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Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells

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Abstract

Secreted IgM and complement are important mediators in the optimal initiation of primary T-dependent humoral immune responses. Secreted IgM serves as a natural adjuvant by enhancing the immunogenicity of protein antigens, perhaps as a result of IgM's ability to facilitate antigen deposition onto follicular dendritic cells (FDCs) and promote rapid germinal center (GC) formation. To understand how IgM enhances adaptive immune responses, we investigated the mechanism by which IgM-containing immune complexes (IgM-IC) are transported to FDCs as a first step in GC formation. We demonstrate that IgM-IC localize first to the splenic marginal zone (MZ) where the IgM-IC bind MZ B cells in a complement and complement receptor (CR1/2) dependent process. MZ B cells then transport the IgM-IC into the follicle for deposition onto FDCs. Mice with reduced numbers of MZ B cells trap IgM-IC on FDC less efficiently, whereas mice with reduced numbers of follicular B cells trap IgM-IC normally. The functional elimination of MZ B cells abrogates the ability of FDCs to trap IgM-IC. Transfer of B cells with associated IgM-IC into naive mice results in deposition of IgM-IC onto FDC by MZ B cells. The results demonstrate an IgM and complement-dependent role for MZ B cells in the fate of antigen early in the initial phases of T-dependent immune responses. The data also establish an important role for CR1/2 on MZ B cells in the efficient binding and transport of IgM-IC to FDCs, which we suggest is an important first step in initiating adaptive immune responses.

Introduction

Secreted IgM plays an important role in the initiation of adaptive humoral immune responses. Mice deficient in secreted IgM ($\mu_s^{-/-}$) have immune defects characterized by their inability to respond to low concentrations of protein antigens, delayed production of protective antibody responses to pathogens, and delayed germinal center formation (1–5). In the absence of secreted IgM, pathogens fail to concentrate into secondary lymphoid organs and instead disseminate into vital organs (6). The adjuvant activity of IgM is complement dependent and, consequently, mice deficient in components of the classical complement pathway or in the complement receptors CR1/2 (CD21/35) (7) have defects similar to those observed in $\mu_s^{-/-}$ mice (8–14). Because specific IgM enhances immune responses to particulate and soluble antigens (5,15–17), the defects in adaptive immune responses in $\mu_s^{-/-}$ mice can be attributed, at least in part, to the failure of other secreted Ig isotypes to compensate for IgM function.

To investigate the role that IgM plays in enhancing immune responses, we established a system to evaluate the immunogenicity and fate of IgM-containing immune complexes (IgM-IC). Antigen associated with IgM, but not antigen alone, stimulated primary immune responses and primed for secondary immune responses (5). IgM-IC localized first in the splenic marginal zone (MZ) and then concentrated onto follicular dendritic cells (FDCs) within 16 h of i.v. injection. The localization of antigen on FDCs has been implicated in optimizing the formation of germinal centers (GC), memory B cell formation and somatic hypermutation (18–21).

The complement receptors CR1/2 are important for the localization of antigen to FDCs (19,22–25). Consistent with this, we found that FDC localization of IgM-IC was not observed in *Cr2*$^{-/-}$ mice or in mice depleted of the complement component C3. Instead, IgM-IC localized exclusively to the MZ where they were bound by the phagocytic marginal zone macrophages (MZM) for removal (5). Interestingly,

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IgM-IC exhibited a different distribution on cells within the MZ in the presence of an intact complement system. In complement and CR1/2 sufficient mice, IgM-IC were not only associated with MZM, but with other cells in the MZ (5). Thus, both complement and CR1/2 play important roles in the fate of the IgM-IC in the MZ and in FDC localization. Collectively, these results suggested that the IgM-IC that were not associated with MZM in complement sufficient mice might instead be associated with a cell or cells responsible for the transport of IgM-IC from the MZ for deposition onto FDCs.

To test this hypothesis, we characterized the cells which bind IgM-IC, determined the mechanism by which IgM-IC are transported to FDCs and further examined the consequences of IgM on immune responses to antigen. We provide evidence that immunization with IgM-IC induces rapid GC formation. We also demonstrate that MZ B cells participate in the initiation of T-dependent immune responses as a result of their capacity to bind and transport IgM-IC into the B cell follicle, and deposit these IgM-IC onto FDCs. While their positioning in the MZ favors their initial interaction with IgM-IC, both *in vivo* and *in vitro* analyses demonstrate that MZ B cells are also particularly adept at binding large amounts of IgM-IC as a result of their increased expression of CR1/2. For binding and transport of IgM-IC, both complement and the CR1/2 are essential, providing a mechanistic basis for the role of complement and CR1/2 in the transport of IgM-IC from the MZ and deposition onto FDCs in the B cell follicle, initiating primary immune responses and facilitating GC formation.

Methods

Mice

C57BL/6J (B6), C57BL/6J-*Igh^{a}Thy1^{a}Gpi1^a* (B6-Igh^a) and CD19-deficient (CD19$^{-/-}$) mice (26) were purchased from Jackson Laboratories (Bar Habor, ME). Breeding pairs of secreted IgM-deficient ($\mu_s^{-/-}$) mice (1) were provided by Dr Jianzhu Chen (Center for Cancer Research, MIT, Cambridge, MA). Breeding pairs of *Cr2*-deficient mice (12) were provided by Dr Ann Marshak-Rothstein (Department of Microbiology, Boston University School of Medicine) from mice originally provided by Dr Michael Carroll (The Center for Blood Research, Harvard Medical School, Boston, MA). IL-7$^{-/-}$ mice (27) were obtained from DNAX Research Institute (Palo Alto, CA). B6-Igh^a, *Cr2*$^{-/-}$, IL-7$^{-/-}$ and $\mu_s^{-/-}$ mice were bred and maintained in the Laboratory Animal Sciences Center, Boston University Medical Center. Mice of both sexes were used at 8–12 weeks of age. C3-depleted mice were produced by treatment with cobra venom factor (CVF) (Quidel Corp., San Diego, CA) as described (5). For *in vivo* depletion of MZ B cells, mice were injected i.v. with 30 ng pertussis toxin (PTx; List Biological Laboratories, Campbell, CA) 2 days before use as described (28).

Preparation and injection of IgM-IC in vivo

Mice were injected i.v. with a mixture of 1 μg each of NP-KLH (Biosearch Technologies, Novato, CA) and affinity purified pentameric B1-8 IgM mAbs or NP-KLH alone, exactly as previously described (5), to monitor GC formation. For immunohistochemistry (IH), 15–20 μg NP_7–BSA–biotin (Biosearch Technologies) in a mixture with equivalent amounts of NP-specific IgM were injected i.v. This increased concentration was required for visualization of the antigen. While IgM-IC efficiently localize to FDC, similar amounts of antigen alone cannot be detected on FDCs (unpublished data) (5).

In vitro IgM-IC binding assay and transfer experiments

B cells were enriched from the spleens of B6 or B6-Igh^a mice by depletion of T cells with mixtures of anti-CD8 and anti-CD4 antibodies and complement (29). Macrophages were depleted by plastic adherence. IgM-IC were prepared by first incubating 1 μg each of NP_7–BSA–biotin and B1-8 pentameric IgM for 30 min at 37°C in DMEM containing 10% freshly isolated mouse serum as a source of complement, or with DMEM containing 10% heat inactivated (56°C, 30 min) mouse serum to deplete complement (30). 10^7 splenic B cells were then resuspended in preformed IgM-IC and incubated an additional 30 min at 37°C. Cells were then washed twice with PBS containing 1% FBS and resuspended for flow cytometry. For the transfer experiment, 5×10^7 cells were incubated with 20–25 μg IgM-IC prepared as described above. After washing cells, the cells were resuspended in 250 μl PBS and injected i.v. into recipient mice as described in Results.

Immunohistochemistry

Staining of spleen sections was performed as previously described (5). Briefly, spleens were removed at the indicated times and snap frozen in O.C.T. compound (Tissue Tek, Torrance, CA) and stored at −80°C. Six-micrometer sections were cut (IEC Microtome, Needham, MA) and fixed in acetone at −20°C for 10 min. For staining, sections were incubated with 0.3% H_2O_2/PBS for 5 min then blocked with anti-Fcγ II/III receptor antibody (2.4G2, BD Biosciences, San Diego, CA) diluted 1/100, or 3% normal rabbit or rat serum. All antibodies were diluted in 5% BSA in PBS. NP_7–BSA–biotin was visualized by alkaline phosphatase (AP)–streptavidin (1/400; Molecular Probes, Eugene, OR) and developed with napthol AS–MX phosphate/Fast blue BB base (Sigma, St Louis, MO). Visualization of antibody stains was accomplished using horseradish peroxidase (HRP)-conjugated secondary antibodies developed with 3-amino-9-ethylcarbazole (Sigma) and H_2O_2. Primary antibodies used included: anti-IgM^a–FITC (1/100), anti-B220–FITC (1/100), anti-CD3–FITC (1/100), anti-CD3–biotin (1/100), anti-CD1d–FITC (1B1; 1/50), anti-CD21/35–FITC (1/50), anti-λ (1/50) (all from BD Biosciences), MOMA-1 (1/25; Serotec, Raleigh NC), ER–TR9 (1/200; BMA Biomedical, Augst, Switzerland), anti-FDC–M1 (1/200; provided by Steve Bogan, Department of Pathology, Boston University School of Medicine) and anti-FDC–M2 (1/200; Immunokontact, Frankfurt, Germany). GCs were identified using FITC-labeled peanut agglutinin (PNA; EY Laboratories, San Mateo, CA). Secondary antibodies included anti-FITC–HRP (1/100; Roche Molecular Biochemicals, Indianapolis, IN), goat anti-rat IgG–HRP (1/500; Jackson Immunoresearch, West Grove, PA) and goat anti-rat IgM $(Fab')_2$–HRP (1/1000; Jackson Immunoresearch). After staining, sections were covered with crystal mount (Biomedia, Foster City, CA) and visualized. Unless otherwise noted, a 10× objective was used for the initial magnification. In all figures, different antibody

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combinations were always performed on serial sections. For immunofluorescence, antibodies included anti-IgM-Texas Red (1/100; BD Biosciences) and anti-laminin (1/500; Sigma) visualized using an anti-rabbit IgG-FITC (1/100; Jackson Immunoresearch).

Flow cytometry

Splenocytes were isolated at the indicated times and stained as follows. The lymphocyte gate (as defined by forward and side scatter characteristics) was utilized for all analysis. Cells were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ) and FlowJo software (Tree Star, Ashland, OR). Antibodies utilized were anti-B220–FITC (1/100), anti-B220–biotin (1/100), anti-B220–PE (1/100), anti-CD21/35–FITC (1/100), anti-CD23–PE (1/100) and anti-CD1d–FITC or PE (1/50) (BD Biosciences). Streptavidin–PE-Cy5 (1/200; BD Biosciences) was used to identify the NP antigen (NP_7–BSA–biotin) associated with lymphocytes, as well as to visualize biotin-labeled antibodies.

Results

IgM-IC promote rapid GC formation

Mice lacking secreted IgM fail to mount rapid antibody responses to antigens and exhibit delayed GC formation (1,2). Because the addition of IgM to T-dependent antigens restores the ability of these mice to mount primary and secondary immune responses (5), we first asked if these mice developed GCs. Mice ($\mu_s^{-/-}$) immunized with IgM-IC, but not antigen alone, developed GC as determined by PNA staining 7 days later (Fig. 1). These GC corresponded with intense regions of staining for the λ light chain, as expected of the clonally restricted response to NP (31) and the pauciclonal nature of GC responses to this hapten (32). Therefore, one of the consequences of IgM-IC immunization is the rapid development of GC which contribute to the evolution of the immune response to T-dependent antigens.

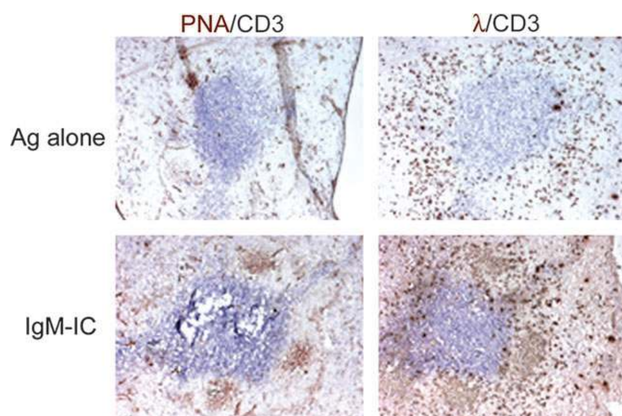

Fig. 1. IgM-IC promote GC formation. $\mu_s^{-/-}$ mice were injected i.v. with 1 μg NP-KLH alone or complexed with 1 μg NP-specific IgM. Spleens were harvested at day 7 and IH was performed using PNA or anti-λ light chain antibody (red-brown stain) and anti-CD3–biotin (blue stain). Mice injected with IgM-IC but not antigen alone have PNA positive cells adjacent to the T cell zone, in areas corresponding to λ light chain positive B cells, expected of the NP-specific antibody response.

IgM-IC associate with MZ B cells in vivo

In complement sufficient mice, IgM-IC are found first in the MZ and then localize to FDCs (5). To investigate how IgM-IC are transported from the MZ to FDCs, IgM-IC were injected into mice and the cell-associated IgM-IC in the spleen was analyzed 1 h later by flow cytometry. As shown in Fig. 2(A), MZ B cells, whether identified by their CD21/CD23 profile ($CD21^{hi}CD23^{lo}$) (33,34) or by CD1d expression (35,36), consistently bound more IgM-IC than the corresponding FO B cells. Neither immature B cells ($CD21^{lo}CD23^{lo}$) nor T cells showed appreciable binding of IgM-IC (data not shown). These results indicate that MZ B cells efficiently bind blood-borne IgM-IC that filter through the spleen. As expected, IH showed that most of the IgM-IC were localized within the MZ 1 h after injection (Fig. 2B). However, some IgM-IC could already be detected inside the marginal sinus (lined by the $MOMA-1^+$ marginal metallophilic macrophages) (37) and within the follicle in the first hour after injection (see arrow). Some of these IgM-IC were already associated with $CD21^{hi}$ cells, consistent with FDC localization.

To determine if IgM-IC remained associated with MZ B cells, spleens of mice were removed 4 h after injection and the amount of cell-associated IgM-IC was assessed. As shown in a representative experiment (Fig. 2C), we have consistently been unable to isolate B cells with associated IgM-IC 4 h after injection. At this time, much of the IgM-IC is found in the follicles associated with FDCs, as determined by IH (Fig. 2D). In other experiments, we have found that IgM-IC are associated with MZ B cells 2 h after injection, but at diminished levels compared with the 1 h time point as assessed by FACS (data not shown).

An intact complement system is required for efficient binding of IgM-IC to MZ B cells

IgM-IC do not localize to FDCs in complement-deficient and *Cr2*$^{-/-}$ mice, but instead are slowly removed from the MZ by MZM (5). We reasoned, therefore, that a major determinant of the initial binding of IgM-IC to MZ B cells might be the interaction of complement components with CR1/2. To test this, C3-depleted or *Cr2*$^{-/-}$ mice were injected with IgM-IC and, 1 h later, the association of IgM-IC with splenic B cells was assessed. As shown in Fig. 3, little if any IgM-IC bound to MZ or FO B cells (distinguished by their CD1d phenotypes) in either the C3-depleted (Fig. 3A) or *Cr2*$^{-/-}$ (Fig. 3C) mice, indicating that C3 and CR1/2 play a crucial role in the binding of IgM-IC to these B cells. In agreement with previous results (5), all of the IgM-IC detected by IH was restricted to the MZ in these mice (Fig. 3B and D). Together, these results indicate that the binding of IgM-IC to MZ B cells is dependent on an intact complement system.

MZ B cells bind more IgM-IC than FO B cells in vitro

The results indicate that IgM-IC associate primarily, if not exclusively, with MZ B cells *in vivo*, and can be interpreted in at least two, non-mutually exclusive, ways. First, MZ and FO B cells might have similar capacities to bind IgM-IC, but the positioning of the MZ B cells within the MZ, the first site of encounter of blood borne IgM-IC, would favor IgM-IC binding to this B cell subset. Alternatively, MZ B cells might have an

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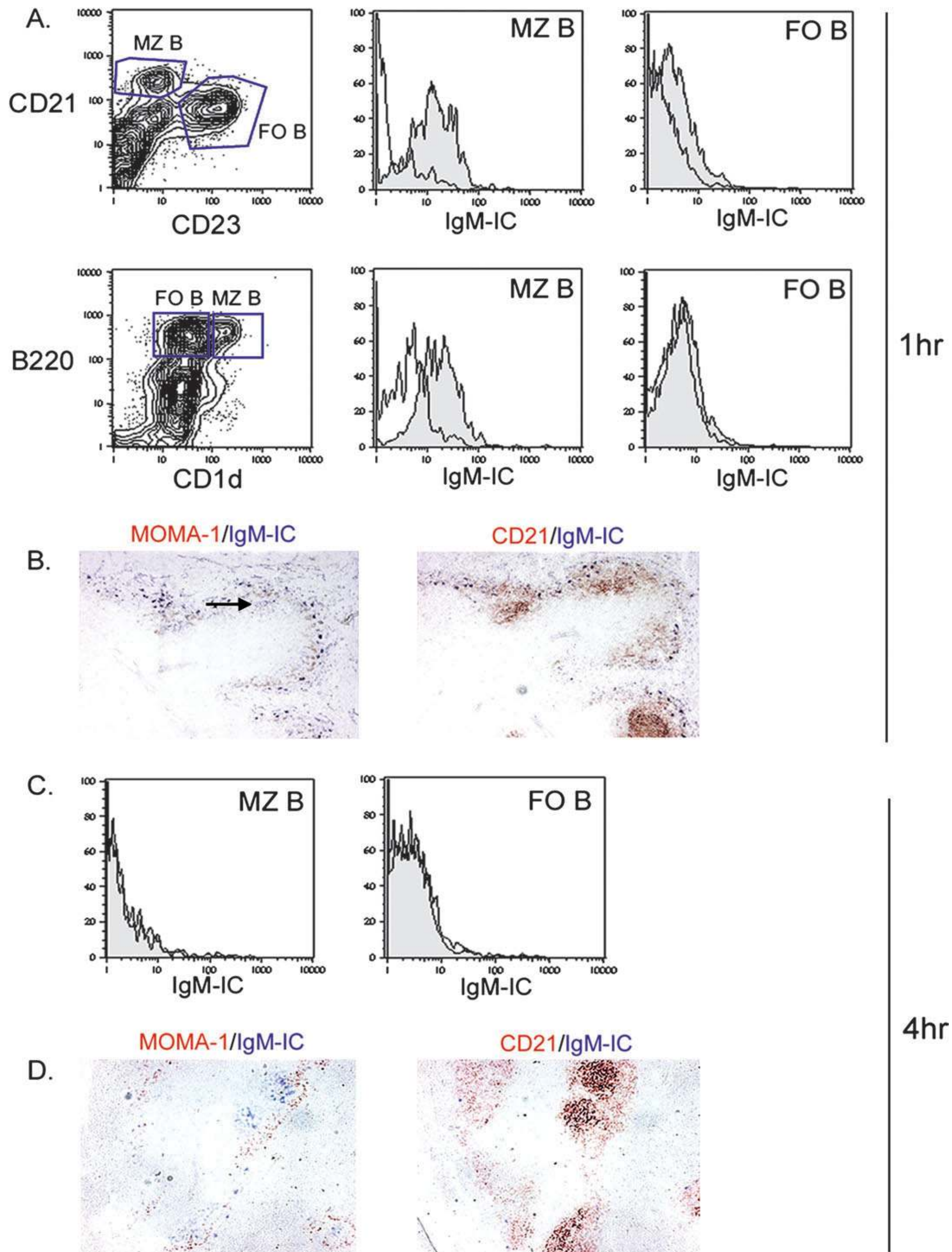

Fig. 2. IgM-IC transiently associate with MZ B cells *in vivo*. $\mu_s^{-/-}$ mice were injected with IgM-IC and spleens were collected 1 and 4 h later with half of the spleen frozen for IH and the other half processed for flow cytometry. (A) FACS analysis 1 h post-injection identifying MZ and FO B cells using a CD21/23 or B220/CD1d expression profile. IgM-IC were detected on these cells by streptavidin–PE-Cy5 which recognizes NP–BSA–biotin in the IgM-IC. The shaded area represents IgM-IC bound to B cells from injected mice while clear regions represent uninjected mice. (B) IH analysis of the other half of the spleen from (A) demonstrates the presence of IgM-IC (stained blue in this and all subsequent figures; see Methods) in the MZ, which lies outside of the MOMA-1$^+$ marginal metallophilic macrophages (stained red-brown) surrounding the B cell follicle. The arrow indicates the presence of IgM-IC within the follicle. (C) Binding of IgM-IC to MZ B cells is not detected 4 h after injection, MZ and FO B cells were defined by the CD21/23 profile and analyzed for IgM-IC binding as described in (A). (D) By 4 h, most of the IgM-IC is concentrated around FDCs, as defined by the intense CD21/35 staining (red-brown). Experiments repeated more than five times gave similar results.

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inherently greater capacity to bind IgM-IC than FO B cells due to their higher level of expression of CR2 (34). To discriminate between these alternatives, the ability of MZ and FO B cells to bind IgM-IC was compared *in vitro*. Since the presence of complement is essential for binding of IgM-IC to MZ B cells *in vivo* (Fig. 3A), IgM-IC were prepared in the presence of fresh mouse serum. As shown in Fig. 4(A), IgM-IC could bind both MZ and FO B cells. However, MZ B cells consistently bound

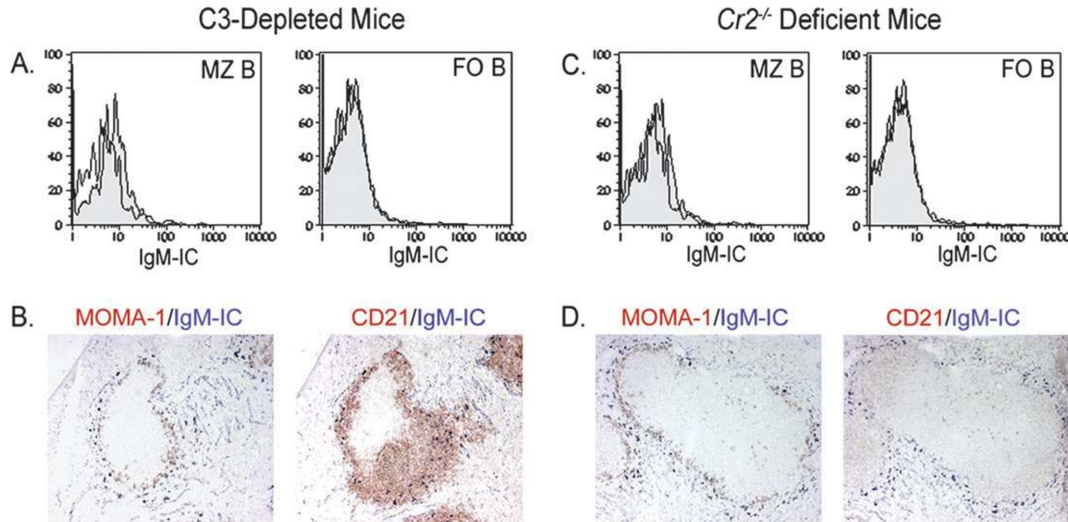

Fig. 3. Complement and complement receptors are essential for the binding of IgM-IC to MZ B cells. (A and C) FACS analysis of splenocytes from a C3-depleted $\mu_s^{-/-}$ mouse (A) or *Cr2*$^{-/-}$ mouse (C) 1 h after injection of IgM-IC. MZ and FO B cells were delineated using B220/CD1d staining and bound IgM-IC was visualized as described in Fig. 2(A). (B and D) IH analysis of the other half of the spleen used in (A) and (C) shows IgM-IC localized to the MZ, but without any IgM-IC inside the MOMA-1$^+$ macrophage ring. This localization contrasts with the pattern in complement sufficient mice (see Fig. 2B).

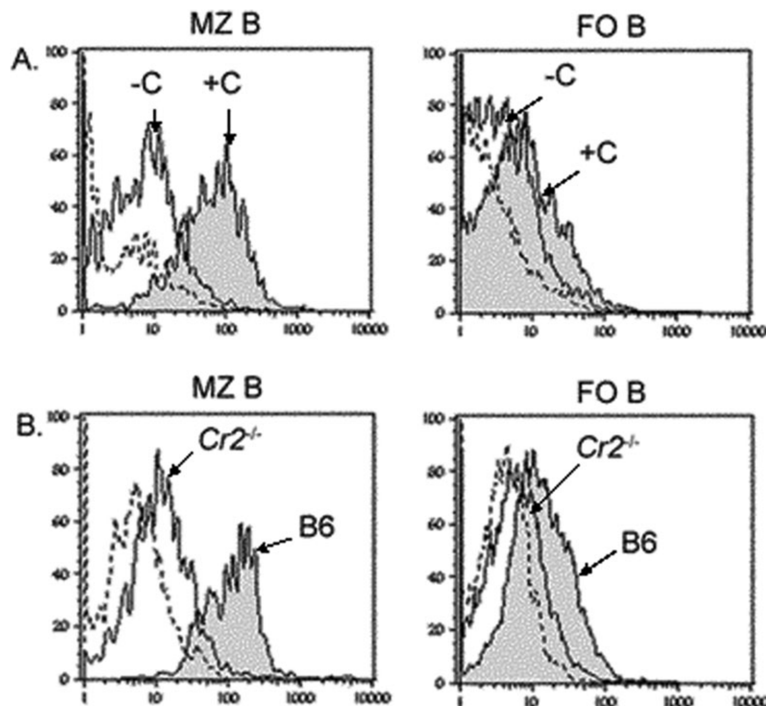

Fig. 4. MZ B cells bind more IgM-IC than FO B cells *in vitro*, and the binding is complement and CR1/2 dependent. (A) Analysis of the ability of MZ and FO B cells to bind IgM-IC *in vitro*. Splenic B cells were incubated with IgM-IC (1 μg each of NP–BSA–biotin and anti-NP IgM) for 30 min at 37°C. The incubations were performed in the presence of fresh mouse serum as a source of complement (shaded area identified by +C), or with heat inactivated serum (clear area identified by –C) as described in Methods. B cells incubated in media alone served as the negative control (represented by the dotted line). Flow cytometry was used to examine IgM-IC association with MZ and FO B cells, defined by their CD21/CD23 profile, as described in Fig. 2. (B) Comparison of the ability of B cells from normal (B6) and *Cr2*$^{-/-}$ mice to bind IgM-IC. B cells were incubated with IgM-IC as described in (A). MZ and FO B cells were defined by CD1d gating on B220$^+$ cells as described in Fig. 2.

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more IgM-IC than FO B cells (estimated to be 5–10-fold more by MFI from >20 independent experiments), despite the fact that MZ B cells represent only a minor subset of splenic B cells. These results demonstrate that MZ B cells have a higher intrinsic capacity to bind IgM-IC.

To confirm the dependence of IgM-IC binding on complement and CR1/2, the ability of B cells to bind IgM-IC was first compared when IgM-IC were prepared in the presence (fresh mouse serum) or absence (heat-inactivated serum) of complement. As shown in Fig. 4(A), the ability of both MZ and FO B cells to bind IgM-IC was severely impaired when IgM-IC were prepared in the absence of complement. In addition, the ability of both MZ and FO B cells from *Cr2*$^{-/-}$ mice to bind IgM-IC was greatly diminished compared with mice expressing intact CR1/2. These results support the *in vivo* data demonstrating an important role for CR1/2 for the binding of IgM-IC by MZ B cells.

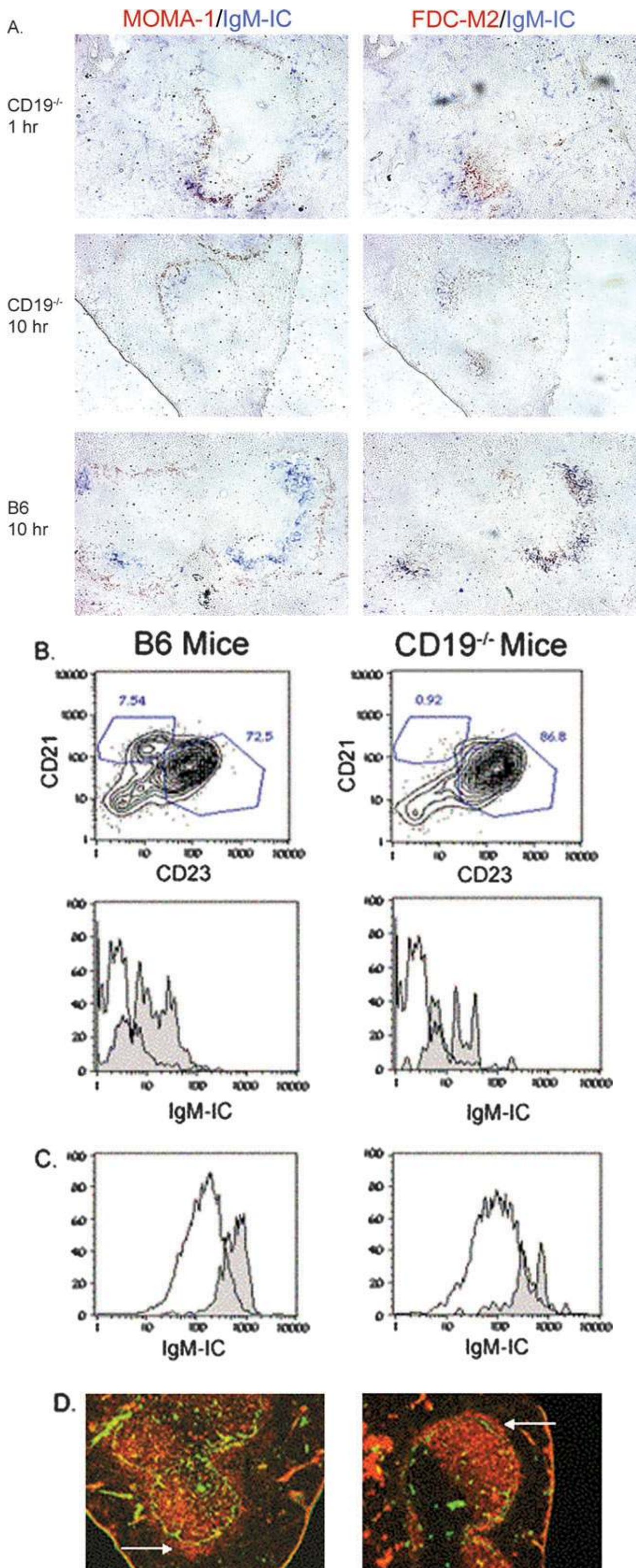

Fig. 5. CD19$^{-/-}$ mice have reduced numbers of MZ B cells and exhibit decreased efficiency in IgM-IC localization on FDC. (A) IH shows IgM-IC localization in CD19$^{-/-}$ mice at 1 and 10 h after injection. At 1 h, IgM-IC localize to the MZ, but fewer IgM-IC localize to FDCs as compared with WT B6 mice. (B) FACS profile of B6 and CD19$^{-/-}$ splenocytes showing association of IgM-IC *in vivo* to MZ B cells, as defined in Fig. 2(A). (C) *In vitro* binding of IgM-IC to B6 and CD19$^{-/-}$ MZ (shaded) and FO (clear) B cells. (D) Immunofluorescence of uninjected B6 and CD19$^{-/-}$ spleen sections with arrows identifying IgM^{+} cells in the MZ, demarcated by laminin staining. IgM appears red and laminin appears green.

Role of MZ B cells for efficient trapping of IgM-IC on FDC

To examine the requirement for MZ B cells in the deposition of IgM-IC on FDC, several approaches were taken. First, we examined the fate of IgM-IC in the spleens of CD19$^{-/-}$ mice, reported to be deficient in MZ B cells (36,38). CD19$^{-/-}$ mice were injected with IgM-IC and spleens examined by IH 1 and 10 h later. As shown in Fig. 5(A), IgM-IC localized first to the MZ of these mice and, within 10 h, some IgM-IC were found on FDCs. However, the intensity of staining of IgM-IC in the splenic follicles of CD19$^{-/-}$ mice was substantially reduced compared to B6 mice, suggesting that much less IgM-IC reached the FDCs of the CD19$^{-/-}$ mice.

To confirm that CD19$^{-/-}$ mice truly lacked MZ B cells, FACS analysis was performed (Fig. 5B). The number of MZ B cells was drastically reduced in these mice. However, some B cells did fall within the MZ gate, and these exhibited high levels of IgM-IC binding, consistent with that of MZ B cells from B6 mice (Fig. 5B, lower panels). This suggested that CD19$^{-/-}$ mice have small numbers of functional MZ B cells. To substantiate their presence and function, an *in vitro* binding assay was performed. B cells from CD19$^{-/-}$ mice that fell within the MZ B cell gate exhibited high levels of IgM-IC binding *in vitro*, at levels similar to that of MZ B cells from control mice (Fig. 5C). Finally, to verify that these putative MZ B cells could be found in the MZ of CD19$^{-/-}$ mice, immunofluorescence was performed using laminin staining to demarcate the MZ (39). As shown in Fig. 5(D), spleen follicles of CD19$^{-/-}$ mice contained IgM^{+} cells in the MZ, although in reduced numbers compared with normal mice. Therefore, although CD19$^{-/-}$ mice have fewer MZ B cells, these can bind high levels of IgM-IC, and appear to be positioned normally around the marginal sinus. This conclusion is consistent with recent results indicating that MZ B cells are generated in CD19$^{-/-}$ mice, but turnover

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rapidly (40). It is likely that these MZ B cells are involved in the deposition of IgM-IC on FDCs in these mice (Fig. 5A).

As an alternative approach, we took advantage of the ability of pertussis toxin (PTx) to functionally deplete MZ B cells (28). Mice were treated with PTx and 2 days later the fate of IgM-IC was examined. The depletion of MZ B cells by PTx was confirmed by reductions in CD21^{hi}CD23^{lo} B cells (Fig. 6A). Small numbers of splenocytes from these mice were found in the MZ gate, and these bound detectable amounts of IgM-IC, as compared with uninjected controls (Fig. 6A, lower panels). We then examined the fate of IgM-IC in the spleens of these mice. Although IgM-IC localized to FDC 18 h after injection in control mice, IgM-IC were completely absent in the spleens of PTx-treated mice (Fig. 6B). FDCs were found in these mice, indicating that their presence was not compromised by PTx treatment. In addition, significant amounts of IgM-IC were localized to the MZ 1 h after injection (Fig. 6B, lower panels), indicating that treatment of mice with PTx did not alter the ability of IgM-IC to localize to the MZ of the splenic follicles. However, the IgM-IC were never observed within the marginal sinus, as determined by MOMA-1 staining. This indicates that, in PTx-treated mice, IgM-IC are trapped normally within the MZ, but do not subsequently migrate into the follicles.

To determine if the presence of MZ B cells is sufficient for FDC trapping, IgM-IC were injected into IL-7-deficient mice, which contain a high frequency of MZ B cells and significantly reduced numbers of FO B cells (41) (Fig. 6C). IgM-IC localized to FDCs in the spleens of these mice, indicating that IgM-IC are deposited onto FDC even when FO B cells constitute only a small percentage of the splenic B cells.

MZ B cells transfer IgM-IC to FDC

While the previous results indicate that IgM-IC are bound by MZ B cells, and that the presence of MZ B cells is required for the deposition of IgM-IC onto FDCs, they do not prove that MZ B cells are actively transporting the IgM-IC later found on the FDC. To examine the ability of MZ B cells to transport IgM-IC,

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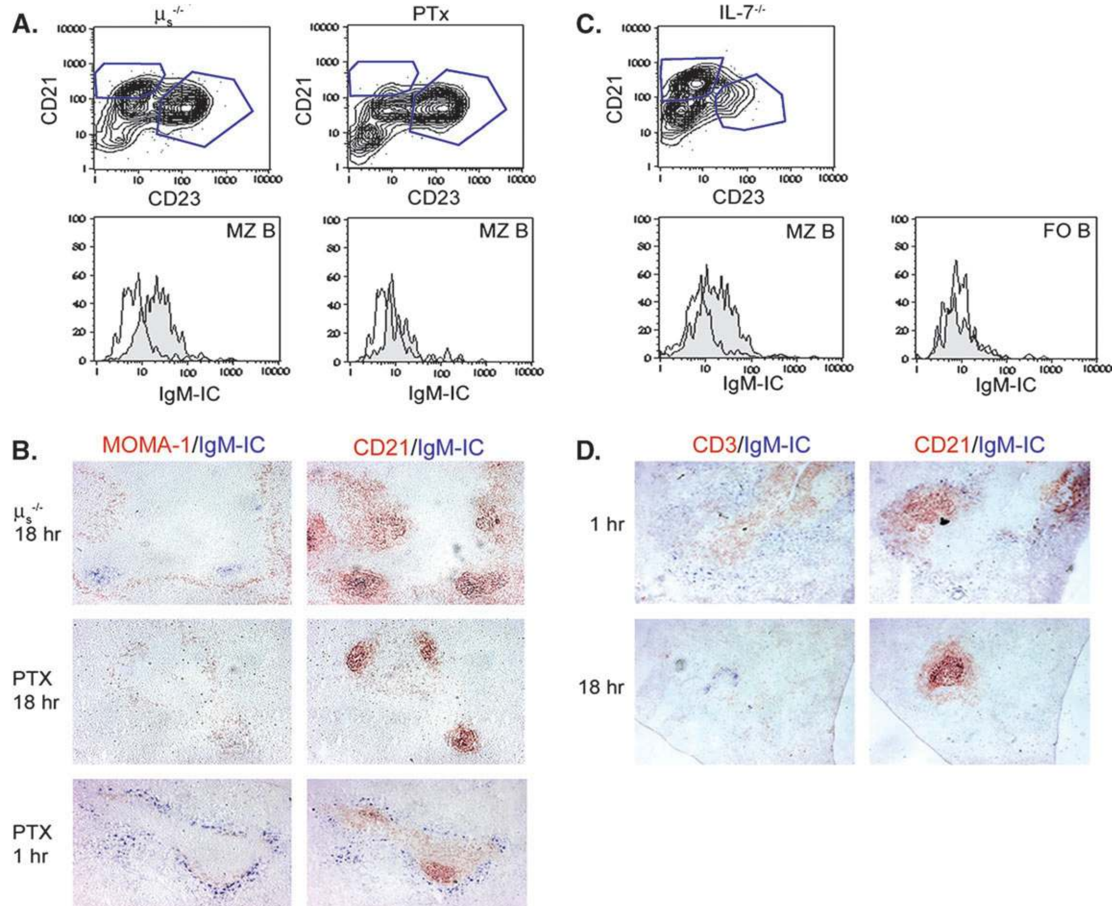

Fig. 6. MZ B cells are required for FDC localization of IgM-IC. (A) $\mu_s^{-/-}$ mice were injected with PTx to deplete MZ B cells or PBS as control. Forty-eight hours later, IgM-IC were injected i.v. Spleens harvested 1 h later were prepared for FACS analysis and IH. The CD21/23 profile shows a selective reduction of MZ B cells while the FO B cell population is unaltered in PTx mouse compared to PBS control. Also shown is IgM-IC association with MZ B cells from each mouse as described in Fig. 2. (B) IH of spleen sections obtained at the indicated times and processed for IH as described. Stains were performed as in Fig. 2. IgM-IC localize to the spleen at 1 h as seen in WT, but fail to deposit onto FDC (as shown by intense CD21 staining) at 18 h despite the presence of FDC. (C) IL-7$^{-/-}$ mice were injected with IgM-IC and spleens were harvested 1 h later for FACS and IH or 18 h later for IH. FACS analysis demonstrates the presence of MZ B cells while the FO B cell population is severely decreased. (D) IH shows IgM-IC localization to the MZ as seen in $\mu_s^{-/-}$ mice (Fig. 2) at 1 h. IgM-IC is deposited onto FDC in the B cell follicle at the later time.

we initially tried to isolate these cells by cell sorting and use them to transfer IgM-IC to recipient mice *in vivo*. However, we have found that the transport and deposition of IgM-IC is inhibited during the sorting process (unpublished observations). As an alternative approach, we used unsorted B cells in an adoptive transfer experiment, and then sought to distinguish the phenotype of the cells that were transferring IgM-IC to FDCs. To distinguish transferred B cells from endogenous B cells, Igh congenic strains of mice were used.

B6-Igh^{a} B cells were isolated and incubated with IgM-IC in the presence of fresh or heat inactivated serum. After IgM-IC binding, B cells were washed to remove unbound IgM-IC, and transferred i.v. into a recipient B6 mouse. Spleens were removed 12 h later and processed for IH. Spleen sections were first examined for the presence of IgM-IC within the B cell follicles. As expected, no IgM-IC were found within follicles of mice injected with B cells incubated with IgM-IC formed in the absence of complement (Fig. 7A). However, IgM-IC were found within the B cell follicles in mice that had received B cells incubated with IgM-IC in the presence of complement. To determine if the transferred B cells homed to the B cell follicle and associated with FDCs, spleen sections from these mice were stained for IgM-IC and for the expression of IgM^{a}, which detects only the transferred B cells. Representative sections are shown in Fig. 7(B). Transferred B cells were not only found within the B cell follicle, but intimately associated with structures having dendritic morphology with associated IgM-IC, consistent with FDCs. Interestingly, the IgM-IC on the transferred B cells was frequently capped on one pole, directed toward the dendrites (Fig. 7B, closed arrow), suggesting that the B cells might be transferring the IC to the FDCs. These data conclusively demonstrate that B cells alone can serve as the source of IgM-IC found subsequently on FDC.

Serial sections from the spleens of mice injected with the transferred cells carrying IgM-IC were next stained with CD1d, to distinguish MZ from FO B cells, and the FDC marker FDC-M2. CD1d staining revealed a characteristic ring around the follicle, as expected for the positioning representative of MZ B cells (Fig. 7C). Since few transferred cells (as defined by IgM^{a} staining) were found in the MZ, these CD1d^{+} cells must represent the endogenous MZ B cells. Higher magnifications of these sections showed the presence of CD1d^{+} cells inside the follicle clustered around FDC, as illustrated in the lower panels. Some of the CD1d^{+} positive cells contained IgM-IC and therefore can clearly be identified as the transferred cells. These cells were juxtaposed with FDCs, as shown by the FDC-M2 staining in the serial section (lower panel, right). Together, these results are consistent with a role for MZ B cells in the transport and deposition of IgM-IC onto FDCs.

While the transfer experiments demonstrate the ability of MZ B cells to transport IgM-IC to FDCs, they do not rule out the possible contribution of FO B cells. To address this issue, B cells from PTx-treated mice, which are deficient in MZ B cells (Fig. 6), were used as a source of donor B cells. As shown in Fig. 8(A), significant amounts of IgM-IC were found on FDCs when normal but not PTx-treated mice were used as a source of B cells. Nevertheless, small amounts of IgM-IC were detectable in some of the follicles of recipients of PTx-treated cells. These IgM-IC could have been transferred (albeit inefficiently) by FO B cells, or they could have been transported to FDCs after transfer to recipient MZ B cells. To distinguish between these two possibilities, the transfer experiment was repeated using PTx-treated mice as recipients. As shown in Fig. 8(B), only B cells from untreated mice were capable of transferring IgM-IC to recipient FDCs. We conclude that MZ B cells are uniquely capable of transporting and depositing IgM-IC to FDCs.

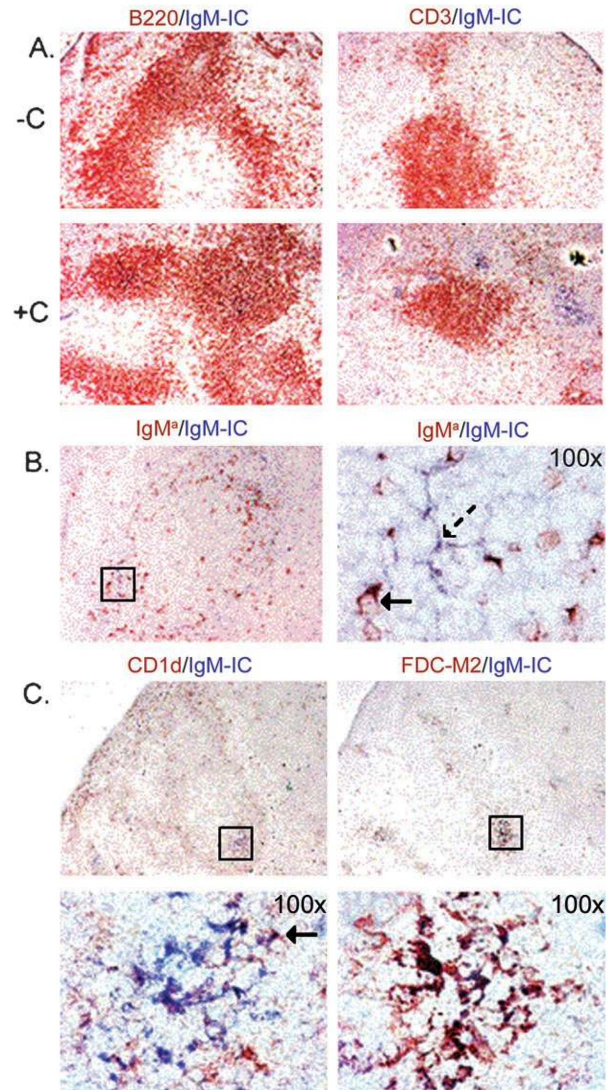

Fig. 7. MZ B cells bind and transfer IgM-IC to FDC. (A) B cells were isolated from Igh^{a} B6 mice and incubated with IgM-IC as explained in Fig. 4(A). IgM-IC laden B cells (4×10^{7}) were transferred i.v. into B6 mice. Spleens were harvested 12 h later and prepared for IH. Stains were performed as described. (B) Transferred B cells deposit IgM-IC onto FDCs. The utilization of Igh^{a} B cells enabled tracking of these cells using an anti-IgM^{a}–FITC antibody. Notice the cluster of IgM^{a+} cells around areas of IgM-IC deposition. Higher magnification (100×) shows FDC dendrites loaded with IgM-IC (identified by dashed arrow) as well as IgM^{a+} cells transferring IgM-IC to an empty FDC dendrite (identified by closed arrow). (C) Stains for CD1d and FDC-M2 to visualize transferred MZ B cells depositing IgM-IC onto FDCs. CD1d^{+} cells encompass the B cell follicle and are found inside the B cell follicle. FDC-M2 stain confirms the presence of IgM-IC on FDC. At higher magnification (100×), MZ B cells (CD1d+) can be observed depositing IgM-IC onto FDC processes (arrows).

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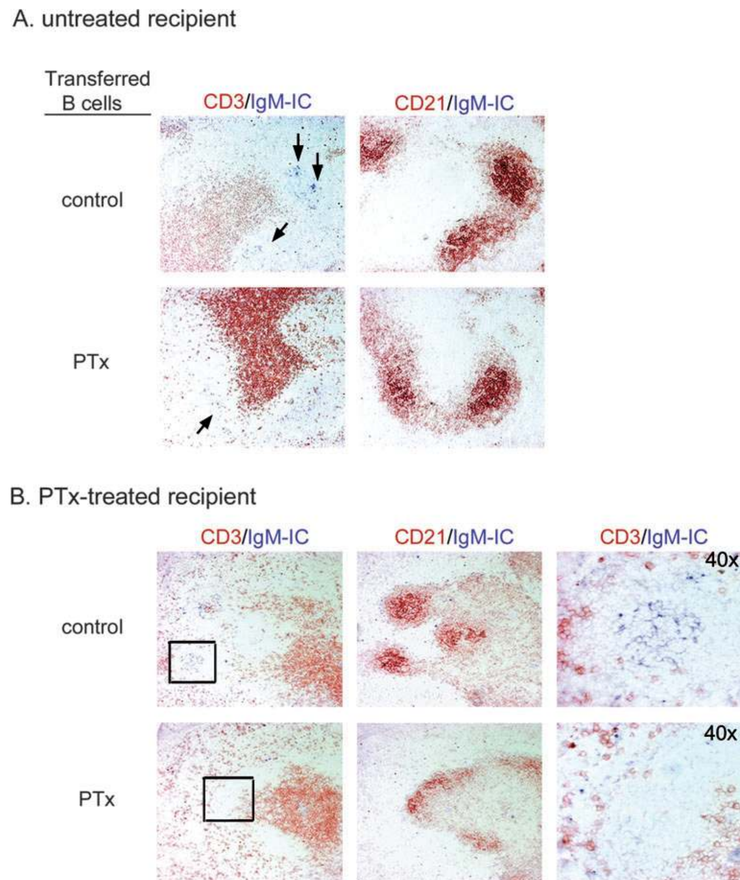

Fig 8. MZ B cells are uniquely capable of transferring IgM-IC to FDCs. B cells were isolated from untreated or PTx-treated mice, incubated with IgM-IC and complement as described in Fig. 4(A) and transferred into untreated (A) or PTx-treated (B) recipient B6 mice. Spleens were removed 12 h (A) or 8 h (B) later and processed for IH. In (B), the boxed sections are shown at higher magnification in the right panels. Arrows identify regions in which IgM-IC are localized to FDCs.

Discussion

One of the hallmarks of T-dependent antibody responses is the development of GC which promote affinity maturation and memory B cell development (20,21). IgM has a potent adjuvant effect by promoting primary immune responses to low doses of antigen that do not normally elicit such responses, and priming for memory B cell formation (5). The IgM-induced response is also characterized by the induction of GC (Fig. 1). One of the earliest events that can be detected in mice immunized with IgM-IC, but not antigen alone, is the deposition of the IgM-IC onto FDCs (5) (Fig. 2), suggesting that this might reflect one of the initial steps in the immunopotentiating effect of IgM.

The trapping of antigen on FDCs has long been thought to play an important role in immune responses (19,42–44). The role of antigen deposition on FDC for GC function has recently been called into question (45) based on studies that show that B cells from mice that lack secreted antibodies (46) or lymphotoxin α, which lack FDCs (47), still undergo somatic hypermutation. However, large doses of antigen with adjuvant were used in these studies, and it has been reported that somatic mutations do not arise in mice lacking FDCs in the absence of adjuvants (47,48). These data argue that FDCs are most important in orchestrating adaptive antibody responses under conditions in which antigen is limiting, such as those used in the current study. Given that responses only initiate when antigen is complexed with IgM, we suggest that the deposition of IgM-IC onto FDCs not only promotes GC formation and function (Fig. 1), but 'jump starts' the immune response as well.

Previous studies have established the requirements for secreted IgM (1–6) and an intact complement system (8–14) in the optimal initiation of adaptive T-dependent immune responses, but the exact mechanism(s) by which these mediators fulfill these functions has remained largely unknown. Our observations link the requirement for IgM and complement in a process in which IgM-IC activate the complement cascade, promoting the binding of these complexes to CR1/2 on MZ B cells. Several lines of evidence indicate that these MZ B cells are then responsible for transporting the complexes to the FDC. Reductions in MZ B cells either significantly (CD19$^{-/-}$) or entirely (PTx-treated mice) prevent IgM-IC deposition onto FDCs. Transfer studies prove that B cells can serve as the sole source of IgM-IC, indicating that they alone bind sufficient IgM-IC for FDC localization (Fig. 7). Furthermore, these transfer experiments demonstrate that the donor B cells must contain functional MZ B cells to enable the efficient transfer of IgM-IC to FDCs (Fig. 8). This supports the idea that only MZ B cells are competent

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to transport IgM-IC to FDCs. Notably, in the transfer experiments, MZ B cells with capped IgM-IC could be found in close juxtaposition with FDC dendrites, supporting a proposed role for MZ B cells in the localization of IgM-IC on FDCs. Interestingly, at times beyond 2 h after i.v. injection, we are unable to recover MZ B cells, or any cells for that matter, with associated IgM-IC (Fig. 2B), even though these cells are clearly present by IH (see Fig. 7). One possible explanation is that these MZ B cells are intertwined within the FDC network and, like FDCs themselves (49), cannot be released into cell suspension without procedures that incorporate enzymatic digestion. Together, these data provide strong evidence for a major role for MZ B cells in the deposition of IgM-IC onto FDCs.

MZ B cells have been established as a major component of the rapid antibody response to both T-independent blood borne pathogens and T-dependent antigens (28,33,50–52). MZ B cells are positioned to interact with these antigens by virtue of their localization around the marginal sinus of the splenic follicle. Our results suggest that MZ B cells not only function in the early phases of the antibody responses to antigens, but also play an important role in the initiation of the T-dependent immune response by virtue of their ability to bind, transport and deposit IgM-IC onto FDCs. Their ability to carry out this function is not only facilitated by their unique localization at the marginal sinus, but by the high levels of CR1/2 present on these cells, which enables them to efficiently interact with blood borne IgM-IC.

CR1/2 appears to be the major receptor on MZ B cells involved in the initial binding of IgM-IC. In the absence of CR1/2, little binding of IgM-IC can be detected over background, suggesting that other potential receptors, such as the Fcα/μ receptor (53) or the B cell receptor (BCR), play limited roles, at least in initiating IgM-IC binding. The deposition of IgM-IC onto FDCs in the absence of the BCR co-receptor, CD19 (54,55), suggests a role for CR1/2 in IgM-IC transport independent of signaling through CD19. Interestingly, the *Cr2*$^{-/-}$ mice used in the current study have a truncated form of the CR2 receptor on the cell surface (56). From our data it is clear that this truncated receptor binds little if any IgM-IC and is not functional. Therefore, we conclude that CR1/2 is the major receptor involved in the binding of the IgM-IC to MZ (and FO) B cells. Our data also suggest that MZ B cells have been able to exploit the elevated levels of membrane CR1/2 to bind larger amounts of IgM-IC, increasing their efficiency in transporting these IC into the B cell follicle for deposition onto FDC.

The idea that specific cells transport IC into splenic follicles is not new. A number of earlier studies examined this issue using aggregated IgG or large IgG-IC, and gave rise to competing theories on the mechanism by which IC reach FDCs. These included passive diffusion (57,58) and active transport by macrophages (59), migrating FDC precursors (60,61), or lymphocytes (62–67). The interactions of the IgG with IgG Fc receptors (68) on various cells types may have complicated the ability to interpret results from these studies. Moreover, in many of these studies, trafficking of the IC was studied in immunized recipients, in contrast with our own studies. Indeed, we have found that IgG antibodies cannot substitute for IgM when using naive mice, suggesting that IgM has a unique, non-redundant role in antigen deposition in non-immune mice (A.R.Ferguson and R.B.Corley, manuscript in preparation). This is consistent with an IgM-specific defect in mice devoid of secreted IgM, but expressing other secreted isotypes (1–4).

Two mechanisms have been proposed to explain the positioning of MZ B cells within the MZ. Cyster and colleagues (69) demonstrated the retention of MZ B cells through integrin-mediated adhesion. Ravetch and colleagues (70) found that MZM expressing MARCO are responsible for the retention of MZ B cells. The BCR-dependent interaction of MZ B cells with antigens has been shown to result in migration of these cells from the MZ to the T–B interface, followed by the subsequent relocation of these cells to the red pulp as differentiated plasmablasts [reviewed in (71,72)]. In the current study, we found that the interaction of MZ B cells with IgM-IC causes their migration from the MZ into the follicle, with subsequent deposition of IgM-IC onto FDCs. The means by which IgM-IC causes the release of MZ B cells from retention and directs trafficking into the follicle is unknown. In this regard, the results using PTx-treated mice are of interest. PTx inhibits G protein signaling involved in chemotaxis (73). While the low residual numbers of MZ B cells in CD19$^{-/-}$ mice enable IgM-IC deposition onto FDCs, this is not the case in PTx-treated mice. This suggests that chemotatic signals might be involved in directing MZ B cells into the follicles, once they are released from retention signals. A delineation of the signals involved in the release and migration of MZ B cells following IgM-IC interactions will not only be important in determining their fate, but will provide insights into how the deposition of IgM-IC onto FDCs plays a role in stimulating the onset of the adaptive humoral immune response.

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