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B7-1/2 (CD80/CD86) Direct Signaling to B Cells Enhances IgG Secretion

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B cell responses are regulated by Ag recognition, costimulatory signals provided by interaction with helper T cells, and by innate signals. We recently provided evidence for a link between the effects of innate and costimulatory signals on B cells during influenza virus infection, by demonstrating that most B cells in the regional lymph nodes of the respiratory tract enhance surface expression of the costimulator B7-2 (CD86) within 24–48 h following infection via a type I IFN-dependent mechanism. A finding we are confirming here. While the role of B7-1/2 for helper T cell activation is well-documented, its role in direct B cell regulation is poorly understood. Here, our in vivo studies with mixed bone marrow irradiation chimeric mice, lacking B7-1/2 only on B cells, demonstrated that B7-1/2 expression is crucial for induction of maximal local, but to a lesser extent systemic, IgG Ab responses following influenza virus infection. In contrast to mice that completely lack B7-1/2 expression, loss of B7-1/2 on B cells alone did not significantly affect germinal center formation or the extent of CD4+ T cell activation and IFN-γ secretion. Instead, our in vitro studies identify a dramatic effect of B7-2 engagement on IgG, but not IgM secretion by already class-switched B cells. Concomitantly, B7-2 engagement induced expression of X-box binding protein 1 (XBP-1) and spliced XBP1, evidence for increased protein synthesis by these cells. Taken together, these results identify direct signaling through B7-1/2 as a potent regulator of IgG secretion by previously activated B cells. The Journal of Immunology, 2009, 183: 7661–7671.

Complex interactions among cells presenting and recognizing Ags are involved in the initiation and regulation of adaptive immune responses (1). T cell-dependent B cell responses require reciprocal interactions between T and B cells that are dependent on engagement of appropriate BCR complexes, costimulatory molecules, and innate signals (1–4). Among the most important costimulatory molecules are those involving the B7 family members B7-1 (CD80) and B7-2 (CD86) (3, 5, 6). These receptors are expressed on APCs (dendritic cells, macrophages, and B cells) and are rapidly up-regulated by inflammatory as well as Ag-specific signals for enhanced interaction with CD28 or CTLA-4 expressed on T cells (3). Whereas costimulatory molecules appear to be required for full B cell activation, the presence of additional “third” signals, that is, innate signals such as TLR agonists (7–9) and/or cytokines such as type I IFN (10–13), seem to control and regulate the magnitude and quality of the specific B cell responses.

We provided evidence for a linkage between the effects of innate and costimulatory signals on B cells during influenza virus infection by demonstrating that most B cells in the regional mediastinal lymph nodes (MedLN) of the respiratory tract enhance surface expression of the costimulator B7-2 within 24–48 h following infection. At that time B7-2 induction is dependent entirely on direct type I IFN-mediated signals to B cells (10, 11). This widespread IFN-driven B7-2 up-regulation is thus one of the first responses of B cells at the local site of infection during early influenza virus infection.

Direct type I IFN-mediated B cell activation significantly affects the quality and magnitude of the antiviral humoral response (10–13). As we and others showed previously, mice deficient in type I IFN or lacking the IFN only on B cells showed reduced virus-specific IgM, IgA, and IgG responses as well as alterations in the isotype profile of those responses that did develop. Specifically, type I IFN affected the isotype profile of the response with a shift in the ratio of IgG2a/IgG1 caused by reduced secretion of IgG2a and enhanced secretion of IgG1 (11).

Studies by others have provided solid evidence that B7/CD28-mediated signaling regulates B cell responses. The blockade of CD28-B7-1/2 interactions using CTLA-4-Ig treatment causes a reduction in overall antiviral Ab production following influenza virus infection (14). Mice deficient in CD28 or in both B7-1 and B7-2 (B7-1/2−/−) lack germinal center formation and induce only limited Ig class-switch recombination, memory formation, and affinity maturation through somatic hypermutation following protein immunization (15–17). B7 costimulation was also shown to influence IgG production in vivo. Following immunization via various routes, Ag-specific IgG1 and IgG2a responses are strongly reduced in B7-1/2−/− gene-targeted mice (15). Given that B7/CD28 signaling is crucial for T cell activation (3, 5, 18), it is important to assess which of the defects in the humoral response are due to a loss of B7/CD28 interaction required for the activation of T cells.
and which are due to the direct loss of B7-1/2 signaling for B cells. This is a focus of the study presented herein.

Recent studies provide evidence that B7 signaling can directly enhance B cell immune responses (3). Ligation of B7-2 on human tonsillar B cells resulted in a modest increase in IgE and IgG4 (14), and mouse TNP-specific B cells increased secretion of IgE and IgG1 following B7-2 ligation in vitro (19). Furthermore, signaling through B7-2 on LPS-stimulated mouse B cells enhanced proliferation and production of IgG1 and IgG2a (20). Studies by Sanders and colleagues suggest that B7-2, in conjunction with β2-adrenergic receptors, transduces positive signals to B cells that increase IgG1 and IgE production (21–24). Taken together, these emerging data indicate that signals through B7-2 bidirectionally affect both T and B cells during T-dependent B cell responses, and that both types of effects could shape the magnitude and quality of B cell responses.

Given the importance of the costimulatory B7 molecules for B cell response regulation and the paucity of in vivo evidence for B7 as providing direct signaling to B cells, we sought here to evaluate the role of B7-1/2 on the regulation of the virus-specific humoral response. Using bone marrow irradiation chimeras in which only B cells lack B7-1/2, we demonstrate direct effects of these molecules on B cells in vivo for the regulation of local virus-specific Ab responses. The effects are directed primarily at the class-switched IgG response without affecting IgM levels or germinal center formation. Our in vitro data support these findings by demonstrating a dramatic effect of B7-2 engagement on inducing/enhancing IgG secretion by already isotype-switched B cells.

Materials and Methods

**Mice**

Female wild-type and Igh-6-deficient BALB/c mice (Igh-a, B7-1/2−/−) were purchased from The Jackson Laboratory. All experimental animals were kept under conventional housing conditions in filter top cages and used at 8–12 wk of age. Age- and sex-matched BALB/c mice deficient in type I IFN receptor (provided by Dr. J. Durbin, University of Ohio) or deficient in both CD80 and CD86 (B7-1/2−/−) (15) were bred and maintained in the mouse barrier facility at the University of California, Davis. B7-1/2−/− breeders were provided by A. Abbas (University of California, San Francisco) with permission from A. Sharpe (Harvard University). All experiments were conducted in accordance with protocols approved by the University of California, Davis, Animal Use and Care Committee.

**Virus**

Virus were infected intranasally under isofluoran anesthesia with a subthalal dose of influenza virus A/PR8 (H1N1, 20 PFU) in 40 μl of PBS per mouse. Virus was propagated in embryonated hen eggs and infectious titers were established as outlined previously (25).

**Serum collection**

Blood samples were collected directly into serum separator tubes (Microtainer; BD Biosciences), and serum was isolated by centrifugation and stored at −20°C until analysis for Ab levels was conducted using ELISA.

**Chimeric mice**

To generate mixed bone marrow irradiation chimeras, BALB/c (B7-1/2−/−) recipient mice received a lethal dose of gamma irradiation (650 rad or 800 rad whole-body irradiation). Twelve to 24 h later they were reconstituted with 2 × 10^6 mixed bone marrow cells. Bone marrow cell mixtures consisted of cells from B cell-deficient (Igh6−/−) BALB/c mice (The Jackson Laboratory) and either wild-type BALB/c (B7-1/2−/−) or BALB/c mice deficient in both CD80 and CD86 (B7-1/2−/−) at different ratios. Chimeras were provided with acidified drinking water for at least 6 wk after irradiation and kept in filter top cages. Six weeks after bone marrow transfer, blood was taken from mice by tail vein, and reconstitution was verified by FACS analysis with Abs to CD19, CD4, and CD8.

**Magnetic cell separation**

B cell isolation was done by magnetic cell separation using an autoMACS (Miltenyi Biotec) according to previous published protocols (10). Purity were >90%. For cell culture and gene expression analysis a variant protocol resulting in higher purities was developed. For that, spleen cell suspensions were stained with a cocktail of biotinylated Abs to GR-1 (RB6-8C5), F4/80 Ag (F4/80) (in-house generated), Th1.2, CD49b (DX-5; both eBioscience), and anti-biotin MACS beads (Miltenyi Biotec). In some experiments anti-IgD biotin (11–26) was added to deplete naive B cells. Purity was >95% as assessed by staining with anti-CD19 (ID3) and for IgD depletion with anti-CD19, anti-IgD, and anti-IgM (33; all in-house generated).

**Tissue cultures**

Induction of CD69 and B7-2 in vitro was determined by culturing 2.5 × 10^6 MACS-purified B cells/ml, with 200 U/ml IFN-β (R&D Systems) and/or 20 μg/ml F(ab')2 goat anti-mouse IgM (Jackson ImmunoResearch Laboratories) in medium (RPMI 1640, 292 μg/ml l-glutamine, 100 μg/ml penicillin/streptomycin, 10% heat-inactivated FCS, 0.03 M 2-ME) for 16 h at 37°C in 5% CO2 before staining for FACS. Similar analysis was conducted to assess B cell expansion on B cells from PBMC of irradiation chimeras, for which cells were stimulated at 1 × 10^7 cells/ml with 20 μg/ml goat anti-mouse IgM F(ab')2, in medium.

To test the effects of B7-2 engagement on Ab secretion and gene expression, MACS-purified total or IgD B cells were stimulated with anti-B7-2 at 10 μg/ml in the presence or absence of IL-4 (15 ng/ml) or IFN-γ (100 ng/ml), IL-5 (2 ng/ml) (all eBioscience) and in some experiments CD40L (0.1 μg/ml; ProSpec) for 24–96 h in 5% CO2. For B7-2 knockout cellular staining of T cell-derived IFN-γ, single-cell suspensions of MedLN were added to anti-CD3-coated tissue culture plates (10 μg/ml mAb clone 145-2C11), and cultured in the presence of monensin (5 μM), for 12 h at 37°C.

**Quantitative RT-PCR analysis**

Real-time RT-PCR was set up to measure expression levels of the following genes: activation-induced cytidine deaminase (AID), X-box-binding protein 1 (XBP-1; ready-by-design assay; Applied Biosystems), and spliced transcripts for IgG1 (forward, 5′-GGCGGGTCTGCTGAG-3′; reverse, 5′-CTATAAGGGAAAGGGAATGCA-3′; probe, 5′-ACAGGCTCTCGT TCCGTGTT; probe, 5′-CACCTCCTCTGCGACAAAGGTCTG TGTGT-3′; reverse, 5′-GGCTAATCTTTGTCGGTGTATGGA-3′; reverse, 5′-6FAM-CACCTCCTCGATGCTGAG-3′; forward, 5′-TATTTCCACAGAAGAAAGGAGACATACAAAG-3′; GAPDH (forward, 5′-GTTGTCGTCGTGATGCTGTA-3′; reverse, 5′-CCTGCTT GACCACCATCTTCTGTATG-3′; probe, 5′-6FAM-TCCTGCTTGGGACAAACTGTGCA-3′) as well as sterile transcripts for IgG1 (for-ward, 5′-CTTGTTT; probe, 5′-6FAM-CACCTCCTCTGCGACAAAGGTCTG TGG-3′; reverse, 5′-GGCCGGGTCTGCTGAG-3′), and spliced transcripts for IgG1 (forward, 5′-GTTGTCGTCGTGATGCTGTA-3′; reverse, 5′-CCTGCTT GACCACCATCTTCTGTATG-3′; probe, 5′-6FAM-TCCTGCTTGGGACAAACTGTGCA-3′) was used as a housekeeping gene to control for RNA input. Total RNA was isolated from B cells after in vitro culture using RNAeasy kit (Qiagen) and cDNA was synthesized with random hexamers and SuperScript II (Invitrogen) following the manufacturer’s instructions. ABI Prism 7700 (Applied Biosystems) was used for amplification, data acquisition, and data analysis. Amplification conditions for use with the Clontech polymerase were: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 s, and 60°C, 1 min. Relative expression levels were calculated following data normalization to GAPDH.

**B cell proliferation assay**

B cell proliferation was assessed using MTT assay. For that, purified B cells were stimulated in quadruplicate wells with or without 200U IFN-β for 16 h followed by washing and incubation with and without 10 μg/ml anti-IgM (Fab′)2 and 1 or 10 μg/ml anti-B-7.2. Every 12 h for 72 h following culture onset B cell expansion was assessed by MTT assay using the cell proliferation kit I (Roche Diagnostics) according to the manufacturer’s instructions. Abundance (595 nm) was measured on a Spectramax M5 reader (Molecular Devices) using a 650-nm reference wavelength.

**FACS staining and analysis**

Single-cell suspensions from spleen and MedLN were prepared as previously described (11, 25). Erythrocytes were lysed with ammonium chloride lysis buffer. PBMC were isolated from heparinized peripheral blood by Ficoll-Hypaque (Amersham Pharmacia) density centrifugation. Live cell counts were obtained by trypan blue exclusion using a hemocytometer. All staining was performed at 2.5 × 10^6 cells/ml in “staining medium” (buffered saline solution: 0.168 M NaCl, 0.168 M KCl, 0.112 M CaCl2, 0.168 M MgSO4, 0.168 M KH2PO4, 0.112 M K2HPO4, 0.336 M HEPES, 0.336 M
M NaOH, containing 3.5% heat-inactivated, filtered newborn calf serum, 1 mM EDTA, 0.02% sodium azide) for 20 min on ice. Dead cells were identified using propidium iodide added at 1 μg/mL immediately before cell analysis. Cells were first incubated with Fc-receptor block (mAb rified B cells from IFNR BC-mediated signals acted independently to induce B7-2, as pu-
ther by BCR stimulation using anti-IgM cross-linking. IFNR- and
quired on a FACSCalibur or a FACSAria instrument (BD Biosciences), the latter of which was equipped with three lasers as described previously (26), and analyzed using FlowJo software (Tree Star).

**ELISPOT and ELISA**

For ELISPOT analysis, MedLN and spleen cells from four to six mice were pooled and 2-fold serially diluted in triplicate into ELISPOT plates (Mul-
biotin, CD8a-Cy5.5PE, CD24-Cy7PE, CD4-allophycocyanin, CD44-allophycocyanin, CD3-Alexa 750-allophycocyanin (BD Biosciences), CD19-Cy5.5-allophy-
cocyanin (Caltag Laboratories), and B7-1PE (eBioscience). Data were ac-
countable spot numbers and calculated as mean numbers ± SD of live B cells present as determined by FACS analysis. Virus-specific total IgG, IgM, IgG2a, and IgG1 serum titers were determined by ELISA on samples from individual mice, as described (27). Serum concentrations of virus-
specific Ig were calculated by comparison to a standard A/PR8 HA-specific IgG Ab (H57-41–7; gift of W. Gerhard, The Wistar Institute).

**Statistical analysis**

Student’s t tests (unpaired, two-tailed) were conducted to determine the level of significance of the data from FACS, ELISA, and ELISPOT. For correlation of B7-2 expression levels and germinal center formation, a

**Results**

**Induction of B7-2 but not B7-1 on most draining lymph node B cells following influenza virus infection**

Our previous studies showed the strong type I IFN-mediated induction of CD69 and B7-2 (CD86) on the cell surface of virtually all B cells in the respiratory tract draining MedLN, but not the spleen, within 24–48 h of infection (11). We confirm the strong up-regulation of both surface molecules on B cells from MedLN, but not spleen, of influenza virus-infected wild-type mice (Fig. 1A) and show that B7-1 (CD80) expression was induced only slightly at that time. As expected, infected and noninfected B7.1/2−/− mice lacked any measurable B cell expression of B7.1 and B7.2. However, they did show up-regulation of CD69, thereby demonstrating a similar level of IFN-mediated B cell activation in the regional lymph nodes of these different strains of influenza-in-

To determine what effects BCR cross-linking (i.e., Ag encounter) may have on the expression of B7-2 on already IFN-stimulated B cells, we conducted in vitro stimulation assays with purified B cells from wild-type and IFN−/− mice. As shown in Fig. 1B, IFN-β-induced increases in B7-2 expression were enhanced further by BCR stimulation using anti-IgM cross-linking. IFN-β and BCR-mediated signals acted independently to induce B7-2, as pu-
ified B cells from IFN−/− mice induced B7-2 expression following anti-IgM stimulation, but not following stimulation with IFN-β. Anti-IgM stimulation, but not stimulation via IFN-β, moder-
etly increased expression of B7-1 (data not shown). As ex-
picted, none of these stimuli induced measurable B7-2 up-regulation on B7-1/2 double gene-deficient B cells (Fig. 1B). Thus, the data suggest that early during influenza virus infection direct type

I IFN-mediated signals act in synergy with BCR-mediated signals to enhance expression of B7-2 on regional B cells above levels achieved by BCR stimulation alone. Since IFN-induced up-regulation of B7-2 is restricted to the site of infection (11), effects of B7-2 stimulation may differ depending on the tissue location.

**Strong reduction in influenza virus-specific Ab responses in the absence of B7-1/2**

Given the well-known importance of B7 family members as co-
stimulatory molecules for T-dependent B cell responses (3, 5, 18)
and the changes in expression induced during early influenza virus infection (Fig. 1 and Refs. 10, 11), we studied the role of B7-1/2 for the influenza virus-specific humoral responses. Since B7-1 and B7-2 share the same ligands, we did this by comparing virus-specific humoral responses in double gene-deficient B7-1/2−/− mice with those of congenic BALB/c wild-type mice. Frequencies of virus-specific IgA, IgG, and IgM Ab-secreting cells were significantly reduced in the regional lymph nodes of B7-1/2−/− mice compared with wild-type mice during the 12-day measuring period (Fig. 2B). We conclude that B7-1/2 expression is necessary for the induction of maximal influenza virus-specific local and systemic humoral responses of all isotypes.

Lack of B7-1/2 expression by B cells results in significant reduction in the virus-specific IgG response to influenza

To determine the extent to which the effects of B7-1/2 on the regulation of the humoral response are due to B cell-direct effects, as opposed to effects of B7-1/2 on cells other than B cells, we generated mixed bone marrow irradiation chimera mice and their respective control mice. Two types of chimeric mice were generated, one in which only B cells were deficient in B7-1/2, and another type in which all bone marrow-derived cells were deficient in B7-1/2. The absence of B7-1/2 on B cells alone caused significant reductions in the influenza virus infection-induced IgG1 and IgG2a responses in both MedLN (Fig. 3A, left panel) and spleen (Fig. 3A, right panel).
right panel) at day 10 of infection. In contrast, frequencies of IgM-secreting B cells in MedLN of these mice were not consistently affected, showing slight enhanced frequencies in one but not the other experiment conducted (Fig. 3 and data not shown). In the spleen, however, virus-specific IgM-secreting B cells were slightly higher in mice that lacked B7-1/2 on B cells compared with the control chimeras. Again, frequencies of B cells in MedLN were comparable between the groups (average of 45% CD19+B cells). Overall, the data show that lack of B7-1/2 only on B cells recapitulates the reduction in virus-specific IgG seen in mice that completely lack these molecules, albeit to a lower degree, whereas the effects on IgM secretion seem largely due to effects of B7-1/2 on cells other than B cells.

Comparisons of the virus-specific serum Ab response in these chimeras were consistent with this finding, as total virus-specific IgG but not IgM titers were reduced in mice that lacked B7-1/2 expression only on B cells 10 days after infection (Fig. 3B). Consistent with the more pronounced reduction of IgG2a compared with IgG1-secreting cells in MedLN and spleen, virus-specific IgG2a titers were significantly reduced in these mice, while serum titers of virus-specific IgG1 appeared largely unaffected at that time point (Fig. 3B). Using similar B71/2-/- mice on a C57BL/6 background others had shown an opposite effect of B7-1/2 on IgG1 and IgG2a titers following influenza infection (14), with stronger effects of global B7-1/2 expression on IgG1 instead of IgG2a. The reasons for these differences are unclear but they might be related to the genetic background of the animals. Collectively, these data indicate that the effects of B7-1/2 direct signaling to B cells are mainly on IgG secretion.

Germinal center formation is independent from B7-1/2 expression by B cells

Previous studies by others (15) had demonstrated a nonredundant role for B7-1/2 signaling in germinal center formation after protein immunization. Therefore, we next tested whether the observed reduction in virus-specific Ab responses in B7-1/2-/- (Fig. 2A) and B cell-only B7-1/2-/- mice (Fig. 3) following influenza virus infection correlated with a lack of germinal center formation. Consistent with this earlier report (15) we observed a strong reduction of germinal center B cells (CD3, CD4, CD8, F4/80-propidium iodide- and CD19+B220high) in MedLN of influenza virus-infected B7-1/2-/- compared with wild-type mice (Fig. 4A). Note, however, that despite this considerable reduction in germinal center B cells (3.3 ± 1.7 in B7-1/2-/- vs 34.4 ± 2.3 in BALB/c mice; p < 0.0001, Fig. 4B), small numbers of germinal center B cells were nonetheless present. In contrast to the strong effects of global B7-1/2 expression, lack of B7-1/2 on B cells only did not significantly (p = 0.25) affect frequencies of germinal center B cell at day 10 after infection (9.98 ± 2.1 vs 14.1 ± 2.1, Fig. 4B).

B7-1/2 expression levels on B cells do not correlate with GC B cell frequencies in MedLN

We noted the relative large variation in the frequency of germinal center B cells in the MedLN of chimeras lacking B7-1/2 only on B cells (Fig. 4B) and sought to determine to what extent this might be due to differences in the number of host-derived “contaminating” B7-expressing B cells in the chimeras (due to incomplete removal of host B cells following irradiation and reconstitution). Thus, we sought to determine whether any effects of B7 expression by B cells on germinal center formation might be masked in these chimeras due to incomplete removal of B7-expressing host B cells. For that, we determined the frequencies of B7-2-expressing B cells in the chimeras by stimulating PBMC from individual mice overnight with anti-IgM F(ab')2 before FACS analysis. Frequencies determined for B7-2-expressing B cells in B7-1/2-/- (0%) and BALB/c (80–86%) mice served as negative and positive controls, respectively (see Fig. 5A). In chimeras reconstituted with B7-1/2-/-
bone marrow, frequencies of B7-2-expressing cells ranged between 0.5 and 32% (Fig. 5A). We then stratified the chimeras according to their frequencies of B7-2-expressing B cells and compared the frequencies of germinal center B cells in a group of mice with low (4–8%) or high (30–32%) frequencies of B7-2 B cells. Results showed no significant correlation between B7-2 expression levels on B cells in the blood and germinal center B cell frequencies in MedLN of individual mice (Fig. 5B). Furthermore, while the highest frequencies of germinal centers appeared to be present in chimeras with B cells expressing relatively high levels of B7-2 per cell, there was not significant correlation between the levels of B7-2 expression per B cells and the frequency of germinal center B cells. B7-2 expression was measured as mean fluorescent intensity B7-2 of total CD19 MedLN B cells (Fig. 5C, left panel) and of CD24^high^CD38^low^ germinal center B cells (Fig. 5C, right panel).

To further confirm that there is no requirement for B cell B7 expression to establish germinal centers, we generated a set of chimeras in which host-cell contamination was at only 1% (Fig. 5D) using increased doses of whole-body irradiation. Analysis of these mice confirmed that while IgG2a responses were significantly reduced in mice lacking B7 only on B cells following influenza virus infection, there was no effect on germinal center formation (Fig. 5D).
CD80/86<sup>−/−</sup> mice were inconclusive, as these mice showed differences in their levels of reconstitution as analyzed by FACS (data not shown). This could explain the apparent discrepancies of our findings to those by Lumsden et al. (44), who found no difference in Ab secretion by B cells from CD80/CD86<sup>−/−</sup> vs wild-type cells in bone marrow chimeras following immunization. We conclude that while B7-1/2 expression on hematopoietic cells is important for germinal center formation, their expression by B cells is largely dispensable and is not likely responsible for the reduction in the IgG responses in mice lacking B7-1/2 only on B cells.

Expression of B7-1/2 by B cells is not required for CD4 T cell activation

B7-1 and B7-2 are important costimulatory molecules for T cell priming (3, 5, 18). Lack of B7-1/2 on all APCs reduces the frequency of activated T cells and thus may indirectly affect the influenza-specific humoral response. We aimed to determine whether the observed reduced virus-specific humoral response to influenza virus infection in B7-1/2<sup>−/−</sup> mice and in chimeras lacking B7-1/2 only on B cells is due to reduced availability of T cell help, and thus whether B cells contribute via B7-1/2 to Th response induction or maintenance. For that, we compared frequencies of activated CD4<sup>+</sup> T cells in MedLN of day 7 influenza virus-infected mice by studying CD11a<sup>high</sup>/CD44<sup>high</sup> expression (28). As expected, B7-1/2<sup>−/−</sup> mice had similar total frequencies of CD4 T cells (Fig. 6A) but fewer were activated in MedLN compared with wild-type controls (Fig. 6B). However, B7-1/2 expression by B cells does not appear to be required for full CD4<sup>+</sup> T cell activation in the regional lymph nodes of influenza-infected mice, as overall CD4<sup>+</sup> T cell numbers and frequencies of activated CD4<sup>+</sup> T cells were similar in the reconstituted chimeras either expressing or lacking expression of B7-1/2 on B cells (Fig. 6B).

Lack of B7-1/2 on B cells does not affect IFN-γ secretion in CD4<sup>+</sup> T cells

IgG2a production by B cells is strongly affected by IFN-γ secretion. To determine whether the strong reduction in IgG2a noted in both B7-1/2<sup>−/−</sup> and B cell chimeras was due to reduced IFN-γ production, we measured its production by MedLN T cells on day 7 after infection with influenza (a time point at which germinal centers begin to appear, data not shown). The results clearly show that similar frequencies of CD4<sup>+</sup> T cells of chimeras reconstituted with B cells from wild-type and B7-1/2<sup>−/−</sup> mice secreted IFN-γ (Fig. 6C). Thus, development of conventional CD4 T cell help, including the development of IFN-γ-secreting CD4 T cells, seemed unaffected by a lack of B7-1/2 on B cells. These data are consistent with a study measuring IL-4<sup>+</sup> T cells following reconstitution of RAG-deficient mice with wild-type and CD86<sup>−/−</sup> B cells.
B7-2 engagement induces IgG secretion by committed B cells

To elucidate potential mechanisms for the B7-mediated direct effects on B cells, we conducted a number of in vitro experiments. First, we determined whether stimulation of purified B cells with anti-CD86 in the absence or presence of anti-IgM and IFN-β affected B cell proliferation, that is, clonal expansion of Ag-specific B cells. MTT proliferation assays with MACS-purified splenic B cells showed that stimulation via anti-B7-2 did not induce measurable proliferation (Fig. 7). This did not change with addition of IFN-β, which we show (Fig. 1) enhances CD86 expression. Furthermore, B7-2 stimulation did not cause a significant enhancement of B cell proliferation above that induced by BCR stimulation with anti-IgM \( F(ab')_2 \) in the absence or presence of IFN-β. In fact, at earlier time points anti-B7-2 seemed to slightly inhibit proliferation. Similar results were obtained by CFSE labeling (data not shown). Additional experiments were conducted in which cells were pretreated for 16 h with IFN-β, washed, and then stimulated with anti-B7-2 with/without anti-IgM to exclude the possibility that IFN-β might have inhibitory effects on B cell proliferation, thereby masking a potential positive effect of anti-B7 on proliferation. Again, we saw no evidence for enhanced proliferation by B7-2 signaling (data not shown).

Next we studied the effects of anti-B7-2 on Ab secretion. For that, MACS-purified total B cells were stimulated during a 4-day period with/without anti-B7-2 in the presence of CD40L, IFN-γ, and IL-5. Cultures were set up in duplicate for each time point, and IgG2a concentrations in supernatants were determined by ELISA every 24 h. Dramatic differences in IgG secretion were noted in these cultures (Fig. 8A). Cultures that had received anti-B7-2 mAb contained strong levels of IgG2a, whereas none of the cultures without anti-B7-2 showed any Ab production (threshold of detection 0.016 ng/ml). Maximal differences were already noted at 24 h after culture onset, and no further increases in IgG2a secretion were observed after that time (Fig. 8A). Very little IgM was present in the culture supernatants and there was no difference between cultures containing or not containing anti-B7-2 (data not shown). Consistent with the results from the proliferation studies (Fig. 7), cell recovery was similar between these cultures.

The results suggested that anti-B7-2 was acting by enhancing IgG production by already committed, class-switched B cells. To test this, cells from these cultures were harvested and analyzed by quantitative RT-PCR for sterile transcript induction of IgG2a as well as expression of AID, an enzyme required for class-switch recombination (29). The results showed that B7-2 stimulation did not affect sterile IgG2a transcript levels and did not consistently enhance AID expression (Fig. 8B). Similar results were obtained when we stimulated the cells with IL-4 and IL-5 alone in the absence of CD40L and indeed even with anti-CD86 stimulation alone and measured sterile transcripts for IgG1 (data not shown). Furthermore, anti-B7-2 stimulation of total B cells and B cells depleted of naive IgD+ cells showed a further enhancement of IgG1 and IgG2a secretion (Fig. 8C), also indicating that anti-B7-2 stimulation induces IgG-committed B cells to secrete Abs. Consistent with the strong B7-mediated increase in IgG production, anti-B7-2 stimulation strongly increased expression of both XBP-1 and spliced XBP-1 (Fig. 8D), transcription factors regulating the unfolded protein response of cells actively secreting Abs (30).

Collectively, the results of this study identify direct B7 signaling to B cells as necessary stimuli for the induction of maximal influenza virus-specific IgG responses. The in vitro studies suggest that B7-direct stimulation acts by inducing maximal IgG secretion by previously activated B cells.

Discussion

This study provides evidence for a nonredundant role of B7-1/2-mediated direct signaling to B cells in the regulation of the virus-specific IgG response following influenza virus infection. We provide a potential mechanism for these observed effects of B7 on Ab production by identifying B7-direct signaling as an extremely potent inducer of IgG secretion by previously activated B cells. We previously showed that influenza virus-induced type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11). Taken together with the present study, the data suggest that type I IFN induces B7-2 expression by regional B cell populations and is required for maximal antiviral Ig production (11).
stimulation of human tonsil B cells activated in vitro with cytokines and anti-CD40 resulted in significant increases in IgE and IgG4 production. In that study, however, increased Ab production was accompanied by increased B cell proliferation in addition to increases in IgG transcript levels.

Our studies are overall consistent with the findings from studies by Sanders et al. by providing evidence that direct B7-2 engagement on B cells will strongly enhance IgG production in vivo. In vitro, the B7-2-induced increase in Ab production was not accompanied by induction of germline IgG1 and IgG2a transcripts or a strong induction of AID (Fig. 8 and data not shown). It also did not affect B cell proliferation (Fig. 7). Instead, it induced the differentiation of already isotype-switched B cells to strong Ab-secreting cells (Fig. 8A). This conclusion was further supported by data showing that enrichment for IgD<sup>-</sup> previously activated B cells increased Ab production following B7 engagement compared with cultures of total B cells (Fig. 8C), and that B7 signaling caused the concomitant up-regulation of the transcription factor XBP-1 and its spliced variant (Fig. 8D), transcription factors that are indispensable for B cell differentiation to Ab-secreting cells (34) by promoting the expansion of the endoplasmic reticulum, an increase in mitochondrial mass and total organelle content, and increased protein synthesis (35).

The in vivo studies supported a direct regulatory role of B7-1/2 for B cell Ab response regulation, as the lack of B7-1/2 on B cells alone reduces overall IgG Ab responses to influenza virus infection (Fig. 3). Consistent with a study by Lumsden et al., where B7-1/2 signaling was blocked with soluble CTLA-4 Ig treatment (14), we show that the complete lack of B7-1/2 dramatically reduced local and systemic Ab production of all isotypes (Fig. 2), and reduced germinal center formation (Figs. 4 and 5), likely due to a strong reduction in CD4 T cell activation (Fig. 6). We found little evidence, however, for a role of B cell-expressed B7-1/2 for either germinal center responses or T cell activation (Figs. 4 – 6). Instead, B cell-expressed B7-1/2 affected Ab production in vivo, as in their absence IgG Ab responses to influenza virus infection were reduced (Fig. 3), and anti-B7-2 strongly enhanced Ab production in vitro either alone or on the presence of cytokines (Fig. 8 and data not shown).

The expression of B7-1/2 on B cells seemed largely dispensable for T cell activation (Fig. 6), including for the activation and development of follicular helper T cells (F. C. Rau and N. Baumgarten, unpublished observations), resulting in germinal center B cell responses that are not significantly different from those of control chimeras (Figs. 4 and 5). We had shown previously that the type I IFN-induced activation of B cells and the up-regulation of B7-2 are not sufficient for mature, naive B cells to prime naive T cells (10), and others concluded that induction of B7-2 on immature B cells was not sufficient to activate T cells (36). A similarly redundant role for helper T cell activation via B cell expression of another otherwise important costimulatory molecule, CD40, was reported by Crawford et al. (37). Taken together, these data are in support of the view (3, 38) that engagement of individual costimulatory molecules during T-B interaction might not be required for...
certain effector functions of CD4 T cells after they are activated and primed by dendritic cells. The data further suggest that a major role of B7-CD28 engagement during B-T interaction is to drive B cell differentiation.

The herein demonstrated predominant role for B7 signaling on previously activated/class-switched B cells is consistent with the fact that memory B cells express higher levels of B7-2 compared with naïve B cells (39), and thus that previously activated cells are in a position to respond to these signals. In the context of influenza virus infection, the type I IFN-induced nonspecific induction of B7-2 on regional lymph node B cells early after infection (Fig. 1) might thus induce or enhance Ig secretion by reactivated memory B cells that are recruited into the draining lymph nodes via further up-regulation of B7-2. Additionally, B7 signaling might facilitate the rapid differentiation of activated virus-specific B cells to Ab-secreting cells following a primary infection. Indeed, a rapid accumulation of Ab-secreting cells in the regional lymph nodes following primary influenza virus infection is a hallmark of the B cell response to live influenza virus (11, 48).

This additional role for B7 signaling in B cell activation provides a further impetus for, and should be taken into account when, interpreting the results of ongoing preclinical and clinical studies aimed at developing therapies that block B7-1/2/CD28 or -CTLA4 interaction (reviewed in Ref. 40). In light of our findings, such therapies might be of particular value for the modulation of autoimmune diseases that are being linked to B cell activation defects, such as systemic lupus erythematosus. Note that systemic lupus erythematosus has also been linked to dysregulation of type I IFN signaling (41, 42).

To summarize, the importance of B7-1/2 costimulation for the induction of both humoral and cellular immune responses has been amply documented in various infectious model systems (14, 43–47). Our data add to these reports by demonstrating a distinct and direct role of B cell-expressed B7-1/2 for the regulation of the isotype-switched humoral response to influenza virus via the induction of Ab secretion by previously activated and class-switched B cells.

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Disclosures

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References


