Title
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Permalink
https://escholarship.org/uc/item/5q10v720

Journal
PloS one, 6(10)

ISSN
1932-6203

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Publication Date
2011

DOI
10.1371/journal.pone.0026536

Peer reviewed
Homeostatic Regulation of Marginal Zone B Cells by Invariant Natural Killer T Cells

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Abstract

Marginal zone B cells (MZB) mount a rapid antibody response, potently activate naïve T cells, and are enriched in autoreactive B cells. MZBs express high levels of CD1d, the restriction element for invariant natural killer T cells (iNKT). Here, we examined the effect of iNKT cells on MZB cell activation and numbers in vitro and in vivo in normal and autoimmune mice. Results show that iNKT cells activate MZBs, but restrict their numbers in vitro and in vivo in normal BALB/c and C57BL6 mice. iNKT cells do so by increasing the activation-induced cell death and curtailing proliferation of MZB cells, whereas they promote the proliferation of follicular B cells. Sorted iNKT cells can directly execute this function, without help from other immune cells. Such MZB regulation by iNKTs is mediated, at least in part, via CD1d on B cells in a contact-dependent manner, whereas iNKT-induced proliferation of follicular B cells occurs in a contact- and CD1d-independent manner. Finally, we show that iNKT cells reduce ‘autoactive’ MZB cells in an anti-DNA transgenic model, and limit MZB cell numbers in autoimmune-prone (NZB × NZW)F1 and non-obese diabetic mice, suggesting a potentially new mechanism whereby iNKT cells might regulate pathologic autoimmunity. Differential regulation of follicular B cells versus potentially autoreactive MZBs by iNKT cells has important implications for autoimmune diseases as well as for conditions that require a rapid innate B cell response.

Introduction

B cells in the spleen anatomically localize in the follicles and marginal zone and are called follicular B cell (FoB) and marginal zone B cell (MZB), respectively [1,2]. The MZBs exhibit unique characteristics not shared by FoBs. Their location in the spleen helps MZBs mount a rapid antibody response to blood-borne antigens independently of T cell help [3]. MZBs continuously shuttle between marginal zone and lymphoid follicles to transport antigens to follicular dendritic cells [4] and potently activate naïve CD4 T cells and quickly differentiate into plasma cells [5]. Rapid and efficient regulatory mechanism(s) must exist to curb such prompt and vigorous responsiveness of MZBs to avoid unnecessary immune activation.

MZBs are phenotypically distinct from other B cells and are identified as CD21hi CD35hi CD23low CD95– IgD–/–IgM+ cells [1,2]. MZBs are also distinct from other B cell subsets through their high expression of CD1d [6]. Since CD1d presents lipid antigens to invariant natural killer T (iNKT) cells [7,8,9], MZBs have been proposed to be important activators of iNKT cells. However, it is unclear whether iNKT cells, in turn, activate or regulate MZBs in a way that is different from their interactions with other B cell populations.

iNKT cells rapidly respond to glycolipid antigens, such as α-galactosylceramide (αGalCer) [10]. These cells trans-activate a variety of other cells, including NK cells, conventional T cells, and B cells [11,12,13,14]. For example, iNKT cells enhance peripheral blood B cell proliferation [15] and enhance immunoglobulin production against T-dependent and T-independent antigens and pathogens [15,16,17,18]. It is unclear whether iNKT cells interact differently with MZBs that express particularly high levels of CD1d compared to FoBs.

MZB cells have several features required to break T cell tolerance. For example, MZB cells can act as robust APCs [5] and can be easily activated by dendritic cells [19]. In fact, several studies have linked MZB cell abnormalities to the development of autoimmune diseases [20,21,22]. MZB cells expand in the non-obese diabetic (NOD) mouse model of type 1 diabetes (T1D) prior to the onset of disease at an early age when autoreactive T cells begin to appear [23,24]. MZB cells are also increased in lupus mice [25,26], and B cells bearing anti-self B cell receptors are enriched in the marginal zone of spleens [20,27]. Thus, understanding mechanisms of MZB cell homeostasis will have important implications for understanding autoimmune diseases.

In this article, we examined the effects of iNKT cells on the activation, proliferation and frequency of major B cell subsets in...
the spleen, MZB and FoB, in vitro and in vivo in normal and autoimmune-prone mice. Our results show that while iNKT cells activate both MZB and FoB, they selectively curtail the proliferation of MZBs and promote their activation-induced cell death (AICD). These results indicate a role of iNKT cells in regulating the homeostasis of MZBs. Such regulation of MZBs might be an important mechanism of controlling autoimmune diseases, since iNKT cells reduce ‘auto-reactive’ anti-DNA MZBs and limit MZBs in lupus-prone NZB/NZW F1 and autoimmune diabetes-prone NOD mice. These data, along with our recent report describing the role of iNKT cells in inhibiting autoantibody production [28], have important implications for the development of iNKT cell-based therapy in autoimmune diseases.

Materials and Methods

Ethics Statement
All of the scientific data were obtained using high scientific, technical, and ethical standards. All animal experiments were approved by UCLA Office of Research Oversight Chancellor’s Animal Research Committee under protocol ARC # 2005-155.

Mice
BALB/cJ, C57BL/6, NOD/ShiLtJ, NZB and NZW mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred locally. R4A-γ2bFcε mice that have increased numbers of IgG2b anti-dsDNA Ab B cells [20] were provided by Dr. B. Diamond. Vx14e [29] and CD1d−/− mice [26] were provided by Dr. A. Bendelac and Dr. L. Van Kaer, respectively. CD1d−/− BWF1 mice were generated by introgressing CD1d-null allele onto NZB and NZW backgrounds for 10 and 12 generations, respectively, and then intercrossing them [30]. The mouse genotype was confirmed by PCR and their phenotype was confirmed by flow cytometry using anti-CD1d antibody (1B1) for CD1d−/− mice and mCD1d/PBS-57 tetramer for Vx14e and CD1d−/− mice [30].

Reagents
The mCD1d/PBS-57 tetramer was obtained from the NIH Tetrامر Core Facility (Emory, GA). Anti-CD93 antibody (AA4.1) was from eBiosciences; anti-IgM antibody was from Jackson ImmunoResearch; all other antibodies were from BD Biosciences (San Diego, CA).

Flow cytometry
Spleen cell suspensions were prepared in staining buffer (0.5% BSA, 0.09% sodium azide) after red blood cell lysis by Tris-NH4Cl (pH 7.2). Cells were incubated with anti-CD16/32 (2.4G2) to block FcγRII/III, followed by staining with conjugated mAbs for mouse antigens, including CD1d (1B1), TCRβ (H57-597), CD86 (GL1), CD69 (H1.2F3), CD95 (Jo2), IgM (μ chain specific), CD19 (1D3), B220 (RA3-6B2), CD21 (7G6), CD23 (B3B4), CD9 (KMC3), active form of caspase3 (C92-405), 7AAD, and mCD1d/PBS-57 tetramer. Flow cytometry analysis was performed using FACSCalibur or FACScan (Becton Dickinson). Data were analyzed using FlowJo software (Ashland, OR) with lymphocyte gate, based on forward and side scatter.

Immunohistochemistry
Spleen was embedded in tissue freezing medium (Fisher Scientific, Pittsburgh, PA) and frozen in liquid nitrogen. Frozen tissue blocks were sectioned using HM550 cryostat (Mikron, San Marcos, CA) and 7 μm sections mounted on Superfrost plus slides (Fisher Scientific). Slides were dried at room temperature (RT), fixed with cold acetone for 2 min, dried at RT and then rinsed three times with PBS. Slides were incubated in a humidified chamber with blocking buffer (20 μg/ml CD16/CD32, 10% normal mouse serum, 2.5% BSA and 0.1% tween-20 in PBS) for 30 min at RT. Blocked sections were stained with biotin-IgM (μ chain specific, Jackson ImmunoResearch) and FITC-conjugated MOMA1 (CD169) (AbD Serotec, Raleigh, NC) at 10 μg/ml on RT for 2 h and then rinsed 5-times with PBS/0.1% Tween20 in PBS. Slides were further incubated with 2 μg/ml of APC-conjugated streptavidin (BD Biosciences) for 1 h at RT and then rinsed 5-times with PBS. Stained slides were mounted with crystal mount (Biomedica, Foster City, CA) and images captured using LEICA DM IRB (Meyer instruments, Houston, TX). Images were analyzed using Leica and Photoshop software.

In vivo immunization
Mice were immunized i.p. with 4 μg of αGalCer or vehicle. Their spleen was collected at various time points after injection for analyses.

Purification of T, B and iNKT cells
Spleen cells were incubated with anti-CD90 or anti-CD19 microbeads (Miltenyi Biotec, Auburn, CA) for 20 min at 4°C to purify T cells and B cells, respectively, using AutoMACS (Miltenyi Biotec). The purity of T cells and B cells ranged from 82–86% and 97–98%, respectively. The iNKT cells were sorted as TCRβ+ mCD1d+/PBS-57 tetramer+ conventional T cells sorted as TCRβ− mCD1d+/PBS-57 tetramer+ cells using FACSaria (Becton Dickinson). The sorted cells were collected in complete RPMI-1640 medium and checked for their purity (≥98%).

Cell culture
Spleen cells at 2×10⁶ per ml in complete RPMI-1640 medium were added to 24-well polystyrene, flat-bottom tissue culture plates (Corning, Costar, NY) and cultured at 37°C in a humidified 5% CO₂ incubator. Purified B cells (1–2×10⁶ per ml) were cultured with or without LPS (10 μg/ml) and αGalCer (50–100 ng/ml). Sorted T cells or iNKT cells were added to these cultures at 0.5–1×10⁶ cells per ml. Cells were collected at the indicated time points, stained with indicated antibodies, and analyzed by flow cytometry.

B cell proliferation assay
Purified B cells in complete RPMI-1640 medium were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) with CellTraceTM CFSE cell proliferation kit (Invitrogen), following the manufacturer’s instructions. LPS, αGalCer, and sorted T cells or iNKT cells were added to cultures, as described above. Cells were collected at the indicated time points, stained with anti-CD19 (PerCP-Cy5.5), anti-CD21 (PE), and anti-CD23 (biotin) antibodies, followed by APC-conjugated streptavidin, and analyzed by flow cytometry.

Statistical analysis
Descriptive statistics are expressed as the mean ± SE values. Comparisons between groups were performed using two-tailed Student t test or Mann-Whitney U test, and a p value of <0.05 was considered significant.

Results
iNKT cells activate MZBs, but restrict their numbers in vivo and in vitro
To determine the effect of iNKT cell activation on different B cell subsets, we injected αGalCer i.p. in BALB/c mice, including
WT, V\(\times\)14\(^{14}\)\(^{+}\) mice that express the canonical TCR \(\alpha\) chain V\(\times\)J\(\times\)10 of iNKT cells [29], and CD1d\(^{-}\) mice that have no iNKT cells [26], and analyzed activation markers on freshly isolated spleen cells. \(\alpha\)GalCer treatment increased CD86 expression on both MZBs and FoBs from WT or V\(\times\)14\(^{14}\)\(^{+}\) mice, but not from CD1d\(^{-}\) mice (Fig. 1A). Unexpectedly, however, in vivo \(\alpha\)GalCer treatment resulted in a marked reduction in MZB frequency and marginal zone IgM\(^{+}\) B cells in V\(\times\)14\(^{14}\)\(^{+}\) and WT mice, but not in CD1d\(^{-}\) mice (Fig. 1B–D, and data not shown).

In vitro studies showed that \(\alpha\)GalCer was as strong as LPS in activating MZBs, and acted synergistically with LPS (Fig. 1E). However, addition of \(\alpha\)GalCer to spleen cell cultures reduced the proportions of MZBs (Fig. 1F, upper panel). Total MZB numbers were also reduced in the presence of \(\alpha\)GalCer (LPS 5.9 \(\times\)10\(^{7}\); LPS+\(\alpha\)GalCer 0.3 \(\times\)10\(^{7}\)). Similar results were obtained using spleen cells from V\(\times\)14\(^{14}\)\(^{+}\) mice (data not shown), whereas no change in MZB numbers we seen using spleen cells from CD1d\(^{-}\) mice (Fig. 1F, lower panel).

Next, we observed that the reduction of MZB cells in the presence of \(\alpha\)GalCer was not due to its direct binding/effector on MZB cells (Fig. 2A, left bars), but was mediated by T cells as the co-culture of B cells with purified T cells from WT mice or V\(\times\)14\(^{14}\)\(^{+}\) mice [that have \(\sim\)50% T cells expressing iNKT cell TCR], but not from CD1d\(^{-}\) mice, reduced MZBs (Fig. 2A, middle and right bars). Strikingly, the presence of sorted iNKT cells alone was sufficient to reduce MZBs, whereas purified conventional T cells had no effect on MZB frequency (Fig. 2B,C). The sorted iNKT cells, in the absence of other T cell subsets, also markedly enhanced CD23 expression. These data clearly show that while iNKT cells can activate MZBs and FoBs, they can directly and selectively restrict the MZB population.

iNKT cells promote AICD of MZBs in vitro and in vivo

Since iNKT cells markedly activate MZBs yet reduce their numbers, we surmised whether iNKT cells induce AICD of MZBs. Consistent with this hypothesis, addition of \(\alpha\)GalCer to spleen cells upregulated the expression of apoptotic marker CD95 (Fas) on MZBs in WT or V\(\times\)14\(^{14}\)\(^{+}\) mice, but not in CD1d\(^{-}\) mice (Fig. 3A, and data not shown), while LPS upregulated CD95 expression both in WT as well as in CD1d\(^{-}\) mice. CD95 expression on MZBs increased in the presence of purified CD90\(^{+}\) T cells from V\(\times\)14\(^{14}\)\(^{+}\) mice, but not from CD1d\(^{-}\) mice in presence of both LPS and \(\alpha\)GalCer (Fig. 3B). Furthermore, sorted iNKT cells alone could directly increase CD95 expression on MZBs, without any help from other immune cells (Fig. 3C).

Similar data were obtained, when we examined the effect of activated iNKT cells on active caspase3, an important mediator of apoptosis pathway downstream of Fas, by intracellular staining. Co-culture of B cells in the presence of T cells from V\(\times\)14\(^{14}\)\(^{+}\) mice or sorted iNKT cells increased caspase3 positive cells among MZBs, but not among FoBs (Fig. 3D,E). Finally, whereas activated iNKT cells did not increase apoptotic FoBs, they increased the proportion of apoptotic MZBs by \(\sim\)6-fold (Fig. 3F).

In search for additional mechanisms of iNKT cell-mediated inhibition of MZBs, we asked if iNKT cells affect the proliferation of MZBs. CFSE-labeled B cells from WT mice were co-cultured with T cells from CD1d\(^{-}\) or V\(\times\)14\(^{14}\)\(^{+}\) mice. Surprisingly, the LPS-induced proliferation of MZBs was profoundly inhibited in cultures containing iNKT cells (Fig. 4A). This inhibitory effect was directly mediated by iNKT cells, as sorted tetramer\(^{+}\) iNKT cells, but not tetramer\(^{+}\) conventional T cells, completely suppressed the proliferation of MZBs (Fig. 4B). In contrast, FoBs robustly proliferated in the presence of iNKT or conventional T cells. Thus, iNKT cells have disparate effects on the proliferation of FoBs versus MZBs.

Innate T Cells Regulate Innate B Cells
Innate T Cells Regulate Innate B Cells

A

MZB

FOB

CD86

WT

CD1d−/−

Vα14Tg

B

Vehicle

αGalCer

CD23

CD21

WT

CD1d−/−

Vα14Tg

C

% MZB

Vehicle

αGalCer

D

Vehicle

αGalCer

20x

40x

20x

40x

E

MFI

CD86

CD69

% of Max

Medium

αGalCer

LPS

LPS + αGalCer

F

CD23

CD1d−/−

CD21

Medium

αGalCer

LPS

LPS + αGalCer

WT
that addition of αGalCer to spleen cell cultures from diseased 7- to 9-mo-old (Fig. 6B) or preclinical 3-mo-old NZB/NZW F1 mice (Fig. 6C) selectively reduced the frequency of MZB cells. The MZB cell changes were not due to direct binding of αGalCer to CD1d on MZB cells, since adding αGalCer to pure B cells alone had no effect on MZB cell frequency in lupus mice (Fig. 6C, lower row). Thus, iNKT cells can regulate MZBs in mice that spontaneously develop autoimmune disease.

Since MZB cells preferentially secrete IgG3 isotype, we asked if the presence of iNKT cells would affect the levels of IgG3 isotype. As shown in Fig. 6D, while serum IgG1 and IgG2a isotype levels were similar between the two groups, serum IgG3 levels were higher in CD1d−/− NZB/NZW F1 mice than in CD1d+/- littermates. Taken together, these results as well as data in Fig. 1, 4, and 5 provide evidence that iNKT cells can influence various functions of MZBs including Ig production, activation and proliferation.

Our results thus far show that iNKT cell effects on MZBs were particularly pronounced under condition, such as in vivo LPS exposure, that is known to cause MZB expansion [1]. Consequently, we asked if iNKT cell activation will restrict MZBs in vivo in animals that spontaneously develop marked MZB expansion. Results show that NOD mice that have reduced numbers of iNKT cells [31] exhibit increased MZB cell proportions and numbers and increased marginal zone B cell area as compared to normal BALB/c (Fig. 7A) and B6 mice (Fig. 7B), as also reported previously [23,24]. To further test the role of this association between reduced iNKT cells and increased MZBs, we treated NOD mice with αGalCer that expands iNKT cells and prevents the development of diabetes [32,33]. As shown in Fig. 7B, αGalCer treatment, as compared to vehicle injection, resulted in a significant reduction in the frequency of MZBs in NOD mice. B cells were also reduced in the marginal zones of spleens in αGalCer-treated NOD mice (Fig. 7C). Thus, iNKT cell activation can reduce MZB cells in vivo in autoimmune mice that have expanded MZB population.

Discussion

We report that while iNKT cells activate the two major subsets of B cells in the spleen, they selectively reduce MZBs in vivo and in vitro. This reduction in MZBs is at least in part due to their increased AICD as well as due to reduced proliferation in a partly CD1d-restricted, contact-dependent manner. In contrast, iNKT cells promote the proliferation and expansion of FoB cells via soluble factors. Thus, iNKT cells interact differently with two major B cell subsets in the spleen, whereby iNKT cells selectively regulate MZB homeostasis.

We demonstrate the iNKT cell-mediated inhibition of MZBs, defined as CD121αCD23− B cells by flow cytometry, under various patho-physiological conditions, including normal BALB/c and B6 mice without or with LPS exposure (Fig. 1B, 7B), anti-DNA+ NZW mice (Fig. 6A), and autoimmune-prone NZB/NZW F1 and NOD mice that exhibit spontaneous MZB expansion (Fig. 6B,C, 7B). Similar observations were made using other markers for MZBs, including CD1d+CD21− or CD1d−CD9+ on gated CD19+ cells, in B6, NOD (Fig. 7B) and BALB/c mice (data not shown). These findings suggest that iNKT cell activation reduces the frequency of MZBs in vivo in mice (Fig. 7A, 7B) and in vitro in B6 (Fig. 6A) and NOD mice (Fig. 6B, C).
Innate T Cells Regulate Innate B Cells

A  

\( CD1^{-/-} \)  

MFI  

19.2  

12.9  

74.4  

74.6  

Medium alone  

\( \alpha \text{GalCer} \)  

LPS  

LPS + \( \alpha \text{GalCer} \)

B

\( T \) cells

MFI  

8.8  

None  

41.9  

11.3  

CD1d \(^{-/-}\)

C

\( T \) cells

MFI  

14.0  

None  

33.4  

19.9  

Tc

D

\( CD1^{-/-} \)  

\( V\alpha 14^{Tg} \)

1.43%  

13.7%  

3.78%  

11.5%

E

Conv-T  

iNK T

MZB  

FOB

F

\( CD1^{-/-} \)  

\( V\alpha 14^{Tg} \)

1.11%  

1.39%

\( CD23 \)

7AAD

G

\( CD95 \)

\( \text{Vehicle} \)  

\( \alpha \text{GalCer} \)

H

\( CD23 \)

7AAD
not shown). Further evidence of reduction in MZBs in vivo was seen by immunohistochemistry showing reduction of B cells in marginal zones of spleen of BALB/c, B6 and NOD mice treated with \(\alpha\)GalCer.

The MZ subset of B cells exhibits several unique features [34]. In the absence of B cell influx from bone marrow, FoBs in the spleen gradually decrease in numbers whereas MZBs are maintained at normal levels [35]. MZBs do not circulate via blood or lymph, and remain in spleen even after depletion of recirculating cells [36]. MZBs are more efficient than FoBs to generate plasma cells after polyclonal in vivo stimulation [37]. Findings in this paper provide evidence for a mechanism whereby such rapid and potent immune responses elicited by MZBs can be regulated by iNKT cells that can also respond and act rapidly.

The iNKT cells are a unique T cell subset that responds rapidly to lipid antigens [8,9,10]. iNKT cells efficiently promote B cell proliferation and Ig production [15,16,17]. Consistent with these observations, we found that iNKT cells enhance activation markers and costimulatory molecules on all B cells (Fig. 1). However, iNKT cells appear to regulate the two major subsets of splenic B cells differently. Whereas FoB cells expand and proliferate in presence of iNKT cells, iNKT cells selectively restrict the proliferation and numbers of MZBs.

We show evidence for at least two mechanisms, namely AICD and suppression of proliferation, whereby iNKT cells can control the homeostasis of MZBs. Cellular and molecular contexts in which these two cells interact are unclear. It has been proposed that MZBs that express high levels of CD1d will efficiently activate iNKT cells [38]. In fact, a recent study showed that MZBs activate iNKT cells in vivo and in vitro [39]. iNKT cells can in turn recognize CD1d on MZB cells and activate them. Activated iNKT cells also express enhanced levels of Fas-L [40], which could engage Fas on activated MZBs leading to their apoptosis. Such feedback inhibitory loop may ensure a homeostasis of these two immune cells after an incidence of rapid cross-activation. In support of this idea, we show that iNKT cells potently inhibit MZB cells if they express CD1d. CD1d expression on MZB or MZB-like cells has also been shown to promote iNKT cell-dependent tolerance [41] and protection from inflammation [42] by facilitating iNKT-B cell interactions. Thus, CD1d-restricted regulation of B cells may be an important mechanism whereby iNKT cells might discriminate between MZB versus other B cells leading to their selective regulation versus activation depending on the context.

iNKT cells can convert tolerogenic B cells into immunogenic APCs that can induce CD8\(^+\) Treg and cytotoxic T cells [43,44], which can potentially regulate autoreactive B cells [43,46]. However, our data show that iNKT cells can directly confer the
regulatory effects on MZBs by themselves, without help from other immune cells. A previous study showed that F4/80^{+}APCs release a chemokine MIP-2 that recruits iNKT cells to the marginal zones of spleen where they can aggregate with MZBs and T cells for at least 7 days [44]. Such close interaction between iNKT cells and MZBs in splenic marginal zone may result in persistent activation of MZBs, ultimately leading to their AICD. Fas-mediated apoptosis is an important negative checkpoint during B cell development to eliminate autoreactive B cells and control B cell homeostasis [47]. We propose that Fas-mediated apoptosis induced by iNKT cells provide an important negative checkpoint at the level of mature MZBs. Since iNKT cells possess cytotoxic abilities [48], they may potentially reduce MZBs by killing them. However, we have so far found no evidence of iNKT cell-mediated direct killing of MZBs (our unpublished data). Although, iNKT cell-mediated inhibition of MZB cells was more potent when the two cells were in contact, some inhibition of MZBs occurred in a contact-independent manner. Cytokines that mediate this inhibition remain to be determined. A recent study has shown that upon stimulation with TLR ligands, peritoneal B1 B cells secrete high levels of IL-10 that then inhibits the proliferation of the same B1 B cells in an autocrine manner [49]. Interestingly, we have recently reported that iNKT cells reduce the numbers of IL-10–secreting B cells [28]. Thus, it would be interesting to investigate whether iNKT cells directly or indirectly inhibit IL-10–producing MZB cells [50], whereas IL-4 produced by iNKT cells might promote the activation and proliferation of FoBs [13]. Alternatively, iNKT cells might affect factors such as Notch2 or its ligands, BAFF or its receptors, chemotactic signals such as sphingosine 1 phosphate receptor (S1pR1), and integrins, which are known to promote the development, differentiation, migration and splenic retention of MZBs (reviewed in [51]).

Studies have linked MZB cell abnormalities to the development of autoimmune diseases [20,21,22,24]. For example, MZB cells increase in lupus-prone [25,26] and NOD mice prior to the onset of T1D [23,24]. B cells with anti-self B cell receptors are enriched in the MZ [20,27]. MZB is also the most potent B cell subset to activate naive CD4 T cells [5]. MZB’s ability to present self-antigens to autoreactive T cells, such as diabetogenic T cells [24], suggests a role of MZBs in the pathogenesis of autoimmune diseases. Interestingly, the MZB expansion correlates with the reduced iNKT cell numbers and functions in NOD mice [31] (Fig. 7). Impairment in iNKT cells prior to the onset of disease is a feature of many animal models of autoimmune diseases, including MRL-lpr and pristane-induced lupus [26,52]. Reduction in NKT cells also associate with autoimmunity in the relatives of lupus patients [53]. Furthermore, germline deficiency of iNKT cells is associated with an expansion of MZB cells in aged mice [54], and CD1d-deficient BALB/c mice injected with a hydrocarbon oil that induces lupus-like disease have more MZB cells than CD1d^{−/−} BALB/c mice injected with PBS [26]. Taken together, it is reasonable to speculate that iNKT cell impairment might be related to MZB expansion in autoimmune conditions.

We provide a direct evidence for regulatory interactions between the two innate immune cells in autoimmunity that iNKT cell activation reduced MZBs in lupus- and autoimmune diabetes-prone mice (Fig. 6 and 7), and specifically inhibited ‘autoreactive’ (anti-DNA) MZB cells (Fig. 6A). In fact, iNKT cells can inhibit autoantibody production, while generally enhancing the normal antibody response in lupus-prone mice [28]. Similar observations have been reported in another model where injections of syngeneic apoptotic cells transiently trigger autoantibody production. In this model of induced autoantibody production, the absence or reduction of iNKT cells leads to increased autoreactive B cell activation without affecting the activation of B cells reactive to...
NP-OVA [55]. Importantly, αGalCer treatment prevents autoimmune disease in many experimental models including NOD mice [32,33]. Such protective effect is believed to be mediated via promotion of Th2 responses [32,33]. However, protection from diabetes in NOD mice is still maintained if iNKT cells are unable to produce or induce IL-4 and other regulatory cytokines (reviewed in [56]). Although, the role of MZBs in the pathogenesis of T1D is not proven yet, reduction of autoreactive MZBs in αGalCer-treated NOD mice should be investigated as an alternative mechanism whereby αGalCer affords protection in T1D.

In summary, iNKT cells regulate homeostasis of MZBs that rapidly respond to blood-borne pathogens and potently activate autoimmune T cells. We provide evidence for AICD and CD1d-restricted inhibition of proliferation as two mechanisms whereby iNKT cells regulate MZB homeostasis. Understanding these mechanisms will open avenues for manipulation of MZBs in human autoimmune diseases, since patients with T1D and SLE and their family members have reduced numbers and/or responses of iNKT cells [53,56]. The iNKT cell-based manipulation of immune responses is particularly appealing, given the limited polymorphism in CD1 genes [8], obviating one of the

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**Figure 6. iNKT cells reduce MZB cells in anti-DNA transgenic and lupus-prone mice.** (A) Spleen cells from 4-mo-old R4A anti-DNA^tg^ NZW mice were cultured with LPS without or with αGalCer for 48 h, and analyzed for MZBs as the % of anti-DNA (IgG2b^hi^) B cells. (B) Spleen cells from 7-mo-old nephritic BWF1 mice were cultured with αGalCer and/or LPS for 18 h, and analyzed for MZBs as the % of IgM^+^ lymphocytes. *Note that the addition of αGalCer to spleen cell cultures reduced the proportion of MZB cells. Bar graphs on the right show results expressed as the mean±SE of three independent experiments performed using pooled cells from 3–4 mice per experiment. #P<0.05 ##P<0.01, compared to LPS alone cultures. Results represent three independent experiments. (D). Serum levels of IgG1, IgG2a and IgG3 isotypes are shown in 8-mo-old CD1d^−/−^ and CD1d^+/+^ BWF1 littermates. *p = 0.04, n = 13 CD1d^−/−^ and 7 CD1d^+/+^ mice, mean ± SE.

doi:10.1371/journal.pone.0026536.g006

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Figure 7. αGalCer treatment limits MZB expansion in NOD mice. A. Spleen cells from NOD or normal BALB/c mice were analyzed for MZBs. Comparison of MZB frequency between NOD and BALB/c mice (n = 6 NOD and 3 BALB/c mice, 8–10-week-old females). Numbers on dot plots indicate MZBs (CD21hiCD23lo/low) as the mean ± S.E. % of CD19+ cells (*p < 0.001). Bar diagram shows the mean ± S.E. absolute numbers of MZBs per spleen (*p < 0.001). B, C. Spleens were harvested from 2-mo-old NOD or C57BL/6 mice at 3 d after a single i.p. injection with αGalCer or vehicle (n = 6 mice per group). B. Cells were analyzed for MZBs as CD21hiCD23lowCD1dhiCD9+ cells on gated CD19+ B cells. Numbers on dotplots indicate MZBs as % of CD19+ B cells. Bar diagrams show the mean ± S.E. % MZBs in NOD mice. Compared to vehicle-injected mice, αGalCer-treated NOD mice had a significant reduction in MZBs, defined as CD19+CD21hiCD23low (p = 0.007), CD19+CD21hiCD1dhi (p = 0.0001), CD19+CD1dhiCD9+ (p = 0.009). C. Frozen spleen sections were stained for APC-IgM (blue) and FITC-MOMA1 (green). Confocal images at 20× and 40× magnification show the reduced thickness of marginal zone (MZ) IgM+ cells (blue) in αGalCer-treated mice (as indicated by blue arrows). Data represent three independent experiments.

doi:10.1371/journal.pone.0026536.g007
major hurdles of therapies aimed at highly polymorphic MHC class I and II system. Enhancing this appeal is our finding that in NKT cells differentially regulate different B cell subsets, thus allowing a selective manipulation of certain B cell functions.

Acknowledgments

We thank Drs. K. Dorskind and R. Halder (both at UCLA) for helpful suggestions, NIH Tetramer Core Facility, Emory, GA for providing mCD1d+/PBS-57 tetramers, and UCLA Flow Cytometry Core for technical help and CNSI Advanced Light Microscopy/Spectroscopy Shared Resource Facility at UCLA for confocal laser scanning microscopy.

Author Contributions

Conceived and designed the experiments: RRS XW JY. Performed the experiments: XW JY. Analyzed the data: XW JY. Contributed reagents/materials/analysis tools: RRS XW JY PJ. Wrote the paper: XW RRS.

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