CD86 and β2-Adrenergic Receptor Signaling Pathways, Respectively, Increase Oct-2 and OCA-B Expression and Binding to the 3′-IgH Enhancer in B Cells*  

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Joseph R. Podolj, Nicholas W. Kin, and Virginia M. Sanders†  
From the Department of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University, Columbus, Ohio 43210

Stimulation of CD86 (formerly known as B7-2) and/or the β2-adrenergic receptor on a CD40 ligand/interleukin-4-activated B cell increased the rate of mature IgG1 transcription. To identify the mechanism responsible for this effect, we determined whether CD86 and/or β2-adrenergic receptor stimulation regulated transcription factor expression and binding to the 3′-IgH enhancer in vitro and in vivo. We showed that CD86 stimulation increased the nuclear localization of NF-xB1 (p50) and phosphorylated RelA (p65) and increased Oct-2 expression and binding to the 3′-IgH enhancer, in a protein kinase C-dependent manner. These effects were lost when CD86-deficient or NF-xB1-deficient B cells were used. CD86 stimulation also increased the level of IgB-α phosphorylation but in a protein kinase C-independent manner. β2-adrenergic receptor stimulation increased CREB phosphorylation, OCA-B expression, and OCA-B binding to the 3′-IgH enhancer in a protein kinase A-dependent manner, an effect lost when β2-adrenergic receptor-deficient B cells were used. Also, the β2-adrenergic receptor-induced increase in the level of mature IgG1 transcript was lost when OCA-B-deficient B cells were used. These data are the first to show that CD86 stimulation up-regulates the expression of the transcription factor Oct-2 in a protein kinase C- and NF-xB1-dependent manner, and that β2-adrenergic receptor stimulation up-regulates the expression of the coactivator OCA-B in a protein kinase A-dependent manner to cooperate with Oct-2 binding to the 3′-IgH enhancer.

Surface receptor stimulation on a B cell that affects class switch recombination (CSR) and production of IgG1 protein

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† Performed this work as part of the dissertation research as predoctoral student in the Department of Cell Biology, Neurobiology, and Anatomy, Loyola University Medical Center, Maywood, Illinois 60153.  
‡ Recipient of National Institutes of Health Training Grant T32 AI55411.  
⊥ To whom correspondence should be addressed: Dept. of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University, 2194 Graves Hall, 333 West 10th St., Columbus, Ohio 43210. Tel.: 614-292-3349; Fax: 614-292-9805; E-mail: Sanders.302@osu.edu.  
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has implications for vaccination protocols, as well as therapies for autoimmune and infectious disease states. The Th2 cell-dependent antibody (Ab) response in vivo is regulated by both immune system- and nervous system-derived ligands that bind to receptors expressed on the surface of a B cell (1). One receptor expressed on the surface of a B cell is CD86 (formerly known as B7-2), which is a costimulatory molecule up-regulated following the stimulation of either the B cell receptor (2, 3), CD40 (4, 5), major histocompatibility complex class II (6), lipopolysaccharide receptor (7, 8), the IL-4 receptor (IL-4R) (9), or the β2-adrenergic receptor (β2AR) (10). CD86 binds to the coreceptor CD28 on an IL-4-producing Th2 cell to increase both the expression of CD40 ligand (CD40L) and the secretion of IL-4. Subsequent engagement of CD40 and the IL-4R on a B cell activates CSR to IgG1 (11–13). Functionally, a decrease in the level of serum IgG1 occurs when the CD86/CD28 interaction is blocked in vivo, an effect initially credited to a lack of CD86 stimulation on a Th2 cell (14, 15). In contrast to this hypothesis, recent in vitro data show that CD86 stimulation on a B cell activated through stimulation of CD40 and the IL-4R increases the level of IgG1 protein (10, 16), mature IgG1 transcript (17), IgE protein (10), and anti-apoptotic genes produced (18). Furthermore, CD86 stimulation increases the level of IgG1 protein and mature IgG1 transcript produced by a CD40L/IL-4-activated B cell via a mechanism that involves an increase in the rate of mature IgG1 transcription, as determined by nuclear run-on, without affecting CSR to IgG1 or mature IgG1 transcript stability (17). In support of these findings, CD86 stimulation does not affect the ability of a B cell to undergo CSR to IgG1, the level of IgG1 produced by donor CD80/CD86-deficient B cells in a chimeric model system in vivo following immunization with a soluble protein antigen was not significantly decreased when compared with recipient wild type B cells (19). In contrast, donor CD40-deficient B cells were unable to undergo CSR to IgG1 in this chimeric model system. These data suggest that the critical signals to induce a B cell to undergo CSR to IgG1 are derived from CD40 and IL-4R stimulation. Taken together, these in vitro and in vivo findings suggest that CD86 stimulation on a B cell increases the level of both IgG1 produced per B cell and B cell survival, without affecting CSR. However, the signaling pathway(s) activated in a B cell following CD86 stimulation remains unknown, even though the short cytoplasmic tail of CD86 contains three putative PKC phosphorylation sites (20).

One nervous system-derived stimulus involved in the regulation of the magnitude of an IgG1 response in vivo is the neurotransmitter norepinephrine (NE), which is released from nerve terminals present in secondary lymphoid tissue following immunization with antigen (21). Norepinephrine binds the β2AR expressed on immune cells (as reviewed by Sanders et al.

23394 This paper is available on line at http://www.jbc.org
This was confirmed recently in vitro when stimulation of the β2AR on a CD40L/IL-4-activated B cell was found to increase the rate of mature IgG1 transcription, without affecting CSR to IgG3 or mature IgG1 transcript stability (17). These findings suggest that βAR stimulation by NE plays a role in regulating B cell activity during a Th2 cell-dependent response in vivo. However, the signaling pathway(s) involved in the β2AR-induced increase in the rate of mature IgG1 transcription remains unknown.

Evidence supports the proposal that the level of mature IgG1 transcript produced by a B cell is controlled by the 3′-IgH enhancer (26), which is composed of four DNAse I hypersensitivity regions, designated hs3A, hs1.2, hs3B, and hs4 (27). Of these four regions, hs1.2 and hs4 appear to have the strongest enhancer activity (28). Contained within both of these hypersensitivity regions are binding sites for a host of transcription factors, including Oct-2, which is a member of the POU family of proteins that bind to an 8-bp octamer sequence (consensus sequence ATGCAAAT) (29). Although Oct-2 by itself is able to activate a limited number of transcriptional activity at an octamer site, full activity occurs only when the coactivator OCA-B is present (28). Oct-2 and OCA-B expression, respectively, are induced in a B cell following CD40 stimulation in an NF-κB-dependent and CREB-dependent manner (30–32), and appear to be necessary for regulating the level of IgG1 produced by a B cell (33–35).

We show here that CD86 and β2AR stimulation, respectively, increase the expression of the transcription factor Oct-2 and its coactivator OCA-B via two distinct signaling pathways but that these two proteins converge at common 3′-IgH enhancer octamer binding sites. Furthermore, we also show that the CD86- and β2AR-induced increase in Oct-2 and OCA-B expression occurs in vivo. The present data are the first to show that CD86 stimulation induces an increase in the nuclear localization of NF-κB (p50) and phosphorylated Rela (p65) and Oct-2 expression via a PKC-dependent pathway. We also show that CD86 stimulation increases IκB-α phosphorylation via a PKC-independent pathway. These data suggest that CD86-induced PKC activity is distal to IκB-α phosphorylation. We also show that β2AR stimulation on a B cell activates CREB and increases OCA-B expression through a PKA-dependent pathway. Taken together, these data show that an immune receptor-induced signaling pathway cooperates with a nervous system receptor-induced signaling pathway to regulate the rate of mature IgG1 transcription.

**EXPERIMENTAL PROCEDURES**

**Animals**—Mice were housed under pathogen-free conditions and were used at 7–8 weeks of age. Female BALB/c (H-2d-restricted), FVB (H-2a-restricted), and Scid/ICR (H-2b-restricted) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, all mice were housed at The Ohio State University in microisolation cages within a laminar flow barrier and provided autoclaved food and water ad libitum. Spleens from OCA-B−/− mice were kindly provided by Dr. Laurel Eckhardt (Hunter College, City University of New York, New York) and by Dr. Michel Nussenzweig (The Rockefeller University). All experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

**Resting B Cell Isolation and Activation**—Spleens were collected from nonimmunized mice, and red blood cells were lysed with 0.4% ammonium chloride solution. Splenocytes were incubated with anti-mouse CD43 magnetic beads following the manufacturer's directions (Miltenyi Biotec, Auburn, CA), and CD43-negative cells were collected by using AutoMacs (Miltenyi Biotec). Resting B cells were cultured at 5 × 10^6 cells/ml of culture medium, which consisted of RPMI 1640 medium (CellGro, Herndon, VA), 10% FBS (Atlas Biologicals, Colorado Springs, CO), 50 mM HEPES, 100 μM penicillin, 100 μM streptomycin, 2 mM glutamine, and 50 μM 2-mercaptoethanol in 24-well plates (Costar, Corning, NY) in a humidified atmosphere at 37°C with 5% CO2. Resting B cells were activated in the presence of CD40 ligand-expressing SF9 cells (CD40L), prepared as described previously (17), at a B cell to SF9 cell ratio of 10:1 and IL-4 (1 ng/ml (eBioscience, San Diego, CA)) in the absence or presence of the β2AR agonist terbutaline (10−4 M (Sigma)). After 16 h, either an anti-CD86 Ab (clone PO3 (eBioscence)) or a species- and isotype-matched control Ab (rat IgG2×, κ, clone A95-1 (Pharmingen, San Diego)) was added at a final concentration of 1 μg/ml. For resting B cell cultures in which the cells received either a βAR-specific agonist or an inhibitor, the cells were pretreated for 30 min at 37°C in a culture medium with or without the specific agonist norepinephrine (10−5 M (Sigma), PKA inhibitors H-89 (5 μM (Biomol, Plymouth Meeting, PA)) and KT5720 (0.5 μM (U. S. Biological, Swampscott, MA)), or PKC inhibitors calphostin C (0.5 μM (Biomol)) or GF-109203X (5 μM, (A. G. Scientific, Inc., San Diego)). B cells were then collected, washed three times with culture medium, and activated as described above. All reagents used for resting B cell isolation and activation were negative for the presence of endotoxin, as determined by Etoxate (Sigma), a Limulus lysate assay with a level of detection <0.1 unit/ml.

In Vivo Activation of B Cells—Chemical sympathectomy was performed at 8 weeks of age. Mice were administered 200 mg/kg 6-hydroxydopamine (6-OHDA) in a volume of 100 μl of 0.9% NaCl containing 100 μM (Sigma) as ascorbate and an antioxidant on three alternating days (days −6, −4, and −2 before cell reconstitution), as described previously (24). Two days following the last administration of 6-hydroxydopamine, all animals received 2 × 10^6 resting B cells in 100 μl of PBS intravenously in the lateral tail vein. Two weeks following B cell reconstitution, mice were administered intra-peritoneally anti-CD40 Ab (500 μg (clone F GK45)) and IL-4 (500 ng (eBioscience)) plus or minus terbutaline (5 μg/ml terbutaline, 100 μg/ml norepinephrine) (Sigma). Mice were then administered orifice samples were collected 7 and 14 days following the initial injection of CD40 Ab and IL-4. The level of serum IgG1 was determined by ELISA in various dilutions of serum samples, as described previously (17).

**Western Blot**—Resting B cells (10 × 10^6 cells) were activated as described above. For the collection of nuclear protein-enriched lysates, B cells were collected, washed three times in PBS, and centrifuged at 500 × g for 5 min at 4°C. The B cell pellets were resuspended by gentle pipetting in cold lysis buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol), and the B cells were allowed to swell for 30 min at 4°C. Plasma membranes were lysed by the addition of 10% IGEPA-CS-630 (Sigma) and vortexed for 15 s, and nuclei were collected by centrifugation for 5 min at 3300 × g. Nuclear pellets were resuspended in lysis buffer B (20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) and centrifuged for 15 min at 4°C. Samples were centrifuged for 5 min at 5000 × g. The nuclear protein-enriched supernatants were collected and frozen at −80°C until analysis. For total cellular protein, cells were collected, washed three times with PBS, lysed with 500 μl of 1× lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol), and 1 mM dithiothreitol was added for 15 min at 4°C. Samples were centrifuged at 5 min for 5000 × g. The nuclear protein-enriched supernatants and dithiothreitol were collected and frozen at −80°C until analysis. Protein samples (5–10 μg) were run on a denaturing 7.5% polyacrylamide gel and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with TBST (20 mM of 5% NaCl, 25 mM of 1× Tris-HCl (pH 7.5), 0.2% of 0.05% Tween-20) for 1 h at room temperature, washed three times with TBST for 5 min at room temperature. Membranes were probed with horseradish peroxidase-labeled secondary antibodies diluted in TBST + 5% dried milk at 1:10000.
room temperature for 1 h and washed three times in TBST. Following the last wash, horseradish peroxidase-labeled antibodies were detected using the LumiGlo Detection Kit (Cell Signaling, Inc., Beverly, MA), and specific bands were visualized on Kodak Biomax MS film using an intensifying screen enabled film cassette. Antibodies used were anti-Oct-2 antibody (C-20), anti-oCA-B antibody (C-20), anti-c-Rel antibody (C-11), anti-Oct-1 antibody (C-21) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IgB-α antibody, anti-phospho-IκB (Ser-32) antibody, or anti-phospho-p65 (Ser-536) (Cell Signaling Technology).

Chromatin Immunoprecipitation (ChIP)—ChIP analysis was carried out essentially as described previously (36). B cells (10 × 10^6 cells) activated as described above were collected on day 3 and fixed for 20 min using 10% formaldehyde. After washing with a PBS buffer (50 mM HEPES (pH 7.6), 1 mM EDTA, 0.7% sodium deoxycholate, 1% Nonidet P-40, 0.25% Triton X-100, and the following protease inhibitors: 1 mg/ml leupeptin and 5 mg/ml aprotinin), and gently rocked for 10 min at 4 °C, the nuclei were pelleted, resuspended, and gently rocked for 10 min at room temperature in buffer 2 (0.2 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM Tris-HCl (pH 8.0)), and protease inhibitors). The nuclei were pelleted again and resuspended in 6 ml of sonication buffer (1 mM EDTA, 0.5 mM EGTA, and 10 mM Tris-HCl (pH 8.0)) and protease inhibitors). The suspension was sonicated 10 times for 30 s, with a 1-min cooling period on ice between times. Debris was removed from samples, and 250 μl was adjusted to 1% Triton X-100, 0.1% sodium deoxycholate, and protease inhibitors in a final volume of 500 μl of TE buffer (10 mM Tris (pH 8) and 1 mM EDTA) and precleared with protein A/protein G-agarose beads that had been blocked with sonicated salmon sperm DNA and 10 mg/ml bovine serum albumin for 3 h with gentle rocking at 4 °C. The beads were removed, and chromatin samples were incubated at 4 °C with various antibodies overnight. Immunocomplexes were precipitated for 3 h by the addition of blocked protein A/protein G-agarose beads. The precipitates were washed seven times for 5 min each with 1 ml of RIPA buffer (50 mM HEPES (pH 7.6), 0.7% sodium deoxycholate, 1% Nonidet P-40, 0.5 mM LiCl, and protease inhibitors) and resuspended in 100 μl of TE buffer. The samples were adjusted to 0.5% SDS, 100 μg/ml RNase A, and 200 μg/ml of protease K and incubated at 55 °C for 3 h, followed by an overnight incubation at 65 °C to reverse the formaldehyde cross-links. The DNA was purified by phenol/chloroform extraction, precipitated in the presence of 20 μg of glycogen, and resuspended in 100 μl of TE buffer.

PCR was done with 2 μl of the immunoprecipitated DNA for 30 cycles (45 s at 95 °C, 45 s at 56 °C, and 2 min at 72 °C), completed by 10 min at 72 °C with various primers. As a control, the PCR was done directly on input DNA purified from chromatin before immunoprecipitation. PCR conditions were resolved on 1.5% agarose gels and visualized with ethidium bromide. The antibodies used were anti-Oct-2 and anti-oCA-B and anti-p50 as a control Ab (Santa Cruz Biotechnology). The level of consens oligonucleotide was labeled with γ−3P in the following labeling reaction: consensus oligonucleotide (5′-AATTGGAGGGCAGGTCGCG-3′, Promega, Madison, WI), as described previously (37). Briefly, the NF-κB-specific consens oligonucleotide was labeled with γ−3P in the following labeling reaction: consensus oligonucleotide (1.75 pmol/μl), T4 polynucleotide kinase 10X buffer, [γ−3P]ATP (2000 Ci/mole at 10 mCi/ml (American Biosciences)), nuclelease-free H2O, and T4 polynucleotide kinase (5–10 units/μl (Promega)) in a final volume of 10 μl. The oligonucleotide labeling reaction was incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.5 μl TE and TE buffer. Nuclear protein samples (5 μg) were incubated in 5X binding buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 mg/ml poly(dI-dC)poly(dI-dC) (Amersham Biosciences) in the absence or presence of either an unlabeled consensus oligonucleotide or NF-κB family member-specific antibody for 15 min at room temperature. Antibodies were used anti-p50 antibody (H-119), anti-p65 antibody (C-20), and anti-c-Rel antibody (N) (Santa Cruz Biotechnology). Labeled consensus oligonucleotide was then added; samples were incubated at room temperature for 20 min, and shift products were separated by electrophoresis and visualized on Kodak Biomax MS film (Fisher) using an intensifying screen-enabled film cassette (Fisher) for 4 h at −80 °C.

Statistics—Data were analyzed by a one-way analysis of variance followed by post hoc analysis to determine whether an overall statistically significant change existed following activation.

RESULTS

CD86 and β2AR Stimulation Increase Oct-2 and oCA-B Expression, Respectively—Previous data from our laboratory using nuclear run-on analysis showed that the rate of mature IgG1 transcription is increased in CD40L/IL-4-activated B cells following either CD86 stimulation with an anti-CD86 Ab or β2AR stimulation with the agonist terbutaline, and that this level is increased further when both receptors are stimulated (17). Because the expression of Oct-2 and its coactivator oCA-B appears to be necessary for IgG1 protein and mature IgG1 transcript production (35), the level of Oct-2 and oCA-B expression was determined following CD86 and/or β2AR stimulation on CD40L/IL-4-activated B cells. When the level of Oct-2 expression was measured in CD40L/IL-4-activated B cells in the absence or presence of an anti-CD86 Ab, the level of Oct-2 was increased 2.5-fold following CD86 stimulation as compared with B cells activated in the presence of a control Ab. Likewise, the level of Oct-2 expression did not increase above the control Ab group in the presence of terbutaline or when CD86−/−B cells were used (Fig. 1, a and b). When both CD86 and the β2AR were stimulated, the level of Oct-2 expression was increased only to the level induced following CD86 stimulation alone.

Although Oct-2 is able to regulate transcriptional activity at octamer sequences, full activity occurs only when oCA-B is present (28). When the level of OCA-B expression was measured in CD40L/IL-4-activated B cells in the absence or presence of terbutaline, the level of OCA-B was increased 2.5-fold following β2AR stimulation as compared with B cells activated in the presence of a control Ab. Likewise, the level of OCA-B expression did not increase above the control Ab group in the presence of an anti-CD86 Ab or when β2AR−/−B cells were
used (Fig. 1, a and c). When both CD86 and the \( \beta_2 \)AR were stimulated, the level of OCA-B expression was increased only to the level induced following \( \beta_2 \)AR stimulation alone. Therefore, stimulation of CD86 and the \( \beta_2 \)AR, respectively, on a CD40L/IL-4-activated B cell appears to increase the expression of Oct-2 and its coactivator OCA-B.

**CD86 and \( \beta_2 \)AR Stimulation, Respectively, Increase Oct-2 and OCA-B Binding to the 3'-IgH Enhancer**—Because Oct-2/OCA-B can bind to octamer sites other than those contained in the 3'-IgH enhancer, we sought to determine whether the increase in Oct-2 and OCA-B protein expression results in increased association of these two proteins to the 3'-IgH enhancer using ChIP. PCR primer sets were designed to amplify regions of DNA that are known to contain octamer sequences and to act as strong enhancer sequences in transient transfection assays when mature B cells were used (28). When the level of Oct-2 or OCA-B bound to octamer sites contained within the 3'-IgH enhancer was analyzed by semi-quantitative PCR, the amount of Oct-2- or OCA-B-specific PCR product for each sample was determined by subtracting out the nonspecific PCR product from samples that received no Ab during the immunoprecipitation and normalized to the amount of input DNA. Data are presented as the mean fold increase in Oct-2 or OCA-B binding ± S.E. from three independent experiments with a representative gel inlay.
affect the level of OCA-B binding and terbutaline did not affect the level of Oct-2. Likewise, when both CD86 and the β2AR were stimulated, the level of Oct-2 or OCA-B was increased only to the level induced following stimulation of either receptor alone. Therefore, CD86 and β2AR stimulation, respectively, on a CD40/IL-4-activated B cell appears to increase the level of Oct-2 and OCA-B that is bound to octamer sites contained within the hsl1,2 and hsl4 regions of the 3′-IgH enhancer.

Lack of OCA-B in B Cells Prevents the β2AR-Induced Increase in Mature IgG1 Transcription—To determine whether the β2AR-induced increase in nuclearly localized OCA-B is necessary for expression of the β2AR-induced increase in mature IgG1 transcript, B cells from wild type and OCA-B−/− mice were activated in vitro. As shown in Fig. 2a, the addition of an anti-CD86 Ab or terbutaline to wild type CD40/IL-4-activated B cells increased the level of mature IgG1 transcription 2-fold above that produced by B cells activated in the presence of a control Ab, as reported previously (17). When B cells from OCA-B−/− mice were activated, stimulation of CD86 induced a lower increase in the level of mature IgG1 transcript as compared with the level induced in wild type B cells, and this level was not increased further by the addition of terbutaline. These results were expected, because the coactivator OCA-B is no longer present to allow for optimal Oct-2 activity. Likewise, the addition of terbutaline to CD40/IL-4-activated OCA-B−/− B cells was unable to increase the level of mature IgG1 transcription above the level induced by CD40/IL-4 and a control Ab alone (Fig. 2a). In contrast, B cells from either wild type or OCA-B−/− mice produced similar levels of IgG1 (Fig. 2b). Therefore, although CD40/IL-4-activated B cells from OCA-B−/− mice in the absence or presence of an anti-CD86 Ab or terbutaline are able to undergo CSR to IgG1, OCA-B appears to be necessary for the optimal level of mature IgG1 transcription following CD86 stimulation and essential for expression of the β2AR-induced increase in the mature IgG1 transcription.

CD86 and β2AR Stimulation, Respectively, Increase Oct-2 and OCA-B Expression in Vivo and in vitro—To assess whether the CD86- and β2AR-induced increase in nuclearly localized Oct-2 and OCA-B protein was because of either an increase in the translocation of pre-existing Oct-2 and OCA-B protein located in the cytoplasm or to newly synthesized protein resulting from CD86- and β2AR-induced Oct-2 and OCA-B transcription, quantitative real time PCR was performed. When the level of Oct-2 or OCA-B transcript was analyzed in CD40/IL-4-activated B cells in the absence or presence of either an anti-CD86 Ab or terbutaline, respectively, the level of Oct-2 transcript increased 2-fold (Fig. 4a) and the level of OCA-B transcript increased 2-fold (Fig. 6a) as compared with B cells activated in the presence of a control Ab. In contrast, neither anti-CD86 Ab nor terbutaline was able to affect the level of the reciprocal transcript, i.e. anti-CD86 Ab did not affect the level of OCA-B transcript and terbutaline did not affect the level of Oct-2 transcript. Likewise, when both CD86 and the β2AR were stimulated, the level of Oct-2 transcript or OCA-B transcript was increased only to the level induced following stimulation of either receptor alone.

To determine whether the stimulation of CD86 and the β2AR induces the same effect on the level of Oct-2 and OCA-B expressed by B cells activated in vivo, we designed a model system in which we controlled the activation of B cells alone. In this model system, scid mice were first depleted of NE to remove the endogenous ligand for the β2AR. These mice were then reconstituted with resting B cells isolated from non-immunized wild type mice. The B cells were activated by the intraperitoneal injection of an anti-CD40 Ab and IL-4, plus or minus an isotype- and species-matched control Ab, an anti-CD86 Ab, and/or terbutaline. When serum was collected on day 14 post-activation, the level of IgG1 was increased 3-fold following CD86 stimulation and 2-fold following β2AR stimulation as compared with mice that received a control Ab, and this level was further increased by 6-fold when both CD86 and the β2AR were stimulated (Fig. 3a). Likewise, the levels of mature IgG1 transcript produced by splenocytes from these mice (Fig. 3b, open bars) positively correlated with the increased level of serum IgG1 protein. The levels of Oct-2 and OCA-B transcript and protein were also analyzed in total splenocytes. The levels of splenic Oct-2 transcript (Fig. 3b, gray bars) and OCA-B transcript (Fig. 3b, black bars) and protein (Fig. 3, c and d) were increased following administration of anti-CD86 Ab or terbutaline, respectively, as compared with the levels present in total splenocytes from mice that received a control Ab. These changes in transcript and protein are B cell specific because Oct-2 and OCA-B are reported to be expressed in B cells only (29, 38). Therefore, the level of mature IgG1 transcript and IgG1 protein increased by anti-CD86 Ab or terbutaline appears to be associated with a similar increase in the level of Oct-2 and OCA-B transcript produced in vitro and in vivo.
CD86-induced Increase in Oct-2 Is PKC-dependent—Because the cytoplasmic tail of CD86 contains three putative PKC phosphorylation sites, we sought to determine whether PKC activation is necessary for CD86 stimulation to induce an increase in Oct-2 transcription (20). When CD40L/IL-4-activated B cells were pretreated with either PKC inhibitors (calphostin C or GF-109203X) or PKA inhibitors (H-89 or KT5720), the CD86-induced increase in Oct-2 transcription was blocked by the pretreatment with the PKC inhibitors and not with the PKA inhibitors (Fig. 4b). To ensure that the lack of an increase in Oct-2 transcription following CD86 stimulation and pretreatment with PKC inhibitors was not because of a decrease in the level of CD86 expression (37), fluorescence-activated cell sorter analysis was used to show that the level of CD86 expression on CD40L/IL-4-activated B cells was similar between wild type and p50−/− B cells (data not shown). Therefore, p50 expression appears to be necessary for the CD86-induced increase in Oct-2 transcription.

Because p50 expression appears to be necessary for the CD86-induced increase in Oct-2 expression and NF-κB remains inactive when bound to unphosphorylated IκB-α, we sought to determine whether stimulation of CD86 on CD40L/IL-4-activated B cells increases the level of IκB-α phosphorylation and subsequent degradation. As shown in Fig. 4d, CD86 stimulation on CD40L/IL-4-activated B cells increases the level of phosphorylated IκB-α over a 45-min period following CD86 stimulation while decreasing the level of total IκB-α over the same period. Because the above data in Fig. 4b showed that PKC appears to be involved in the CD86-induced increase in the level of Oct-2 transcript, B cells were pretreated with PKC inhibitors and then activated in the absence or presence of an anti-CD86 Ab and CD40L/IL-4. The addition of either the PKC inhibitor GF-109203X (Fig. 4d) or calphostin C (data not shown) was unable to block completely the increase in the level of phosphorylation and degradation of IκB-α induced by CD86 stimulation. Collectively, these data show that the CD86-induced increase in Oct-2 transcript appears to be dependent upon p50 expression and PKC activation, whereas the CD86-induced activation of phosphorylation and degradation of IκB-α is PKC-dependent.

CD86 and β2AR stimulation, respectively, increase serum IgG1 and mature IgG1 transcript in vivo. Scid mice that were depleted of peripheral NE by chemical sympathectomy were reconstituted with 2 × 10⁶ resting splenic B cells. B cells were activated by the intraperitoneal administration per mouse of anti-CD40 Ab (500 μg) and IL-4 (500 ng) in the absence or presence of terbutaline (5 mg) in PBS. Twenty-four hours following anti-CD40 Ab/IL-4/Terb administration either an anti-CD86 Ab (clone GL1) or a species- and isotype-matched control Ab (500 μg) was injected intraperitoneally. a, serum samples were collected on day 14 following the administration of anti-CD40 Ab/IL-4/Terb, and the level of serum IgG1 was determined by enzyme-linked immunosorbent assay. b, on day 14 following the administration of anti-CD40 Ab/IL-4/Terb, RNA was isolated from total splenocytes, and the levels of mature IgG1 transcript, Oct-2 transcript, and OCA-B transcript were analyzed by real time PCR. Data are presented as the fold increase in transcript above B cells activated with an anti-CD40 Ab, IL-4, and a species- and isotype-matched control Ab. In contrast to wild type B cells, when B cells from above that produced by B cells activated in the presence of a control Ab. To ensure that the lack of an increase in Oct-2 transcription following CD86 stimulation was not because of a decrease in the level of CD86 expression (37), fluorescence-activated cell sorter analysis was used to show that the level of CD86 expression on CD40L/IL-4-activated B cells was similar in either the absence or presence of the PKC inhibitors (data not shown).

Because Oct-2 mRNA expression has been reported to be regulated by NF-κB activation (30), we sought to determine whether the CD86-induced increase in Oct-2 transcription is dependent upon NF-κB1 (p50) expression. As shown in Fig. 4c, the addition of an anti-CD86 Ab to wild type CD40L/IL-4-activated B cells increased the level of Oct-2 expression ~2-fold above that produced by B cells activated in the presence of a control Ab. In contrast to wild type B cells, when B cells from either CD86−/− or p50−/− mice were activated in a similar manner, the addition of an anti-CD86 Ab was unable to induce an increase in the level of Oct-2 transcript above that expressed by CD40L/IL-4-activated B cells that received a control Ab. To
induced increase in the phosphorylation and degradation of IκB-α appears to be independent of PKC activation. Therefore, these findings suggest that the CD86-induced increase in the level of Oct-2 expression may involve the activation of two independent pathways in which there is a PKC-dependent step that is distal to IκB-α phosphorylation.

**CD86-induced Increase in NF-κB1 (p50) and Phosphorylated RelA (p65) Nuclear Localization Is PKC-dependent**—Because the above data in Fig. 4b showed that PKC appears to be involved in the CD86-induced increase in the level of Oct-2 transcript, and because the data in Fig. 4d showed that PKC appears to not be involved in the CD86-induced increase in IκB-α phosphorylation, we sought to determine whether PKC activation following CD86 stimulation was indeed distal to IκB-α phosphorylation. As shown in Fig. 5a, the overall level of NF-κB present in the nucleus of B cells activated in the presence of CD40L/IL-4 is either in the presence of an anti-CD86 Ab or a control Ab appears to be relatively equal for both treatment groups. In contrast, when the individual NF-κB family members were analyzed by supershift, a differential ratio of the NF-κB family members present in the nucleus was found. CD86 stimulation on CD40L/IL-4-activated B cells increases the level of p50 and p65 present in the nucleus 30 min following CD86 stimulation, as indicated by the disappearance of a shift product when the nuclear protein samples were incubated with either an anti-p50 Ab or anti-p65 Ab. In contrast, B cells that did not receive an anti-CD86 Ab had a higher level of c-Rel present in the nucleus, as compared with B cells that did receive an anti-CD86 Ab. When B cells were pretreated with the PKC inhibitor GF-109203X, the inhibition of PKC was able to block the CD86-induced increase in p50 and p65 nuclear localization. Published data indicate that although p65/p50 is released from IκB-α and translocates to the nucleus, p65 must be phosphorylated in order to have transactivating activity (39). As shown in Fig. 5, b and c, stimulation of CD86 induces an increase in the level of p50 and phosphorylated p65 located in the nucleus, an effect lost when B cells were pretreated with the PKC inhibitor GF-109203X. To confirm further the findings in Fig. 5a, the level of nuclearly localized c-Rel was also analyzed. As shown in Fig. 5, b and c, the level of nuclearly localized c-Rel is not increased following CD86 stimulation. As a loading control for the nuclear protein samples, the level of the ubiquitously expressed transcription factor Oct-1 was analyzed (40). Therefore, these findings suggest that although the CD86-induced increase in IκB-α phosphorylation is PKC-independent, the CD86-induced increase in nuclearly localized p50 and phosphorylated p65 appears to be PKC-dependent.

**β2AR-induced Increase in OCA-B Is PKA-dependent**—Because βAR stimulation is known to activate PKA (23), we sought to determine whether the β2AR-induced increase in OCA-B transcription is PKA-dependent. When CD40L/IL-4-activated B cells were pretreated with either of the PKA inhibitors (H-89 or KT5720) or the PKC inhibitors (calphostin C or GF-109203X), the β2AR-induced increase in OCA-B transcription was blocked only with the pretreatment with the PKA inhibitors (Fig. 6b). Because the activity of the OCA-B promoter is known to be dependent on CREB activation, and because CREB activation can occur via PKA, we sought to determine whether β2AR stimulation induced an increase in CREB phosphorylation in a PKA-dependent manner. As shown in Fig. 6c, the level of phosphorylated CREB (pCREB) is increased 30 min after the addition of terbutaline to CD40L/IL-4-activated B cells, as compared with B cells activated in the presence of the control Ab or an anti-CD86 Ab. Likewise, when both CD86 and the β2AR were stimulated, the level of pCREB was increased only to the level induced following β2AR stimulation alone. Because the data in Fig. 6b showed that PKA appears to be involved in the β2AR-induced increase in the level of OCA-B transcript, B cells were pretreated with PKA inhibitors or the βAR antagonist naldol and then activated with CD40L/IL-4 in the absence or presence of terbutaline. As shown in Fig. 6d, the addition of terbutaline to CD40L/IL-4-activated B cells in-
creases the level of pCREB in a concentration-dependent manner. In contrast, when CD40L/IL-4-activated B cells were pretreated with the βAR-antagonist nadolol, or the PKA inhibitors H-89 or KT5720, the β2AR-induced increase in pCREB was blocked. Therefore, β2AR stimulation of CD40L/IL-4-activated B cells appears to increase the level of pCREB and OCA-B transcription in a PKA-dependent manner.

DISCUSSION

Previous data from our laboratory showed that stimulation of CD86 and/or the β2AR increases the rate of mature IgG1 transcription and the level of protein produced, without affecting CSR (17). The present in vitro and in vivo data show that stimulation of CD86 or the β2AR, respectively, on a CD40L/IL-4-activated B cell induces an increase in Oct-2 and OCA-B binding to the 3′-IgH enhancer, a process known to regulate mature IgG1 transcription (26, 28). Evidence to show that Oct-2 and OCA-B expression and 3′-IgH enhancer activity selectively affect mature IgG1 transcription versus CSR to IgG1 includes the findings that Oct-2 and OCA-B expression and 3′-IgH enhancer activity selectively affect mature IgG1 transcription versus CSR to IgG1; includes the findings that Oct-2 and OCA-B expression and 3′-IgH enhancer activity selectively affect mature IgG1 transcription versus CSR to IgG1, the CD86- and β2AR-induced effect on mature IgG1 transcription is
Fig. 6. β2AR stimulation increases OCA-B transcription and CREB phosphorylation. Resting B cells (5 × 10⁵ cells/ml) were incubated in culture medium alone or in the presence of either the βAR-selective antagonist nadolol (10⁻⁶ M), PKA inhibitors (H-89 (5 μM) or KT5720 (0.5 μM)), or PKC inhibitors (calphostin C (0.5 μM) or GF-109203X (5 μM)). Cells were collected after 30 min and then activated in the presence of SF9/CD40L cells (1:10) and IL-4 (1 ng/ml) in the absence or presence of terbutaline (Terb, 10⁻⁶ M) at time 0. Sixteen hours following the CD40L/IL-4/terbutaline activation, either an anti-CD86 Ab (clone PO3) or a species- and isotype-matched control Ab (1 μg/ml) was added. a and b, on day 2 following the CD40L/IL-4/terbutaline activation, total RNA was collected and analyzed by quantitative real time PCR for the presence of OCA-B. Data are presented as the mean fold increase in OCA-B expression ± S.E. from three independent experiments. An asterisk indicates a p value <0.05 in comparison to the control Ab group. c, nuclear enriched protein samples were collected 30 min following the CD40L/IL-4/terbutaline activation (10⁻⁶ M). The level of nuclear enriched protein was collected 30 min following stimulation of the β2AR and CD86, and the level of phosphorylated CREB (pCREB) was analyzed. Data are presented as the mean fold increase in pCREB ± S.E. from three independent experiments. d, nuclear enriched protein samples were collected 30 min following the CD40L/IL-4/terbutaline activation (10⁻⁶ M). The level of pCREB was analyzed as stated above.

Our data also show that the CD86-induced increase in Oct-2 and the β2AR-induced increase in OCA-B are not merely in vitro artifacts. As mentioned in the Introduction, the loss of β2AR stimulation in vivo decreases the serum level of antigen-specific IgG₁, the formation of germinal centers in the spleen, and the level of antigen-induced up-regulation of CD86 on splenic B cells (24), a molecule that may also influence the magnitude of the IgG₁ response (10, 16–19). For example, previous reports (42) indicate that CD86 expression by a B cell is necessary for the formation of germinal centers so that optimal IgG₁ production will occur in vivo. Thus, the mechanisms responsible for the decrease in serum antigen-specific IgG₁ in mice depleted of NE (24, 37) may include either a loss of the direct effect of β2AR stimulation to increase mature IgG₁ transcription, a loss of the indirect effect of β2AR stimulation to up-regulate CD86 expression and subsequent germinal center formation, or a combination of both. However, another possible mechanism that may explain the decrease in serum IgG₁ in mice depleted of NE is also suggested by the present data. If our in vivo data are correct that CD86 stimulation increases Oct-2 expression and β2AR stimulation increases OCA-B expression, then the level of Burkitt’s lymphoma receptor-1 (b1r-1) may be decreased in the absence of NE. Burkitt’s lymphoma receptor-1 is a chemokine receptor whose expression is regulated by Oct-2 and OCA-B, and b1r-1 expression has been shown to be necessary for germinal center formation (43, 44). If Oct-2 and OCA-B are required to up-regulate b1r-1 expression, then the loss of the β2AR-induced increase in OCA-B expression, as well as the loss of the indirectly induced increase in Oct-2 by β2AR-induced up-regulation of CD86, may be related to a lower level of b1r-1 expression and, consequently, the decrease in germinal center formation. Therefore, the loss of β2AR stimulation in the absence of NE would prevent optimal OCA-B, Oct-2, and CD86 expression from occurring and thus may prevent optimal germinal center formation, CSR to IgG₁, and mature IgG₁ transcription and protein production in vivo.
We show that the stimulation of the β2AR on a CD40L/IL-4-activated B cell in vitro and in vivo induced a direct signal to a B cell to activate PKA, phosphorylate CREB, and increase OCA-B expression and binding to the 3′-IgH enhancer. OCA-B is a transcription factor coactivator that is unable to bind DNA and regulate transcriptional activity at an octamer site in the absence of a transcription factor such as Oct-2 (28). Because stimulation of both CD86 and the β2AR induced an additive increase in the rate of mature IgG1 transcription (17), and because β2AR stimulation increased the expression of the coactivator OCA-B, we reasoned that the stimulation of CD86 might induce an increase in Oct-2 expression. The current data show that this is true. However, the question remains as to how the independent stimulation of either CD86 or the β2AR on a CD40L/IL-4-activated B cell induces an increase in the rate of mature IgG1 transcription because the stimulation of one receptor increases the level of the transcription factor Oct-2, whereas the stimulation of the other receptor increases the level of the coactivator OCA-B. The answer may lie in the fact that both Oct-2 and OCA-B expression are induced following CD40 stimulation alone (32, 35), and therefore, CD40 signaling may provide a non-rate-limiting supply of Oct-2 and OCA-B in CD40 stimulation alone (32, 35), and therefore, CD40 signaling that both Oct-2 and OCA-B expression are induced following activation. The finding that CD40L/IL-4-activated B cells begin to undergo CSR to IgG1 in vitro is primarily because of the inclusion of an agonist to stimulate the β2AR family members p50, p65, c-Rel, and RelB (45). Although all of the NF-κB family members appear to be activated following CD40 stimulation, the RelB/p50 heterodimer appears to regulate IκB1 transcription in conjunction with STAT6 (11–13). Therefore, our data indicate that CD86 stimulation does not induce the NF-κB family members that regulate IκB1 transcription. The difference between the ability of CD40 and CD86 stimulation to regulate IκB1 versus Oct-2 production may also be related to the time at which CD86 is stimulated on a B cell in relation to when CD40 and the IL-4R are stimulated. Because we add the anti-CD86 Ab at 16 h following the initial activation of the B cell with CD40L/IL-4, this amount of time between the CD40- and CD86-induced NF-κB may be long enough to dissociate the CD40-induced effect on IκB1 from the CD86-induced effect on Oct-2 expression. The finding that CD40L/IL-4-activated B cells begin to undergo CSR to IgG1 by the time the anti-CD86 Ab is added in the present study (46) supports this possibility further.

The contribution of the present findings to the understanding of IgG1 production in vivo is primarily because of the inclusion of an agonist to stimulate the β2AR and an anti-CD86 Ab to stimulate CD86 on a B cell in vitro. In this manner, the endogenous microenvironment in which a B cell is activated in vivo is more closely mimicked in vitro. For example, during a Th2 cell-dependent Ab response in vivo, CD40L is up-regulated and IL-4 is secreted by a Th2 cell to stimulate CD40 and the IL-4R, respectively, on a B cell. Also during a Th2 cell-dependent Ab response in vivo, NE is released from sympathetic nerve terminals in the spleen following antigenic challenge (21) to stimulate the β2AR expressed on a B cell (22). As mentioned above, β2AR stimulation on a B cell increases the expression of

![Fig. 7. Model. Stimulation of CD86 increases the phosphorylation/degradation of IκBα, which is independent of PKC activation. In contrast, the CD86-induced increase in p50 and phosphorylated p65 nuclear localization and Oct-2 expression is dependent upon PKC activation, suggesting that CD86 stimulation may activate two separate signaling pathways. Stimulation of the β2AR has been shown to increase the level of intracellular cAMP. We show here that stimulation of the β2AR increases the level of phosphorylated CREB (pCREB) and OCA-B expression, which is dependent upon PKA activation. The increase in Oct-2/OCA-B expression correlates with an increase in their binding to hs1.2 and hs4 octamer sites.](http://www.jbc.org/)

![Image](http://www.jbc.org/)
CD86, which our data suggest would not only allow for increased costimulation of a Th2 cell but would also allow for an enhanced direct signal to be delivered to a B cell through CD86. Previously reported data from our laboratory and others show that stimulation of either CD86 or the $\beta_2$AR alone on a CD40L/IL-4-activated B cell increases the amount of IgG1 produced per B cell by ~2-3-fold (10, 17, 19) and that an additive increase occurs when both receptors are stimulated (10, 17). This finding may explain why a direct relationship is found to exist between the level of Ab produced by a B cell and the level of protection afforded against a specific antigen in vivo. For example, although low level changes in IgG1 may appear to be modest, it may reflect the subtle mechanisms that are called into play in vivo to maintain immune homeostasis.

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CD86 and \( \beta_2 \)-Adrenergic Receptor Signaling Pathways, Respectively, Increase Oct-2 and OCA-B Expression and Binding to the 3'-'IgH Enhancer in B Cells

Joseph R. Podojil, Nicholas W. Kin and Virginia M. Sanders

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