Toll-like receptor 9 antagonizes antibody affinity maturation

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Key events in T cell–dependent antibody responses, including affinity maturation, are dependent on the B cell’s presentation of antigen to helper T cells at critical checkpoints in germinal-center formation in secondary lymphoid organs. Here we found that signaling via Toll-like receptor 9 (TLR9) blocked the ability of antigen-specific B cells to capture, process and present antigen and to activate antigen-specific helper T cells in vitro. In a mouse model in vivo and in a human clinical trial, the TLR9 agonist CpG enhanced the magnitude of the antibody response to a protein vaccine but failed to promote affinity maturation. Thus, TLR9 signaling might enhance antibody titers at the expense of the ability of B cells to engage in germinal-center events that are highly dependent on B cells’ capture and presentation of antigen.

Although TLR signaling has the potential to amplify humoral immune responses in vivo, it remains unknown whether TLR signaling occurs in secondary lymphoid organs and is dependent on the stable interaction of antigen-primed helper T cells with activated antigen-specific B cells through complexes of peptide and major histocompatibility complex (MHC) class II presented on the B cell surface. Depending in part on the quality of the B cell–helper T cell interaction, B cells either enter germinal centers (GCs) or differentiate into short-lived plasma cells (PCs) and GC-independent memory B cells. Within GCs, the competitive process of affinity selection occurs on the basis of the ability of B cell receptors (BCRs) to capture, process and present antigen to follicular helper T cells (TFH cells). The B cells’ successful presentation of antigen to TFH cells ultimately results in the differentiation of GC B cells into long-lived memory B cells and PCs.

B cells also express germline-encoded Toll-like receptors (TLRs) that respond to microbial products that have pathogen-associated molecular patterns. The dual expression of the BCR and TLRs appears to modulate the outcome of antigen encounter in the presence of pathogens. Indeed, TLR9 signaling has been shown to enhance the response of B cells to antigens coupled to the TLR9 agonist CpG, in terms of proliferation and differentiation into antibody-secreting cells both in vitro and in vivo. In addition to signaling for B cell activation, the BCR functions to transport bound antigen to specialized intracellular compartments in which the antigen is degraded and the resulting peptides are bound to MHC class II molecules that are presented on the cell surface for recognition by antigen-specific helper T cells. At present, whether TLR signaling influences the processing and presentation of antigen by the BCR remains unknown.

Although TLR signaling has the potential to amplify humoral responses to antigen, B cell–intrinsic TLR signaling does not seem to be required for adjuvant-enhanced T cell–dependent antigen-specific antibody responses in vivo. Although adjuvants can influence several qualities of antibody responses, including kinetics, magnitude and breadth; the ability of adjuvants to augment B cell GC responses, including somatic hypermutation events, has remained unexplored until recently. BCR responses specific to the human immunodeficiency virus envelope protein in nonhuman primates responding to immunization with envelope protein formulated with eight different adjuvants, including ones that target TLR pathways, have now been compared. The adjuvants increased the titer of antibodies specific to envelope protein but, remarkably, did not increase the frequency of somatic hypermutation essential for the development of broadly neutralizing antibodies in this system.

Here we provide evidence that although TLR9 signaling enhanced BCR-induced B cell proliferation and differentiation into antibody-secreting cells, TLR9 signaling reduced the ability of B cells to capture, process and present antigen and to interact with and activate antigen-specific helper T cells in vitro, which was detrimental to the establishment of high-affinity, long-lived antibody responses in vivo.

Results

The effect of TLR9 signaling on BCR-dependent activation of B cells. We assessed the effect of CpG on several antigen–driven BCR signaling functions in mouse splenic B cells purified by negative selection and treated with soluble polyclonal F(ab’), antibody specific for immunoglobulin M (anti-IgM), CpG or anti-IgM plus CpG. The effects assessed included the following: phosphorylation of kinases in the BCR signaling pathway (Fig. 1a–d and Supplementary Fig. 1a–c); Ca2+ responses (Fig. 1e and Supplementary Fig. 1d); the expression of cytokine-encoding mRNA at 3 h after stimulation (Fig. 1f) and of cytokines (as protein)
Fig. 1 | The effect of TLR9 signaling on the outcome of B cell responses to antigen. Analysis of purified mouse splenic B cells stimulated in vitro with anti-IgM (2 μg/ml (a–d) or 5 μg/ml (e–i, l–m)) or CpG (1 μM) alone or in combination. a–d, Abundance of phosphorylated kinases in individual wild-type B cell samples fixed and barcoded with combinations of B220-specific antibodies19, pooled, permeabilized and stained with mAbs specific for the phosphorylated (p-) kinases Syk (a), Btk (b), p38 (c) and Akt (d); results are presented as mean fluorescent intensity (MFI) in stimulated B cells relative to that in unstimulated B cells. e, Calcium flux (measured by flow cytometry) in wild-type B cells loaded with the Ca²⁺ sensor dyes Furo-red and Fluo-4 and stimulated (key). f, Expression of mRNA encoding various cytokines (horizontal axis) in wild-type B cells stimulated for 4 h (key); results are presented relative to those of unstimulated B cells. g, ELISA of cytokines (below plot) in the culture supernatants of wild-type (WT) or TLR9-deficient (KO) B cells (horizontal axis) left unstimulated (US) or stimulated in vitro for 18 h (for analysis of IL-6) or for 24 h (for analysis of TNF, IL-2 and IL-10) with anti-IgM (2 μg/ml (g–j) or 5 μg/ml (g, j)) or CpG (1 μM) (g–j); results are presented relative to those of unstimulated B cells. h, Proliferation of wild-type or TLR9-deficient B cells (bracketing at right end of lines) stimulated in vitro with a sub-optimal concentration (1 μg/ml) of anti-IgM (or no anti-IgM) and increasing concentrations (0–3 μM) of CpG (key); results are presented as mean and s.d. in h–m. Each symbol (g–j) represents an individual biological replicate; dashed horizontal lines (f) indicate the mean. *P < 0.01; ***, 0.001 < P ≤ 0.001; ****, 0.0001 < P ≤ 0.0001; *****P < 0.0001 (two-sided unpaired t-test). Data are representative of three independent experiments with biological duplicates (a–d, mean and s.d.) or biological triplicates (f–m; mean and s.d. in h–m).
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Internalization and trafficking of soluble antigen by the BCR. We assessed the ability of the BCRs to internalize soluble antigen, either anti-IgM (internalized by C57BL/6 B cells) or hen egg lysozyme (HEL) (internalized by HEL-specific B cells from MD4 mice, which have transgenic expression of a HEL-specific BCR), in the presence or absence of CpG. CpG did not affect the rate or magnitude of the internalization of antigen by BCRs in either case (Supplementary Fig. 2a,b). We characterized the intracellular vesicles into which anti-IgM was internalized by incubating B cells with anti-IgM-coated electron-dense metal particles and imaging the cells by transmission electron microscopy and tomography. Quantification of three-dimensional reconstructions of the images showed anti-IgM+ particles in morphologically similar compartments in CpG-treated cells and untreated cells (Supplementary Fig. 2c–g).

Once internalized into early endosomes, the BCR traffics antigen to increasingly acidic late endosomes. We measured the fluorescence intensity (FI) of HEL-specific MD4 B cells internalizing soluble HEL conjugated to a pH-sensitive dye, the FI of which increases with decreases in pH. For both CpG-treated B cells and untreated B cells, the FI increased at the same rate for the first 20 min at 37 °C (Fig. 2a,b and Supplementary Fig. 3a). In the absence of CpG, the FI of the B cells continued to increase with time. In contrast, the FI of the CpG-treated B cells increased little after 30 min, and the block in trafficking was TLR9 dependent (Fig. 2a,b).

We further characterized, by confocal microscopy, the endosomal compartments into which the BCR internalized rhodamine-conjugated HEL in the presence or absence of CpG (Fig. 2c–e). Cells were fixed and then were stained with mAbs specific for CD71 (HEL), LAMP1 (a marker of late endosomes), or H2M (a marker of early endosomes; LAMP1, a marker of late endosomes; or H2M, a marker of early endosomes). As a result of treatment with both anti-IgM and CpG, the B cells exhibited a punctate pattern of HEL fluorescence (Fig. 2c–e). The HEL fluorescence in the untreated B cells was relatively evenly distributed throughout the perinuclear area, whereas in the CpG-treated B cells, the fluorescence was more concentrated in distinct punctate structures. These structures were also observed in cells treated with anti-IgM, but the pattern of fluorescence was more diffuse, with less defined punctate structures.

Together these results provided evidence that TLR9 signaling had the potential to drive B cells toward PC differentiation and away from GC responses.

Fig. 2 | TLR9 signaling antagonizes the trafficking of BCR-bound antigen into late endosomal compartments. a, MFI of the pH-sensitive fluorescent dye pHrodo (measured by flow cytometry) in HEL-specific MD4 B cells incubated for 0–160 min (horizontal axis) at 37 °C with pHrodo-conjugated HEL in the presence or absence of CpG (key); results are presented relative to those obtained before incubation. b, MFI of pHrodo in TLR9-sufficient (‘wild-type’) MD4 and TLR9-deficient MD4 B cells (horizontal axis) incubated for 10, 60 or 90 min (above plot) as in a (key); results presented as in a. c–e, Confocal microscopy (left) of MD4 B cells that were immunized on coverslips and incubated with rhodamine-conjugated HEL in the presence or absence of CpG (1 μM) (left margin), then fixed, permeabilized and stained with mAbs specific for CD71 (HEL), LAMP1 (d) or H2M (e). Right, three-dimensional (3D) colocalization indices of HEL with CD71 at 10 min (c), of HEL with LAMP1 at 60 min (d) or of HEL with H2M at 60 min (e) in cells as at left (n=25 cells per group). Scale bars (left), 1 μm. Each symbol (c–e) represents an independent biological replicate; dashed horizontal lines (c–e) indicate the mean. P values (above plots, c–e), two-sided Mann–Whitney U-test. Data are representative of three independent experiments (mean ± s.d. in a).

at 18–24 h (Fig. 1g); the proliferative responses of B cells (Fig. 1h); and concentration of IgM (Fig. 1i) and IgG (Fig. 1j) in culture supernatants over 7 d. We determined that although treatment with anti-IgM or CpG resulted in different patterns of kinase phosphorylation, the result of treatment with both anti-IgM and CpG was additive and was dependent on the concentration of anti-IgM and, for CpG responses, on TLR9 expression (Fig. 1a–c and Supplementary Fig. 1a–c). In contrast, B cells underwent Ca2+ flux only in response to anti-IgM (Fig. 1e and Supplementary Fig. 1d), and this response was unaffected by CpG. CpG and anti-IgM acted in synergy to induce high expression of the cytokines IL-2 and TNF at the level of both mRNA and protein, whereas the CpG-induced expression of IL-6 and IL-10 was substantially antagonized by anti-IgM (Fig. 1f,g). CpG enhanced, in a TLR9-dependent fashion, the proliferation of B cells treated sub-optimally with anti-IgM (Fig. 1h and Supplementary Fig. 1e,f). CpG induced the secretion of IgM by B cells that was weakly antagonized by anti-IgM (Fig. 1i), whereas anti-IgM enhanced the CpG-induced secretion of IgG by B cell populations depleted of IgG+ B cells (Fig. 1j and Supplementary Fig. 1g). Together these results demonstrated the ability of CpG to alter the B cell response to BCR crosslinking and the ability of BCR crosslinking to alter the B cell response to CpG.

We also measured by qPCR the transcription of genes encoding three factors that influence the PC fate of B cells1. CpG alone and CpG plus anti-IgM decreased the expression of Bcl6 (which encodes a key transcriptional repressor of PC differentiation whose expression is critical for the maintenance of B cell GC reactions) (Fig. 1k), but such treatment increased the expression of Predm1 (which encodes BLIMP-1, a transcription factor that promotes PC differentiation) (Fig. 1l) and Aicda (which encodes the deaminase AID and is upregulated when B cells differentiate toward PCs) (Fig. 1m).

Together these results provided evidence that TLR9 signaling had the potential to drive B cells toward PC differentiation and away from GC responses.
Fig. 3 | The effect of TLR9 signaling on B cell responses to membrane bound antigen. a. Total internal reflection fluorescence microscopy of HEL-specific MD4 B cells left untreated (No CpG) or pretreated for 20 or 60 min with 1µM CpG (left margin), labeled with DyLight 649–Fab anti-IgM and placed for 0–306 s (above images) on planar lipid bilayers containing Alexa Fluor 488–HEL (Supplementary Video 1a–c). Scale bar (below images), 12.45 µm.
b, Quantification of the contact area of B cells pretreated as in a (above plots) or left untreated (key) and placed for 0–630 s (horizontal axis) on a HEL-containing planar lipid bilayer as in a. c, d, Accumulation of the BCR (c) and HEL (d) in the area of contact of the B cell with the planar lipid bilayer as in b. e, Confocal z-stack images (reconstructed as an x and z side view by maximal projection along the y axis) of HEL-specific MD4 B cells labeled with DyLight 649–Fab anti-IgM and left untreated (HEL) or treated with 1µM CpG (HEL + CpG), then placed for 30 min at 37 °C on a CM-DiI-labeled PMS (green) containing Alexa Fluor 488–HEL (magenta; internalized HEL, cyan). Scale bars, 2 µm.
f, Proportion of HEL in the bilayer as in e that was internalized (left), and total internalized HEL, presented as total fluorescence intensity (TFI) (right). g, Three-dimensional (3D)-reconstructed image of confocal z-stacks showing the BCR (cyan), HEL (magenta) and the PMS (yellow) (Supplementary Video 2a,b) in cells as in e. Scale bars, 2 µm. h, i, Quantification of the colocalization of the BCR and HEL (h) and HEL and the PMS (i) in cells in e. Each symbol (f,h,i) represents an individual cell; dashed horizontal lines indicate the mean. P values (above plots, c,d,f,h,i), two-sided quadratic regression (c,d) or two-sided Mann-Whitney U-test (f,h,i). Data represent three independent experiments.
Fig. 4 | The effect of TLR9 and BCR signaling on B cell transcription and the expression of B cell surface proteins. a. Quantification of genes with expression (by RNA-seq analysis) differentially affected in purified B cells left unstimulated or stimulated for 4 h in vitro with CpG (1µM) or anti-IgM (5µg/ml) or a combination of those (along perimeter), showing the overlap of those groups. b. Principle-component analysis of gene expression as in a, c, Ingenuity Pathway Analysis of gene expression (key) in cells as in a (below plot), relative to that of unstimulated B cells, presented as log2-scale enhancement and diminished transcriptional regulation of various pathways (left margin) in response to such stimulation. d. Surface expression of various proteins (assessed with fluorophore-conjugated antibodies) in purified B cells cultured for 24 h as in a (below plot) and barcoded, showing results for proteins with an increase in expression of threefold or more or a decrease in expression of 30% or more, relative to that of untreated B cells (log, scale). e, Flow cytometry of HEL–I-Ak in HEL-specific MD4 B cells stimulated for 24 h with HEL (1µg/ml) or HEL plus CpG (1µM) (above plots), assessed with phycoerythrin (PE)-labeled mAb AW3.18 (specific for HEL–I-Ak) or isotype-matched control mAb (Isotype) (key). f, MFI of HEL–I-Ak in HEL-specific MD4 B cells left unstimulated or stimulated for 24 h with HEL (1µg/ml) or CpG (1µM) or both (key), presented as the ratio of the MFI of cells stained with mAb AW3.18 to that of cells stained with the isotype-matched control mAb (AW3.18/isotype). g, Surface expression of MHC class II (MHCII) (g) and CD86 (h) on B cells left unstimulated or stimulated with CpG (1µM) or anti-IgM (5µg/ml) (key) in the presence or absence of the following kinase inhibitors or activator (horizontal axis): 50µM PD98059 (inhibits MEK1 and MEK2), 10µM SB202198 (inhibits p38), 30µM SP600125 (inhibits JNK), 5µM Akt IV (inhibits Akt), 10µM MHY1485 (activates mTOR; inhibits autophagy), 20µM perifosine (inhibits Akt), 100nM rapamycin (inhibits mTOR) or 50nM wortmannin (inhibits PI3K). Each symbol (f,g,h) represents an individual biological replicate. *, 0.01<P≤0.05; **, 0.001<P≤0.01; ***, 0.0001<P≤0.001; ****P≤0.0001, compared with the condition of no inhibitor or activator (two-sided unpaired t-test). Data are representative of one experiment in triplicate (a-c), two independent experiments in triplicate (d,g,h) or two experiments (e,f, mean in f).
Fig. 5 | TLR9 signaling decreases the ability of antigen-specific B cells to interact with and activate antigen-specific helper T cells in response to soluble antigen. **a–c.** Duration of B cell–T cell colocalization (**a**), T cell track speed (**b**) and T cell track length (**c**) for CMTPX-stained 3A9 T cells cultured (at a ratio of 1:1) with CMFDA-stained HEL-specific MD4 B cells in the presence or absence (US) of CpG (1µM) and/or HEL (1µg/ml) (key), imaged for 24 h at rate of 1 frame per 10–20 min. **d,e.** CD44 expression (**d**) and proliferation (assessed with the cell-proliferation dye e450) (**e**) of HEL-specific 3A9 T cells stimulated for 72 h with various combinations (grid at left) of HEL (0.5 or 2.0µg/ml), CpG (0.2 or 2.0 µM) and HEL-specific MD4 B cells. APC, allophycocyanin. **f,g.** Proliferation (**f**) and CD44 expression (**g**) of wild-type or TLR9-deficient HEL-specific 3A9 CD4+ T cells cultured for 72 h with wild-type or TLR9-deficient HEL-specific MD4 B cells (above plots) in the presence or absence of HEL (1µg/ml) with or without CpG (1µM) (key). AF700, Alexa Fluor 700. **h.i.** CD44 expression (**h**) and proliferation (**i**) of CD4+ T cells left unstimulated or stimulated for 72 h with anti-CD3 with or without anti-CD28 (right margin) in the presence or absence of 1µM CpG (key). Each symbol (**a–c**) represents an individual event; dashed horizontal lines indicate the mean. P values (above plots, **a–c**), two-sided Welch’s t-test. Data represent two independent experiments in triplicate (**f.g**) or are representative of three experiments (**a–e**) or three independent experiments in triplicate (**h.i**).

three-dimensional microscopy (Fig. 2c–e and Supplementary Fig. 3b,c). HEL colocalized with CD71 in early endosomes at 10 min, and treatment with CpG had no significant effect on this colocalization (Fig. 2c). The colocalization of HEL with either LAMP1 or H2M increased from 10 min (Supplementary Fig. 3b,c) to 60 min, and treatment with CpG significantly diminished the colocalization at 60 min (Fig. 2d,e). Together these data indicated that TLR9 signaling compromised the BCR’s delivery of antigen into late endosomes and the antigen-processing compartments.
TLR9 signaling affects B cell responses to membrane-bound antigen. We assessed the effect of CpG treatment on B cell responses to antigen incorporated into fluid planar lipid bilayers. HEL-specific MD4 B cells were labeled with Fab anti-IgM conjugated to the fluorophore DyLight 649 and were placed on bilayers containing HEL labeled with the fluorescent dye Alexa Fluor 488, and live-cell imaging was carried out by total internal reflection fluorescence microscopy (Fig. 3a and Supplementary Video 1a–c). When pretreated for 20 min with CpG, the B cells spread more robustly over the HEL-containing bilayer, covering larger areas, relative to the spreading of untreated B cells (Fig. 3a,b), but they did not form a well-organized central synapse and accumulated less BCR (Fig. 3c) and HEL (Fig. 3d) in the contact area. B cells pretreated for 60 min with CpG acted similarly, but they contracted after 90 s and accumulated less BCR than the untreated B cells did but accumulated amounts of HEL comparable to those accumulated by the untreated B cells (Fig. 3d), suggestive of an antigen-independent component of BCR accumulation, as described earlier.

We also assessed the BCR-dependent internalization of HEL incorporated into plasma membrane sheets (PMSs) by HEL-specific MD4 B cells. B cells were labeled with DyLight 649–Fab anti-IgM and were placed for 30 min at 37 °C on PMSs labeled with fluorescent dye CM-Dil (used for monitoring cell movement), into which Alexa Fluor 488–HEL was incorporated. Confocal z-stack images were obtained and reconstituted as an x view and a z view (Fig. 3e) with software program codes that quantify only the internalized antigen, excluding antigen tethered on the PMS surface (Fig. 3f). In the absence of CpG, B cells internalized HEL from the PMS, but in the presence of CpG, the internalization of HEL was much less, as measured by either the proportion of the HEL in the bilayer that was internalized or the total HEL internalized (Fig. 3f). We also determined the degree of colocalization of the BCR and HEL, and of HEL and fragments of the PMS, in three-dimensional reconstructed images of the confocal z-stacks of B cells placed on HEL-containing PMSs for 30 min (Fig. 3g and Supplementary Video 2a,b). HEL, BCR and fragments of the PMS above the contact area with the PMS surface indicated internalization (Fig. 3g). In the absence of CpG, B cells were frequently observed in contact areas from which the PMS had been cleared, in contrast to CpG-treated B cells, which were observed in areas covered by the PMS. Videos of three-dimensional surface images showed that for both CpG-treated cells and untreated cells, little antigen was retained on the B cell surface. In the xy and xz cross sections of B cells not treated with CpG, the BCR and HEL and PMS fragments to which HEL was bound were evident outside the cell (Supplementary Video 2a,b) where the BCR and HEL colocalized (Fig. 3h), as did HEL and PMS fragments (Fig. 3i). However, for CpG-treated B cells, although the BCR and HEL were present intracellularly, very few PMS fragments were evident (Fig. 3g). The colocalization of HEL and the BCR in CpG-treated B cells was similar to that in untreated cells (Fig. 3h); however, the colocalization of HEL and fragments of the PMS was significantly lower in CpG-treated B cells than in untreated cells (Fig. 3i). The lack of PMS fragments in CpG-treated B cells suggested that antigen taken up by BCRs in the presence of CpG did not involve pulling the antigen and attached PMS into the cell but was probably the result of the internalization of antigen that was not tightly attached to the PMS.

Gene transcription and the expression of B cell surface markers. RNA-based next-generation sequencing (RNA-seq) analysis was carried out on untreated B cells and B cells treated for 4 h with anti-IgM or CpG alone or together. Venn diagramming (Fig. 4a) and principle-component analysis (Fig. 4b) of the results showed notable separation of the transcription profiles of B cells under the different stimulation conditions. Thus, treatment of B cells with anti-IgM and/or CpG resulted in the activation of distinct transcriptional programs. An Ingenuity Pathway Analysis of the genes expressed differentially in B cells treated with anti-IgM or CpG (alone or together) relative to their expression in unstimulated cells showed that a variety of pathways were affected, including those related to cell proliferation and apoptosis, signal transduction, responses to cytokines and cytoskeleton organization (Fig. 4c).

We also quantified by flow cytometry the B cell surface expression of approximately 250 mouse cell-surface proteins using a bar-coding strategy and a kit with fluorophore-conjugated antibodies for the screening of cell-surface molecules (Fig. 4d). The expression of 63 surface proteins changed significantly after treatment relative to their expression on unstimulated cells. We observed synergy (up to 55-fold) between CpG and anti-IgM that induced an increase in 48% of the surface proteins; antagonism of anti-IgM responses by CpG for 9.5% of markers; and antagonism of CpG responses by anti-IgM for 8% of surface proteins. The expression of 30% of the surface proteins decreased in response to CpG and/or anti-IgM. Together these results supported the conclusion that signaling through the TLR9 alone, TLR9 alone or both together resulted in distinct outcomes.

We further analyzed the effect of CpG and anti-IgM on expression of the early activation marker CD69 and three cell-surface molecules that are critical to B cell antigen presentation and activation of helper T cells: MHC class II and the costimulatory molecules CD86 and CD80 (Supplementary Fig. 4a–g). The surface expression of MHC class II increased approximately eight- to tenfold at 24–48 h after B cells were treated with anti-IgM alone; this was antagonized by the addition of CpG, via a TLR9-dependent process (Supplementary Fig. 4c,f). However, treatment with CpG alone induced a four- to fivefold increase in the surface expression of MHC class II (Supplementary Fig. 4c,f), which indicated that CpG initiated at least two signaling pathways through TLR9: one that antagonized BCR-induced expression of MHC class II, and one that independently enhanced the expression of MHC class II. We also assessed the effect of CpG on the ability of HEL-specific B cells from MD4 mice to process HEL and present complexes of HEL peptide and the MHC class II molecule I-Ak (HEL–I-Ak) on the B cell surface; for this, we used a monoclonal antibody (mAb) specific for HEL–I-Ak. After 24 h of incubation with HEL, HEL-specific B cells significantly increased their cell-surface abundance of HEL–I-Ak by approximately 2.5-fold, and the addition of CpG blocked this increase (Fig. 4e,f).

Neither treatment with CpG nor treatment with anti-IgM had a large effect on CD80 expression at 24 h (Supplementary Fig. 4b); however, after 48 h, the cell-surface expression of CD80 increased approximately twofold for cells treated with CpG alone, anti-IgM alone or both together, and this showed neither antagonism nor synergy (Supplementary Fig. 4b). In contrast, treatment with anti-IgM induced a large increase in the expression of CD86 that was blocked by CpG (Supplementary Fig. 4a,e). The regulation of the surface expression of CD69 showed a pattern consistent with a simple additive effect of anti-IgM and CpG (Supplementary Fig. 4d,g).

We assessed the effects of several inhibitors of kinases in the BCR and TLR9 signaling pathways on the induced expression of MHC class II and CD86. The most consistent effects of the inhibitors were on BCR-induced changes in the expression of both MHC class II and CD86, although these effects were only partial (Fig. 4g,h). In contrast, the TLR9-induced changes in the expression of MHC class II and CD86 were unaffected by the inhibitors, which suggested, in both cases, the possibility of redundancy in the signaling pathways.

Together these results provided evidence that signaling through the BCR, TLR9 or both triggered distinct transcriptional programs and had differential effects on the expression of cell-surface markers. Of greatest interest was the antagonistic effect of TLR9 signaling on BCR-induced increases in the abundance of peptide–MHC class II complexes and the expression of CD86.
Fig. 6 | TLR9 signaling decreases the ability of antigen-specific B cells to activate antigen-specific helper T cells in response to membrane bound antigen. a, b, Surface expression of the HEL–CD4 chimeric membrane protein on NIH3T3-HEL or NIH3T3-mock cells (key), quantified with an Alexa Fluor 647 (AF647)-labeled mAb specific for CD4 (a) or an allophycocyanin (APC)-labeled mAb specific for HEL (b) or the corresponding isotype-matched control mAb (key). c, Flow cytometry of the acquisition of HEL by HEL-specific MD4 B cells or nonspecific (WT) B cells (right margin) incubated for 20 or 40 min (left margin) with NIH3T3-mock or NIH3T3-HEL cells (right margin), then fixed, permeabilized and stained with a HEL-specific mAb. d, Expression of CD86, MHC class II and CD69 (below plots) by HEL-specific MD4 B cells incubated for 24 h with NIH3T3-mock or NIH3T3-HEL cells (key) in the presence (+) or absence (−) of 1 μM CpG (horizontal axis); results are presented relative to those of B cells cocultured with NIH3T3-mock in the absence of CpG. e–g, Proliferation (e) and CD44 expression (f, g) of HEL-specific 3A9 CD4+ T cells cultured for 72 h with HEL-specific MD4 B cells and either NIH3T3-mock or NIH3T3-HEL cells in the presence or absence of CpG (1 μM). Each symbol (d, g) represents an individual biological replicate. P values (above plots, d, g), two-sided unpaired t-test. Data are representative of three independent experiments in triplicate (mean values).

CpG blocks B cell–helper T cell interactions in vitro. We assessed the effect of CpG on the length of time antigen-specific B cells and T cells interacted in vitro in the presence of antigen. We labeled HEL-specific T cells from 3A9 mice (which have transgenic expression of a HEL-specific TCR) with the cell-tracking fluorescent dye CMTPX and labeled HEL-specific B cells from MD4 mice with cell-tracking fluorescent dye CMFDA, then incubated those cells in culture with HEL alone, CpG alone or HEL plus CpG, imaging the cells continuously over a period of 24 h at a rate of 1 frame per 10–20 min. HEL induced a significant increase in the duration of colocalization of T cells and B cells, and this was antagonized by CpG (Fig. 5a). The presence of HEL also resulted in a decrease in both the speed and the length of the tracks of T cells, an effect that was mitigated by CpG (Fig. 5b, c).

We assessed the effect of TLR9 signaling on the ability of antigen-specific B cells to activate antigen-specific T cells in vitro. In the presence of HEL-specific MD4 B cells and HEL (at a concentration of either 0.5 μg/ml or 2.0 μg/ml), HEL-specific 3A9 T cells were induced to increase their expression of the activation and memory marker CD44 (Fig. 5d and Supplementary Fig. 4h) and proliferate (Fig. 5e). The addition of CpG to the cultures (at a concentration of either 0.5 μM or 2.0 μM) significantly reduced both CD44 expression and T cell proliferation. In the absence of B cells, T cells did not respond to either HEL or CpG. The ability of CpG to antagonize the activation of HEL-specific T cells by HEL-specific B cells was dependent on the expression of TLR9 by B cells but not on the expression of TLR9 by T cells (Fig. 5f, g). TLR9-sufficient (‘wild-type’) or TLR9-deficient 3A9 T cells cultured with wild-type MD4 B cells and HEL proliferated and increased their expression of CD44; this was blocked by the presence of CpG. In contrast, CpG had no effect on the proliferation or CD44 expression of either wild-type 3A9 T cells or TLR9-deficient 3A9 T cells induced by TLR9-deficient MD4 B cells. CpG had no effect on the ability of wild-type CD4+ T cells to increase their expression of CD44...
Fig. 7 | B cell-intrinsic expression of MyD88 affects the outcome of T cell dependent antibody response in vivo. a, Flow cytometry of splenocytes from chimeric mice with MyD88-deficient B cells (μMT-MyD88-KO) or wild-type B cells (μMT-WT) (above plots) (Supplementary Fig. 5a) on day 14 after intraperitoneal immunization with NP-CGG (100 μg per mouse) adsorbed onto alum (100 μg per mouse) and CpG (65 μg per mouse) (gating strategy, Supplementary Fig. 5c). Numbers adjacent to outlined areas indicate percent GC B cells (CD95+/CD138+) among B cells as in a (key). b, Total GC B cells (b), frequency of NP-specific GC B cells among all GC B cells (c) and total NP-specific GC B cells (d) from chimeras as in a (above plots). Numbers adjacent to outlined areas indicate percent IgG1/IgD– B cells among B cells (top row) or NP-specific cells among those cells (bottom row). e, Total IgG1/IgD– cells (f), frequency of NP-specific B cells among all IgG1/IgD– B cells (g) and total NP-specific IgG1/IgD– B cells (h) from chimeras as in e (key). i, Flow cytometry of cells from chimeras as in a (above plots). Numbers above outlined areas indicate percent cells of the plasma cell lineage (CD138+/CD138+). j, k, Total PC-lineage cells (j) and NP-specific IgG-secreting cells (k) among cells from chimeras as in i (key). l, ELISPOT analysis of cells from chimeras as in k (left margin). m–o, NP-specific IgM (m), NP-specific IgG (n) and high-affinity NP-specific IgG (o) in serum from chimeric mice as in a (key) on days 7–91 after immunization as in a (horizontal axis), presented as arbitrary units (AU) calculated from serial dilutions of pooled serum. Each symbol (b–d,f–h,j,k) represents an individual mouse; dashed horizontal lines indicate the mean. P values (above plots in b–d,f–h,j,k): NS, not significant (P > 0.05); *, 0.01 < P ≤ 0.05; **, 0.001 < P ≤ 0.01; and ***, 0.0001 < P ≤ 0.001 (two-sided Welch’s t-test). Data are pooled from two independent experiments (a–l) or represent two independent experiments (m–o; mean and s.d. of n = 8 mice per group).
(Fig. 5b) or proliferate (Fig. 5i) in response to stimulation with plate-bound anti-CD3 and anti-CD28.

We also assessed the effect of CpG on the ability of B cells to respond to and capture HEL from cell surfaces and to present it to HEL-specific 3A9 T cells using NIH3T3 mouse fibroblasts stably expressing a chimeric membrane protein of HEL and the monomorphic co-receptor CD4 via a retroviral vector (NIH3T3-HEL cells) or transduced with a mock vector (NIH3T3-Mock cells) (Fig. 6a,b). HEL-specific MD4 mouse B cells (but not nonspecific wild-type B cells) captured HEL, as detected by flow cytometry with a HEL-specific mAb (Fig. 6c), and increased their expression of CD86, MHC class II and CD69 (Fig. 6d). For both CD86 and MHC class II, the presence of CpG blocked this increase, but it had a synergistic effect on the increase in CD69 expression (Fig. 6d). Co-culture of HEL-specific MD4 B cells and 3A9 T cells in the presence of NIH3T3-HEL cells resulted in the activation of T cells to proliferate (Fig. 6e) and increase their expression of CD44 (Fig. 6f,g), but such co-culture in the presence of NIH3T3-Mock cells did not, and both of those effects were diminished in the presence of CpG.

Together these results demonstrated that in the presence of CpG, antigen-specific B cells were unable to present soluble antigen or antigen captured from cell surfaces and activate antigen-specific helper T cells.

The effect of CpG on B cell responses in vivo. To investigate the effect of CpG on B cell responses in vivo, we generated chimeric mice in which the B cells were either wild-type or deficient in the TLR9 adaptor MyD88 and all other hematopoietic cells were wild-type (Supplementary Fig. 5a). Reconstitution in the two chimeras was similar (Supplementary Fig. 5b). MyD88 deficiency in B cells is not predicted to affect the intrinsic ability of B cells to participate in T cell–dependent antibody responses, as robust T cell–dependent antibody responses are elicited by immunization of MyD88-deficient mice in vivo11. We immunized the chimeric mice with alum-adsorbed nitrophenyl–chicken γ-globulin (NP-CGG) plus CpG, harvested the spleens on day 14 after immunization and analyzed the cells by flow cytometry according to the appropriate gating strategy (Supplementary Fig. 5c). Although the spleen of chimeras with wild-type cells had a larger total number of GC B cells than that in the spleen of chimeras with MyD88-deficient cells (Fig. 7a,b), the frequency and total number of GC B cells that were NP specific was significantly higher in chimeras with MyD88-deficient cells than in chimeras with wild-type cells (Fig. 7c,d). Similarly, the total number of IgG-class-switched B cells was higher in chimeras with wild-type cells than in chimeras with MyD88-deficient cells (Fig. 7e,f), but the opposite was true for the frequency and total number of NP-specific B cells (Fig. 7g,h). The total number of cells of the PC lineage was greater in chimeras with wild-type cells than in chimeras with MyD88-deficient cells (Fig. 7i,j), but the number of NP-specific antibody–secreting cells was greater in the spleen of chimeras with MyD88-deficient cells than in that of chimeras with wild-type cells (Fig. 7k,l). We also determined the total number of NP-specific antibody–secreting cells.
of T<sub>H</sub> cells (CD4<sup>+</sup>CXCR5<sup>+</sup>PD1<sup>+</sup>) in the spleen of the chimeric mice at 14 d after immunization and found no significant difference between the chimeras in the number of T<sub>H</sub> cells, although the chimeras with wild-type cells tended to have more T<sub>H</sub> cells (Supplementary Fig. 5d,e). However, without tools to determine the frequency of T<sub>H</sub> cells that were antigen specific, that observation was difficult to interpret, given the differences observed for total GC B cells versus antigen-specific GC B cells, isotype-switched B cells and PCs (Fig. 7).

Chimeras with wild-type B cells showed higher NP-specific IgM serum responses than those of chimeras with MyD88-deficient B cells (Fig. 7m). For total NP-specific IgG, although the peak levels at day 30 after immunization were not significantly different in immunized chimeras (Fig. 7n), the levels of NP-specific IgG dropped faster in chimeras with wild-type B cells than in those with MyD88-deficient B cells. The most notable differences were in the levels and persistence of high-affinity NP-specific IgG (Fig. 7o), which reached higher peak levels and persisted for weeks longer in chimeras with MyD88-deficient B cells than in those with wild-type B cells (Fig. 7o). Thus, CpG drove the production of IgM and short-lived antigen-specific IgG responses that did not undergo affinity maturation in vivo.

The effect of CpG on antibody affinity in humans. A longitudinal study has been done of the antibody response to a candidate malaria vaccine administered with or without CpG to healthy malaria-naive people in the US (trial NCT00320658 at https://www.clinicaltrials.gov)<sup>20</sup>. The vaccine was composed of recombinant Plasmodium falciparum apical membrane protein 1 (PfAMA1) formulated on the adjuvant aluminum-hydroxide gel and mixed with 564 µg of CpG 7909. The inclusion of CpG in the vaccine resulted in significantly higher titers of PfAMA1-specific IgG but did not have an effect on the longevity of the responses<sup>20</sup>.

Using serum samples from that trial<sup>20</sup>, we determined the effect of the inclusion of CpG in the vaccine on the affinity of the IgG response. Serum was collected at 70 d after vaccination from ten people who received the vaccine alone and ten who received the vaccine containing CpG, and IgG was purified on protein G columns (Fig. 8). The chromatographic profiles of IgG from all people were similar, and the IgG antibodies seemed to be very pure (Fig. 8a). The purified IgG antibodies were analyzed by BIACore with PfAMA1 recombinant protein adsorbed onto CM5 chips. There was no difference between people who received the vaccine alone and those who received the vaccine containing CpG, in terms of apparent affinity (Fig. 8b,c) or off rate (Fig. 8b,d). Thus, although the inclusion of CpG resulted in an increase in the levels of antigen-specific antibody<sup>20</sup>, CpG caused no detectable increase in the affinity of these antibodies.

Discussion

It was established over 30 years ago that the processing and presentation of antigen to CD4<sup>+</sup> helper T cells by antigen-specific B cells is highly efficient<sup>21</sup>. However, although it was clear that antigen-specific B cells are able to efficiently process and present antigen, the role of antigen processing and presentation by B cells in T cell–dependent antibody responses was not elucidated until recently. Several studies have now provided evidence that the ability of B cells to process and present antigen to antigen-activated helper T cells and to T<sub>H</sub> cells provides critical checkpoints for the entry of B cells into the GC and for the affinity selection of B cells expressing somatically hypermutated immunoglobulin-encoding genes in the GC<sup>22,23</sup>. The quality of the B cell’s presentation of antigen to antigen-activated T cells at the T cell–B cell border in lymphoid organs determines, in part, if a B cell will enter the GC or differentiate along a GC-independent pathway to become short-lived PCs or GC-independent memory B cells<sup>2</sup>. In the GC light zone, selection by T<sub>H</sub> cells is dependent on the density of peptide–MHC complexes on the B cell surface, reflective of the B cell’s affinity-dependent ability to compete for the capture of antigen from follicular dendritic cells<sup>1</sup>. Thus, the processing and presentation of antigen by B cells controls, to a large extent, the clonal selection of B cells.

Given the central role of the gathering, processing and presentation of antigen by B cells in the generation of high-affinity, long-lived antibody responses, it is likely that this B cell function is regulated to ensure appropriate and timely responses to antigenic challenge, particularly during infection. However, little is known about the role of receptors of the innate immune system that respond to microbial products containing pathogen-associated molecular patterns in regulating these processes. We found that TLR9 signaling promoted B cell proliferation and the differentiation of B cells into antibody secreting cells, as previously shown<sup>12,22,23</sup>, and, in addition, that it triggered changes in gene expression, including the downregulation of the gene encoding BCL6, a transcriptional repressor of PC differentiation, and upregulation of the gene encoding BLIMP1, a transcription factor that promotes PC differentiation. However, TLR9 signaling greatly diminished the ability of antigen-specific B cells to gather, process and present antigen and to interact with and activate antigen-specific CD4<sup>+</sup> T cells in vitro. In the presence of CpG, antigen-specific B cells also failed to upregulate their expression of important costimulatory ligands for T cells, including CD86. The ability of CpG to diminish the ability of B cells to capture antigen from membranes and cell surfaces, as shown here, is particularly relevant to the regulation of B cell responses in vivo, where the B cells in lymphoid organs activate most antigens on the surface of follicular dendritic cells<sup>24</sup>.

Such findings have implications for the outcome of T cell–dependent antibody responses in vivo. Our data suggest that if B cells encounter TLR9 ligands during an infection, the B cells would have diminished ability to gather, process and present antigen to antigen-activated helper T cells at the T cell–B cell border of lymphoid organs. As a consequence, B cells would be directed to GC-independent pathways and would yield ‘affinity-unselected’ PCs and GC-independent memory B cells. It might be advantageous to rapidly produce antibodies with a wide range of affinities in response to activation of TLR9 during an acute infection and to leave an unselected broad B cell repertoire for future antigen challenge<sup>1</sup>. Such a mechanism might underlie the observation that, in a nonhuman primate model, although adjuvants that stimulate receptors of the innate immune system, including TLRs, were effective in enhancing the magnitude of antibody responses to protein immunogens, remarkably, these were not able to increase somatic hypermutation<sup>25</sup>.

We have presented evidence both in mice and in humans consistent with that model. We compared the responses of chimeric mice containing wild-type B cells with the responses of chimeric mice containing MyD88-deficient B cells after immunization with an alum-adsorbed protein antigen (NP-CGG) given together with CpG. The ability of B cells to respond to CpG resulted in short-lived low-affinity IgG responses and reduced numbers of antigen-specific GC B cells. We provided evidence that in humans, the inclusion of CpG in a recombinant protein vaccine formulated on alum resulted in increased levels of antigen-specific antibodies but had no detectable effect on the apparent affinity of the antibodies, relative to the response of people who received the vaccine alone. To our knowledge, the studies presented here and a published study<sup>15</sup> are the only ones to address the effect of TLR ligands as adjuvants on antibody affinity maturation. It will be of interest to determine the generality of these findings for other vaccines and vaccine regimens.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41590-018-0052-z.
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Author contributions
M.A. and S.K.P. conceived of the project, designed the experiments and wrote the manuscript; M.A., B.A., A.S.K., P.M., H.S., M.P., A.S.R., B.P.T., T.H. and J.L. carried out the experiments; M.A., B.A., A.S.K., P.M., H.S., J.K., J.L., D.W.D., E.D., J.S., L.H.M. and S.K.P. analyzed the data; and S.K.P. secured funding.

Competing interests
The authors declare no competing financial interests.

Additional information
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**Methods**

**Mice.** C57BL/6, B10.BR-H2K2H2-TIa8a/SgnSnArc, B cell-deficient (B6.129S2-Ighmtm1Cgn/J) (µMT) mice, B6.129S2-Ighmtm1Cgn/J, MyD88-deficient mice and B6 CD45.1+ congenic wild-type mice were purchased from Jackson Laboratory. HEL-specific BCR transgenic mice (MD4) were purchased from S. Akira (Osaka University, Osaka, Japan). Mice with transgenic expression of a HEL-specific, MHC class II I-A<sup>+</sup>-restricted TCR (3A9)<sup>-</sup>, were provided by L. Gery (NEI/NIH, Bethesda, MD, USA). Both MD4 mice and 3A9 mice were kept hemizygous for the transgenic allele in an MHC class II I-A<sup>+</sup>-I-A<sup>−</sup> haplotype by crossing 3A9 MD4 mice with wild-type mice of the relevant haplotype. TLR9-deficient MD4 and 3A9 mice were generated by crossing the MD4 and 3A9 mice with TLR9-deficient mice for at least three generations. TLR9-deficient mice on the MHC class II I-A<sup>+</sup>-haplotype were generated as the F2 generation of a cross between TLR9-deficient mice (I-Ab) and B10.BR-H2K2H2-TIa8a/SgnSnArc (I-A<sup>−</sup>). These TLR9-deficient I-A<sup>+</sup>- and I-A<sup>−</sup>-mice were further processed as four generations of TLR9-deficient MD4 and 3A9 mice to obtain TLR9-deficient MD4 or 3A9 mice in the I-A<sup>+</sup>-haplotype. Mice were bred and maintained in NIH animal facility according to Animal Care and Use Committee Standards.

**Antibodies and proteins.** Antibodies specific for the following mouse molecules were purchased from BioLegend: CD19 (Clone:RA3-6B2) (conjugated to Alexa Fluor 647 or Alexa Fluor 488) and polyclonal F(ab′)<sub>2</sub> anti-mouse IgG (conjugated to Alexa Fluor 647 or Alexa Fluor 488) of which 90% were from µMT mice and 10% were from either MyD88-deficient mice or wild-type mice. Mice were allowed to reconstitute for at least 8 weeks before intraperitoneal immunization with both 100 µg NP<sub>e</sub>–CGG (Biosearch Technologies) adsorbed into 100 µl Alum (ThermoFisher) in PBS and 65 µg CpG B (ODN 1826) (Invivogen) in PBS.

**Flow cytometry.** Flow cytometry was performed on a BD LSRII flow cytometer and data were analyzed in Flowjo software (Tree Star). During analysis, dead cells were excluded by staining with LIVE/DEAD fixable dead cell stain kits (ThermoFisher).

**Cells.** B cells and naive CD4<sup>+</sup> T cells were isolated from mouse spleen using mouse B cell and naive T cell isolation kits (Miltenyi Biotech) according to previously published optimized protocols<sup>59,60</sup>. NIH3T3 mouse fibroblast cell line (ATCC CRL-1658) was provided by O. Voss. Phoenix Ecolin retroviral packaging cell line (ATCC CRL-3214) was purchased from American Type Culture Collection (ATCC). NIH3T3 cell cultures maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 µM 2-mercaptopetoan, 50 µM penicillin, 50 µM streptomycin, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 mM HEPES unless specified otherwise. The pathway inhibitors SB600125, PD98059, Wortmannin, Rapamycin, SB202190 were purchased from Invivogen. MYH1485 and Akt Inhibitor IV (Akt IV) were purchased from Sigma-Aldrich.

**B cell--T cell co-culture.** B cells were isolated from the spleens of MD4 mice on the MHC class II I-A<sup>+</sup>-haplotype. Naïve T cells were isolated from the spleen and lymph nodes of 3A9 mice. Both cells were labeled with Cell Proliferation Dye eFluor 450 (ThermoFisher), mixed at 1:1 ratio (7.5 x 10<sup>5</sup> each) in complete medium and incubated in 96-well plates at 37°C in the presence of HEL (1 µg/ml) and/or CpG B (10 µM). Stimulation by mitomycin C and HEL-loaded antigen was carried out by incubating NIH3T3 cells, transfected to stably express HEL or mock transfected, in 96-well plates, 5 x 10<sup>4</sup> cells per well, 1 d before adding the B cell--T cell mixture.

Visualization of real-time interactions between B cells and T cells was carried out by labeling B cells and T cells with CellTracker Green CFMFA and CellTracker Red CMFDA (ThermoFisher), respectively. Labeled cells were washed and plated into 96-well plates at 1:1 ratio (1 x 10<sup>5</sup> each) and stimulated as indicated above. Interactions between cells were recorded as 10- to 20-min time-lapse photos by IncuCyte ZOOM system (Essen Bioscience) for 24 h. Image analysis was performed using Imaris Software.

**Human study population and vaccination procedures.** Serum was obtained 70 d after immunization from malaria-naïve adults enrolled in a phase 1 clinical trial of the blood-stage malaria vaccine candidate AMA1-C1 (80 µg), formulated on Alhydrogel and mixed with CpG 7909 (36 µg]<sup>3</sup> (trial NCT00320658 at https://www.clinicaltrials.gov) as detailed elsewhere<sup>3</sup>. The trial was conducted under Investigational New Drug Applications reviewed by the US Food and Drug Administration and was reviewed and approved by the National Institute of Allergy and Infectious Diseases Review Board and the University of Rochester Institutional Review Board and funding agency. Written informed consent was obtained from all participants. Serum samples from 20 subjects were randomly selected for analysis; half of the subjects had been vaccinated with CpG7909-containing vaccine and half had been vaccinated with the vaccine alone.

**Generation of stable cell lines expressing HEL chimeric protein.** NIH3T3 cells stably expressing HEL on their surface were generated by transfection with a construct encoding a protein chimera containing on its amino terminus the entire HEL protein (NCBI protein database accession code AAA48943.1), followed by a serine-tyrosine linker sequence and then by the third and fourth transmembrane and intracellular domains of rat CD4 (residues 206–457) (NCBI protein database accession code P05540.1). A codon optimized final construct was generated by Geneart Gene Synthesis (ThermoFisher) and was then subcloned into a pFB-Hygro vector<sup>3</sup>. Transduction of NIH3T3 cells using the Phoenix Eco system and selection of the stably transfected cells were done as previously described<sup>61</sup>.

**Bone marrow chimeras and immunizations.** To generate mice with B cell lineage–specific TLR9-deficiency, lethally irradiated 7- to 8-week-old C57BL/6 mice (CD45.1<sup>+</sup> haplotype) were reconstituted with 1 x 10<sup>7</sup> bone marrow cells, of which 90% were from µMT mice and 10% were from either MyD88-deficient mice or wild-type mice. Mice were allowed to reconstitute for at least 8 weeks before intraperitoneal immunization with both 100 µg NP<sub>e</sub>–CGG (Biosearch Technologies) adsorbed into 100 µl Alum (ThermoFisher) in PBS and 65 µg CpG B (ODN 1826) (Invivogen) in PBS.

**Signaling pathway analysis.** B cells isolated from mouse spleen were cultured in 96-well plates at 37°C with the appropriate stimulus. At each time point, cells were fixed with 4% PFA containing HBSS buffer for 10 min at 37°C, followed by permeabilization on ice using pre-chilled Perm Buffer III (BD Biosciences) and/or 10% bovine serum albumin in PBS for 10 min at 4°C. Cells were washed and cultured at 37 °C, and aliquots were taken with time and transferred to ice for RNA isolation. Following barcoding as described<sup>19</sup>, samples from the same time point, each having 1 x 10<sup>7</sup> bone marrow cells, were amplified using phosphorylated kinase–specific antibodies (identified above). For flow cytometry, each sample was gated based on the barcodes. For each phosphorylated kinase–specific antibody, the net mean fluorescence intensity (MFI) was calculated by subtraction of the MFI of the fluorescence-minus-one (FMO) control from the MFI of the actual samples. The change in the phosphorylation content (fold value) was determined as the ratio of the net MFI of a stimulated condition to the net MFI of the unstimulated sample at the same time point. Changes in calcium influx into the cell as a response to different stimulation conditions were analyzed using Fluo-4 and Fura red dyes (ThermoFisher) as explained<sup>66</sup>.
incubated on ice with pHrodo-HEL for 30 min, and then were washed and cultured.

BCR-bound HEL was internalized, the pH-sensitive fluorescent dye pHrodo was bound to lipoidal bilayers (PLBs) containing biotin (Avanti Polar Lipids). HEL was incorporated into the PLB by adding 10 μM HEL 488-labeled biotinylated HEL into PLB-containing chambers, incubating for 30 min at RT, and then washing to remove unbound HEL.

Plasma membrane sheets (PMSs) were prepared as described. In brief, 1.5×10^6 293A cells were seeded into a chamber of an eight-chamber Lab-Tek II chambered (1.5 mm thick coverglass chamber (Nunc) and were cultured overnight in complete medium containing Rhodamine-conjugated HEL (Nanocs) with or without CpG for increasing times. Cells were fixed with fixation buffer (4% PFA in PBS at 37 °C) for 10 min, permeabilized with PBS containing 0.5% saponin and 5% BSA at RT for 20 min, washed and incubated with PBS containing 10% normal rat serum and 0.5% saponin at RT for 1 h. Cells were stained with fluorescent mAb specific for CD71, LAMP1 and H2M (identified above) and were stained for early (CD71^hi) and late (LAMP1^hi) endosomes, as well as for the antigen-processing compartment (H2M^hi), using fluorescein-labeled monoclonal antibodies diluted in blocking buffer mounted on microscope slides using Pro-Long Gold Antifade Mountant with DAPI (Thermo Fisher). A Zeiss LSM780 microscope equipped with a 63× A Plan Apochromat 63× oil-immersion objective was used for image acquisition. Three-dimensional colocalization of rhodamine-HEL with the fluorescent antibodies was calculated using Imaris software.

Electron microscopy. For transmission electron microscopy (TEM) and electron tomography (ET), B cells were fixed by suspension in modified Karnovsky’s fixative prepared with 10 mM PFA (4%), 20 mM sodium phosphate buffer (0.2 M), 2 ml glutaraldehyde (50%) and 8 ml distilled water (Electron Microscopy Sciences). Samples were processed using microwave irradiation as previously described, with the following exceptions: between each step, cells were centrifuged for 5 min at 800× g. Fixed and dehydrated cells were infiltrated with Araldite resin (SPI). Sections for tomography were cut at a thickness of 200 nm and were imaged without post-section staining. Tomographic tilt series were collected on an Ultrascan 4000 camera (Gatan), using SerialEM acquisition and control software (University of Colorado, Boulder CO^m). Volumetric reconstructions were performed using Batchrunтомo script (University of Colorado). Particle counts and volumetric measurements were conducted using IMOD software (University of Colorado)^m. Volumetric models were rendered using Amira for Biosciences (FEI). ELISAs and ELISPOT. Concentrations of cytokines (IL-2, IL-6, IL-10 and TNF) and immunoglobulins (IgG and IgM) secreted by purified splenic B cells in culture were measured by analysis of the supernatants with relevant Ready-Set-GO! ELISA kits (ThermoFisher) according to the manufacturer’s guidelines.

The relative affinities of NP-specific antibodies were determined by the application of 100 μl diluted mouse serum (1:1,000 to 1:100,000) onto Nunc MaxiSorp 96-well flat-bottom plates (ThermoFisher) previously coated with 10 μg per well of either NP4-BSA (to capture high affinity antibodies) or NP30-BSA (to capture low affinity antibodies) and were stained with 1.0 mg/ml 3,3’-tetramethylbenzidine (TMB) substrate (ThermoFisher). The reaction was quenched using a stop agent purchased from Sigma-Aldrich. The OD at 450 nm was measured on a Spectramax plate reader (Molecular Devices). ELISPOT analysis of NP-specific antibody–secreting cells were carried out according to the protocol explained elsewhere, and plates were evaluated and imaged by Zellnet Consulting.

Quantitative RT-PCR (qPCR) and RNA-seq analyses. For qPCR analyses, RNA was isolated from mouse splenic B cells stimulated in vitro using RNAeasy mini or micro Kits (Qiagen) according to manufacturer’s guidelines. cDNA was generated using iScript Reverse Transcription Supermix (Bio-Rad). qPCR assays containing primers and Taqman probes were purchased from Integrated DNA Technologies, qPCR assays containing primers only were purchased from Eurofins MWG Operon. Sequences of these primers and probes are in Supplementary Table 1. qSYBR Green Supermix (Bioutronic) or Platinum Quantitative PCR SuperMix-UDG (ThermoFisher) were used for amplification of target genes. The change in gene expression (fold value; target vs control) was calculated as previously shown. For RNA-seq analysis, 250 μl of purified mouse B cells cultured in full medium for 18–48 h were mixed with 750 μl of Zymo lysis buffer (Zymo Research) and processed according to the manufacturer’s recommendations. Total RNA was prepared for next-generation sequencing using a TruSeq Stranded mRNA-Seq library preparation kit (Illumina). The quality and size of the final purified libraries were tested on Bioanalyzer DNA 1000 chips (Agilent Technologies). Libraries were quantified by qPCR using a Kapa Quantification Kit Illumina sequencing (Kapa Biosystems) and the Kapa Green Master Mix 2.0 (Kapa Biosystems) and were normalized to 2.0 μM stocks. These samples were pooled equally and were clustered on the HiSeq 2500 (Illumina) using 12 pM of template for a 2 × 100 bp rapid sequencing run. Raw next-generation
sequencing reads were first prepped by removal any illumina adaptor sequences using Cutadapv 1.12.16 and then were trimmed and filtered for quality, q = 18, and length, min length 35 bp using FastX Tool Kit v0.0.13 (Hannon Lab, Cold Spring Harbor Laboratory). Trimmed reads for each replicates were then mapped to Mus musculus mm10 genome using TopHat2 v2.1.17 set to report only matched pairs. Final transcripts based on the standardized replicates, and then differentials for each comparison were generated with the Cufflinks suite18 using pooled normalization for each experimental condition. Changes in the transcriptional regulation of various pathways were analyzed using Ingenuity Pathway Analysis software (Qiagen).

**Purification and surface plasmon resonance (BIACore) binding analysis of antibodies to ßAMA1.** Total serum IgG antibodies from ßAMA1-immunized subjects were purified by protein G affinity chromatography. In brief, 0.5 ml serum from each individual subject was loaded onto a 5 ml HiTrap Protein G column (GE healthcare) and was subjected to a standard elution with a glycine pH gradient. The eluted IgG proteins were pooled together and dialyzed against 10 mM HEPES (pH 7.5) and 0.15 M NaCl for binding studies.

Surface plasmon resonance measurements were performed using BIACore 3000 instrument and were analyzed with BIAevaluation 4.1 software (Biacore AB). To measure the affinity, ßAMA1 protein was immobilized on carboxylated dextran CM5 chips (Biacore AB) to 500–1,500 response units using a primary amine coupling and a flow rate of 10 µl/min in 10 mM sodium acetate (pH 4.5). The analyte consisted of serial dilutions of IgG antibodies between 16.0 nM and 75 nM in a buffer containing 10 mM HEPES (pH 7.4) and 0.15 M NaCl. The dissociation constants were obtained by kinetic curve fitting using BIAevaluation 4.1 (BIACore).

**Statistical analysis.** The statistical significance of differences between experimental groups was measured using GraphPad Prism software. The methods used for each experiment and the P value ranges were indicated in the relevant figure legends.

**Life Sciences Reporting Summary.** Further information on experimental design and reagents is available in the Life Sciences Reporting Summary.

**Data availability statement.** The data that support the findings of the study are available from the corresponding author upon request. RNA-seq data were uploaded to the SRA database under project number PRJNA422889.

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Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

1. Sample size
   Describe how sample size was determined. Based on earlier studies

2. Data exclusions
   Describe any data exclusions. No data was excluded

3. Replication
   Describe whether the experimental findings were reliably reproduced. Experiments were reliably reproduced

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups. For chimeric mouse experiments mice were randomly allocated to groups

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis. Our animal technician, who did the injections did not know the details of the experiment.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).
   - n/a Confirmed
   - The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including \textit{central tendency} (e.g. median, mean) and \textit{variation} (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

See the web collection on \textit{statistics for biologists} for further resources and guidance.

7. Software
   Describe the software used to analyze the data in this study. Statistical significance was analyzed in GraphPad Prism Versions 6 and 7.
Flow cytometry data was analyzed in Flowjo ver.10. Imaging data were analyzed in Imaris and ImageJ softwares.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

### Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

| There are no restrictions |

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

| Antibody specificity, commercial source, clone name and fluorescent dye conjugates of antibodies were clearly identified in the methods section. Antibodies were selected from clones that are regularly used for similar studies. |

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

| NIH3T3 (ATCC-obtained from a colleague), Phoenix Eco (ATCC) |

b. Describe the method of cell line authentication used.

| No authentication was made |

c. Report whether the cell lines were tested for mycoplasma contamination.

| Yes. They were free of mycoplasma |

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

| No misidentified cell lines were used. |

### Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

| Female mice 8-12 weeks were used for experiments except for bone marrow chimeric mice. C57BL/6 (JAX0664), MyD88 KO (JAX 09088), B10.BR (JAX 0465), MuMT (JAX002288), CD45.1+ WT mice (JAX 2014) were purchased from Jackson Laboratories. TLR9 KO mouse in BL/6 background was obtained from Dr. Shizuo Akira, 3A9 mouse was obtained from Dr. Igal Gery, MD4 mice were purchased from Taconic farms. Related breedings were carried out at NIAID animal facilities and genotypings were done according to previously published protocols. |

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

| Human participants were as detailed in the original manuscript as well as online in the following location: www.clinicaltrials.gov no. NCT00320658 |
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. Spleens were harvested, mashed and RBCs were lysed. Splenocytes were used either as is or after B cell purification and culturing. Details in the methods section.

6. Identify the instrument used for data collection. BD LSR-II

7. Describe the software used to collect and analyze the flow cytometry data. Flow JO version 10

8. Describe the abundance of the relevant cell populations within post-sort fractions. For experiments involving purified B cells, B cell purity was checked by staining the cells with live/dead CD19 and B220. Then gating for CD19+ B220+ cells among the singlet live population.

9. Describe the gating strategy used. FSC-A SSC-A was used to gate the splenocytes/B cells (depending on experiment) FSC-H/ FSC-A was used to gate the singlets. FSC-H/ Live Dead was used to gate the alive cells. Rest depends on the experimental set up. Gating strategy is exemplified in Figure 7 and also additional information was provided in the relevant methods, results and figure legends sections.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ✗