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A Protease-Dependent Mechanism for Initiating T-Dependent B Cell Responses to Large Particulate Antigens

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Ab production is critical for antimicrobial immunity, and the initial step in this process is the binding of Ag to the BCR. Recognition of Ag by the BCR results in a cascade of protein tyrosine phosphorylation events that promotes the expression of costimulatory and homing molecules, such as CD86, CD40, and CCR7 (1, 2). Activated B cells migrate to the border between the follicle and the T cell area where they display Ag-derivated peptides, in the context of MHC class II (MHC II) molecules, to activated CD4⁺ T cells (3). After receiving signals from T cells, B cells divide and switch isotypes (4). Some of the progeny establish residence in the paracortex and become Ab-secreting plasmablasts, whereas others migrate back to the follicle and participate in germal center reactions, where they undergo affinity maturation (5).

Ags must be carried from tissues to secondary lymphoid organs where naive lymphocytes reside before the aforementioned processes can occur. Following infection of tissue, microbes and their secreted proteins are carried through afferent lymphatic vessels and then deposited into the subcapsular and paracortical sinuses of the draining lymph node (6, 7). From here, soluble proteins or small viruses can enter the conduit network (8), which serves as a series of tubes that connect the subcapsular sinus to the paracortical space, as well as a scaffolding on which T cells migrate through the lymph node (9). The conduit network consists of a collagen core, wrapped within a microfibrillar matrix and basement membrane, and surrounded by fibroblastic reticular cells (10). Lymph node-resident dendritic cells (DCs) are found between the fibroblastic reticular cells, which line the conduits (10); they take up Ag from the conduits via macrophagocytosis and present it in the form of peptide:MHC II complexes to T cells (10, 11). Large particulate Ags, such as vaccinia virus, bacteria, and multicellular parasites, are presumably excluded from the conduit network, but they can be presented to T cells by tissue DCs that acquire Ag at the site of infection and then migrate into the T cell zone of the lymph node (11).

Ag acquisition by follicular B cells is more complex. In contrast to T cells, the majority of B cells reside in the follicles. Follicular B cells can acquire soluble proteins directly as they diffuse into the follicles (12), possibly from gaps in the floor of the subcapsular sinus (13–15) or more likely through a novel follicular conduit network that seems to be analogous to the paracortical conduit network (16). These mechanisms are ineffective for delivery of large particulate Ags to the follicle because of conduit size constraints; thus, various types of Ag transport cells have been proposed to carry particulate Ags to B cells (17, 18). DCs are one candidate, because they were shown to internalize and store native Ag via an FcγRIIB-dependent pathway (19–22). Although B cell follicles do not contain many DCs, newly arriving blood-borne B cells must traverse the DC-rich T cell zone on their way to the follicles, and they can acquire Ag from Ag-pulsed DCs in vivo (23). Subcapsular sinus macrophages, some of which protrude from the subcapsular sinus into lymph node follicles, were shown to translocate 0.2-μm microspheres (7), immune complexes (24), and virus particles (25) across the sinus floor and into the follicle. However, it is unknown whether these macrophages are capable of transporting larger particles, such as bacteria or multicellular parasites.

To investigate the mechanism by which naive lymph node B cells initiate T-dependent immune responses to particulate Ags, we constructed a 1-μm fluorescent particle decorated on the surface with an Ag containing T cell and B cell epitopes. We then tracked Ag-specific B cells following s.c. injection of the particles.

Materials and Methods

Animals and adoptive transfer

C57BL/6 recipient mice (6–8 wk old) and TCR-α knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TgEa TCR transgenic RAG-deficient mice expressing the CD90.1 allele (26), anti-HEL knock-in mice (4), and CD11c-DTR mice (27) were bred on site.
Mice were housed in a specific pathogen-free facility at the University of Minnesota. Experiments were conducted in accordance with federal and institutional guidelines with the approval of the University of Minnesota Institutional Care and Use Committee.

Spleen and lymph node cell suspensions from naive TCR transgenic and BCR knock-in donor mice were prepared for adoptive transfer, as previously described (28), and injected into the tail veins of recipient mice. Approximately $2 \times 10^7$ TCR transgenic and BCR knock-in donor cells were injected into each recipient for flow cytometry experiments, whereas $1 \times 10^6$ BCR knock-in cells were injected for immunohistochemistry experiments. For some experiments, anti-HEL B cells were labeled with 50 μM CellTracker Blue 7-aminomethylcoumarin (CMAC) (Invitrogen, Carlsbad, CA) at 37°C for 15 min prior to injection. Polyclonal B cells were purified from the spleens of B6 mice using a B cell isolation kit (Miltenyi Biotec, Auburn, CA) and labeled with 10 μM CellTracker Orange CMTPX (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) (Invitrogen) at 37°C for 30 min prior to injection.

**Ags and injections**

LPS was purchased from List Biological Laboratories (Campbell, CA); hen egg lysozyme (HEL) was purchased from Biozyno Laboratories (San Diego, CA); and eGFP, containing DIVSA Green (Diversa, San Diego, CA), was obtained from Amgen (Thousand Oaks, CA). The recombinant protein was purified from bacterial lysates and covalently linked to HEL using maleimide-based chemistry (Pierce, Rockford, IL), as previously described (11, 12). For construction of microsphere-linked Ags, various sizes of amino-modified white, red fluorescent, or green fluorescent latex microspheres were purchased from In vitro Cytokinesis (Warrington, PA). Protein Ags were covalently linked to microspheres using glutaraldehyde or maleimide-based chemistry (as described by Polysciences or Pierce) and validated using spectrophotometry and flow cytometry to detect GFP. The covalent linkage of EozGFP-HEL to the microspheres was tested by incubating them in PBS at 37°C overnight and demonstrating that anti-HEL B cells could not acquire any green Ag from the PBS supernatant. Recipient mice were immunized with $1 \times 5 \times 10^6$ microspheres (or $1 \times 5 \mu g$ Ag) intradermally in the ear, 24 h after adoptive transfer of cells and, where indicated, with 2 μg LPS as an adjuvant. In some cases, mice received 10 μl PBS or clodronate liposomes intradermally in the ear. Clodronate was a gift of Roche Diagnostics, Mannheim, Germany, and was encapsulated in liposomes, as described previously (29). Diphtheria toxin (DT; Sigma-Aldrich, St. Louis, MO) was administered by i.p. injection at 4 mg per gram body weight. Pertussis toxin (PTx; List Biological Laboratories) was administered by i.p. injection at 5 μg per dose.

For the in vivo protease-inhibition experiment, protease inhibitor mixture (cat. #539131, Calbiochem, San Diego, CA) was prepared in 400 μl PBS such that it contained 125 mM N-(2-aminoethyl)benzenesulfonfyl fluoride, 37.5 μM aprotinin, 250 μM E-64 protease inhibitor, 125 mM EDTA, and 250 μM leupeptin. BODIPY TR-X casein from the EnzChek Protease Assay Kit (cat. #E6639, Molecular Probes, Eugene, OR) was reconstituted by the manufacturer (cat. #539131, Calbiochem). Final concentrations were as follows: 500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 150 mM aprotinin, 1 μM E-64 protease inhibitor, 0.5 μM EDTA, and 1 μM leupeptin. The resulting supernatants were passed through a 0.2-μm filter and incubated with anti-HEL B cells on ice for 30 min.

**Flow cytometry**

Lymph nodes were minced in collagenase and EDTA solutions, passed through fine mesh, washed, and resuspended in culture supernatant containing 24G2 (American Type Culture Collection, Manassas, VA) plus mouse and rat serum to block FcRs. In experiments with white latex microspheres, cell suspensions were incubated with Pacific blue-labeled anti-CD11c, Alexa Fluor 700-labeled anti-Y-Ae, 50 μg/ml HEL, followed by APC-conjugated anti-CD11c and biotin-labeled Y-Ae mAb (31) followed by PE-conjugated streptavidin (BioSource, San Diego, CA). The cells were then fixed in formaldehyde, permeabilized in 0.5% saponin, and incubated with biotin-labeled anti-IgM followed by PerCP-conjugated streptavidin in experiments with red fluorescent microspheres, cell suspensions were incubated with Pacific blue-labeled anti-B220, Alexa Fluor 700-labeled IgM*, 50 μg/ml HEL followed by Pacific orange-labeled anti-HEL, and biotin-labeled Y-Ae mAb followed by APC-conjugated streptavidin. For CD11c–DTR experiments, DC ablation was assessed by enriching DCs from inguinal lymph nodes by FcR blocking solution and avidin and biotin. Anti-HEL B cells were detected with biotin-labeled anti-IgM*, followed by HRP-conjugated streptavidin and TSA-direct Coumarin-tyramide (PerkinElmer, Wellesley, MA), as directed by the manufacturer. T cell areas were detected with biotin-labeled anti-CD3 or anti-CD4, followed by HRP-conjugated streptavidin and Cy5-tetramethylrhodamine. For assessing the presence of immunohistochemistry.

**Immunohistochemistry**

Lymph nodes were harvested and frozen in OCT freezing medium (Sakura Finetek, Torrance, CA). Cryosections (8–10 μm) were fixed in 1% formaldehyde and blocked with 3% H2O2, FcR-blocking solution and avidin and biotin. Anti-HEL B cells were detected with biotin-labeled anti-IgM*, followed by HRP-conjugated streptavidin and TSA-direct Coumarin-tyramide (PerkinElmer, Wellesley, MA), as directed by the manufacturer. T cell areas were detected with biotin-labeled anti-CD3 or anti-CD4, followed by HRP-conjugated streptavidin and Cy5-tetramethylrhodamine. For assessing the presence of

![Figure 1](http://www.jimmunol.org/) Tracking Ag-specific B cell responses to particulate Ags. A, B6 mice injected with $1 \times 10^6$ TEa T cells or uninjected TCR−α-deficient mice were left untreated (No Ag) or were immunized with 3 μg soluble EozGFP-HEL plus LPS (Soluble) or $3 \times 10^6$ μg EozGFP-HEL linked to 1-μm microspheres plus LPS (Particulate). Twelve days later, serum was harvested and assayed for the presence of GFP-specific IgG (IgG1+IgG2a+IgG2b) Abs. Each mouse is represented by a dot. The data are representative of two independent experiments. B, B6 mice were injected with $2 \times 10^6$ anti-HEL B cells and then left untreated or immunized with 1-μm microsphere-linked EozGFP-HEL plus LPS. Draining lymph nodes were harvested 24 h later. The plots on the left show surface HEL (detected by the addition of soluble HEL followed by anti-HEL mAb staining) and intracellular IgM* staining on gated B220+ B cells. The plots in the middle and on the right show peptide:MHC II complexes (Y-Ae) and GFP Ag on gated anti-HEL B cells or gated HEL−negative endogenous B cells, respectively. Numbers indicate the percentage of cells in each gate. Data shown are representative density plots from three independent experiments.
subcapsular sinus macrophages, cryosections were stained with FITC-labeled anti-B220 and purified rat anti-MOMA-1 (Abcam), followed by biotin-labeled donkey anti-rat IgG, HRP-labeled streptavidin, and Coumarin-tetramide. For the in vivo protease cleavage assay, cryosections were analyzed immediately without hydration or fixation. Images were captured with an ×10 or ×20 objective lens on a Leica DM5500B automated upright microscope with a high-precision motorized x/y stage and a Leica DFC340FX digital camera. Photoshop (Adobe, San Jose, CA) was used to process the images for display.

Multiphoton laser scanning microscopy acquisition and analysis
A custom resonant-scanning instrument, based on published designs, containing a four-photon multiplier tube operating at video rate was used for two-photon microscopy (32, 33). B6 recipients of 1 × 10^7 CMTMR-labeled polyclonal B cells were injected with PBS or PTx 18 h prior to imaging. Inguinal lymph nodes were harvested from recipients and immobilized on coverslips, with the hilum facing away from the objective. Lymph nodes were maintained at 36°C in RPMI 1640 medium bubbled with 95% O2 and 5% CO2 and were imaged through the capsule distal to the efferent lymphatic. Samples were excited with a 10-W Mai Tai Ti: Sapphire laser (Spectra-Physics, Mountain View, CA) tuned to a wavelength of 810 nm, and emission wavelengths of 567–640 nm (for CMTMR) and 380–420 nm (for detection of second harmonic emission) were collected. Images acquired were 50–200 μm below the lymph node capsule identified by the second harmonic signal. Each z plane spanned 192 × 160 μm at a resolution of 0.4 μm per pixel, and images of 44–46 z planes with 2-μm z-spacing were created by averaging 10 video frames every 30 s for 10–30 min. Images were acquired by Video Savant software (IO Industries, London, Ontario, Canada). The maximum intensity z-projection time-lapse image sequences were generated with MetaMorph software (Molecular Devices, Sunnyvale, CA). Three-dimensional rotations and time-lapse image sequences were generated in Imaris 5.7.2 x64 (Bitplane, St. Paul, MN). Semiautomated cell tracking in three dimensions was verified manually with Imaris 5.7.2 x64. Tracking data were analyzed in Microsoft Excel. Statistical analysis of the data was done with GraphPad Prism 4.0 software (GraphPad, San Diego) for comparisons of two groups, and p values were calculated by the unpaired Student t test.

ELISA
Sera were titrated in 96-well plates (Costar, Cambridge, MA) coated with 20 μg/ml EoGFp-HEL and blocked with 1% BSA. Plate-bound Ig was revealed by incubating the wells sequentially with a mixture of biotinylated anti-IgG2a, IgG1 plus IgG2b (BD Pharmingen, San Diego, CA), HRP-labeled streptavidin (Sigma-Aldrich), and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The titer at both the maximal OD was calculated for each sample.

Results
Tracking Ag-specific B cell responses to particulate surface Ags following s.i. immunization
Particulate Ag was produced by covalently linking 1-μm microspheres with the model Ag EcoGFp-HEL, which consists of a fusion protein of Diversa Green protein (Diversa) and the Eo peptide (pEa) from the I-E MHC II protein (34), covalently linked to HEL. To test whether the Ag-linked 1-μm microspheres could induce T-dependent B cell responses, B6 mice, which received pEo-I-A^b-specific TEastern CDR4 T cells (31) to provide abundant help, or, TCR-α–deficient mice, which lack T cells, were immunized s.c. with 3 μg of microsphere-linked Ag plus LPS or 3 μg of soluble Ag plus LPS. LPS was included as an adjuvant to ensure optimal activation of helper T cells (35). GFP-specific IgG, which was undetectable in unimmunized mice, was observed in B6 mice 12 d after immunization with soluble Ag or microsphere-linked Ag (Fig. 1A). TCR-α–deficient mice did not have detectable GFP-specific IgG following immunization with either form of Ag. Thus, Ag-linked 1-μm microspheres induced T cell-dependent immune responses.

An adoptive transfer system was then used to assess the capacity of Ag-specific B cells to acquire Ag from the microspheres. B cells from anti-HEL BCR transgenic knock-in mice, specific for HEL, were injected into B6 recipients and tracked via HEL binding and an Ab specific for the IgM1 allotypic determinant present in the anti-HEL Ig H chains but not in H chains of B6 origin (4, 12). Ag uptake by anti-HEL B cells was visualized using the fluorescent protein, and Ag presentation was measured using the Y-Ae mAb, which is specific for pEo-I-A^b complexes (31). B220^+ IgM^+ , and anti-HEL B cells were detected in the draining lymph nodes of recipient mice by flow cytometry before or 24 h after the injection of EcoGFp-HEL–linked microspheres plus LPS (Fig. 1B, left panels). In contrast, GFP and pEo-I-A^b complexes were detected on the anti-HEL B cells after the injection of EcoGFp-HEL–linked

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**FIGURE 2.** Particulate Ag is rapidly acquired by follicular B cells. B6 recipients of 2 × 10^6 anti-HEL B cells were immunized with 1-μm red fluorescent microspheres plus LPS (No Ag) or 1-μm red fluorescent microsphere-linked EcoGFp-HEL plus LPS. Draining lymph nodes were harvested at the indicated times after immunization. The anti-HEL B cells were gated as shown in Fig. 1B. A, Representative flow cytometry showing the acquisition of green Ag and expression of peptide:MHC II complexes. B, Representative flow cytometry showing the percentage of anti-HEL B cells that acquire the red fluorescent microspheres. C, Percentages of anti-HEL B cells that acquired Ag and microspheres over time. Error bars represent the mean ± SEM. As a control for cells that acquired microspheres during the harvesting steps, an animal that did not receive transgenic B cells was immunized with 1-μm red fluorescent microsphere-linked EcoGFp-HEL plus LPS, and the anti-HEL B cells were added during processing (4-h Ctl). The percentage of anti-HEL B cells that acquired Ag at 4 h was significantly different relative to the 4-h Ctl sample (*p = 0.003), whereas the percentage of anti-HEL B cells that acquired microspheres at 4 h was not significant relative to the 4-h Ctl sample. Data are representative of two independent experiments.
microspheres plus LPS but not before (Fig. 1B, middle panels). Further, green fluorescence and pE:α:1-Ab complexes were not detected on endogenous B cells in mice immunized with EoGFP-HEL-linked microspheres plus LPS (Fig. 1B, right panels) or anti-HEL B cells in mice immunized with LPS alone (data not shown). Therefore, the early Ag uptake and peptide:MHC II production by Ag-specific B cells could be visualized with this system.

Surface Ags are rapidly separated from microspheres and acquired by Ag-specific follicular B cells

The acquisition of the microspheres, GFP, and pE:α:1-Ab complexes by the anti-HEL B cells was studied over time to investigate the mechanism by which Ag-specific B cells obtained microsphere-linked Ag. For this study, recipient mice were immunized with EoGFP-HEL-linked 1-µm red fluorescent microspheres plus LPS. Heterogeneous amounts of GFP were detected by flow cytometry on the anti-HEL B cells at 15 and 30 min after immunization. Heterogeneous expression of GFP was also observed at 24 h, but not before (Fig. 2A, 2C). pE:α:1-Ab complexes were first detected at 1 h, peaked at ~24 h, and then declined (Fig. 2A and data not shown). Despite the efficient acquisition of Ag by all of the anti-HEL B cells, only ~10% of anti-HEL B cells acquired the fluorescent microspheres (Fig. 2A, 2C). The numbers of anti-HEL B cells that acquired microspheres did not increase over time; the acquisition of microspheres occurred to some extent, even if mice were immunized with Ag-free microspheres (Fig. 2C). Some, but not all, of the microsphere binding by anti-HEL B cells occurred during the processing of the lymph node tissue for flow cytometry, because a control in which the anti-HEL B cells were added during the processing step also revealed a small amount of microsphere acquisition (Fig. 2C, 4-h control [4-hCtl]). Together, these data show that most Ag-specific B cells rapidly acquired microsphere-linked Ag, without acquiring the microsphere itself.

A caveat to assaying Ag acquisition by flow cytometry is that it does not reveal the location where this occurs. To address this issue, B cell Ag acquisition was tracked by microscopy in draining lymph node tissue sections over a similar time course. Lymph node follicles were identified by the presence of the transferred IgM⁺ anti-HEL B cells and a lack of CD3 staining (Fig. 3A). The transferred IgM⁺ anti-HEL B cells were pseudo-colored red, and the red fluorescent microspheres were pseudo-colored blue in the images shown. Microspheres appear dark blue if they were not coated (Fig. 3B) and aqua if they were coated with green Ag (Fig. 3C–E). Following injection, the majority of Ag-coated microspheres were confined to the subcapsular sinus (Fig. 3C–E). None of the anti-HEL B cells had acquired green Ag 15 min after injection (Fig. 3C). However, a band of anti-HEL B cells nearest the subcapsular sinus was detected at 30 min (Fig. 3D). By 4 h, nearly all of the anti-HEL B cells in sections had acquired Ag (Fig. 3E). Display of the yellow signal from these images against a black background reveals the location of anti-HEL B cells that have acquired Ag as visualized by the microspheres. Data are representative of three independent experiments and >100 individual follicles. A–H, Original magnification ×20.
background with an outline of the subcapsular sinus emphasized the pattern of Ag acquisition (Fig. 3F–H). Most notably, nearly all of the anti-HEL B cells acquired green Ag without acquiring a fluorescent microsphere, agreeing with measurements obtained by flow cytometry and confirming that the Ag and microspheres become separated after immunization.

**DCs and macrophages are not required for acquisition of particulate Ags by Ag-specific B cells**

It was possible that DCs or macrophages residing in the subcapsular sinus removed the Ag from the particles and transported it across the floor of the sinus or extended cellular processes into the follicles and transferred Ag from the microspheres to the anti-HEL B cells. CD11c-DTR mice, in which GFP and human DTR are expressed under the control of the CD11c promoter (27), were used to determine whether DCs were required for the acquisition of Ag by the anti-HEL B cells. Administration of DT to these mice resulted in a 96% reduction in the number of DCs in the lymph node (Fig. 4A), as previously described (12, 27, 36). CD11c-DTR mice were injected with anti-HEL B cells and DT and immunized the next day with microsphere-linked EsOgp-HEL plus LPS. A second dose of DT was given at the time of microsphere injection. Twenty-four hours after immunization, the anti-HEL B cells in the draining lymph nodes were examined by flow cytometry for acquisition of Ag and the expression of peptide:MHC II complexes. The anti-HEL B cells acquired similar amounts of GFP and displayed similar amounts of pEsOgp-I-A^k complexes (Fig. 4B), regardless of whether the DCs had been depleted. These data demonstrated that DCs were not required for B cell acquisition of microsphere-linked Ags.

To address the role of macrophages, clodronate liposomes (37) were used to deplete phagocytic cells from the subcapsular sinus before immunization. Subcutaneous injection of clodronate liposomes 48 h prior to the injection of microspheres resulted in the depletion of the MOMA-1^+ macrophages in the subcapsular sinus, whereas injection of PBS liposomes had no effect (Fig. 4C). However, the absence of MOMA-1^+ macrophages had no effect on GFP acquisition or pEsOgp-I-A^k complex production by anti-HEL B cells (Fig. 4D). The finding was not explained by a reduction in the barrier function of the subcapsular sinus because it has been shown that the subcapsular sinus ultrastructure is not altered by clodronate treatment (25) and 1-μm microspheres were still primarily confined to the subcapsular sinus 4 h after injection into clodronate-treated mice (data not shown). Therefore, these data show that MOMA-1^+ subcapsular macrophages are not required for the acquisition of microsphere-linked Ag by Ag-specific follicular B cells.

**Particulate Ag acquisition by Ag-specific B cells does not require migration to the subcapsular sinus**

Although DCs and MOMA-1^+ macrophages in the subcapsular sinus were not required, it was still possible that the surface of the 1-μm microspheres were exposed to follicular B cells through small pores or gaps in the lining of the subcapsular sinus. Ag-specific B cells that migrated to the subcapsular sinus could then acquire Ag from the exposed surface of the particles. If this were the case, then B cell motility within the follicle would be required for significant acquisition of Ag from the microspheres. The role of B cell motility in Ag acquisition was tested using PTx, which was shown to prevent T lymphocyte motility in lymph nodes (38). To confirm that PTx treatment prevents motility of B cells in lymph nodes, multiphoton microscopy was used to measure the velocity and displacement of injected, fluorescently labeled polyclonal B cells. As shown for T cells, the velocity and mean displacement of the labeled B cells were almost completely inhibited by PTx treatment (Fig. 5A, 5B; Supplemental Videos 1, 2). B6 mice that received anti-HEL B cells were then treated with PTX and subsequently immunized with 1-μm EsOgp-HEL–linked microspheres plus LPS. Despite PTx treatment, the anti-HEL B cells present in the draining lymph nodes 3 h after microsphere injection acquired a similar amount of GFP as control mice that were not treated with PTx (Fig. 5C). These data demonstrate that...
motility was not required for B cell acquisition of microsphere-linked Ags, making it unlikely that Ag-specific B cells migrated to the subcapsular sinus to remove Ag from the microsphere surface. A more likely explanation is that soluble Ag becomes separated from the microspheres in the subcapsular sinus prior to entry into the follicles where B cells reside.

FIGURE 5. B cell motility is not required for acquisition of particulate Ag. B6 recipients of $1 \times 10^7$ CMTMR-labeled, polyclonal B cells were treated with PBS or 5 µg of PTx 18 h prior to the excision of inguinal lymph nodes for multiphoton microscopy. Velocity measurements (A) and 10-min displacement measurements (B) of labeled B cells from PBS- or PTx-treated mice are shown. C, B6 recipients of $2 \times 10^7$ anti-HEL B cells were left untreated or were treated with 5 µg PTx and then immunized 18 h later with 3 µg of 1-µm microsphere-linked EosGFP-HEL plus LPS. An untreated B6 recipient mouse that received no Ag was included as a control. Three hours after immunization, Ag acquisition by anti-HEL B cells was measured by flow cytometry. Each dot represents the mean fluorescent intensity of the anti-HEL B cells from one lymph node. The results from two independent experiments are shown.

FIGURE 6. Effect of proteases on B cell Ag acquisition. A, PBS (ctl), plasma dilutions (1:250–1:8,000), or neat plasma were incubated with 1-µm microspheres linked with EosGFP-HEL, passed through a filter, and then incubated with anti-HEL B cells. Flow cytometry was used to measure acquisition of green fluorescence by the anti-HEL B cells shown on the bar graph as GFP mean fluorescence intensity. B, PBS (black line), a 1:1000 dilution of plasma alone (blue line), or a 1:1000 dilution of plasma mixed with protease inhibitors (red line) was incubated with microspheres linked with EosGFP-HEL and assayed as described above. The curves represent EosGFP-HEL acquisition by the transgenic B cells. C and D, B6 recipients of $2 \times 10^7$ CMAC-labeled anti-HEL B cells were immunized with 1-µm microsphere-linked EosGFP-HEL plus BODIPY TR-X casein in the presence or absence of protease inhibitor mixture; the draining lymph nodes were harvested 30–40 min later. Whole lymph node images of cryosections show anti-HEL B cells (red) and cleaved protease substrate (green) in control (C) and inhibitor-treated (D) lymph nodes. Scale bar, 200 µm. E–G and I–K, High-magnification views of the subcapsular sinus overlaying B cell follicles containing anti-HEL B cells (red) and EosGFP-HEL Ag (green). H and L, To emphasize the anti-HEL B cells that acquired Ag, the yellow pixels in the region underlying the subcapsular sinus in G and K were enlarged, filled, and moved to a black background. The white line roughly defines the floor of the subcapsular sinus, as visualized by the microspheres. Scale bar, 25 µm. C–L, Original magnification ×20. M, Ratios of fluorescent protease substrate signal relative to total lymph node area ($n = 4$ lymph nodes per sample pooled from two independent experiments; error bars show SD; $p = 0.03$). N, Percentage of Ag-positive anti-HEL B cells from control or protease inhibitor-treated lymph node follicles ($n = 1200$–1600 B cells from four lymph nodes per sample pooled from two separate experiments; error bars show SD; $p = 0.002$).
A protease-dependent mechanism for separating Ags from Ag-linked microspheres

We next tested the possibility that the Ag was being actively cleaved from the surface of the microspheres prior to acquisition by follicular anti-HEL B cells. This mechanism would require the presence of a protease in the plasma or lymph. To investigate this possibility further, serial dilutions of plasma from naïve B6 mice were incubated with Ag−linked microspheres. After removal of the microspheres, the resulting supernatants were subsequently incubated with anti-HEL B cells. In contrast to the PBS control supernatant, the plasma supernatants contained cleaved EoGFP-HEL as evidenced by the dose−dependent acquisition of GFP by the anti-HEL B cells (Fig. 6A). The anti-HEL B cells did not acquire green fluorescence when EoGFP−linked or HEL−linked microspheres were used (data not shown). Furthermore, the cleavage of Ag was not unique to the glutaraldehyde linkage method used to attach Ag to the microspheres, because Ag that was attached via passive absorption or a maleimide linker could also be cleaved in the presence of plasma (data not shown). Finally, Ag cleavage from the microsphere by mouse plasma could be inhibited in vitro by a commercial mixture of protease inhibitors (Fig. 6B), supporting the role of a protease.

To address the relevance of protease-mediated generation of B cell Ags in vivo, B cell Ag acquisition and protease activity were imaged simultaneously within the same lymph node. BODIPY TR-X casein, a protease substrate that fluoresces red upon cleavage, was used to visualize in vivo protease activity. Naïve mice were first injected with CMAC-labeled anti-HEL B cells and then immunized with a mixture of BODIPY TR-X casein plus EoGFP-HEL−linked microspheres that did or did not contain a protease inhibitor mixture. LPS was not added to the mixtures in these experiments, because it was not required for Ag acquisition by the B cells. Protease activity was readily detectable in the subcapsular sinus of the draining lymph nodes 30−40 min after injection of the substrate/microsphere mixture, demonstrating the presence of a protease or proteases in this region of the lymph node (Fig. 6C). The level of protease activity in the draining lymph nodes was greatly reduced when the substrate−Ag mixture contained the protease inhibitor mixture (Fig. 6D, 6M). Ag acquisition by the follicular anti-HEL B cells was assessed between 30 and 45 min after injection, because peak inhibition of protease activity occurred during this time (data not shown). EoGFP-HEL−linked microspheres (green) were observed in the subcapsular sinus of the draining lymph nodes of mice that were immunized with the Ag−substrate mixture, regardless of the presence of protease inhibitors (Fig. 6E, 6F). However, Ag acquisition by anti-HEL B cells (red) was greatly reduced in the lymph nodes from mice that received protease inhibitors (Fig. 6G, 6H, 6K, 6L, 6N). These data provide a causal link between protease activity and acquisition of Ag by B cells and support a mechanism by which a protease or proteases act on particles to generate soluble Ags that enter follicles.

**FIGURE 7.** Separation of Ag from the microspheres is required for functional T cell−dependent B cell response

If B cells acquire Ag that is released from particles, then they should produce peptide:MHC II complexes from the released Ag specific for their BCR but not from other Ags present on the particle. Such a mechanism predicts that B cells could only receive T cell help and produce a productive immune response to released Ags that contain the B cell epitope and a T cell epitope. To address this question, a variety of Ag−linked microspheres were made (Fig. 7A) and used to immunize mice that had been transferred with anti-HEL B cells and TEa TCR transgenic CD4+ T cells. TEa cells are specific for the pEoc1-A\(^2\) complexes (31) and provide help for anti-HEL B cell clonal expansion and differentiation in vivo (12). Some mice were immunized with microsphere−linked EoGFP-HEL, whereas other mice were immunized with microspheres that had EoGFP and HEL bound to their surface individually (referred to as EoGFP+HEL microspheres). As controls, mice were immunized with microsphere−linked EoGFP, microsphere−linked HEL, or a mixture of the two types of microspheres. If separation of Ag from the microspheres is required for initiating a T−dependent immune response, EoGFP uptake and pEoc1-A\(^2\) complex formation by the anti-HEL B cells will not occur because HEL and EoGFP will be cleaved individually from the EoGFP+HEL microspheres. Thus, anti-HEL B cells in mice immunized with EoGFP+HEL microspheres will not acquire help from the pEoc1-A\(^2\)−specific T cells and should fail to...
undergo clonal expansion and differentiation. However, if separation of Ag from the microspheres is not required and B cells acquire the intact microsphere containing both epitopes, then immunization with EoGFp+HEL microspheres should result in the clonal expansion and differentiation of the anti-HEL B cells.

The efficiency of Ag linkage to the microspheres was measured by flow cytometry (Fig. 7A). Although all of the microspheres were linked to the expected proteins, the microspheres linked with EoGFp alone had slightly lower levels of green fluorescence than did microspheres linked with EoGFp+HEL or EoGFp-HEL, whereas microspheres linked with HEL alone had more HEL than did those linked with EoGFp+HEL or EoGFp-HEL (Fig. 7A). Despite the difference in EoGFp levels, all of the microspheres that were linked with EoGFp simulated similar amounts of T cell response by day 5 following immunization (Fig. 7B). To determine whether the Ag-specific B cell response differed following immunization with the various Ags, isotype-switched IgG2a anti-HEL B cells were quantified on day 5 (Fig. 7C). Only when EoGFp-HEL was linked to the microspheres did the anti-HEL B cell produce robust IgG2a responses (Fig. 7C). Recipient mice immunized with EoGFp+HEL microspheres or a mixture of EoGFp and HEL microspheres had significantly lower levels of anti-HEL IgG2a B cells than did recipient mice immunized with EoGFp-HEL microspheres (p < 0.01). Immunization with EoGFp microspheres, which lacked the appropriate B cell Ag, and immunization with HEL microspheres, which contained large amounts of HEL (Fig. 7A) but lacked the T cell epitope needed to induce help, did not result in IgG2a anti-HEL B cell responses.

Thus, Ag-specific B cells underwent T-dependent isotype switching if T and B cell epitopes were present on a single Ag but not if the T and B cell epitopes were cleaved individually from the same particle. These results provide functional evidence that the B cells took up Ags that were released from the microspheres rather than the microspheres themselves.

Discussion

Our results demonstrate that immunization with 1-μm particles results in rapid acquisition of a linked Ag by Ag-specific follicular B cells without uptake of the particle. An important consequence of this Ag uptake mechanism was that the B cells only produced an optimal isotype-switched Ab response when the released Ag also contained a T cell epitope.

The mechanism for generating humoral responses to the Ag-linked 1-μm microspheres is distinct from that previously described for 0.2-μm microspheres. Carrasco et al. (7) showed that the majority of Ag-specific follicular B cells acquired Ag-linked 0.2-μm microspheres within 6 h following immunization. This did not occur in the case of 1-μm Ag-linked microspheres, in all likelihood because the 1-μm microspheres were less efficient at entering follicles and, thus, could not be readily accessed by Ag-specific follicular B cells. Although the prevailing view is that subcapsular sinus macrophages are important for the transfer of particulate Ags across the subcapsular sinus floor to migrating follicular B cells (7, 24, 25), this has primarily been studied in the context of smaller Ags, such as viral particles, that could easily be translocated along the macrophage cell surfaces. In contrast, we found no evidence that MOMA-1+ subcapsular sinus macrophages or CD11c+ DCs played such a role with bacteria-sized particles. Although the possibility remains that a different cell is important for the transport of 1-μm Ags into the follicles, the inefficient entry of particles into the follicular space, coupled with the acquisition of Ag by almost all of the Ag-specific follicular B cells, makes this scenario unlikely.

Another potential mechanism for B cell Ag acquisition was the selective removal of Ag from the surface of the particle by the B cell via its high-affinity BCR. In this case, B cells would have to migrate from random positions in the follicle to the floor of the subcapsular sinus and absorb the Ag from the exposed area of the particle. Our finding that Ag-specific B cells acquired Ag from the particles, even when their motility was blocked with PTx, suggests that this was not the case.

Rather, our findings are consistent with a model in which proteases liberate Ags from the surface of the microspheres in the subcapsular sinus or the injection site, thereby allowing these proteins to move into the follicles for uptake by Ag-specific B cells (12, 16). This mechanism could explain the previous finding that serum proteases can affect antigenicity following immunization with model Ags (39). Although our studies were done with latex microspheres, the natural extension of this work would be to identify an Ag on the surface of a bacterial cell that is subject to proteolytic cleavage. Currently, the tools available for such studies are limited, because the necessary BCR transgenic mice specific for native bacterial surface Ags are not available. However, several reports in the literature suggest that a proteolysis mechanism may contribute to Ag presentation from other microbes. For example, Junt et al. (25) demonstrated that viral proteins reached follicular B cells, even when subcapsular sinus macrophages were depleted with clodronate liposomes. Although the investigators attributed this observation to free viral proteins in the inoculums, it could also be explained by protease-mediated cleavage of viral proteins in vivo. Such a process may also explain the perplexing findings of Crotty and colleagues on the Ab response to vaccinia virus (40). These investigators found that prior immunization with a single T cell epitope (peptide H1121–35) from the vaccinia virus I1 protein enhanced the Ab response to I1, but not to other viral Ags, following infection with the intact virus. This is not the result one would have predicted if Ag-specific B cells acquire intact viral particles, because any B cell to take up the viral particle would produce H1121–35:MHC II complexes and receive enhanced help from the expanded H1121–35:MHC II-specific T cell population. In contrast, if vaccinia virus particles, which may be too large to enter the conduit network, were degraded by proteases into separate protein fragments, then only I1-specific B cells would be expected to take up I1 and receive the enhanced T cell help, as observed by Crotty and colleagues (40). Thus, the advantage that the host gains from this mechanism is the production of Abs against the surface Ags of microbes that are too large to enter the lymph node follicles where naive B cells reside.

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