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CD21 Signaling via C3 Regulates Purkinje Cell Protein 4 Expression

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Abstract

Complement receptor proteins CR2 (CD21) and CR1 (CD35) have been identified as components of the murine B cell co-receptor complex. Gene expression profiles between naïve WT, C3^{-/-}, and CD21/35^{-/-} B cells demonstrate enhanced expression of a Ca²⁺-modulating gene, *Pcp4*, in WT mice compared to the complement-deficient animals. Increased expression of *Pcp4* is also coincident with B cell maturation into end stage phenotypes. Prolonged activation of B cells via crosslinking of the BCR (but not CR1/CR2 alone) leads to increased expression of *Pcp4* and suppressed Ca²⁺ release. In total these data demonstrate that the expression of *Pcp4* in naïve resting mature B cells is dependent upon tonic stimulation from the CR1/CR2 proteins via a C3 ligand, and that antigen specific B cell activation can also elevate *Pcp4* expression that is coincident with suppression of calcium-dependent responses.

1. Introduction

Signals through the B cell antigen receptor regulate B lymphocyte development and function (Tedder et al., 1997). B cell receptor engagement activates signaling pathways by protein tyrosine kinases (PTKs) including members of the Src-family of PTKs, Syk, and Btk, and results in Ca²⁺ mobilization. After receptor activation, the release of Ca²⁺ from endoplasmic reticulum (ER) stores into the cytosol is the first detectable increase in cytosolic Ca²⁺. Once ER stores are depleted, BCR signals are able to sustain intracellular Ca²⁺ ([Ca²⁺]_i) levels via store-operated calcium entry (SOCE) that allows Ca²⁺ to pass from the extracellular environment to the cytosol (Cahalan et al., 2001; Engelke et al., 2007). Negative regulation of BCR-induced Ca²⁺ signaling can occur through co-ligation with either the low-affinity Fc receptor for IgG (FcγRII) or CD22 with the BCR (Nitschke et al., 1997; Sato et al., 1998; Wilson et al., 1987). Modifications of Ca²⁺ signaling in B cells contribute to B cell activation and maturation, differentiation, and gene expression.

Complement receptor protein CD21 (CR2) along with CD19, CD81, and Leu13 have been identified as components of the human B cell co-receptor complex. The mouse CD35 (CR1) protein is also part of this complex on murine B cells (reviewed in (Jacobson and Weis, 2008)). Co-ligation of CD21 and the BCR via complement-bound antigens results in a higher level of cellular activation, elevation of intracellular Ca²⁺ and activation of mitogen-activated protein kinases than BCR signaling alone (Carter et al., 1988; Luxembourg and Cooper,

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1994). The CD21/35 proteins also enhance complex-bound antigen uptake and processing, assist in the localization of the BCR to lipid rafts, and function in transferring immune complexes from marginal zone B cells to follicular dendritic cells (FDCs) (Cherukuri et al., 2001; Whipple et al., 2004). Enhanced B cell activation by CD21/35 cross-linking is primarily considered to be dependent upon its association with CD19. After BCR and CD21/35/CD19 ligation, phosphorylation of CD19 recruits the Src family kinase, Lyn, resulting in amplified Lyn kinase activity (Fujimoto et al., 1999b; Hasegawa et al., 2001). This action facilitates CD19 interactions with phosphoinositide 3-kinase (PI3K) and Vav, initiate downstream signaling events and results in augmentation of $[Ca^{2+}]_i$ responses (Buhl et al., 1997; Fujimoto et al., 1999a; Sato et al., 1997).

In the mouse, CD21/35 proteins are encoded by the same *Cr2* locus and co-expressed on B cells and FDCs (Kurtz et al., 1990). The role for complement C3 cleavage products, such as the ligands for CD21/35, as regulators of B cell signaling and humoral immunity is well appreciated. As such, animals lacking the CD21/35 proteins generate modest antibody responses to both T cell-dependent and T cell-independent antigens (Ahearn et al., 1996; Haas et al., 2002; Molina et al., 1996). It is important to note that although C3 breakdown products positively regulate B cell activation through the CD19/CD21 complex, excess CD21 ligation has also been shown to attenuate Ca^{2+} responses (Chakravarty et al., 2002; Lee et al., 2005).

Even though antibody responses are depressed, B cell activation and maturation occurs in the absence of CD21/35 proteins (Haas et al., 2002; Jacobson et al., 2008). To better understand splenic B cell activation in the presence or absence of CD21/35 co-ligation *in-vivo*, we analyzed gene expression profiles from splenocytes of naïve and immunized WT and CD21/35^{-/-} mice. Of the various genes differentially expressed, we choose to further analyze one gene of interest, that encoding the purkinje cell protein 4 (*Pcp4*). *Pcp4* (also known as *Pep19*) belongs to a family of proteins involved in calcium transduction signals. Initially identified in neurons, *Pcp4* modulates Ca^{2+} by binding to calmodulin via an IQ motif (Johanson et al., 2000). More relevant to our studies, gene expression profiling identified *Pcp4* as a transcript increased in anergic B cells (Glynne et al., 2000). We have found the expression of *Pcp4* was increased 6 fold in splenocytes from immunized WT mice compared to splenocytes from immunized CD21/35^{-/-} animals. These data were consistent with previous gene expression profiles between naïve WT and CD21/35^{-/-} splenocytes (Jacobson et al., 2008) indicating that the altered expression was due to the lack of the CD21/35 proteins, not the effect of immunization. Additionally, transcript analysis from C3-deficient splenocytes also demonstrated decreased levels of *Pcp4* expression (compared to wild type), implicating a role for tonic stimulation of the B cell via C3-CD21/35 interaction *in-vivo*. Finally, *in-vitro* studies demonstrated that BCR activation independent of CD21/35 crosslinking induced *Pcp4* expression coincident with suppression of Ca^{2+} responses. These data suggest that *Pcp4* may be a novel regulator of BCR/CD21 induced Ca^{2+} signaling in splenic B cells.

2. Materials and Methods

2.1. Mice

Female BALB/c and C57BL/6 mice were obtained from the National Cancer Institute. CD21/35^{-/-} mice were generated as described (Haas et al., 2002) and backcrossed 10 times onto BALB/c or C57BL/6 backgrounds and used for the experiments described. C57BL/6 C3^{-/-} mice (Wessels et al., 1995) were purchased from Jackson Labs. Animals were housed in the Animal Resource Center (University of Utah Health Science Center, Salt Lake City, UT) according to the guidelines of the National Institute of Health for the care and use of laboratory animals.

2.2. Immunizations

Mice 8 wk old were immunized by i.p injection of 85 µg of trinitrophenyl keyhole limpet hemocyanin precipitated in aluminum hydroxide gel (Sigma). Spleens were harvested for RNA 4 days post injection.

2.3. Gene Expression Analysis

Equal amounts of total RNA from the spleens of 5 immunized (see above) female 8-wk-old BALB/c or C57BL/6 WT or CD21/35^{-/-} mice were pooled into a sample that was prepared for Affymetrix array hybridization. cDNA was synthesized from 8 µg of total RNA, and each sample was hybridized to a single GeneChip Mouse Genome 430.2 Array (Affymetrix). Data was processed as described in (Jacobson et al., 2008). Gene expression analysis between strains was performed in duplicate.

2.4. RNA isolation and quantitative RT-PCR

Total RNA was isolated from spleen tissue using CsCl guanidine extraction. After RBC lysis, single cell populations of splenocytes were labeled with B220 magnetic microbeads (Miltenyi Biotech) and separated according to manufacturer's protocol. Total RNA was isolated from cells using Amersham RNA mini-spin kit (GE Healthcare). RT-PCR on 2 µg of total RNA was preformed using random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). cDNA levels were assessed using quantitative PCR on the LightCycler (Roche) as in (Jacobson et al., 2008). β-Actin: forward, 5'-gtaacaatgccatgttcaat-3'; reverse, 5'-ctccatcgtggcgcgctctag-3', 135bp. PCP4: forward, 5'-cagccaccctgagcattg-3'; reverse, 5'-ttgtctttccgttggtgc-3', 108bp. CD19: forward, 5'-aggaaaaggaagcgaatgact-3'; reverse, 5'-gcctggccagaggtatgta-3', 125bp.

2.5. Cell culture

Single-cell suspensions were made from spleens of 6-to 8wk-old mice, and RBCs were lysed with ACK lysing buffer. Total splenocytes or B220⁺ sorted cells were cultured at 5 X 10⁶ cells/ml in RPMI 1640 supplemented with 10% FBS (Invitrogen Life Technologies), L-glutamine, 50 µM 2-ME, 100U/ml penicillin, and 100 µg/ml streptomycin in the presence or absence of 10 µg/ml Goat F(ab')₂ anti-mouse IgM (Southern Biotech), 5 µg/ml rat anti-mouse CD21/35 (BD Pharmingen), or Rat IgG2b (BD Pharmingen).

2.6. Ca²⁺ measurements and immunofluorescence analysis

A fraction of purified B cells from 48 hour cultures were analyzed for IgM expression levels using flouorochrome-Cy5-conjugated anti-IgM (Biomedex Corp). Cell staining and analysis was performed as described previously (Jacobson et al., 2008). Remaining B cells were incubated for 45 minutes at 37° with the Ca²⁺-sensitive dye Fluo-4 (3 µg/ml; Molecular Probes) in RPMI medium containing 10% FBS. Lymphocytes were washed and incubated in the dark for 20 minutes at 37° and then analyzed on a FACS Caliber flow cytometer (BD Bioscience). Baseline Ca²⁺ was measured for 30s, followed by the addition of 10 µg/ml F(ab')₂ anti-mouse IgM or 1 µg/ml ionomycin. Ca²⁺ was measured for 5 minutes.

2.7. Statistical analysis

Data are given as means ± SEM. Student's *t* test was used to determine whether there were significant differences between sample means.

3. Results

3.1. Differential *Pcp4* expression between WT, CD21/35^{-/-}, and C3^{-/-} splenocytes

The murine CD21/35 proteins are members of the B cell co-receptor complex such that cross-linking the BCR along with CD21/35 results in a higher level of cellular activation than signaling through the BCR alone. To investigate differences downstream of B cell receptor signaling between WT and CD21/35^{-/-} mice during the early events of an immune response, gene expression profiles from total spleen RNA of immunized WT or CD21/35^{-/-} were obtained using Affymetrix GeneChip Microarrays. Analysis of the microarray data from these immunized mice indicated that a variety of genes are differentially expressed between WT and CD21/35^{-/-} splenocytes on both the BALB/c and C57BL/6 strain background. Of the 25 genes with increased expression levels in WT compared to CD21/35^{-/-} on both BALB/c and C57BL/6 background, *Pcp4* (increased 2–6 fold) was of particular interest (Table 1). It has been previously reported that *Pcp4* expression is increased in anergic B cells compared to naïve B cells (Glynn et al., 2000) suggesting elevated levels of *Pcp4* may inhibit B cell activation.

Previous gene expression profile analysis between naïve WT and CD21/35^{-/-} splenocytes also demonstrated elevated expression of *Pcp4* in WT cells indicating depressed *Pcp4* expression in CD21/35^{-/-} mice was not due to the immunization regimen (Table 1) (Jacobson et al., 2008). Additionally, past analysis of maturing B cells demonstrated increasing *Pcp4* expression with differentiation (Debnath et al., 2008). *Pcp4* expression differences were confirmed by RT-PCR for both BALB/c and C57BL/6 WT and CD21/35^{-/-} naïve (non-immunized) spleen samples (Fig. 1A). To determine if decreased *Pcp4* expression from CD21/35^{-/-} cells could be due to a lack of C3 cross-linking of CD21, *Pcp4* transcripts were also analyzed from splenocytes from naïve C3^{-/-} mice. Compared to C57BL/6 WT, *Pcp4* mRNA was decreased 2 fold in C3^{-/-} splenocytes (Fig. 1A).

Because the gene expression analysis was performed with total splenocyte populations, *Pcp4* expression was confirmed in B cells by transcript quantification of B220⁺ and B220⁻ sorted splenocyte populations. As shown in Fig. 1B, *Pcp4* expression was predominantly found in B220⁺ cells compared to B220⁻ cells in the spleen. In total, these data demonstrate that the lack of the CD21 proteins and/or C3 depress expression of *Pcp4* in murine splenic B cells obtained from either naïve or immunized animals. Thus tonic stimulation of CD21/35 via C3 intermediates on naïve, resting B cells is required for the normal expression level of *Pcp4*.

3.2. *Pcp4* transcript increases with B cell maturation

CD21/35 transcript and surface expression levels increase as B cells develop from the bone marrow to mature splenic B cells. Furthermore, our previous gene expression data suggested an increase in *Pcp4* expression throughout B cell maturation that paralleled the increased expression of CD21 (Debnath et al., 2008). To determine if *Pcp4* transcripts were increased in mature, CD21/35^{-/-} expressing B cells, we analyzed *Pcp4* expression differences between developing and mature B cells from BALB/c WT or CD21/35^{-/-} mice. Bone marrow B cells from 2-week-old animals, in which all of the B cells are immature and CD21/35 negative, demonstrated barely detectable *Pcp4* expression levels (Fig. 2). Transitional, maturing B cells from the spleens of 2wk old animals had a slight increase in *Pcp4* mRNA, whereas *Pcp4* displayed the highest expression level in mature B cells from the spleens of WT animals (Fig. 2). These data show that *Pcp4* expression levels from developing WT B cells correlates with the level of maturation of the B cell. When coupled with the decrease in *Pcp4* mRNA from splenocytes of CD21/35^{-/-} and C3 deficient mice, these data further suggest a role for the CD21/35 proteins in regulating *Pcp4* expression levels in maturing B cells.

3.3. BCR cross-linking induces *Pcp4* expression

The strength of the B cell signal throughout the course of development is important for B cell survival and selection. The finding that anergic B cells demonstrate elevated levels of *Pcp4* transcripts suggests that the *Pcp4* protein may serve to suppress B cell activation. These data led us to hypothesize that *Pcp4* expression may also be influenced by BCR signaling. Splenic B cells were held in culture for either 5 min or 48hr prior to RNA isolation. The expression level of *Pcp4* drops dramatically to about the same level as the CD21/35^{-/-} cells when they are held in culture without any additional stimulation (WT media controls) (Fig. 3). When the BCR was cross-linked with F(ab')² anti-IgM in culture, *Pcp4* expression was increased compared to un-treated splenocyte cultures (Fig. 3). Cross-linking the BCR with CD21/35 led to a slight increase in *Pcp4* transcription over BCR cross-linking alone suggesting that, *in vitro*, B cell receptor activation dependent induction of *Pcp4* transcription may be augmented with CD21/35 signaling. Cross-linking the BCR with anti-IgM maintained *Pcp4* expression in CD21/35^{-/-} B cells as well (Fig. 3), indicating that there is not a developmental or intrinsic defect in these cells to induce *Pcp4* transcripts upon BCR mediated activation.

3.4. Increased *Pcp4* expression decreases intracellular calcium flux after BCR cross-linking

To date, the identified function for *Pcp4* is as a calmodulin(CaM)-binding protein in neurons, acting as a modulator of Ca²⁺-dependent activation of CaM kinase II (Johanson et al., 2000). The known role for *Pcp4* in regulating calcium signaling (in neurons) and its induction downstream of BCR cross-linking (see above) prompted us to determine if *Pcp4* is a modulator of BCR-dependent calcium signaling in B cells. We induced *Pcp4* expression in B cell cultures by BCR cross-linking with anti-IgM, similar to above, except 24 hrs after induction, cells were placed in fresh media in the absence of stimulus for the remaining 24hrs of culture. RT-PCR analysis of cultured B cells again showed an increase in *Pcp4* expression 48 hours after BCR cross-linking (Fig. 4A). To determine if *Pcp4* expression levels altered BCR-induced intracellular calcium ([Ca²⁺]_i) flux, we loaded media-treated (low *Pcp4*) and BCR cross-linked (high *Pcp4*) B cells with a Ca²⁺- sensitive dye, stimulated the cells with anti-IgM and measured [Ca²⁺]_i flux by flow cytometry. B cells possessing increased levels of *Pcp4* responded to BCR stimulation with a lower [Ca²⁺]_i flux than those without a prior BCR stimulation (and thus lower expression levels of *Pcp4*) (Fig. 4C, upper panel). Ionomycin stimulation yielded similar [Ca²⁺]_i release from both cell populations controlling for equivalent dye loading (Fig. 4C, lower panel). Furthermore, cells stimulated with anti-IgM demonstrated increased amounts of IgM on the cell surface, indicating that the decrease in [Ca²⁺]_i flux was not due to unavailability of BCR to stimulate (Fig. 4B). These data suggest that previous BCR stimulation leads to *Pcp4* induction and dampened [Ca²⁺]_i release after BCR cross-linking.

4. Discussion

In this report, we have identified a novel, potential regulator of BCR-induced Ca²⁺ responses. Gene expression analysis between immunized and resting, naïve WT and CD21/35^{-/-} splenocytes indicated elevated *Pcp4* expression in WT spleens, which we demonstrate was produced in mature B cells. A similar depression of expression of *Pcp4* in naïve, resting B cells was also found in animals lacking C3 suggesting tonic stimulation of mature, naïve B cells via CD21/35 is required for normal expression of the *Pcp4* gene. Interestingly, expression of *Pcp4* could be elevated over WT base line levels by crosslinking the BCR. This elevated expression was slightly enhanced with additional CD21 crosslinking although CD21 crosslinking alone did not maintain *Pcp4* transcription to the same level as BCR activation. These data suggest two thresholds of *Pcp4* expression may exist in mature B cells: the first level, dictated by tonic stimulation of resting B cells by the CD21/35 proteins (via a C3 ligand) and a second level within activated B cells that is dependent upon BCR signaling. Finally, consistent with its role as a calmodulin-binding protein in neurons, *Pcp4* expression may have

a negative influence on $[Ca^{2+}]_i$ flux as cells with increased *Pcp4* expression levels via BCR-cross-linking mounted decreased $[Ca^{2+}]_i$ responses in response to additional anti-IgM stimuli.

The data presented in this report suggest two, if not three, mechanisms regulating *Pcp4* expression: tonic via CD21/35 crosslinking, increased expression with increased maturation of the B cell (which is coincident with increased expression levels of CD21/35), and the elevated expression levels after BCR crosslinking. CD21/35^{-/-} B cells produced *Pcp4* transcripts in similar quantity to WT after BCR stimulation, indicating that cells from CD21/35-deficient animals do not have an intrinsic defect in *Pcp4* transcription or signaling via the BCR. Other B cell stimuli, such as LPS through toll-like receptor 2, or CD40L did not enhance *Pcp4* transcripts alone, or in conjunction with BCR cross-linking (data not shown). Collectively, these data suggest that signaling through the B cell receptor alone is sufficient to induce the elevated levels of *Pcp4* transcripts.

The observations that *Pcp4* levels are depressed (compared to WT) in naïve, resting B cells obtained from both CD21/35^{-/-} and C3- strains of mice suggests that tonic stimulation via C3 ligands through the CD21/35 signal transduction pathway is required for normal *Pcp4* expression. However an alternative model for the role of CD21/35 and C3 proteins in inducing *Pcp4* expression in B cells *in-vivo* implicates the function of these proteins on FDCs. The role for FDCs and complement receptors in antigen acquisition in the spleen is well appreciated (reviewed in (Roozendaal and Carroll, 2007)). For example, complement-coated antigen bound by CD21/35 on FDCs is required for efficient maintenance of antibody producing cells (Barrington et al., 2002; Fang et al., 1998). Within follicles of lymphoid organs, C3-coated immune complexes bound by CD21/35 on FDCs can serve as means of cell-to-cell interaction with B cells. Thus, a function for C3/CD21/35 in regulating steady state *Pcp4* expression by B cells *in-vivo* may be via a FDC interaction of B cells with complement-coated antigen complexes. This is an environment not reproduced in our culture system. Furthermore, unstimulated CD21/35^{-/-} B cells produced similar levels of *Pcp4* transcripts as WT B cells after 48 hrs in culture, compared to decreased relative transcript levels after 5 min in culture (Fig. 3). These data suggest that after 48 hours of culture signals, such as low level BCR-activation, required for normal induction of *Pcp4* are absent and that the B cell micro-environment may influence *Pcp4* expression.

Our data indicated that cells with increased *Pcp4* expression responded with decreased $[Ca^{2+}]_i$ responses to BCR ligation. From these data we can infer a role for negative regulatory role of PCP4 in B cell $[Ca^{2+}]_i$ responses. The increased expression of *Pcp4* observed in anergic B cells (and the link of *Pcp4* expression to C3/CD21/CD35 in WT B cells) is interesting in light of the report that B cell anergy can be broken by the crosslinking of the CD21 proteins on anergic B cells (Lyubchenko et al., 2007). Putkey and colleagues have defined a role for Pcp4 (and similar IQ motif proteins) to accelerate the rates of association and dissociation of Ca^{2+} to sites II and IV in the C-domain of calmodulin (Putkey et al., 2003; Putkey et al., 2008). Additionally, Pcp4 can alter the function of free calmodulin as well as that bound to calmodulin-dependent protein kinase II. These data suggest that modulation of *Pcp4* expression in B cells could alter the function of calmodulin in such cells, altering their steady state and activation dependent Ca^{2+} responses. Modulating the expression of *Pcp4* in naïve and anergic B cells should help define the role of this protein in regulating B cell activation and anergy, and autoimmunity.

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Fig. 1.

RT-PCR confirmation of *Pcp4* expression in splenocytes. RNA from total splenocytes from BALB/c WT and CD21/35^{-/-} mice and C57BL/6 WT, CD21/35^{-/-}, and C3^{-/-} mice was collected and quantified by RT-PCR for *Pcp4* transcripts (A). Transcripts are shown relative to β-actin and n≥5 for each group. In B, *Pcp4* expression was identified as a B cell-specific gene by RT-PCR from B220-positively selected splenocytes from BALB/c WT mice.

Fig. 2.

Pcp4 expression increases with B cell maturation. Total RNA from the bone marrow (BM) and spleens (SPL) of 2 week old and spleens of 8wk old BALB/c WT and CD21/35^{-/-} mice was collected and *Pcp4* transcripts were quantified by RT-PCR. Transcripts are relative to CD19 to control for B cell numbers in samples. CD21/35 cell surface expression increases as B cells develop from immature B cells in the 2 wk BM, transitional B cells in 2wk spleens, to mature B cells in 8 wk spleens as depicted in the figure.

Fig. 3.

Pcp4 transcript is induced after B cell receptor cross-linking. *Pcp4* transcripts in cultured splenocytes were quantified with RT-PCR. A, Single cell populations of total splenocytes from WT mice were cultured in the presence or absence of 10 $\mu\text{g/ml}$ (Fab')₂ anti-mouse IgM, cells were collected at various time points, RNA was harvested and transcript quantified. B, Single cell populations of WT or CD21/35^{-/-} splenocytes were cultured in the presence or absence of 10 $\mu\text{g/ml}$ (Fab')₂ anti-mouse IgM, 5 $\mu\text{g/ml}$ anti-CD21/35, or 5 $\mu\text{g/ml}$ Rat IgG2b isotype control. Cell RNA was collected at 5 min or 48 hours as indicated and analyzed by RT-PCR.

Fig. 4.

Induction of *Pcp4* expression reduces BCR-induced intracellular calcium responses. B220-positively sorted splenocytes were cultured for 48 hours in the presence or absence of 10 μ g/ml anti-mouse IgM. After 24 hours of culture, cells were washed and replaced with fresh media. After 48 hours total of culture, cells were harvested. A, *Pcp4* RT-PCR analysis of cultured cells in the presence or absence of BCR activation. B, Cells cultured with anti-mouse IgM (dark line) for the first 24 hours of culture have increased levels of surface IgM as shown by FACS analysis (compared to isotype stimulated control: grey filled profile). C, 48 hour cultured B cells were loaded with the calcium indicator Fluo-4 and analyzed for BCR-induced, or ionomycin-induced $[Ca^{2+}]_i$ release.

Table 1
Pcp4 (probe ID 1460214) Fold Change Expression from Mouse Splenocytes

Genotype ^a	Cell Type	Fold change relative to WT	Fold change relative to 2wk B220+ bone
marrow			
naive BALB/c CD21/35 ^{-/-}	Total Spleen	-6.6 ^d	
immunize BALB/c CD21/35 ^{-/-}	Total Spleen	-6.1	
immunize C57BL/6 CD21/35 ^{-/-}	Total Spleen	-2.2	
AJ ^b	2wk B220+ Splenic B cells		5.2 ^c
AJ ^b	8wk B220+ Splenic B cells		24.2 ^c

^a Genotype as described in text

^b B220+ sorted splenocyte samples

^c Referenced in Supplemental Data in Debnath et al., *Genes Immun.* (2008)

^d Referenced in Supplemental Data in Jacobson et al., *J. Immunol.* (2008)