Published in final edited form as:
J Immunol. 2009 December 15; 183(12): 7661–7671. doi:10.4049/jimmunol.0803783.

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

Friederike C. Rau[†,§], **Jacquelyn Dieter**[†], **Zhang Luo**[†], **Stephen O. Priest**[†,Ω], and **Nicole Baumgarth**[†,Ω,¶,∫]

[†] Center for Comparative Medicine, University of California, Davis, Davis, CA 95616

[Ω] Graduate Group in Immunology, University of California, Davis, Davis, CA 95616

[¶] Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, Davis, CA 95616

Abstract

B cell responses are regulated by antigen-recognition, co-stimulatory 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 co-stimulatory 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 co-stimulator B7-2 (CD86) within 24–48h following infection via a type I IFNR-dependent mechanisms, 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 antibody 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 XBP-1 and sXBP1, evidence for increased protein synthesis by these cells. Together, these results identify direct signaling through B7-1/2 as a potent regulator of IgG secretion by previously activated B cells.

Introduction

Complex interactions among cells presenting and recognizing antigens 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

*This work was supported by a grant from the NIH/NIAID (AI051354 to N.B.). F.C.R. was supported in part by the Deutsche Forschungsgemeinschaft (RA1373). Abbreviations: AID, activation-induced deaminase; XBP-1, X-box binding protein 1; MedLN, mediastinal lymph nodes.

∫Correspondence: Nicole Baumgarth, DVM, PhD, Center for Comparative Medicine, University of California, Davis, County Road 98 and Hutchison Drive, Davis, CA95616. nbaumgarth@ucdavis.edu, Phone: 530-754 5813, FAX: 530–752 7914.
§**Current address:** Small Animal Clinic, Surgery, Justus-Liebig-Universitaet Giessen, Germany

appropriate B cell receptor complexes, costimulatory molecules and innate signals (1–4). Among the most important co-stimulatory molecules are those involving the B7 family members B7-1 (CD80) and B7-2 (CD86) (3,5,6). These receptors are expressed on antigen presenting cells (DC, macrophages and B cells) and are rapidly up-regulated by inflammatory as well as antigen-specific signals for enhanced interaction with CD28 or CTLA-4 expressed on T cells (3). Whereas co-stimulatory molecules appear to be required for full B cell activation, the presence of additional "third" signals, i.e. innate signals such as Toll-like Receptor (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 24–48h following infection. At that time B7-2 induction is dependent entirely on direct type I IFNR-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 IFNR or lacking the IFNR 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 CTLA4-Ig treatment causes a reduction in overall antiviral antibody 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 co-stimulation was also shown to influence IgG production in vivo. Following immunization via various routes, antigen-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 here.

Recent studies provide evidence that B7-signaling can directly enhance B cell immune responses (3). Ligation of B7-2 on human tonsillar 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 beta2 adrenergic receptors, transduces positive signals to B cells that increase IgG1 and IgE production (21–24). Together, these emerging data indicate that signals through B7-2 bi-directionally 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 co-stimulatory B-7 molecules for B cell response regulation and the paucity of in vivo evidence for B-7 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 antibody 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.

Material and Methods

Mice

Female wildtype and Igh-6 deficient BALB/c mice (Igh-a, B7-1/2^{+}) were purchased from Jackson Laboratories. All experimental animals were kept under conventional housing conditions in filtertop cages and used at 8–12 weeks of age. Age and sex-matched BALB/c mice deficient in type I IFN receptor (kindly provided by Dr. Joan 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 kindly 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

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

Serum collection

Blood samples were collected directly into serum separator tubes (Microtainer, BD Bioscience), and serum was isolated by centrifugation and stored at −20°C until analysis for antibody 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 twenty-four hours later they were reconstituted with 2×10^6 mixed bone marrow cells. Bone marrow cell mixes 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 weeks 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 antibodies to CD19, CD4, and CD8.

Magnetic cell separation

B cell isolation was done by magnetic cell separation using an *auto*-MACS (Milteny Biotec) according to previous published protocols (10). Purities 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 antibodies to GR-1 (RB6-8C5), F4/80 antigen (F4/80) (in-house generated), Thy1.2, CD49b (DX-5, both e-Bioscience) and anti-biotin MACS-beads (Milteny Biotec). In some experiments anti-IgD biotin (11–26) was added to deplete naïve 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 (331, 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 200U/ml IFNβ (R&D Systems) and/or 20μg/ml $(Fab)_2$ goat anti-mouse IgM (Jackson Immuno Research Laboratories) in medium (RPMI 1640, 292ug/ml L-Glutamine, 100ug/ml Penicillin/Streptomycin, 10% heat inactivated fetal calf serum, 0.03M 2-ME) for 16h at 37°C in 5% CO_2 prior to staining for FACS. Similar analysis was conducted to assess B7-2 expression on B cells from PBMC of irradiation chimeras, for which cells were stimulated at 1×10^7 cells/ml with 20μg/ml goat anti-mouse IgM $(Fab)_2$ in medium.

To test the effects of B7-2 engagement on antibody secretion and gene expression, MACS-purified total or IgD- B cells were stimulated with anti-B7-2 at 10μg/ml in the presence or absence of IL4 (15ng/ml) or IFN-γ (100ng/ml), IL5 (2ng/ml) (all e-biosciences) and in some experiments CD40L (0.1μg/ml, Prospec) for 24h–96h in 5% CO_2 at 37°C. For intracellular staining of T cell-derived IFN-γ, single cell suspensions of MedLN were added to anti-CD3 coated tissue culture plates (10μg/ml mAb clone 145-2C11), and cultured in the presence of monensin (5 μM), for 12h at 37°C.

Quantitative RT-PCR analysis

Real-time RT-PCR was set up to measure expression levels of following genes: Activation-induced cytidine deaminase (AID), X-box binding protein 1 (XBP-1; ready-by-design assay; Applied Biosystems, Foster City CA); and spliced XBP-1 (forward: 5′-GGCCGGGTCTGCTGAG-3′; reverse: 5′-CTGAAGAGGCAACAGTGTCAGAGT-3′; probe: 5′-6FAM-CGCAGCAGGTGCAGGCCCA-3′) as well as sterile transcripts for IgG1 (forward: 5′-CATATGATGGAAAGAGGGTAGCA; reverse:5′-CAGCCGTCTCTGTTCCTGTTT; probe:5′-6FAM-CACCTCTCTGGGACAAAGGCTGTGACTC and IgG2a (forward:5′-CTACCTGCAGCCTGGGATCA-3′; reverse: 5′ GCTTACTCTGGTTGTCTGTATGTGACA; probe:5′-6FAM-TTCCCACACACAGAAGAACGGAACACTAAAG-3′). GAPDH (forward: 5′-TGTGTCCGTCGTGGATCTGA-3′; reverse: 5′-CCTGCTTCACCACCTTCTTGAT-3′; probe: 5′-CCGCCTGGAGAAACCTGCCAAGTATG-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, Valencia CA) and cDNA was synthesized with random hexamers (Promega, San Luis Obispo CA) and Superscript II (Invitrogen Life Technologies, Carlsbad CA) following the manufacturer's instructions. ABI Prism 7700 (Applied Biosystems, Foster City CA) was used for amplification, data acquisition and data analysis. Amplification conditions for use with the Clontech polymerase were: 50C°, 2 min; 95C°,10 min; (40 cycles): 95C° 15sec, 60C°, 1min. 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 16h followed by washing and incubation with and without 10μg/ml anti-IgM $(Fab)_2$ and 1 or 10μg/ml anti-B-7.2. Every 12h for 72h following culture onset B cell expansion was assessed by MTT assay using the cell proliferation kit I (Roche Diagnostics, Indianapolis, IN) according to the manufacturers instructions. Absorbance (595nm) was measured on a Spectramax M5 reader (Molecular Devices) using a 650nm 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 2.5×10^7 cells/ml in "staining medium" (Buffered saline solution: 0.168M NaCl, 0.168M KCl, 0.112M $CaCl_2$, 0.168M $MsSO_4$, 0.168M KH_2PO_4, 0.112M K_2HPO_4, 0.336M HEPES, 0.336M NaOH, containing 3.5% heat inactivated, filtered newborn calf serum, 1mM EDTA, 0.02% sodium azide) for 20 minutes on ice. Dead cells were identified using propidium iodide added at 1μg/ml immediately prior to cell analysis. Cells were first incubated with Fc-receptor block (mAb 2.4.G2 at 10μg/ml). PBMC were then stained with in-house generated antibodies against CD4 (FITC), CD3 (Cy5PE), CD19(allophycocyanin) and B7-2(PE; eBiosciences) to evaluate B7-2 expression on B and T cells. The following antibody conjugates were in-house generated, unless otherwise indicated, and used at previously determined optimal concentrations for FACS analysis: B220-Pacific Blue, CD38-FITC, CD138-PE, CD3− CD19− and CD11b-Cy5PE, CD8-Alexa610-PE (CALTAG), CD3− CD4− CD8− and F4/80-biotin, CD8a− and CD24-Cy5.5PE, CD11a-Cy7PE, CD4− and CD44-allophycocyanin, CD3-Alexa750-allophycocyanin (BD Biosciences), CD19-Cy5.5-allophycocyanin (CALTAG) and B7-1PE (eBiosciences). Data were acquired on a FACSCalibur or a FACSAria instrument (BD Biosciences), the latter equipped with three lasers as described previously (26), and analyzed using FlowJo software (Tree Star Inc).

ELISPOT and ELISA

For ELISPOT analysis MedLN and spleen cells from 4–6 mice were pooled and 2-fold serially diluted in triplicate into ELISPOT plates (MultiScreen HA Filtration; Millipore). Analysis for determination of virus-specific IgG and isotype-and virus-specific B cell secretion frequencies were performed exactly as described previously (25). Spots were counted from all wells containing 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 antibody (H37-41-7; kind gift of Walter Gerhard, The Wistar Institute).

Statistical Analysis

Student's *t* tests (unpaired, two-tailed) were carried out 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 linear regression analysis was performed. All analyses were done with help of the Prism4 software (GraphPad Software Inc.) and data were regarded as statistically significant at $p < 0.05$.

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 24–48h 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 wildtype mice (Fig. 1A) and show that B7-1 (CD80) expression was induced only slightly at that time. As expected, infected and non-infected 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-infected mice (Fig. 1A).

To determine what effects BCR cross-linking, i.e. antigen-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 wildtype and IFNR$^{-/-}$ mice. As shown in Figure 1B, IFNβ-induced increases in B7-2 expression were enhanced further by BCR stimulation using anti-IgM cross-linking. IFNR and BCR-mediated signals acted independently to induce B7-2, as purified B cells from IFNR−/− mice induced B7-2 expression following anti-IgM stimulation, but not following stimulation with IFNβ. Anti-IgM stimulation, but not stimulation via IFNβ, moderately increased expression of B7-1 (data not shown). As expected 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 upregulation 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 antibody 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 (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 that of congenic BALB/c wildtype mice. Frequencies of virus-specific IgA, IgG and IgM antibody-secreting cells were significantly reduced in the regional lymph nodes of B7-1/2$^{-/-}$ mice compared to BALB/c controls on day 12 after infection as determined by ELISPOT (Fig. 2A left panel). Among IgG, IgG1 and IgG2a subtypes were affected, consistent with earlier reports on mice treated with CTLA-4-Ig prior to influenza virus infection (14). The reductions in the frequencies of antibody-secreting cells in B7-1/2$^{-/-}$ mice were not due to differences in the frequencies of B cells accumulating in the regional lymph nodes, as they were comparable in B7-1/2$^{-/-}$ and BALB/c mice (25 ± 4.2% and 26 ± 1.6% CD19+ B cells, respectively).

Similar strong reductions in the frequencies of virus-specific antibody-secreting cells of all measured Ig isotypes were observed also in the spleen (Fig. 2A right panel) of B7-1/2$^{-/-}$ mice compared to wildtype mice. Infection-induced, IFNR-dependent B7-2 induction is not observed at that site (Fig. 1 and (11)), further suggesting that the effects of type I IFN and B7-2 on B cell response regulation to influenza virus infection are distinct. Consistent with the reductions in the frequencies of antibody-secreting cells in MedLN and spleen in the absence of B7-1/2 (Fig. 2A), virus-specific serum IgG responses were significantly lower in B7-1/2$^{-/-}$ mice compared to wildtype 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

In order 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) 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 (Figure 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 only on B cells compared to 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.

Comparison of the virus-specific serum antibody 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 to 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 might be related to the genetic background of the animals. Together these data indicate that the effects of B7-1/2 direct signaling to B cells affect mainly IgG secretion.

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

Previous studies by others (15) had demonstrated a non-redundant role for B7-1/2 signaling in germinal center formation after protein immunization. Therefore, we next tested whether the observed reduction in virus-specific antibody 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 negative and CD19^{+} B220^{high} CD24^{high} CD38^{low}) in MedLN of influenza virus-infected B7-1/2$^{-/-}$ compared to wildtype mice (Fig. 4A). It is of note, however, that despite this considerable reduction in germinal center B cells (3.3± 1.7 in B7-1/2−/− versus 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 versus 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 aimed 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, 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 $F(ab)_2$ prior to 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 control, respectively (Fig. 5A). In chimeras reconstituted with B-71/2^{-} bone marrow, frequencies of B7-2-expressing cells ranged between 0.5–32% (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 GC 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$^+$ B cells (Fig. 5C, left panel) and of CD24^{hi} CD38^{lo} 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 around 1% (Fig. 5D) using increased doses of whole-body irradiation. Analysis of these mice confirmed that while IgG2a responses was significantly reduced in mice lacking B7 only on B cells following influenza virus infection, there was no effect on germinal center formation (Fig. 5D). Attempts to reconstitute SCID mice with similar numbers of allotype-mismatched B cells from wildtype and CD80/86−/− 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 antibody secretion by B cells from CD80/CD86$^{-/-}$ versus wildtype 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 unlikely is 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 co-stimulatory molecules for T cell priming (3,5,18). Lack of B7-1/2 on all antigen-presenting cells 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$^{-/-}$ mice and in chimeras lacking B7-1/2 only on B cells is due to reduced availability of T cell help, thus whether B cells contribute via B7-1/2 to T helper response induction or maintenance. For that we compared frequencies of activated CD4$^+$ T cells in MedLN of day 7 influenza virus infected mice by studying CD11a^{high} CD44^{hi} expression (28). As expected B7-1/2$^{-/-}$ mice had similar total frequencies of CD4 T cells (Fig. 6A), but fewer were activated in MedLN compared to wildtype controls (Fig. 6B). However, B7-1/2 expression by B cells does not appear to be required for full CD4$^+$ T cell activation in the regional lymph nodes of influenza infected mice, as overall CD4$^+$ T cell numbers and frequencies of activated CD4 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$^+$ 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$^{-/-}$ 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$^+$ T cells of chimeras reconstituted with B cells from wildtype and B7-1/2$^{-/-}$ 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. This data is consistent with a study measuring IL-4+ T cells following reconstitution of RAG-deficient mice with wildtype and CD86−/− B cells and immunization with TNP-KLH (21). In addition, we had shown previously that type

I IFN-direct signaling is a necessary and sufficient principal signal for the early induction of CD86 on B cells following influenza virus infection (10,11). Thus indicating that IFN-γ does not regulate IgG2a production indirectly via up-regulation of B7 following influenza virus infection.

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, i.e. clonal expansion of antigen-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$(Fab)_2$ 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 pre-treated for 16h 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 antibody secretion. For that MACS-purified total B cells were stimulated over 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 timepoint and IgG2a concentrations in supernatants were determined by ELISA every 24h. 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 antibody production (threshold of detection 0.016 ng/ml). Maximal differences were already noted at 24h 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 qRT-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, stimulation of total B cells and B cells depleted of naïve IgD+ cells with anti-B7-2 showed a further enhancement of IgG1 and IgG2a secretion (Fig. 8C), also indicating that anti-B-2 stimulation induces IgG-committed B cells to secrete antibodies. 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 un-folded protein response of cells actively secreting antibodies (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 non-redundant 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 mechanisms for these observed effects of B7 on antibody 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). Together with the current study, the data suggest that type I IFN, through its effects on increasing B7-2 expression by local B cell populations (Fig. 1), might enhance antibody production in vivo in part by facilitating increased B cell-signaling through B7-2. Overall, the study suggests that the modulation of B7-ligand interaction is a mechanism by which an innate signal directly affects local T-dependent B cell responses to influenza virus.

Studies by Sanders and colleagues have provided solid evidence for a direct signaling role of B7-2 on activated B cells (19,21–24,31,32). Specifically, they showed that engagement of B7-2 alone or in conjunction with stimulation of the β2-adrenergic receptor on IL-4/CD40-stimulated B cells affects their activation status in vitro causing increased Oct-2 expression, and increased the activity of NF-κB and the 3′-IgH enhancer (19,22,31). Their data further suggest that cells stimulated in vitro in such a way increased antibody production per cell, rather than the rate of class switch recombination (23). A study by Jeannin et al (33) found that B7-2 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 antibody production was accompanied by increased B cell proliferation in addition to increases in Ig epsilon 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 antibody 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 antibody secreting cells (Fig. 8A). This conclusion was further supported by data showing that enrichment for IgD^{-} previously activated B cells increased antibody production following B7-engagement compared to cultures of total B cells (Fig. 8C) and that B7-signaling caused the concomitant upregulation of the transcription factor XBP-1 and its spliced variant sXBP-1 (Fig. 8D); transcription factors that are indispensable for B cell differentiation to antibody-secreting cells (34) by promoting the expansion of the ER, 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 antibody response regulation, as the lack of B7-1/2 on B cells alone reduces overall IgG antibody 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 antibody 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 antibody production in vivo, as in their absence IgG antibody

responses to influenza virus infection were reduced (Fig. 3), and strongly enhanced antibody 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. R. and N. B., unpublished), 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 is not sufficient for mature, naive B cells to prime naïve 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 co-stimulatory molecule, CD40, was reported by Crawford et al (37). Together these data are in support of the view (3,38) that engagement of individual co-stimulatory 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. It further suggests that a major role of B7-CD28 engagement during B–T interaction is to drive B cell differentiation.

The here 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 to 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 non-specific 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. In addition, B7-signaling might facilitate the rapid differentiation of activated virus-specific B cells to antibody secreting cells following a primary infection. Indeed, a rapid accumulation of antibody-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) and K. Rothaeusler and N.B., submitted).

This additional role for B7-signaling in B cell activation provide 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 (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 (SLE). It is of note that SLE has also been linked to disregulation of type I IFN signaling (41,42).

To summarize, the importance of B7-1/2 co-stimulation 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 antibody secretion by previously activated and class-switched B cells.

Acknowledgments

We thank Abigail Spinner for help with flow cytometry, Adam Treestar for Flow Jo software as well as Dr. Kristina Abel (University of North Carolina) for critical evaluation of the manuscript.

References

1. Kelsoe G. Studies of the humoral immune response. Immunol Res 2000;22:199–210. [PubMed: 11339356]
2. Bishop GA, Hostager BS. B lymphocyte activation by contact-mediated interactions with T lymphocytes. Curr Opin Immunol 2001;13:278–285. [PubMed: 11406358]

3. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005;23:515–548. [PubMed: 15771580]
4. McHeyzer-Williams LJ, Malherbe LP, McHeyzer-Williams MG. Helper T cell-regulated B cell immunity. Curr Top Microbiol Immunol 2006;311:59–83. [PubMed: 17048705]
5. Greenfield EA, Nguyen KA, Kuchroo VK. CD28/B7 costimulation: a review. Crit Rev Immunol 1998;18:389–418. [PubMed: 9784967]
6. McAdam AJ, Schweitzer AN, Sharpe AH. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. Immunol Rev 1998;165:231–247. [PubMed: 9850864]
7. Chiron D, Bekeredjian-Ding I, Pellat-Deceunynck C, Bataille R, Jego G. Toll-like receptors: lessons to learn from normal and malignant human B cells. Blood. 2008
8. Gerondakis S, Grumont RJ, Banerjee A. Regulating B-cell activation and survival in response to TLR signals. Immunol Cell Biol 2007;85:471–475. [PubMed: 17637697]
9. Meyer-Bahlburg A, Rawlings DJ. B cell autonomous TLR signaling and autoimmunity. Autoimmun Rev 2008;7:313–316. [PubMed: 18295736]
10. Chang WL, Coro ES, Rau FC, Xiao Y, Erle DJ, Baumgarth N. Influenza virus infection causes global respiratory tract B cell response modulation via innate immune signals. J Immunol 2007;178:1457–1467. [PubMed: 17237394]
11. Coro ES, Chang WL, Baumgarth N. Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. J Immunol 2006;176:4343–4351. [PubMed: 16547272]
12. Fink K, Lang KS, Manjarrez-Orduno N, Junt T, Senn BM, Holdener M, Akira S, Zinkernagel RM, Hengartner H. Early type I interferon-mediated signals on B cells specifically enhance antiviral humoral responses. Eur J Immunol 2006;36:2094–2105. [PubMed: 16810635]
13. Le Bon A, Thompson C, Kamphuis E, Durand V, Rossmann C, Kalinke U, Tough DF. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. J Immunol 2006;176:2074–2078. [PubMed: 16455962]
14. Lumsden JM, Roberts JM, Harris NL, Peach RJ, Ronchese F. Differential requirement for CD80 and CD80/CD86-dependent costimulation in the lung immune response to an influenza virus infection. J Immunol 2000;164:79–85. [PubMed: 10604996]
15. Borriello F, Sethna MP, Boyd SD, Schweitzer AN, Tivol EA, Jacoby D, Strom TB, Simpson EM, Freeman GJ, Sharpe AH. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. Immunity 1997;6:303–313. [PubMed: 9075931]
16. Ferguson SE, Han S, Kelsoe G, Thompson CB. CD28 is required for germinal center formation. J Immunol 1996;156:4576–4581. [PubMed: 8648099]
17. Shahinian A, Pfeffer K, Lee KP, Kundig TM, Kishihara K, Wakeham A, Kawai K, Ohashi PS, Thompson CB, Mak TW. Differential T cell costimulatory requirements in CD28-deficient mice. Science 1993;261:609–612. [PubMed: 7688139]
18. Abbas AK. The control of T cell activation vs. tolerance. Autoimmun Rev 2003;2:115–118. [PubMed: 12848951]
19. Kasprowicz DJ, Kohm AP, Berton MT, Chruscinski AJ, Sharpe A, Sanders VM. Stimulation of the B cell receptor, CD86 (B7-2), and the beta 2-adrenergic receptor intrinsically modulates the level of IgG1 and IgE produced per B cell. J Immunol 2000;165:680–690. [PubMed: 10878340]
20. Suvas S, Singh V, Sahdev S, Vohra H, Agrewala JN. Distinct role of CD80 and CD86 in the regulation of the activation of B cell and B cell lymphoma. J Biol Chem 2002;277:7766–7775. [PubMed: 11726649]
21. Kin NW V, Sanders M. CD86 Regulates IgG1 Production via a CD19-Dependent Mechanism. J Immunol 2007;179:1516–1523. [PubMed: 17641017]
22. Podojil JR, Kin NW, Sanders VM. CD86 and beta2-adrenergic receptor signaling pathways, respectively, increase Oct-2 and OCA-B Expression and binding to the 3′-IgH enhancer in B cells. J Biol Chem 2004;279:23394–23404. [PubMed: 15024018]
23. Podojil JR V, Sanders M. Selective regulation of mature IgG1 transcription by CD86 and beta 2-adrenergic receptor stimulation. J Immunol 2003;170:5143–5151. [PubMed: 12734361]
24. Podojil JR V, Sanders M. CD86 and beta2-adrenergic receptor stimulation regulate B-cell activity cooperatively. Trends Immunol 2005;26:180–185. [PubMed: 15797507]

25. Doucett VP, Gerhard W, Owler K, Curry D, Brown L, Baumgarth N. Enumeration and characterization of virus-specific B cells by multicolor flow cytometry. J Immunol Methods 2005;303:40–52. [PubMed: 16045923]
26. Rothaeusler K, Baumgarth N. Evaluation of intranuclear BrdU detection procedures for use in multicolor flow cytometry. Cytometry A 2006;69:249–259. [PubMed: 16538653]
27. Baumgarth N, Herman OC, Jager GC, Brown L, Herzenberg LA. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. Proc Natl Acad Sci U S A 1999;96:2250–2255. [PubMed: 10051627]
28. Baumgarth N, Egerton M, Kelso A. Activated T cells from draining lymph nodes and an effector site differ in their responses to TCR stimulation. J Immunol 1997;159:1182–1191. [PubMed: 9233612]
29. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 2000;102:553–563. [PubMed: 11007474]
30. Iwakoshi NN, Lee AH, Vallabhajosyula P, Otipoby KL, Rajewsky K, Glimcher LH. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. Nat Immunol 2003;4:321–329. [PubMed: 12612580]
31. Kin NW V, Sanders M. CD86 stimulation on a B cell activates the phosphatidylinositol 3-kinase/Akt and phospholipase C gamma 2/protein kinase C alpha beta signaling pathways. J Immunol 2006;176:6727–6735. [PubMed: 16709832]
32. Kohm AP, Mozaffarian A, Sanders VM. B cell receptor- and beta 2-adrenergic receptor-induced regulation of B7-2 (CD86) expression in B cells. J Immunol 2002;168:6314–6322. [PubMed: 12055247]
33. Jeannin P, Delneste Y, Lecoanet-Henchoz S, Gauchat JF, Ellis J, Bonnefoy JY. CD86 (B7-2) on human B cells. A functional role in proliferation and selective differentiation into IgE- and IgG4-producing cells. J Biol Chem 1997;272:15613–15619. [PubMed: 9188449]
34. Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, Friend D, Grusby MJ, Alt F, Glimcher LH. Plasma cell differentiation requires the transcription factor XBP-1. Nature 2001;412:300–307. [PubMed: 11460154]
35. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A, Hurt EM, Petroulakis E, Sonenberg N, Yewdell JW, Calame K, Glimcher LH, Staudt LM. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. Immunity 2004;21:81–93. [PubMed: 15345222]
36. Chung JB, Wells AD, Adler S, Jacob A, Turka LA, Monroe JG. Incomplete activation of CD4 T cells by antigen-presenting transitional immature B cells: implications for peripheral B and T cell responsiveness. J Immunol 2003;171:1758–1767. [PubMed: 12902475]
37. Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. J Immunol 2006;176:3498–3506. [PubMed: 16517718]
38. Schweitzer AN, Sharpe AH. The complexity of the B7-CD28/CTLA-4 costimulatory pathway. Agents Actions Suppl 1998;49:33–43. [PubMed: 9426826]
39. Good KL, Avery DT, Tangye SG. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. J Immunol 2009;182:890–901. [PubMed: 19124732]
40. Linsley PS, Nadler SG. The clinical utility of inhibiting CD28-mediated costimulation. Immunol Rev 2009;229:307–321. [PubMed: 19426230]
41. Ronnblom L, Pascual V. The innate immune system in SLE: type I interferons and dendritic cells. Lupus 2008;17:394–399. [PubMed: 18490415]
42. Thibault DL, Graham KL, Lee LY, Balboni I, Hertzog PJ, Utz PJ. Type I interferon receptor controls B-cell expression of nucleic acid-sensing Toll-like receptors and autoantibody production in a murine model of lupus. Arthritis Res Ther 2009;11:R112. [PubMed: 19624844]
43. Fuse S, Obar JJ, Bellfy S, Leung EK, Zhang W, Usherwood EJ. CD80 and CD86 control antiviral CD8+ T-cell function and immune surveillance of murine gammaherpesvirus 68. J Virol 2006;80:9159–9170. [PubMed: 16940527]

44. Lumsden JM, Williams JA, Hodes RJ. Differential requirements for expression of CD80/86 and CD40 on B cells for T-dependent antibody responses in vivo. J Immunol 2003;170:781–787. [PubMed: 12517941]

45. McAdam AJ, Farkash EA, Gewurz BE, Sharpe AH. B7 costimulation is critical for antibody class switching and CD8(+) cytotoxic T-lymphocyte generation in the host response to vesicular stomatitis virus. J Virol 2000;74:203–208. [PubMed: 10590107]

46. Thebeau LG, Morrison LA. Mechanism of reduced T-cell effector functions and class-switched antibody responses to herpes simplex virus type 2 in the absence of B7 costimulation. J Virol 2003;77:2426–2435. [PubMed: 12551980]

47. Zhang P, Martin M, Yang QB, Michalek SM, Katz J. Role of B7 costimulatory molecules in immune responses and T-helper cell differentiation in response to recombinant HagB from Porphyromonas gingivalis. Infect Immun 2004;72:637–644. [PubMed: 14742503]

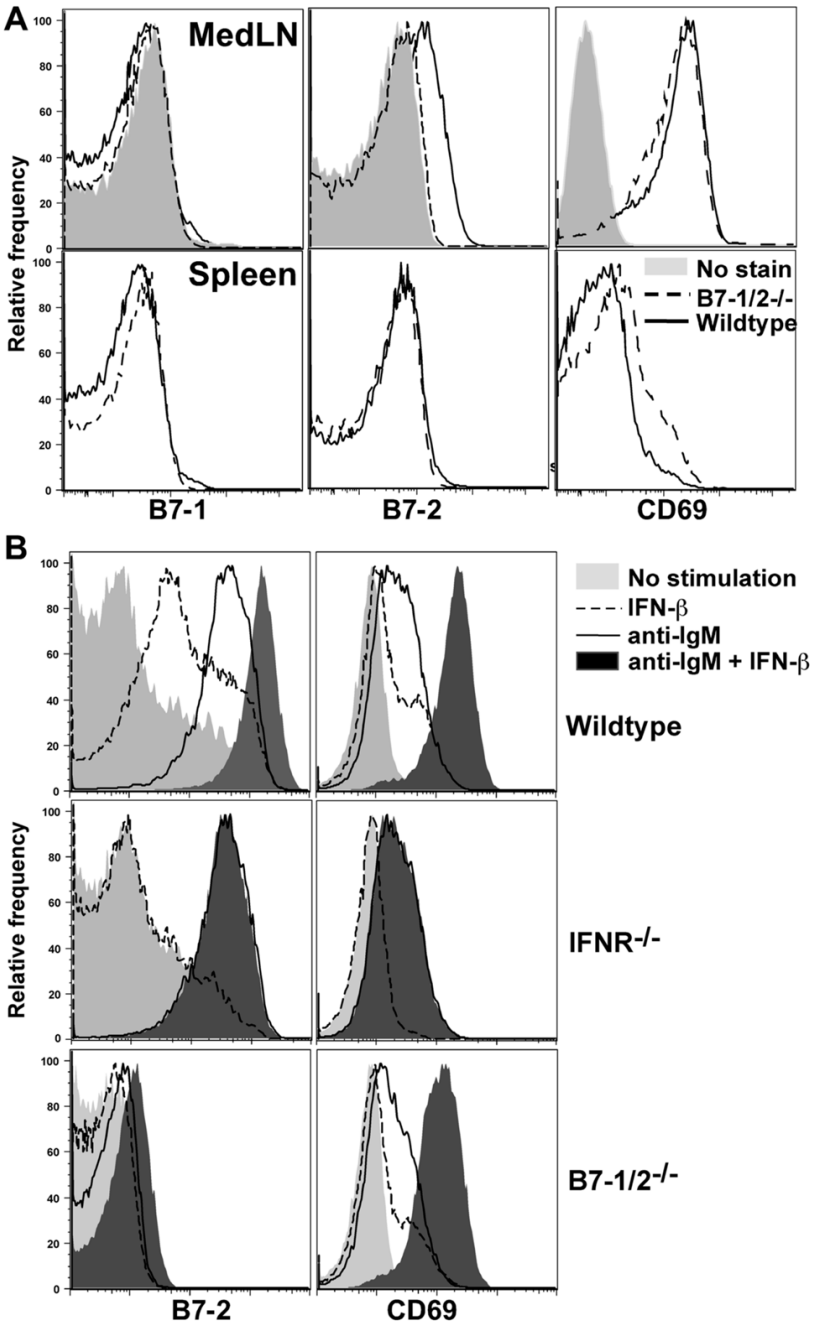

Figure 1. Type I IFN and BCR-mediated stimuli act synergistically to induce B-7-1/2

(A) Multicolor flow cytometric analysis for expression of the indicated surface molecules was conducted on B cells from mediastinal lymph nodes and spleen cells of 2-day influenza virus A/PR8-infected BALB/c mice (open histogram, solid line) or gene-targeted B7-1/2−/− mice (dashed line, open histogram). For lymph nodes, a sample was stained with all markers, except the one displayed (solid grey) to serve as a background "fluorescence minus one" control. Overlaid histograms are from live B cells, identified by their FSC/SSC, expression of CD19, exclusion of propidium iodide and lack of staining for CD3, 4, 8, F4/80 and GR-1 (not shown). (**B**) Similar flow cytometric analysis conducted on MACS-purified splenic B cells of non-infected BALB/c (top), IFNR$^{-/-}$ (middle) and B7-1/2$^{-/-}$ mice (bottom panels) following

culture for 16h in the absence (solid light grey) or presence of the following stimuli: $(Fab)_2$ goat-anti-mouse IgM at 20μg/ml (solid line, open histogram); 200U IFN-β (dashed line, open histogram); and anti-IgM plus IFN-β (solid dark grey).

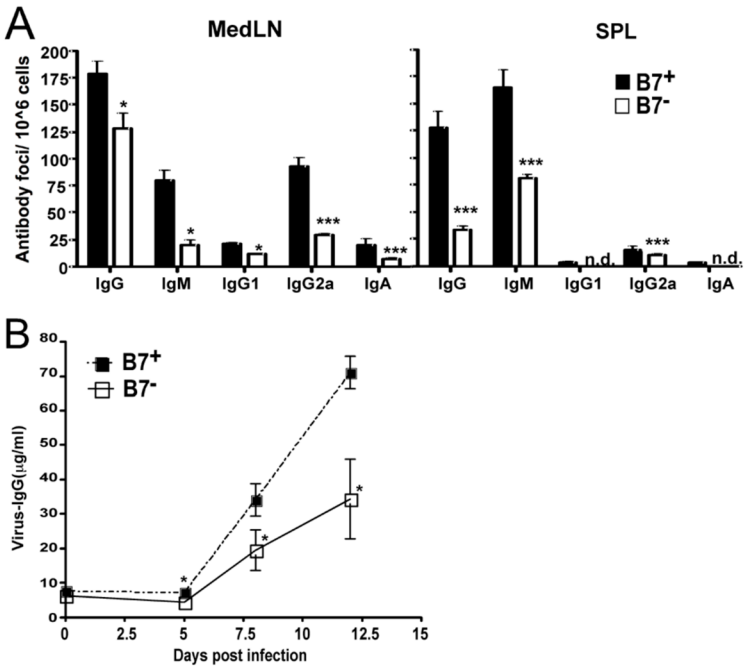

Figure 2. B7-1/2 expression required for maximal virus-specific antibody responses to influenza virus infection

(A) Frequencies of virus-specific Ig-secreting cells were determined by ELISPOT on day 12 after influenza virus infection. Shown are mean counts ± SD from 2-fold titrated duplicate cultures of MedLN and triplicate cultures of spleen cells from pooled (n=6) BALB/c ($B7^+$) mice (filled bars) and B7-1/2 $^{-/-}$ mice ($B7^-$, open bars), respectively. Virus-specific IgG1 and IgA secreting spots were not detected (n.d.) in spleen of $B7^-$ mice. Results are a representative from two independent experiments. **(B)** Virus-specific Ig serum response was measured in BALB/c ($B7^+$) (filled symbols) and B7-1/2$^{-/-}$ mice ($B7^-$, open symbols) by ELISA at indicated time points after influenza virus infection. Data represent mean ± SD concentrations (μg/ml) of virus-specific IgG from 6 individual mice per group. $^{*}p<0.05$; $^{**}p< 0.01$; $^{***}p<0.001$.

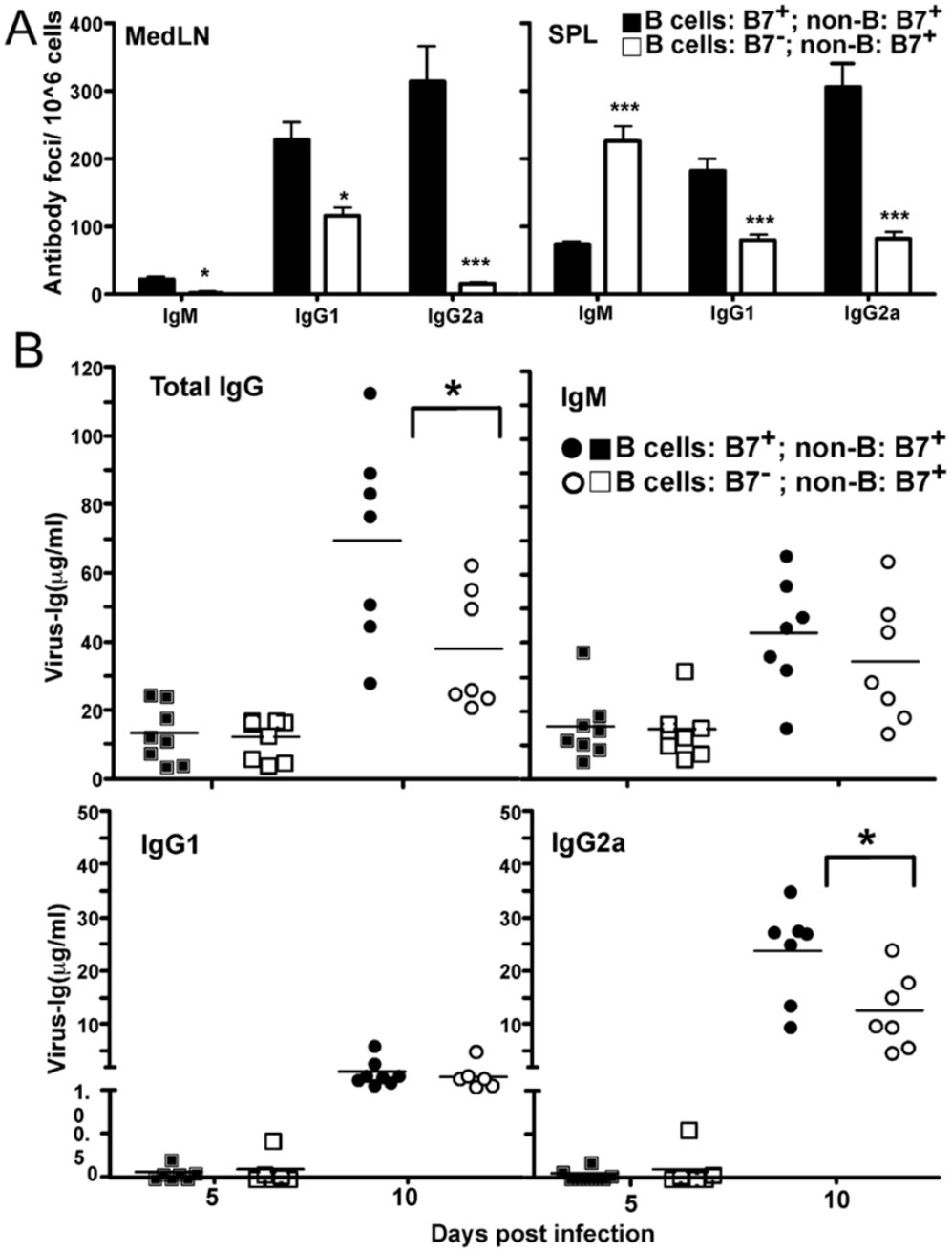

Figure 3. B7-1/2 expression by B cells affects virus-specific antibody responses

(A) Virus-specific antibody production was assessed in mice that lacked B7-1/2 expression only on B cells (open bars) and compared to controls with B7-expressing wildtype B cells (filled bars). Mice were mixed bone marrow irradiation chimeras generated by reconstituting lethally irradiated wildtype (WT) BALB/c mice with a mix (75%/25%) of bone marrow from B cell deficient mice and either B7-1/2 $^{-/-}$ or WT BALB/c mice. MedLN and spleens from mice (n = 6/group) were pooled and analyzed by ELISPOT 10 days after infection with influenza A/PR8. Shown are mean frequencies of antibody-secreting foci ± SD calculated from two-fold titrated duplicate cultures of MedLN and triplicate cultures of spleen cells. The data are a representative of two independent experiments yielding comparable

results. $^{*}p<0.05$, $^{**}p< 0.01$, $^{***}p<0.001$. Reduction in IgM production by B cell B71/2^{-}/$^{-}$ mice in MedLN in (A) was only observed in one of two experiments. **(B)** Virus-specific serum total IgG, IgM as well as IgG1 and IgG2a levels in chimeras with wildtype (filled symbols) and B7-1/2−/− B cells at indicated times after infection with influenza A/PR8 as measured by ELISA. Each symbol represents data from one animal. The horizontal lines represent the geometric mean for each group. $^{*}p < 0.05$.

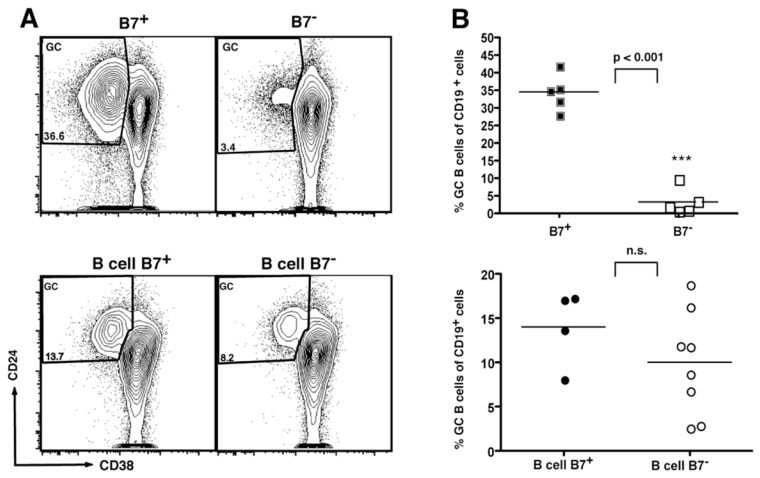

Figure 4. Germinal center formation is not dependent on B7-1/2 expression by B cells

(A) Shown are representative contour plots (5%) with outliers from FACS analyses of MedLN from wildtype (wt) and B7-1/2$^{-/-}$ mice (upper panels) and of chimeras lacking B cell-expression of B7-1/2 and their controls (lower panels) at days 10 (top) and 12 (bottom) after influenza infection, respectively. Germinal center B cells (GC) were identified as CD24^{hi} CD38^{lo} B cells as shown, following gating on live B cells (CD3, CD4, CD8, F4/80- propidium iodide negative and CD19^{+} B220^{high}). Numbers indicate frequencies among CD19^{+} B220^{hi} B cells. Shown are representative samples from the respective groups, consisting of 4–8 mice each. **(B)** Summary of the results from the FACS analysis for individual mice analyzed as shown in (A). The horizontal lines represent the geometric mean for each group. ***p<0.001; n.s., not significant.

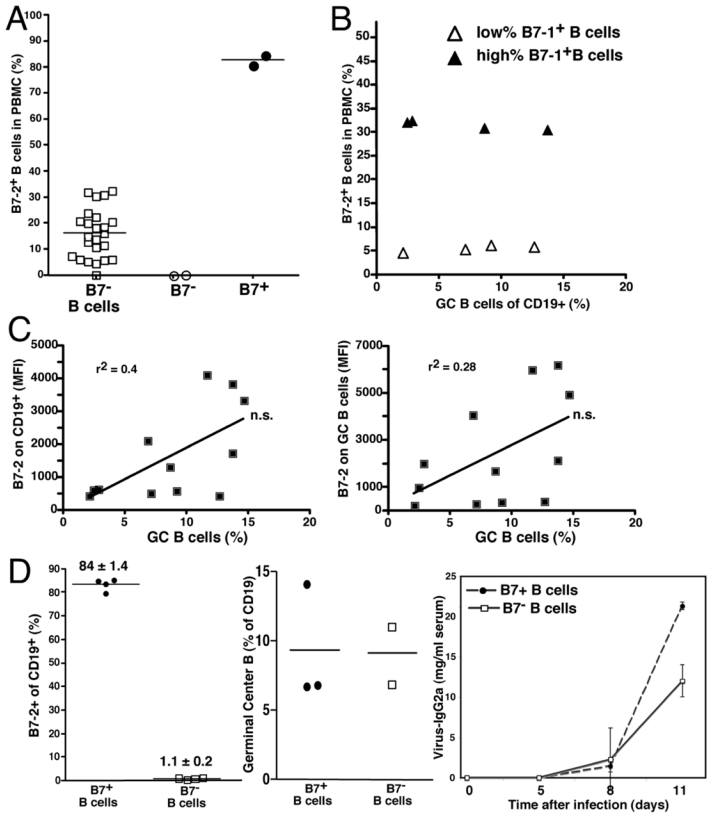

Figure 5. Lack of correlation between B7-2 expression levels on B cells and germinal center B cell frequencies in MedLN

(A) Shown are frequencies of B7-2 expressing peripheral blood B cells of individual mixed bone marrow irradiation chimeras following overnight in vitro stimulation of PBMC with anti-IgM $(Fab)_2$ (open boxes). B7-1/2$^{-/-}$ (open circles) and BALB/c mice (filled circles) were used as negative and positive controls, respectively. Chimeras were generated by reconstituting lethally irradiated BALB/c mice (650rd whole body irradiation) with a mix (75%/25%) of bone marrow from B cell-deficient mice and B7-1/2$^{-/-}$ mice. In the chimeras, B7-2 expressing B cells are presumably radio-resistant and host-derived. **(B)** To determine whether a correlation exists between expression of B7-2 on B cells and germinal center formation, two groups of chimeras (n= 4) differing in the frequencies of peripheral B7-2 B cells were infected for 10 days and frequencies of germinal center B cells were determined by FACS. Chimeras were stratified in groups according to their frequencies of B cells expressing B7-2 (4–8 % open triangles; 30–32%, filled triangles) Note the lack of correlation between frequencies of B cells expressing B7-2 and germinal center B cell frequencies. **(C)** Levels of B7-2 expression (MFI) on CD19 + MedLN B cells (left panel) and on MedLN germinal center B cells (right panel) of individual mice were correlated with germinal center B cell frequencies. No significant correlation was observed ($p > 0.05$). **(D)** Chimeras generated with B cells from either B7+ or B7− mice as in A, but using 800rd whole body irradiation were analyzed for expression of B7-2 after in vitro stimulation of PBMC as in A (n = 4 per group, left panel). Data are mean values ± SD. Mice were infected for 11 days with influenza A/PR8 and frequencies germinal center B cells in regional lymph nodes assessed by flow cytometry (middle panel). ELISA on sera taken from mice at indicated time points were analyzed for virus-specific IgG levels. Shown are mean concentrations (μg/ml) ± SD.

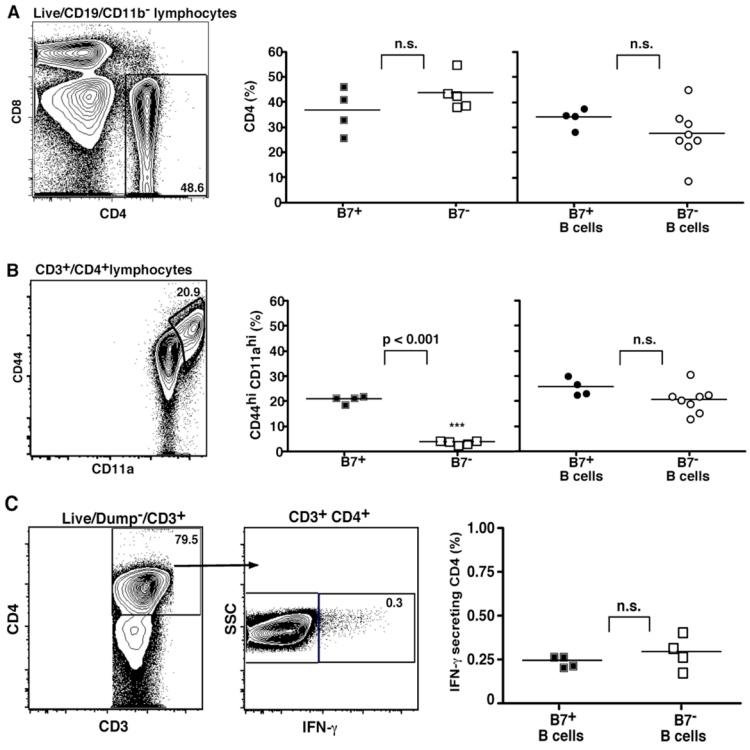

Figure 6. Reduced CD4 T cell activation in B7-1/2−/− mice is due to expression of B7-1/2 on cells other than B cells

Shown are representative FACS contour plots (5%) with outliers to demonstrate the gating strategies used to determine the frequencies of **(*A*)** total and **(B)** activated (CD11a ^{hi}/CD44^{hi}) CD4^{+} T cells in MedLN. Results are from chimeras lacking B7-1/2 on B cells only and their controls (far right panels) infected for 10 days with influenza virus, and BALB/c (B7+) and B7-1/2$^{-/-}$ mice (B7−, middle panels), infected for 12 days with influenza A/PR8 virus. Each symbol represents the result from an individual mouse. The horizontal lines represent the geometric mean for each group. Data represent results from one of at least three independent experiments performed. *** p<0.001. (**C**) Shown is the gating strategy used to determine frequencies of IFN-γ secreting CD4^{+} T cells in MedLN 7 days following influenza virus infection. Frequencies of IFN-γ producers of live T cells (CD3+, CD4+ or CD8+, CD11b^{-}, CD19^{-} and excluding propidium iodide) were determined by intracytoplasmic staining following overnight re-stimulation with plate-bound anti-CD3 (clone 145-2C11) in the presence of monensin. Results from individual mice lacking B7-1/2 only on B cells (B cell B7^{-}; *n* = 4) and controls (B cell B7^{+}; *n* = 4) are summarized in the right panels. The horizontal lines represent the geometric mean for each group. **p<0.01. Results are from one of two experiments that yielded similar results.

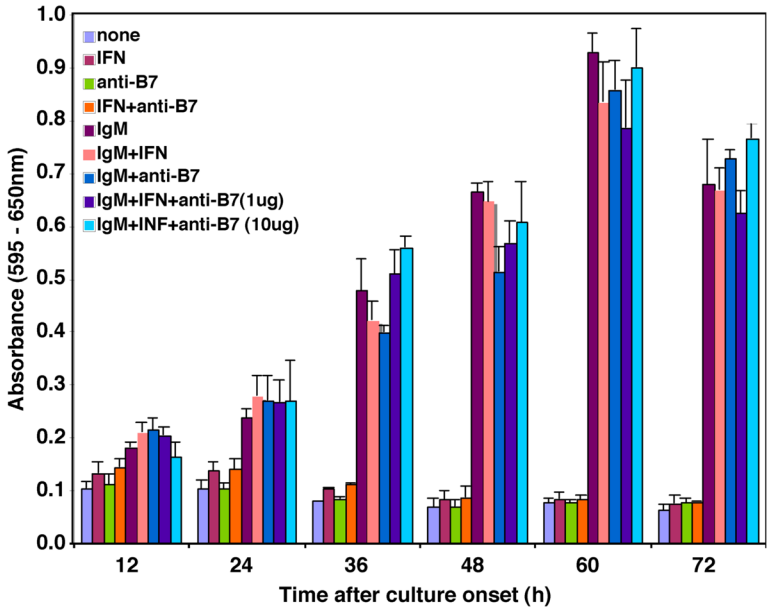

Figure 7. B7-2 direct stimulation does not affect B cell proliferation

Shown are results from a proliferation assay of MACS-enriched splenic B cells stimulated with/or without a combination of the following: IFN-b (200U/ml), anti-B7-2 (10 μg/ml unless otherwise noted), anti-IgM(Fab)$_2$ (20 μg/ml). At indicated times after culture onset cells were analyzed by MTT assay for relative numbers of cells in each well (measured as adsorbance at 590–650 reference wavelengths). Stimulation of B cells with IFN-b for 16h followed by washing of the cells and then stimulation with anti-B7-2 gave similar results (data not shown). Results are from one of three experiments done that gave similar results.

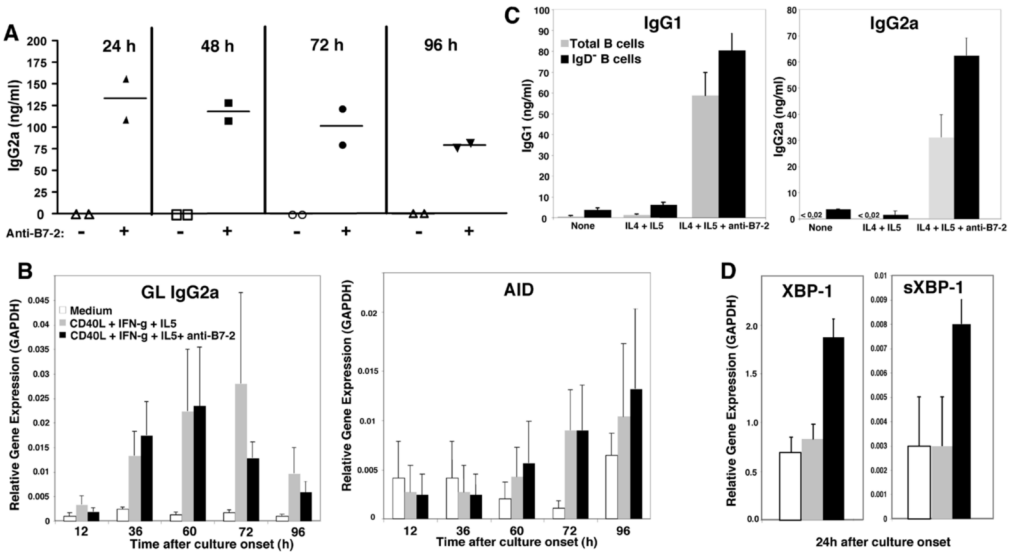

Figure 8. B7-2 stimulation induces IgG production by class-switched B cells

(A) MACS-enriched splenic B cells were cultured in duplicate for indicated times in the presence of CD40L (0.I mg/ml), IFN-g (15ng/ml) and IL-5 (2ng/ml) with/without anti-B7-2 (10 μg/ml). Supernatants were harvested and IgG2a protein levels measured by ELISA. Horizontal lines indicate mean of 2 cultures. IgG2a levels in cultures without anti-B7-2 were below the threshold of detection (0.0165 ng/ml) at all time points analyzed. **(B)** Cells from similar cultures as in (A), set-up in triplicate were harvested at indicated timepoints after culture onset and RNA was extracted for qRT-PCR analysis of germline IgG2a sterile transcripts and AID expression. No significant differences in gene expression were noted between cultures in the presence/absence of anti-B7-2 at any time point. Similar results were obtained when germline IgG1 transcripts were measured in cultures containing IL-4 instead of IFN-g (data not shown). **(C)** Total splenic B cells and splenic B cells in which naïve IgD+ cells were removed by MACS-depletion were cultured in quadruplicate in the presence of IL-4 and IL-5 with/without anti-B7-2. IgG1 and IgG2a protein levels in supernatants were measured by ELISA 24h after culture onset. **(D)** Shown are relative expression levels of XBP-1 and sXBP-1 from qRT-PCR analysis of RNA extracted from B cells cultured for 24h as outlined in (A). Results are representative of 2–5 experiments conducted for each analysis.