Marginal Zone B Cells Transport IgG3-Immune Complexes to Splenic Follicles

Lu Zhang, Zhoujie Ding, Hui Xu and Birgitta Heyman

*J Immunol* 2014; 193:1681-1689; Prepublished online 11 July 2014;
doi: 10.4049/jimmunol.1400331
http://www.jimmunol.org/content/193/4/1681
Marginal Zone B Cells Transport IgG3-Immune Complexes to Splenic Follicles

Lu Zhang, Zhoujie Ding, Hui Xu, and Birgitta Heyman

Ag administered together with specific IgG3 induces a higher Ab response than Ag administered alone, an effect requiring the presence of complement receptors 1 and 2 (CR1/2). In this study, we have investigated the fate of Ag, the development of germinal centers (GCs), and the Ab response after i.v. administration of IgG3 anti-trinitrophenyl (TNP) in complex with OVA-TNP. After 2 h, OVA-TNP was detected on marginal zone (MZ) B cells, and a substantial amount of Ag was detected in splenic follicles and colocalized with follicular dendritic cells (FDCs). After 10 d, the percentage of GCs and the IgG responses were markedly higher than in mice immunized with unmatched OVA-TNP. The effects of IgG3 were dependent on CR1/2 known to be expressed on B cells and FDCs. Using bone marrow chimeric mice, we demonstrate that an optimal response to IgG3-Ag complexes requires that CR1/2 is expressed on both cell types. These data suggest that CR1/2+ MZ B cells transport IgG3-Ag-C complexes from the MZ to the follicles, where they are captured by FDCs and induce GCs and IgG production. This pathway for initiating the transport of Ags into splenic follicles previously known B-cell dependent pathways where Ag is transported by 1) MZ B cells, binding large Ags-IgM-C complexes via CR1/2; 2) recirculating B cells, binding Ag via BCR; or 3) recirculating B cells, binding IgE-Ag complexes via the low-affinity receptor for IgE, CD23. The Journal of Immunology, 2014, 193: 1681–1689.

Antibodies administered together with their specific Ag can feedback regulate the Ab response, resulting in complete suppression or several 100-fold enhancement (1–3). Ab-mediated feedback regulation is Ag but not epitope specific, that is, Abs binding to one epitope will usually suppress or enhance responses also to other epitopes on the same Ag. The most well-known feedback regulation is IgG-mediated suppression of responses to erythrocytes. This has been observed successfully in the clinic for prevention of hemolytic disease of the fetus and newborn, resulting from Rh incompatibility between mother and fetus (4, 5). Interestingly, erythrocyte-specific IgM has the opposite effect from IgG and enhances Ab responses to erythrocytes both in humans and animals (4, 6). IgM-mediated enhancement requires the presence of C3 and complement receptor (CR)1/2 (7–9) and that IgM is able to activate C (7, 10, 11). It is well known that lack of C1, C2, C3, C4, and CR1/2 leads to impaired primary as well as secondary Ab responses also to Ag administered alone (12). The observation that primary Ab responses depend on C activation via the classical pathway, which to a large extent is initiated by Ab-Ag complexes, seemed paradoxical because little specific Ab is present in naive animals. The idea that natural IgM, via C activation, may initiate the classical pathway (13) was tested in knockin mice with a point mutation in the Cµ H chain, leading to production only of IgM unable to activate C (14). Surprisingly, these animals did not have severely impaired Ab responses, suggesting that other factors activating the classical pathway play a role.

When specific Abs are administered with soluble proteins in physiological salt solutions, they generally act to enhance the Ab responses. IgE-Ag complexes are captured by circulating B cells via the low-affinity receptor for IgE, CD23, and rapidly transported to splenic B cell follicles (15, 16). Subsequently, both Ab and CD4+ T cell responses are enhanced (17–19). All IgG subclasses, including IgG3, have dual effects and can suppress responses to particulate Ags, for example erythrocytes, and enhance responses to soluble protein Ags (20–24). Murine IgG1, IgG2a, and IgG2b require the presence of FcγRs, but not C, and most likely operate by increasing the uptake of IgG-Ag complexes by FcγR+ dendritic cells, causing efficient proliferation and activation of CD4+ Th cells (3, 25–27).

IgG3 constitutes only a small part of the response to T-dependent Ags but is the predominant subclass responding to T cell–independent type 2 Ags (28, 29) and plays an important role in the defense against bacterial and fungal infections (30–32). IgG3 has unique physicochemical properties, allowing it to self-associate and in experimental autoimmune hemolytic anemia relies on C activation (32, 40). IgG3 is a potent activator of the classical C pathway (39, 40), and, interestingly, also the role of IgG3 in the defense against disseminated candidiasis and in experimental autoimmune hemolytic anemia relies on C activation (32, 40). In this study, we have investigated the mechanism behind IgG3-mediated enhancement of Ab responses by studying how IgG3-Ag complexes are transported in vivo, their capacity to induce germinal centers (GCs), and whether CR1/2
expression on B cells and/or follicular dendritic cells (FDCs) is required for induction of optimal Ab responses to IgG3-Ag.

Materials and Methods

Mice

BALB/c mice (wild-type [WT]) were originally obtained from Bonn mice (Ry, Denmark). Cr2 knockout (KO) mice (lacking CR1/2) (41) and CD23 KO mice (lacking CD23) (17) were backcrossed for 10 generations to BALB/c mice. All mice were bred and maintained in the animal facilities at the National Veterinary Institute (Uppsala, Sweden) by skilled personnel under the supervision of a veterinarian. All animal studies were carried out with the approval of Uppsala Animal Ethics Committee. Mice aged 6 wk or more were used and matched for age and sex within each experiment.

Antigens

OVA and 2,4,6-trinitrophenyl (TNP) were purchased from Sigma-Aldrich (St. Louis, MO). OVA-TNP conjugation was performed, as described, and a batch with 2.5 TNP molecules/OVA molecule was used (42). Biotinylation of OVA-TNP was performed, as described (15), with the difference that 4.8 mg sulfosuccinimidyl-6-(biotinamido)-hexanoate (sulfo-NHS-LC-biotin; Pierce, Rockford, IL) per 10 mg OVA-TNP was used. The reaction took place in the dark at room temperature for 2 h and then at 4˚C overnight. Biotinylated OVA-TNP was dialyzed against PBS, sterile filtered, and stored at 4˚C in the dark.

Abs for flow cytometry and confocal microscopy

For flow cytometry, rat IgG2ak anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen, San Diego, CA) were used as Fc block. FITC-labeled rat IgG2ak anti-human/mouse CD45R (B220) (clone RA3-6B2; eBioscience, San Diego, CA) and allophycocyanin-labeled streptavidin (SA; eBioscience) were used to detect Ag bound to B cells in circulating blood; Alexa Fluor 700–labeled rat IgG2ak anti-mouse CD45R (B220) (clone RA3-6B2; BD Pharmingen), PE–labeled rat IgG2ak anti-mouse CD23 (clone B3B4; eBioscience), FITC–labeled rat (LEW) IgG2ak anti-mouse CD16/CD32 (clone 2.4G2; BD Pharmingen), and SA–allophycocyanin (eBioscience) were used to detect Ag bound to B cells in the spleen. Alexa Fluor 647–labeled rat (LOU) IgMk anti-mouse T and B cell activation Ag (clone GL7; BD Pharmingen); Pacific Blue–labeled rat IgG2ak anti-mouse CD45R (B220) (clone RA3-6B2; BD Pharmingen), and biotinylated peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA), followed by FITC–labeled SA (eBioscience) were used to identify GC B, B cells in confocal immunofluorescence staining. Pacific Blue–labeled rat IgG2ak anti-mouse CD45R (B220) (clone RA3-6B2; BD Pharmingen), FITC–labeled rat IgG2a anti-mouse CD169 (clone MOMA-1; AbD Serotec, Oxford, U.K.), and SA–allophycocyanin (eBioscience) were used to detect Ag localization in the follicles; Pacific Blue–labeled rat IgG2ak anti-mouse CD45R (B220) (clone RA3-6B2; BD Pharmingen), FITC–labeled rat IgG2a anti-mouse CD169 (clone MOMA-1; AbD Serotec), rabbit anti-OVA (generated in house from hyperimmune sera using affinity chromatography on protein A–Sepharose; GE Healthcare, Uppsala, Sweden), followed by Alexa Fluor 647–labeled goat anti-rabbit IgG (H+L; Life Technologies, Carlsbad, CA), and purified rat IgG2a anti-mouse CD35/CR1 (clone SC12 (43); generated in house from hyperimmune sera using affinity chromatography on protein A–Sepharose; GE Healthcare, Healthcare, followed by PE–labeled goat anti-rat IgG (BioLegend, San Diego, CA) were used to detect Ag deposition on FDCs; Pacific Blue–labeled rat IgG2ak anti-mouse CD45R (B220) (clone RA3-6B2; BD Pharmingen), FITC–labeled rat IgG2a anti-mouse CD169 (clone MOMA-1; AbD Serotec), and rabbit anti-OVA (generated in house), followed by Alexa Fluor 647–labeled goat anti-rabbit IgG (H+L; Life Technologies) were used to detected Ag localization in 2-amino-2-[4-(octylphenyl)ethyl]-1,3-propanediol hydrochloride (FTY720–treated mice; Pacific Blue–labeled rat IgG2ak anti-mouse CD45R (B220) (clone RA3-6B2; BD Pharmingen), FITC–labeled rat IgG2a anti-mouse CD169 (clone MOMA-1; AbD Serotec), and biotinylated PNA (Vector Laboratories) together with SA–allophycocyanin (eBioscience) were used to detect GCs.

Immunization and blood sampling

IgG3 and IgE used for immunization were derived from B cell hybridomas producing murine IgG3 anti-TNF (clone IM-F10 and IM-H11) (22) or IgE anti-TNF (clone CD11/1a) (44). Clones IM-F10 and IM-H11 are easily inducible and interchangeable. They were both able to enhance Ab responses (22) and had the same effect in the assays used in this work (see figure legends). mAbs were purified, stored, and measured, as described (19, 22). OVA–TNP or biotinylated OVA-TNP was mixed with IgG3 or IgE in PBS at room temperature within 1 h before immunization. A total volume of 0.2 ml/mouse was administered in the tail vein. To induce reversible dislocation of marginal zone (MZ) B cells, mice were injected i.p. with 1 mg/kg mouse body weight of FTY720 (Cayman Chemical, Ann Arbor, MI) in 0.6% ethanol 4 h before immunization. Controls were given 0.6% ethanol alone. The amount of Ag and Ab used is indicated in each figure legend. Blood samples were collected through tail bleedings.

Flow cytometry

Blood and spleen samples were prepared and stained, as described (11). Briefly, cell suspensions were treated with hypotonic buffer to deplete the RBC, washed in PBS, and resuspended in FACS buffer. Fc block was used before staining. Stained cells were fixed with 4% paraformaldehyde in PBS before analysis. For each sample, 1–4 × 10⁵ events of lymphocytes (gated according to forward- and side-scatter properties; Figs. 1, 2, 5) or 2–4 × 10⁵ total events (Fig. 6) were acquired on a LSR II cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Confocal microscopy

Spleens were harvested, prepared, and stored, as described (11). Briefly, spleens were embedded in optimal cutting temperature embedding media (Sakura Finetek, Alphen aan den Rijn, the Netherlands), snap frozen in liquid nitrogen, and stored at −80˚C. Sections (8 m) were cut with Thermo Scientific CryoStar NX70 Cryostat (Thermo Scientific, Waltham, MA) and stored at −80˚C. Before staining, slides were fixed in 50% aceton 30 s, followed by 100% acetone for 5 min (Figs. 3–5) or 4% paraformaldehyde for 15 min, rehydrated in PBS, and blocked using 5% horse sera (Sigma-Aldrich) for 30 min (Fig. 6). Slides were then stained, as indicated in each figure. Finally, slides were washed twice in PBS before they were mounted with Fluoromount G (Southern Biotech, Birmingham, AL). All slides were analyzed in a LSM 700 confocal microscope (Carl Zeiss, Thornwood, NY) and quantified with ImageJ (National Institutes of Health).

Bone marrow transplantation

WT and Cr2 KO mice were irradiated and reconstituted with bone marrow from either WT or Cr2 KO mice, as described (9). Briefly, female WT and Cr2 KO mice were whole body irradiated with 7.5 Gy. Twenty-four hours later, 5–10 × 10⁵ bone marrow cells from the hind legs of nonirradiated WT or Cr2 KO mice were transferred to irradiated mice, resulting in four groups, as follows: mice expressing CR1/2 on both FDCs and B cells (WT→WT), on either FDCs (Cr2 KO→WT) or B cells (WT→Cr2 KO), or on neither cell type (Cr2 KO→Cr2 KO). Mice were rested for 6 wk before immunization.

ELISA

Sera prepared from blood samples obtained from tail veins were tested for OVA–specific IgG in an ELISA, using hyperimmune mouse polyclonal IgG anti-OVA as a standard (19). Briefly, 96-well plates (Costar 96-well enzyme immunoassay/RIA; Sigma-Aldrich) were coated with 50 µg/ml OVA in PBS at 4˚C overnight. Plates were blocked with 5% dry milk at room temperature for 2 h before adding serum samples and then incubated at 4˚C overnight. After washing, alkaline phosphatase–conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added, and the plates were incubated at room temperature for 3 h before adding substrate. Construction of standard curves and calculations were made with SOFTmax software (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Statistical differences between groups were determined by unpaired Student t test, as follows: *p < 0.05 (not significant [ns]), **p < 0.05, ***p < 0.01, or ****p < 0.001.

Results

IgG3 does not increase binding of Ag to B220+ cells in the blood

Ag administered i.v. together with specific IgE is captured by B cells in the blood and rapidly transported to splenic follicles, where it can be found after 30 min (15). Because IgG3–mediated enhancement of Ab responses is dependent on CR1/2 (22), which are expressed on B cells, we wanted to determine whether IgG3-
complexed Ag is also captured by circulating B cells. To this end, biotinylated OVA–TNP– and TNP-specific IgG3 were given alone or in combination to WT and Cr2 KO mice. For comparison, OVA–TNP was administered alone or together with TNP-specific IgE to WT and CD23 KO mice. Five minutes after immunization, blood samples were collected and the geometric mean of the fluorescence intensity of Ag bound to B220+ cells was quantified. A representative histogram is shown (Fig. 1A). Ag administered alone at low levels to B220+ cells (Fig. 1), but not to B220+ cells (data not shown). Part of the binding of uncomplexed Ag to B220+ cells may be explained by binding to CR1/2 (Fig. 1C), but we do not know what explains the remaining binding. Possibly, biotinylated OVA–TNP is recognized via BCRs on naive B cells. As expected, IgE-Ag complexes bound well to peripheral WT B cells, but not to B cells from CD23 KO mice (Fig. 1B). Binding of IgG3-Ag complexes to B cells was not higher than binding of Ag alone, neither in WT nor in Cr2 KO mice (Fig. 1C). In summary, IgE facilitates binding of its specific Ag to peripheral B cells via CD23, whereas IgG3 does not enhance binding of Ag to peripheral B cells.

IgG3 increases binding of Ag to MZ B cells via CR1/2

MZ B cells do not recirculate but reside in the spleen and express more CR1/2 than recirculating/follicular (FO) B cells (45). Therefore, it seemed likely that IgG3-Ag complexes would be

FIGURE 1. IgG3 does not increase binding of Ag to B220+ cells in the blood. (A and B) BALB/c (WT) and CD23 KO mice were immunized with 50 μg IgE anti-TNP alone (n = 2), 150 μg biotinylated OVA–TNP (Ag) alone (n = 5), a mixture of 50 μg IgE anti-TNP and 150 μg biotinylated OVA–TNP (n = 5), or left unimmunized (nil, n = 1-2). (A and C) WT and Cr2 KO mice were immunized with 50 μg IgG3 anti-TNP (IM-F10) alone (n = 2), 150 μg biotinylated OVA–TNP (Ag) alone (n = 5), a mixture of 50 μg IgG3 anti-TNP and 150 μg biotinylated OVA–TNP (n = 5), or left unimmunized (n = 2). Binding of Ag to peripheral B cells was analyzed in blood obtained 5 min after immunization. Cells were stained with anti-B220 FITC (B cells) and SA-allophycocyanin (Ag). (A) Representative histograms show binding of Ag to B220+ cells in untreated WT mice (gray area), WT mice given Ag alone (gray line), WT mice given IgE-Ag complexes (black line), and WT mice given IgG3-Ag complexes (dotted line). (B and C) The geometric mean of fluorescence intensity (MFI) of Ag bound to B220+ cells. **p < 0.01, ***p < 0.001. Data are representative of four independent experiments (two with clone IM-F10 and two with IM-H11).

FIGURE 2. IgG3 increases binding of Ag to MZ B cells via CR1/2. WT and Cr2 KO mice were immunized with 50 μg IgG3 anti-TNP (IM-F10) alone (n = 2), 150 μg biotinylated OVA–TNP (Ag) alone (n = 5), a mixture of 50 μg IgG3 anti-TNP and 150 μg biotinylated OVA–TNP (n = 5), or left unimmunized (nil, n = 2). Spleens were harvested 2 h after immunization. Single-cell suspensions were prepared from half of each spleen and stained with B220-Alexa Fluor 700, CD23-PE, CD1d-FITC, and SA-allophycocyanin. B220+ cells were gated as MZ B cells (CD23lowCD1dhigh cells) or FO B cells (CD23high CD1dlow). (A–C) Representative dot plots are shown (Ag+B220+ cells, blue; B220+ cells, red). Geometric mean fluorescence intensity (MFI) of Ag bound to splenic B220+ cells (D), MZ B cells (E), and FO B cells (F) was calculated. p > 0.05 (not significant [ns]), *p < 0.05, ***p < 0.001. Data are representative of four independent experiments (three with clone IM-F10 and one with IM-H11).
captured by MZ B cells rather than by recirculating B cells. To test this, spleens from mice immunized, as described above, were harvested 2 h after immunization, and single-cell suspensions were analyzed. Representative dot plots are shown (Fig. 2A–C). A small increase in binding of IgG3-Ag to splenic B220+ cells from WT, but not from Cr2 KO, mice was seen (Fig. 2D). IgG3 significantly increased the amount of Ag bound to MZ B cells in WT mice (Fig. 2E). No increase was seen in Cr2 KO mice (Fig. 2E), and IgG3 had no effect on Ag binding to FO B cells from either mouse strain (Fig. 2F). These results demonstrate that IgG3 increases Ag binding to MZ, but not to FO B cells, and that this binding requires CR1/2. Interestingly, the opposite results have been reported for binding of IgE-Ag complexes, showing preferential binding to splenic FO B cells and little binding to MZ B cells (15). These observations were confirmed in this study (data not shown) and are probably explained by the fact that MZ B cells express high levels of CR1/2 and low levels of CD23, whereas the situation for FO B cells is the opposite (45).

IgG3-mediated localization of Ag to splenic follicles is dependent on CR1/2

The other halves of the spleens analyzed in flow cytometry (Fig. 2) were subjected to confocal microscopy. In WT mice given IgG3-Ag, Ag deposition was seen in FDCs in the follicular areas (Fig. 3A–D). In Cr2 KO mice, Ag deposition was seen in the MZ areas, but not in the follicular areas (Fig. 3E–H). These results demonstrate that IgG3-mediated localization of Ag to splenic follicles is dependent on CR1/2. The other halves of the spleens analyzed in flow cytometry (Fig. 2) were subjected to confocal microscopy. In WT mice given IgG3-Ag, Ag deposition was seen in FDCs in the follicular areas (Fig. 3A–D). In Cr2 KO mice, Ag deposition was seen in the MZ areas, but not in the follicular areas (Fig. 3E–H). These results demonstrate that IgG3-mediated localization of Ag to splenic follicles is dependent on CR1/2.

FIGURE 3. IgG3-mediated localization of Ag to splenic follicles is dependent on CR1/2. WT and Cr2 KO mice were immunized with 50 μg IgG3 anti-TNP (IM-F10) alone (n = 2), 150 μg biotinylated OVA-TNP (Ag) alone (n = 3), a mixture of 50 μg IgG3 anti-TNP and 150 μg biotinylated OVA-TNP (Ag) alone (n = 5), or left unimmunized (nil, n = 2). Spleens were harvested 2 h after immunization. The other half of each spleen analyzed in flow cytometry (Fig. 2) was sectioned and stained with B220-Pacific Blue (B cells; blue), MOMA-FITC (metallophilic macrophages; green), and SA-allophycocyanin (Ag; red). One longitudinal section/each half spleen was analyzed by confocal microscopy. Images show representative FO areas (250 μm × 250 μm) from each group of the 8–13 follicles per mouse that were analyzed. (A–D) All colors included. (E–H) Blue color subtracted. (I and J) Quantification of Ag was done in B220+ areas with the border against the MZ defined by MOMA+ cells. p > 0.05 (not significant [ns]), *p < 0.05. Data are representative of four independent experiments (three with clone IM-F10 and one with IM-H11).

FIGURE 4. IgG3 enhances deposition of Ag on FDCs. WT mice were immunized with 50 μg IgG anti-TNP (IM-F10) and 150 μg biotinylated OVA-TNP (n = 3) or with 150 μg biotinylated OVA-TNP alone (n = 3). Spleens were harvested 2 h (A, B, E, F, I, and J) or 8 h (C, D, G, H, K, and L) after immunization and sectioned and stained with B220-Pacific Blue (B cells; blue), MOMA-FITC (metallophilic macrophages; gray), purified rat IgG2a anti-mouse CD35/CR1 followed by PE-labeled goat anti-rat IgG (FDCs; red), and rabbit anti-OVA followed by Alexa Fluor 647-labeled goat anti-rabbit IgG (Ag; green). One longitudinal section/each half spleen was analyzed by confocal microscopy. Images show representative FO areas (354 μm × 354 μm) from each group of the two to three follicles per mouse that were analyzed. (A–D) All colors except green included. (E–H) Blue and red colors subtracted. (I–L) Red and green colors combined (yellow indicates colocalization); the magnified square = 47 μm × 47 μm. Representative of two experiments at 2 h and one at 8 h (clone IM-F10).
Ag, substantial amounts of Ag had entered the follicle after 2 h (Fig. 3B, 3F). Very little Ag was found in follicles of WT mice given Ag alone (Fig. 3A, 3E) as well as in follicles of Cr2 KO mice, regardless of whether they had been immunized with IgG3-Ag or Ag alone (Fig. 3C, 3D, 3G, 3H). Quantification of the percentage of the Ag+ area within the total B220+ area showed that WT mice given IgG3-Ag complexes had 4.7-fold more Ag in the follicles than WT mice given Ag alone (Fig. 3I, 3J). Determining the amount of IgG3-Ag in follicles from WT mice at various time points, we found that Ag was clearly detectable 1 h after immunization, peaked after 2 h, decreased after 4 h, and was almost undetectable after 16 h (data not shown). To determine whether Ag colocalized with FDCs, splenic sections obtained from WT mice 2 and 8 h after immunization were stained with the mAb 8C12, specific for CR1/CD35 (43) primarily expressed on FDCs (46). In mice given Ag alone, very little Ag was found in follicles after 2 h (Fig. 4A, 4E, 4I). After 8 h, Ag could be detected in these groups, but did not localize to FDCs (Fig. 4C, 4G, 4K). Possibly, the small OVA-TNP molecules entered the follicles directly via conduits (47, 48). IgG3-Ag colocalized with FDCs at both time points, as shown by the yellow areas (Fig. 4B, 4F, 4J and 4D, 4H, 4L), whereas very little colocalization was seen in mice immunized with Ag alone (Fig. 4A, 4E, 4I and 4C, 4G, 4K). Interestingly, colocalization was very prominent in the periphery of the follicles already after 2 h, whereas colocalization appeared more centrally and was dimmer after 8 h. This could perhaps be explained by internalization of immune complexes by FDCs (49). In summary, IgG3 enhances entry of Ag into the splenic follicles, where it becomes associated with FDCs. This process requires CR1/2.

Dislocation of MZ B cells from the MZ results in impaired binding of IgG3-Ag to MZ B cells and impaired localization of IgG3-Ag in follicles

Positioning of MZ B cells in the MZ depends on the sphingosine 1-phosphate receptor S1P1. When exposed to the S1P1 antagonist FTY720, MZ B cells cannot localize to the MZ but are instead found in the follicles (50). To test whether IgG3-Ag depended on MZ B cells for transport into follicles, mice were treated with FTY720 or buffer alone. Four hours later, when the MZ were devoid of MZ B cells, the animals were immunized with Ag alone or IgG3-Ag, and their spleens were analyzed after 2 h. FO B cells bound very low levels of IgG3-Ag (Fig. 5A). As expected, MZ B cells bound IgG3-Ag complexes well in mice treated with buffer alone (Fig. 5B). In FTY720-treated mice, this binding was significantly decreased, albeit not completely abrogated (Fig. 5B). In splenic sections from mice treated with buffer alone, IgG3-Ag localized efficiently to the follicles, whereas this localization was almost completely abrogated in FTY720-treated mice (Fig. 5C–G). These observations strongly suggest that IgG3-Ag complexes in the MZ are captured by MZ B cells, which subsequently transport them into the follicles.

IgG3 enhances Ab responses and development of GC in WT mice

To determine whether IgG3 could enhance GC responses and whether such enhancement required CR1/2, WT and Cr2 KO mice were treated with buffer or FTY720 4 h prior to immunization with IgG3 anti-TNP IM-H11 and biotinylated OVA-TNP (n = 6), biotinylated OVA-TNP alone (n = 6), or left untreated (nil, n = 2). Spleens were harvested 2 h after immunization. One half was analyzed in flow cytometry (A and B) and the other in confocal microscopy (C–G). Single-cell suspensions were stained with B220-Pacific Blue, CD23-PE, CD1d-FITC, and SA-allophycocyanin. (A) Geometric mean fluorescence intensity (MFI) of Ag bound to FO B cells was calculated. (B) Geometric MFI of Ag bound to MZ B cells was calculated. (C) Quantification of the percentage of the Ag area within the B220+ area in follicles (defined as in Fig. 3I). One longitudinal section/each half spleen was analyzed by confocal microscopy. Images show representative FO areas (250 μm × 250 μm) from each group of the five follicles per mouse that were analyzed (D–G). **p < 0.01, ***p < 0.001. Data are representative of three independent experiments (two with clone IM-F10 and one with IM-H11).
were immunized with OVA-TNP alone or together with IgG3 anti-TNP. Spleens and blood samples were collected 10 d after immunization. IgG3 induced a ~5-fold increase in the percentage of GC B cells (B220+PNA+GL7+) among total B220+ cells in WT mice (Fig. 6B). Also in Cr2 KO mice, IgG3 enhanced the percentage of GC B cells, but the increase was considerably lower than in WT mice (Fig. 6B). In parallel, the development of GC structures was studied in confocal microscopy. IgG3 increased the percentage of PNA+ follicles by ~20-fold in WT mice, whereas no significant increase was seen in Cr2 KO mice (Fig. 6C, 6D). IgG3 also induced a strong enhancement of the OVA-specific IgG response in WT mice but only a small enhancement in Cr2 KO mice (Fig. 6E). In summary, IgG3 administered with its specific Ag enhances the development of GCs as well as the IgG response. The enhancement of both responses was impaired in Cr2 KO mice.

**CR1/2 expression on both FDCs and B cells is required for optimal Ab responses to IgG3-Ag**

The experiments described above show that IgG3-Ag complexes bind to CR1/2+ B cells (Fig. 2) and that induction of GCs by IgG3 is impaired in Cr2 KO mice (Fig. 6). CR1/2 are expressed on both B cells and FDCs, and, according to recent data, FDCs primarily express CR1 (46). Because murine CR1 and CR2 are derived by alternative splicing from the same gene, both receptors are absent in Cr2 KO mice (51). To investigate whether CR1/2 on FDCs and/or B cells are required for IgG3-mediated enhancement of Ab responses, bone marrow chimeric mice were generated. Mice expressing CR1/2 on both FDCs and B cells (WT→WT), on B cells only (WT→Cr2 KO), on FDCs only (Cr2 KO→WT), or on neither cell type (Cr2 KO→Cr2 KO) were immunized with OVA-TNP alone, IgG3 anti-TNP alone, or a mixture of IgG3 anti-TNP and OVA-TNP. As expected, the highest Ab responses to IgG3-Ag were seen in WT→WT mice (Fig. 7A). A small response was seen also in Cr2 KO→Cr2 KO mice (Fig. 7D). In mice expressing CR1/2 on either FDCs alone (Fig. 7B) or B cells alone (Fig. 7C), the responses to IgG3-Ag were higher than in mice completely lacking CR1/2, but lower than in mice expressing CR1/2 on both cell types (Fig. 7A). In conclusion, CR1/2 expression on both FDCs and B cells is required for an optimal Ab response to IgG3-Ag. The fold increase, which would establish the relative IgG3-mediated increase in Ab responses in the different groups, is difficult to establish accurately because the amount of IgG anti-OVA is frequently below detection level in the groups immunized with OVA-TNP alone.

**Discussion**

We have here shown that IgG3-complexed proteins are captured by MZ B cells and can be found in splenic follicles 2 h after immunization, where they colocalize with FDCs (Figs. 2–4). In mice in which MZ B cells were dislocated from the MZ by treatment with FTY720, very little Ag was found in the follicles (Fig. 5).
The percentage of follicles that have developed GCs after 10 d was 20-fold higher in mice given IgG3-Ag than in mice given Ag alone (Fig. 6D). At this time, the Ab response in IgG3-Ag–immunized mice was also dramatically increased (Fig. 6E). All effects were either completely abolished or severely reduced in Cr2 KO mice. Why a residual enhancement of Ab responses and a low induction of GC B cells are seen also in Cr2 KO mice is not known, but similar effects on Ab responses in mice immunized with IgG3-Ag complexes are indicated without parentheses/brackets; p values for comparison of responses between mice immunized with Ag alone or IgG3-Ag complexes (within each type of chimera) are indicated within brackets ([ ]) in (A) versus (B), within angle brackets ([A]) versus (C); < >, or within parentheses [(A)] versus (D); ( ), p > 0.05 (not significant [ns]), *p < 0.05, **p < 0.01, ***p < 0.001. Data are representative of three independent experiments (two with clone IM-F10 and one with IM-H11).

Although IgG3 and IgM seem to regulate immune responses in a similar way, they are specialized in different types of Ags. IgM can only enhance responses to large particulate Ags such as erythrocytes, keyhole limpet hemocyanin, and malaria parasites (6, 7, 10, 11, 56). IgG3, in contrast, enhances responses to small proteins such as OVA and BSA (22, 38). The reason that IgM preferably enhances responses to large Ags is probably its strategy for C activation. To bind C1q, IgM must change its conformation from the stellar form, assumed in solution, to a spider shape. Most likely, this can only take place by binding to an Ag larger than the IgM molecule itself. IgG Abs use another strategy to bind C1q, which enhances IgG-dependent localization of Ag and that we have observed in this study. It suggests that CR1/2 play a dual role, first on MZ B cells in the transport of immune complexes and then on FDCs for capture and presentation of immune complexes to GC B cells. It has been shown in vitro that cocross-linking of the CR2/CD19/TAPA-1 coreceptor with the BCR lowers the threshold for B cell activation (55). Such cross-linking could be induced by IgG3-Ag-C complexes and thus be part of the explanation for IgG3-mediated enhancement of Ab responses. However, because localization of IgG3-Ag was abrogated by FTY720 treatment and because FO B cells are poor binders of IgG3-Ag, we find that the MZ B cell–mediated transport of IgG3-Ag-C into follicles is more likely to explain the effect than increased B cell signaling.

An important step in generation of adaptive Ab responses is the development of GCs in which affinity maturation and class switch
recombination take place. Important cells in the formation of GCs are the FDCs that express Fc and C receptors and bind immune complexes that are then internalized and displayed periodically on the surface for recognition by GC B cells (49). Essential for development of GCs is that Ag can get access to the follicles, and various pathways for this have been identified. Small Ags can be transported to splenic and lymph node follicles via small channels, conduits (47, 48). Larger Ags are transported to lymph node follicles by subcapsular sinus macrophages (48, 57–59) and to splenic follicles by a subgroup of dendritic cells (60) or by B cells (61). Currently, different ways for B cells to transport Ag to splenic follicles are known, as follows: specific B cells can capture Ag in the lung via the BCR (62), peripheral B cells can capture IgE-Ag complexes via CD23 in the blood (15, 16), and, finally, MZ B cells can capture and transport C-opsonized Ag from the MZ to FDCs (53, 54). We have shown in this work that IgG3 plays an important role in opsonizing small Ags for binding to CR1/2 and subsequent transport by MZ B cells to splenic follicles in which efficient induction of GCs takes place. In addition, we have confirmed that recirculating B cells capture IgE-Ag complexes in peripheral blood and that this process requires CD23.

Acknowledgments

We thank Annika Westin for excellent technical support, Ingela Stake and colleagues at National Veterinary Institute for excellent care of the mice, and the SciLifeLabBioVis platform at Uppsala University for providing flow cytometry facilities.

Disclosures

The authors have no financial conflicts of interest.

References

6. Henry, C., and N. K. Jerne. 1968. Competition of 19S and 7S antigen receptors in immunoglobulin production in mice deficient for complement receptors 1 and 2 can be induced by anti-IgG/Ag and IgE/Ag complexes, but not IgM/Ag complexes. Scand. J. Immunol. 32: 2328–2337.


