B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help *in vivo*

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Highly regulated activation of B cells is required for the production of specific antibodies necessary to provide protection from pathogen infection. This process is initiated by specific recognition of antigen through the B cell receptor (BCR), leading to early intracellular signaling followed by the late recruitment of T cell help. In this study we demonstrate that specific BCR uptake of CD1d-restricted antigens represents an effective means of enhancing invariant natural killer T (iNKT)-dependent B cell responses *in vivo*. This mechanism is effective over a wide range of antigen affinities but depends on exceeding a tightly regulated avidity threshold necessary for BCR-mediated internalization and CD1d-dependent presentation of particulate antigenic lipid. Subsequently, iNKT cells provide the help required for stimulating B cell proliferation and differentiation. iNKT-stimulated B cells develop within extrafollicular foci and mediate the production of high titers of specific IgM and early class-switched antibodies. Thus, we have demonstrated that in response to particulate antigenic lipids iNKT cells are recruited for the assistance of B cell activation, resulting in the enhancement of specific antibody responses. We propose that such a mechanism may operate to potentiate adaptive immune responses against pathogens *in vivo*.

innate | B cell activation | antigen affinity

As antibodies are designed to specifically recognize and eliminate invading antigens, they are effective weapons used by the immune system to combat infection. To elicit antibody production, B cells must be activated in a process that is initiated through specific antigen recognition by the B cell receptor (BCR) (1). Specific antigen engagement initiates two BCR-mediated processes: the transmission of intracellular signals regulating entry into cell cycle (2, 3) and antigen internalization before its processing and presentation in association with MHC to specific T cells (4). T cell-stimulated B cells can either differentiate into extrafollicular (EF) plasma cells (PCs) or develop into germinal center B cells. Short-lived EF PCs mediate the secretion of the first wave of predominantly low-affinity antibodies, some of which may have undergone class switch (5). Alternatively, germinal center B cells undergo somatic hypermutation and affinity maturation, leading to the selection of high-affinity B cell clones that differentiate into either long-lived PCs or memory B cells (6).

During the development of immune responses BCR-mediated uptake of antigen allows for its concentration and delivery to specialized late endosomes containing newly synthesized MHC class II molecules (7). For many years, peptides were assumed to be the only antigenic determinant for initiating T cell responses. However, it is now evident that T cells are also able to recognize and respond to antigenic lipids and glycolipids, presented by CD1 molecules (8). The human CD1 gene family is composed of five nonpolymorphic genes (*CD1A*, *CD1B*, *CD1C*, *CD1D*, and *CD1E*) (9), whereas mice express only CD1d molecules. In a manner similar to MHC class II molecules, CD1 proteins mediate the presentation of antigenic lipids on the surface of antigen-presenting cells (APCs) after they are loaded or processed in intracellular compartments (10). As CD1d is expressed by B cells, it is conceivable that BCR-mediated internalization could also play a role in CD1d-dependent presentation of antigenic lipids to T cells. However, such a pathway and its potential impact on the development of B and T cell responses remain poorly characterized.

T cells recognize a diverse range of potential antigens through their highly polymorphic T cell receptor (TCR). A subset of T cells known as invariant natural killer T (iNKT) cells are defined by their expression of a restricted TCR repertoire, consisting of a canonical Vα14–Jα18 or Vα24–Jα18 chain in mice and humans, respectively. iNKT cells recognize and become activated in response to self or foreign antigenic lipids presented by nonpolymorphic CD1d molecules expressed on the surface of APCs (8, 11). iNKT cells are activated in response to a variety of infections and during inflammatory and autoimmune diseases (12, 13). α-Galactosylceramide (αGalCer), a marine sponge-derived glycolipid, remains the best-characterized iNKT cell antigen to date, with proven capacity to stimulate strongly both murine and human iNKT cells (14, 15).

iNKT cells provide a means of linking and coordinating innate and adaptive immune responses, as their stimulation can induce the downstream activation of dendritic cells (DCs), NK, B, and T cells (11). It has been demonstrated *in vitro* that iNKT cells stimulate B cell proliferation and antibody production (16), although this activation appears independent of the presentation of exogenous iNKT cell ligands and BCR specificity. As the effective operation of the immune system requires the tightly regulated control of B cell activation *in vivo*, it would be expected that iNKT-mediated activation must be subject to stringent regulation; however, the mechanism by which this occurs remains uncharacterized. In this study we demonstrate that specific BCR internalization enhances B cell presentation of particulate lipid antigens to iNKT cells *in vivo*. Subsequently, activated iNKT cells help specific B cell proliferation, differentiation to EF PCs, and secretion of high titers of specific IgM and early class-switched antibodies. Thus, we propose that specific BCR internalization of particulate iNKT antigens may play a major role in the modulation of iNKT-dependent early antibody responses *in vivo*.

Results

We aimed to investigate the impact of targeting iNKT cell help to antigen-specific B cells during the development of an immune

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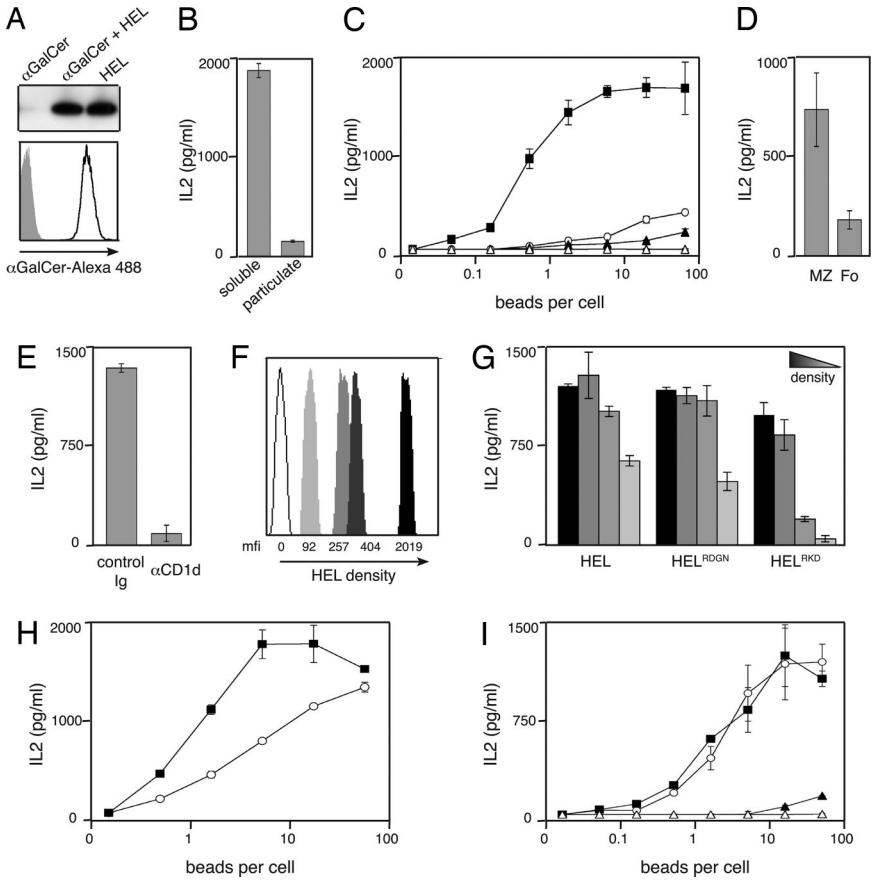

Fig. 1. B cells internalize particulate conjugates through the BCR and present αGalCer to iNKT cells. (*A*) (*Upper*) Silica beads were coated with αGalCer-containing liposomes (αGalCer), αGalCer-containing liposomes and HEL (αGalCer+HEL), or HEL alone (HEL). The binding of HEL to the beads was detected by Western blot with an anti-HEL antibody. (*Lower*) αGalCer-Alexa 488-coated beads were detected by FACS (black) compared with nonlabeled liposome-coated beads (gray). (*B–H*) B cell presentation of particulate αGalCer to iNKT cells. (*B*) MD4 B cells were stimulated with soluble αGalCer (15 ng/ml) or 0.1 μl of αGalCer-containing particles and cultured with iNKT cells. αGalCer presentation was measured by IL-2 production. (*C*) MD4 B cells were stimulated as in *B* with particles containing αGalCer (○), HEL (▲), or HEL-αGalCer (■). Control WT B cells were stimulated with HEL-αGalCer particles (△). (*D*) MD4 marginal zone (MZ) and follicular (Fo) B cells were sorted by FACS and stimulated with particulate αGalCer-HEL before culture with iNKT cells. (*E*) MD4 B cells were primed with HEL-αGalCer particles and preincubated with CD1d-blocking antibody (αCD1d) or an isotype control (control Ig). (*F*) FACS analysis of αGalCer-containing particles coated with different HEL densities, expressed as mean fluorescence intensity (mfi). (*G*) MD4 B cells were stimulated with particles containing αGalCer and different densities of HEL, HEL^{RDGN}, or HEL^{RKD} (gray scale) before culture with iNKT cells. (*H*) Presentation of HEL-αGalCer conjugates by B cells expressing D1.3 IgM HEL-specific BCR (■) or a D1.3 IgM/H2 chimera (○). (*I*) MD4 B cells were stimulated with particles containing HEL-αGalCer (■), αGalCer (▲), HEL-Gal(α1→2)αGalCer (○) or Gal(α1→2)αGalCer (△) before culture with iNKT cells.

response. However, as B cells take up antigenic lipid relatively nonselectively, we sought to achieve specificity in internalization through the use of particulate antigen. To this end we coated silica beads (100 nm) with liposomes containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and biotinyl-phosphatidylethanolamine (PE-biotin) in the presence of αGalCer (Fig. 1*A*). Alexa 488-labeled-αGalCer was used to quantify the amount of αGalCer loaded on the beads, and we found this to be 150 ng per μl of beads (10^8 beads per μl). To assess the capacity of B cells to present αGalCer *in vitro*, B cells were stimulated for 20 h with particulate or soluble αGalCer before their incubation with iNKT cells derived from a mouse hybridoma (DN32.D3). The secretion of IL-2 into the culture medium by iNKT cells was used as a measure of αGalCer presentation. In agreement with a previous report (17), soluble αGalCer was efficiently presented by B cells and induced IL-2 production (Fig. 1*B*). In contrast, on stimulation with particulate αGalCer we observed a severe attenuation of IL-2 production from iNKT cells. In addition, IFN-γ production was detected after activation of purified murine iNKT cells in response to soluble αGalCer presentation by B cells (data not shown). Thus, fundamental to our investigation, B cells were highly inefficient in the internalization of particulate antigenic lipid through the nonselective manner observed for soluble αGalCer.

CD1d-Dependent Presentation of Particulate Antigenic Lipids to iNKT Cells Is Enhanced by BCR-Mediated Uptake. To achieve specific and enhanced delivery of particulate iNKT cell ligand, we took advantage of the exquisite discriminatory capacity and internalization of the BCR. Accordingly, we bound biotinylated hen egg lysozyme (HEL), through a streptavidin linker to particles loaded with or without αGalCer. The total amount of bound protein was estimated by Western blotting (Fig. 1*A*). To assess the ability of B cells to mediate BCR-specific uptake and presentation of these particles, we used HEL-specific transgenic primary B cells (MD4) and DN32.D3 cells. MD4 B cells stimulated with particulate HEL-αGalCer induced strong iNKT cell activation (Fig. 1*C*). In contrast, IL-2 production was not detected after incubation with particulate HEL or αGalCer alone. In addition, no iNKT cell activation was observed after stimulation of WT B cells with particulate HEL conjugated with αGalCer. Interestingly, marginal zone MD4 B cells were more efficient in presenting particulate HEL-αGalCer to

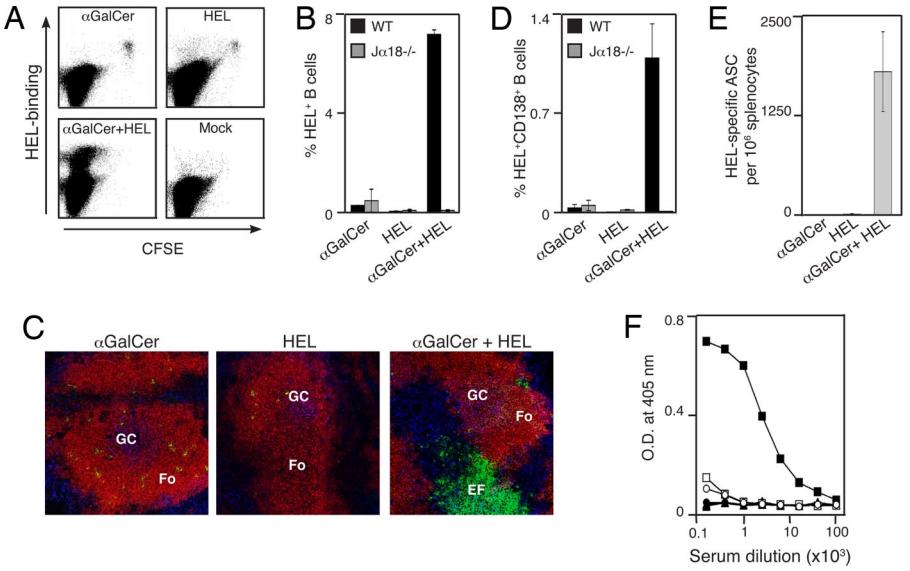

Fig. 2. iNKT cells help antigen-dependent B cell proliferation *in vivo*. MD4 B cells (or no cells, in mock) were transferred into WT mice challenged with particles containing αGalCer and/or HEL. (*A*) Proliferation of donor HEL-binding cells in the spleen was measured by FACS as CFSE dilution after 5 days. (*B* and *D*) Transfer experiments were also performed by using J$\alpha18^{-/-}$ mice as recipients. Percentage of HEL$^+$ (*B*) and CD138$^+$HEL$^+$ cells (*D*) recovered from recipient J$\alpha18^{-/-}$ (gray bars) or WT (black bars) spleens are depicted. (*C*) Immunofluorescence microscopy was used to detect splenic follicular B cells (Fo, B220, red), HEL-binding cells (intracellular HEL, green), germinal centers (GC, PNA, blue), and EF PCs (EF) 5 days after MD4 cell transfer to recipient mice. (Magnification: ×10.) (*E*) ELISPOTS were used to detect HEL-specific IgMa secreting splenic cells in WT recipients. (*F*) Specific anti-HEL IgMa was measured in the sera of WT (filled symbols) and J$\alpha18^{-/-}$ (unfilled symbols) recipient mice challenged with HEL-αGalCer particles (squares), αGalCer (triangles), or HEL (circles).

iNKT cells than follicular B cells (Fig. 1*D*). The activation of iNKT cells is completely blocked by preincubating B cells with a mAb against CD1d, indicating that this process absolutely depends on CD1d-mediated presentation (Fig. 1*E*). These observations demonstrate that specific antigen recognition by BCR can dramatically enhance presentation of particulate antigenic lipid to iNKT cells.

Given the importance of BCR-antigen recognition in driving particulate αGalCer presentation by B cells, it would be expected that the avidity of conjugated antigen, a function of affinity and density, would influence iNKT cell activation. To explore the effect of altering the antigen affinity we used various HEL proteins representing a wide range of affinities for the MD4 BCR (HEL^{WT}, $K_a = 2.1 \times 10^{10}\ M^{-1}$; HEL^{RDGN}, $K_a = 5.2 \times 10^{7}\ M^{-1}$; HEL^{RKD}, $K_a = 8.0 \times 10^{5}\ M^{-1}$) (18). At the highest density of antigen (Fig. 1*F*) we observed similar levels of iNKT cell activation regardless of the affinity of antigen conjugated with particulate αGalCer (Fig. 1*G*). Interestingly, even the low-affinity HEL^{RKD} antigen enhanced the presentation particulate αGalCer to iNKT cells. However, decreasing the density of this antigen on the particle surface ≈20-fold abolished the presentation of particulate αGalCer (Fig. 1*G*). Thus these findings indicate that even very low-affinity antigens can induce efficient particulate αGalCer presentation providing they exceed a tightly regulated avidity threshold for BCR stimulation.

Although BCR antigen recognition is necessary, it is not sufficient to drive optimal B cell presentation. We observed that transgenic B cells expressing a signaling-deficient BCR (IgM-H2) with high affinity for HEL exhibited diminished ability to induce iNKT activation (Fig. 1*H*). In line with these findings, particulate HEL conjugated with either αGalCer or Gal(α1→2)αGalCer gave rise to equally potent activation of iNKT cells (Fig. 1*I*). As Gal(α1→2)αGalCer requires intracellular processing before it can be effectively recognized by iNKT cells (19), it can be concluded that lipid-containing microspheres are internalized by B cells before presentation to iNKT cells. Thus, BCR-mediated antigen recognition and internalization are required for B cell-mediated presentation of particulate αGalCer to iNKT cells.

BCR-Mediated Uptake of Particulate αGalCer Leads to Recruitment of iNKT Cell Help, Resulting in Extensive B cell Proliferation and Antibody Production *in Vivo*. Having demonstrated that BCR-mediated uptake of particulate antigenic lipid leads to efficient αGalCer presentation *in vitro*, we were keen to assess how this process would affect B cell activation and fate *in vivo*. To this end, carboxyfluorescein succinimidyl ester (CFSE)-labeled HEL-specific B cells from MD4 mice were adoptively transferred into C57BL/6 recipients challenged i.v. with particulate HEL with or without conjugated αGalCer, in absence of any other adjuvant. Five days after stimulation, spleens of recipient mice were harvested and the dilution of CFSE in the HEL-specific B cell population was used as a measure of B cell proliferation.

As shown in Fig. 2*A*, extensive proliferation of the HEL-specific B cells in response to particulate HEL conjugated with αGalCer was observed; indeed, after 5 days MD4 B cells comprised >7% of the total splenic lymphocytes. These HEL-specific B cells originated from the adoptively transferred MD4 cells, as they were not detected after particulate HEL-αGalCer stimulation of C57BL/6 mice (mock). Importantly, no proliferation was detected after challenge with either particulate αGalCer or HEL alone, as HEL itself is incapable of eliciting a T cell-dependent response on the C57BL/6 background (20). In addition, HEL-specific B cell proliferation depended on the presence of iNKT cells, as it was not observed in similar adoptive transfer experiments with J$\alpha18^{-/-}$ mice as recipients (Fig. 2*B*).

Immunohistological analysis of spleen sections confirmed expansion of HEL-specific B cells in response to HEL-αGalCer particles (Fig. 2*C*). Interestingly, these expanded B cells were predominantly located as EF foci in the bridge channels and red pulp of the spleen and exhibited an intense cytoplasmic HEL staining characteristic of PCs (Fig. 2*C*). The presence of specific PCs was confirmed by flow cytometry, on the basis of high intracellular HEL staining and CD138 expression (Fig. 2*D*). PC differentiation was accompanied by the production of elevated anti-HEL IgMa titers (Fig. 2*F*). These antibodies were derived exclusively from the transferred MD4 B cells, as antibodies produced by C57BL/6 mice would be of the IgH^b allotype. HEL-specific PCs or antibodies were not detected in mice challenged with particulate HEL or αGalCer alone (Fig. 2 *D* and *F*). Antibody-secreting cells were also identified by HEL-specific ELISPOT only in recipients challenged with HEL-αGalCer-containing particles (Fig. 2*E*). In addition, splenic HEL$^+$ CD138$^+$ cells were not present after particulate HEL-αGalCer stimulation of MD4 B cells adoptively transferred into J$\alpha18^{-/-}$ mice (Fig. 2 *D* and *F*). Hence PC differentiation of HEL-specific B cells in response to particulate HEL-αGalCer absolutely depended on the presence of iNKT cells.

Notably, and in line with our *in vitro* observations, B cell proliferation and antibody production depended on the avidity of the BCR for the antigen present on the particles (Fig. 3 *A–C*). A

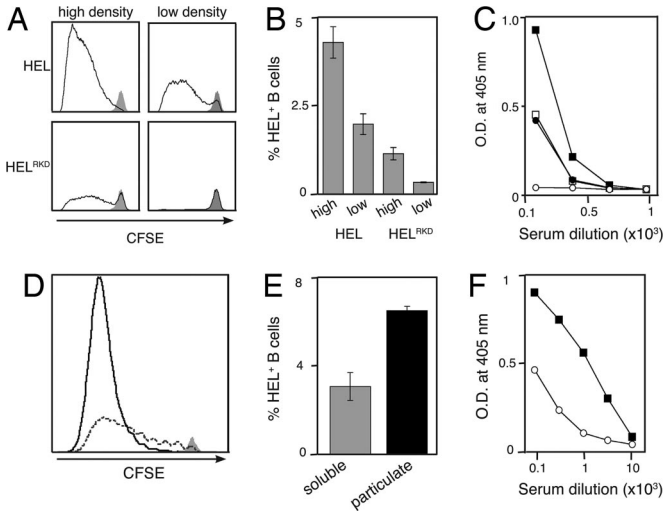

Fig. 3. Antigen avidity modulates iNKT-dependent B cell proliferation and antibody production. MD4 transfer experiments were performed as in Fig. 2. WT recipient mice were challenged with particles containing αGalCer and HEL or HEL^{RKD} at different densities. (*A*) MD4 proliferation was detected as CFSE dilution in the HEL-binding population. (*B*) Percentage of HEL^+ cells recovered from recipient spleens (high or low density). (*C*) Anti-HEL specific IgMa was detected on day 5 in the sera of recipient mice challenged with beads containing αGalCer and HEL high density (■), HEL low density (□), HEL^{RKD} high density (●), HEL^{RKD} low density (○). (*D–F*) WT mice received MD4 cells and particles containing HEL-αGalCer or HEL particles and soluble αGalCer. (*D*) Donor cell proliferation was detected as CFSE dilution after challenge with HEL-αGalCer conjugates (black line), HEL particles and soluble αGalCer (dotted line), or no beads (solid gray). (*E*) Percentage of HEL^+ donor cells in mice receiving soluble (gray bar) or particulate (black bar) αGalCer-HEL. (*F*) Anti-HEL-specific IgMa in recipient mice that received beads containing HEL-αGalCer (■) or HEL beads plus soluble αGalCer (○).

reduction of the affinity or density of HEL on the particulate αGalCer resulted in diminished B cell proliferation and antibody production. Thus an avidity threshold for the BCR-mediated internalization of particulate αGalCer is also present *in vivo*. However, it is evident that even low-affinity antigen can efficiently induce αGalCer presentation to iNKTs, allowing stimulation of B cell responses.

Direct linkage of protein and lipid antigens within the particles gave rise to greatly enhanced B cell proliferation and antibody production than observed for particulate HEL alone administered with soluble αGalCer (Fig. 3 *D–F*). This finding indicates that specific BCR uptake of antigenic lipids represents a more efficient means of internalization than that used by soluble αGalCer, resulting in greater stimulation of iNKT-mediated specific B cell responses. Thus we have identified a strategy involving particulate antigenic lipid to enable enhanced and specific B cell proliferation and development of functional EF PCs *in vivo*.

Immunization with Particulate Antigen Conjugated with αGalCer Enhances Specific Antibody Responses. We sought to investigate the ability of particulate antigen-αGalCer conjugates to induce a systemic immune response *in vivo*. To assess this we used a single-dose i.p. immunization strategy, using chicken gamma globulin (CGG) as antigen. It is known that CGG induces strong T cell-dependent responses in mice. C57BL/6 and $J\alpha18^{-/-}$ mice were challenged with particulate CGG conjugated with αGalCer, and after 7 and 14 days specific antibody responses were analyzed by ELISA.

We detected specific anti-CGG antibody production, comprising high titers of IgM and class-switched IgG, as early as 7 days after immunization of C57BL/6 mice with particulate CGG conjugated with αGalCer (Fig. 4*A*). No specific antibodies were detected at that time point in mice immunized with particulate CGG or αGalCer alone.

Interestingly, at 14 days we observed that whereas stimulation with CGG alone induced a classic T helper 2 (Th2) response (IgG1 production), conjugation with αGalCer yielded a Th0-like response with increased levels of IgG1, IgG2b, and IgG2c. Immunization of both C57BL/6 and $J\alpha18^{-/-}$ mice with particulate CGG gave rise to similar specific antibody responses (Fig. 4*B*). In contrast, immunization with particulate CGG conjugated with αGalCer induced a dramatic increase in specific CGG antibody production in C57BL/6 mice compared with $J\alpha18^{-/-}$ mice. This observation demonstrates the requirement of iNKT for specific antibody production in response to particulate αGalCer conjugated with antigen. Importantly, the same pattern of class-switched antigen-specific antibodies observed by using CGG as antigen was detected in response to immunization of C57BL/6 mice with particulate HEL conjugated with αGalCer (Fig. 4*C*). Hence, after immunization iNKT cells can efficiently activate specific antibody production and class-switch without the recruitment of specific $CD4^+$ helper T cells.

Thus far we have demonstrated that specific BCR recognition is required for the efficient B cell presentation of particulate antigenic lipids to iNKT cells, and that the recruitment of iNKT cell help can modulate the outcome of B cell activation. However, we were keen to assess the impact of couptake of antigen and αGalCer in the production of specific antibodies during the development of a

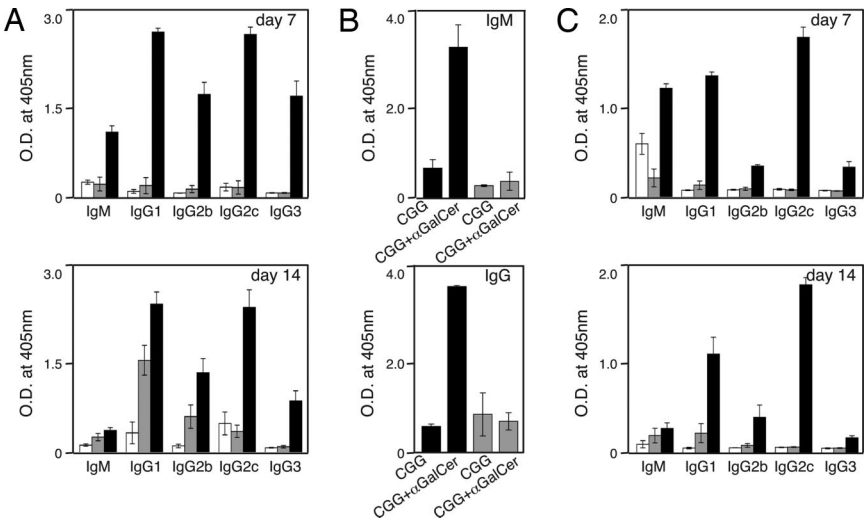

Fig. 4. Immunization with particulate antigen-αGalCer induces IgM and early class-switched specific antibodies. (*A*) C57BL/6 mice (four mice per group) were immunized with 10 μl of particles containing αGalCer (white bars), CGG (gray bars), or CGG-αGalCer (black bars). Specific CGG antibodies were detected in mice sera at 7 and 14 days after immunization. (*B*) WT (black bars) and $J\alpha18^{-/-}$ (gray bars) mice (three mice per group) were immunized with particles containing CGG or CGG/αGalCer. (*C*) C57BL/6 mice (four mice per group) were immunized with 10 μl of particles containing αGalCer (white bars), HEL (gray bars), or HEL-αGalCer (black bars). Specific HEL antibodies were detected in mice sera at 7 and 14 days after immunization.

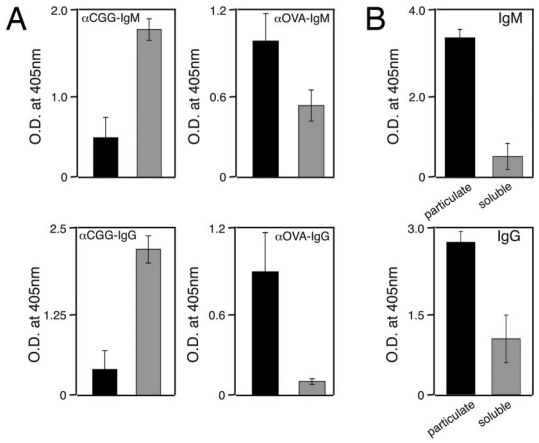

Fig. 5. Cross-link of antigen and αGalCer enhances specific antibody responses. (*A*) C57BL/6 mice (four mice per group) were immunized with 1 μl of CGG-αGalCer-coated particles plus 1 μl of OVA-coated particles (gray bars) or 1 μl of OVA-αGalCer-coated particles plus 1 μl of CGG-coated particles (black bars). CGG- and OVA-specific antibodies were measured on day 7. (*B*) Specific CGG antibodies detected in C57BL/6 mice (three mice per group) immunized with 1 μl of particles containing CGG-αGalCer (black bars) or 1 μl of CGG-particles plus 150 ng of soluble αGalCer (gray bars). Specific CGG antibodies were detected on day 7.

systemic immune response. To address this we immunized C57BL/6 mice with two different combinations of particles: a first group of mice was immunized with particulate CGG conjugated with αGalCer alongside particulate ovalbumin (OVA), and a second group received particulate OVA conjugated with αGalCer alongside particulate CGG (Fig. 5*A*). Production of anti-OVA and anti-CGG antibodies was measured in both groups of mice 7 days after immunization. Increased levels of specific antibodies were generated in each group in response to the antigen conjugated with αGalCer, showing that couptake of antigen-αGalCer enhances specific B cell activation and antibody production (Fig. 5*A*). Notably, we also observed an enhancement in the production of specific antibodies in response to immunizations with particulate CGG conjugated with αGalCer compared with particulate CGG and soluble αGalCer (Fig. 5*B*). This result, in line with our *in vivo* proliferation experiments, demonstrates that BCR-mediated uptake of particulate αGalCer is more efficient than that of soluble αGalCer uptake, resulting in enhanced specific B cell responses.

Discussion

In this study we show that BCR-mediated uptake allows efficient presentation of particulate antigenic lipids to iNKT cells. This mechanism permits CD1-mediated presentation of lipids after BCR recognition of even low-affinity antigen, as long as a defined avidity threshold is surpassed. As a result, activated iNKT cells provide help for specific B cell proliferation, EF plasma B cell differentiation, and production of high titers of specific IgM and early class-switched antibodies.

APCs accumulate antigen from their extracellular environments through different mechanisms. Internalization of antigenic lipids by DCs occurs predominantly through processes mediated by receptors such as the LDL receptor. LDL receptor binds apolipoprotein E in very low-density lipoprotein particles facilitating their internalization and subsequent presentation of antigenic lipids to iNKT cells (21). However, the mechanism by which this uptake occurs in B cells is not clear at this stage. Here, we used the BCR both as a means of achieving the selectivity in delivery and efficient internalization of particulate antigenic lipids by specific B cells. Although we have demonstrated that BCR-mediated internalization of αGalCer leads to CD1d presentation of antigenic lipids, the underlying mechanisms by which these processes occur in B cells have not been identified. Further insight into these mechanisms could be provided through the use of B cells deficient in proteins previously implicated in similar pathways in other cell types, such as saposins (19). The ability of B cells to internalize and process particulate antigen has been described by several groups (22–24). We have demonstrated that BCR-mediated internalization of particulate antigen can occur *in vivo* even in response to low-affinity antigen, provided a defined avidity threshold is exceeded. This observation is most likely interpreted as a requirement for a minimum degree of BCR clustering necessary for triggering internalization of the particulate antigenic lipids. This finding may be of particular relevance in a primary immune response against pathogens where B cells can recognize low-affinity antigen on the surface of viruses or microbes, provided that antigenic epitopes are present at sufficient density. In line with this idea, many acute infectious agents exhibit highly repetitive antigen determinants in their envelope, for example, the cellular wall of *Sphingomonas* is comprised of arrays of glycosphingolipids capable of activating murine and humans iNKT cells (25, 26).

The ability of iNKT cells to induce B cell proliferation has been characterized predominantly *in vitro* (16). Here, we found that, even in the absence of specific $CD4^+$ T cells, iNKT cells could help B cell proliferation and antibody production *in vivo*. Our observations are in line with previous investigations where immunization of MHC II-deficient mice with antigen and αGalCer induced detectable levels of IgG, indicating that iNKT cells can substitute for $CD4^+$ Th functions (27). iNKT cells have an antigen-experienced phenotype and can respond very rapidly to CD1d-presented antigens without the need for clonal expansion. We have demonstrated that direct iNKT cell help after BCR engagement leads to early Th0-like antibody production when compared with the more characteristic later Th2 responses mediated by $CD4^+$ T cells. In addition, iNKT cell help induced the generation of EF PCs, previously described as responsible for the generation of the initial wave of antibodies in a T cell-dependent response (5). These antibodies can have neutralizing effects for protection against viruses and bacteria, which suggests a main role for direct iNKT cell help to B cells in the induction of early immune responses against different pathogens. In line with this, iNKT-deficient mice exhibit severe defects in the clearance of several microorganisms, including *Streptococcus pneumoniae*, *Sphingomonas* ssp., and *Plasmodium berghei* (28). Interestingly, iNKT cells have been suggested as significant players in the development of early antibody responses after infection with *P. berghei* in the model for cerebral malaria (29). In this case, specific antibody production in $CD1^{-/-}$ mice is particularly reduced at early time points after parasitic challenge. Thus, it is apparent that iNKT cells can play a critical role in shaping early antibody responses *in vivo* and thus offer enhanced protection from invading pathogen.

Although αGalCer has proved to be an exceedingly useful tool for the characterization of iNKT cells both *in vitro* and *in vivo*, it is, however, not a natural ligand for iNKT cells. Thus, what is the functional significance of our observations in the context of an immune response? iNKT cells are activated by glycolipids from LPS-negative bacteria like *Sphingomonas*, *Erlichia*, and *Borrelia* (25, 26, 30). Alternatively, iNKT cells can recognize an as-yet-undefined endogenous lipidic ligand, via CD1d presentation, up-regulated by DCs in response to TLR signaling (25, 31, 32). Although all B cells are known to express CD1d, this expression is enhanced in marginal zone B cells, such that they present a $CD21^{high}$ $CD23^{low}$ $CD1d^{high}$ phenotype. This phenotype suggests that marginal zone B cells may play an important role in CD1d-dependent iNKT activation after infection. Indeed, these cells are localized in the marginal sinus of the spleen where they can provide early immune

responses to bloodborne particulate antigen (33, 34). We postulate that marginal zone B cells expressing BCR specific for bacterial glycolipids allow for the more efficient recruitment of iNKT cell help and associated generation of specific antibody responses. Alternatively, B cells capable of internalizing particulate microbes may receive TLR signals and subsequent iNKT cell help after the up-regulation of a CD1d-restricted endogenous ligand.

Our results identify BCR internalization of particulate antigenic lipids as a means of modulating iNKT-mediated B cell responses *in vivo*. The collaboration between B and iNKT cells leads to the development of early specific antibody responses, emphasizing the importance of iNKT cells in coordinating innate and adaptive immune responses.

Materials and Methods

Antigens, Lipid Preparation, and Microsphere Coating. The antigens HEL and OVA (both from Sigma), and CGG (Jackson Immuno Research) were used and where required were biotinylated by sulfo-NHS-LC-LC-biotin (Pierce). For the preparation of liposomes containing DOPC and *N*-Cap PE-biotin (both from Avanti Polar Lipids), DOPC/PE-biotin (98/2, m/m) or DOPC/PE-biotin/αGalCer (88/2/10, m/m/m), lipids were dried under argon and resuspended in 25 mM Tris, 150 mM NaCl, pH 7.0 with vigorous mixing. αGalCer was purchased from Alexis Biochemical. The synthesis of αGalCer-Alexa 488 was based on the methodology used for the synthesis of biotinylated αGalCer (35). αGalCer-Alexa 488-containing liposomes were used to quantify the amount of αGalCer bound to particles by using an EnVision Multilabel Reader to record relative fluorescence intensity. For coating, silica microspheres (100 nm; Kisker GbR) were incubated with liposomes followed by streptavidin and biotinylated proteins. Biotinylated (Fab'_2)-F10 anti-HEL was used to bind HEL of different affinities to the particles. For different densities of HEL, binding was carried out in the presence of competing biotinylated CGG and quantified by using FACS with an anti-HEL HyHel10 monoclonal. The binding HEL to liposome-coated beads was detected by Western blot.

Mice and Cell Lines. MD4, D1.3, D1.3-H2, and $J\alpha18^{-/-}$ mice were bred and maintained at the animal facilities of Cancer Research UK and John Radcliffe Hospital. C57BL/6 mice were purchased from Charles River. All experiments were approved by the Cancer Research UK Animal Ethics Committee and the United Kingdom Home Office. iNKT hybridoma DN32.D3 was kindly provided by A. Bendelac (University of Chicago, Chicago).

B Cell Purification and Presentation Assays. Splenic B cells were enriched by negative selection to >99% purity by using a B cell purification kit (Miltenyi Biotec). For analysis of αGalCer presentation, B cells were incubated with particles, washed, cultured at 5×10^4 cells per well with equal numbers of DN32.D3 cells, and IL-2 was measured in the supernatant of the cocultures. For blocking experiments MD4 B cells were preincubated with anti-CD1d (25 μg/ml; clone 1B1) before exposure to iNKT cells. After sorting by FACS, 2×10^4 cells per well MZ ($CD21^{high}$ $CD23^{low}$ $B220^+$) and Fo ($CD21^{int}$ $CD23^{high}$ $B220^+$) B cells were used in the presentation assay.

Adoptive Transfer and FACS Analyses. A total of $5–10 \times 10^6$ MD4 B cells were labeled with 2 μM CFSE (Molecular Probes) and adoptively transferred by tail-vein injection into WT C57BL/6 or $J\alpha18^{-/-}$ mice together with particles containing αGalCer and/or HEL. Five days later, spleens from recipient mice were harvested and splenocytes were stained for surface molecules and intracellular HEL binding as described (36).

Immunizations. Mice were immunized i.p. with 1–10 μl of beads containing different antigens and/or αGalCer, and antigen-specific Ig levels were determined in mice sera by ELISA.

ELISA and ELISPOT. IL-2 concentration was determined by ELISA, by using the JES6–1A12 for capture and biotinylated JES6–5H4 for detection (both from BD Pharmingen). Sera antibodies were measured by ELISA by using antigen (HEL, OVA, or CGG) for capture and biotin-labeled goat anti-mouse IgM, IgG, IgG1, IgG2b, IgG2c, or IgG3 for detection (all from BD Pharmingen).

For ELISPOT, single-cell suspensions of splenocytes were incubated for 15–18 h in HEL-coated multiscreen filtration plates (Millipore) and subsequently stained with goat anti-mouse biotinylated IgMa followed by streptavidin-peroxidase and 3-amino-9-ethyl-carbazole (both from Sigma).

Immunohistochemistry. Cryostat splenic sections (10 μm thick) were fixed and stained with rat anti-mouse CD45R/B220 (BD Biosciences). HEL^+ cells were detected by the addition of HEL (200 ng/ml) and anti-HEL F10 antibody-Alexa 488, and germinal centers were stained with peanut agglutinin-biotin (Vector Labs) and streptavidin-Alexa 633.

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