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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 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 0 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

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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., 2012a,b), 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., 2012a,b).

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 μl. Immunization with APC was i.p. injection of 30 μg APC (ProZyme Inc., Hayward, CA) in 200 μl 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 0.15 M $NaHCO_3$ buffer at 1 mg/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 h 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 13,000 rpm 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 h 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/trimethroprim 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., 2012a,b). Total splenocytes were isolated by straining spleens through 100 μm mesh strainers into ice cold 0.5% BSA 2 mM EDTA PBS (FACS buffer), pelleted by centrifugation, and resuspended in ACK red blood cell lysis buffer. Cells were repelleted and washed with 10 ml of ice cold PBS. Cells were then stained with 2.5 μg/ml APC (ProZyme Inc., Hayward, CA) in 2% rat serum PBS with rat anti-CD16/32 (Fc block) for 30 min on ice in the dark. A 12.5 ml volume of FACS buffer was added to the cell mix and the cells were pelleted by centrifugation. APC-stained cells were incubated in a 500 μl volume of FACS buffer with 50 μl of anti-APC microbeads (Miltenyi Biotec Inc., Aubrun, CA), covered in the dark for 15 min. Cells were washed with an additional 12.5 ml of