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ICOS-Induced B7h Shedding on B Cells Is Inhibited by TLR7/8 and TLR9

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We report in this study that B7h, the ligand for the ICOS costimulatory receptor, is rapidly shed from mouse B cells following either ICOS binding or BCR engagement. Shedding occurs through proteolytic cleavage that releases the extracellular ICOS-binding region of B7h. Prior exposure of B7h-expressing APCs to ICOS-expressing cells inhibits their subsequent ability to costimulate IFN-γ and IL-4 production from CD4+ T cells. Shedding is regulated as TLR7/8 and TLR9 ligands inhibit B7h shedding. A shedding-resistant B7h mutant elicits greater costimulation of IFN-γ production from CD4+ T cells than does wild-type B7h. These data define shedding of B7h as a novel mechanism for controlling costimulatory signaling by B7-CD28 family members that is regulated on B cells by TLR signaling. The Journal of Immunology, 2006, 177: 2356–2364.

The ICOS receptor (1) expressed on activated T cells and its ligand B7h (2) (also known as ICOSL (3), B7RP-1 (4), B7-H2 (5), and GL50 (6)), expressed on B cells, dendritic cells, and macrophages are members of the CD28-B7 costimulatory family that regulate activation, differentiation, and effector functions of T cells (7–9). ICOS costimulation regulates production of multiple cytokines, including IL-4, IL-10, and IFN-γ (1, 4, 10, 11), and is critical in Ab responses to T-dependent Ags (11–15). ICOS costimulation regulates effector functions of both Th2 and Th1 cells (16), as well as CD8+ T cells (17, 18). Recent studies also implicate ICOS-B7h in maintaining the balance between effector and regulatory T cells in mouse models of asthma (19, 20) and autoimmune diabetes (21).

ICOS plays a critical role in the production of autoimmune Abs by self-reactive B cells. In mouse models of rheumatoid arthritis and systemic lupus erythematosus (SLE), blockade of ICOS–B7h interactions resulted in reduced autoantibodies and disease progression (22, 23). Enhanced ICOS levels also are associated with disease progression in human SLE patients (24), as well as the autoimmune phenotype of sanracco mice (25).

During T-dependent Ab responses, ICOS signaling is regulated by limited access to B7h on activated B cells (26). Although B7h is highly expressed on naive B cells, B7h is transcriptionally extinguished on B cells following activation through either BCR or IL-4R (26). However, transcription can be reestablished by costimulation through CD40 receptor, suggesting that CD40L expressed on activated T cells also regulates B7h expression. Thus, the level of B7h on activated B cells is tightly regulated and appears to serve as a control point for regulating T-dependent Ab responses.

We report in this study that B7h expression also is limited through the novel mechanism of ectodomain shedding. Although many cell surface ligands and receptors, including inflammatory molecules such as TNF-α, TNFR, and IL-6R (27, 28) undergo ectodomain shedding, members of the B7/CD28 costimulatory family have not been identified previously as susceptible to shedding. The TNF superfamily member CD40 is one of the few costimulatory molecules known to undergo ectodomain shedding (29). Ectodomain shedding often is induced in response to receptor/ligand engagement or treatment with phorbol esters (27, 28). Shedding of B7h is rapidly induced on B cells by both cell-intrinsic and cell-extrinsic signals. Activation of B cells through either BCR cross-linking or ICOS binding induces B7h shedding.

Ectodomain shedding of B7h is functionally significant because it can limit the ability of APCs to costimulate cytokine production from CD4+ T cells. B7h down-regulation and shedding also are regulated by TLRs and are inhibited by exposure of B cells to either TLR7/8 or TLR9 ligands. Thus, in addition to the cell-autonomous role for TLR9 in activation of autoreactive B cells (30), TLR9 also stabilizes B7h, which regulates subsequent interactions of B cells with T cells during Ab responses. This stabilization of B7h is likely to enhance ICOS costimulation as a shedding-resistant B7h molecule enhanced cytokine production from CD4+ T cells. Thus, B7h shedding defines a novel immunoregulatory mechanism by which TLR7/8 and TLR9 signals can enhance T cell help during Ab responses.

Materials and Methods

Flow cytometry

Before staining with specific mAbs, cells were incubated with anti-FcR (2.4G2) hybridoma supernatant. Subsequent staining was performed in staining buffer (1× HBSS, 2% FBS, 10 mM HEPES, and 0.1% NaN3) using the following first-step reagents: biotinylated anti-B7h mAb (clone 22D7 or 13H12), biotinylated anti-B7.2 mAb (GL1; BD Pharmingen), ICOS-Ig (R&D Systems), PE-conjugated anti-I-A/E (M5/114.15.2; BD Pharmingen), and FITC-conjugated anti-B220 mAb (Caltag) to gate on B cells in coculture experiments. Streptavidin-PE was used as a second-step reagent. All experiments involving B7h staining were performed using 22D7 and were repeated using 13H12. Cells were analyzed using a Coulter EPICS XL, and data were plotted using FlowJo software (Tree Star).

Mice

C57BL/6 mice (The Jackson Laboratory), B7h−/− mice (a gift from T. Mak, University of Toronto, Toronto, Canada), MyD88−/− mice (a gift from A. Winoto, University of California, Berkeley, CA), and B7-1−/−/− mice were used. Cells were incubated with anti-FcR (2.4G2) hybridoma supernatant. Subsequent staining was performed in staining buffer (1× HBSS, 2% FBS, 10 mM HEPES, and 0.1% NaN3) using the following first-step reagents: biotinylated anti-B7h mAb (clone 22D7 or 13H12), biotinylated anti-B7.2 mAb (GL1; BD Pharmingen), ICOS-Ig (R&D Systems), PE-conjugated anti-I-A/E (M5/114.15.2; BD Pharmingen), and FITC-conjugated anti-B220 mAb (Caltag) to gate on B cells in coculture experiments. Streptavidin-PE was used as a second-step reagent. All experiments involving B7h staining were performed using 22D7 and were repeated using 13H12. Cells were analyzed using a Coulter EPICS XL, and data were plotted using FlowJo software (Tree Star).
B7-2−/− mice (The Jackson Laboratory) on a C57BL/6 background were all used between 5 and 8 wk of age. DO11.10 TCR-transgenic mice (The Jackson Laboratory) were used within 6 mo of age. All animal procedures were performed in accordance with regulations set by the University of California, Berkeley, Animal Care and Use Committee.

**Lymphocyte activation**

Rested effector and activated DO11.10-transgenic T cells were isolated after either 7–9 or 3–4 days, respectively, from bulk splenocyte cultures incubated with 0.3 μM OVA peptide, as described previously (2). Rested effector T cells were purified by passage over a nylon wool column. Both rested effector and activated T cells cultures were subject to complement lysis with a mixture of anti-MHC (28.16.8S and BP107), anti-heat-stable Ag (JD11), and anti-FcR (2.4G2) mAbs. Splenic B cells were purified as described previously (26). For Fig. 1, purified B cells were incubated overnight in 12-well plates (2 × 10^6 cells per well) in Medium or activated with either 2 μg/ml anti-CD40 (3/23; BD Pharmingen), 10 μg/ml LPS (Sigma-Aldrich), 5 μg/ml CpG oligonucleotide 1826 (5′-TCCATGACGTTCCCTGACGGACT-3′), 5 μg/ml non-CpG oligonucleotide 1982 (5′-TCCAGGACTTCTCTCAGGTT-3′), 2 μg/ml lipoteichoic acid or 25 μg/ml poly(I:C) (gift from A. Winoto), and 500 ng/ml BLP or R-848 (gift from G. Barton, University of California, Berkeley, CA). Activated or naive B cells were stimulated for 3 h in a 96-well round-bottom plate (1 × 10^6 cells per well) with 250 ng/ml PMA, 5 μg/ml anti-IgM Fab′, or cocultured with activated DO11.10-transgenic T cells at a 1:1 ratio. For Figs. 2B and 3C, the B7h-Chinese hamster ovary (CHO):T cell ratio was 1:5. For Fig. 4, the metalloprotease inhibitor GM6001 and the GM6001 negative control compound (CabiBiochem) were used at 30 μM. For Figs. 1E, 5D, and 6, the B7h-3T3 cells:T cell ratio was 1:10. After 3 h, supernatants were harvested for the analysis of soluble B7h (sB7h) release, and cells were stained with mAbs for anti-B220-FITC mAb and anti-B7h mAb (13H12 or 22D7).

**Microscopy**

In Fig. 2A, the B7hGFP CHO cells were grown on a coverslip in a 12-well plate and incubated for 3 h with activated ICOS+ T cells that were labeled with a PKH26 red fluorescent cell linker kit (Sigma-Aldrich). Cells were fixed with 1% paraformaldehyde (PFA) and analyzed using a Nikon Eclipse E800 fluorescence microscope. Image processing and analysis were performed using SlideBook 2.5 software (Intelligent Imaging Innovations).

**FIGURE 1.** B7h is rapidly removed from the cell surface of B cells following BCR engagement, PMA treatment, or binding to ICOS on activated T cells. For flow cytometric histograms, B7h staining with treatment (shaded) and without treatment (heavy dashed line) are shown with background staining levels (light dashed line). Except in B, all treatments are for 3 h. A, Specific down-regulation of B7h, but not B7-2, on B cells. B7h and B7.2 expression were examined after treatment of naive or activated B cells with PMA, anti-IgM Ab, or coculture at a 1:1 ratio with activated T cells as described in Materials and Methods. Mean fluorescence intensity (MFI) of B7h on naive B cells treated with PMA and anti-IgM was 6.57, 1.75, and 2.12 following treatment. MFI of B7h on activated B cells treated with PMA and anti-IgM was 10.2, 2.16, and 3.27 following treatment. MFI of B7h on naive and activated B cells cocultured with T cells was 14.2 and 19.2, respectively, and 2.45 and 2.24 following coculture. MFI of B7h on activated B cells treated with PMA, anti-IgM and activated T cells. B, New protein synthesis is not required for B7h down-regulation. B7h down-regulation was examined in the presence of the protein synthesis inhibitor cyclohexamide by flow cytometry at 0.5 and 3 h after treatment. MFI of untreated B7h was 14.6 and reduced to 4.9 at 0.5 h and 4.28 at 3 h with PMA and reduced to 2.01 at 0.5 h and 1.41 at 3 h with activated T cells. C, ICOS is required for B7h down-regulation. B7h expression on B cells was examined after coculture at a 1:1 ratio with either activated WT or ICOS−/− T cells in the presence of the blocking 22D7 mAb or a control hamster IgG mAb. MFI of B7h used with activated WT T cells was 4.38 and after coculture was 0.26, 3.79, and 0.29 with no mAb, anti-B7h mAb, and control mAb, respectively. MFI of B7h used with activated ICOS−/− T cells was 9.89, and after coculture was 8.81. D, ICOS expression on fixed cell membranes induces B7h down-regulation. B7h expression on B cells was examined after coculture at 1:1 ratio with CHO cells, CHO cells expressing ICOS, or fixed CHO cells expressing ICOS. MFI of B7h was 8.07 and was reduced to 7.55, 2.71, and 2.94 following coculture with CHO cells, live ICOS-CHO cells, or fixed ICOS-CHO, respectively. E, B7h expressed on dendritic cells and nonlymphoid CHO and 3T3 cells also is down-regulated. Down-regulation of B7h on retrovirally transduced 3T3 and CHO cells and endogenous B7h on CD11c+ dendritic cells was examined after coculture at ratios of 1:10, 1:5, and 1:1, respectively, with activated T cells. B7h-3T3 cells, B7h-CHO cells, and CD11c+ dendritic cells before coculture had B7h MFI of 14.9, 19.0 and 6.01, which was reduced to 3.52, 1.10, and 1.59, respectively, following coculture.
FIGURE 2. T cell-induced removal of cell surface B7h does not occur through internalization or degradation of B7h. A, A B7h-GFP fusion protein localizes to the cell surface of CHO cells. CHO cells expressing a B7h fusion protein with a C-terminal cytoplasmic GFP tag (green) were cocultured for 3 h with activated T cells (red) and visualized by fluorescence microscopy. Representative GFP expression on CHO cells with and without interactions with activated T cells are shown. B, Cell surface B7h is removed extracellularly. Fixed or unfixed CHO cells expressing a B7h-GFP fusion protein were cocultured for 3 h at a 1:5 ratio with activated T cells. Both GFP expression and staining for extracellular B7h expression with the 13H12 mAb are shown with activated T cells (shaded), without treatment (heavy dashed line), along with background staining levels (light dashed line). MFI of GFP was 31.0 and 29.4 on live and fixed cells and following coculture was 23.7 and 29.7, respectively. MFI of B7h was 119 and 95.4 on live and fixed cells and was reduced to 9.55 and 7.85 following coculture.

Retroviral constructs

For Fig. 5, the B7h/B7.2 chimeras were generated by an overlapping PCR strategy, sequence confirmed, and cloned into an MSCV-IRES-GFP vector. B7.2h-C contains B7.2 residues 1–135 and B7h residues 164–322 (VIAN-FSTP junction). B7.2h-M contains B7.2 residues 1–244 and B7h residues 278–322 (TYWK-LKVL junction). B7.2h-S contains B7.2 residues 1–237 from B7.2 and B7h residues 262–322 (QEFP-FTGN junction). B7.2h-SAT contains B7.2 residues 1–237 and B7h residues 262–303. B7.2h-S contains B7.2 residues 1–237 and B7h residues 238–309 (SQAE-SPQT junction). B7h.2-C contains B7h residues 1–163 and B7.2 residues 136–309 (VAAN-FSEP junction). In Figs. 2, 3C, and 3D, the triple-Flag epitope and GFP tags were added to the wild-type (WT) B7h gene at residue 322 and cloned into an MSCV-IRES-Thy1.1 retroviral vector. In Figs. 3D and 5, the retroviral supernatants were prepared and transduced into primary B cells stimulated for 24 h with 30 μg/ml LPS as described previously (31).

sB7h ELISA

For Figs. 3, A and B, 4A, 5D, and 7C, plates were coated overnight with purified 13H12 anti-B7h mAb (5 μg/ml) in PBS. Culture supernatants were spun at high speed for 20 min to remove membrane-bound B7h. A standard curve with B7hIg was used to establish the concentration of sB7h in supernatants. Bound sB7h was detected using a biotinylated 22D7 anti-B7h mAb. Streptavidin-peroxidase was used to develop the reaction with ABTS substrate solution.

Immunoblot

In Fig. 3, C and D, B7h-myc-expressing CHO cells or B7h-myc-infected primary B cells (~30% infection) were incubated for 3 h with activated DO11.10 T cells or PMA (250 ng/ml), respectively. Cells were washed and incubated in NP40 lysis buffer (1% NP40, 150 mM NaCl2, 50 mM Tris-Hcl (pH 8.0)) with protease inhibitors. Lysates were preclared with protein G-agarose, followed by immunoprecipitation using 5 μg of an anti-myc mAb (9E10). Immunoprecipitates were washed extensively, run on a 15% polyacrylamide gel, and blotted onto a nitrocellulose membrane. Membranes were incubated with an anti-myc rabbit polyclonal IgG primary (Santa Cruz Biotechnology) and a mouse anti-rabbit HRP secondary (Jackson Immunoresearch Laboratories) and were visualized by ECL kit (Western Lightning; PerkinElmer).

Intracellular cytokine analysis

In Figs. 6 and 8, the 3T3 APCs (1 × 104 cells per tube) were incubated in round-bottom tubes with rested DO11.10 T cells (5 × 105 cells per tube), OVA peptide, and BD GolgiPlug (BD Pharmingen) for 5 h. Cells were
B7h is rapidly down-regulated by activated T cells or BCR ligation

We reported previously that expression of B7h on activated B cells is extinguished over several days through shut-off of B7h gene expression (26). Although expression of B7h on activated B cells is thought to be critical for T-dependent Ab responses, activation of B cells through either BCR or IL-4R paradoxically extinguishes B7h mRNA expression. Because B7h transcription was reestablished by an activating anti-CD40 mAb, we proposed that CD40L expressed on activated T cells could restore B7h expression on cognate B cells and serve as a potential in vivo checkpoint for controlling Ab responses. Surprisingly, in testing whether activated T cells expressing CD40L were capable of restoring B7h expression on activated B cells, we found that cell surface levels of B7h were rapidly down-regulated within several hours following coculture with activated T cells (Fig. 1A).

This rapid down-regulation of B7h on activated B cells induced by coculture with activated T cells was detected by flow cytometry using three separate staining reagents: both blocking (22D7) and nonblocking (13H12) mAbs (26) that recognize epitopes on the V-like domain of B7h which mediates ICOS binding, as well as an ICOS-Ig fusion protein. Down-regulation of B7h was unique to B7h and was not observed with B7.2, the other principal B7 ligand expressed on activated B cells. Thus, B7h expression is limited on activated B cells, not only through transcriptional extinguishment, but also through an additional protein-mediated mechanism that occurs rapidly following coculture with activated T cells.

In addition to coculture with activated T cells, BCR ligation also induced rapid down-regulation of B7h protein on B cells (Fig. 1A). Again, this rapid down-regulation was unique to B7h and also was induced by pharmacological activation of B cells with the phorbol ester PMA. The rapid down-regulation of B7h was not a result of enhanced basal turnover of B7h protein, because B cells treated with the protein synthesis inhibitor cyclohexamide (Fig. 1B) or the protein transport inhibitor brefeldin A (data not shown) did not show any reductions in cell surface B7h expression over the 3-h period when down-regulation was assayed. B7h down-regulation induced by BCR or ICOS also was unaffected by cyclohexamide treatment, indicating that new protein synthesis was not required, and down-regulation occurred rapidly, with the majority of down-regulation occurring within 30 min.

ICOS mediates T cell-induced down-regulation

We next sought to clarify how activated T cells were inducing rapid and specific down-regulation of B7h on B cells. The addition of a mAb against B7h that blocks ICOS binding also blocked B7h down-regulation (Fig. 1C). This dependence on ICOS was consistent with the inability of both resting T cells that lack ICOS expression (data not shown) or activated T cells from ICOS-/- mice to down-regulate B7h. ICOS-induced down-regulation also did not appear to depend on other signaling or membrane relocalization events that occur during synapse formation with T cells. Using CHO cells that did not induce B7h down-regulation (Fig. 1D), we found that retroviral expression of ICOS on CHO cells was sufficient to induce B7h down-regulation, and that fixation of these CHO-ICOS cells with PFA did not alter the ability of these ICOS-expressing cells to down-regulate B7h on activated B cells. These results indicate that ICOS binding is sufficient to induce down-regulation of B7h, and that membrane relocalization of ICOS is not required for rapid down-regulation.

Down-regulation occurs on multiple cell types

B7h expressed on other cell types also was subject to rapid down-regulation by coculture with activated T cells (Fig. 1E). Endogenous B7h expressed on CD11c+ dendritic cells, as well as B7h that was ectopically expressed on 3T3 and CHO cells, were all susceptible to down-regulation of B7h following coculture with activated T cells. Thus, the ability of activated T cells to induce rapid B7h down-regulation is a property of many different cell types, including dendritic cells and B cells that regulate ICOS+ T cell responses in vivo.

The decrease in cell surface B7h levels was occurring either extracellularly or intracellularly through internalization or protein degradation of B7h. To address the mechanism of B7h down-regulation, we cocultured activated T cells with CHO cells expressing a B7h-GFP fusion protein, where a GFP tag was added to the
cytoplasmic tail of B7h. Irrespective of whether these CHO cells were complexed with activated T cells, the GFP marker still appeared highly localized to the cell surface of CHO cells (Fig. 2). Flow cytometric analysis of GFP expression and anti-B7h mAb staining (Fig. 2B) revealed a striking difference between intracellular GFP and extracellular B7h expression following coculture with activated T cells. Although coculture substantially reduced extracellular mAb staining of B7h, intracellular GFP expression was only minimally reduced, indicating that down-regulation of cell surface B7h did not involve protein degradation. B7h-GFP CHO cells also were fixed with PFA to prevent both intracellular internalization of cell surface proteins and transport of intracellular proteins to the cell surface. Fixation completely prevented the minor decrease in expression of intracellular GFP induced by activated T cells, but, strikingly, also resulted in the same decrease in mAb staining of extracellular B7h that was observed on live B7h-GFP CHO cells. These results indicate that B7h down-regulation does not involve intracellular internalization or require the relocalization of B7h within the cell membrane. Thus, rapid B7h down-regulation induced by activated T cells occurs entirely through an extracellular process.

**ICOS-binding region of B7h is shed by proteolytic cleavage**

To examine whether the extracellular portion of B7h was inducibly shed from the cell surface of B cells, we used a sandwich ELISA for sB7h that detects intact ICOS-binding fragments of B7h (Fig. 3A). The sB7h was basally shed from resting WT B cells, but not from resting B7h−/− B cells, and could also be readily detected in serum from WT mice, but not from B7h−/− mice (Fig. 3B). Treatment with anti-IgM Abs, PMA, and activated T cells all resulted in enhanced release of sB7h. Thus, the extracellular portion of B7h that binds to ICOS is inducibly shed into the supernatant following either BCR activation or engagement of ICOS on activated T cells.

A cleavage remnant containing the transmembrane region and cytoplasmic tail of B7h also was detected in B7h-expressing cells after down-regulation induced by ICOS or BCR (Fig. 3, C and D). Washed whole-cell lysates from CHO cells expressing a B7h protein that was myc-tagged on the cytoplasmic tail were immunoprecipitated and immunoblotted with anti-myc mAbs. A band corresponding to intact B7h-myc was readily detected in B7h-myc CHO cells, but not in control CHO cells. Following coculture with activated T cells, immunoblots revealed a smaller m.w. band that was specific to coculture with B7h-myc CHO cells and not with CHO cells. Transduced splenic B cells expressing the B7h-myc fusion protein also were treated for 3 h with and without PMA, and washed whole-cell lysates were immunoprecipitated and immunoblotted with anti-myc mAbs. Again, specific bands corresponding to the intact B7h-myc protein and cleavage product were detected. These data indicate that shedding of B7h by both activated T cells and PMMA occurs through specific proteolytic cleavage that generates a cleavage product of B7h that is no longer capable of binding to ICOS.
B7h cells was 45.4 and 3.49 after down-regulation. To map the domains of B7h that regulated both PMA- and ICOS-induced B7h shedding on B cells, we generated a series of chimeric molecules between the two major structurally related B7 ligands that are expressed on activated B cells as monomers (32): B7h that undergoes shedding and B7.2 that is resistant to shedding (Fig. 5A). Using structural data for B7.2 and sequence homology for B7h, structural domains for these molecules were identified. Chimeric molecules with swaps between the V-like Ig domain, C-like Ig domain, stalk peptide, transmembrane, and cytoplasmic tail regions of B7h and B7.2 were retrovirally expressed in either cocultured with activated T cells showed only a minimal block by GM6001 treatment that correlated with only a minimal inhibition of sB7h release by either PMA or anti-IgM Ab treatment (shaded) and without treatment (heavy dashed line). In contrast, sB7h release was determined by ELISA with and without PMA treatment for 3 h as described in Materials and Methods. Each bar is the mean of triplicate samples with error bars showing SD.

**FIGURE 6.** ICOS-induced shedding reduces the ability of APCs to co-stimulate IFN-γ and IL-4 cytokine production. Rested effector DO11.10 T cells were incubated with matched A4 and A4/B7h 3T3 cells in the presence of 0.3 μM OVA peptide and analyzed for intracellular IFN-γ and IL-4 cytokine production after 6 h as described in Materials and Methods. A4 and B7h expression (heavy dash) on 3T3 cells and background staining (light dash) are shown before coculture with rested effector DO11.10 T cells. Matched A4 and A4/B7h 3T3 cells were cocultured with ICOS-CHO cells for 3 h, and ICOS-CHO cells were removed to <1% of purified cells by positive MACS bead sorting using a Thy1.2 marker on CHO cells. Levels of A4 and B7h on 3T3 cells are shown after down-regulation with ICOS and before coculture with rested effector DO11.10 T cells. The MFI for A4 was 39.1 and 39.2 on A4 cells and A4/B7h cells before down-regulation and 42.1 and 37.4 after down-regulation. MFI for B7h on A4/B7h cells was 45.4 and 3.49 after down-regulation.

**FIGURE 7.** Ligands for TLR9 and TLR7/8 inhibit B7h down-regulation and shedding on B cells. For flow cytometric histograms, B7h staining with treatment (shaded) and without treatment (heavy dashed line) are shown with background staining levels (light dashed line). A. CpG DNA and compound R-848 specifically inhibit B7h down-regulation. B cells were cultured for 24 h in medium or with medium containing either activating anti-CD40 mAb, non-CpG, CpG DNA, compound R-848, BLP, LPS, LTA, or poly(I:C), as described in the Materials and Methods. Before down-regulation, B7h MFI on these B cell populations was 8.08, 19.3, 14.2, 10.2, 5.1, 3.74, 9.46, 14.9, and 13.9, and after 3 h of PMA treatment, MFI was 2.33, 7.14, 6.61, 7.55, 3.77, 1.51, 5.09, 6.32, and 5.46. B. CpG DNA stabilizes cell surface B7h expression through a MyD88-dependent pathway. B cells from WT or MyD88−/− mice were treated with and without CpG DNA for 24 h. Down-regulation of B7h on these B cells was assessed by flow cytometry 3 h after treatment with either PMA, anti-IgM Ab, or coculture with activated T cells. MFI of B7h on WT B cells, CpG-treated WT B cells, MyD88−/− B cells, and CpG-treated MyD88−/− B cells was 5.20, 6.64, 4.68 and 5.06, respectively, and following PMA treatment, MFI was 2.06, 5.7, 2.09, and 2.12. Following anti-IgM treatment, MFI was 2.41, 6.89, 2.33, and 2.38, whereas after coculture with activated T cells, MFI was 1.12, 2.91, 1.02, and 1.02. C. CpG treatment inhibits basal and induced B7h release. B cells were treated with and without CpG DNA for 20 h, and sB7h release was determined by ELISA with and without PMA treatment for 3 h as described in Materials and Methods. Each bar is the mean of triplicate samples with error bars showing SD.

Differential inhibition of ICOS- and BCR-induced shedding

Because many shedding events that are induced by phorbol esters such as PMA can be differentially inhibited by hydroxamate compounds that bind to the active sites of metalloproteases, we examined whether shedding of B7h could be inhibited by the GM6001 hydroxamate inhibitor that inhibits a broad spectrum of metalloproteases, including the TNF-α converting enzyme (TACE/ADAM17), which cleaves CD40 on B cells (29). Basal shedding of sB7h from unstimulated B cells was completely inhibited by treatment with GM6001, but not a control compound (Fig. 4). Induction of sB7h release by either PMA or anti-IgM Ab treatment also was completely inhibited by GM6001 treatment and correlated with a complete block in B7h down-regulation observed by flow cytometric analysis. In contrast, sB7h release from B cells cocultured with activated T cells showed only a minimal block by GM6001 treatment that correlated with only a minimal inhibition of cell surface B7h down-regulation detected by flow cytometry. Thus, induction of B7h shedding by BCR activation and ICOS binding differed in their sensitivity to inhibition by the hydroxamate compound GM6001. Although BCR- and ICOS-induced shedding appear to involve different sheddases, further insight will require studies using additional protease inhibitors.

**Extracellular stalk peptide regulates shedding**

To map the domains of B7h that regulated both PMA- and ICOS-induced B7h shedding on B cells, we generated a series of chimeric molecules between the two major structurally related B7 ligands that are expressed on activated B cells as monomers (32): B7h that undergoes shedding and B7.2 that is resistant to shedding (Fig. 5A). Using structural data for B7.2 and sequence homology with B7h, structural domains for these molecules were identified. Chimeric molecules with swaps between the V-like Ig domain, C-like Ig domain, stalk peptide, transmembrane, and cytoplasmic tail regions of B7h and B7.2 were retrovirally expressed in either...
B7h−/− or B7.1−/− B7.2−/− B cells to eliminate background staining from endogenous molecules and assayed for down-regulation by flow cytometry.

Chimeric B7.2 molecules could be induced to undergo down-regulation by PMA if they contained the stalk peptide of B7h (Fig. 5B). In chimeric B7.2 molecules retaining the V- and C-like Ig domains of B7.2, the B7.2h-M molecule with the transmembrane and cytoplasmic tail of B7h was still resistant to down-regulation, whereas the B7.2h-S and B7.2h-SΔT molecules with the stalk peptide and transmembrane region of B7h were now down-regulated by PMA treatment. Reciprocally, the chimeric B7h molecules B7h.2-S and B7h.2-C that lack the stalk peptide of B7h were now completely resistant to down-regulation by PMA treatment. These data indicate that the extracellular stalk peptide of B7h is necessary and sufficient to confer down-regulation of B7h and B7.2 by PMA treatment of activated B cells.

When the same chimeric B7 molecules were used to study T cell-induced shedding on B cells (Fig. 5C), none of the chimeric B7.2 molecules that were down-regulated by PMA treatment were down-regulated by coculture with activated or naive T cells expressing CD28. Because these chimeric B7.2 molecules bound to both anti-B7.2 mAbs and CTLA-4-Ig, these data suggest that CD28–B7.2 interactions were unable to substitute for ICOS–B7h interactions in inducing shedding. Although, we do not understand the basis for this difference in activation of shedding, modeling and biophysical characterization indicate that the ICOS–B7h binding surface differs significantly from the CD28–B7.2 binding surface (33), and that ICOS is likely to be functionally bivalent, whereas CD28 is functionally monovalent (32). Analysis of chimeric B7h molecules also showed the requirement for different regions of B7h in ICOS-mediated down-regulation. Replacement of the stalk peptide that rendered the B7h.2-S chimera completely resistant to PMA-induced down-regulation, only partially inhibited ICOS-induced down-regulation by (Fig. 5C). More complete inhibition was achieved only by extending the swap to include the C-like Ig domain of B7.2 in the B7h.2-C chimeric mutant. These results indicate that both the C-like Ig domain and the extracellular stalk peptide of B7h are involved in down-regulation induced by ICOS binding.

The B7h.2-S and B7h.2-C mutants that were progressively resistant to down-regulation on B cells cocultured with activated T cells were also inhibited in release of sB7h (Fig. 5D). Because of differences in the expression levels and transduction efficiency of chimeric molecules on primary B cells, we examined sB7h release using 3T3 cells expressing chimeric molecules where expression levels were carefully matched. In comparison with WT B7h, both basal and ICOS-induced shedding of B7h were inhibited with the B7h.2-S chimera and even further inhibited with the B7h.2-C chimera that displayed the greatest inhibition of B7h down-regulation by flow cytometry.

Shedding reduces costimulation by APCs

To test whether shedding of B7h could play a functional role in regulating costimulatory signals through the ICOS receptor, we first examined whether prior shedding reduced the subsequent ability of APCs to costimulate intracellular cytokine production from resting effector transgenic DO11.10 T cells. B7h expression elicited ICOS-dependent costimulation of both IFN-γ and IL-4 when 3T3 cells expressing equivalent levels of A4d with and without B7h expression were used as APCs (Fig. 6). However, when these A4d- and A4d/B7h-expressing 3T3 cells were first cocultured with ICOS+ CHO cells to induce B7h shedding and restet after purification for their ability to costimulate IFN-γ and IL-4 production, costimulation by A4d cells remained unchanged before and after coculture with ICOS+ cells, whereas costimulation by A4/B7h cells was markedly reduced. These results indicate that decreases in B7h levels induced by shedding are functionally significant and can inhibit the subsequent ability of APCs to costimulate cytokine production from rested effector T cells.

TLR7/8 and TLR9 inhibit shedding

We also examined whether the stability of B7h was regulated by innate signals using a panel of TLR ligands. Treatment with TLR ligands did not markedly alter B7h levels on B cells consistent with a prior study by Zhou et al. (34). However, we identified two ligands that stabilized B7h on B cells: CpG DNA, which activates TLR9 (35), and compound R-848, which activates TLR7/8 (36) (Fig. 7A). B cells pretreated with CpG DNA and R-848 were now resistant to PMA-induced down-regulation, as well as ICOS- and BCR-induced down-regulation of B7h (Fig. 7B) and data not shown). This stabilization of B7h was dependent on TLR signaling, because no stabilization was observed in MyD88−/− B cells treated with CpG DNA. Consistent with the stabilization of cell surface B7h, CpG-treated B cells showed reduced levels of basal and BCR-induced release of sB7h (Fig. 7C). Thus, rapid down-regulation and shedding of B7h on B cells induced by both BCR
activation and coculture with activated T cells can be inhibited by pretreatment with ligands for TLR7/8 and TLR9.

**Shedding-resistant B7h enhances costimulation**

To examine the functional significance of TLR stabilization of B7h, we tested whether ICOS costimulation was enhanced by a shedding-resistant mutant of B7h. We compared intracellular IFN-γ cytokine production from rested effector DO11.10 T cells cocultured with 3T3 cells expressing Aβ and equivalent levels of either WT B7h or the B7h.2-C chimera that was resistant to ICOS-mediated down-regulation and shedding (Fig. 8A). APCs expressing the shedding-resistant B7h.2-C chimera showed enhanced costimulation of intracellular cytokine production over APCs expressing WT B7h. This increase in costimulation of intracellular IFN-γ T cells resulted from expression of stabilized B7h, because addition of a blocking Ab to B7h reduced costimulation by APCs to the level observed with just Aβ expression alone. Interestingly, the enhancement of IFN-γ costimulation by the shedding-resistant B7h was greater at lower concentrations of OVA peptide (an 86% increase over WT at 0.5 μM vs a 40% increase at 2 μM), indicating that inhibition of B7h shedding was more significant at lower Ag concentrations (Fig. 8B). These data suggest that stabilization of B7h on TLR7/8- and TLR9-activated B cells can enhance ICOS receptor signaling on T cells and lead to increased production of IFN-γ.

**Discussion**

Multiple mechanisms have been identified that allow exquisite temporal and spatial control of CD28-B7 costimulatory molecules both before and during T cell–APC contacts (8, 37, 38). In contrast with costimulatory receptors, the study of B7 molecules on APCs has focused to a greater extent on transcriptional regulation that occurs before contact with T cells (26, 39–42), and it is only recently that the regulation of B7 molecules on APCs during T cell contacts has been shown to be a dynamic contributor to modulating costimulatory signals (43). Our results define B7h shedding as a novel mechanism for regulation of CD28-B7 molecules on APCs that occurs during T cell contacts and limits the expression of B7h by proteolytic cleavage.

Shedding of B7h can occur either through a cell-intrinsic pathway following BCR activation or through a cell-extrinsic pathway following binding to ICOS on T cells. B7h ectodomain shedding provides a mechanism that may account for previously reported results showing B7h down-regulation upon ICOS binding and increased B7h levels on human B cells in ICOS-deficient patients (24, 44, 45). Although BCR signaling and ICOS binding both induce rapid shedding of B7h, these pathways differ in terms of their sensitivity to protease inhibitors and their structural requirement for different domains of B7h. Shedding of a cell surface molecule through activation of different sheddase pathways is not unprecedented as TNFR-I is cleaved by both a metalloprotease (TACE) and a serine protease (elastase) (46) in response to TNF-α binding. Further understanding of the mechanistic differences between BCR- and ICOS-induced shedding will require identification of the proteases that regulate B7h shedding.

Our results examining the functional significance of B7h shedding indicate that shedding limits the ability of APCs to costimulate cytokine production from rested effector CD4+ T cells. Prior contact with ICOS+ cells can reduce levels of B7h on APCs sufficiently to markedly inhibit the subsequent ability of these APCs to costimulate IFN-γ and IL-4 production from additional ICOS+ T cells. B7h shedding also plays a functional role during T cell–APC contacts, because a shedding-resistant B7h allowed for enhanced costimulation of IFN-γ production from rested effector CD4+ T cells, compared with WT B7h. These results indicate that rapid shedding of B7h limits the ability of APCs to continue to activate ICOS signaling on T cells, and suggest that sB7h is limited in its ability to activate ICOS signaling. Although IOS is functionally bivalent, B7h, like B7.2, is thought to be present on the cell surface of APCs as a monomer (32). Thus, sB7h would be monovalent and unlikely to activate ICOS signaling through cross-linking.

Our results demonstrating that B7h shedding can modulate functional T cell responses are consistent with studies that report ICOS receptor expression on T cells is correlated with effector function. ICOS expression appears to differ between T cell subsets with CD4+ T cells having higher levels than CD8+ T cells and Th2 cells having higher levels than Th1 cells (11, 47). Further studies have indicated that ICOS-expressing cells can be categorized into low, medium, and high expressers with very different cytokine profiles (48). High-expressing cells were linked to IL-10 cytokine production, with medium expressors linked to IL-4, IL-5, and IL-13 production and low expressors with IL-2, IL-3, IL-6, and IFN-γ, suggesting that ICOS levels may influence the cytokine profile of effector Th cells and their responsiveness to B7h levels on APCs. Thus, it will be of interest to examine whether regulation of B7h expression through shedding also regulates differentiation of Th cells, as well as regulatory T cells and CD8+ T cells.

Both DNA containing unmethylated CpG motifs that activates TLR9 and single-stranded RNA that activates TLR7/8 have been implicated in the production of autoantibodies (30, 49–51). The ability of TLR9 and TLR7/8 to stabilize B7h on B cells is likely to result in the enhanced ability of B cells to induce ICOS signaling during B cell–T cell interactions, because a stabilized mutant of B7h was found to costimulate IFN-γ more effectively than WT B7h. Thus, the inhibition of B7h shedding by TLR9 and TLR7/8 suggests a B cell-extrinsic mechanism that may contribute to the TLR-driven emergence of autoantibodies.

CpG DNA has been shown to directly activate B cells (30). However, this direct activation of B cells is not enough to explain the emergence of pathogenic autoantibody production in disease models. In both SLE and rheumatoid arthritis, pathogenic autoantibodies show the hallmarks of T cell help, including both affinity maturation and class switching (52). Thus, TLR9 and TLR7/8 signaling must involve not only direct cell–intrinsic B cell activation (30, 51) but also cell-extrinsic enhancement of T cell help during autoantibody formation. Our results suggest that this enhancement can occur by TLR-mediated stabilization of B7h.

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**References**


