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Cross-reactive antigen expressed by B6 splenocytes drives receptor editing and MZ differentiation of IgG2a-reactive AM14 Vκ8 B cells

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Abstract

The AM14 BCR, derived from an autoimmune MRL/lpr mouse, binds autologous $IgG2a^{a/j}$ with low affinity, and as a result AM14 B cells only proliferate in response to IgG2a immune complexes that incorporate DNA, RNA, or nucleic acid binding proteins that serve as autoadjuvants. As such, AM14 B cells have served as a useful model for demonstrating the importance of BCR/TLR co-engagement in the activation of autoreactive B cells. We now show that the same receptor recognizes an additional murine encoded antigen, expressed by B6 splenocytes, with sufficient avidity to induce a TLR-independent proliferative response of BALB/c AM14 Vκ8 B cells both in vivo and in vitro. Moreover, detection of this cross-reactive antigen by B6 AM14 Vκ8 B cells promotes an anergic phenotype as reflected by suboptimal responses to BCR-crosslinking and the absence of mature B cells in the bone marrow. The B6 antigen further impacts B cell development as shown by a dramatically expanded MZ compartment and extensive receptor editing in B6 AM14 Vκ8 mice, but not BALB/c AM14 Vκ8 mice. Despite their anergic phenotypes, B6 AM14 Vκ8 B cells can respond robustly to autoantigen/autoadjuvant immune complexes and could therefore participate in both autoimmune responses and host defense.

Introduction

B cells expressing the transgene-encoded AM14 BCR, specific for IgG2a, have provided a critical tool for defining the role of nucleic acid sensing TLRs in the activation of autoreactive B cells (1–5). The AM14-producing hybridoma was originally isolated from a diseased MRL/lpr mouse and this antibody is considered a rheumatoid factor since it binds to autologous MRL IgG2a, as well as BALB/c IgG2a (6–8). AM14 binds monomeric IgG2a

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with relatively low affinity (Kd=~2.2×10^{-6} M, (7), and as a result, AM14 B cells are essentially non-responsive (ignorant) to normal circulating levels of IgG2a (6) and do not proliferate or exhibit any evidence of anergy in non-autoimmune mice. Immune complexes (ICs) consisting of IgG2a-bound proteins also fail to induce AM14 B cell proliferation. By contrast, IgG2a autoantibody/autoantigen (autoAbs/autoAgs) ICs that incorporate DNA or RNA and therefore carry their own autoadjuvant are potent inducers of AM14 B cell proliferation (1, 2, 5). We have extensively characterized B cells derived from BALB/c AM14 Vκ8 site-directed transgenic mice and shown that these highly allelically restricted B cells are limited to the follicular (FO) B cell compartment; hence these mice lack marginal zone (MZ) B cells (9). The IgG2a monoclonal autoAb PL2–3 binds to both DNA and RNA associated proteins and induces proliferation of $Tlr9^{-/-}$ and $Tlr7^{-/-}$, but not double deficient, AM14 Vκ8 B cells. Intriguingly, BCR/TLR7 and BCR/TLR9 activation of AM14 Vκ8 B cells leads to distinct functional outcomes (3). To better define the receptor specific signaling cascades, we backcrossed the AM14 and Vκ8 transgenes to C57BL/6J (B6) mice so that we could take advantage of tissue specific deleter strains only available on a B6 background. Unexpectedly, we found that B6 AM14 Vκ8 B cells are hyporesponsive to anti-IgM stimulation, readily differentiate to a MZ B cell phenotype, and undergo extensive receptor editing. These outcomes reflect the capacity of the AM14 Vκ8 BCR to respond to a cross-reactive antigen expressed by B6 but not BALB/c mice.

Materials and Methods

Animals

BALB/c AM14 Vκ8 site-directed transgenic (sdTg) mice have been described previously (9, 10). BALB/c and C57BL/6J (B6) wild type mice, BALB/c $Rag2^{-/-}$ and B6 $Rag1^{-/-}$, BALB/c and B6 CD45.1 mice were obtained from Jackson Lab. B6 mice homozygous for the AM14 heavy and heterozygous for the Vκ8 light chain (Vκ8/+) were provided by Dr. M. Shlomchik (University of Pittsburgh). These mice were intercrossed to generate AM14 homozygous and Vκ8 homozygous (Vκ8/ Vκ8) mice. AM14 Vκ8/ Vκ8 mice were crossed to B6 $Rag1^{-/-}$ (Jackson Lab), B6 $Btk^{-/-}$ (11) or B6 $Unc93B1^{3d/3d}$, kindly provided by Dr. B. Beutler (12). F1 offspring were intercrossed to obtain B6 AM14 Vκ8/+ $Rag1^{-/-}$, AM14 Vκ8/+ $Btk^{-/-}$ and AM14 Vκ8/+ $Unc93B1^{3d/3d}$. All mice were bred and maintained at the Department of Animal Medicine of the University of Massachusetts Medical School in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

Cell culture

Splenic B cells were positively selected with B220 magnetic beads (BD Biosciences) and then cultured in RPMI/5% heat-inactivated FCS as described previously (9) with the following ligands: 1 μg/ml CpG 1826 (s-oligodeoxynucleotide, Idera Pharmaceuticals), 15 μg/ml goat anti-mouse IgM $F(ab')_2$ (Jackson ImmunoResearch), or 1 μg/ml mAb PL2–3 (13). B cell proliferation was assessed by ^{3}H-thymidine incorporation at 30 hr or fluorescent dye dilution at 72 hr. BLyS was provided by Human Genome Sciences and added to selected experimental groups as mentioned at a final concentration of 50 ng/ml. Inhibitor (Inh) 18 (3’

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CCT GGA TGG GAA CTT ACC GCT GCA 5'), an inhibitory ODN, was used at 1 μg/ml to block TLR9 activation (14).

Flow cytometric analysis

Splenic B cell subsets were identified as FO B cells (B220$^+$ AA4.1^{neg} CD23^{hi} CD21^{lo}) and MZ B cells (B220$^+$ AA4.1^{neg} CD23^{lo} CD21^{hi}) using these antibodies: violetflour450 anti-CD45R/B220 (RA3–6B2, Tonbo), Alexafluor 647 anti-CD93 (AA4.1, Invitrogen), PE anti-CD21 (eBio8D9, eBioscience), FITC or APC anti-CD23 (B3B4, Biolegend), FITC polyclonal rat anti-λ light chain (SouthernBiotech). The following B cell subsets, Fraction B-D (B220^{lo}IgM^{neg}, proB and preB), Fraction E (B220^{lo}IgM$^+$, immature B) and Fraction F (B220^{hi}IgM$^+$, recirculating B) were determined using violetfluor450 anti-CD45R/B220 (RA3–6B2) and PE-Cy7 anti-IgM (II/41, eBioscience). In vitro plasma cells (CD22.2^{lo} CD138$^+$ CD44$^+$, PCs) were detected with FITC anti-CD22.2 (Cy34.1, BD Pharmingen), PE anti-CD138 (281–2, BD Pharmingen), APC-Cy7 anti-CD44 (IM7, Tonbo) (9). Receptor edited cells in the different B cell compartments were detected by staining with FITC anti-λ (Southern Biotech). AM14 Vκ8$^+$ B cells were detected with biotinylated anti-idiotype 4G7 (7) in combination with streptavidin-PerCP-Cy5.5. B cell proliferation was assessed by dilution of CFSE (Life Technologies) or VPD450 (BD Biosciences) (9). Dead cells were distinguished with TO-PRO-3 (Life Technologies) (9). Flow cytometric analysis was carried out using a BD LSR II with Diva Software (BD) and analysis was conducted with FlowJo software (Tree Star).

Immunofluorescence

Spleens were embedded in OCT (TissueTek) and frozen in ice-cooled butanol. Cryostat sections (7–10 μm) were stained with FITC anti-MOMA-1, Alexa 647 anti-λ, BV421 anti-B220 as described previously (15). AM14 Vκ8-expressing B cells were detected with biotinylated anti-idiotype 4–44 (7)and streptavidin-Alexa 555 (Invitrogen). Slides were mounted with Prolong Antifade (Molecular Probes). Images were captured with a 10x lens on a Nikon Eclipse Ti automated wide-field microscope with a QImaging Retiga 200R CCD camera using NIS Elements software. Images were processed with Adobe Photoshop software.

Homeostatic proliferation assay and in vitro coculture

B220 purified B cells were stained with either CFSE or VPD450 as described (3). Cells were resuspended in PBS and 1×10^7 cells were injected i.v. into BALB/c Rag2$^{-/-}$ and B6 Rag1$^{-/-}$. Rag hosts were depleted of NK cells using 20 μl of anti-Asialo GM1 (eBioscience) 2 days before B cell transfer. Spleens were harvested 10 days after B cell transfer and proliferation was assessed by dye dilution using flow cytometry. For the co-culture experiments, 0.2×10^6 B6 or BALB/c antigen presenting cells and 0.2×10^6 CFSE or VPD450 labeled AM14 Vκ8 B cells per well were added to a 96 well plate in a final concentration of 50 ng/ml BLyS. Proliferation of AM14 Vκ8 B cells was measured by fluorochrome dilution at day 4. In some experiments antigen presenting cells and B cells were separated by a 0.3 um transwell insert (Corning). An AM14 Vκ8 IgM monoclonal antibody (mAb) was isolated from the fusion of LPS-activated BALB/c AM14 Vκ8 B cells and used at 30 μg/ml to block the in vitro response of AM14 Vκ8 B cells to the B6 cross-reactive antigen.

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ELISA

Serum IgG2a levels were measured by ELISA. Plates were first coated with 2 μg/ml polyclonal goat IgG specific for mouse IgG2a (SouthernBiotech) overnight at 4°C, and blocked with PBS/BSA. Serum samples diluted in PBS/BSA were added to the wells. Bound antibody was detected with biotinylated polyclonal goat $F(ab')_2$ anti-IgG2a (SouthernBiotech) and streptavidin-HRP (BD Bioscience) and then developed with TMB liquid substrate system (Sigma-Aldrich).

Statistical Analysis

Statistical analyses were conducted with the Graphpad Prism7 software. Comparisons between two groups were performed by unpaired Students *t*-test for normally distributed data. One-way ANOVA including Sidak's multiple comparisons test was used for multiple group comparisons. A *P* value <0.05 was considered significant. *P* values are denoted as follows: * *P* 0.05, ** *P* 0.01, *** *P* 0.005, **** *P* 0.001.

Results

Distinct features of BALB/c and B6 AM14 Vκ8/Vκ8 B cells

All AM14 Vκ8 B cells used in this study express the same heavy and light chain site directed transgenes (10, 16, 17). We previously reported that BALB/c AM14 Vκ8/Vκ8 BCR Tg mice develop higher than normal numbers of follicular B cells, all of which express the AM14 Vκ8 receptor as shown by staining with the clonotypic antibody 4G7 (9). Since the AM14 targeting construct incorporated the $IgG2a^a$ constant region domains, we expected that both BALB/c and B6 AM14 Vκ8 B cells would differentiate in the context of the same circulating levels of $IgG2a^{\mathbf{a}}$, and therefore develop the same B cell subset distribution and respond comparably to anti-IgM stimulation. Despite similar levels of serum IgG2a (Fig. 1A) and comparable numbers of total B cells (Fig. 1B), we found that B6 mice homozygous for both the AM14 heavy chain and Vκ8 light chain (AM14 Vκ8/Vκ8) were hyporesponsive to anti-IgM $F(ab')_2$ stimulation despite normal responses to the TLR9 ligand CpG 1826 (Fig. 1C). To better understand the reason for the decreased BCR induced proliferative response, we analyzed the splenic B cell compartments and found a striking difference in mature B cell subsets. As reported earlier (9), all BALB/c AM14 Vκ8/Vκ8 B cells differentiate into FO B cells; there are no MZ B cells. However, ~70% of B6 AM14 Vκ8/Vκ8 mice had substantial numbers of MZ B cells as shown by the canonical $CD23^{int}$ $CD21^+$ phenotype (Fig. 1D).

Since immature and MZ B cells proliferate poorly in response to goat anti-mouse IgM $F(ab')_2$ crosslinking (18), we speculated that the increased frequency of MZ B cells might account for the decreased response of B6 AM14 Vκ8/Vκ8 B cells to anti-IgM activation. To address this possibility, we isolated FO B cells using CD23-specific magnetic beads. The B6 $CD23^+$ AM14 Vκ8/Vκ8 B cells still responded poorly to BCR crosslinking when compared to BALB/c $CD23^+$ AM14 Vκ8/Vκ8 B cells despite a normal response to TLR ligand (Fig. 1E), indicating that B6 AM14 Vκ8/Vκ8 FO B cells per se are hyporesponsive to BCR cross-linking.

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Unresponsiveness to BCR crosslinking is often a sign of anergy, one of the mechanisms in place to ensure that self-reactive B cells remain tolerant of self-antigens (selfAgs) (19, 20). Another feature of anergic B cells is decreased surface expression of the BCR (19). We measured surface expression levels of AM14 Vκ8 BCR using the clonotypic mAb 4G7 that recognizes a combined AM14 heavy and Vκ8 light chain idiotype (7). The MFI of AM14 Vκ8 BCR on B6 AM14 Vκ8/Vκ8 FO B cells was ~25% lower than on BALB/c AM14 Vκ8/Vκ8 B cells (Fig. 1F). Usually the decrease in sIg is due to a decrease in sIgM expression while sIgD stays the same (19–21). Surprisingly, we found that the decrease in surface AM14 BCR expression reflected a specific loss of IgD on the surface, but surface IgM (sIgM) levels stayed constant (Fig. 1F).

Mature B cells recirculate back to the bone marrow and can be distinguished from the developing B cells in the bone marrow by their increased expression of B220 and IgM (Fraction F, (22). However, anergic B cells do not recirculate back to the bone marrow (23). We isolated cells from the bone marrow of BALB/c and B6 AM14 Vκ8/Vκ8 mice and assessed B220 and IgM expression by flow cytometry. While the frequency of recirculating Fraction F cells in the bone marrow of BALB/c AM14 Vκ8/Vκ8 mice was greater than or equal to that of non-Tg BALB/c mice, the number of recirculating B cells was reduced in the bone marrow of B6 AM14 Vκ8/Vκ8 mice, consistent with an anergic phenotype (Fig. 1G).

AM14 Vκ8/Vκ8 B cell detection of a non-IgG2a cross-reactive antigen present in B6 but not BALB/c mice leads to anergy

Both anergy and MZ development have been associated with recognition of selfAgs and together, these data were consistent with the notion that the AM14 Vκ8 BCR cross-reacts with a self-Ag expressed on the B6 but not BALB/c background. AM14 Vκ8/Vκ8 B cells developing in BALB/c mice should not encounter a B6-restricted self-Ag, and therefore we reasoned that the transfer of naïve BALB/c AM14 Vκ8/Vκ8 B cells into B6 mice should result in the activation of these cells by the B6 antigen. Purified splenic B cells from either BALB/c AM14 Vκ8/Vκ8 or BALB/c non-Tg mice were CFSE labeled and injected into either BALB/c Rag2$^{-/-}$ or B6 Rag1$^{-/-}$ mice. The recipient mice were treated with NK-depleting antibody 2 days before injection of B cells to avoid rejection of the allogeneic B cells by Natural Killer (NK) cells. Recipient spleens were harvest on day 10 after injection and the in vivo proliferative response of the B cells was assessed by CFSE dilution. As expected, the B cells from the wildtype (WT) control underwent homeostatic proliferation in either Rag deficient background (Fig. 2A, (24). However, the BALB/c AM14 Vκ8/Vκ8 B cells were essentially non-responsive in the BALB/c recipients, indicating that homeostatic proliferation depends on a diverse BCR repertoire and is not simply due to a need to fill “empty space”. By contrast, the BALB/c AM14 Vκ8/Vκ8 B cells injected into the B6 Rag1$^{-/-}$ mice proliferated much more extensively than the non-Tg control, consistent with the idea that BALB/c AM14 Vκ8/Vκ8 B cells recognize a B6-restricted self-Ag.

To further explore the nature of this self-Ag, we used an in vitro proliferation assay. CFSE-labeled BALB/c AM14 Vκ8/Vκ8 or non-Tg B cells were co-cultured with RBC-depleted splenocytes obtained from either BALB/c or B6 Rag-deficient mice, and CFSE dilution was analyzed after 4 days in culture. Similar to the in vivo results, BALB/c AM14 Vκ8/Vκ8

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B cells only proliferated when co-cultured with B6 splenocytes not when cultured with BALB/c splenocytes (Fig. 2B). Since Rag-deficient mice lack B and T cells, we were interested to know if the self-Ag was restricted to the myeloid/stroma cell lineage or was also present on lymphocytes. To this end, we used purified B cells, T cells or lymphocyte-depleted cells from WT CD45.1 B6 and BALB/c spleens as activator cells in our in vitro assay. The BALB/c AM14 Vκ8/Vκ8 B cells only proliferated in response to lymphocyte-depleted cells not to B or T cells, suggesting that the self-Ag is not present on lymphocytes (Fig. 2C). Recognition of selfAgs plays an important role in B cell development in the bone marrow as well as in the spleen. We therefore were interested to know if the cross-reactive antigen was restricted to the spleen or is also present in the bone marrow. Hence, we activated BALB/c AM14 Vκ8/Vκ8 B cells with either bone marrow cells or splenocytes from BALB/c or B6 Rag-deficient mice. Interestingly, the AM14 Vκ8/Vκ8 B cells proliferated only in response to the B6-derived splenocytes, not to B6 derived bone marrow cells. This suggests that the cross-reactive Ag is restricted to the periphery.

We next asked whether the response to B6 antigen was contact dependent or due to a soluble factor produced by B6 cells present in the co-cultures. BALB/c AM14 Vκ8/Vκ8 B cells separated from the B6 splenic 'activator' cells using a transwell system did not proliferate (Fig. 2E). In addition, we were able to block the BALB/c AM14 Vκ8/Vκ8 B cell response against the B6-derived antigen by adding an AM14 Vκ8 IgM mAb, but not an IgM isotype control, to the culture system (Fig. 2F). We conclude from these studies that the activation of AM14 Vκ8 B cells is cell contact dependent and due to a molecule recognized by the AM14 antibody.

Detection of cross-reactive antigen induces receptor editing

B cells that recognize a self-Ag during development can also be tolerized by secondary rearrangement of variable domain genes, a process referred to as receptor editing (25–27). Receptor editing primarily involves rearrangement of the light chain V locus. Since mice homozygous for the Vκ8 light chain Tg are unable to undergo receptor editing (28), we generated BALB/c and B6 AM14 Vκ8/+ mice (heterozygous for Vκ8) and then analyzed their B cell compartments. As with the AM14 Vκ8/Vκ8 mice, MZ B cells developed only on the B6 background (Fig. 3A). However, total B cell numbers as well as the % of FO B cells were significantly decreased in the B6 AM14 Vκ8/+ mice (Fig. 3B). This decrease in FO B cells was not due to an increased % of MZ B cells since the percentage of MZ B cells was comparable between the B6 AM14 Vκ8/Vκ8 and AM14 Vκ8/+ mice. The loss of FO B cells is more likely due to deletion of cells with unproductive receptor editing events. To test for receptor editing, we stained splenic B cells with the 4G7 anti-idiotype and an antibody specific for λ light chain. This staining scheme distinguished unedited B cells ($4G7^{hi}$ λ^{neg}) from B cells that had undergone a secondary κ chain gene rearrangement ($4G7^{neg}$ λ^{neg}) and B cells that had undergone a secondary λ chain gene rearrangement ($4G7^{neg}$ λ^{+}). As expected, the vast majority of BALB/c AM14 Vκ8/+ B cells were $4G7^{+}$ and λ^{-} and therefore likely to be unedited. By contrast, many of B6 AM14 Vκ8/+ B cells underwent receptor editing, as shown by expression of λ light chain and/or loss of the 4G7 idiotype (Fig. 3A). We observed receptor editing in both, FO and MZ B cells, but receptor editing was more extensive in the MZ compartment. Furthermore, the % of MZ B cells and

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the % λ^+ MZ B cells increased with age suggesting accumulation of MZ and receptor edited B cells overtime (Fig. 3C). We confirmed the development of MZ B cells and the extent of receptor editing by immunofluorescence staining of splenic sections from BALB/c and B6 AM14 Vκ8/+ mice (Fig. 3D).

Marginal zone development and receptor editing is independent of TLR signaling

Consistent with the role for Rag recombinase in receptor editing (29), rag-deficient B6 AM14 Vκ8/+ mice developed a MZ compartment but did not show evidence of receptor editing (Fig. 4A+D). Receptor editing requires the engagement of the full BCR signaling cascade (30). To address the contribution of BCR signaling to receptor editing observed in B6 AM14 Vκ8/+ B cells, we crossed the B6 AM14 Vκ8/Vκ8 mice with btk-deficient mice. As expected, male btk-/y × B6 AM14 Vκ8/+ B cells, but not the corresponding female btk−/+ F1 B cells, failed to undergo receptor editing, even though they still developed a MZ compartment (Fig. 4B+D). However, receptor editing of B6 AM14 Vκ8/+ B cells did not require co-engagement of either TLR7 or TLR9 as Unc93B1-deficient B6 AM14 Vκ8/+ B cells underwent extensive receptor editing in both the FO and MZ subsets (Fig. 4C+D).

BCR/TLR crosslinking activates anergic B6 AM14 B cells

The AM14 Vκ8 BCR recognizes IgG2a ICs and BALB/c AM14 Vκ8/Vκ8 B cells are activated by ICs that also incorporate nucleic acids (1, 2, 5, 9). To determine whether anergic B6 AM14 Vκ8/Vκ8 B cells could respond to IC activation despite their relatively weak response to BCR-crosslinking, we stimulated BALB/c and B6 AM14 Vκ8/Vκ8 FO B cells with the $IgG2a^a$ autoAb PL2–3. PL2–3 activates BALB/c AM14 Vκ8/Vκ8 B cells through a BCR and TLR7/9 dependent mechanism (1–3, 5). We found that anergic B6 $4G7^+$ AM14 Vκ8/ Vκ8 B cells proliferated as well as BALB/c AM14 Vκ8/Vκ8 B cells in response to PL2–3, despite their inability to respond to anti-IgM $F(ab')_2$ (Fig. 5A).

Moreover, we previously showed that BCR/TLR7 engagement, in the absence of BCR/TLR9 engagement (TLR9-deficiency or TLR9 inhibitor) leads to PL2–3 driven plasma cell (PC) differentiation of BALB/c AM14 Vκ8/Vκ8 B cells within 3 days of in vitro activation (9). We repeated this assay with B6 AM14 Vκ8/Vκ8 B cells to determine whether anergic B cells have the capacity to differentiate into PCs. Under these conditions, both B6 AM14 Vκ8/Vκ8 B cells and BALB/c AM14 Vκ8/Vκ8 B cells readily differentiated into PCs (Fig. 5B).

Next, we reasoned that responsiveness of B cells to anti-IgM stimulation in AM14 Vκ8 B6 mice should be restored in cells that lost Vκ8 expression, and therefore reactivity to the cross-reactive antigen, as a result of receptor editing. To test this idea, we stimulated CFSE-labeled B cells from BALB/c and B6 mice with anti-IgM or PL2–3 and then assessed proliferation by CFSE dilution 3 days later. As expected, most of the BALB/c AM14 Vκ8 B cells were 4G7+ and proliferated in response to anti-IgM and PL2–3 (Fig, 5C). Most of the B6 B cells that responded to anti-IgM were $4G7^{neg}$ and therefore edited. A small % of the B6 B cells responded to PL2–3, and this included a high % of 4G7+ (unedited) cells, as well as some $4G7^{neg}$ cells, that presumably retained reactivity to IgG2a (Fig. 5C). Taken together, our data suggests that anergic B6 AM14 Vκ8 B cells can respond to PL2–3

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even though they are non-responsive to IgM-crosslinking, and that receptor editing restores anti-IgM responsiveness in cells that can no longer detect the B6 cross-reactive antigen.

Discussion

In the current study, we demonstrate that the presence of a B6 derived cross-reactive self-Ag, detected by AM14 Vκ8 expressing B cells, induces an anergic B cell phenotype as defined by decreased responsiveness to BCR crosslinking by anti-IgM, decreased levels of surface BCR and loss of recirculating B cells. The existence of this B6 restricted cross-reactive antigen is strongly supported by: (a) the *in vivo* proliferation of BALB/c AM14 Vκ8/Vκ8 B cells in B6, but not BALB/c, Rag-deficient mice; (b) the development of MZ B cells, a subset enriched for autoreactive clones, in B6 but not BALB/c AM14 Vκ8 mice; and (c) extensive receptor editing in the FO as well as MZ B cell compartment in only B6 AM14 Vκ8/+ mice. Intriguingly, these anergized B cells are still fully capable of responding to BCR/TLR co-engagement and could therefore theoretically participate in host defense or autoimmune disease.

Homeostatic expansion of B cells in a lymphopenic mouse is commonly thought to depend on an empty B cell niche and elevated serum titers of survival factors such as BLyS (31). However, if these were the only requirements for homeostatic expansion then the specificity of the BCR should be irrelevant and proliferation should be independent of self-reactivity. Hence, B cells with a fixed BCR repertoire and B cells with a diverse repertoire should undergo comparable levels of homeostatic proliferation. Our findings were more consistent with studies by Freitas and colleagues who showed that the survival of a specific BCR clone depends on competition for self-Ag specific resources (32). In fact, BALB/c AM14 Vκ8/Vκ8 B cells completely failed to undergo homeostatic proliferation in BALB/c Rag-deficient hosts, but exhibited extensive proliferation in B6 Rag-deficient mice. Together our data indicate that self-Ag, and not just the host environment, promote homeostatic proliferation (Fig. 2A). They furthermore argue that the self-Ag necessary for selection and survival may be different from the known binding specificity of the BCR, since both B6 and BALB/c AM14 Vκ8/Vκ8 mice have comparable levels of $IgG2a^a$.

Although we have not identified the B6 specific target antigen, it is not likely to be an antibody-associated epitope since AM14 Vκ8 B cells respond to splenocytes from B6 $Rag1^{-/-}$ mice that lack B cells and they fail to respond to either B6 BM cells or splenic B cells (Fig. 2A–D). There are numerous examples of unanticipated cross-reactivities of monoclonal antibodies (mAbs) (33–35), the reason why tissue cross-reactivity studies are a routine step in the development of therapeutic mAbs (36). Therefore, potential cross-reactivities of autoAbs on diverse genetic backgrounds warrant further consideration. Due to the stochastic nature of BCR gene rearrangement, around 70% of newly emerging B cells are thought to recognize self-Ags (37) and this self-reactivity plays a key role in clonal diversification by promoting receptor editing (25, 27).

The activation of FO AM14 Vκ8 B cells by autologous IgG2a-containing ICs requires co-engagement of the BCR and the endosomal TLRs, TLR7 or TLR9 (1–3, 5), presumably due to the low affinity of the AM14 Vκ8 BCR for autologous IgG2a. Also, the MZ has

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been shown to be enriched for autoreactive B cell clones (38) and human studies further point to a role for TLR signals in MZ B cell development and selection (39). To address the role of endogenous TLRs in MZ B cell development of B6 AM14 Vκ8 B cells, we generated B6 AM14 Vκ8/+ Unc93B1-deficient mice. Our data show that the response to the B6-encoded cross-reactive antigen does not require an endosomal TLR ligand since MZ B cell development and the extent of receptor editing appear comparable in Unc93B1-sufficient and Unc93B1-deficient B6 AM14 Vκ8/+ mice (Fig. 4C). Therefore, it is possible that the AM14/B6 antigen interaction is either higher avidity or depends on a different autoadjuvant, an avenue that can be further explored when the B6 antigen is identified.

Btk is an important signaling molecule downstream of the BCR as well as TLRs (40, 41). Btk-deficient mice are known to have increased frequency of MZ B cells due to preferential death of FO B cells (11, 42, 43). We previously reported that btk-deficient BALB/c × CBA/N AM14 Vκ8 mice develop a MZ B cell compartment while btk-sufficient BALB/c × CBA/N AM14 Vκ8 mice lack a MZ B cell compartment (9). Based on these findings, we expected btk-deficient B6 AM14 Vκ8/+ mice to have an even greater % of MZ B cells than btk-sufficient B6 AM14 Vκ8/+ mice. However, the size of the MZ B cell compartment in btk-deficient AM14 Vκ8/+ mice was comparable to the B6 AM14 Vκ8 WT controls (Fig. 4B). Even more unexpectedly, we found a total loss of λ expressing B cells and $4G7^{neg}$ λ^{neg} (kappa edited) B cells in both the FO and MZ B cell compartments of btk-deficient B6 AM14 Vκ8/+ mice. Together, these data suggest that btk plays an important role in the signaling cascade responsible for receptor editing driven by the B6-derived epitope in our mouse model. This is in contrast to a previous study that concluded btk was dispensable for receptor editing in the 3–83 (anti-H-2K) Tg mouse model (44). A possible explanation for the difference between the two models is the site of Ag expression. In our case the cross-reactive antigen seems to be restricted to the periphery (Fig. 2D) while the Ag (MHC) for 3–83 is present in the BM during B cell development. 3–83 is also likely to be a higher avidity interaction due to high levels of MHC class I on the majority of cell types.

Depending on the context of self-Ag encounter, anergic B cells can display a range of defects (45). One common criteria for assessing anergy is responsiveness to BCR-crosslinking by antigen or anti-IgM $F(ab')_2$. In some cases, anergic B cells do not proliferate at all in response to BCR crosslinking (19, 46, 47). In other cases, such as B cells expressing 3H9/ Vκ8 (DNA-reactive) or VH2–12 (SmRNP-reactive) receptors, anergic B cells exhibit weak to intermediate proliferation in response to anti-IgM crosslinking (46, 48). B6 AM14 Vκ8 B cells fall into this hyporesponsive category. Furthermore, anergic B6 AM14 Vκ8 B cells respond normally to TLR dependent activation as was shown for most anergic B cell models (45).

Anergy is also frequently accompanied by decreased surface expression of the BCR (19). We show here that B6 AM14 Vκ8/Vκ8 B cells have slightly reduced levels of sIg, as tested by staining with the AM14 Vκ8 specific anti-clonotype 4G7 (Fig. 1F). Several reports showed that anergic B cells usually have a decrease in sIgM levels while surface IgD (sIgD) levels stay the same (49–52). Downregulation of IgM is thought to limit responses of FO B cells to self-Ags (52), however more recent studies point to a tolerogenic role for IgD (51, 53). Interestingly, B6 AM14 Vκ8/Vκ8 B cells have decreased sIgD levels suggesting that

these cells might respond more rapidly to activation by endogenous ligands. Indeed, anergic B6 AM14 Vκ8/Vκ8 B cells proliferated extensively in response to nucleic acid containing ICs (Fig. 5A).

Goodnow and colleagues have reported that anergic B cells are capable of participating in germinal center (GC) responses (54, 55). Participation of anergic B cells in a GC response poses the potential risk of accumulation of autoreactive PCs and autoimmune diseases. Surprisingly, anergic B cells in the HEL model system undergo extensive mutations that decreases their affinity to the self-Ag and increases affinity to a foreign Ag (54). We have shown here that activation of anergic AM14 Vκ8/Vκ8 B cells by dual engagement of the BCR and TLR can readily induce PC formation and therefore break tolerance (Fig. 5B). It is well established that endosomal TLRs play a key role in the activation of autoreactive B cells (56). Hence, the role of anergic B cells in GC responses may need to be further addressed using more physiologically relevant model systems.

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Abbreviations used in this paper

autoAbs	auto-antibodies
autoAgs	auto-antigens
BLyS	B Lymphocyte Stimulator
FO	follicular
GC	germinal center
IC	immune-complex
MZ	marginal zone
PC	plasma cell
selfAgs	self-antigens
sIg	surface Ig
SLE	systemic lupus erythematosus

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KEY POINTS

- The AM14 Vκ8 BCR recognizes a B6 derived cross-reactive antigen.
- This B6 "self-Ag" induces anergy, receptor editing and MZ B cell development.
- Anergic AM14 Vκ8 B cells can be fully activated by nucleic acid containing ICs.

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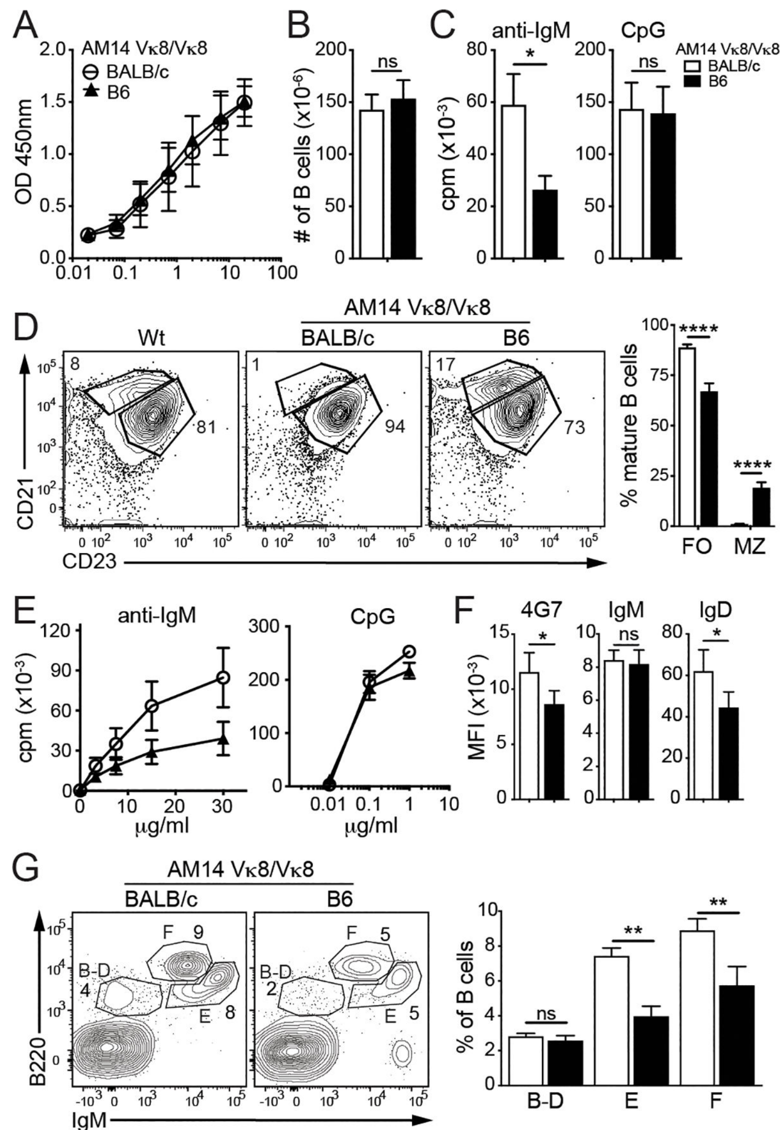

Figure 1.

B6 AM14 Vκ8 B cells have an anergic phenotype

A. Serum IgG2a levels of AM14 Vκ8/Vκ8 BALB/c (○) and B6 (π) mice were measured by ELISA (n=5 BALB/c, n=11 B6, mean +/− SEM).

B. Number of B220+ B cells in the spleen of AM14 Vκ8/Vκ8 BALB/c (white bar, n=19) and B6 (black bar, n=9). Mean +/− SEM is shown.

C. Proliferation of B220-purified splenic B cells in response to 15 μg/ml goat anti-IgM F(ab')$_2$ (left) or 1 μg/ml ODN1826 (right) was measured by ^{3}H-thymidine uptake after 30h (n=7 BALB/c and B6, mean +/− SEM).

D. Distribution of CD23^{hi} CD21^{lo} FO and CD23^{lo} CD21^{hi} MZ B cells of B220$^+$ AA4.1$^-$ mature B cells in the spleen was ascertained by flow cytometry (representative flow plots are shown, summary plot of n=19 BALB/c, n=9 B6 is shown on the right, mean +/− SEM).

E. CD23-purified splenic B cells from AM14 Vκ8/Vκ8 BALB/c (○) and B6 (π) mice were stimulated with anti-IgM (left) and ODN1826 (right) and proliferation was measured as in C (n=9 BALB/c, n=7 B6, mean +/− SEM).

F. Surface expression levels of the AM14 Vκ8 BCR (4G7), IgM and IgD on AM14 Vκ8/Vκ8 BALB/c B cells (white bar) and AM14 Vκ8/Vκ8 B6 (black bar) were measured by flow cytometry. Mean fluorescent intensity (MFI) was calculated using FlowJo software. The mean +/− SEM of n=6 mice is shown.

G. B cell subsets in the bone marrow of AM14 Vκ8/Vκ8 BALB/c and B6 mice were analyzed using flow cytometry according to Hardy fractions B-D ($B220^{low}IgM^{neg}$), E ($B220^{low}IgM^{+}$) and F ($B220^{hi}IgM^{+}$). A summary graph of the different subsets is shown on the right for AM14 Vκ8/Vκ8 BALB/c mice (white bar, n=16) and AM14 Vκ8/Vκ8 B6 mice (black bar, n=14). Mean +/− SEM is shown.

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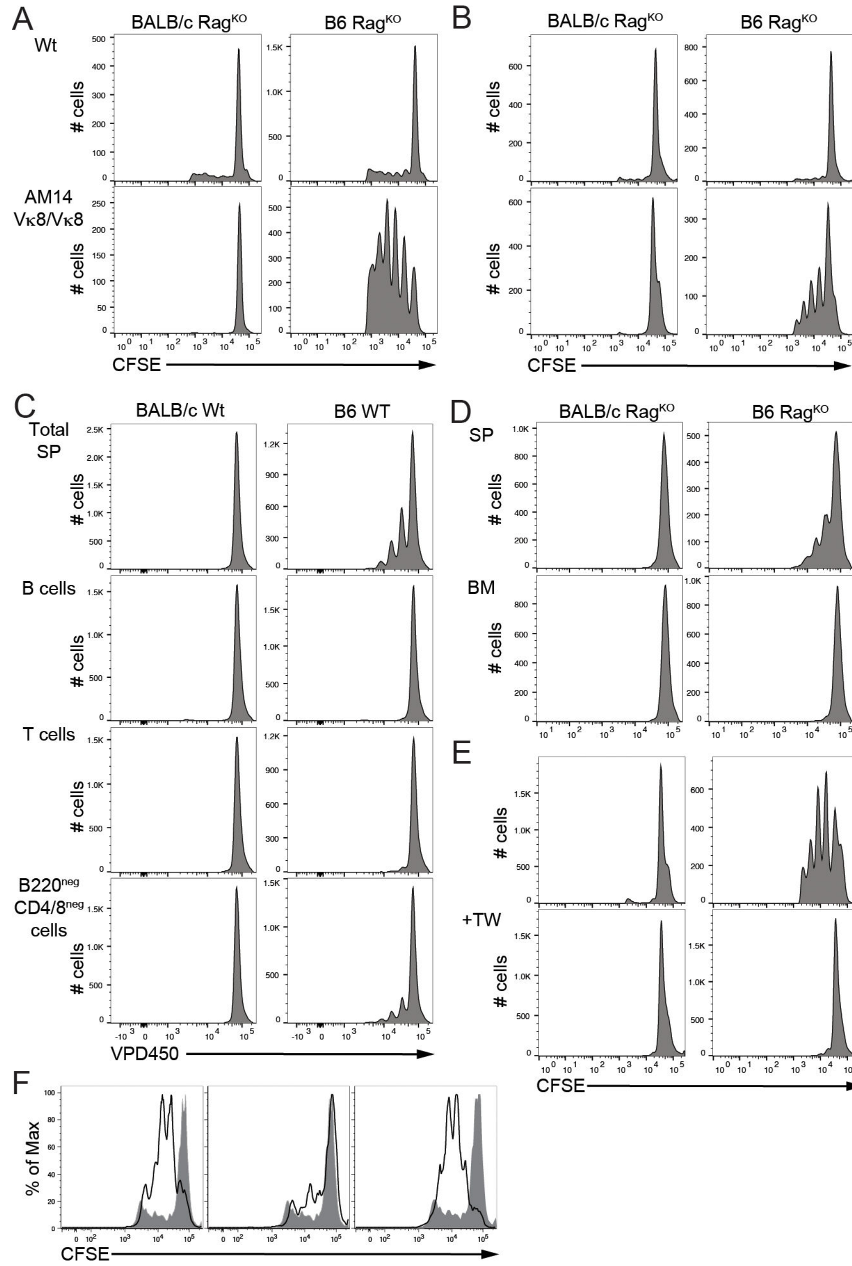

Figure 2.

AM14 Vκ8/Vκ8 BCR recognizes a B6 derived cross-reactive antigen

A. B220+ purified B cells from BALB/c WT or AM14 Vκ8/Vκ8 spleens were injected into NK cell-depleted BALB/c or B6 Rag-deficient mice. Proliferation was measured by CFSE dilution at day 10 (n=6).

B. Splenic BALB/c WT or AM14 Vκ8/Vκ8 B cells were cocultured in vitro with splenocytes from BALB/c or B6 Rag−/− mice in the presence of 50 ng/ml BLyS. Proliferation was measured by CFSE dilution at 72h (n=8).

C. CFSE labeled BALB/c AM14 Vκ8/Vκ8 B cells were cocultured with either total splenocytes, B220+ purified B cells, CD4+ and CD8+ T cells, or B220/CD4/CD8 depleted splenocytes as described in B. Proliferation was measured at 72h (n=2).

D. BALB/c AM14 Vκ8/Vκ8 B cells were cocultured with total splenocytes or bone marrow cells from Rag-deficient mice as described above (n=2).

E. BALB/c AM14 Vκ8 B cells were separated from Rag-deficient splenocytes by a 0.3 μm transwell chamber and proliferation of B cells was measured as described (n=5).

F. B6 Rag1$^{-/-}$ splenocytes were pre-incubated with 30 μg/ml of an AM14 Vκ8 IgM monoclonal antibody or 36–54 (irrelevant IgM) for 30 min. CFSE-labeled BALB/c AM14 Vκ8/Vκ8 B cells were added to the wells and proliferation was measured at 72h (n=4).

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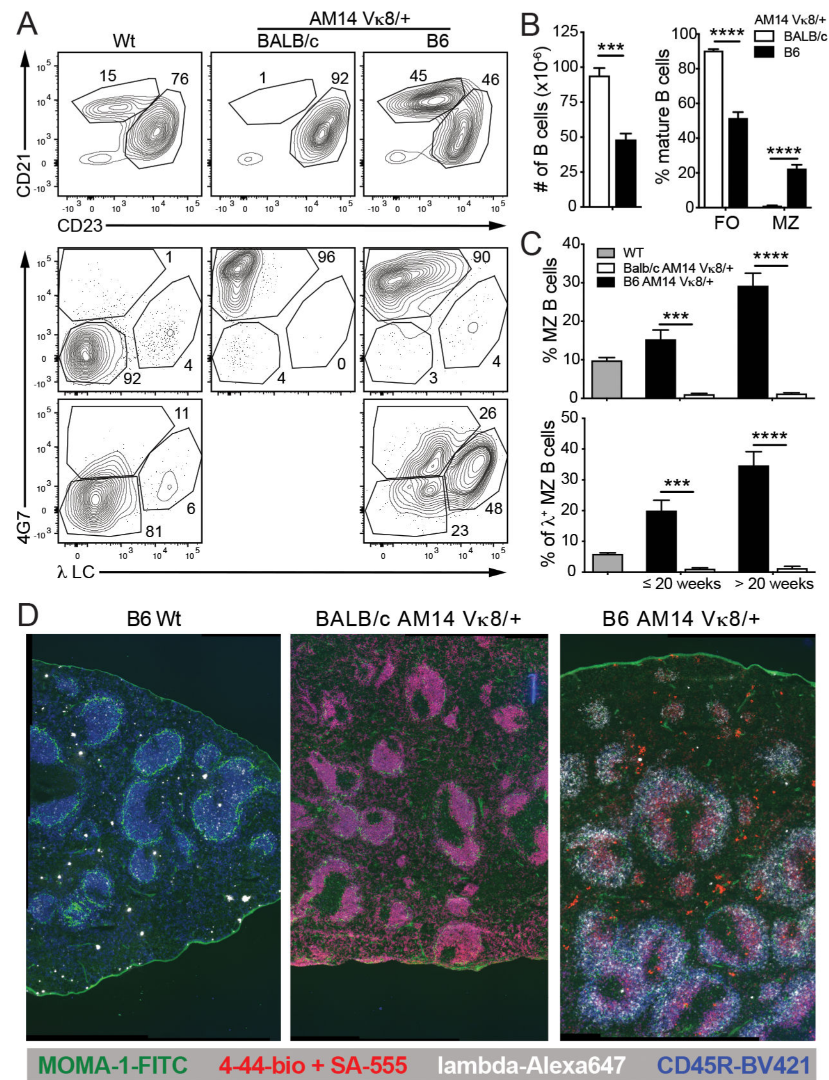

Figure 3.

B6-derived cross-reactive Ag induces receptor editing

A. Distribution of FO ($CD23^{+}CD21^{lo}$) and MZ ($CD23l°CD21^{+}$) B cell subsets in BALB/c or B6 AM14 Vκ8/+ mice were measured by flow cytometry. Receptor editing in FO and MZ was assessed by expression of λ light chain and loss of 4G7 clonotype. Representative plots are shown.

B. Summary plot showing total B cell numbers (left) and percentages (right) of FO and MZ B cells in BALB/c (white bars) and B6 (black bars) AM14 Vκ8/+ mice. (mean +/− SEM is shown, BALB/c n=25, B6=49)

C. Age dependent accumulation of MZ and λ+ B cells in spleens from BALB/c and B6 AM14 Vκ8/+ mice as detected by flow cytometry (BALB/c n=19, B6 n=34, mean +/− SEM).

D. Splenic architecture of BALB/c and B6 AM14 Vκ8/+ mice was examined by confocal imaging.

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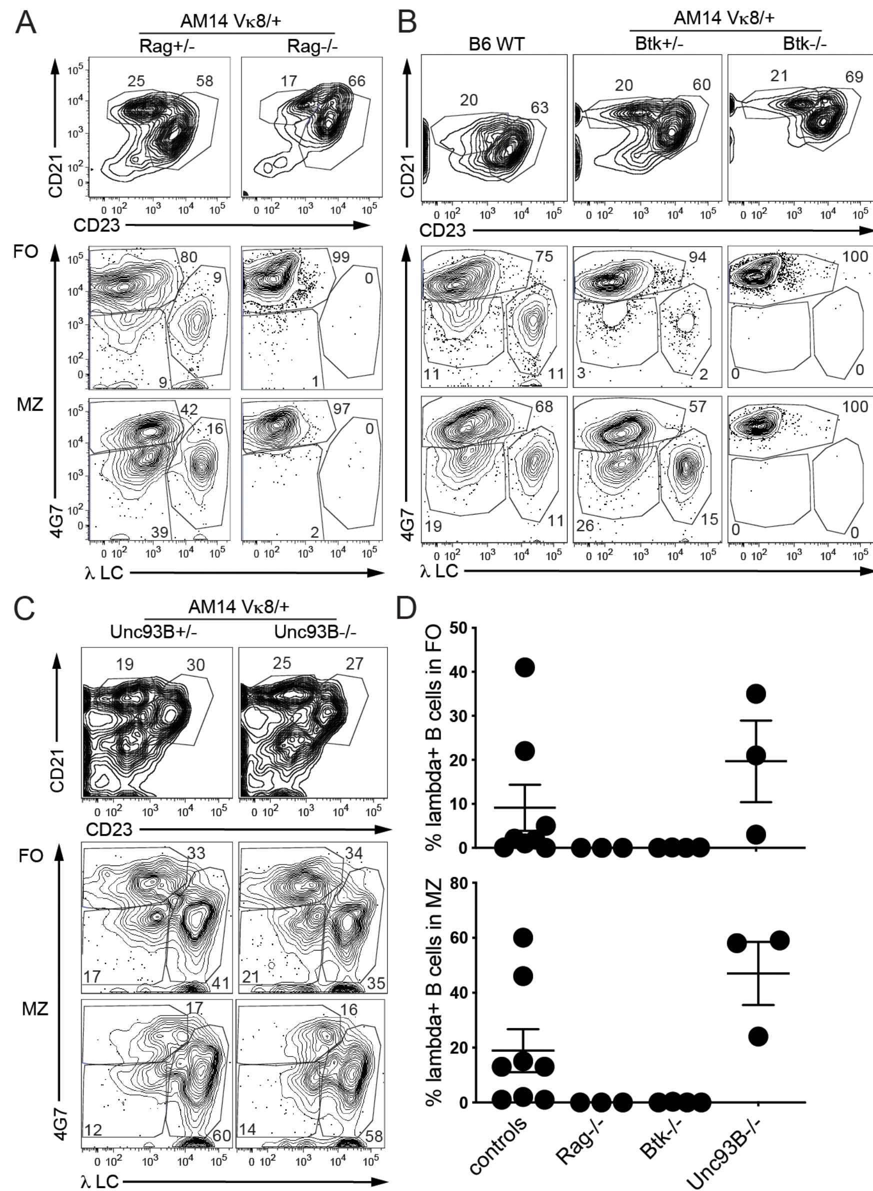

Figure 4.

MZ B cell development is independent of receptor editing and TLR signaling.

A-C. B cell subset distribution and receptor editing in B6 AM14 Vκ8/+ Rag1$^{-/-}$ (A), Btk$^{-/-}$ (B) and Unc93B1$^{3d/3d}$ (C) mice and littermate controls was ascertained by flow cytometry (n=3–4/group).

D. Summary plots showing the % λ^{+} B cells in FO (top) and MZ (bottom) compartments of the different KO mice (mean +/– SEM).

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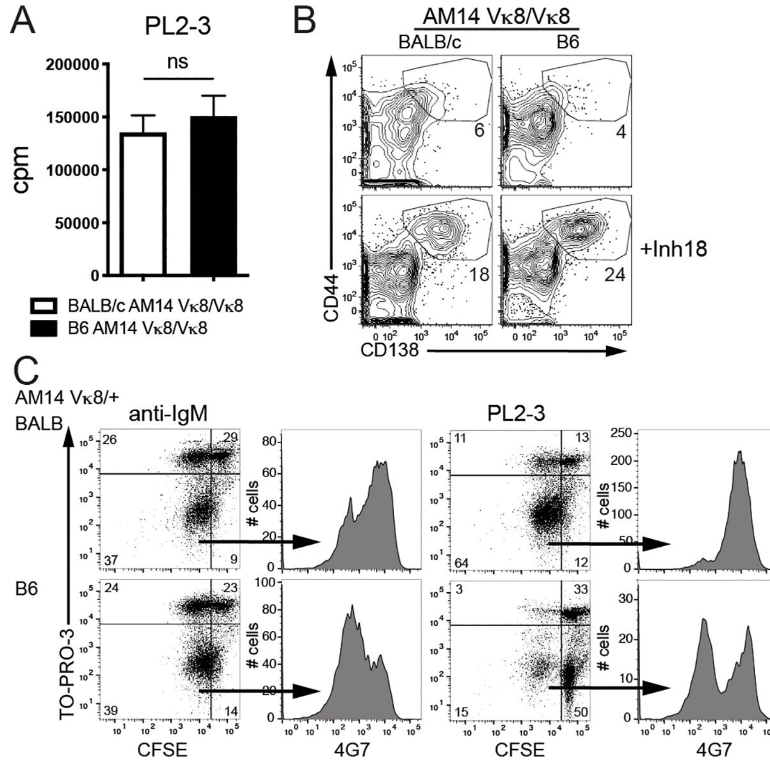

Figure 5.

BCR/TLR co-engagement activates anergic B cells

A. CD23-purified BALB/c and B6 AM14 Vκ8/Vκ8 B cells were stimulated with 1μg/ml PL2–3 for 30h. Proliferation was measured by 3H-thymidine uptake for the last 6h of incubation (BALB/c n=6, B6 n=8, mean +/− SEM).

B. PL2–3 activated BALB/c and B6 AM14 Vκ8/Vκ8 B cells were cultured with or without Inhibitor 18 and stained for plasmablasts ($CD22^{lo}CD44^{+}CD138^{+}$) at 72h (n=3).

C. CFSE-labeled BALB/c and B6 B cells isolated from AM14 Vκ8/+ mice were stimulated with either 15 μg/ml goat anti-IgM $F(ab')_2$ or 1 μg/ml PL2–3. At 72h, cells were stained with anti-idiotype 4G7 and AM14 Vκ8 expression levels of cells in the live divided (TO-PRO-3^{neg} $CFSE^{lo}$) quadrant are depicted in the histogram. Representative plots of 5 independent experiments are shown.