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Optimal Germinal Center B Cell Activation and T-Dependent Antibody Responses Require Expression of the Mouse Complement Receptor Cr1

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Follicular dendritic cells (FDCs) and complement receptor (Cr)1 and complement receptor (Cr)2 are important for the generation of humoral immunity. Cr1/2 expression on B cells and FDCs was shown to provide a secondary signal for B cell activation, to facilitate transport of Ag in immune follicles, and to enhance retention of immune complexes by FDCs. We show in this study that murine B cells predominantly express the Cr2 product from the Cr2 gene, whereas FDCs almost exclusively express the Cr1 isoform generated from the Cr2 gene. To define the specific role of Cr1, we created an animal that maintains normal cell-restricted expression of Cr2 but does not express Cr1. Cr1-deficient (Cr1KO) mice develop normal B1 and B2 immature and mature B cell subsets and have normal levels of naive serum Abs but altered levels of natural Abs. Immunization of the Cr1KO animal demonstrates deficient Ab responses to T-dependent, but not T-independent, Ags. Germinal centers from the immunized Cr1KO animal possess a deficiency in activated B cells, similar to that seen for animals lacking both Cr1 and Cr2 or C3. Finally, animals lacking only Cr1 respond similarly to wild-type animals to infections with Streptococcus pneumoniae, a pathogen to which animals lacking C3 or both Cr1 and Cr2 are particularly sensitive. Altogether, these data suggest that the production of Cr1, primarily by FDCs, is critical in the generation of appropriately activated B cells of the germinal center and the generation of mature Ab responses. The Journal of Immunology, 2013, 191: 434–447.

Antibodies (Igs) are the major effectors of the adaptive immune response, and different isotype classes of Ab are produced to achieve different effects with Ab of the same specificity. Ig class-switch recombination is a hallmark of B cell responses to T-dependent (TD) Ags (1). These reactions occur within immune follicles of secondary immune structure, such as the spleen and lymph nodes (2). Central to follicles are follicular dendritic cells (FDCs), which establish the zonal identity (3) and act as a concentrated depot of Ag. In the course of an immune challenge, Ag is trafficked to the FDCs (4–6), where follicular B cells are recruited for surveillance of retained Ag. BCR specificity for an Ag retained on an FDC results in internalization and processing for presentation to T cells. The B cell then migrates to the T cell zone and presents the processed peptide to Th cells in the context of class II MHC. Upon a secondary signal from a peptide-specific Th cell, this B cell undergoes somatic hypermutation and isotype switch. Activated germinal center (GC) B cells must then retest their new Ig before undergoing clonal expansion and differentiation to either a plasma or memory cell. FDCs play an integral role in retention of Ag for this test of the new Ig, as well. Transport to and capture of Ag by FDCs in a primary immune response uses the protein products of the mouse Cr2 gene: complement receptor (Cr)1 and complement receptor (Cr)2 (4).

The predominant role of the complement cascade is detection of danger signals via the classical, mannose-binding lectin and alternative pathways and targeting of bound cells for lytic killing by the membrane attack complex (7–9). However, in addition to targeting foreign cells for membrane attack complex lysis, opsonization by the protein C3 can be used in transport to an FDC, phagocytosis, secondary signals through various complement receptors, and activation of more complement. These outcomes are dependent on the cleavage fragment of C3 and the corresponding cell receptor that they encounter. C3 is central to all three complement pathways; upon activation, it is cleaved into C3b and C3a. C3a is a potent anaphylatoxin that diffuses away to recruit and activate cells, whereas C3b remains bound to the foreign molecule and forms a C3 convertase complex that cleaves more C3. Alternatively, in the presence of the complement regulator, factor I, and one of the cofactors—factor H, Crry, or Cr1—C3b can be cleaved into one of the enzymatically inactive fragments: iC3b or C3d(g). Activation of the complement pathway can modulate humoral immunity (10) through the complement receptors 1 and 2 (Cr1 and Cr2) (11–14). Both Cr1 and Cr2 can bind the terminal cleavage products of C3: iC3b, and C3d(g). In addition, Cr1 is capable of binding the enzymatically active C3 convertase subunit C3b and acting as a cofactor for factor I cleavage of C3b to iC3b or C3d(g) (15).

The mouse differs from the human in that the single mouse Cr2 gene encodes both Cr1 and Cr2 via alternative splicing, whereas primates use distinct genes for the CR1 and CR2 proteins (16). Expression of the mouse Cr2 gene by B cells and FDCs has long been held under the assumption that the two different isoforms, Cr1 and Cr2, are produced equally in both of these distinct cell

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Abbreviations used in this article: C3KO, complement component C3 knockout; Cr1, complement receptor 1; Cr2, complement receptor 2; Cr1KO, complement receptor 1 knockout; Cr1/2KO, complement receptor 1 and 2 knockout; dpi, days postinfection; DZ, dark zone; FDC, follicular dendritic cell; FOB, follicular mature B cell; GC, germinal center; KLLH, keyhole limpet hemocyanin; LZ, light zone; MFI, mean fluorescent intensity; MZB, marginal zone B cell; PBT, 0.1% Tween-20 in 1× PBS; T1, transition 1; T2, transition 2; TD, T dependent; T1, T independent; TNP, trimethoprim; WT, wild-type.
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types. Functionally, mouse knockout models addressed the loss of both Cr1 and Cr2 when critical sequences of the Cr2 gene were deleted (12, 13). These studies did not delinate the specific functions of the Cr1 and Cr2 proteins on B cells and FDCs, and elevated surface expression of CD19 on Cr1/2-deficient (Cr1/2KO) B cells was proposed to lead to B cell anergy (17). Additional studies also used Cr1/2KO animals to examine the function of human Cr1 or Cr2 via transgenic-expression models of these proteins using Ig gene promoters (18, 19). In these studies, however, premature expression of the transgenes (compared with native Cr2), lack of FDC expression, expression in inappropriate cell types (e.g., T cells), and the structural distinctness of human Cr1 compared with the mouse Cr1 protein introduces critical variables. In light of these variables, neither of the human Cr1 or Cr2 transgenic models elucidates the individual functions of the Cr1 and Cr2 proteins (mouse or human) in the context of their autochthonous immune response.

To directly assess the phenotype of an animal lacking Cr1, but expressing Cr2 under the endogenous transcriptional controls of the Cr2 gene, we created a novel mouse Cr1 knockout model (Cr1KO) in which the exons encoding the domains unique to the Cr1 protein were removed, forcing Cr2 gene transcripts to splice from the exon encoding the signal sequence to the exon encoding the first domain of the Cr2 protein. The validation of the Cr1KO animal showed a complete lack of that protein. Comparison of this animal with wild-type (WT) mice demonstrated the Cr1 protein on FDCs and Cr2 protein on B cells as the dominant Cr2 gene isoforms on these cell types in the native animal. Cr1KO mice display numerous phenotypes that are different from WT and Cr1/2KO mice. Cr1KO mice do not exhibit the Ab response deficiencies to T-independent (TII) and low-dose TD Ags that are hallmarks of C57BL/6-derived embryonic stem cells by electroporation, and TK1TK2 vectors were obtained from the University of Utah Transgenic and Gene Targeting Mouse Core. The pACN and neomycin-containing pACN positive-selection vector (20). Targeted homology-directed exons 2 through 8 while leaving the sequence for splicing of the Cr1 and Cr2 proteins (mouse or human) in the context of their autochthonous immune response.

Materials and Methods

Generation of Cr1KO mice

A 21-kb construct was built in the pBluescriptII KS+ vector. Homologous recombination was targeted to the intronic EcoRV restriction fragment of the Cr2 gene 5’ to exon 2 and the NheI restriction fragment 3’ to exon 8 to delete exons 2 through 8 while leaving the sequence for splicing of C2 (Fig. 1A). These fragments were fused to the germine self-deleting neomycin-containing PACN positive-selection vector (20). Targeted homologous recombination was enhanced by the flanking TK1TK2 thymidine kinase positive-selection vector. The pACN TK1TK2 vectors were obtained from the University of Utah Transgenic and Gene Targeting Mouse Core. The linearized construct was targeted to mouse strain 129–derived embryonic stem cells by electroporation, and 192 clones were screened by Southern blot for homologous recombination of the Cr1KO construct. Chimeras were generated via injection of a positive clone into blastocyst and surrogate implantation. Electroporation, blastocyst injection, and chimera generation were performed by the University of Utah Transgenic and Gene Targeting Mouse Core. The Cr1KO construct insertion was tracked via PCR of DNA isolated from tail biopsies. Primers flanking exon 2, upstream of the EcoRV site (#4150 5’-TAGTGGTGGACGCGAACTTTACACA3’), and exon 8, downstream of the NheI site (#4316 5’-CAGCCTGACAGTATTTGTTAGAC3’), were designed to amplify a 483-bp fragment upon recombination for identification of the Cr1KO condition. The WT allele was identified with a primer from coding sequences in exon 8 (#4404 5’-TGGAAATATAAAGTCTTCCGTCGGTTG-3’) and a primer in the adjacent intron between exons 8 and 9 but upstream of the NheI site (#4422 5’-AGAATCTTCCAGTAAAGAGGATTG-3’) that generated a 350-bp product. All experiments were carried out using Cr1KO mice backcrossed to C57BL/6 or BALB/c mice for at least five generations. WT C57BL/6 and BALB/c mice, as well as complement component 3–deficient (C3KO) mice, were purchased from The Jackson Laboratory (Bar Harbor, ME) or obtained from colonies bred on location. C57BL/6-derived (Cr1KO, Cr1/2KO, C3KO, and WT) (12) 8–16-wk-old male and female mice were used in all experiments, unless otherwise specified. Mice were sex matched across genotypes. All mice were housed at the Comparative Medicine Center (University of Utah Health Sciences Center) in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were housed in an Animal Biosafety Level 2 protocol–approved area for all Streptococcus pneumoniae infection experiments.

Total spleen protein isolation

Spleen protein isolation, with FDCs included, was performed by extracting approximately two thirds of a spleen (RNA was isolated from the remaining third using the method described above) from a mouse and disrupting it with a razor blade in 5 ml ice-cold PBS. The dissociated spleen was then pipetted into a 15-ml conical tube and centrifuged at 300 × g, and the supernatant was discarded. The dissociated spleen was resuspended in 300 μl RIPA buffer containing protease inhibitors (complete, mini, EDTA-free, ultra tablets; Roche Diagnostics, Indianapolis IN), transferred to a 1.5-ml microcentrifuge tube, and placed in a rotator at 4°C for 45 min. The solubilized protein was isolated by centrifuging the lysate at high speed (4°C) in a microcentrifuge for 10 min and collecting the supernatant. The resulting protein isolate was stored at −70°C until Western blot analysis.

Bone marrow transplantation

Transplant recipients were lethally irradiated 24 h prior to transplantation with two doses of 500 cGy with an x-ray irradiator. Bone marrow donor mice were sacrificed, and marrow was isolated from the femurs and tibias. RBCs were lysed with ACK lysis buffer, and the remaining cells were washed, strained through 100-μm mesh, and resuspended in ~300 μl PBS/ mouse. Each recipient mouse was anesthetized with isoflurane and given 100 μl sex-matched bone marrow retro-orbitally. One donor’s marrow was transplanted into up to three recipients. Mice were maintained on antibiotic water, and engraftment was allowed for 6 wk.

Quantitative RT-PCR

Total spleen RNA was isolated using the CSCL centrifugation method, as described previously (21). Briefly, spleens were isolated from mice, homogenized using a tissuemizer in 4 M guanidine isothiocyanate solution, and centrifuged at 30,000 rpm overnight on a 5.7-M CsCl gradient. The RNA pellet was recovered and resuspended in 400 μl nuclelease-free water with 1 M NaCl, and ethanol was precipitated for 3 h. The RNA was pelleted at high speed in a microcentrifuge, washed with 70% ethanol, and resuspended in 50 μl nuclelease-free water. cDNA was generated as previously described (22). Quantification of transcript levels for Cr1, Cr2, C3, C5, C3KO, and β-actin was measured using a Roche LightCycler, as described previously (21, 23). Primers for PCR of total Cr1/2 transcript were #4824 5’-AAGAGAAAGAAAATGTTCGTCGGTC-3’ and #4825 5’-CCCCGCAACAAACTGTCAGAC-3’. Primers for PCR of Cr1- and Cr2-specific transcripts were as previously published (22, 24, 25). PCR primers for quantification of Al0x5, Dhp, Cstg, Fv, Lcn2, Krl18, and β-actin were as previously published (25).

Western blot analysis

Western blot analysis was performed as previously described (22). For Cr1/2 protein detection, the membrane was blocked in 5% milk 0.2% Tween-20 in TBS. Cr1/2 were detected with goat polyclonal anti-CD21 (Santa Cruz Biotechnology; #sc-7027) and visualized with the HRP-conjugated secondary Ab bovine anti-goat IgG (Jackson ImmunoResearch; #805-035-180).

Immunohistochemistry

Spleens were fixed by freezing in 4% paraformaldehyde and dehydrating in 5, 15, and 30% sucrose in PBS. Spleens were then frozen on dry ice in OCT embedding medium (Sakura Finetek USA, Torrance, CA) and stored at −70°C. Frozen sections were sectioned on a cryostat at a thickness of 10–12 μm. Spleen sections were rehydrated in 0.1% Tween-20 in PBS, washed, strained through 100-μm mesh, and resuspended in ~300 μl PBS. Each recipient mouse was anesthetized with isoflurane and given 100 μl sex-matched bone marrow retro-orbitally. One donor’s marrow was transplanted into up to three recipients. Mice were maintained on antibiotic water, and engraftment was allowed for 6 wk.
washed three times with PBT. Sections were then incubated with a 1:1000 dilution of streptavidin conjugated to HRP to HRP in 1% BSA PBT for 1 h at room temperature. Sections were washed with PBT, stained with 3, 3’-diaminobenzidine (Vector Laboratories, Burlingame, CA) per the manufacturer’s protocol, and counterstained with hematoxylin QS (Vector Laboratories). Sections were then overlaid with VectaMount AQ (Vector Laboratories) preservation reagent, cover slipped, and sealed with fingernail polish.

ELISA

ELISAs to determine total serum Ab titers and Ag-specific Ab titers were performed using the same basic blocking and detection protocol. ELISA plates were prepared as follows for Ag-specific Ab quantification: 5 μg/ml trinitrophenyl (TNP)-BSA (TNP-LPS and TNP-keyhole limpet hemocyanin [KLH] Ab-response measurement), 5 μg/ml DNP-BSA (DNP-AECM–Ficol Ab response measurement), 5 μg/ml phosphorylcholine-BSA (phosphorylcholine natural Ab quantification) (all conjugates from Biosearch Technologies), or 5 μg/ml KLH (Sigma-Aldrich) in 1× PBS was prepared and dispensed in volumes of 100 μl/well onto Immunol 4 HBX (Thermo Scientific) plates. Sandwich ELISA plates for total serum Ab quantification were prepared by incubating plates with 5 μg/ml anti-mouse IgM–IgG–IgA Ab (Pierce #31171) in carbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, 3.1 mM NaN3). Polysorp plates (Thermo Scientific) for detection of chitin/ chitosan-specific Ab were prepared as described (26). Maxisorp plates (Thermo Scientific) for DNA-specific Ab were coated overnight with 10 μg/ml calf thymus DNA in 1× PBS at 4°C. Plates were covered and incubated overnight at 4°C. Solution was discarded, and all wells were blocked with 200 μl 5% BSA 0.1% Tween-20 in 1× PBS blocking buffer for 1 h at room temperature. Blocking buffer was discarded, and wells were washed three times with 0.1% Tween-20 in 1× PBS ELISA wash buffer. Serum samples were then applied serially to wells in 5% BSA in 1× PBS as follows: natural Ab detection, 1:50, 1:100, 1:200, and 1:400; total serum IgG, IgM, 1:4,000, 1:8,000, 1:16,000, and 1:32,000; and Ag-specific Ig, 1:200, 1:400, 1:800, and 1:1,600. Serum was incubated in wells at room temperature for 1.5 h. Serum was discarded, and all wells were washed three times with ELISA wash buffer. Secondary Ab was diluted 1:2000 in 5% BSA in 1× PBS, and 100 μl was distributed to each well and incubated for 1.5 h at room temperature. All secondary Abs were conjugated to HRP: rabbit anti-mouse IgM (Jackson ImmunoResearch #315-035-049), rabbit anti-mouse IgG1 (Zymed #61-0120), rabbit anti-mouse IgG2b (Zymed #61-0320), rabbit anti-mouse IgG2c (Jackson ImmunoResearch #115-035-206), and goat anti-mouse IgG3 (AbD Serotec #STAR6P). The secondary solution was then discarded, and the wells were washed six times with ELISA wash buffer. Bound secondary Ab was then detected by exposing to 100 μl of o-phenylenediamine and 6.6 μl H2O2 in 20 ml citrate buffer and stopped with 100 μl 1 N HCl, and the absorbance was read at 490 nm with a BioTek plate reader. The absorbance values were calculated by subtracting the absorbance reading at 490 nm of a well without serum treatment. Positive control Abs were used as IgM (eBioscience clone #11E10), purified mouse myeloma IgG1 (Invitrogen #2062100), mouse IgG2b (eBioscience clone 192-121-15F9; eBioscience), anti-CD16/32 (FcR block, clone 93; eBioscience), and rat IgM–Alexa Fluor 488 isotype control (eBMR; eBioscience). Cells were stained through 100-μm mesh to disrupt cell clumps, vortexed gently with 1.5 μl 1 mM DAPI for live/dead discrimination, and analyzed using a FACS Canto II (BD Biosciences). FACS data were quantified using FlowJo software, version 8.8.7 (TreeStar).

S. pneumoniae culture and preparation

S. pneumoniae was generously provided by Kristiana Pierce (University of Utah Medical Technology Laboratory). It was presumptively reconfirmed via exhibition of α-hemolysis and optochin sensitivity (Taxo P Discs; BD Biosciences). The isolate was determined to be serotype 3 by the Statens Serum Institute (Copenhagen, Denmark). S. pneumoniae was passaged once through mice by isolating viable S. pneumoniae from the spleen of a lethally infected C57BL/6 mouse. Isolation was performed by staining the spleen through a 100-μm strainer into sterile 5% BSA PBS and growing serial dilutions on 5% Sheep Blood Columbia Plates (BD Biosciences). The isolate was determined to be serotype 3 by the Statens Serum Institute (Copenhagen, Denmark). S. pneumoniae was passaged once through mice by isolating viable S. pneumoniae from the spleen of a lethally infected C57BL/6 mouse. Isolation was performed by staining the spleen through a 100-μm strainer into sterile 5% BSA PBS and growing serial dilutions on 5% Sheep Blood Columbia Plates (BD Biosciences). The isolate was determined to be serotype 3 by the Statens Serum Institute (Copenhagen, Denmark).

Immunizations and S. pneumoniae infections

All immunizations and infections were given i.p. to 2–3-mo-old mice. Doses for T1 and TD Ag-specific Ig response curves were 50 μg TNP-LPS, 25 μg DNP–AECM–Ficol, 10 μg TNP-KLH, and 100 μg TNP-KLH (all from Biosearch Technologies, Novato, CA). Serum for Ig titering was obtained by collecting tail vein blood into heparinized capillary tubes, centrifuging at 13,000 rpm in a microcentrifuge for 6 min, and collecting the supernatant. Serum was collected every 7 d for 21 d. The low- and high-dose (10 and 100 μg) TD primary immunizations were followed 21 d later by a secondary boost of the same quantity, and an additional 28 d serum collection was done to measure the secondary Ig response.

For GC B cell–activation experiments, mice were injected with 2×106 SRBCs (Innovative Research, Novi, MI). Seven days later the mice were sacrificed, and splenocytes were analyzed via FACS.

Results

Creation and characterization of a Cr1-deficient mouse

The functions of Cr1/2 have been well established to be important in humoral immunity. To investigate the independent roles of Cr1 and Cr2 in a cell- and stage-specific manner for the generation of functional Ab responses, we generated a Cr1-deficient, but Cr2-sufficient, Cr1KO mouse line. To delete Cr1-specific transcripts from Cr2 without disrupting the Cr2 locus, a construct was produced in which DNA from the Cr2 gene promoter, transcription start site, and the
first coding exon (encoding the signal sequence) was fused to the germline sequences possessing the C-terminal exons specific for the Cr2 protein (Fig. 1A). Care was taken to include flanking intronic DNA so as not to disrupt sequences required for the appropriate splicing of Cr2 transcripts. The region between these two genomic sequences (occupied by Cr1-encoding exons in the native gene) was replaced with a neomycin selection cassette that would self-delete in the sperm of targeted animals, leaving behind a single Lox site. Analysis of Cr1 and Cr2 splenocyte transcripts in the Cr1KO animal was performed by quantitative RT-PCR using oligonucleotide sets specific for the Cr1-encoding transcript, a second set specific for the Cr2 product, and a third set that includes sequences common to both Cr1 and Cr2 products. As expected, Cr1KO mice lacked Cr1-specific transcripts and expressed higher levels of Cr2-encoding transcripts than did WT mice (data not shown).

The absence of the Cr1 protein in the Cr1KO animal was verified by immunoblot analysis of total splenocytes and B220+ splenocytes (Fig. 1B) using a polyclonal Ab with specificity for Cr1 and Cr2. The WT samples showed higher quantities of Cr2 compared with Cr1, whereas the Cr1KO animal only expressed Cr2 protein. FACS analysis of the expression of the Cr1 and Cr2 proteins on splenic B220+ B cells was done using the Cr1-specific Ab 8C12 and the 7G6 Ab, which recognizes both the Cr1 and Cr2 proteins (Fig. 1C). These data demonstrate the absence of the Cr1 protein (8C12) and the elevated surface expression of the Cr2 protein (7G6) on the Cr1KO cells compared with WT cells. Similar data were obtained using the anti-Cr1/Cr2 Abs eBio8D9 and 7E9 (data not shown), indicating that Cr1 deletion results in a filling of the Cr1/2 niche with Cr2. It should be noted that the preparation of isolated splenocytes for analysis in Fig. 1B and 1C was accomplished by straining mechanically disrupted spleens through 100-μm cell strainers prior to analysis, which results in the loss of FDCs from these cell populations.

**Phenotypic analysis of the Cr1KO mouse**

The mouse Cr1 and Cr2 proteins form complexes with CD19 on the surface of mouse B cells. The absence of Cr2 gene products on the surface of Cr1/2KO B cells was linked to the elevated expression of CD19 that is proposed as one mechanism leading to reduced B cell responses to Ag (17). To determine whether Cr2 sufficiency returned CD19 expression on B cells to normal in Cr1KO mice, we measured the mean fluorescent intensity (MFI) of CD19 staining on live B220+ cells from the spleen (Supplemental Fig. 1B). The MFI of CD19 staining on the Cr1KO cells was somewhat

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Cr1-specific deletion by targeted homologous recombination. (A) Diagram of the Cr2 gene and the construct used for targeted deletion of the Cr1-specific exons. Exons are denoted 1–14 and are not inclusive of all of the 3’ Cr2 gene exons. The coding sequences for the amino terminal SCR domains are also noted, with the Cr1KO deletion resulting in the deletion of exons encoding the first six SCRs of the Cr1 protein. (B) Western blot analysis of strained spleen lysates (no FDCs) from B220+–sorted, B220−–sorted, or total spleen cells. (C) FACS analysis of CD19+B220+ mature splenic B cells for the expression of Cr1 and Cr2 (left panel; 7G6 Ab) and Cr1 (right panel; 8C12 Ab).
matched 8–12-wk-old mice; C57BL/6 background. ***

were first segregated as live (DAPI−), pre-B cells (CD43+ B220+), immature B cells (CD43− IgMhi B220+), and mature (CD43− IgMlo B220+) B cells. All cells were first segregated as live (DAPI−). (B) Quantification of the frequency of pre-, immature, and mature B cells from WT, Cr1KO, and Cr1/2KO bone marrow, as defined by the gating strategy in (A). Pre, immature, and mature B cell populations were not significantly different by ANOVA. 

Analysis of bone marrow B cell populations from WT, Cr1KO, and Cr1/2KO mice. (A) Representative gating for delineating pro-B cells (B220+ CD43+), pre-B cells (CD43− IgMhi B220+), immature B cells (CD43− IgMhi B220+), and mature (CD43− IgMlo B220+) B cells. All cells were first segregated as live (DAPI−). (B) Quantification of the frequency of pro-, pre-, immature, and mature B cells from WT, Cr1KO, and Cr1/2KO bone marrow, as defined by the gating strategy in (A). Pre, immature, and mature B cell populations were not significantly different by ANOVA. 

FIGURE 2. Analysis of bone marrow B cell populations from WT, Cr1KO, and Cr1/2KO mice. (A) Representative gating for delineating pro-B cells (B220+ CD43+), pre-B cells (CD43− IgMhi B220+), immature B cells (CD43− IgMhi B220+), and mature (CD43− IgMlo B220+) B cells. All cells were first segregated as live (DAPI−). (B) Quantification of the frequency of pro-, pre-, immature, and mature B cells from WT, Cr1KO, and Cr1/2KO bone marrow, as defined by the gating strategy in (A). Pre, immature, and mature B cell populations were not significantly different by ANOVA. 

Analysis of B cell subsets from Cr1KO mice identified no significant differences compared with WT mice. B cells in the marrow of adult mice were examined (Fig. 2) based upon the differential expression of B220, CD43, and IgM (Fig. 2A) to delineate pro-B, pre-B, immature, and mature B cells. The frequencies of each of these B cell subsets in Cr1KO and Cr1/2KO mice were not altered compared with WT mice based on ANOVA (Fig. 2B). The expression level of CD19 was also quantified on these B cell subsets (Supplemental Fig. 1A) and was shown to be identical for the four animals (Supplemental Fig. 1B).

Cr1KO mice were not altered compared with WT mice (Fig. 2). Interestingly, a significant (p < 0.001) reduction in the frequency of B1a cells in Cr1/2KO mice was observed (Fig. 3B). This decrease in B1a B cells had not been documented in Cr1/2KO animals (12, 13), but it was described for the hypomorphic Cr1/2-deletion animal (31, 32). A decrease in B1a cells in Cr1KO mice was not observed. Quantification of the MFI of CD19 on the surface of the B1a and B1b cells from Cr1KO animals did not show any significant difference compared with WT or Cr1/2KO animals (Supplemental Fig. 1B).

The numbers of mature B cell subsets in the spleen of Cr1KO mice were also analyzed based upon differential staining of B200, CD23, CD24, and CD21/35 (Cr1/2) (Fig. 4). Comparable transition 1 (T1), transition 2 (T2), follicular mature B cell (FOB), and marginal zone B cell (MZB) subset frequencies were found in the spleens of Cr1KO mice as in WT and WT/Cr1KO heterozygote animals (ANOVA). The expression of Cr2 in the Cr1KO animal was consistent with the generation of T2 B cells at the same point in B cell maturation as WT B cells. Therefore, the loss of Cr1 on the surface of murine B cells did not alter their development or their localization within the splenic B cell populations.

We showed previously that the spleens of Cr1/2KO animals displayed signatures of a more highly inflamed environment than did WT mice, presumably due to the lack of control of complement convertases within immune follicles (25). The analysis of total splenic transcripts for inflammatory response genes also demonstrated altered expression of inflammatory mediator genes in the Cr1KO spleen compared with WT and Cr1/2KO animals (Supplemental Fig. 2).

The Cr1 isoform is the dominant Cr2 gene product on FDCs

The two major cell types of the mouse that express the Cr2 gene are the B cell and the FDC. As described above, the Cr1 and Cr2 proteins are generated from alternative splice isoforms from the Cr2 gene. As shown previously (Fig. 1B), immunoblot analysis of total splenic cell populations (isolated by straining free cells away from the FDC-enriched stromal matrix) for the total Cr2 gene

FACS analysis of the B220+ CD11b+ and CD5+ (B1a) and CD5− (B1b) cells of the peritoneal cavity revealed that they were not present at different frequencies in Cr1KO mice compared with WT mice (Fig. 3). Interestingly, a significant (p < 0.001) reduction in the frequency of B1a cells in Cr1/2KO mice was observed (Fig. 3B). This decrease in B1a B cells had not been documented in Cr1/2KO animals (12, 13), but it was described for the hypomorphic Cr1/2-deletion animal (31, 32). A decrease in B1a cells in Cr1KO mice was not observed. Quantification of the MFI of CD19 on the surface of the B1a and B1b cells from Cr1KO animals did not show any significant difference compared with WT or Cr1/2KO animals (Supplemental Fig. 1B).

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The two major cell types of the mouse that express the Cr2 gene are the B cell and the FDC. As described above, the Cr1 and Cr2 proteins are generated from alternative splice isoforms from the Cr2 gene. As shown previously (Fig. 1B), immunoblot analysis of total splenic cell populations (isolated by straining free cells away from the FDC-enriched stromal matrix) for the total Cr2 gene

FACS analysis of the B220+ CD11b+ and CD5+ (B1a) and CD5− (B1b) cells of the peritoneal cavity revealed that they were not present at different frequencies in Cr1KO mice compared with WT mice (Fig. 3). Interestingly, a significant (p < 0.001) reduction in the frequency of B1a cells in Cr1/2KO mice was observed (Fig. 3B). This decrease in B1a B cells had not been documented in Cr1/2KO animals (12, 13), but it was described for the hypomorphic Cr1/2-deletion animal (31, 32). A decrease in B1a cells in Cr1KO mice was not observed. Quantification of the MFI of CD19 on the surface of the B1a and B1b cells from Cr1KO animals did not show any significant difference compared with WT or Cr1/2KO animals (Supplemental Fig. 1B).

The numbers of mature B cell subsets in the spleen of Cr1KO mice were also analyzed based upon differential staining of B200, CD23, CD24, and CD21/35 (Cr1/2) (Fig. 4). Comparable transition 1 (T1), transition 2 (T2), follicular mature B cell (FOB), and marginal zone B cell (MZB) subset frequencies were found in the spleens of Cr1KO mice as in WT and WT/Cr1KO heterozygote animals (ANOVA). The expression of Cr2 in the Cr1KO animal was consistent with the generation of T2 B cells at the same point in B cell maturation as WT B cells. Therefore, the loss of Cr1 on the surface of murine B cells did not alter their development or their localization within the splenic B cell populations.

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products using a polyclonal rabbit anti-Cr1/2 Ab demonstrated a much higher quantity of Cr2 versus Cr1. A similar pattern is also obtained from the analysis of purified B220+ B cells. FACS analysis of B cells with saturating levels of the Cr1-specific Ab (8C12) in contrast to Abs recognizing both Cr1 and Cr2 (7G6, 7E9, eBio8D9) also demonstrates that the MFI of Cr2 staining is much higher than that of Cr1 (data not shown). Altogether, these data suggest that the predominant isoform of the Cr2 gene expressed by B cells is the Cr2 protein. Additionally, immunohistochemical staining for Cr1 (Cr1-specific Ab clone 8C12) of splenic or lymph node cross-sections has been used by many investigators to definitively mark FDCs. This suggested to us that FDC expression of Cr1 is not equal to B cell expression and that the Cr1/Cr2 ratio may not be equal in these two cell types. In light of these observations, we chose to definitively delineate Cr1/2 expression in FDCs and B cells.

Initially, we used the same immunohistochemical staining strategy as other investigators to screen splenic cross-sections from Cr1KO mice. As shown in Fig. 5A, staining WT, Cr1KO, and Cr1/2KO spleen sections with the anti-Cr1 (CD35) Ab 8C12 demonstrates strong Cr1 expression on the FDCs of WT mice but an absence of Cr1 expression on the two mouse mutant strains. Thus, the Cr1KO mouse also shows the expected loss of Cr1 expression by FDCs. Staining of parallel sections with an Ab that recognizes both Cr1 and Cr2 (CD21/35; Ab 7E9) demonstrated a virtually identical pattern of B cell staining evident in WT and Cr1KO sections that was absent from the Cr1/2KO section (Fig. 5B). However, this staining did not highlight the FDCs in the WT sample in a similar fashion as did that with the anti-Cr1 Ab.

To further evaluate this question, quantitative RT-PCR and immunoblot analysis were performed on dissociated total unstrained spleen samples from bone marrow–transplant experiments in which hematopoietic cells from Cr1/2KO mice were transplanted into lethally irradiated WT host mice (Cr1/2KO→WT), as well as WT→WT, WT→Cr1/2KO, and Cr1/2KO→Cr1KO. Total replacement of the hematopoietic lineage of host mice, with or without donor Cr1/2KO B cells, was confirmed by FACS, and all mice had <1% of B cells expressing the host Cr1/2 phenotype (Fig. 6A). Transcript analysis from these chimeric mice revealed that the Cr1 isoform is more highly expressed than that of Cr2.
in the Cr1/2KO→WT chimera (p < 0.001, Fig. 6B). This was in contrast to WT→Cr1/2KO and WT→WT mice, neither of which displayed a significant difference in the expression of Cr1 versus Cr2. It should be noted that, although some Cr1/2 transcript is detected in Cr1/2KO mice using primers common to both Cr1/2, this is a distal portion of the transcript (3') that cannot be translated into any portion of Cr1 or Cr2 (12). Comparison of the ratio of relative Cr1 transcript/Cr2 transcript demonstrated that the Cr1/2KO→WT chimera, in which only FDCs transcribe the Cr2 locus, expresses Cr1 at an ~3.5-fold greater level than Cr2. This is in contrast to the significantly lower 1:1 ratio displayed by the WT→Cr1/2KO and WT→WT chimeras, in which B cells are the most common cell type expressing the Cr2 locus (p < 0.001 and p < 0.01, respectively, Fig. 6C). Western blot analysis of total spleen lysate (inclusive of both B cells and FDCs) from the same reconstituted mice revealed that Cr1 is nearly the exclusive product of the Cr2 gene by FDCs, because WT irradiated mice reconstituted with Cr1/2KO bone marrow primarily express the Cr1 protein (Fig. 6D, left panel). In contrast, Western blot analysis of total spleen lysates from either irradiated WT or Cr1/2KO hosts given WT bone marrow transplants shows that the Cr2 protein is the predominant product of B cells (Fig. 6D, left panel). Interestingly, although Cr1 and Cr2 transcript data suggest that FDCs in the Cr1/2KO→Cr1KO chimeras produce only the Cr2 transcript, this transcript was not translated by FDCs into appreciable levels of Cr2 protein, in contrast to the Cr2 protein produced by Cr1KO B cells (Fig. 1B, 1C). Total spleenic lysates from Cr1/2KO and WT animals are shown for comparison (Fig. 6D, right panel).

Immunoblot analysis for Cr1/2 was also performed on lymph node and spleen lysates from mice 2 d postirradiation. Such peripheral immune organs are virtually devoid of B cells 2 d after irradiation (data not shown). Spleens were disrupted, single cells were strained away from the splenic matrix, and the resulting matrix material was solubilized in RIPA detergent for immunoblot analysis. As shown in Fig. 6D (center panels), the irradiated splenic tissue matrix was highly enriched for the Cr1 protein, as opposed to the Cr2 protein seen in the samples enriched for B cells.

Altogether, the data detailed above describe the Cr1KO mouse as lacking Cr1 expression on B cells and FDCs. However, Cr2 expression on mouse B cells is very similar to that seen for WT B cells. Additionally, although the Cr2 gene is still transcriptionally active in the FDCs of Cr1KO mice, as measured by the production of transcripts specific for Cr2, there is little to no Cr2 protein expression by these cells (as is also shown for WT FDCs, which do possess Cr1 protein but not Cr2). These findings suggest that the effect of losing the Cr1 protein in the Cr1KO animal is likely to be most pronounced for functions of the FDCs and not the B cells, for which the major Cr2 gene product is Cr2 protein.

Ab deficiencies of Cr1KO mice

To identify possible effects of Cr1 deficiency on humoral immunity, we measured the quantities of total and Ag-specific Abs of IgM, IgG1, IgG2b, IgG2c, and IgG3 in naive and immunized mice. The levels of circulating Abs in naive Cr1KO animals were compared with that of WT and Cr1/2KO animals. As shown in Fig. 7, the levels of circulating IgGs and IgM in Cr1KO mice are not significantly different from those of WT mice. However, Cr1KO mice (as well as WT mice) have statistically higher levels of IgG2b, IgG2c, and IgG3 than do Cr1/2KO mice (p < 0.01, p < 0.001, and p < 0.001, respectively).

Within the naive Ab repertoire is a collection of Igs (IgM isotype), known as natural Abs, which is specific for conserved epitopes often found on apoptotic cells and pathogens. Many epitopes recognized by natural Abs have been identified, such as phosphorylcholine, chitosan/chitin, and dsDNA. To test the effect of Cr1 deficiency on the production of natural Abs, we quantified the titers of IgM specific for phosphorylcholine, chitosan/chitin, or dsDNA in the serum of naive mice. Anti-phosphorylcholine Ab was found at significantly reduced titers in Cr1KO mice compared with WT mice (p < 0.05), with a magnitude similar to the reduction seen in Cr1/2KO mice (p < 0.001, Fig. 8A). This pattern of
natural Ab deficiency was not the same for anti-chitosan/chitin and anti-dsDNA natural Abs. Anti-chitosan/chitin Abs from Cr1KO animals were equivalent to WT levels (but statistically significantly reduced in Cr1/2KO mice) (Fig. 8B), whereas natural Abs specific for dsDNA were statistically significantly reduced in Cr1KO mice compared with WT and Cr1/2KO mice (Fig. 8C).

Ags are generally split into three categories defined by the secondary signal that tunes B cell activation upon BCR cross-linking: TI-1, provided by TLR ligands; TI-2, provided by large repetitive Ags, such as polysaccharides; and TD, provided by coincident recognition of protein Ag by CD4 T cells. To test the contribution of Cr1 in Ab responses to such Ags, we immunized Cr1KO, WT, and Cr1/2KO mice with the model Ags TNP-LPS (TI-1), DNP-Ficoll (TI-2), or TNP-KLH (TD) and quantified the Ag-specific response of different isotypes of Ig.

Cr1KO mice generated similar Ab responses to TI-1, TI-2, and low-dose TD Ag relative to WT mice (Fig. 9A, Supplemental Fig. 3). In contrast, Cr1/2KO mice were generally determined to have lower detectable levels of Ig against TI-2 and low-dose TD Ag than were WT mice, as previously observed. A similar reduction for Cr1KO and Cr1/2KO animals was observed for the Ag-specific IgG3 isotype in response to high-dose TNP-KLH immunization (days 7, 14, and 21). Intriguingly, both Cr1KO and Cr1/2KO mice displayed a significant decrease in Ag-specific IgM following high-dose TNP-KLH for days 7, 14, and 21 but a significant expansion of Ag-specific IgM (compared with WT mice) following the day-21 boost with TNP-KLH.

Follow-up analysis of Ig produced against the KLH carrier of the TNP-KLH hapten-carrier conjugate demonstrated a reduction in Ag-specific IgM produced by Cr1KO and Cr1/2KO mice, although the levels of Ag-specific IgG to KLH in Cr1KO mice was very similar to that of WT mice (Fig. 9B). Cr1KO animal responses to immunization with whole SRBCs also tracked similarly to WT animals (both IgM and IgG), whereas the SRBC-specific IgG response of Cr1/2KO mice was significantly less than that of WT mice (Fig. 9C).
The generation of activated GC B cells is reduced in Cr1KO mice

Because of the altered response to the T cell-dependent Ag in Cr1KO mice, we questioned whether GC B cells were effectively activated in such mice. To test this, we immunized mice with $2 \times 10^8$ SRBCs and used FACS to assay splenic B cells for activated GC B cell markers. Activated GC B cells are identified by the increased expression of Fas and reduced expression of IgD, as well as increased expression of the glycan epitope recognized by the Ab clone GL7 (3, 33–35). Cr1KO, WT, and Cr1/2KO mice were immunized with SRBCs, and their spleens were isolated 7 d later. Splenocytes were B220+ FACS sorted for B cells and then identified as GC B cells by IgDint Fas+. Similar to Cr1/2KO mice, Cr1KO mice consistently possessed a significantly ($p < 0.001$) reduced population of GC B cells in response to SRBC immunizations compared with WT mice (Fig. 10). GL7 expression was equivalent in the various IgDint Fas+ populations, indicating that when GC cells were activated in the Cr1KO mouse (and Cr1/2KO animal), the level of activation was equivalent to WT mice (data not shown). To determine whether the lack of activated GC B cells in Cr1KO (and Cr1/2KO) animals was complement (C3) dependent, we analyzed the generation of GC B cells in SRBC-immunized C3-deficient animals. As shown in Supplemental Fig. 4, C3KO mice generated significantly reduced GC B cell populations. However, the small subset of cells from the C3KO animal that did gate in the activated GC population also expressed elevated levels of GL7 (data not shown). Therefore, the full activation of GC B cells requires C3 in addition to expression of the Cr1 protein.

Cr2 sufficiency rescues Cr1KO mortality during S. pneumoniae infection

Cr1/2KO mice generate a deficient primary immune response to the pathogen S. pneumoniae and a subsequent poor secondary immune response to infection. To determine whether the susceptibility of Cr1KO mice to S. pneumoniae was similar to that of the Cr1/2KO animal (along with WT controls), mice were immunized with 10,000 heat-killed S. pneumoniae and infected 10 d later with 1000 CFU of live bacteria. Survival of Cr1KO, Cr1/2KO, C3KO, and WT mice was analyzed using the log-rank test ($p < 0.01$); survival of Cr1KO mice (85%, 2 d postinfection [dpi]) was not significantly less than the 100% survival of WT mice (Fig. 11). In contrast to Cr1KO animals, Cr1/2KO mice were significantly ($p < 0.05$, 55%, 3 dpi) more susceptible to mortality from the infection. Consistent with the critical role for the complement cascade in control of S. pneumoniae infections, none of the C3KO mice survived beyond 3 dpi.

Discussion

The complement pathway is a critical component of the innate and acquired immune response. The generation of Ab responses, both T cell dependent and independent, was demonstrated to be influenced by complement products and their cellular receptors. Animals lacking the Cr2 gene products, the Cr1 and Cr2 receptors, have defined deficiencies in Ab responses (both natural and as the result of specific immunizations), heightened sensitivity to bacterial pathogens (e.g., S. pneumoniae), and a lack of optimal B cell activation (12, 13). The Cr2 gene is transcriptionally active in B cells and FDCs and, via alternative splicing, creates the transcripts specific for Cr1 and Cr2. Gene-knockout strategies in the past eliminated the ability of B cells and FDCs to make any of the Cr2 gene products; thus, it has been impossible to delineate the specific functions of either of the individual proteins. In this study, we describe the creation of a new mouse line, the Cr1KO mouse, in which the exons unique to the Cr1 protein have been deleted by targeted homologous recombination, creating a gene very similar to that of human CR2, which possesses the vestiges of the Cr1-like exons but only produces the CR2 protein (36).

Characterization of the Cr1KO animal led us to focus upon Cr1 and Cr2 protein production by B cells and FDCs. It had always been a question why immunostaining of splenic GCs with an Ab specific for Cr1 identified FDCs, whereas similar staining with an Ab that recognizes both Cr1 and Cr2 (there are no specific anti-Cr2 Abs) primarily identifies B cells (Fig. 5). By teasing apart the protein contributions of the Cr2 gene in B cells and FDCs, we found that the Cr2 gene product produced in FDCs is almost exclusively that of Cr1, whereas B cells primarily splice to form the Cr2 protein (Fig. 6). Even when the Cr2 gene is forced to only splice to the Cr2 isoform in the Cr1KO animal, the quantity of Cr2 produced by FDCs is minimal (Cr1/2KO→Cr1KO bone marrow chimera), whereas that of B cells is normal (Fig. 6D). Thus, the deficiency of the Cr1KO animal could be expected to have a greater impact upon FDC functions compared with B cell activation/Ag acquisition.
The comparison of Ab titers in naive and immunized Cr1KO and Cr1/2KO mice demonstrated a more dramatic deficiency when both Cr1 and Cr2 are absent (Figs. 7–9). Although the naive Cr1/2KO mouse has depressed circulating levels of total IgG2b, IgG2c, and IgG3, the Cr1KO titers for these Ab types are virtually identical to those in WT mice. The responses to both TI and TD Ags from the Cr1KO mouse, which was virtually identical to that for WT mice. However, the decreased anti-KLH and anti-SRBC IgM responses in both the Cr1KO and Cr1/2KO animal lines, compared with WT, suggest that Cr1, expressed by either B cells or FDCs, may be playing a role in this initial Ag response. Previous work on an independently generated Cr1 and Cr2–deficient mouse by Molina et al. (13) described SRBC-specific IgM primary responses that were very similar to those demonstrated in this study.

Our IgG analysis of the Cr1KO animal may also be compared with bone marrow chimera studies in which Ab responses were analyzed in animals lacking functional Cr2 gene expression in either bone marrow lineages or FDCs.

One such study analyzed responses to immunization with SRBCs and KLH; it showed that, although the absence of Cr1 gene products expressed by FDCs had a profound impact upon Ab generation, the generation of an optimal response also needed B cell Cr2 gene products (37). The demonstration of the predominance of expression of Cr1 on FDCs suggest that the IgG response to SRBCs or KLH in Cr1KO mice should mirror those of bone marrow chimeras in which complement receptor expression is limited to B cells, as described in that report. Our data on the IgG responses to SRBC immunization demonstrate there is not a significant difference in response to these immunogens, suggesting that bone marrow chimeras may not precisely define the phenotype of Cr1 deficiency. Furthermore, despite the lack of significance between the Cr1KO and WT IgG responses to SRBCs, the similarity of the pattern of IgG responses to KLH by Cr1KO mice relative to WT and Cr1/2KO mice (Fig. 9B, 9C, right panels) suggests that these differences are more than random variation. A more recent bone marrow chimera analysis (using Cr1/2 deficiency bred upon a BALB/c background) demonstrated that the presence or absence of Cr2 gene products on B cells did not alter the generation of various IgG isotopes to immunizations with SRBCs; instead, Cr2 gene expression by FDCs was critical for such responses (14). Intriguingly, this study also demonstrated that immunization with IgM–SRBC complexes required Cr2 gene products on both B cells and FDCs for optimal Ab responses. Our data generated using the Cr1KO animal fundamentally agrees with the conclusions reached by Rutemarck et al. (14); the ability of B cells to efficiently class switch from IgM to IgG isotopes (whether they express Cr2 gene products or not) is compromised in the absence of the Cr1 protein expressed by the FDCs. However, there are specific points of variance between our studies and those using bone marrow chimera models that may be
FIGURE 9. TI-1, TI-2, and TD Ag-specific Ig response. (A) Deviation from WT in production of Ag-specific Ig of the isotypes IgM, IgG1, IgG2b, IgG2c, and IgG3 by Cr1KO and Cr1/2KO mice. Ig in response to DNP or TNP after immunization with the model immunogens TNP-LPS (TI-1), DNP-Ficoll (TI-2), TNP-KLH (10 μg), or TNP-KLH (100 μg) was measured at 0, 7, 14, and 21 d after primary immunization. Secondary immunizations were given to TNP-KLH–immunized mice at 21 d, and 28-d serum samples were also measured. Graphs show difference in absorbance of each mouse from the average WT absorbance at 490 nm for each day. TI-1, n = 7–10; TI-2, n = 9; TD (low), n = 7; TD (high), n = 10; 8–12-wk-old sex-matched mice; all data represent a composite of two independently performed immunizations; see Supplemental Fig. 3. (B) ELISA analysis of KLH-specific IgM (Figure legend continues)
a consequence of the experimental procedures. For example, bone marrow chimera studies require irradiation and bone marrow reconstitution that may result in an immune environment that is not functionally equivalent to the environment found in a nonirradiated animal (in our case, the Cr1KO line). Additionally, in removing the ability of B cells to produce the Cr1 protein in the Cr1KO animal, all Cr2 gene transcripts are committed to producing the Cr2 protein, which is evidenced by the increased MFI of Cr2 expression by B cells of the Cr1KO mouse compared with WT mice. This increase in Cr2 protein may heighten the sensitivity of the activation of B cells, reducing the difference between Cr1KO and WT mice. Alternatively, Cr1 expressed by B cells may suppress B cell activation. Mouse strain differences may also play a role; our Ab analyses, as well as those of Fang et al. (37), used the C57BL/6 background, whereas those of Rutemark et al. (14) used the BALB/c background. Mouse strain differences may also play a role; our Ab analyses, as well as those of Rutemark et al. (14) used the C57BL/6 background, whereas those of Fang et al. (37), used the BALB/c background. Further analysis of bone marrow chimera models described above using marrow reconstitution from the Cr1/2KO mouse possess B cells expressing increased levels of CD19. However, the analysis of B cells obtained from the Cr1KO animal showed a lower level of CD19 than did those from WT mice, thus mitigating the concerns about CD19-dependent B cell anergy in the Cr1KO mouse.

The generation of activated GC B cells requires the interaction of the Ag-specific B cell with Ag and the cognate T cell. It was shown that the number of GC B cells after immunization was reduced in Cr1/2KO mice (38); this was presumed to be due, in part, to the loss of Cr2 signaling on the B cell as part of the BCR coreceptor complex. Interestingly, we found that the Cr1KO animal also does not generate activated B cells (Fig. 10) based upon the IgD and Fas expression status (Fig. 10). Indeed, the immunized Cr1KO and Cr1/2KO animals had the equivalent percentages of activated B cells as did the unimmunized WT control. The movement of B cells from the region of high B cell proliferation, known as the dark zone (DZ), to the FDC-localized light zone (LZ) of the GC (interzonal migration), has been defined as “cyclic re-entry”; Ag-specific B cells are selected for by binding to Ag held by the FDCs, followed by encounter with Ag-specific T cells and migration to the DZ for proliferation (39–41). After activation and proliferation in the DZ (accompanied by somatic hypermutation and isotype switching), the Ag-specific B cells then cycle back into the LZ for another round of positive Ag selection on the FDC. The absence of such activated B cells in the Cr1KO animal suggests that Ag bound by the FDC Cr1 protein is critical for this pathway to proceed in an optimal manner, and C3 is a required ligand. Further analysis of bone marrow chimera animals, as well as characterization of DZ- and LZ-associated B cells (via differential expression of CXCR4 and CD83) (42), will help us to determine the specific signaling defect associated with the absence of Cr1.

Functions associated with the Cr2 gene products have also been implicated in the development and progression of systemic lupus erythematosus, although the specific pathway(s) of Cr1/Cr2 control have not been described (43, 44). The loss of Cr1 on the surface of FDCs, resulting in the loss of positive selection of ac-

and IgG from TNP-KLH (100 μg)–immunized mice shown in (A) (n = 10; 8–12-wk-old sex-matched mice). (C) ELISA analysis of SRBC-specific IgM and IgG from mice immunized i.v. with 1 × 10^9 SRBCs on days 0 and 21 (n = 6; sex-matched 8–10-wk-old mice). *WT versus Cr1KO, †WT versus Cr1/2KO, and /p < 0.05, **/p < 0.01, ***/p < 0.001; one-way ANOVA was performed for each day, and Tukey multiple-comparison posttest results are shown for all time points for which p ≤ 0.05 by ANOVA.
tivated B cells, may allow such cells to escape into the periphery. The mutations in the VDJ region associated with the presence of the B cell in the D2 is random, such that the generation of autoreactive B cells could easily proceed. In fact, the Ig genes obtained from autoreactive B cells demonstrate a high level of VDJ somatic hypermutation (43–46). If the absence of Cr1 expression by the FDCs allows such cells to exit the GC without selection, then the dissemination of autoreactive B cells into the periphery of the animal may be enhanced. Experiments to test this hypothesis are underway.

In summary, these data provide two new findings in support of the model that expression of Cr2 gene products by FDCs and B cells is required for optimal Ag-specific B cell activation and Ab production to TD Ags: the novel discovery that Cr1 is preferentially expressed by FDCs, whereas Cr2 is preferentially expressed by B cells, and the fact that Cr1 is more integral to GC B cell activation and response to TD Ags than TI responses. The similarity and differences between deletion of Cr1 alone or Cr1/2 together highlight the importance and functional independence of these similar proteins and emphasize the significance of the new Cr1KO mouse line. These findings are relevant for the optimization of adjuvants targeted to FDCs for the enhancement of humoral immunity (47).

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental. Optimal Germinal Center B cell Activation and T-dependent Antibody Responses Require the Expression of the Mouse Complement Receptor Cr1

Supplemental Figure 1. Representative histograms of CD19 expression on bone marrow, peritoneal, and splenic B cells. A and B, Total bone marrow, peritoneal cavity, or splenic cells were isolated from naïve WT (filled histogram/black bars), Cr1/2KO (gray line histogram/gray bars), and Cr1KO (black line histogram/white bars) mice and surface CD19 expression was analyzed by FACS. A, Representative histograms of CD19 expression on pro-B, pre-B, immature, and mature B cells (top four plots); B1a and B1b cells from the peritoneal cavity (middle two plots); and total splenic B cells (bottom plot). B, Quantification of the geometric mean fluorescent intensity (gMFI) of CD19 staining by FACS analysis on B cell populations from the bone marrow, peritoneal cavity, and total splenic B cells. Bars represent the average gMFI for each genotype. CD19 gMFI was analyzed by ANOVA for each population and determined to be at least p<0.05 where significance is shown pairwise (* =p<0.05, ** = p<0.01, and ***=p<0.001 by the student’s t-test; n=3,4; sex matched 8-12 week old mice).

Supplemental Figure 2. RT-PCR quantification of splenic inflammatory gene expression. Intron spanning primer sets specific for arachidonate 5-lipoxygenase (Alox5), D site albumin promoter binding protein (Dbp), cathepsin G (Ctsg), coagulation factor V (Fv), lipocalin 2 (Lcn2), and killer cell lectin-like receptor, subfamily A, member 18 (Klra18) were used to quantify gene expression in mRNA obtained from total splenic extracts. All sample quantifications were normalized to 1000 β-actin transcripts. Bars represent the mean fold change from WT (center line) for Cr1/2KO (black) and Cr1KO (white). (WT n=3, Cr1KO and Cr1/2KO n=4; 12-16 week old female mice; BALB/C background; Error bars represent SEM; *=p<0.05, **=p<0.01, ***=p<0.001 by student’s t-test).

Supplemental Figure 3. TI-1, TI-2, and TD antigen specific immunoglobulin responses. Experiments were performed in which WT, Cr1/2KO, and Cr1KO mice
were immunized with the model antigens (A) TNP-LPS (TI-1) and (B) DNP-AECM-Ficoll (TI-2), as well as (C) TNP-KLH (TD) at both a low dose immunization (10µg) and high dose immunization (100µg). All mice that were immunized with TNP-KLH were administered a secondary immunization at 21 days. Serum samples were collected at seven day intervals post-immunization, and antigen specific immunoglobulin (IgM, IgG1, IgG2b, IgG2c, and IgG3) abundance was measured by ELISA. All data graphed here are included in the larger data set shown in Fig.9. (TI-1 n=4-7; TI-2 n=6; TD (low) n=4; TD (high) n=5; 8-12 week old sex matched mice; * = p<0.05; ** = p<0.01 significance between WT and Cr1KO by student’s t-test).

**Supplemental Figure 4.** FACS analysis of C3KO GC B cells following SRBC immunization. Percentage of live B220+ splenocytes that are IgDint Fas+ in SRBC immunized WT and C3KO mice compared to mock PBS immunized mice and representative dot plots. (n=8 immunized, n=2 PBS; Error bars represent SEM; ns=not significant; *=p<0.05, **=p<0.01, ***=p<0.001 by student’s t-test).
Supplemental Figure 1

A

Pro-B cells

Immature B cells

Pre-B cells

Mature B cells

CD19

% of Maximum

Bone Marrow

Peritoneal cavity

Spleen

B

MFI (Geometric)

Pro-B

Pre-B

Immature B

Mature B

CD19

% of Max

0

20

40

60

80

100

Total Live B220+

CD19 gMFI

***

WT

Cr1/2KO

Cr1KO

B1a

B1b

CD19 MFI

1000

1500

2000

WT

Cr1/2KO

Cr1KO

***

CD19 gMFI

50

100

150

***

WT

Cr1/2KO

Cr1KO
Supplemental Figure 2
Supplemental Figure 3

A  TI-1  TNP-LPS

Absorbance (490nm)

IgM  IgG1  IgG2b  IgG2c  IgG3

B  TI-2  DNP-AECM-Ficoll

Absorbance (490nm)

IgM  IgG1  IgG2b  IgG2c  IgG3

C  TD  TNP-KLH (10µg)

Absorbance (490nm)

IgM  IgG1  IgG2b  IgG2c  IgG3

TD  TNP-KLH (100µg)

Absorbance (490nm)

IgM  IgG1  IgG2b  IgG2c  IgG3

WT  Cr1/2KO  Cr1KO  Cr1K
Supplemental Figure 4

The figure shows a flow cytometry analysis comparing wildtype (WT) and C3KO genotypes under different treatments (SRBC and PBS). The x-axis represents Fas expression, while the y-axis represents IgD expression. The graph depicts the percentage of IgD^int Fas^+ cells for each genotype and treatment group. The black bar represents WT - SRBC, the white bar represents C3KO - SRBC, the gray bar represents WT - PBS, and the gray-filled bar represents C3KO - PBS. Significant differences are indicated by asterisks (* and **) on the graph.