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Murine complement receptor 1 is required for germinal center B cell maintenance but not initiation

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Abstract

Germinal centers are the anatomic sites for the generation of high affinity immunoglobulin expressing plasma cells and memory B cells. The germinal center B cells that are precursors of these cells circulate between the light zone B cell population that interact with antigen laden follicular dendritic cells (FDC) and the proliferative dark zone B cell population. Antigen retention by follicular dendritic cells is dependent on Fc receptors and complement receptors, and complement receptor 1 (Cr1) is the predominant complement receptor expressed by FDC. The newly created *Cr1KO* mouse was used to test the effect of Cr1-deficiency on the kinetics of the germinal center reaction and the generation of IgM and switched memory B cell formation. Immunization of *Cr1KO* mice with a T cell-dependent antigen resulted in the normal initial expansion of B cells with a germinal center phenotype however these cells were preferentially lost in the *Cr1KO* animal over time (days). Bone marrow chimera animals documented the surprising finding that the loss of germinal center B cell maintenance was linked to the expression of Cr1 on B cells, not the FDC. Cr1-deficiency further resulted in antigen-specific IgM titer and IgM memory B cell reductions, but not antigen-specific IgG after 35-37 days. Investigations of nitrophenyl (NP)-specific IgG demonstrated that Cr1 is not necessary for affinity maturation during the response to particulate antigen. These data, along with those generated in our initial description of the *Cr1KO* animal describe unique functions of Cr1 on the surface of both B cells and FDC.

Introduction

The generation of high affinity antibody producing memory B cells and plasma cells requires the generation and then selection of antigen activated B cells within structures in immune organ follicles known as germinal centers (GCs). These GC B cells are initiated quickly within five to seven days of an infection or immunization, and generally peak within

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two weeks (Victora and Nussenzweig, 2012, Shinall, et al., 2000). GCs form around the aptly named follicular dendritic cells (FDCs), which coordinate the formation, organization, and maturation of GCs through production of cytokines, and although there is some debate about the necessity of antigen retention, very likely through concentration of antigen within the follicle (Haberman and Shlomchik, 2003, Kosco-Vilbois, 2003). It is apparent that the selection of high affinity antibody producing clones from activated B cells that have undergone somatic hypermutation requires the formation of GCs. The procession of class switch recombination for the production of switched immunoglobulin antibodies is however less dependent on formation of GCs.

The complement system and the complement receptors, Cr1 and Cr2, have been implicated in the proper generation of GC B cells, memory B cell responses and affinity maturation in mouse model systems. Studies directly assessing the ability of *Cr2* hypomorphs (mice in which the *Cr2* gene produces low quantities of smaller Cr1/2 proteins (7, 8) and *Cr2*^{-/-} mice (which lack expression of both the Cr1 and Cr2 proteins) has supported a role for Cr1/2 in the generation of memory B cells (Brockman, et al., 2006, Fernandez Gonzalez, et al., 2008, Barrington, et al., 2002, Molina, et al., 1996, Wu, et al., 2000, Fang, et al., 1998). The inhibition of the generation of normal responses in such mice has been attributed to the deficiency of expression of Cr1/2 in the stromal compartment, most notably the FDC. FDC are responsible for the trapping of antigen via C and Fc receptors (Tew, et al., 1997, Roozendaal, et al., 2009) and for orchestrating the GC reaction (Wang, et al., 2011, Donius, et al., 2013). The recent development of a mouse specifically deficient for the Cr1 isoform of *Cr2*, the *Cr1KO* mouse, and the revelation that Cr1 is the nearly exclusive isoform expressed by the stromal compartment FDCs, suggested that the Cr1-deficiency (*Cr1KO*) may result in a significant reduction in GC output of high affinity antibody producing cells and memory B cells (Donius, et al., 2013, Michel, et al., 2012).

Naïve mice have been shown to contain thousands of B cells specific for large multi-epitope proteins (Pape, et al., 2011). Immunization with such proteins can produce tens of thousands of long-term memory B cells from this pool. Phenotypically these cells are nearly indistinguishable from naïve cells by cell surface markers, but they consist of a heterogeneous population of B cells possessing various immunoglobulin classes and antibody affinities for antigen. Memory B cells have been primarily described as emerging from the follicular (FO) B cell subset (B2 B cells) however memory has also been shown to be generated from the B1b pool (Alugupalli, et al., 2004) and the marginal zone (MZ) B cell pool (Phan, et al., 2005). Despite the considerable work being done on B cell responses and generation of immunological memory, the mechanistic details of these functions are still not fully understood. B cell markers such as CD35, CD80, and CD73 have been used to delineate switched from unswitched B cells, and germinal center (GC) derived from non-GC-derived B cells (Anderson, et al., 2007, Taylor, et al., 2012), however, memory B cells still must be tracked by other means. Recently, in a very innovative series of experiments, such memory B cells have been quantified and tracked via their binding to the immunizing antigen, the fluorophore allophycocyanin (APC)(Pape, et al., 2011, Taylor, et al., 2012, Taylor, et al., 2012).

Production of antibody can differ by quantity and quality. Frequently the quantity of antigen-specific antibodies is measured by ELISA, however this method does not provide any information on the quality of these antibodies. It is possible however to determine the binding affinity of the polyclonal repertoire present by measuring the quantity of antibody remaining in the presence of increasingly stringent binding conditions (Macdonald, et al., 1988). Previous work by others has found that depending on the immunization conditions, affinity maturation may or may not be affected in the absence of Cr1 and Cr2 (Wu, et al., 2000, Chen, et al., 2000). The reduction of GC B cells in *Cr1KO* mice led us to test affinity maturation following immunization of *Cr1KO* mice.

In this manuscript we elaborate on our previous findings on Cr1-deficiency in mice, especially in regards to the demonstrated GC B cell deficiencies and their consequences (Donius, et al., 2013). In light of the *Cr1KO*'s GC B cell deficiencies we test here the requirement for Cr1 in the production of memory B cells and high affinity antibody, while further investigating effects seen during different routes of immunization and the dependence on FDC or B cell expression of Cr1.

Materials and Methods

Mice and immunizations

All mice used were six to twelve weeks of age. *Cr1KO* mice were at least N=6 generations backcrossed on C57BL6/J and derived from those described previously (Donius, et al., 2013). *Cr1/2KO* mice bred on site were *Cr2*-null mice on the C57BL6 background (Haas, et al., 2002). *C3KO* mice bred on site were progeny of *C3*-null mice purchased from The Jackson Laboratory (Bar Harbor, ME) and extensively backcrossed on C57BL/6 in our facility to remove the significant contamination of 129/sv sequences. All WT C57BL6/J mice were purchased from The Jackson Laboratory or bred on site.

Sheep red blood cell (SRBC) (Innovative Research Inc., Novi, MI) immunizations utilized SRBC washed three times with cold PBS and resuspended in PBS just before use. SRBC immunizations were 2×10^8 SRBC delivered i.p. in 200 μ l. Immunization with APC was i.p. injection of 30 μ g APC (ProZyme Inc., Hayward, CA) in 200 μ l of an emulsion of 1:1 complete Freund's adjuvant. PBS controls were done identically with no APC.

Nitrophenyl (NP) affinity experiments were performed using NP-coated SRBCs. To conjugate NP to SRBC the molecule NP-Osu (Biosearch Technologies, Inc. Petaluma, CA) was dissolved in 0.15M NaHCO₃ buffer at 1mg/ml. SRBC that had been washed twice in PBS and resuspended in PBS to the original concentration of purchased SRBC were then gently mixed with an equal volume of NP-Osu solution by inversion. The SRBC/NP-Osu mix was then incubated 2 hours with occasional mixing by inversion. The NP-SRBC were then washed three times with PBS, counted, and 2×10^8 NP-SRBCs were injected i.p. Pre- and post-immune blood samples were collected via tail bleeding and serum was obtained by isolating the supernatant after centrifuging the blood at 13,000rpm in a microcentrifuge. An NP-SRBC boost was administered i.p. at day 21.

Bone marrow chimera mice

Bone marrow chimera mice were generated as described previously (Donius, et al., 2013). Briefly, one day prior to transplant two doses of 550 cGy (4 hours apart) were delivered to host mice using an x-ray irradiator. Bone marrow was isolated into PBS from femurs and tibias of donor mice. WT and *Cr1KO* bone marrow was pooled respectively and split into a ratio of one donor to three host mice. The lethally-irradiated mice were anesthetized with isoflurane (VetOne, Meridian, ID) and the bone marrow transplant was administered retro-orbitally. Chimeras were administered sulfamethoxazole/trimethoprim via drinking water for 21 days and full reconstitution was allowed for six weeks.

Flow cytometry

Cell staining and flow cytometric analysis of dark zone, light zone, and total GC B cells were performed exactly as described previously (Donius, et al., 2013). The following antibodies from BioLegend (San Diego, CA) were used: rat anti-CD83 Alexafluor647 (clone: Michel-19), rat anti-B220 APC/Cy7 or BV785 (RA3-6B2), rat anti-CD38 PE or PE/Cy7. The following antibodies from eBioscience (San Diego, CA) were used: rat anti-GL7 Alexafluor488, rat anti-CXCR4 PerCP/Cy5.5 (2B11), rat anti-IgM PE (eB121-15F9), and purified rat anti-CD16/32 as Fc block. The following antibodies from BD Biosciences (San Jose, CA) were used: rat anti-CD35(Cr1) biotin (8C12), rat anti-CD95 (Fas) PE/Cy7 (Jo2), rat anti-CD4 PE (GK1.5), and rat IgG2a, kappa PE (Catalog #553930) as an isotype control for anti-IgM. Rat anti-IgM was proactively determined to be negative for cross-reactivity with rat IgM anti-GL7. Data acquisition was performed on a FACS CantoII (BD Biosciences, San Jose, CA) and data analysis was performed using FlowJo version 8.8.7 (Tree Star, Inc., Ashland, OR).

APC-positive cell enrichment

The protocol for enrichment of APC⁺ cells from total splenocytes was adapted from methods described by others (Pape, et al., 2011, Taylor, et al., 2012). Total splenocytes were isolated by straining spleens through 100µm mesh strainers into ice cold 0.5% BSA 2mM EDTA PBS (FACS buffer), pelleted by centrifugation, and resuspended in ACK red blood cell lysis buffer. Cells were repelleted and washed with 10ml of ice cold PBS. Cells were then stained with 2.5µg/ml APC (ProZyme Inc., Hayward, CA) in 2% rat serum PBS with rat anti-CD16/32 (Fc block) for 30 minutes on ice in the dark. A 12.5ml volume of FACS buffer was added to the cell mix and the cells were pelleted by centrifugation. APC-stained cells were incubated in a 500µl volume of FACS buffer with 50µl of anti-APC microbeads (Miltenyi Biotec Inc., Auburn, CA), covered in the dark for 15 minutes. Cells were washed with an additional 12.5ml of FACS buffer and pelleted by centrifugation. The cells were then resuspended in 500µl FACS buffer and retained on a pre-washed, magnetized LS column (Miltenyi Biotec Inc., Auburn, CA) followed by three washes with 3ml of FACS buffer before removing the column from the magnet and flushing the retained cells free with 5ml of FACS buffer. Total cell numbers for APC-enriched and APC⁻ fractions were determined by trypan blue cell counts. All APC-enriched cells ($1-3 \times 10^6$ viable cells) and $2-4 \times 10^6$ viable APC⁻ cells were aliquotted and stained for flow cytometric analysis. Total APC⁺ cell numbers were calculated after flow cytometry by determining the total APC⁺

population of interest from enriched and negative (usually negligible) sorts and adding the two together. Total memory B cell numbers were determined by subtracting the average number of APC⁺ cells in PBS/CFA immunized mice from APC/CFA immunized counts.

ELISA

Immulon 4HBX (Thermo Fisher Scientific Inc., Waltham, MA) plates were coated by applying 100µl of 5µg/ml APC in PBS, sealing, and incubating overnight at 4°C. For ELISAs detecting NP-specific antibody Immulon 4HBX plates were pre-coated with 100µl aliquots of 5µg/ml NP-BSA (Biosearch Technologies Inc., Petaluma, CA) with either a binding ratio of 4 NP to 1 BSA or 32 NP per BSA. Plates were washed three times by flooding with 0.1% Tween/PBS (wash buffer) and discarding. Plates were blocked with 200µl volumes per well of 1% BSA/0.1% Tween/PBS for 1.5 hours at room temperature. Serial dilutions of serum were prepared in 1% BSA/PBS in 96-well polystyrene plates. For IgM, dilutions were started at 1:10 and 7 serial dilutions of 1:2 were performed to a maximum of 1:1280. IgG assessment was done identically with 8 dilutions from 1:100 to 1:12800. Plates were washed once. Diluted samples were applied to plates in 100µl volumes after blocking and incubated for 1.5 hours at room temperature. Plates were washed three times. Horseradish peroxidase conjugated sheep anti-mouse IgG (#515-035-071, Jackson ImmunoResearch, West Grove, PA) or rabbit anti-mouse IgM (#315-035-049, Jackson ImmunoResearch, West Grove, PA) was diluted 1:2000 in 1% BSA/PBS and wells were incubated with 100µl volumes for 1.5 hours at room temperature. Plates were washed six times with wash buffer and horseradish peroxidase was visualized by exposure to o-Phenylenediamine solution. Reaction was stopped with 1N HCl and absorbance was measured at 490nm with a plate reader (BioTek Instruments Inc., Winooski, VT). Serial diluted standards on capture antibody were included on each plate to assure plate to plate consistency and non-specific IgM or IgG was used to assure secondary specificity. Reciprocal titers were determined by calculating the best fit logarithmic trendline for each sample's serial dilution and calculating the reciprocal dilution necessary for a specified absorbance (OD=0.4 for IgM and OD=1 for IgG). SRBC-specific ELISAs were performed as described previously (Donius, et al., 2013).

Ammonium thiocyanate elution ELISA

Denaturing ELISAs using ammonium thiocyanate (NH₄SCN) to measure the affinity of antibody produced were performed based on methods described by others (Macdonald, et al., 1988, Boulianne, et al., 2013). Specifically, reciprocal titers producing an absorbance of 1 for the NP(4) specific ELISAs were used to determine dilutions for all samples to give similar concentrations of antibody. NP(4)-BSA-coated Immulon 4HBX plates were prepared as described above, and columns of wells were plated with 100µl aliquots of the diluted serum antibody after blocking. Further steps were performed as described for antigen-specific IgG ELISA above with the exception of the treatment of serum bound wells for 15 minutes with serial dilutions of NH₄SCN (Sigma-Aldrich Inc., St. Louis, MO). This elution step was preceded by and followed by three washes with wash buffer and was performed after the 1.5 hour serum binding step. Primary response serum antibody was eluted with serial dilutions of NH₄SCN from 1M to 0.0156M and 0M control for determination of maximum binding. The secondary response was eluted with dilutions from 4M to 0.0625M.

To determine the affinity index the log10(% absorbance of maximum at 0M NH₄SCN) was plotted for each dilution and the curve was fit with a third-order polynomial using Prism (GraphPad Software, Inc). The affinity index was then calculated as the NH₄SCN dilution at log10(50%), or 1.699, by using the equation of the polynomial and Microsoft Excel's "Goal Seek" function.

Statistical analysis

Statistical analysis and graphs were generated using Prism version 5.0c (GraphPad Software, Inc).

Results

Activation of germinal center B cells in the *Cr1KO* animal

Previous work from our group characterizing the *Cr1KO* mouse revealed that the most striking phenotype of that mouse was a reduction in the percentage of GC B cells following immunization (Donius, et al., 2013). That study also revealed the surprising finding that the Cr1 and Cr2 isoforms are differentially expressed on B cells and FDCs with a preference for Cr2 on the former and near exclusivity of Cr1 on the latter. In light of these findings we sought to further investigate the effects and extent of the GC B cell reduction after immune induction, and the dependence of B cell and/or FDC-expression of Cr1.

We initially designed experiments to define the extent of the previously described GC B cell deficit. B cells expressing an activated, GC B cell phenotype are commonly seen as early as 4 days and peak at 8 to 14 days after immunization with sheep red blood cells (SRBCs) (Shinall, et al., 2000), a trend which is seen with most immunizations (Shlomchik and Weisel, 2012). Our previous work found that Cr1-deficiency resulted in a reduced frequency of GC B cells at 7 days post-immunization (dpi) despite normal SRBC-specific IgG titers suggesting normal plasma cell generation. We considered the possibility that GC B cells were not depressed equally at all time points and may even recover after 7 days. To visualize the progression of the GC B cell response we immunized *Cr1KO*, WT, *Cr1/2KO*, and *C3KO* mice intraperitoneally (i.p.) with 2×10^8 SRBCs, harvested spleens at 5, 7, 9, 11, and 13 dpi and analyzed for the presence of activated GC B cells (Fas^{HLGL7^{HL}}) (Fig. 1A and B). Intriguingly the expansion of B cells with an activated GC phenotype appeared to be initiated equally as well in WT as the *Cr1KO* animals (dpi 5,7) however *Cr1KO* mice showed a dramatic loss of activated cells in the later time points (dpi 9, 11, 13). As controls, the *Cr1/2KO* and *C3KO* animals demonstrated a marked loss of B cell activation in all of the measured time points emphasizing the importance of the complement pathway in the initiation of these responses. The early activation kinetics seen in the *Cr1KO* animals suggested that the primary activation state evident in the dpi5/7 time points is independent of Cr1 expression on either the FDC and/or B cells.

To further quantify the relative contributions of Cr1 expression by B cells or FDC in the generation of activated GC B cells, we created a series of bone marrow chimera animals between the *Cr1KO* and WT mouse strains. Mouse FDC are radioresistant while B cells and other hematopoietic stem cell lineages are ablated following irradiation. WT bone marrow

was transplanted into irradiated *Cr1KO* hosts (WT-->*Cr1KO*) and *vice versa* for comparison to control WT-->WT and *Cr1KO*-->*Cr1KO* chimeras. After reconstitution (6wks) such mice were immunized with 2×10^8 SRBCs and analyzed for GC B cell populations 9 dpi. WT marrow introduced into either WT mice or *Cr1KO* mice produced the highest level of GC B cell generation, with the introduction of *Cr1KO* marrow into WT or *Cr1KO* animals providing the lowest (Fig. 2A, left panel and B). To confirm that reconstitution of host mice with donor bone marrow was appropriate we tracked Cr1-expression on B cells (Fig. 2A, **right panel**). As expected only the animals reconstituted with WT bone marrow possessed B cells that expressed Cr1. These data thus suggest that Cr1 expression by B cells is more important than Cr1 expression by FDC for the generation of activated GC B cells following inoculation with SRBC.

Analysis of germinal center constituents in Cr1KO animals

GC reactions have been defined as containing two GC B cell subsets, centroblasts and centrocytes. The highly proliferative centroblasts are found in the visibly darker “dark zone” (DZ) and the centrocytes are found in the visibly lighter “light zone” (LZ) in close interaction with antigen laden FDCs (MacLennan, 1994). Recently the Nussenzweig group identified markers (CXCR4 and CD83) that delineate DZ and LZ B cells from within the GC B cell niche (Victora, et al., 2010). We hypothesized that the reduction in GC B cells found in the absence of Cr1 may be due to a loss or accumulation of GC B cells in the DZ or LZ compartments. Assessment of the percentage of GC B cells ($CD38^-/CD4^+ \text{ Fas}^+$) that were found in the LZ ($CXCR4^{LO} CD83^{HI}$) compartment versus the DZ ($CXCR4^{HI} CD83^{LO}$) compartment was not affected by Cr1 deficiency (Fig. 3A and B). This trend of equivalent percentages of DZ and LZ cells did not skew demonstrably at any time point assessed from 5 to 13 dpi. It should be noted, however, that as subsets of the total GC B cell population, the actual number of DZ and LZ B cells is reduced in the *Cr1KO* compared to WT mice.

Antibody responses in the Cr1KO animal

Our previous *Cr1KO* study demonstrated that T cell-dependent antigen-specific IgM and IgG3 titers were significantly reduced compared to WT when mice were immunized with soluble TNP-KLH (Donius, et al., 2013). The reduction of GC B cells in the *Cr1KO* animal allowed for the hypothesis that the generation of antigen specific IgG isotypes following recall immunizations would be reduced in the *Cr1KO* animal compared to WT. However, we considered the possibility that 21 dpi may still be too early to identify reductions in the GC-derived immunoglobulin. To investigate this possibility we measured the total allophycocyanin (APC) specific IgM, as well as total IgG, antibody titer from *Cr1KO* and WT serum samples collected 35 to 37 days following immunization with 30 μ g APC emulsified in complete Freund’s adjuvant (CFA). There was a significant difference in antigen-specific IgM produced between the WT and *Cr1KO* animals (Fig. 4A); however, the anti-APC IgG titers were strikingly similar among the *Cr1KO* and WT mice (Fig. 4B), suggesting that the greatest deficiency of the immunization response in the *Cr1KO* animal was in the initial B cell activation step.

In light of the differences between the antibody responses to the injection of SRBC (Donius, et al., 2013) versus soluble protein antigen emulsified in CFA, we decided to test the possibility that the mode of immunization was responsible for the discordance in responses noted between the GC B cell and antibody data. We therefore tried a different strategy by evaluating the antibody response to nitrophenyl (NP) that was conjugated directly to SRBC. This experimental design would thus allow for the quantification of an antigen-specific antibody response using the same SRBC immunogen delivery to follicles utilized in the GC B cell time course experiment. In this experiment WT, *Cr1KO*, and *Cr1/2KO* mice were immunized with NP-coated SRBC (with no additional adjuvant) at day 0 and boosted at day 21. Serum samples were obtained via tail bleeding at 0, 6, 14, 21, 28, and 35 dpi. Antibody titers were determined as the reciprocal of the titer required for an absorbance of 1. Quantification of the NP-specific IgG response was determined using two different NP-BSA complexes, one with a low NP-BSA ratio, NP(4) to quantify high affinity antibody, and a second with a high NP-BSA ratio, NP(32) to quantify low affinity antibody in two different parallel ELISA reactions (Kim, et al., 2011). The low affinity, NP(32) signals were stronger than the high affinity NP(4) signals during the primary response for the three mouse strains analyzed (Fig. 5A). Interestingly, however, the *Cr1KO* animal generated more high affinity antibody (days 6, 14 and 21 dpi) (anti-NP(4)) than either the WT or *Cr1/2KO* strains. Following the day 21 boost, high affinity antibodies were evident in all three strains (days 28, 35 dpi). It was surprising, however, to find the *Cr1/2KO* animals responding with anti-NP titers similar to that of WT animals. The *Cr1/2KO* mice are well documented to have a reduced response to SRBC compared to WT mice (Molina, et al., 1996, Donius, et al., 2013, Carlsson, et al., 2009, Rutemark, et al., 2012). To determine if the *Cr1/2KO* SRBC-specific IgG response was depressed despite the WT-like response to the NP epitope we performed an ELISA to quantify the SRBC-specific IgG at 14 dpi. As expected the SRBC-specific response was significantly reduced in the *Cr1/2KO* mice compared to WT and the *Cr1KO* mice (Fig. 5B). Additional dilutions of these sera also demonstrated the same trends relative to WT for each genotype and treatment (data not shown).

Analysis of memory B cell generation and affinity maturation in the *Cr1KO* animal

GC B cells are predominantly the precursors of high affinity and class-switched memory B cells. To test the effect the reduced GC B cell number in *Cr1KO* mice had on memory B cells we used the recently described method of immunizing mice with APC and tracking the generation of APC⁺ cells (Pape, et al., 2011, Taylor, et al., 2012). WT, *Cr1KO* and *C3KO* animals were immunized with APC and assayed 35 to 37 dpi by incubating their splenocytes with APC and using anti-APC magnetic beads to enrich for APC⁺ cells on a magnetized column. These cells were then analyzed by flow cytometry for their differential expression of CD38 and GL7 (CD38⁺ GL7⁻, indicative of the naïve/memory B cell phenotype) (Taylor, et al., 2012) and the percent of APC⁺ cells from the IgM⁺ and IgM⁻ (isotype switched) fraction was assessed (Fig. 6A). Using the percentages from the enriched and depleted fractions the number of APC⁺ memory B cells was calculated in comparison to the number of APC⁺ naïve B cells in the PBS/CFA immunized mice. The number of naïve APC⁺ cells in the IgM⁺ and IgM⁻ populations were not found to significantly differ between the *Cr1KO*, WT, and *C3KO* mice (Fig. 6B). Following immunization approximately 20,000 WT APC⁺ IgM⁺ cells were generated and remained constant at 35-37 dpi. The *Cr1KO* and

C3KO mice generated only about 5000-10,000 APC⁺ IgM⁺ B cells. No significant difference between *Cr1KO* or *C3KO* and the WT mice was identified in the IgM⁺ population of cells although a downward trend was noted in the *Cr1KO* and *C3KO* animals compared to WT. These data thus suggest that the absence of Cr1 (and C3) suppresses the generation of memory B cells specific for T cell-dependent antigens such as APC.

The loss of activated GC B cells as well as the suppression of antigen-specific memory cells in the *Cr1KO* animal led us to question if the generation of high affinity antibodies via affinity maturation in these animals was also compromised. Others had previously demonstrated reduced affinity maturation (to NP) in the *Cr1/2KO* animal in the absence of adjuvants but the restoration of WT affinity maturation with adjuvants (Wu, et al., 2000). In order to functionally assess antigen-specific antibody affinity we performed ELISAs on the serum antibody quantified in Figure 5A to determine their ability to bind NP(4)-BSA at increasing NH₄SCN concentrations (Macdonald, et al., 1988). As the concentration of NH₄SCN increases, only the high affinity antibodies can continue to bind to the hapten in the ELISA reaction. Following elution of serum samples with NH₄SCN the percent of the absorbance signal for each dilution was calculated compared to the maximum absorbance signal found in the absence of NH₄SCN. The log₁₀ value of this percentage was determined and plotted versus NH₄SCN concentration in Fig. 7A and B. These affinity ELISAs were performed for the dpi displayed and by using the equation of the line fit to the points for given mice in each genotype an NH₄SCN molarity that gives log₁₀(50%) was determined. The molarity value is the affinity index and the mean of these values for each *Cr1KO* (dotted line and squares), *Cr1/2KO* (dashed/dotted line and triangles), and WT (solid line and circles) mice is plotted (Fig. 7C). Evaluation of the affinity indices of *Cr1KO* and WT at individual time points by unpaired t-tests did not identify any significant differences suggesting that affinity maturation of NP-specific antibodies in the *Cr1KO* animal (and the *Cr1/2KO* animal) did not differ significantly from that of WT.

Discussion

The data in this report support the importance of Cr1 for GC B cell and memory B cell development, but not the generation of antigen-specific antibody to particulate and adjuvant emulsified antigens. Figures 1 and 2 demonstrate the surprising finding that GC B cell generation is relatively normal in the *Cr1KO* animal in the early, but not late, time points, and that this dependence is primarily upon Cr1 expression by B cells, not FDC. These findings suggest that B cells can adopt the surface markers of a GC-activated B cell early in the immunization stages presumably independent of contact with the FDC. Cr1 has long had a suspected role as a factor for I-mediated degradation of C3 to the terminal ligands that optimally activate Cr2. The absence of Cr1 on the surface of the B cells may reduce the availability of Cr2-activating ligands on the surface of the immunogen, thus limiting B cell activation. Additionally, Cr1 may be expressed at lower levels than Cr2 on the surface of B cells because its co-factor role in helping degrade C3 ligands to forms accessible by Cr2 is transitory while the role of Cr2 is of an activating co-receptor, a pathway that may be dispensable for FDC. These findings were surprising to us considering our findings here, and previously described, in which we documented the predominance of Cr1 on FDCs as opposed to B cells. The loss of maintenance of GC B cells, but not induction, initially

suggested that the reduction was due to FDC expression of Cr1. While not excluding an FDC role for Cr1 it is apparent that the GC B cell phenotype in *Cr1KO* mice is due to the removal of a B cell intrinsic role (Fig. 2).

In light of the demonstrated Cr1-dependent reduction in GC B cells it is surprising that total antigen-specific IgG was not affected long-term or short-term for particulate or adjuvant enhanced immunizations. Our efforts to determine if the difference lay in the affinity of the antibody produced rather than the quantity did not find any appreciable deficiency in the *Cr1KO* mice compared to WT (Fig. 7). We had hypothesized that low affinity B cells could fill B cell niches when high affinity GC B cells are not being generated; however, the finding that polyvalent antibody against NP and APC (data not shown) was generated with identical affinities in *Cr1KO* and WT animals indicated that affinity maturation is not compromised in the absence of Cr1.

The *Cr1KO* reduction in IgM⁺ but less extensively IgM⁻ memory B cells, as well as the loss of antigen-specific IgM but not IgG, is in apparent opposition to what would be predicted in light of the reduction of GC B cells. To us these results demonstrate that GCs promote the expansion of an excessive number of antigen-specific GC B cells capable of filling GC-dependent niches (Fig. 4, 5, 6, and 7) despite GC-deficiencies in the absences of Cr1 (Fig. 1). It is possible that this is a result of the complexity of SRBCs and APC as antigens or that NP is an antigen for which antigen-specific B cells are common. Nonetheless the intermediate number of GC B cells maintained during the immune response by *Cr1KO* mice is an interesting contrast to the near absence in *Cr1/2KO* and *C3KO* mice.

The GC is the location at which both plasma and memory cells are first generated. The *Cr1KO* animal (and *C3KO* mouse) demonstrated significantly reduced numbers of memory IgM⁺ B cells (compared to WT) and also reduced numbers of IgM⁻ memory B cells although these latter observations did not reach statistical significance. The IgM⁺ memory cell population is normally considered to be GC-independent again suggesting a vital role of Cr1 on the surface of B cells in optimal B cell priming and activation. Interestingly, Cr1 has recently been identified as a marker for germline Ig memory B cells (Anderson, et al., 2007).

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Figure 1. GC B cell maintenance is Cr1-dependent but C3 and Cr2 are required for their generation

(A) Representative flow cytometric analysis of GC B cells, identified as the Fas⁺ GL7⁺ cells in the B220⁺ subset, from mice 5, 7, 9, 11, and 13 days post-immunization (dpi) with 2×10^8 SRBCs (administered i.p.). (B) Quantification of the average number of splenic GC B cells for WT, *Cr1KO*, *Cr1/2KO*, and *C3KO* mice represented in (A). Data represents a compilation of three independent experiments (n=6, days 7, 9 and 11; n=4, days 5 and 13 and all *C3KO*). Significance shown *p<0.05, **p<0.01, and ns=not significant by unpaired t-test. *Cr1/2KO* and *C3KO* significantly reduced compared to WT on all days. Error bars represent SD.

Figure 2. B cell but not FDC-expression of Cr1 is required for GC B cell production

Bone marrow chimera mice (Donor→Host) were generated between all possible pairings of *Cr1KO* and WT mice. SRBC immunizations (2×10^8) were administered i.p. following bone marrow reconstitution of lethally irradiated hosts (6wks). (A) Analysis of the Fas⁺ GL7⁺ (GC B cells) from the total B220⁺ population of the spleen 9 days following SRBC immunization.

Plots are shown for each of the three mice from the groups of chimera mice. Representative histograms confirming Cr1-expression or –absence on B cells from chimeras is shown in the column on the right. All mice exhibited Cr1 expression consistent with the marrow transplant administered. (B) Analysis of the percentage of GC B cells from the chimera animals displayed in (A). Points represent individual mice with the mean and SEM for each group shown. Filled circles represent SRBC-immunized mice and open circles denote PBS controls. Brackets designate statistical analysis by unpaired t-test; * $p < 0.05$. All mice were age matched male mice; $n = 3$ or 4. Some females used for WT SRBC and PBS-controls.

Figure 3. Dark zone and light zone B cells are proportionately represented in the absence of Cr1

(A) Gating example showing the strategy used to identify dark zone (DZ) versus light zone (LZ) B cells from the GC B cell compartment. Example shown is from 9 dpi. GC B cells were sorted from the total B220⁺ population of splenic B cells as Fas⁺ and CD38⁻/CD4⁻. DZ B cells are characterized as CXCR4^{HI} and CD83^{LO} and LZ B cells are CXCR4^{LO} and CD83^{HI}. (B) Average percent of GC B cells that are DZ or LZ B cells from WT and *Cr1KO* mice 5, 7, 9, 11, and 13 dpi with 2×10^8 SRBCs (administered i.p.). All days and genotypes represent compilation of two independent experiments (n=4 for all days) and are representative of three independent experiments. Error bars represent SD.

Figure 4. The IgG response to APC is not affected by *Cr1*-deficiency despite a significant reduction in anti-APC natural antibody of the IgM isotype

(A) ELISA analysis of serum from *WT* and *Cr1KO* mice 35-37 days after i.p. immunization with 30 μ g APC emulsified in CFA (APC/CFA) versus negative control PBS/CFA immunized mice. Reciprocal anti-APC IgM required to produce an absorbance of OD=0.4. (B) Reciprocal titers of anti-APC IgG from the same serum samples shown in (A). Each symbol represents one mouse with the mean shown by the bar. Error bars represent SEM. * $p<0.05$, ** $p<0.01$, and ns=not significant by unpaired t-test.

Figure 5. The IgG anti-NP response generated by immunization with NP-conjugated SRBC

(A) The reciprocal titers of total IgG specific for NP from WT, *Cr1KO*, and *Cr1/2KO* mice immunized with 2×10^8 NP-conjugated SRBCs at 0 and 21 dpi. Reciprocal titers were determined via ELISA with NP(4)-BSA (low conjugation ratio for high affinity antibody quantification) or NP(32)-BSA (high conjugation ratio for low affinity antibody quantification) coated plates. Each point represents an individual mouse with the mean shown as the center bar and the error bars representing SEM. All genotypes represented by n=6 females mice of 8-12 weeks of age. (B) SRBC-specific IgG response to SRBC from the same mice at 14dpi. Antibody titers significantly different by one-way ANOVA. Bars represent significance between individual genotypes/treatments by Tukey's post-test; ns=not significant, **p<0.01, ***p<0.001.

Figure 6. IgM memory B cells are reduced in the absence of Cr1

APC positive B cells from WT, *Cr1KO*, and *C3KO* mice were quantified 35-37 days after i.p. immunization with 30µg APC/CFA. Prior to flow cytometric analysis total splenocytes were incubated with APC and enriched by magnetic column isolation.

(A) Naïve and memory B cells were identified using flow cytometry to sort out B220⁺ CD38⁺ GL7⁻ subset and to assess the IgM and APC expression. IgM⁻ cells were considered to be Ig switched. (B) The average number of naïve IgM⁺ and Ig switched APC⁺ cells is shown with error bars showing SEM. The number of naïve cells was calculated by determining the number of these cells present in PBS/CFA immunized mice. Memory B cells for APC are the number of additional APC⁺ cells found in APC/CFA-immunized mice over the average number of APC⁺ cells from PBS/CFA-immunized mice. Data are representative of two experimental replicates. Symbols represent individual mice; *p<0.05 and ns=not significant by unpaired t-test.

Figure 7. NP affinity quantification by NH_4SCN elution serum antibody

(A and B) Dissociation curves of primary (A) and secondary (B) response serum antibody on NP(4)-BSA coated plates were generated by eluting with increasingly stringent NH_4SCN concentrations. Graphs of the average log_{10} (percent of maximum absorbance) for each genotype are shown with error bars representing SEM. WT (squares and solid line), *Cr1KO* (circles and dotted line), *Cr1/2KO* (triangles and dotted/dashed line), mouse IgG on anti-mouse IgG capture antibody (black boxes and gray line), and naïve WT serum (gray circles and gray dashed line). Curves are best fit third-order polynomial curves representing points shown. The dotted line at 1.699 marks the log_{10} (50%) at which the affinity index is determined. (C) Average affinity index of WT (circles and solid line), *Cr1KO* (squares and dotted line), and *Cr1/2KO* (triangles and dashed/dotted line). WT and *Cr1KO* trends are significantly different by two-way ANOVA. Genotypes are represented by n=6 8-12 week old female mice.