatest counts of germinal centers compared to FO or MZ B cells. Therefore, MZPs serve as the critical link that mediate type I IFN-induced germinal center reaction.
GENERAL LIST OF REFERENCES


APPENDIX

APPROVAL FORM FROM INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)
NOTICE OF APPROVAL WITH STIPULATIONS

DATE: January 6, 2008

TO: John Mountz, M.D., Ph.D.
    SHEI 330 2102
    FAX 975-0646

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee

SUBJECT: Title: Suppression of Pathogenic Autoantibodies in Lupus by Inhibition of AID
         Sponsor: NIH
         Animal Project Number: 0801686350

On December 20, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>C</td>
<td>60</td>
</tr>
<tr>
<td>Mice</td>
<td>B</td>
<td>580</td>
</tr>
<tr>
<td>Mice</td>
<td>A</td>
<td>854</td>
</tr>
</tbody>
</table>

Approval is granted with the following stipulation(s):

Animal procurement and initiation of studies may not commence until the Animal Use Safety Information Sheet is authorized by OHS. Once the AUSI is authorized, you will be contacted by Earle Durbin (934-3538) to discuss specific safety precautions which may be necessary for the ARP care staff. Animal procurement and use of potentially hazardous agents in live animals may not occur until Mr. Durbin has informed the IACUC Office that a satisfactory discussion has occurred.

Animal use is scheduled for review one year from January 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 0801686350 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.