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Innate Immunity Mediates Follicular Transport of Particulate but Not Soluble Protein Antigen

Alexander Link,* Franziska Zabel,* Yvonne Schnetzler,* Alexander Titz,* Frank Brombacher,† and Martin F. Bachmann*

Ag retention on follicular dendritic cells (FDCs) is essential for B cell activation and clonal selection within germinal centers. Protein Ag is deposited on FDCs after formation of immune complexes with specific Abs. In this study, by comparing the same antigenic determinant either as soluble protein or virus-like particle (VLP), we demonstrate that VLPs are transported efficiently to murine splenic FDCs in vivo in the absence of prior immunity. Natural IgM Abs and complement were required and sufficient to mediate capture and transport of VLPs by noncognate B cells. In contrast, soluble protein was only deposited on FDCs in the presence of specifically induced IgM or IgG Abs. Unexpectedly, IgG Abs had the opposite effect on viral particles and inhibited FDC deposition. These findings identify size and repetitive structure as critical factors for efficient Ag presentation to B cells and highlight important differences between soluble proteins and viral particles. The Journal of Immunology, 2012, 188: 3724–3733.

A daptive immune responses require the encounter of Ag with rare Ag-specific immune cells. Secondary lymphoid organs provide a framework to maximize the probability of such an interaction. Processed Ag is transported to secondary lymphoid organs from the periphery by APCs for the activation of T cells. Humoral immunity, in contrast, requires the transport of intact Ag to B cell follicles where it is deposited as immune complexes (ICs) on follicular dendritic cells (FDCs) (1–3). Ag retention on FDCs is thought to be essential for B cell activation and clonal selection within germinal centers (GCs) (4).

How ICs of proteins reach B cell follicles has been a long-standing question as they do not travel freely into the parenchyma of lymphoid tissues (5, 6). Recent advances in high-resolution imaging techniques have provided insights into the mechanism by which ICs are relayed to FDCs. ICs drain to lymph nodes (LN), engulfs the receptor and lymph and is captured and displayed on the surface of CD169+ subcapsular macrophages (7, 8). These macrophages extend cell protrusions through the subcapsular sinus wall into the follicles. Noncognate B cells then pick up the ICs in a complement-dependent manner and deliver them to FDCs. The higher expression of complement receptors 1 (CD35) and 2 (CD21) by FDCs (9–11) and shuttle them to FDCs (9, 12).

Alternative pathways for follicular transport were proposed for small Ags. Proteins below 40 kDa were shown to gain access to B cell follicles via a system of conduits throughout the B cell follicles (13). In addition, cognate B cells were found to acquire small protein Ag directly from subcapsular sinus macrophages (14) or from dendritic cells (DCs) (15) via binding to the BCR. Particulate Ags, such as vesicular stomatitis virus (16) or protein-coated beads (17), were also shown to be acquired by cognate B cells from subcapsular sinus macrophages. However, whether binding by cognate B cells leads to Ag deposition on FDCs remains to be evaluated.

In this study, we have taken advantage of the ability to express the coat protein of the bacteriophage Qβ either as 28-kDa dimeric protein (Qβ-dimer) or as virus-like particles (Qβ-VLPs) of 2.5 MDa to study the influence of Ag structure on follicular transport. We demonstrate that in contrast to Qβ-dimer, prior immunity was not required for the deposition of Qβ-VLPs on FDCs. Instead, low-affinity natural IgM (nIgM) Abs and complement were sufficient to mediate binding by noncognate B cells and transport of Qβ-VLPs. The more efficient transport of Qβ-VLPs correlated with stronger induction of GC responses compared with that of Qβ-dimer. In contrast, deposition of Qβ-dimer on FDCs was dependent on the formation of ICs with specific anti-Qβ IgM or IgG Abs. Hence, during the primary immune response, the innate humoral immune system mediates deposition of particulate but not soluble proteins on FDCs.

Materials and Methods

Mice

Wild-type C57BL/6 and BALB/c mice were purchased from Harlan (Horst, The Netherlands), and Ly5.1 mice were obtained from The Jackson Laboratory and bred at BioSupport (Schlieren, Switzerland). Mice deficient
complement receptors CR1 and CR2 (C\textsubscript{R}2/\textsubscript{a}, C\textsubscript{R}2/\textsubscript{b}) (22), complement components C3 (C\textsubscript{3}) and C4 (C\textsubscript{4}), and IgM (C\textsubscript{M}) and JH (C\textsubscript{H}) were generated as described. The C\textsubscript{R}2/\textsubscript{a} and C\textsubscript{R}2/\textsubscript{b} were reported to express a truncated version of CD21 (27). We do not expect this, however, to influence our results as C3 binding by FDCs or MZ B cells was reported to be completely abrogated (27). Mice were housed in germ-free conditions at the BioSupport animal facility, and all experiments conformed to ethical principles and guidelines approved by the Swiss Federal Veterinary Office.

Production of QB-VLPs, QB-dimer, and RNA-free QB-VLPPG

QB-VLPs were expressed in Escherichia coli using the vector PQ810 and purified as described previously (28). During the self-assembly of the QB coat protein into VLPs, E. coli-derived mRNA (ssRNA) is packaged into the virus-like particles (VLPs); the amount of packaged ssRNA is 300 µg ssRNA per milligram QB. QB-dimer was produced by dissembling VLPs in PBS containing 2 mM DTT, 2 mg/ml Rnasase A, and 1 M NaCl and purification by ion-exchange and size-exclusion chromatography. To produce QB devoid of packaged innate stimuli, VLPs were reassembled in the presence of the polyanionic molecule polyglutamic acid. The resulting VLPs were devoid of RNA (QB-VLPPG) but exhibited the shape and size of the original QB-VLPs as demonstrated by electron microscopy (29). QB-VLPs and QB-dimer were conjugated to Alexa 488 or Alexa 647 dyes using Alexa Fluor Protein Labeling Kits (Invitrogen) according to the manufacturer’s instructions.

Immunization

Mice were immunized with QB-VLPs, QB-VLPPG, or QB-dimer either i.v. into the tail vein or s.c. into both sides of the abdomen. For i.v. immunization, QB was diluted in PBS to a final administration volume of 100 µl and for s.c. immunization to a volume of 200 µl.

In vivo generation of ICs

Wild-type mice were immunized with 50 µg QB-VLPs s.c. and bled 5 d later to prepare immune sera containing mostly anti-QB IgM and only a small fraction of anti-QB IgG anti-bodies. To obtain polyclonal anti-QB IgG Abs, mice were immunized s.c. three times with 50 µg QB-VLPs in biweekly intervals. Four weeks after the last immunization, IgM Abs were purified from serum by affinity chromatography using a Sepharose Fast Flow Protein G (GE Healthcare) column. To generate ICs in vivo, mice were given 200 µg polyclonal anti-QB IgG Abs or 250–500 µl of naive or day 5 immune sera i.p. 24 h before s.c. injection of either QB-VLPs or QB-dimer in the flank draining to the inguinal LN. For s.c. injection, mice were anesthetized with isoflurane. Similar deposition of QB-ICs was achieved with in vitro-formed QB-ICs (data not shown).

ELISA

For determination of QB-VLP–specific IgM titers, ELISA plates (Nunc Immuno MaxiSorp, Rochester, NY) were coated overnight with 100 µl QB-VLPs (1 µg/ml), and binding of serum Abs was detected by HRP-conjugated goat anti-mouse IgM (Jackson ImmunoResearch). Absorbance readings at 450 nm of the 1,2-phenylenediamine dihydrochloride color reaction were graphed.

ELISPOT assay

QB-VLP–specific Ab-forming cell (AFC) frequencies were determined as described (30). Briefly, 24-well plates were coated with 10 µg/ml QB-VLPs. Splenocytes were added in DMEM containing 2% FCS, 10 mM HEPES, and penicillin/streptomycin and incubated for 5 h at 37°C. Cells were washed off, and plates were incubated with goat anti-mouse IgG (EY Labs), followed by alkaline phosphatase-conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch) before development of alkaline phosphate color reactions.

Flow cytometry

To detect cells in association with QB-VLPs in the blood, mice were immunized i.v. with 50 µg QB-VLPs labeled with Alexa 488. Sixteen hours postimmunization, single-cell suspensions of spleens and draining LNs were prepared using 70-µm cell strainers (BD Falcon). Cells were blocked with rat anti-mouse CD16/32 (1/200; 2.4G2) and then labeled with PE-conjugated or PerCP–Cy5.5-conjugated rat anti-mouse CD19 (1/500; BD Pharmingen) as well as allophycocyanin-conjugated hamster anti-CD11c and rat anti-CD11b, anti-CD4, and anti-FDS (1/400; all BD Pharmingen) and PE-conjugated anti-CD45.2 (1/40; ebioscience) in 100 µl PBS containing 2% (v/v) FBS and 2 mM EDTA. Dead cells were excluded by propidium iodide staining. Data were acquired on FACSCanto or FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

Immunofluorescence

Freshly dissected spleen and LNs were embedded in optimum cutting temperature compound (Sakura) without prior fixation. Cryostat sections (8 µm in thickness) on Super Frost/Plus glass slides (Fisher Scientific) were air-dried overnight and then fixed for 10 min in ice-cold acetone. After rehydration, sections were blocked with 1% (v/v) BSA and 1% (v/v) normal mouse serum. Immunofluorescence labeling was done with Abs against 0.1% (v/v) PBS containing 1% (v/v) normal rabbit serum. QB Ag was detected by incubating sections with rabbit anti-QB anti-serum (1/1000; produced by RCC, Itingen, Switzerland), followed by Alexa 488-conjugated goat anti-rabbit Ab (1/1000; Molecular Probes). B cell follicles were identified with Alexa 647 rat anti-B220 (1/200; BD Pharmingen), FDCs with anti-CD35 biotin (1/100; BD Pharmingen), and macrophages with anti–MOMA-1 biotin (1/50; Abcam). Peaun agglutinin (PNA)–binding B cells were detected with biotinylated PNA (1/800; Vector Laboratories). The CR–Fc reagent, which was a gift from Dr. Kosco-Vilbois (31), was detected with goat anti-human IgG biotin (Jackson ImmunoResearch). DCs were labeled using a biotinylated hamster anti-CD11c (HL3; BD Pharmingen) Ab. Biotinylated Abs were detected with Alexa 546-labeled streptavidin (1/1000). Images were acquired on an Axioplan microscope with an Axiocam MRm (Zeiss) camera using Open laboratory software (Improvement) and edited with Adobe Photoshop software. Accumulation of QB Ag in B cell follicles was quantified using National Institutes of Health ImageJ software. Background was subtracted from single-channel images, and mean fluorescence intensity of QB fluorescence was determined as mean gray value in B220+ or IgD+ regions.

Colocalization image analysis

Dual-channel colocalization analysis was performed on B220+ B cell follicles as region of interest of the images represented in Fig. 6A using the National Institutes of Health Image J software. The colocalization threshold and the intensity correlation analysis plug-ins were used to perform intensity correlation analysis and generate Mander’s overlap (R) and Pearson’s (r) correlation coefficients as described (32, 33). Background was corrected by subtraction of a selected region of interest. The intensity correlation analysis plugin also calculates Mander’s colocalization coefficients for channel 1 (M1, red, QB) and channel 2 (M2, green, CD35) representing the number of colocalized pixels expressed as a fraction of the number of the pixels within the respective channels. Zero-zero pixels are ignored for purposes of this calculation. The Mander’s coefficients range from 0 to 1 with 1 being high colocalization, zero being low, and are independent of the pixel intensities within the individual channels (33). Pearson’s correlation coefficient (r) was determined by both plug-ins and matched well. It ranges from 1 to −1 with a value of 1 representing perfect colocalization and zero representing random localization.

Radiation bone marrow chimeras

Wild-type Ly5.1 mice were lethally irradiated with 950 rad and were reconstituted with a mixture of 20% bone marrow cells from Cr2-deficient donors and 80% from JH-deficient donors. Prior to cell transfer, T cells were depleted from the bone marrow cell suspension using a MACS-bead CD4 T cell isolation kit (Miltenyi Biotec). Control chimeras were made by transferring 20% of bone marrow cells from C57BL/6 donors and 80% from JH-deficient donors. Engraftment of chimeric mice with Cr2-deficient B cells was confirmed 8–10 wk later by flow cytometry using a PE-conjugated anti-CR1/2 Ab (1/100; BD Pharmingen). Mice were then immunized with 50 µg QB-VLPs i.v.

Gel filtration of QB-VLPs and QB-dimer

Gel filtration was performed on a Sephadex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated with PBS. Approximately 500 µg QB-dimer and 300 µg QB-VLPs were loaded onto the column and eluted with PBS at a flow rate of 2.5 ml/min via an Astra Purifier system (GE Healthcare). The m.w. of the eluted peaks was calculated from the retention time using a protein standard.

Statistics

Statistical analysis was performed with a Kruskal–Wallis test for multiple group comparisons or else with a two-sided Mann–Whitney U test using GraphPad Prism software (GraphPad Software).
Results

Particulate Ags are transported to FDC in the absence of specific Abs

To study the influence of Ag size and structure on trafficking, we used VLPs derived from the bacteriophage Qβ (Qβ-VLP). The gene encoding the 14-kDa capsid protein can be expressed in E. coli, resulting in spontaneous assembly into VLPs. The 30-nm icosahedral particles consist of 180 coat protein monomers with a mass of 2.54 MDa as verified by HPLC–size-exclusion chromatography (Fig. 1A). Qβ-VLPs are morphologically and immunologically indistinguishable from the parental phage (28) and encapsulate E. coli-derived RNA, which is necessary for particle assembly (34). Qβ-VLPs can be disassembled, and, upon removal of the RNA, the coat protein can be kept in solution as a non-covalently stabilized dimer (Qβ-dimer) (Fig. 1B). The protein composition of both Qβ-VLPs and Qβ-dimer is, however, identical as evidenced by the 14-kDa band on a reducing SDS-PAGE gel (Fig. 1C). 

To analyze Ag trafficking during the primary immune response, naive wild-type mice were challenged with Qβ-VLPs or Qβ-dimer, and Ag deposition on FDCs was determined 24 h after challenge. Equimolar amounts of protein, calculated on the basis of total weight and therefore containing the same number of Qβ monomers, were injected. Spleen sections were labeled with polyclonal anti-Qβ rabbit serum to detect Ag. B cell follicles were labeled with Abs against IgD and MZ macrophages with MOMA-1 to illustrate splenic architecture. Consistent with previous reports on other soluble protein Ags (5, 7, 19, 20), Qβ-dimer was undetectable inside B cell follicles in naive mice. In contrast, in VLP form, Qβ was readily detected in a reticular pattern inside B cell follicles in naive mice (Fig. 2A). In a second experimental setup, wild-type mice were passively immunized with polyclonal mouse anti-Qβ IgM serum to allow the in vivo formation of ICs. They were then i.v. challenged with Qβ-VLPs or Qβ-dimer and Ag deposition on FDCs analyzed 24 h after challenge. As expected for ICs, Qβ-dimer was now readily transported to B cell follicles as were Qβ-VLPs (Fig. 2A). Quantitative analysis of the fluorescence images confirmed the accumulation of Qβ inside IgM+ B cell follicles in the case of Qβ-VLPs but not Qβ-dimer in the absence of specific IgM Abs (Fig. 2B).

To determine the transport kinetics of particulate Ag into B cell follicles, naive wild-type mice were immunized with Qβ-VLPs either i.v. or s.c., and spleen or draining LNs, respectively, were analyzed for deposition of Qβ Ag at different time points after challenge. Ag was again detected with polyclonal anti-Qβ rabbit serum. B cell follicles were labeled with Abs against IgD and FDCs with CD35 to localize the Ag. Qβ-VLPs were efficiently trapped in the splenic MZ and were detectable 4 h after i.v. challenge on CD35+ FDCs where they accumulated over time (Fig. 2C). The yellow color hereby results from the colocalization of the red fluorescence of Qβ and the green fluorescence of CD35. After s.c. challenge, most of the Qβ in the draining LN was found in the subcapsular sinus already 1 h after challenge. Over time, Qβ-VLPs accumulated on FDCs with the highest intensity observed at the 24-h time point. At 4–8 h, however, a large part of the Ag was detected in the medulla where it is likely to be degraded by macrophages (Supplemental Fig. 1). This kinetic is comparable to what has been found with ICs of proteins (8, 13).

In summary, Qβ-VLPs were quickly and efficiently relayed to FDC networks even in the absence of specific immunity. This is in contrast to Qβ in soluble dimeric form, which failed to deposit on FDCs in naive mice unless it was given as an IgM IC.

Qβ in particulate form induces a stronger GC response

Efficient transport of Ag to B cell follicles has been shown to be important for GC reactions and affinity maturation (7). We therefore compared the ability of Qβ-VLPs and Qβ-dimer to induce GC responses. To produce Qβ-VLPs without packaged RNA, which serves as a stimulator of innate immunity, VLPs were disassembled and reassembled in the presence of the polyanionic molecule polyglutamic acid. This resulted in VLPs devoid of RNA (Qβ-VLPPPG) (29). MyD88-dependent cytotoxic T cell priming with Qβ-VLPs has previously been shown to be abrogated with Qβ-VLPPPG indicating that innate stimuli were removed and only the protein component of Qβ was present (29). In addition, Qβ-VLPPPG was deposited on FDCs as efficiently as Qβ-VLP in naive mice (data not shown).

Wild-type mice were challenged with Qβ-VLPPPG or Qβ-dimer. Ag doses containing both equal molar amounts of Qβ monomer molecules and therefore the same total protein weight. Ten days after challenge, spleen sections were analyzed for the formation of GCs by labeling with Abs against PNA, B220, and Qβ. Qβ-VLPs (Qβ-VLPPPG) strongly induced PNA+ GCs in which Qβ-antigen was readily detectable. Immunization with equal amounts of Qβ-dimer, in contrast, failed efficiently to induce Qβ+ GCs, and only low amounts of Qβ-antigen were detectable inside B cell follicles (Fig. 3A).

The expansion of Ag-specific B cells was analyzed by ELISPOT assay. Naive mice were immunized with Qβ-VLPPPG or Qβ-dimer Ag. Eleven days after challenge, the numbers of anti-Qβ IgG AFCs were measured in LNs and spleen. Immunization with Qβ-VLPPPG induced higher numbers of anti-Qβ AFCs compared with that of equal molar amounts of Qβ-dimer (Fig. 3B).

In summary, the more efficient transport of particulate Qβ-VLPs correlates with the induction of stronger GC responses and higher numbers of Ag-specific AFCs compared with those of Qβ-dimer.

Transport of VLP Ag depends on complement and B cells

Follicular transport of protein ICs requires the presence of B cells and the components of the complement system (8, 9). Because...
VLPs. Spleen sections were labeled with Abs against Q5 and Q6 of specific Abs. (FIGURE 2. Viral Ags are quickly transported to follicles in the absence of specific Abs. (A) C57BL/6 mice were passively immunized i.p. with 250 μl of day 5 anti-Qb immune serum containing anti-Qb IgM. After 24 h, immunized as well as naive mice were challenged i.v. with 50 μg Qb-dimer or Qb-VLPs. Spleen sections were labeled with Abs against Qb (red), MOMA-1 (green), and IgD (blue) 24 h after challenge. Letters indicate splenic architecture: B, B cell follicles; T, T cell zone; MZ, marginal zone; RP, red pulp. Arrowheads indicate Qb staining inside B cell follicles. Scale bar, 100 μm. (B) Quantification of the accumulation of Qb inside B cell follicles. Images represented in (A) were analyzed for mean fluorescence intensity (MFI) of the red Qb channel inside IgD+ areas. Values are indicated as percentages of maximum intensity (MFI) of the red Qb alone. Scale bar, 100 μm. (C) Naive C57BL/6 mice were challenged with 50 μg Qb-VLPs i.v., and spleen sections were labeled at different time points after challenge with Abs against Qb (red), CD35 (green), and IgD (blue). Black-and-white images show Qb staining alone. Scale bar, 100 μm. Data are representative of at least three independent experiments.

To test the functional consequence of Qb-VLP binding to B cells, we made mixed bone marrow chimeras where only B cells lacked complement receptors (Cr2−/−JH−/→ B6). All other hematopoietic cell types can be reconstituted with wild-type complement receptors from the JH hematopoietic cell types can be reconstituted with wild-type complement receptors 1 (CD35) and 2 (CD21) (Cr2−/−) as well as mice deficient in the complement components C3 (C3−/−) and C1q (C1q−/−) with Qb-VLPs and analyzed Ag deposition on splenic FDCs by histology 24 h after challenge. Whereas wild-type controls showed efficient deposition on the FDC network, no Qb-VLP Ag could be detected on FDCs in Cr2− as well as C3-deficient mice. In the latter mice, Qb was detected only in the MZ and the red pulp. In the case of C3−/− mice, Ag was barely detectable even in the MZ in agreement with results on ICs, which showed that Ag is rapidly degraded in these mice (35). Notably, also in C1q-deficient mice, which fail to activate the classical pathway of complement activation, we observed a complete block of VLP deposition on FDC networks despite the absence of specific Abs (Fig. 4A). Quantitative analysis of the fluorescence images confirmed the strong reduction in follicular accumulation of Qb-VLPs in Cr2−, C3−, and C1q-deficient mice (Fig. 4B).

B cells have been reported to shuttle ICs from the MZ to follicles (12). We therefore tested whether B cells were also able to bind VLP Ag in the absence of specific Abs. We immunized wild-type mice with Alexa 488-labeled Qb-VLPs and analyzed binding of Qb-VLP Ag to splenic B cells by flow cytometry 16 h after challenge. Plots were pregated on propidium iodide (PI)− lymphocytes and show Alexa 488 fluorescence for CD19+ and CD19− cells. More than 20% of splenic CD19+ B cells from wild-type mice were positive for Alexa 488 indicating binding of Qb-VLPs to noncognate naive B cells (Fig. 4C). The CD19− cells showing Alexa 488 fluorescence were mostly DCs or macrophages; T cells were negative for Alexa 488 (data not shown). Mice deficient in complement receptors 1 and 2 (Cr2−/−) as well as complement components C3 and C1q were also injected with Alexa 488-labeled Qb-VLPs, and binding to B cells was analyzed 16 h after challenge. Histograms were pregated on CD19+ B cells and show the fluorescence intensity for Alexa 488. Alexa 488 labeling was reduced on B cells from C1q-deficient mice and almost completely abrogated in C3- and Cr2-deficient mice compared with that of B cells from unchallenged naive wild-type mice (Fig. 4D). Unlabeled B cells from Ly5.1 mice served as internal control.

To exclude formally that other hematopoietic cell types, such as DCs or macrophages, could contribute to Cr2-dependent transport, FDCs still expressed complement receptors and were able to bind ICs (data not shown). Nevertheless, Qb deposition on FDCs was completely abrogated in the chimeras, in contrast to B6/JH−/→ mice.
Data are representative of three independent experiments.

FIGURE 3. Particulate Ag induces strong GC responses. (A) Immuno-fluorescence of spleen sections from C57BL/6 mice 10 d after i.v. immunization with 50 μg Qb-VLPPG or Qb-dimer. Sections were labeled with Abs to Qb (red), B220 (blue), and PNA (green). Scale bar, 100 μm. (B) ELISPOT analysis of the number of Qb-specific B cells in mLNs and spleen 11 d after s.c. immunization with 50 μg Qb-VLPPG or Qb-dimer. Data are representative of three independent experiments.

B6 control animals, which showed normal follicular Ag deposition (Fig. 4E). These results demonstrate that noncognate B cells play a crucial role for transport of non-immunocomplexed VLP Ag.

To determine whether B cells are able to bind Qb-dimer, we immunized mice with Alexa 488-labeled Qb-VLPs as well as Alexa 488-labeled Qb-VLPs and analyzed binding of Qb-antigen to splenic B cells by flow cytometry 16 h after challenge. Histograms were pre gated on CD19+ B cells and show the fluorescence intensity for Alexa 488. In Qb-dimer immunized mice, Alexa 488 fluorescence on B cells was comparable to the naive background in contrast to Qb-VLP immunized mice indicating that B cells were not able to efficiently bind Qb-dimer in naive mice (Fig. 4F).

In summary, particulate Ag is transported by noncognate B cells in a complement-dependent fashion, despite the absence of specific Abs.

Natural Abs mediate transport of particulate Ag

The physiologically most important activation of the classical complement pathway is initiated by binding of the globular domains of C1q to Ag-bound IgG or IgM. In the absence of specific immunity, the classical pathway can be activated by so-called natural IgM (nIgM) Abs that are produced without previous exposure to foreign Ag. Several reports have shown that sera from naive mice contain low-affinity nIgM Abs that bind to viruses and bacteria and are important for neutralizing these pathogens (36–39). The reduced Ag deposition in C1q-deficient mice suggested that nIgM may also play a role in Ag transport.

We immunized IgM-deficient (IgM−/−) mice (25) with Qb-VLPs and analyzed follicular Ag deposition on splenic sections 24 h after challenge. These mice lack nIgM Abs in serum, but B cells still express IgD and are able to undergo class-switching. Hence, all other Ig isotypes are present in serum. Compared with BALB/c control mice, Qb-VLP deposition on FDCs was completely abrogated in IgM-deficient mice. Passive transfer of anti-Qb IgM immune sera or sera from naive wild-type mice was able to restore follicular Ag transport in IgM-deficient mice (Fig. 5A). This is confirmed by quantitative analysis of the fluorescence images, which indicates that transfer of anti-Qb IgM immune sera and even sera from naive wild-type mice significantly restores follicular Qb deposition compared with IgM-deficient mice (Fig. 5B).

In accordance with these data, we detected binding of nIgM to Qb by ELISA in sera of naive wild-type mice but not in sera from IgM-deficient mice (Fig. 5C).

We further analyzed whether these nIgM Abs play a role in Ag binding to B cells. To this end, we immunized IgM-deficient mice i.v. with Alexa 488-labeled Qb-VLPs and analyzed binding of Qb-antigen to splenic B cells by flow cytometry 16 h after challenge. Histograms were pre gated on CD19+ B cells and show the fluorescence intensity for Alexa 488. Qb-VLP binding to B cells was reduced in mice lacking nIgM (Fig. 5D). Passive transfer of naive serum to IgM−/− mice was not able to restore Ag binding by B cells likely due to the lower concentration of transferred natural Abs in the host compared with the wild-type situation (data not shown). However, passive immunization of IgM−/− mice with anti-Qb IgM serum prior to challenge restored B cell binding to wild-type levels (Fig. 5D).

Together, these results suggest that in addition to their role in virus neutralization, nIgM Abs enhance adaptive immune responses by mediating efficient Ag transport to B cell follicles.

IgG Abs target viral particles for phagocyte uptake and inhibit FDC deposition

Ag in the form of ICs is typically more potent in promoting Ab responses than free protein Ag (40). With IgG Abs, this enhancement was mainly observed with soluble protein Ags, whereas responses against particulate Ags were suppressed (41). This dual effect was dependent on the nature of the Ag, as the same anti-hapten Ab was able to enhance or suppress Ab responses when the hapten was conjugated to a soluble protein or particulate Ag, respectively (42). The reason for this dual effect has remained unclear.

To assess the effects of IgG Abs on Ag trafficking, we passively immunized C57BL/6 mice with purified anti-Qb IgG Abs and challenged them i.v. with Qb-VLPs or Qb-dimer. Ag deposition on splenic FDCs was analyzed by histology 24 h postchallenge. As previously shown for other soluble proteins (5, 7, 19), ICs of Qb-dimer with anti-Qb IgG were efficiently transported to FDC networks in contrast to Qb-dimer alone (Fig. 6A). Qb-dimer–IgG ICs, therefore, behave similarly to Qb-dimer–IgM ICs (Fig. 2A). Particulate Qb-VLPs, however, were only deposited on FDCs in naive mice. In mice immunized passively with anti-Qb IgG Abs, Qb-VLPs could not be detected on FDCs (Fig. 6A). Instead, Qb-VLPs were found more strongly trapped by cells in the MZ. Magnification images show that some Qb was found in B cell follicles but did not colocalize with CD35 (Fig. 6B). Table I summarizes statistical colocalization data of a dual-channel colocalization analysis of Qb and CD35 fluorescence. Statistical analysis of the images represented in Fig. 6A demonstrates high colocalization of Qb-VLP with CD35+ FDCs indicated by values for Pearson’s and Manders’ coefficients close to one. Coinjection of anti-Qb IgG Abs, however, completely abrogates colocalization indicated by Pearson’s and Manders’ coefficients close to zero. This is not the case for Qb-dimer where anti-Qb IgG enhances accumulation of Qb on FDCs (Table I).

Flow cytometric analysis of binding of Qb-VLP–Alexa 488 to B cells in mice passively immunized with anti-Qb IgG Abs showed a clear reduction in Qb-VLP binding to B cells compared with that in naive mice (Fig. 6C). Instead, the follicular Qb colocalized largely with a subtype of CD11c+ DCs identified by a fusion protein of the cysteine-rich portion of the mouse mannose receptor and the Fc portion of human Ig (CR–Fc) (Fig. 6D). CR–
and B220 (blue). The arrowhead indicates Qb after Qb and complement receptors 1 and 2 (Cr2) were analyzed for mean fluorescence intensity (MFI) of the red Qb in the accumulation of Qb inside B cell follicles of the wild-type control. Scale bar, 100 μm.

**Figure 4.** Expression of complement receptors 1 and 2 by B cells is required for Ag capture and FDC deposition. (A) Immunofluorescence of spleen sections from C57BL/6 wild-type mice and mice deficient in complement components C3 and C1q, as well as mice deficient for complement receptors 1 and 2 (Cr2−/− mice). Spleen sections were labeled with Abs against Qb (red), MOMA-1 (green), and B220 (blue). The arrowhead indicates Qb staining inside the B cell follicle of the wild-type control. Scale bar, 100 μm. (B) Quantification of the accumulation of Qb inside B cell follicles. Images represented in (A) were analyzed for mean fluorescence intensity (MFI) of the red Qb channel inside B220+ areas. Values are indicated as percentages of maximum ± SEM. (C and D) Flow cytometric analysis of Qb-binding by splenic B cells from wild-type, C3-deficient, C1q-deficient, and Cr2-deficient mice 16 h after i.v. challenge with Qb-VLP–Alexa 488. (E) Immunofluorescence of spleen sections from mixed bone marrow chimeric mice with Cr2 deficiency only on B cells (Cr2−/−/JH−/− → B6) and wild-type controls (B6/JH−/− → B6). Spleen sections were labeled with Abs against Qb (red), CD35 (green), and B220 (blue). Arrowheads indicate the CD35+ area in the black-and-white images showing only Qb staining. Scale bar, 100 μm. Data are representative of six mice, analyzed in two experiments. (F) Flow cytometric analysis of Qb binding by splenic B cells 16 h after i.v. challenge with Qb-VLP–Alexa 488 and Qb-dimer–Alexa 488. Histograms show intensity of Alexa 488 signal on CD45.1+ B cells from challenged and naive control mice.

**Discussion**

Many studies have reported that Ag transport and retention on FDCs require the formation of ICs (9, 19–21). Protein Ag is not detected on FDCs in the absence of specific Abs. Therefore, efforts to elucidate where B cells encounter Ag have focused on transport in the setting of a secondary immune response; that is, in the presence of either specific Abs (5, 7–9, 12, 18, 19) or large numbers of transgenic cognate B cells (14–17). Our findings demonstrate that in contrast to model protein Ags, prior immunity is not required for the capture of viral particles in lymphoid organs and delivery to FDCs. Characteristics of viral Ags such as their particulate nature and repetitive surface structure may overcome the requirement for prior immunity for efficient Ag transport.

Multiple pathways transporting Ag to FDCs have been described. ICs with proteins are captured in the subcapsular sinus of LNs or the splenic MZ. B cells then bind the ICs via complement receptors and deliver them to FDCs (8, 9, 12). Small, uncomplexed proteins have been shown to use a different pathway. They are able to drain passively into B cell follicles through a system of conduits (13). However, retention on FDCs also requires the presence of specific Abs. Particulate Ags such as VSV or hen-egg lysosome-coated particles can be directly acquired from subcapsular sinus macrophages by cognate B cells (16, 17). Direct interaction of a protein Ag with its cognate B cells has also been reported (14). In a naive situation, however, very few specific B cells are present, and the contribution of this mode of Ag transport is likely to be small. Surprisingly, in one of these studies, the observation was made that fluorescent-labeled beads accumulated on FDCs despite the absence of cognate B cells (17). How the beads were transported in the absence of specific B cells was, however, not determined. Our findings demonstrate that naive B cells are able to bind particulate Ag in a complement receptor-dependent fashion and to deliver it to FDCs in the absence of prior Fc has been previously shown to label a population of DCs that do not degrade Ag and migrate to the follicle upon activation (31).

In summary, Ag-specific IgG Abs enhanced transport of Qb to FDCs when injected as a small dimeric protein but inhibited FDC deposition of Qb-VLPs. Instead, in the presence of IgG, particulate Qb-VLPs were trapped by phagocytic cells such as macrophages and CR-Fc+ DCs in the MZ. CR-Fc+ DCs were able to transport Qb-VLP Ag into follicles but did not appear to transfer the Ag to FDCs. Transport of VLPs therefore shows important differences compared with the often studied protein–IgG ICs.
immunity. Neither cognate B cells nor specific Abs were required. This suggests that complement-dependent transport by naive B cells may be a general pathway for transporting not only ICs but also viral particles.

In the absence of specific Abs, complement needs to be activated by other means. We provide evidence that the reason for the much more efficient transport of viral particles lies in the presence of natural Abs. Unspecific nIgM Abs present in serum of naive animals have been shown to bind a number of pathogens (36–38, 43). They represent an innate defense mechanism and play an important role in protection from viral and bacterial infection. Besides direct neutralization of pathogens, they critically influence adaptive immune responses. In the absence of secreted IgM, IgG Ab responses are strongly reduced despite normal CD4 T cell responses (44–46). Our results demonstrate that low-affinity nIgM Abs are sufficient to activate the classical complement pathway thereby promoting FDC delivery of viral particles by noncognate naive B cells. Avidity effects are likely to permit binding of nIgM Abs to repetitive viral surfaces explaining why the same antigenic determinant is transported efficiently as a VLP but not as a dimeric protein. In addition, complement can also be activated via the alternative or lectin pathways. Mannan-binding lectin and its receptor DC-SIGN have been implicated in the uptake of influenza viral particles by medullary DCs (47). The partial reduction of B cell binding in C1q-deficient mice suggests that these pathways might contribute. This may explain why we found only a partial reduction of B cell binding in C1q- and IgM-deficient mice compared with the complete abrogation in C3- and Cr2-deficient mice. Notably, the reduced B cell capture appeared to be biologically important as FDC deposition of Qβ-VLPs was completely blocked in these mice. Natural Abs therefore allow viral particles to be transported by the same mechanisms that have been reported for ICs of protein Ags without the requirement of previously induced Ag-specific immunity.

Recently, the role of complement activation by IgM for enhancing immune responses has been called into question. Mice lacking complement-activating IgM, owing to a point mutation in the μ H chain, showed a normal Ab response to sheep RBCs (48). Upon injection of specific IgM Abs, other mechanisms besides classical pathway activation might therefore also play a role for IgM-mediated Ag trafficking despite the requirement for C1q. Our findings of strong defects in Ag presentation on FDCs, however, agree well with the strongly reduced Ab responses reported by earlier studies in the absence of nIgM and of components of the classical pathway (4, 40). Regardless of the exact mechanism, enhanced Ag presentation of particulate Ags therefore appears to be closely associated with the crucial roles that complement and nIgM play for the induction of IgG responses (44–46) and for the maintenance of long-term memory to viral pathogens (49).

Specific Abs have a profound influence on Ab responses (40, 41). Protein Ag in the form of ICs with IgM Abs is typically more potent than free Ag in promoting Ab responses (40). The mechanism for the IgM-mediated enhancement of the IgG response has remained unclear. Direct stimulation of B cells by cross-linking BCRs or by stimulating complement receptors have been proposed. A role for Ag localization has also been suggested based on the correlation between the magnitude of Ab responses and the total amount of Ag trapped in the spleen in the presence of specific IgM (50). Our data extend these findings suggesting that not just trapping of Ag in the spleen but also enhanced transport into follicles, in the form of IgM ICs, is critical. The efficient delivery of particulate Ag to follicles even in the absence of specific Abs might therefore be an important reason for the high immunogenicity that viral Ags generally have. IgG Abs, in contrast, can have enhancing or suppressing effects on Ab responses. Generally, they enhance the immunogenicity of protein Ags but reduce it for particulate Ags (40, 41). Our findings demonstrate that this dual effect of IgG Abs correlates with their influence on Ag trafficking. This also extends the study by Dennert (50), which compared the total amount of Ag–IgG complexes in the spleen with the magnitude of the immune response and
failed to detect a correlation. Consistent with previous results on IgG ICs with protein Ag (5, 7, 19, 20), anti-Q\(b\) IgG Abs enhanced FDC deposition of Q\(b\)-dimer. However, our results demonstrate that the influence of IgG is dramatically changed when viral particles are used for immunization. In the presence of anti-Q\(b\) IgG Abs, a major fraction of the Q\(b\)-VLP Ag is trapped in the MZ, and delivery to FDCs is blocked. Epitope masking or Fc\(\gamma\)RIIB-mediated inhibition of B cell responses may explain the suppressing effects of IgG Abs. However, the small amount of IgG Abs that is sufficient to inhibit Ab responses to erythrocytes suggests that epitope masking is most likely not the only explanation for this effect (40). In our hands, the amount of IgG was also insufficient completely to mask Q\(b\) epitopes as we were still able to detect Q\(b\)-antigen with Abs by histology. Fc\(\gamma\)RIIB-mediated B cell inhibition is likely also not involved because IgG Abs are able efficiently to suppress primary Ab responses in Fc\(\gamma\)RIIB-deficient mice (51). It would also not explain why IgG Abs should in some instances enhance and in others suppress immune responses. Instead, our findings reveal a correlation between follicular Ag transport and the reported effects of IgG Abs on immunogenicity. Changes in Ag presentation may explain the opposite effects that the same Ab can have on the immune re-

**Table I.** Quantitative colocalization analysis of Q\(b\) fluorescence (red) with CD35 (green) inside B220\(^+\) B cell follicles of immunofluorescence data shown in Fig. 6A

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Q(b)-dimer</th>
<th>Q(b)-Dimer Plus Anti-Q(b) IgG</th>
<th>Q(b)-VLP</th>
<th>Q(b)-VLP Plus Anti-Q(b) IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson’s correlation coefficient, (r^a)</td>
<td>0.34 ± 0.07</td>
<td>0.72 ± 0.02</td>
<td>0.69 ± 0.04</td>
<td>-0.02 ± 0.06</td>
</tr>
<tr>
<td>Overlap coefficient according to Manders, (R)</td>
<td>0.39 ± 0.05</td>
<td>0.72 ± 0.02</td>
<td>0.76 ± 0.04</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>Colocalization coefficient M1</td>
<td>0.91 ± 0.04</td>
<td>0.97 ± 0.004</td>
<td>0.94 ± 0.03</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>Colocalization coefficient M2</td>
<td>0.24 ± 0.04</td>
<td>0.57 ± 0.01</td>
<td>0.75 ± 0.08</td>
<td>0.22 ± 0.1</td>
</tr>
</tbody>
</table>

Values are indicated ± SEM. M1, Manders’ coefficient for channel 1; M2, Manders’ coefficient for channel 2. M1 and M2 represent the number of colocalized pixels in the respective channel expressed as a fraction of the total number of non zero-pixels in that channel. Manders’ coefficients range from 0 (no colocalization) to 1 (100% colocalization) and are independent of the pixel intensities within respective channels.

\(^a\)Pearson’s correlation coefficient, \(r\) (the standard Pearson’s correlation coefficient ranges from \(-1\) to \(+1\) and is a general indicator for pattern similarity between two images).
FOLLICULAR TRANSPORT OF PARTICULATE BUT NOT SOLUBLE Ag

response toward protein versus VLP Ags. This further underlines the importance of efficient Ag delivery to FDCs for the induction of humoral immune responses.

Although FDC deposition of Qβ-VLP Ag was blocked in the presence of IgG Abs, some Ag reached B cell follicles. However, inside the follicles, Qβ-VLPs were principally associated with CR–Fc+ DCs. This population of DCs has previously been shown to capture and retain unprocessed Ag (31, 52). In contrast to conventional DCs that activate T cells, CR–Fc+ DCs have been reported to migrate to follicles within days upon encountering Ag and to activate B cells, although the kinetics of this event is considerably slower compared with the several hours required for B cell-dependent transport (31). DCs residing along the medullary sinus have also been shown to capture viral Ag via SIGN-R1 and start migrating toward follicles (47). Whether medullary DCs alone are sufficient to transport Ag to follicles or whether B cells are required to act as intermediaries has not been investigated. Together, our results suggest that follicular Ag transport by DCs may represent a pathway independent from transport by B cells. Prior immunity (i.e., the presence of Ag-specific IgG Abs) determines which pathway is preferentially taken. Further studies will be needed to determine the roles of the different Ag transport pathways in B cell activation during primary and secondary Ab responses.

The size and structure of Ags have a strong influence on B cell responses. When compared with B cell responses generated by small proteins, antiviral B cell responses have been shown to be more rapidly induced, to reach higher titers, and to be efficiently generated without adjuvants (53, 54). Viral Ags lead to faster antibody affinity maturation.


Figure S1. Kinetic of Qβ-VLP transport into lymph nodes of naïve mice after subcutaneous injection.

Naïve C57BL/6 mice were challenged with 50 μg Qβ-VLPs s.c. and sections of the draining inguinal lymph nodes were labelled at different time points after challenge with antibodies against Qβ (red), CD35 (green) and IgD (blue). Black and white images show Qβ-staining alone. Arrow-head indicates Qβ-positive staining inside B cell follicles. Scale bar, 100 μm. Data are representative of at least 3 independent experiments.