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Functional Activity of Natural Antibody is Altered in Cr2-Deficient Mice[1]

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The major source of natural IgM Abs are B-1 cells, which differ from conventional B cells in their anatomic location, cell surface phenotype, restricted usage of particular V_H genes and limited use of N-region addition during V-D-J rearrangement. The origin of B-1 cells is unclear. However, they are capable of self-renewal and their development is sensitive to signaling via the B cell receptor, as genetic defects that impair the strength of the signal often result in limited development. These findings suggest that B-1 cells require either an intrinsic signal, or contact with Ag, for positive selection and expansion and/or maintenance in the periphery. In support of interaction with cognate Ag, deficiency in the complement receptors CD21/CD35 results in a 30–40% decrease in the CD5$^+$ B-1 population. To determine whether this reduction reflects a loss of certain specificities or simply a proportional decline in the repertoire, we examined peritoneal B cells isolated from Cr2$^+$ and Cr2^{def} mice for recognition of a B-1 cell Ag, i.e., phosphatidylcholine, and assayed for injury in an IgM natural Ab-dependent model of reperfusion injury. We found a similar frequency of phosphatidylcholine-specific CD5$^+$ B-1 cells in the two strains of mice. By contrast, the Cr2^{def} mice have reduced injury in the IgM-dependent model of reperfusion injury. Reconstitution of the deficient mice with pooled IgM or adoptive transfer of Cr2$^+$ peritoneal B cells restored injury. These results suggest that complement receptors CD21/CD35 are important in maintenance of the B-1 cell repertoire to some, but not all, specificities. ***The Journal of Immunology,* 2002, 169: 5433–5440.**

The existence of Abs in the sera of normal animals, termed "natural Abs," (1) has been acknowledged for decades, yet the overall function and biological relevance of these molecules remains unclear. The products of germline Ig gene expression, natural Abs have been shown to have a multispecificity for self- and foreign Ags (2, 3). Natural Abs are involved in various potential roles in homeostasis, such as senescent red cell removal (4), regulation of autoimmunity (5–8), and primary immune defenses (9–13). Qin et al. (13) have suggested that B-1 cells undergo a secondary V(D)J recombination of Ig genes within the periphery as a mechanism to limit autoreactivity. Thus, given the predisposition of B-1 cells for self-Ags, these cells undergo receptor editing in the periphery to escape self-reactivity.

Natural Abs are difficult to define due to their characteristically low affinity and polyreactivity. All B cell subsets are capable of producing natural Abs; CD5$^+$ B-1 cells have been identified as a major source in humans (14) and mice (15). CD5$^+$ B-1 cells are readily distinguishable from "conventional" B-2 cells by their surface phenotype, their ability for self-renewal, their anatomic location (peritoneum vs spleen) and their restricted usage of particular V_H genes (16). For example, over 90% of the natural Ig product against phosphatidylcholine (PtC),[4] a ubiquitous self-Ag, is derived from CD5$^+$ B-1 cells through restricted V_H/V_κ unmutated germline genes (V_H11-$V_\kappa 9$ or V_H12-$V_\kappa 4$) (17).

Despite these unique features, little is certain regarding B-1 cell ontogeny, development, and function. Analyses of knockout mice reveals that defects in B cell receptor (BCR) signaling have a more pronounced affect on the survival of B-1 cells than on conventional B-2 cells. For example, a deficiency in BCR signaling protein CD19 (18, 19), Bruton's tyrosine kinase (20), or *vav* (21, 22), or chronic treatment of mice with the Ab specific for CD19 (from birth), leads to a loss of B-1 cells (23). Moreover, segregation of PtC-specific B-1 cells into the B-1 subset occurs after BCR expression (24). These findings suggest that B-1 cells require intrinsic BCR signaling for maintenance in the repertoire. Alternatively, B-1 cells might require an encounter with cognate Ag in their initial positive selection, expansion, or survival (24, 25). Examination of knockout mice homozygous-deficient in complement receptors CD21/CD35 (Cr2^{def}) revealed a partial deficiency in the CD5$^+$ subpopulation of B-1 cells (26), although in another line of Cr2^{def} mice, no reduction in B-1 cells was identified (27). Although both lines have a similar defect in binding activation products of complement C3 (26–30), the former appears to express a low level of the truncated form of the receptor (31).

CD21 and CD35 are expressed primarily on B lymphocytes and follicular dendritic cells (FDC) and are encoded at a single locus (*Cr2*) (32, 33). Both receptors bind complement ligands iC3b and C3d and form a coreceptor signaling complex on B cells along with CD19 and CD81 (34). Mice deficient in CD21/CD35 have impaired humoral responses to thymus-dependent (26–29, 35, 36)

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[4] Abbreviations used in this paper: PtC, phosphatidylcholine; BCR, B cell receptor; FDC, follicular dendritic cell; I/R, ischemia/reperfusion; RAG, recombination-activating gene; WT, wild type; PI, permeability index; PEC, peritoneal exudate cells.

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and -independent Ags (30). This defect is largely due to the absence of coreceptor signaling, although receptor expression on FDCs is also important in humoral immunity (37). B cell encounter of C3-coated Ag complexes results in coligation of the BCR and CD21/CD19/CD81 coreceptor, the effect of which not only lowers the threshold for B cell activation (38), but also provides a survival signal to germinal center B cells (39).

The reduction in the frequency of $CD5^+$ B-1 cells in $Cr2^{def}$ mice may stem from a general requirement for complement during the initial expansion or maintenance of the cell. Alternatively, complement and coreceptor signaling might be required only for certain low affinity Ags. Given this apparent reduction in $CD5^+$ B-1 cells and the relative importance of CD21/CD35 in regulation of humoral responses, we examined serum IgM and $CD5^+$ B-1 cells isolated from $Cr2^{def}$ mice for binding of PtC and injury in vivo using a model for IgM-dependent ischemia-reperfusion (I/R) injury (40, 41). I/R injury represents an inflammatory response following ischemia in diverse tissues such as the intestine, hind limb, or myocardium (42). Injury is largely dependent on complement, as treatment of mice with a soluble inhibitor of complement C3 blocks full injury (43). Mice deficient in C3 are also protected from full injury (40). Complement appears to be activated by the classical pathway, as mice deficient in C4 are protected to a degree similar to that of $C3^{def}$ mice (40). The finding that mice totally deficient in lymphocytes (recombination-activating gene (RAG)-1^{def}) are also protected, and that reconstitution with pooled IgM restores injury, supports an important role for natural IgM (41). Although the initiating Ag/Ags expressed on ischemic tissues are unknown, the I/R model provides a functional assay to evaluate the repertoire of natural IgM in $Cr2^{def}$ mice.

Examination of serum IgM and $CD5^+$ B-1 cells isolated from $Cr2^{def}$ mice for binding of PtC and intestinal I/R injury in vivo revealed a similar frequency of PtC^+ $CD5^+$ B-1 cells in $Cr2^+$ and $Cr2^{def}$ mice. By contrast, $Cr2^{def}$ mice were protected from reperfusion injury, despite normal levels of serum IgM. Full sensitivity to reperfusion injury was restored following reconstitution of the deficient mice with pooled IgM from wild-type (WT) mice or adoptive transfer of $Cr2^+$ peritoneal B cells. Furthermore, in the I/R model, a direct comparison of RAG-2^{def} mice reconstituted with IgM prepared from either WT or $Cr2^{def}$ mice revealed reduced pathogenic activity in the later.

Materials and Methods

Mice

The construction and generation of $Cr2^{def}$ mice was achieved through embryonic stem cell gene targeting as described (26). To verify deficiency, we tested tail DNA from the parents of all mice by Southern blot analysis. Male mice, 5–8 wk old, were used in all intestinal I/R experiments. WT control mice represent C57BL-6/129 $Cr2^{+/+}$ littermates generated by crossing C57BL-6/129 Cr2 heterozygotes. Mice serving as donors for analysis of peritoneal B-1 cells were 4- to 5-wk-old female $Cr2^+$ and $Cr2^{def}$ mice crossed onto a C57BL/6 background for 10 generations. Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School (Boston, MA) and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Department of Health, Education and Human Services, publication no. 85-23 (National Institute of Health), revised 1985).

Model of intestinal I/R injury

Surgically induced intestinal I/R has been described elsewhere (44). Briefly, mice anesthetized with pentobarbital i.p. (90 mg/kg) underwent laparotomy. Ligation of the jejunum and associated mesenteric vessels was achieved by application of a 10-cm loop tourniquet composed of 3-0 Prolene suture (Ethicon, Somerville, NJ) and 1-cm lengths of intramedic polyethylene tubing (BD Biosciences, Parsippany, NJ) used as a snugger. Ischemia was confirmed by lack of mesenteric pulsations and intestinal pallor. Incisions were closed and ischemia proceeded for 40 min. Each mouse received 1 μCi of ^{125}I-labeled BSA i.v. 5 min before the reperfusion phase. Soluble complement receptor type 1-treated animals received 20 mg/kg protein simultaneously with radio-labeled BSA. After reopening the incisions and removing the tourniquets, mice were kept warm with heating pads for 3 h. All mice received 0.2 ml of saline i.v. 1 h after initiation of reperfusion. Sham-treated controls underwent a similar procedure without jejunal ligation.

Following euthanasia, blood was collected via cardiac puncture and jejunal loops were harvested, counted for radioactivity, and dried to constant weight for 3 days at 90°C. The intestinal permeability index (PI) was determined using the following ratio: (cpm/gram jejunal loop)/(cpm/gram blood). Mice were followed for 5 days after treatment for survival studies.

Reconstitution of $Cr2^{def}$ mice with purified WT IgM

The purification of normal mouse IgM from whole serum has been described previously (11). An immunoaffinity approach was used. Whole serum, obtained from either inhouse C57BL-6/129 mice or from commercial sources (EQUITECH-BIO, West Ingram, TX), was first precipitated by $(NH_4)_2SO_4$ (50% saturation). The precipitate was dissolved in 1× PBS (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.25) and dialyzed against 100 volumes of the same buffer. Recovered dialysate was then filtered through a 0.45-μm syringe filter (Acrodisc; Gelman Sciences, Ann Arbor, MI) and applied to a 2-ml protein G column (Gammabind Sepharose; Pharmacia Biotech, Uppsala, Sweden). After several passages over protein G, the flow-through was applied to a 4–8 ml anti-IgM agarose column (Sigma-Aldrich, St. Louis, MO). Eluates were obtained (0.1 M glycine-HCl, 0.15 M NaCl, pH 2.5) and neutralized. Peak fractions were pooled, dialyzed, and concentrated against 1× PBS using vacuum concentration (Schleicher & Schuell, Keene, NH). Simultaneous purification of murine IgG was achieved by elution of bound material from the protein G column in a similar fashion. Samples were assessed by 10% SDS-PAGE (45) for purity.

Before ischemic challenge, $Cr2^{def}$ mice were reconstituted with 0.35–0.4 mg of purified WT IgM via the retro-orbital plexus. Control $Cr2^{def}$ mice received an equal volume of HBSS (Sigma-Aldrich) i.v., serving as mock-reconstituted controls. A third group received 0.35–0.4 mg of WT IgG.

Reconstitution of $Cr2^{def}$ and RAG-1^{def} mice with peritoneal B cells

Peritoneal B cells were obtained via peritoneal lavage of WT mice. Following euthanasia of 2- to 3-mo-old C57BL-6/129 mice, peritoneal lavage was performed using 5–10 ml ice-cold HBSS/1% BSA per mouse. The recovered cells were pooled, washed, and resuspended in lavage buffer before reconstitution. Trypan blue (Sigma-Aldrich) counterstained cells were counted via hemocytometer for viability. Approximately 1×10^6 cells were injected i.p. into 3- to 4-wk-old $Cr2^{def}$ male mice in a volume of 0.5 ml/mouse. Age-matched RAG-1^{def} recipients (C57BL6/129 background) were used to determine the efficiency of reconstitution.

To verify successful reconstitution of CD21/CD35-deficient mice, peritoneal exudate cells (PEC) were harvested from reconstituted mice at 6–8 wk time points, erythrocyte-depleted via Ficoll-Hypaque separation (Ficoll PLUS; Pharmacia Biotech), washed with HBSS/10 mM EDTA, and incubated with biotin-conjugated B220 and 7E9, a monoclonal directed against CD35.

Immunohistological analysis of jejunal sections following I/R injury

Cryostat serial sections of intestinal tissue from saline-reconstituted, IgM-reconstituted, or peritoneal cell-reconstituted CD21/CD35-deficient mice were analyzed via immunoperoxidase labeling with goat anti-mouse IgM and C3 (5 μg/ml) (Organon Teknika, Durham, NC) and a previously described avidin-biotin protocol (46). WT, sham-treated WT, and $Cr2^{def}$ mice served as controls.

Flow cytometry analysis

PEC isolated from $Cr2^+$ and $Cr2^{def}$ mice were obtained as previously described (26). Approximately 1×10^6 cells were washed twice in PBS containing 2% FCS and 0.02% sodium azide and subsequently incubated with a mixture containing either FITC-encapsulated anti-IgM, PE-conjugated anti-CD19 mAb (CD19-PE; BD PharMingen, San Diego, CA), and allophycocyanin-conjugated anti-CD5, or FITC-conjugated liposomes containing PtC- (a gift of Dr. L. A. Herzenberg, Stanford University, Palo Alto, CA), PE-conjugated CD19, and allophycocyanin-conjugated CD5 for 30 min on ice. After washing, cells were incubated with propidium iodide to identify dead cells. A fluorescence analysis was performed with a FACSCalibur (BD Biosciences) as described (26).

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Statistics

PIs represent mean ± SD calculated using the Student *t* test. Comparison of survival following I/R over a 5-day period was performed by the Mantel-Cox rank test. Student's *t* test was used to evaluate differences between the frequencies of peritoneal $CD5^+/CD19^+$ B-1 cells in $Cr2^{def}$ vs WT controls. Differences were considered statistically significant when $p < 0.05$.

Results

I/R injury is significantly reduced in $Cr2^{def}$ mice

Although $Cr2^{def}$ mice bear normal levels of serum IgM and serum complement proteins, we proposed that the specificity of natural IgM might be altered given the general impairment in B cell activation and the apparent reduction in $CD5^+$ B-1 cells. To test this hypothesis, deficient mice were analyzed in an intestinal I/R model and compared with mice deficient in complement proteins C3 ($C3^{def}$) or C4 ($C4^{def}$). In this model, ischemia is induced by ligating the jejunum and mesenteric vessels for 40 min with a tourniquet as described (36). Before release of the tourniquet, mice were injected with radio-labeled albumin. The extent of injury was evaluated after 3 h of reperfusion based on increased permeability, i.e., the ratio of radio-labeled protein in the blood vs dried tissues. Injury was also assessed by histopathology. As expected, injury was reduced in $C3^{def}$ and $C4^{def}$ mice relative to WT controls (Fig. 1*a*) (35, 36). Interestingly, mice deficient in Cr2 were also partially protected, as the PI of the mutant mice was ~43% that of $Cr2^+$ controls. These findings correlate with a significant increase in 5-day survival following treatment, i.e., 80 and 20% for $Cr2^{def}$ and $Cr2^+$, respectively (Fig. 1*b*).

To examine direct binding of complement and IgM to intestinal tissue following reperfusion of ischemic tissues, an immunohistological approach was followed. Microscopic examination of the intestinal mucosa harvested from treated $Cr2^{def}$ mice demonstrated normal villous architecture with intact brush borders (Fig. 2, *c* and *d*). By contrast, the mucosa of $Cr2^+$ controls was characterized by significant necrosis (Fig. 2, *a* and *b*). Immunohistological analysis of tissues revealed abundant deposits of C3 and IgM on the vascular endothelium of treated $Cr2^+$ mice. By contrast, both proteins were significantly reduced on tissues from $Cr2^{def}$ mice (Fig. 2, *c* and *d*). Thus, deficiency in complement receptors CD21/CD35 was protective in the intestinal I/R model.

Reconstitution of $Cr2^{def}$ mice with purified IgM restores injury

To determine whether protection is due to a cellular or a serum defect, $Cr2^{def}$ mice were reconstituted with 0.4 mg of pooled IgM i.v. before treatment. A similar concentration of IgM, which is below physiologic levels in normal serum (~1–2 mg), was previously shown to restore injury in treated $RAG\text{-}1^{def}$ mice (41). Reconstitution of $Cr2^{def}$ animals resulted in a reversal of protection equivalent to that of $Cr2^+$ animals, based on PIs of the two groups (Fig. 3) and histopathology of the jejunal mucosa (Fig. 2, *e* and *f*). Most interestingly, reconstituted $Cr2^{def}$ mice exhibit C3 and IgM deposits on the vascular endothelium of ischemic tissues (Fig. 2, *e* and *f*). It is important to note that IgM is the isotype of natural Ab required to produce injury, as IgG reconstitution of deficient mice had no pathogenic effect (Fig. 3). These analyses provide firm evidence that nonimmune $Cr2^+$ serum contains a natural IgM that

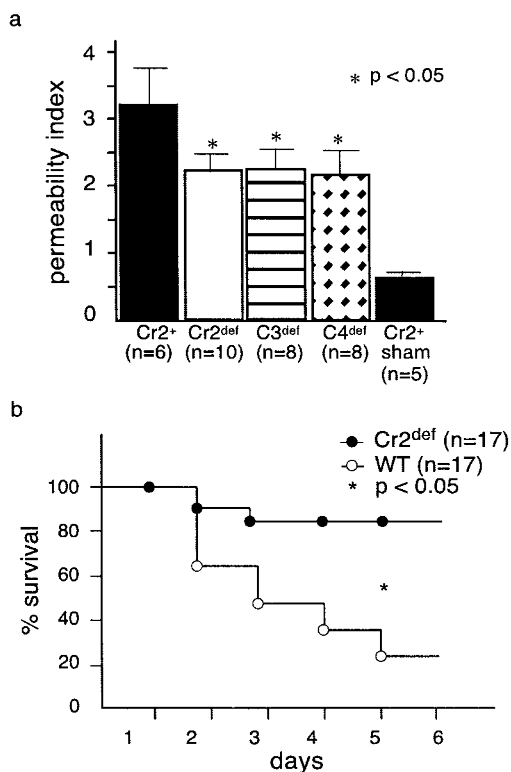

FIGURE 1. *a*, Mice deficient in complement receptors CD21/CD35 are protected from intestinal I/R to the same degree as C3- and C4-deficient mice. Mice were subjected to intestinal reperfusion as described in *Materials and Methods*. The level of injury was evaluated by PI (see *Materials and Methods*). Error bars represent means ± SE; n = number of mice in experimental groups; *, significance at $p < 0.05$. *b*, Survival of $Cr2^+$ mice (WT) following intestinal I/R is significantly decreased compared with $Cr2^{def}$ mice. Mice were treated in the intestinal I/R protocol and assessed for 5-day survival. Statistical comparison was performed by the Mantel-Cox rank test; *, significance at $p < 0.05$.

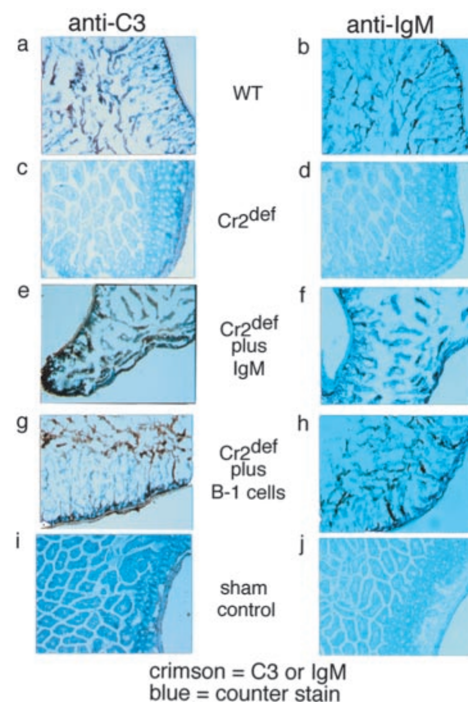

FIGURE 2. Representative cryosections of intestinal tissues harvested from experimental mice or sham controls following intestinal I/R. *Left* and *right panels* were stained with anti-C3 or anti-IgM, respectively. *a* and *b*, WT ($Cr2^+$) mice treated for I/R; *c* and *d*, $Cr2^{def}$ mice treated for I/R; *e* and *f*, $Cr2^{def}$ mice reconstituted with 0.4 mg pooled IgM each before I/R; *g* and *h*, $Cr2^{def}$ mice reconstituted with peritoneal B-1 cells 6–8 wk before I/R; *i* and *j*, WT sham control-no treatment.

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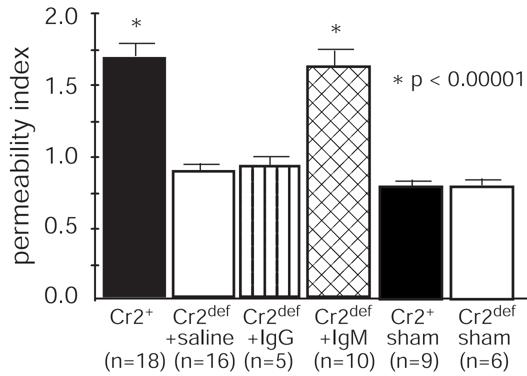

FIGURE 3. Intestinal permeability of inbred mice after intestinal I/R or no injury (sham). Cr2^{def} mice were reconstituted with pooled IgG or IgM or saline control and then subjected to the intestinal I/R. Error bars represent means ± SE; n = number of mice in experimental groups; *, significance at $p < 0.05$.

is specific against postischemic tissue, and is missing in Cr2^{def} mice despite their normal level of IgM.

Cr2^{+} peritoneal B cells restore injury in Cr2^{def} mice

To determine whether Cr2^{+} peritoneal B-1 cells are a source of pathogenic autoantibody in the intestinal I/R model, peritoneal B cells from adult Cr2$^{+/+}$ mice were harvested and transferred i.p. into 3- to 4-wk-old MHC-gender- and age-matched Cr2^{def} (WT PEC→Cr2^{def}) or RAG-1^{def} (WT PEC→RAG-1^{def}) mice. Because RAG-1^{def} mice are totally deficient in B lymphocytes and serum Ig, they served as controls for ascertaining transfer of peritoneal B cells and confirming that B-1, but not B-2, cells make up the dominant population of B lymphocytes. Indeed, within 6 wk after engraftment of 1×10^{6} cells, the levels of serum IgM in RAG-1^{def} recipients were ~50% that of normal (data not shown). To confirm that B-1 cells were the major B cell subset, spleen, peritoneal, and mesenteric lymph node cells were harvested from the WT PEC→RAG-1^{def} chimeric mice 6–8 wk following transfer. Monocytic cells were treated with mAbs specific for IgM, CD11b (B-1 cell marker), and CD23 (B-2 cell marker) and examined by FACS. As expected, the majority of the surviving IgM^{+} B cells expressed the B-1 cell phenotype, i.e., IgM^{+}/CD11b^{+}/CD23^{-} and <2% of B cells stained positive for CD23; i.e., IgM^{+}/CD11b^{+}, 95.7 ± 1.9% SD and IgM^{+}/CD23^{+}, 1.2 ± 0.9% SD for WT PEC→RAG-1^{def} vs IgM^{+}/CD11b^{+}, 49.6 ± 5.3% SD and IgM^{+}/CD23^{+}, 43.9 ± 5.5% SD for B6 (Fig. 4*a*). Forster et al. (47) made a similar observation reporting that Ly-1^{+} (CD5), but not Ly-1^{-}, peritoneal B cells were long-lived and self-replenishing. Thus, Cr2^{def} chimeric mice (WT PEC→Cr2^{def}) were considered reconstituted primarily with donor Cr2^{+} B-1 cells. The presence of donor B-1 cells in chimeras was confirmed by two-color flow cytometric analysis following treatment with mAbs specific for B220 and CD21/CD35. By 6 wk posttransfer, >50% of the CD5^{+} peritoneal B cells stained positive for CD21/35 (Fig. 4*b*).

Characterization of the WT PEC→Cr2^{def} chimeric mice in the reperfusion model revealed a dramatic increase in injury, equivalent to WT and IgM-reconstituted knockout mice, and significantly higher than untreated Cr2^{def} mice (Fig. 4*c*). Restoration of injury was confirmed by histological examination of the intestinal tissue of treated chimeric mice. The pathology observed in treated WT PEC→Cr2^{def} chimeras was similar to that of IgM recipients and WT controls; murine jejunal mucosa was marked by necrosis as well as the deposition of C3 and IgM (Fig. 2, *g* and *h*). Presumably, IgM detected by immunoperoxidase staining is derived from Cr2^{+}

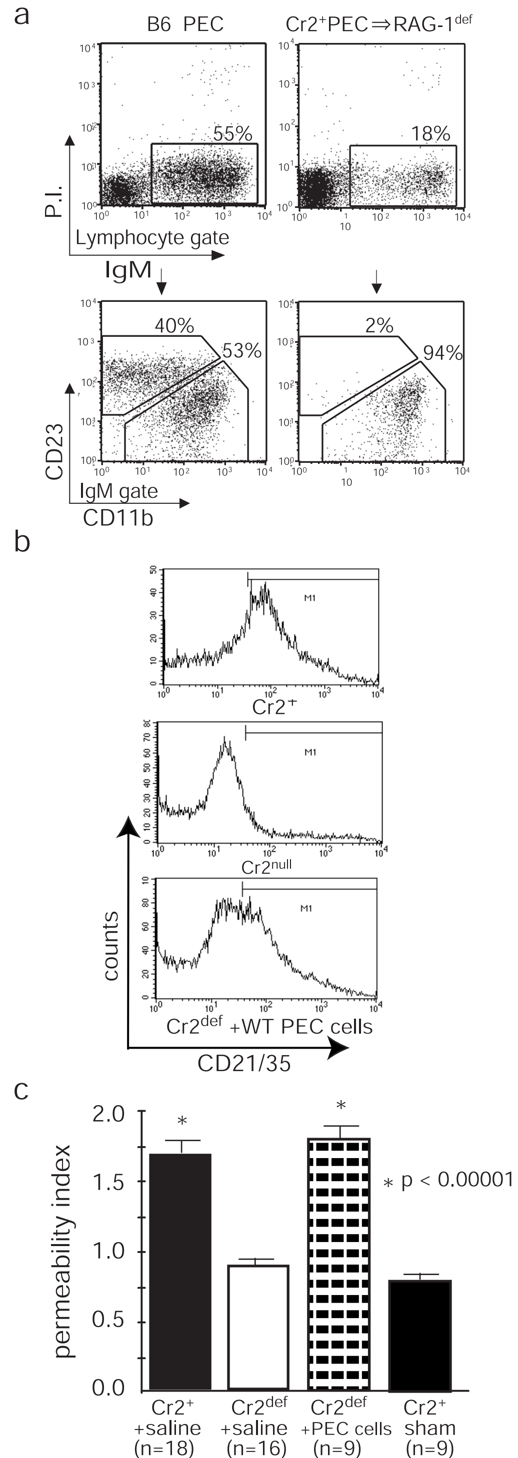

FIGURE 4. Adoptive transfer of PEC cells into Cr2^{def} mice restores reperfusion injury to WT levels. *a*, Adoptive transfer of PEC (C57BL/6) into B6.RAG-1^{def} mice restores IgM^{+}CD11b^{+} B-1, but not IgM^{+}CD23^{+}, compartment. PEC were harvested from C57BL/6 mice and Cr2^{+}PEC→RAG-1^{def} chimeras 6–8 wk following transfer and analyzed by FACS for B-1 (IgM^{+}/CD11b) and B-2 (IgM^{+}/CD23^{+}) B populations. *b*, Analysis of peritoneal B cells by FACS identifies Cr2^{+} B cells in WT PEC→Cr2^{def} chimeras. PEC were harvested, treated with anti-B220, and CD21/CD35 Ab and analyzed by FACS. Histograms identify gated B220^{+} cells analyzed for expression of CD21/CD35 and are representative of the nine chimeric mice analyzed. *c*, Cr2^{def} mice reconstituted with WT PEC (WT PEC→Cr2^{def}) develop injury after intestinal I/R model. Mice were treated in the intestinal I/R model as described (*Materials and Methods*). Tissues were harvested and analyzed for PI. Error bars represent means ± SE; n = number of mice in experimental groups; *, significance at $p < 0.05$.

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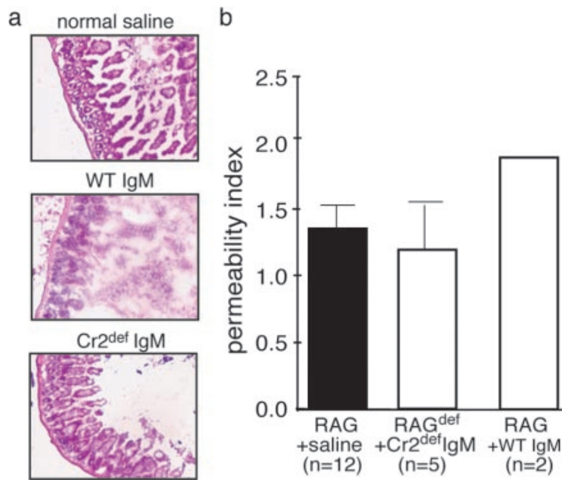

FIGURE 5. Serum IgM of Cr2^{def} mice does not restore full I/R injury in RAG-1^{def} mice. *a*, RAG-1^{def} mice were reconstituted with either normal saline or 0.4 mg of purified IgM isolated from sera of WT or Cr2^{def} mice. Mice were treated for I/R injury (see *Materials and Methods* and *Results* for description) and inflammation was assessed by histopathology of cryosections prepared from jejunum tissue. Results indicate apparent absence of injury in saline and Cr2^{def} IgM-treated mice. By contrast, extensive necrosis is observed in mice treated with WT IgM, as reported earlier (36). Results are representative of at least three mice in each group. *b*, RAG-1^{def} mice were reconstituted as in *a* and treated for I/R injury. Following reperfusion, jejunum tissues were evaluated for permeability (see *Materials and Methods* and *Results* for description). Reconstitution of RAG-1^{def} mice with purified WT IgM resulted in increased PI, as previously reported (36). By contrast, reduced injury is observed in jejunum samples harvested from mice receiving either Cr2^{def} IgM or saline. Results represent mean PI with error bars indicating SE of the mean; n = number of mice used in each experiment.

peritoneal CD5$^+$ B-1 cells, as ungrafted mice fail to stain positive (Fig. 2*d*). We concluded that Cr2$^+$ donor B-1 cells are a major source of the I/R pathogenic autoantibody that is missing or reduced in the Cr2^{def} animals.

IgM isolated from Cr2^{def} mice is not pathogenic

To directly assess the presence of pathogenic IgM in the sera of Cr2^{def} mice, RAG-1^{def} mice were reconstituted with pooled IgM and tested in the intestinal I/R model. Ig-deficient RAG-1^{def} mice were reconstituted with 0.4 mg of purified IgM isolated from the sera of Cr2^{def} or Cr2$^+$ mice. Injury was evaluated by PI and histopathology as described above. The PI index of RAG-1^{def} mice administered WT IgM was increased relative to saline controls, as previously reported (36), while the PI of RAG-1^{def} mice receiving pooled IgM prepared from Cr2^{def} mice was similar to that of saline controls (Fig. 5*b*). Examination of tissue sections from the region of ischemia revealed extensive necrosis of the villous architecture in RAG-1^{def} mice reconstituted with IgM prepared from WT, as reported (36). By contrast, negligible injury was observed in mice reconstituted with saline or Cr2^{def} IgM (Fig. 5*a*). Thus, based on two different assays, the IgM isolated from Cr2^{def} mice does not appear to induce I/R injury.

Normal frequency of PtC-specific CD5$^+$ B-1 cells in Cr2^{def} mice

A common specificity of B-1 cells is PtC; 10–15% of mouse peritoneal B-1 cells are specific for this common membrane Ag (48, 49). To directly assess the frequency of peritoneal CD5$^+$ B-1 cells in Cr2$^+$ and Cr2^{def} mice, PECs were isolated from 4- to 5-wk-old female mice and treated with mAbs in combination with PtC-bear-

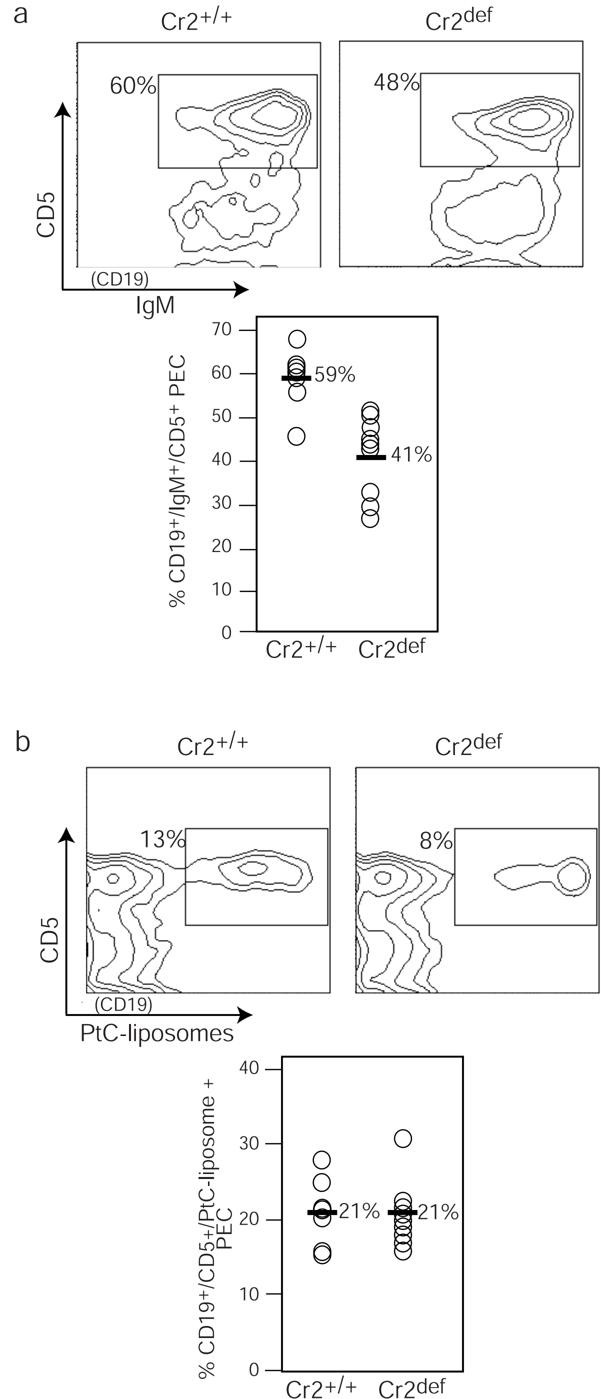

FIGURE 6. Relative frequency of PtC CD5$^+$ B-1 cells is not affected by absence of CD21/CD35. *a*, The contour plots are representative of lymphocytes gated for expression of the pan-B cell marker CD19 and negative for propidium iodide to exclude dead cells. PECs were stained with Abs specific for CD19, IgM, and CD5 to determine frequency of CD5$^+$ B-1 subset. Percentages indicate frequencies of gated cells of all CD19$^+$ peritoneal cells from Cr2$^+$ or Cr2^{def} mice. Scatter plot identifies a significant ($p < 0.0005$) reduction in CD5$^+$ B-1 cells in Cr2^{def} relative to Cr2$^+$ mice on a C57BL/6 background (10 generations). *b*, The frequency of PtC-specific cells was determined by staining PEC with CD19, CD5, and FITC$^+$ PtC-liposomes. The scatter plot indicates a similar frequency of PtC-binding CD5$^+$ B-1 cells in Cr2$^+$ and Cr2^{def} mice. Relative frequency of PtC-binding cells among CD5$^+$ B-1 cells was calculated by dividing mean frequency of CD5$^+$/PtC$^+$ cells by the frequency of CD19$^+$/IgM$^+$/CD5$^+$ PEC for each strain. Each circle represents a single mouse (4- to 5-wk-old female mice; n = 10); horizontal bar represents mean ± SE. *, Statistical significance.

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ing liposomes encapsulated with FITC. As previously reported (26), a significant decrease in the mean frequency of CD5$^+$ B-1 cells in Cr2^{def} mice was observed, i.e., 59 vs 41% in Cr2$^+$ and Cr2^{def}, respectively ($p < 0.0005$; Fig. 6*a*). Three-color FACS analysis of the CD5$^+$ B-1 population (CD5$^+$/CD19$^+$) for PtC-liposome binding predictably revealed that the CD5$^+$ B-1 subpopulation bound PtC, whereas negligible binding was observed in the CD5$^-$ B-1 and conventional B-2 populations (Ref. 49; Fig. 6*b*). Interestingly, comparison of the frequency of PtC$^+$ CD5$^+$ B-1 cells between the two groups of mice indicated a similar mean frequency of PtC-binding B cells (Fig. 6*b*). Thus, despite an overall reduction in CD5$^+$ B-1 cells in the Cr2^{def} mice, the frequency of PtC-binding B cells among the CD5$^+$ population was similar, i.e., 21% for both.

Discussion

The in vivo experimental system of I/R injury revealed that mice deficient in CD21/CD35 failed to mount an inflammatory response when compared with Cr2$^+$ controls, as assessed by PIs (Fig. 1*a*), survival (Fig. 1*b*), and histopathology (Fig. 2). Reconstitution of Cr2^{def} or RAG-1^{def} mice with purified WT IgM, but not IgG, restored I/R injury (Figs. 2, *e* and *f*, and 3). Therefore, WT serum contains an autoantibody (or autoantibodies) that recognizes ischemic endothelium and initiates injury via classical pathway complement. Interestingly, 0.35–0.4 mg of IgM, <40% of the normal level of serum IgM, was sufficient to restore injury in CD21/CD35-deficient animals. Therefore, the 40–45% reduction in CD5$^+$ B-1 cells observed in Cr2^{def} mice is an unlikely explanation for reduced injury. Our results suggest that the natural Ab repertoire is altered in the deficient mice.

An earlier report identified a significant reduction in peritoneal CD5$^+$ B-1 cells in Cr2^{def} mice on a mixed C57BL-6/129 background (26), although in a similar study using the same line of mice, no reduction in this subset of peritoneal B-1 cells was reported (31). In the current study, analysis of Cr2^{def} mice bred onto a B6 background for 10 generations also revealed a significant reduction (~30–40%) in peritoneal CD5$^+$ B-1 cells, i.e., 59 vs 41% mean frequencies for Cr2$^+$ and Cr2^{def}, respectively. Given the reduction in CD5$^+$ B-1 cells in the Cr2^{def} mice, it seemed likely that B-1 cells were a possible source of pathogenic natural IgM. Adoptive transfer of 1×10^6 peritoneal B cells from unimmunized Cr2$^+$ mice to Cr2^{def} mice fully restored normal levels of injury (Figs. 2, *g* and *h*, and 4*c*). Chimerism was confirmed by expression of CD21/CD35 on the majority of CD5$^+$ B-1 cells harvested at 6–8 wk posttransfer (Fig. 4*b*). Analysis of lymphoid tissues harvested from WT PEC→RAG-1^{def} chimeras 6–8 wk following transfer revealed that CD11b$^+$ B-1 cells were the major subset of surviving B cells and that <2% were B-2 (Fig. 4*a*). Because full injury is restored in the WT PEC→Cr2^{def} chimeric mice, we concluded that B-1 cells are a primary source of I/R pathogenic Ab, although we cannot rule out a role for conventional B-2 cells in normal mice. Studies by Forster and Rajewsky (47), through transfer of CB.20 peritoneal cells to the allotype-specific congenic mouse strain BALB/c, revealed that the percent of donor-derived natural IgM in recipient mice posttransfer was 30% at week 10 and 50% at week 20. Similarly, reconstitution of RAG-1^{def} mice with 1×10^6 peritoneal B cells led to serum IgM concentrations ~50% those of normal by 6 wk posttransfer (R. R. Reid and M. C. Carroll, unpublished observation). Considering the kinetics of natural Ab production by donor cells, and the dramatic reversal of protection seen in Cr2^{def} chimeric mice, it can be surmised that only minimal concentrations of specific natural Ab are required for the induction of injury.

The finding that there was no difference in the frequency of PtC-binding CD5$^+$ B-1 cells isolated from Cr2$^+$ and Cr2^{def} mice suggests that complement is not involved in the development or maintenance of this population. Although considered primarily a common self-Ag, PtC or cross-reacting Ags are also common among bacteria. Mice deficient in IgM are highly susceptible to acute septic peritonitis, but reconstitution with a PtC-specific hybridoma is partially protective (9).

Reperfusion injury represents an acute inflammatory response following ischemia that can affect both local and remote tissues (42). In at least four of the tissues examined, i.e., intestinal (41, 50–53), hind limb (40, 44), myocardium (54), and CNS (55), injury is complement-dependent. Complement appears to be activated by the classical pathway and injury appears to be IgM-dependent in at least the hind limb (40) and intestinal models (41, 56). However, other mediators, such as mannan-binding protein, can also activate early complement components C1-C3 and could be involved in pathology following formation of the initial cellular injury by natural Ab (57). Classical pathway activation of C3 could trigger the alternative pathway and might be important in amplification of the cascade (51). It will be important to examine injury in mice bearing a defect in alternative pathway components, such as factor D-deficient mice (58).

The finding that Cr2^{def} mice are protected, but that reconstitution with normal IgM restores injury, supports the model that pathogenic IgM is specific and not the result of binding of polyspecific natural Ab. Identification of a specific clone or clones of B cells that secrete pathogenic IgM will be important in the development of a possible therapy to block injury and to identify tissue Ags involved in injury. Whether the specificity of pathogenic IgM is similar for all tissues or varies for each tissue type, i.e., intestine, myocardium, or hind limb, cannot be determined from these studies. Identification of the specific ischemia Ag or specific B cells will hopefully resolve this important question.

In a recent study, Holers and colleagues (56) reported that their line of Cr2^{def} mice was protected from injury in the intestinal I/R model based on necrosis of intestinal mucosa, infiltration of neutrophils, and release of inflammatory mediators such as leukotriene β_4 and peroxidase. In their study, restoration of full injury required both IgM and IgG. They also found that based on pathology and C3 deposition, reconstitution with normal IgM alone restored injury, in concordance with observations in our study. However, normal IgG was required for neutrophil influx and release of mediators such as peroxidase and leukotriene β_4. The latter parameters were not examined in our study. Thus, both lines of Cr2^{def} mice appear to have an altered repertoire of natural Ab.

The combined results from the analysis of two B-1 cell assays support the hypothesis that the complement system is important in shaping the repertoire of IgM natural Ab to certain, but not all, Ags. Complement and its receptors CD21/CD35, could be involved at several distinct stages in B-1 cell development. B-1 cells are thought to derive from a positive selection event at the immature B cell stage in the fetal liver and early neonatal period (24, 25). Because they are self-renewing, contact with cognate Ag may be important for B-1 cell expansion and/or maintenance within the repertoire. CD21/CD35 receptors are expressed primarily on B cells (late transitional and mature stages) and FDCs (44, 59). Because repopulation of Cr2^{def} mice with Cr2$^+$ B-1 cells restores I/R pathogenic IgM, and these cells are maintained in the Cr2^{def} chimeras, it seems unlikely that CD21/CD35 expression on stromal (FDC) and myeloid cells is required for the maintenance of B-1 cells. Therefore, it is more probable that the role of complement is to enhance early positive selection or expansion of B-1 cells during neonatal development.

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In summary, the repertoire of natural Ab in Cr2^{def} mice is limited by at least one specificity, i.e., I/R pathogenic Ab. Adoptive transfer of peritoneal B cells or passive transfer of pooled IgM can restore the deficiency. By contrast, no reduction in the frequency of CD5^{+} PtC-binding CD5^{+} B-1 cells is observed in the deficient mice. We propose that the complement system is critical in shaping the repertoire of IgM natural Ab to certain, but not all, Ags.

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