Antigen (Ag) targeting is a method to efficiently induce immune responses by delivering Ags directly to APCs such as DCs by coupling them to antibodies (Abs) specific for APC-restricted surface molecules (Caminschi et al., 2009; Caminschi and Shortman, 2012). Many Ag-targeting approaches have directed Ags to DC subsets via mAbs specific for C-type lectin receptors (Sancho and Reis e Sousa, 2012). This method of immunization reduces the amount of Ag required and directs the immune response toward certain effector cell functions. Depending on the cell surface receptor targeted by an mAb, a different kind of immune response may be induced. For example, delivery of Ag to Dectin-1 induces strong CD4+ T cell responses if administered with adjuvant (Carter et al., 2006); targeting to DEC205 induces strong CD8+ T cell responses with adjuvant (Dudziak et al., 2007); targeting to DCIR2 in the absence of adjuvants generates strong CD4+ T cell help and extrafollicular (EF) IgG1 Ab responses (Chappell et al., 2012); and targeting to Clec9A generates CD8+ T cell responses with adjuvant and efficiently activates Tfh follicular helper cells for Ab production without adjuvant (Lahoud et al., 2011).

In this study, we investigated the effect of targeting Ags to a receptor expressed on both DCs and B cells. We selected the 95-kD B cell–associated surface molecule CD180 (also called Bgp95 or RP105) as a target because (a) ligating CD180 with mAb triggers B cell activation and proliferation (Valentine et al., 1988; Miyake et al., 1994) and (b) CD180 is an orphan member of the TLR family most closely related to TLR4 (Miyake et al., 1995), an effective target for adjuvants (Alving et al., 2012). Although CD180, unlike TRL4 and other TLRs, lacks a cytoplasmic TIR domain, it initiates a BCR-like signaling cascade that does not use TLR signaling adaptors (Valentine et al., 1988; Miyake et al., 1994; Chan et al., 1998; Yazawa et al., 2003; Hebeis et al., 2004, 2005); (c) CD180 internalizes after ligation, suggesting that Ag-αCD180 might be processed by DCs and/or B cells and activate CD4+ T cell helper cells; and (d) we previously found that inoculation of mice with a high dose of CD180 Ag targeting also induced IgG Abs in BAFF-R KO mice lacking mature B cells and in mice deficient in interferon signaling. Targeting Ag to CD180 may be useful for therapeutic vaccination and for vaccinating the immune compromised.

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of anti-CD180 induces extremely rapid and robust polyclonal IgG production, even in the absence of CD40 signaling or T cells (Chaplin et al., 2011).

We evaluated whether Ag delivery to CD180 was able to induce Ag-specific IgG responses and found that mice inoculated i.v. with Ag-αCD180 rapidly produced Ag-specific IgG responses that were greater than mice immunized with Ag in alum. Remarkably, targeting Ags via CD180 in a single inoculation without adjuvant primed mice to mount secondary immune responses, even in CD40-deficient mice. The powerful adjuvant effect of Ag-αCD180 required B cells to express both an Ag-specific BCR and CD180. Thus, coupling Ags to anti-CD180 is an effective means for rapidly raising Ag-specific IgG responses that may find efficacy for both therapeutic and prophylactic vaccines.

RESULTS

Targeting Ag to CD180 rapidly induces strong Ag-specific IgG responses

Administration of a high dose of anti-CD180 mAb induced >15-fold increases in serum IgG through polyclonal Ig production both in WT mice and in CD40- and T cell–deficient mice (Chaplin et al., 2011). Given this B cell stimulatory effect and the fact that CD180 is internalized after ligation by mAbs (unpublished data), we examined whether Ag coupled to anti-CD180 could induce Ag-specific IgG responses in normal and immunodeficient mice. We first conjugated the hapten 4-hydroxy-3-nitro-phenacetyl (NP) to anti-CD180 (NP-αCD180) or to a nonbinding rat IgG2a isotype control (NP-isotype) mAb and administered them in graded doses i.v. to WT mice. Doses ranging from 10 to 100 µg NP-αCD180 induced significant NP-specific IgG responses in a dose-dependent manner (Fig. 1 A, bottom), with little or no polyclonal Ig production compared with unimmunized mice (pre-bleed for 100 µg NP-αCD180 group) or mice injected with 100 µg NP-isotype (Fig. 1 A, top). Anti-NP Abs were not observed in mice immunized with anti-CD180 mAb alone (Fig. 1 B); therefore, the Ag-specific Ab response to NP-αCD180 was caused by targeting of Ag rather than by a product of polyclonal Ig production. Strong Ag-specific Ab responses to NP-αCD180 were also induced when conjugates were inoculated i.p. (not depicted); in subsequent experiments, we inoculated mice via the i.v. route.

Targeting Ag to CD180 also induced Ag-specific IgM and IgG production in both CD40 KO mice and T cell–deficient (TCRβ/δ KO) mice (Fig. 1 B). Ag-specific IgM levels were similar in the WT and immunodeficient mice, but Ag-specific IgG levels were significantly lower in both CD40 KO and

Figure 1. Targeting to CD180 induces Ag-specific IgG production that is partially TI. (A) WT mice were inoculated i.v. with either 100 µg NP-isotype control mAb or graded doses of NP-αCD180 mAb (10, 30, or 100 µg); mice were pre-bled at days 0 (100 µg NP-αCD180 group only) or day 10, and total serum Ig (top) or anti-NP specific (bottom) IgM, IgG1, and IgG3 levels were determined by ELISA. (B) WT, CD40 KO, or TCR KO mice were inoculated with 100 µg NP-αCD180 mAb or NP-isotype mAb or αCD180, bled at day 10, and analyzed for NP-specific IgM, IgG1, and IgG3 responses. (C) WT or CD40 KO mice were treated as in B and bled at days 0 and 10, and sera were analyzed for levels of NP-specific Abs (IgM and IgG subclasses). Data are representative of three (A and C) or four experiments (B) using three mice/group and are presented as mean ± SEM. **, P < 0.01; and ***, P < 0.001.
CD180 targeting rapidly induces higher levels of Ag-specific IgG than Ag inoculated in alum

We next determined the kinetics of Ag-specific IgG production after NP-αCD180 inoculation. We immunized WT or CD40 KO mice i.v. with either NP-αCD180 or NP-isotype or i.p. with the NP-isotype Ag precipitated in alum. In WT mice, NP-αCD180 induced a rapid anti-NP IgG response that peaked 10 d post immunization (p.i.) as compared with Ag in alum, which peaked on day 21. Mice inoculated with NP-isotype alone did not produce >2 µg/ml anti-NP Ab at any time point (Fig. 2 A, left). As expected, CD40 KO mice immunized with Ag in alum did not make an NP-specific IgG response; however, they did develop a significant and continually increasing amount of NP-specific IgG after CD180 targeting (Fig. 2 A, right).

Targeting to CD180 induces anti-protein IgG responses and requires covalently linked Ag

We next determined whether the strong Ab response to NP-αCD180 was also induced when we targeted protein Ags to CD180. We coupled whole OVA to anti-CD180 (OVA-CD180) and isotype mAb (OVA-isotype) and immunized WT mice i.v. with one of these Ags or i.p. with OVA-isotype in alum (Fig. 2 B). As with NP-αCD180, OVA-αCD180 induced a strong Ag-specific IgG response with concentrations of nearly 2 mg/ml IgG anti-OVA at day 14 p.i.

Anti-CD180 alone can stimulate B cells and thus has the potential to convert B cells into efficient APCs so they could present Ag even if it were administered in an unlinked fashion. To test this possibility, we inoculated mice with two different Ags with only one Ag coupled to αCD180: NP-αCD180 + soluble OVA or OVA-αCD180 + soluble NP-isotype. As expected, mice inoculated with only NP-isotype in alum or OVA in alum produced IgG only against NP or OVA, respectively (Fig. 2 C). Mice inoculated with NP or OVA coupled to anti-CD180 together with soluble OVA or soluble NP-isotype only made Abs against the Ag coupled to anti-CD180 and not to the soluble, unlinked Ag. We conclude that during Ag targeting to CD180, only B cells specific for the Ag attached to anti-CD180 are driven to produce Ab.
Figure 3. CD180 targeting induces affinity maturation, EF responses, GC formation, and immunological memory. (A) Sera from WT (C57BL/6) or CD40 KO mice immunized with 100 µg NP-αCD180 or 10 µg NP-αDCIR2 were analyzed for affinity to NP on days 5 and 7 p.i. (B) WT mice were inoculated with 100 µg NP-αCD180 alone or with the indicated adjuvant (50 µg CpG-A, 50 µg CpG-B, 20 µg R848, or 4 µg LPS) and then bled at days 7 and 28; sera were analyzed for affinity against NP. Controls included mice inoculated with NP-iso, NP-iso + alum, or NP-αDCIR2. (C) WT mice were inoculated with 50 µg NP-αCD180 alone or with the indicated adjuvants as in B and bled at days 7, 14, 21 and 28; sera were analyzed for levels of NP-specific IgM (left) or IgG (right) Abs. Data are presented as mean ± SEM. A representative experiment of three experiments each for A–C is shown. (D–F) 2 × 10^5
affinity, whereas the other adjuvants did not (Fig. 3 B, right). Unlike in WT mice, the affinity of the IgG Abs induced in CD40 KO mice did not increase above the levels of the negative controls (Fig. 3 A).

To follow expansion and differentiation of Ag-specific B cells after immunization with NP-αCD180, we adoptively transferred splenocytes containing NP-specific B cells from Ly5.1+ B1-8hi mice (Shih et al., 2002) into Ly5.2+ WT hosts. Spleens were harvested at day 4 or 7 after inoculation with NP-αCD180 or NP-isotype control and analyzed by flow cytometry using sequential gating for B220+, Ly5.1+, and NP-APC binding. NP-isotype–treated mice showed no expansion of Ag-specific B220hi B cells, whereas NP-αCD180–treated mice showed an ~20-fold expansion at both time points (Fig. 3 D). This expansion included GL7+ PNA+ GC B cells, which increased in number by day 7 (Fig. 3 E). By day 7, the NP-αCD180–treated mice also had significant numbers of CD138+ Ab-forming cells (AFCs) in the spleen (Fig. 3 F). These results suggested that NP-αCD180 induces both EF Ab responses and GC formation. Indeed, by day 4, the spleens of NP-αCD180–treated mice had large numbers of Ag-specific B cells in EF sites, and by day 7, PNA+ GCs were evident (Fig. 3 G). In comparison, GCs induced by NP-αCD180 were generally smaller (Fig. 3 G) and present in fewer numbers (Fig. 3 H) than those induced by NP–chicken gamma globulin (CGG) plus alum.

The presence of GCs in NP-αCD180–treated mice suggested that the Ag-specific B cell expansion induced by Ag-αCD180 leads to the development of immunological memory, which is characterized by the ability to rapidly generate Ag-specific AFCs in response to soluble Ag rechallenge. To test this, we immunized groups of WT and CD40 KO mice with NP–conjugated mAbs as above or with NP–CGG in alum as a positive control. After 10 wk, mice were boosted with matched soluble Ag (NP–isotype or NP–CGG) or with PBS as a negative control. On day 4 after boost, spleens were harvested and the number of IgG-producing AFCs was assessed using an NP-specific IgG ELISPOT assay. As expected, WT mice primed with NP–CGG in alum produced significant numbers of IgG–producing AFCs when boosted with soluble Ag (Fig. 3 I, left). In contrast to mice primed with NP–isotype, NP–αCD180–primed mice contained low but detectable numbers of Ag-specific long-lived plasma cells in the spleen 10 wk p.i. (0.0 vs. 5.7 ± 0.8 SEM per 10^6 splenocytes). Levels of Ag-specific AFCs increased ~10-fold upon rechallenge with soluble Ag (56 ± 8.7 SEM per 10^6 splenocytes). The number of AFCs generated in mice primed with NP–αCD180 in the absence of adjuvant was roughly one third that of NP–CGG in alum primed mice. This result is in accord with smaller GC induction by NP–αCD180 compared with NP–CGG plus alum (Fig. 3, G and H). However, the spot size was three times as large as spots from mice primed with NP–CGG (not depicted), suggesting that the amount of NP-specific IgG produced per AFC was greater. Addition of the adjuvant R848 during the primary immunization with NP–αCD180 neither increased the spot number nor spot size upon Ag rechallenge compared with mice primed with NP–αCD180 alone.

Surprisingly, after rechallenge with soluble Ag, we also detected some NP-specific IgG-secreting AFCs in CD40 KO mice primed with NP–αCD180 (Fig. 3 I, right). Although the number of NP-specific IgG-secreting AFCs in CD40 KO mice was roughly 1/15 the number in WT mice, this number was significantly higher than in PBS–boosted CD40 KO mice or CD40 KO mice primed with Ag in alum. Thus, CD180 targeting effectively primes for immunological memory in WT mice, and to a lesser extent even in the absence of CD40.

**Ag-specific B cells are efficiently activated by linking BCR and CD180 stimuli**

The fact that specific Ag must be linked to anti-CD180 to induce IgG Ab responses (Fig. 2 C) suggested that both BCR and CD180 ligation on the same cell are required for specific Ab to be produced. To test this possibility, first we compared the activation of B cells after stimulation in vivo through the BCR, CD180, or through both receptors. We used B1-8hi mice that contain NP-specific B cells (Shih et al., 2002); groups of these mice were injected with either 100 μg NP–αCD180 or NP–isotype, and spleens were harvested 24 h later. The NP-specific B cells (6–10%) were distinguished from total CD19+ B cells by staining with NP–APC. Four groups of CD19+ B cells were then analyzed ex vivo for their expression of CD69, CD86, MHC class II, and the receptor, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI): unstimulated B cells (NP– B cells from NP–isotype–treated mice), BCR-stimulated B cells (NP– B cells from NP–isotype–treated mice), CD180-stimulated B cells (NP– B cells from NP–CD180–treated mice), and B cells stimulated through both the BCR and CD180 (NP– B cells from NP–αCD180–treated mice). Compared with unstimulated...
Figure 4. Ag-specific B cells are efficiently activated in vivo by combined signaling through the BCR and CD180. (A and B) Groups of B1-8<sup>hi</sup> mice (nine mice/group) were inoculated with either 100 µg NP-isotype or NP-αCD180, and spleens were harvested 24 h later (A and B) or 48 and 72 h later (not depicted). The NP-specific B cells (6–10%) were distinguished from total CD19<sup>+</sup> B cells by staining with NP-APC. Four groups of CD19<sup>+</sup> B cells were then analyzed ex vivo for their expression of CD69, CD86, MHC class II, and TACI: unstimulated B cells (NP<sup>−</sup> B cells from NP-isotype–treated mice; gray in A), BCR-stimulated B cells (NP<sup>+</sup> B cells from NP-isotype–treated mice), CD180-stimulated B cells (NP<sup>−</sup> B cells from NP-αCD180–treated mice), and B cells stimulated through both the BCR and CD180 (NP<sup>+</sup> B cells from NP-αCD180–treated mice). (A) Histograms show CD86 and TACI expression on B220<sup>+</sup> B cells stimulated as indicated 24 h p.i. (B) Mean fluorescent intensities (MFI) at 24 h p.i. are plotted for the indicated surface markers relative to unstimulated control B cells (value 1.0). Similar data were obtained at 48 and 72 h p.i. A representative experiment of three experiments is shown. Data are presented as mean ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.

B cells, B cells stimulated via either Ag or αCD180 had increased expression of CD86 and TACI (Fig. 4 A). However, over a series of experiments, the levels of CD69, CD86, and TACI were significantly higher on B cells stimulated through both the BCR and CD180 at 24 h (Fig. 4, A and B) and later time points (not depicted). Thus, the combination of BCR and CD180 signaling in vivo appears to be more effective at activating B cells than either signal alone.

B cell expression of CD180 is necessary and sufficient for Ag-αCD180–driven Ab responses

The data in Fig. 4 suggest that the powerful adjuvant effect of Ag-αCD180 may be mediated by the combination of BCR and CD180 signaling of B cells. However, because CD180 is expressed on both B cells and non-B cells, Ab responses induced by CD180 targeting may be mediated by either delivery of both Ag-mediated BCR signaling together with CD180 signals to Ag-specific B cells and/or CD180 delivery and signaling to non-B cells, which then in turn stimulate Ag-specific B and T cell responses. To distinguish these possibilities, we performed adoptive transfers to establish mice that express CD180 only on B cells, only on non-B cells, or on both B and non-B cells (Fig. 5 A). B cell–deficient μMT mice were inoculated with CD180 KO B cells to create mice in which CD180 was expressed only on non-B cells. These mice failed to make Ag-specific IgG after inoculation with NP-αCD180 (Fig. 5 A), demonstrating that CD180 expression on B cells is necessary to generate an Ab response after CD180 targeting. CD180 KO recipients, into whom purified CD180<sup>+</sup> B cells were transferred, expressed CD180 only on B cells and not on non-B cells. After immunization with NP-αCD180, these mice produced high levels of Ag-specific IgG. These data show that CD180 expression on B cells is sufficient for CD180–based targeting. B cell–deficient (μMT) mice into which CD180<sup>+</sup> B cells were transferred so that CD180 was expressed on both B cells and non-B cells made somewhat more NP-specific IgG than mice not expressing CD180 on non-B cells (Fig. 5 A). This suggests that CD180 expression on non-B cells such as DCs, although neither sufficient nor essential for Ag targeting, influences the extent of IgG production.

When anti-CD180 mAb is inoculated i.v. into mice, it binds to CD180<sup>+</sup> CD19<sup>+</sup> B cells and to other CD180<sup>+</sup> cells in the spleen, including CD11c<sup>+</sup> DCs and F4/80<sup>+</sup> macrophages, but not to CD3<sup>+</sup> T cells, which do not express CD180 (not depicted). To determine which APCs were most effective at priming T cells after targeting to CD180, WT mice were inoculated with either OVA-isotype or OVA-αCD180; 16 h later, B cells and DCs were purified by negative selection and co-cultured with CFSE-labeled OVA-specific OT-II CD4<sup>+</sup> or OT-I CD8<sup>+</sup> T cells. After 72 h, the levels of CFSE in the OVA-specific T cells were measured by flow cytometry (Fig. 5, B and C). OVA-αCD180–targeted B cells, unlike B cells from OVA-isotype–treated control mice, clearly induced proliferation of Ag-specific CD4<sup>+</sup> T cells. However, OVA-αCD180–targeted DCs were much more effective on a per cell basis at stimulating OT-II proliferation. OVA-αCD180–targeted B cells, unlike OVA-αCD180–targeted DCs, failed to induce any proliferation of OVA-specific OT-I CD8<sup>+</sup> T cells (Fig. 5 C), consistent with the poor cross-presentation of Ag by B cells compared with DCs. Thus, although DCs are not required for the Ag-specific Ab responses induced by Ag-αCD180, they may function to stimulate Ag-specific CD4<sup>+</sup> helper T cells required for optimal IgG production.

**IL4, IFN-α/β signaling, and mature B cells are not required for Ag targeting to CD180**

Type I IFN has been shown to act directly on B cells and promote Ab responses (Fink et al., 2006; Le Bon et al., 2006). Thus, we compared IgG responses of type 1 IFN–α/β receptor (IFN-α/βR) KO and WT mice after inoculating NP-αCD180; abrogating signaling through the IFN-α/βR, if anything, increased anti-NP IgG production, suggesting that type I IFNs may normally restrain Ab responses induced via CD180. Mice deficient in MHC class II (MHC II KO) after
immunization with NP-αCD180 had a more severe reduction in anti-NP IgG production than either CD40 or TCR KO mice (Fig. 6 A). The reason for this is not clear as B cell levels are normal in MHC II KO mice and MHC II B cells respond normally to TI Ags (Markowitz et al., 1993). Another TD B cell activator, αIgD, requires the action of IL-4 (Finkelman et al., 1986), so we compared Ab responses of WT, IL4 KO, and OX40L KO mice inoculated with NP-αCD180. After CD180 targeting, both IL-4 KO and OX40L KO mice produced anti-NP IgG at levels similar to WT controls (Fig. 6, A and B), demonstrating that the IL-4–Th2 pathway is not required during CD180 targeting.

To assess a possible role for the cytokine BAFF in CD180 targeting, we immunized BAFF-R KO mice, which have a near complete block in mature B cell development (Sasaki et al., 2004). To our surprise, BAFF-R KO mice produced normal levels of NP-specific IgG Abs after targeting to CD180 (Fig. 6 A). This suggests that although CD180+ B cells are required for Ag-αCD180 targeting, mature B cells may not be necessary. Indeed, we observed significant increases in both FO (B220+CD23loCD21hi) and transitional 1 (T1)/T2 (B220+CD23hiCD21loCD93+) B cells on days 1 and 3 p.i. in NP-αCD180– compared with NP-isotype–injected animals (Fig. 6 C). In contrast, B cells with an MZ phenotype (B220+CD23loCD21hiCD93−) showed a marked decrease after NP-αCD180 administration. These results demonstrate that NP-αCD180 expands some but not all splenic B cell subsets. Because BAFF can bind to TACI and BCMA as well as BAFF-R (Rickert et al., 2011), it remains possible that signaling through TACI or BCMA contributes to Ag-αCD180–driven IgG responses.

DISCUSSION
Collectively, our data indicate that targeting Ags to CD180 induces rapid activation of Ag-specific B cells, leading to significant IgG production within 7 d. Remarkably, a single injection of Ag-αCD180 without any additional adjuvant also led to the development of both Ab affinity maturation and immunological memory (Fig. 3). Furthermore, although severely impaired, Ag-specific IgG production and responses to secondary immunizations were retained in CD40 KO mice (Fig. 3 H), even though CD40 KO mice did not make Ag-specific IgG or develop memory Ab-producing cells in response to Ag in alum, as reported previously (Kawabe et al., 1994). The Ab responses induced required the Ags to be attached to anti–CD180 and could be induced to both haptons and protein Ags.

Why is this mode of immunization so effective in rapidly raising IgG Ab responses? Previous studies showed that i.p. inoculation of high doses of αCD180 could induce increases in plasma cells (500 µg) and polyclonal Ig production (250 µg; Nagai et al., 2005; Chaplin et al., 2011). When not coupled to Ags, free αCD180 mAbs have either no effect or reduce Ag-specific Ab responses, and this only occurs when inoculated at very high doses (Chaplin et al., 2011). Rather, several lines of evidence suggest that it is the combination of simultaneous signaling of Ag-αCD180 through both the BCR and CD180 on B cells that promotes the rapid Ab responses. First, effective induction of IgG by Ag-αCD180 required CD180 to be expressed on B cells and not on other cells. Second, although Ab responses to linked Ag occurred with both NP-αCD180 and OVA-αCD180, there was little or no response to soluble Ags co-administered at the same time. Third, B cells activated in vivo by stimulating the Ag receptor and CD180...
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(Kawamura and Berzofsky, 1986; Denis et al., 1993). However, Ab responses induced by Ag–anti-Ig were weaker than those induced by targeting Ag to the pan-APC marker MHC II (Berg et al., 1994), whereas other B cell targets tested, B220 and FcγRII, proved ineffective at generating Ab responses (Snider and Segal, 1989). The higher efficacy of Ag delivery to DCs has led to the majority of Ag-targeting approaches being focused on targeting myeloid cell subsets (Caminschi and Shortman, 2012; Sancho and Reis e Sousa, 2012). It is worth noting that the B cell surface molecules chosen in prior studies were (a) not known to signal (B220), (b) inhibitory receptors (FcγRIIb), or (c) BCR components (IgD), such that targeting Ag to them was unlikely to produce additional stimulation beyond what Ag already provided.

together expressed higher levels of activation markers than B cells triggered by either stimulus alone (Fig. 4). Indeed, the greater induction of CD86 expression after co-ligation of the BCR and CD180 may well be a critical feature of targeting Ag to CD180, as CD86 is necessary for IgG responses to nonadjuvanted Ag (Borriello et al., 1997). The TACI receptor was also induced to higher levels after CD180 targeting, and TACI plays a role in class switching and IgG production (He et al., 2010). Further studies are required to define how delivering the CD180 stimulus together with Ag produces a substantially different activation signal than Ag and anti-CD180 in combination.

Early Ag targeting approaches used anti-Ig mAb to deliver Ag to B cells and speed expansion of Ag-specific CD4+ T cells (Kawamura and Berzofsky, 1986; Denis et al., 1993). However, Ab responses induced by Ag–anti-Ig were weaker than those induced by targeting Ag to the pan-APC marker MHC II (Berg et al., 1994), whereas other B cell targets tested, B220 and FcyRII, proved ineffective at generating Ab responses (Snider and Segal, 1989). The higher efficacy of Ag delivery to DCs has led to the majority of Ag-targeting approaches being focused on targeting myeloid cell subsets (Caminschi and Shortman, 2012; Sancho and Reis e Sousa, 2012). It is worth noting that the B cell surface molecules chosen in prior studies were (a) not known to signal (B220), (b) inhibitory receptors (FcγRIIb), or (c) BCR components (IgD), such that targeting Ag to them was unlikely to produce additional stimulation beyond what Ag already provided.

Figure 6. MHC class II is required for Ag targeting to CD180 but not BAFF-R, IFN-α/βR, IFN-γR, or OX40L. (A and B) WT C57BL/6 mice and the indicated KO mice (A) or BALB/c mice (B) were inoculated with 100 µg NP-αCD180 or NP-isotype and bled at day 10; levels of NP-specific IgG Abs were determined by ELISA. Data are representative of two experiments for both A and B using three mice/group and are presented as mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001 as determined by one-way ANOVA with Bonferroni post tests by comparing with WT controls. (C) Groups of WT mice were immunized with 100 µg NP-αCD180 or NP-isotype and sacrificed on days 1 and 3 p.i. for analysis of splenic B cell subsets. Flow cytometry plots show gating strategy used to enumerate FO (B220+CD23hiCD21int), MZ (B220+CD23loCD21hiCD93−) and T1/T2 transitional (B220+CD23loCD21hiCD93+) B cells (day 1 group shown). Bar graphs depict total number of cells in the spleen (mean ± SEM) of each subset. Number on graphs indicate fold increase in cell subsets from NP-αCD180–immunized mice compared with isotype controls. Data are from one experiment using three to four mice/group/time point. *, p < 0.05; **, p < 0.01; ***, p < 0.001 as determined by one-way ANOVA with Bonferroni post tests.
Ag targeting to CD180, while requiring B cells, does not appear to require mature B cells: BAFF-R KO mice mainly have T1 B cells; they have a fivefold reduction in T2 B cells and are almost completely deficient of mature follicular and marginal zone B cells (Sasaki et al., 2004). Nevertheless, inoculation of Ag-αCD180 into BAFF-R KO mice produced as much Ag-specific IgG as in WT mice. This suggests that T1 B cells are a major target for Ag-anti-CD180. Although T1 B cells readily apoptosis after BCR stimulation alone, they do not die when signaled via BCR and a second signal (Kövesdi et al., 2004); T1 B cells also constitutively express activation-induced deaminase (AID; Han et al., 2007; Ueda et al., 2007; Kuraoka et al., 2009) and can rapidly produce large quantities of IgG and undergo somatic mutation when triggered with a combination of BCR and TLR stimuli (Mao et al., 2004; Han et al., 2007; Ueda et al., 2007; Capolunghi et al., 2008; Aranburu et al., 2010; Kuraoka et al., 2011). Furthermore, inoculation of unconjugated anti-CD180 dramatically increases numbers of transitional B cells more so than mature B cells (Chaplin et al., 2011). Thus, AID+ T1 B cells signaled through both the BCR and CD180 may rapidly switch and mature into IgG-producing plasma cells. Further studies are in progress to define the B cell subsets and signaling pathways responsible for the rapid IgG response.

Our results indicate that Ag-αCD180 targeting generates long-lived plasma cells and switched memory B cells in both WT and CD40 KO mice. First, the t1/2 of Ag-specific IgG in immunized WT mice was ~38 d (based on the kinetics in Fig. 2 A), whereas catabolism of a discrete burst of IgG from a short-lived AFC response would have a t1/2 of 21 d. Additionally, Ag-specific IgG levels in CD40 KO mice continue to rise over time. Both of these results require continual IgG production to slow or offset the constant elimination IgG, implying that some Ab-producing cells are retained. Second, Ag-specific GL7+ PNA+ GC B cells were evident by day 7 p.i. with NP-αCD180. This GC phenotype suggests memory B cell precursors were being generated. Third, both WT and CD40 KO mice had significantly more AFCs after Ag boost than mice primed with Ag-isotype or mice not boosted (Fig. 3 B). Many studies have implicated CD40 signals in the induction of memory B cells by TD or TI-2 Ag (Kaji et al., 2011). MATERIALS AND METHODS

**Mice.** C57BL/6, CD40 KO, OT-I OVA-specific CD8+ TCR transgenic, OT-II OVA-specific CD4+ TCR transgenic, B cell-deficient (μMT), and T cell-deficient (TCRB-6 KO) mice were purchased from the Jackson Laboratory. All strains were on the C57BL/6 background unless otherwise noted. CD180 KO, MHC II KO, and IFN-α/βR KO mice were gifts from S. Skerrett, P. Fink, and K. Murali-Krishna, respectively (University of Washington, Seattle, WA). OX40L KO mice were gifts from S. Skerrett, P. Fink, and K. Murali-Krishna, respectively (University of Washington, Seattle, WA). OX40L KO mice were a gift from A.H. Sharpe (Harvard University, Cambridge, MA). BAFF-R KO mice were a gift from K. Rajewsky (Harvard Medical School, Boston, MA). B6.SJL-B1-8KO mice were a gift from M. Nussenzweig (Rockefeller University, New York, NY). IL-4 KO mice on the BALB/c background were a gift from S. Ziegler (Benaroya Research Institute, Seattle, WA), and WT control BALB/c mice were purchased from the Jackson Laboratory. All mice were sex and age matched and used at 6–10 wk of age. The University of Washington Institutional Animal Care and Use Committee approved all animal work.

**Cell preparation and adoptive transfers.** Total splenocytes were processed by mechanical disruption and erythrocytes were depleted by Gey’s lys. For adoptive transfer experiments in Fig. 3, splenocytes from B1-8* IgH transgenic mice were labeled with PE-conjugated NP and anti-B220-FITC to determine the frequency of Ag-specific B cells by flow cytometry. Total splenocytes by mAb (unpublished data), it was likely that CD180 targeting could deliver Ag both to Ag-specific B cells as well as to DCs that don’t bind Ag. Indeed, this was the case: DCs targeted in vivo via αCD180 were more efficient than targeted B cells in stimulating CD4 T cell proliferation. Although Ab responses induced by αCD180 only required CD180 expression on B cells, it appears that DC-mediated T cell priming helped promote a greater response to CD180 targeting in WT mice than if Ag were solely directed to B cells (Fig. 5 A).

Anti-CD180 activates human B cells in vitro to enter cell cycle, express activation markers and produce IL-6 (Clark et al., 1989; Clark and Shu, 1990), suggesting Ag-αCD180 may stimulate human B cells to produce Ab. Further studies are in progress to assess how simultaneous signaling of the BCR and CD180 affects human B cells. However, the combined Ag targeting/adjuvant method described here has the potential to find utility in human vaccines. Most vaccines do not induce protective immunity in all individuals, and most vaccines do not induce lasting immunity. Furthermore, vaccination of immunocompromised individuals requires special considerations and approaches (Rappuoli et al., 2011; Miller and Rathore, 2012). Targeting to CD180 induces IgG responses and some immunological memory even in CD40 KO mice and, remarkably, induces high levels of IgG Abs even in mature B cell–deficient BAFF-R KO mice and IFN signaling–deficient IFN-α/βR KO mice. Thus, a CD180-based vaccine platform may find utility for immunizing immunocompromised people, including the elderly. In addition, most vaccine strategies require more than one injection to produce sufficient circulating levels of protective Abs. Single dose vaccines provide several advantages (Bowick and McAuley, 2011; Levine, 2011), and one injection of Ag attached to anti-CD180 induces a rapid and strong IgG response. Thus, a single inoculation of a CD180-based vaccine could produce protective humoral immunity and be a particularly attractive approach for therapeutic vaccination shortly after an exposure to a pathogen.
containing 2 × 10^6 NP-binding B cells were transferred i.v. to individual B6 recipients 24 h before immunization. For experiments in Fig. 5A, splenic B cells from WT or CD180-deficient mice were isolated by three rounds of negative selection enrichment (STEMCELL Technologies). 10 × 10^6 purified B cells of appropriate genotype were transferred i.v. to recipients as indicated 24 h before immunization. For experiments in Fig. 5 (B and C), CD4 and CD8 T cells from OT-II or OT-I TCR transgenic mice, respectively, and DCs or B cells from immunized C57BL/6 mice were isolated by three rounds of negative selection enrichment using the appropriate kit (STEMCELL Technologies). Purities for all cell enrichments were ≥99% as determined by flow cytometry for CD19 (B cells), CD4 or CD8 (T cells), or CD11c (DCs). Frequencies of OT-I and OT-II T cells were determined within the CD3^+ T cell population by staining for Vα2 and used to determine final numbers for cell culture.

In vitro CFSE proliferation assay. 5 × 10^5 B cells or DCs from immunized mice were enriched as described above and co-cultured with titrating numbers of CFSE-labeled Vα2^+ OT-I or OT-II T cells in 96-well round-bottom plates for 3 d at 37°C, 5% CO2, as previously described (Chaplin et al., 2011). CFSE (fluorescein) labeling was performed as previously described (Chaplin et al., 2011).

ELISA and ELISPOT assays. For ELISA assays, polystyrene plates were coated with either 2 µg/ml anti–mouse IgG (H+L; Jackson Immunoresearch Laboratories, Inc.) for total IgG, 20 µg/ml NiP-BSA (Biosearch Technologies) for NP-specific Ab, or 20 µg/ml OVA (Sigma-Aldrich). Affinity determinations were performed as described previously (Herzenberg et al., 1980; Chappell et al., 2012), using custom NiP2- and NiP2-BSA prepared by conjugation to the succinimidyl ester of NiP (Biosearch Technologies) according to manufacturer’s instructions. Detection and analyses were performed as previously described (Chaplin et al., 2011). ELISPOT assays were performed as previously described (Goms et al., 2010). Spot number and size were quantified using a CTL-ImmuNoSpot S5 Core Analyzer ELISPOT reader with ImmunoSpot Academic version 5.0 software (Cellular Technology Ltd.).

Flow cytometry. Flow cytometry analyses were performed on a FACS-Canto (BD). A minimum of 30,000 cells of the final gated population was used for all analyses. Data analyses were performed with FlowJo (Tree Star) software. Stainings were performed for: CD3, CD80, and CD95 (BD mAbs used for all analyses. Data analyses were performed with FlowJo (Tree Star) software. Stainings were performed for: CD3, CD80, and CD95 (BD mAbs used for all analyses. Data analyses were performed with FlowJo (Tree Star) software.}

Other Abs and reagents. The anti-CD180 (RP/14) hybridoma was a gift from K. Miyake (University of Tokyo, Tokyo, Japan), and the rat IgG2a isotype control (9D6) hybridoma was a gift from R. Miller (Emory University, Atlanta, GA). To ensure equivalence, these mAbs were sequentially purified on the same protein G column and tested for endotoxin by LAL gel-clot assays in GlucaShield from K. Miyake (University of Tokyo, Tokyo, Japan), and the rat IgG2a isotype control (9D6) hybridoma was a gift from R. Miller (Emory University, Atlanta, GA). To ensure equivalence, these mAbs were sequentially purified on the same protein G column and tested for endotoxin by LAL gel-clot assays in GlucaShield from K. Miyake (University of Tokyo, Tokyo, Japan).

Inmunohistochemistry. 8-µM frozen spleen sections obtained from mice immunized 4 or 7 d previously with 100 µg NP-αCD180, NP-isotype, or NP-CGG plus alum were stained with anti-B220-eFluor450 (eBioscience), PNA-FITC (Vector Laboratories), and NP-PE as previously described (Chappell et al., 2011). Immunofluorescence labeling was performed as previously described (Chaplin et al., 2011).

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