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Selective Regulation of Mature IgG1 Transcription by CD86 and β_2-Adrenergic Receptor Stimulation[1]

Joseph R. Podojil[2] and Virginia M. Sanders[3]

Stimulation of CD86 and the β_2-adrenergic receptor (β_2AR) on a B cell, either alone or together, is known to increase the level of IgG1 protein produced by a CD40 ligand/IL-4-activated B cell. It is also known that the mechanism by which CD40 and IL-4R stimulation on a B cell increases the level of IgG1 protein is by increasing germline γ1 transcription, IgG1 class switching, and mature IgG1 transcription, while the molecular mechanism responsible for mediating the CD86- and β_2AR-induced effect remains unknown. In the present study using real-time PCR we show that the level of mature IgG1 transcription increases in CD40 ligand/IL-4-activated B cells following stimulation of either CD86 and/or β_2AR, and that this increase reflects the increase in IgG1 protein. Furthermore, we show that the CD86- and/or β_2AR-induced increase in mature IgG1 transcript is due to an increase in the rate of mature IgG1 transcription, as determined by nuclear run-on analysis. This effect is additive when both receptors are stimulated and is lost when B cells from CD86- and β_2AR-deficient mice are used. In contrast, the level of germline γ1 transcription, the stability of mature IgG1 transcript, the number of IgG1-positive B cells, and the number of IgG1-secreting B cells did not change. These results provide the first evidence that CD86 and/or β_2AR stimulation on a CD40 ligand/IL-4-activated B cell increases the level of IgG1 protein produced per cell by increasing the rate of mature IgG1 transcription. ***The Journal of Immunology,*** **2003, 170: 5143–5151.**

The clearance of a T cell-dependent Ag is mediated by the successful interplay between a B cell that produces Ab and a Th cell that secretes a specific cytokine profile (1, 2). The resting B cell and naive $CD4^+$ T cell differentiate into effector cells by the binding of either whole Ag or an antigenic peptide in the context of MHC class II to the B cell receptor (BCR)[4] and TCR, respectively. In addition, full activation of these cells requires the binding of costimulatory molecules expressed on one cell to costimulatory molecules expressed on the other (3, 4). One such costimulatory molecule is CD86 (B7-2), which is expressed at a low level on a resting B cell (5, 6), but is up-regulated following the stimulation of BCR (7, 8), CD40 (9, 10), MHC class II (11), LPS receptor (5, 7, 12, 13), IL-4R (14, 15), and/or β_2-adrenergic receptor (β_2AR) (16, 17). CD86 binds to the costimulatory molecule CD28 on the Th cell to increase the expression of the costimulatory molecule CD40 ligand (CD40L) on the Th cell and also to increase the level of cytokine produced by the Th cell (13, 15, 18). Subsequently, CD40L and the secreted cytokine interact with CD40 and a cytokine receptor on the B cell to activate the process of Ab production and to regulate the process of Ig isotype class switching from IgM to another isotype that is used to mediate specific immune effector functions (19, 20).

While the positive regulatory effects of CD28 stimulation on the Th cell have been studied extensively (reviewed in Ref. 21), emerging data also suggest that CD86 is able to generate an intracellular signal within the B cell itself. For example, in vitro, an anti-CD28 blocking Ab decreases the level of IgG1 produced in a T/B cell coculture system (22). Likewise, in vivo studies using either CD28-deficient mice or wild-type mice that had received a blocking Ab to inhibit CD86/CD28 interaction have shown a decrease in the level of serum IgG1 compared with wild-type controls (23, 24). Although these findings were attributed to a lack of CD28 costimulation on the Th2 cell that resulted in a decrease in the level of IL-4 and CD40L costimulation provided to the B cell, these findings also suggested that CD86 costimulation might generate a signal within the B cell to regulate the level of a Th cell-dependent Ab response. Recent in vitro data show that the stimulation of CD86 on a B cell activated through CD40 and the IL-4R with or without BCR stimulation increases the level of IgG1 and IgE produced (16, 25) and differentially affects the level of anti-apoptotic and proapoptotic molecules expressed (26). Likewise, both host and donor B cells from chimeric mice that received donor cells from CD80/CD86-deficient mice and were challenged with a protein Ag were able to produce Ag-specific IgG1, but the level of IgG1 produced by the CD80/CD86-deficient cells was decreased compared with that of the host B cells (27). These in vivo data show that CD86 stimulation is not necessary for a B cell to class switch to IgG1, but instead that CD86 stimulation may increase the level of IgG1 produced per cell. Taken together these findings suggest that CD86 expressed on a B cell not only stimulates CD28 on a Th cell, but also transduces a positive regulatory signal directly to the B cell to enhance B cell activity via an unknown intracellular mechanism.

Likewise, stimulation of the β_2AR on a B cell, which serves as the receptor for the neurotransmitter norepinephrine that is released in the B cell microenvironment within lymphoid organs (28), increases the level of CD86 expressed on and IgG1 produced

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[4] Abbreviations used in this paper: BCR, B cell receptor; β_2AR, β_2-adrenergic receptor; CD40L, CD40 ligand; Iγ1, germline intervening γ1 transcript; OCA-B, Oct coactivator from B cells.

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by a B cell activated in vitro in the presence of a Th2 cell or CD40L/IL-4 (16). Since the Th2 cell did not express β_2AR, it was concluded that stimulation of β_2AR on a B cell most likely had a direct effect on IgG1 production. Thus, to eliminate any indirect contribution of β_2AR stimulation on a B cell due to an affect on Th2 cell activity, the Th2 cell was removed from the in vitro culture system. Resting B cells were activated in the presence of Ag with and without a β_2AR agonist before activation with CD40L and IL-4 with or without an anti-CD86 Ab. Using this model system, the stimulation of either CD86 or β_2AR increased the level of IgG1 produced, and this effect was increased further when both receptors were stimulated. Although these data showed that both CD86 and the β_2AR were able to positively regulate the level of IgG1 protein, the mechanism by which these two receptors increased the level of IgG1 remained unknown.

In contrast, the mechanism by which CD40 and IL-4R stimulation on a B cell regulates IgG1 production at the level of class switching (19, 29) and/or mature IgG1 transcript (30–32) is well known. The class switching event is regulated by CD40- and IL-4R-induced NF-κB and STAT-6 binding to the germline γ1 promoter, respectively, to activate transcription of germline γ1 (referred to as intervening γ1 (Iγ1)) (33–39). The production of mature IgG1 transcript is regulated by an increase in Oct coactivator from B cells (OCA-B) expression following CD40 stimulation to regulate the activity of the 3′ IgH enhancer (40). Although B cells in which the 3′ IgH enhancer has been deleted are able to class switch to IgG1, the ability to attain normal levels of secreted IgG1 and mature IgG1 transcript is lost (41). Although much has been learned about the mechanisms by which the CD40 and the IL-4R activation signals regulate the process of IgG1 production, other endogenous immune and nonimmune molecules that may regulate the intensity of this response, such as CD86 and β_2AR, are only beginning to be elucidated.

In this study our goal was to determine at the molecular level whether the increased level in IgG1 protein produced following CD86 and/or β_2AR stimulation on CD40L/IL-4-activated B cells was due to either an increase in class switching to IgG1 and/or an increase in the level of mature IgG1 transcript produced. The present data are the first to show at the molecular level that the stimulation of CD86 and β_2AR, alone or together on a CD40L/IL-4-activated B cell, increases both the level and the rate of mature IgG1 transcription without affecting transcript stability or class switching to IgG1.

Materials and Methods

Animals

Mice were housed under pathogen-free conditions and were used at 7–8 wk of age. Female BALB/c (H-2^d-restricted) and female FVB (H-2^q-restricted) mice were purchased from Taconic Farms (Germantown, NY). Female CD86 knockout (CD86$^{-/-}$) mice (H-2^d-restricted) (42) were provided by Dr. A. Sharpe (Brigham and Woman's Hospital, Boston, MA), and β_2AR$^{-/-}$ mice (H-2^q-restricted) (43) were provided by Dr. B. Kobilka (Stanford University, Stanford, CA). All mice were bred and housed within the pathogen-free facility at Taconic Farms until 7–8 wk of age and then housed at Ohio State University in microisolator cages and provided autoclaved food and water ad libitum.

Resting B cell isolation and culture

Spleens were collected from unimmunized wild-type, CD86$^{-/-}$, or β_2AR$^{-/-}$ mice. After RBC lysis with 0.4% ammonium chloride, splenocytes were incubated with rat anti-mouse CD43 Ab (clone S7; BD PharMingen, San Diego, CA) in HBSS/5% FCS for 1 h at 4°C. The cells were washed three times with HBSS/5% FCS and incubated with sheep anti-rat IgG Dynabeads M-450 (Dynal Biotech, Lake Success, NY) for 30 min at 4°C. CD43-positive cells were eliminated using magnetic separation, and the remaining CD43-negative cells were collected, analyzed by FACS analysis, and found to be >98% B220$^+$, CD24^{high}, CD43$^-$, and CD86^{low}. Resting B cells were cultured at a final volume of 2.5×10^5 cells in 1 ml of complete RPMI, which consisted of RPMI 1640 medium (CellGro, Herndon, VA), 10% FBS (Atlas Biologicals, Colorado Springs, CO), 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μM 2-ME, in 24-well plates (Costar, Corning, NY) at 37°C in 5% CO_2. Resting B cells were activated in the presence of CD40L-expressing Sf9 cells (provided by Dr. M. Berton, University of Texas Health Science Center, San Antonio, TX) (38) at a ratio of 10/1 to 80/1 and IL-4 (eBioscience, San Diego, CA; 0.1–10 ng/ml) in the presence or the absence of the β_2AR agonist terbutaline (Sigma-Aldrich, St. Louis, MO; 10^{-9}–10^{-6} M). After 16–24 h, either an anti-CD86 Ab (clone PO3; eBioscience) or a species- and isotype-matched control Ab (rat IgG2b, κ, clone A95-1; BD PharMingen) was added at a final concentration of 0.1–10 μg/ml. All reagents used for resting B cell isolation and activation tested negative for the presence of endotoxin using etoxate (Sigma-Aldrich), a *Limulus* lysate assay with a level of detection <0.1 U/ml. Although the level of endotoxin in the B cell cultures was found to be below the level of detection, the level of IgG1 protein produced by resting B cells from Toll4-deficient mice (strain C.C3-Tlr4$^{Lps\text{-}d}$; The Jackson Laboratory, Bar Harbor, ME) was analyzed to confirm that the increase in IgG1 following CD86 and/or β_2AR stimulation was not due to nonspecific activation by low levels of endotoxin. The level of IgG1 protein produced by Toll4-deficient cells was similar to the level produced by BALB/c resting B cells under identical activation/stimulation conditions (data not shown).

IgG1 ELISA

B cell culture supernatants were collected on days 2–7 after the initial activation. Supernatant was frozen immediately at −80°C until analyzed by ELISA, as described in detail previously (16). A standard curve for IgG1 was prepared using known quantities of the myeloma protein MOPC-21 (IgG1; Sigma-Aldrich). Color development was determined on a Spectramax Plus microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm. The lower limit of detection for IgG1 was <4 ng/ml.

Flow cytometry

The number of IgG1-positive B cells was determined by FACS analysis, as described in detail previously (16). Briefly, B cells were collected on day 6 after initial activation and stained with PE-conjugated rat anti-mouse B220 Ab (clone RA3-6B2; BD PharMingen) and FITC-conjugated rat anti-mouse IgG1 Ab (clone A85-1; BD PharMingen). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The FACSCalibur was calibrated daily using Rainbow Calibration Particles (Sherotech, Libertyville, IL), and the data were analyzed using CellQuest software (BD Biosciences).

Quantitative real-time PCR

Total cellular mRNA was isolated from the B cells using TRIzol (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol, quantified by measuring the absorbance at 260 nm, and stored at −80°C until analysis. RNA integrity was monitored by running 1 μg of each sample on a 1.5% agarose gel. Before RT-PCR, all RNA samples were treated with 1 μg of DNase I (Life Technologies)/1 μg of RNA. RT was preformed on 2.5 μg of DNase-treated RNA/sample using a common master mix (50 U of Moloney murine leukemia virus reverse transcriptase, 2.5 μM random hexamer, 20 U of RNase inhibitor, 1 mM dNTPs, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl; PerkinElmer, Norwalk, CT), and the samples were incubated at 25°C for 10 min, 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. In addition, 2.5 μg of RNA from each sample was not reverse transcribed to ensure the effectiveness of the DNase treatment.

For quantitative real-time PCR, a common master mix (LightCycler-FastStartDNA SYBR Green I (Roche, Mannheim, Germany), 2 mM $MgCl_2$, 0.5 μM gene-specific primer) and 1.5 μl of cDNA for a final reaction volume of 15 μl was used. Each gene considered used the following cycling protocol: 95°C for 10 min, followed by 35 cycles of 95°C denaturing for 15 s, gene-specific annealing temperature for 2 s, and 72°C extension for 20 s. The concentration of gene-specific cDNA was quantified by comparison with a standard curve of gene-specific PCR product diluted 1/10 for concentrations ranging from 1 ng/ml to 1 fg/ml. Actin primers served as an internal control to ensure the efficiency of the RT and the amount of RNA used in each reaction, and the concentration of actin cDNA in each sample was used to normalize the gene-specific concentrations. The following primers were used for actin: top primer, 5′-TACAGCTTCACCACCACAGC-3′; and bottom primer, 5′-AAGGAAG GCTGGAAAAGAGC-3′ (annealing temperature of 60°C). The following

primers were used for germline γ1 transcript (Iγ1): top primer, 5′-CATCCTATCACGGGAGATTGGG-3′; and bottom primer, 5′-ATCCTCGGGGCTCAGGTTTG-3′ (annealing temperature of 65°C). The following primers were used for mature IgG1 transcript: top primer, 5′-TATGGACTACTGGGGTCAAG-3′; and bottom primer, 5′-CCTGGGCACAATTTTCTTGT-3′ (annealing temperature of 63°C). After each real-time reaction a melting curve was generated, and samples were run on a 1.2% agarose gel to ensure that only one gene-specific PCR product was generated. Real-time PCR was preformed using the Roto-Gene 2000 Real-Time Cycler (Phenix Research Products, Hayward, CA).

Determination of mature IgG1 stability

On day 6 following the initial activation of resting B cells, 20 μg/ml actinomycin D (Sigma-Aldrich) was added to each culture to stop the further production of mature IgG1 transcript. B cells were collected from the cultures over a 16-h time course following the addition of actinomycin D, cell viability was analyzed by trypan blue exclusion, and total mRNA was isolated. The level of mature IgG1 transcript was quantified by real-time PCR as described above.

Nuclear run-on

The rate of mature IgG1 transcription was determined by nuclear run-on as described in detail previously (44). Briefly, 20×10^6 B cells were collected on day 6 following initial cell activation in the absence or the presence of CD86 and/or β_2AR stimulation and were washed twice with PBS before resuspension in 5 ml of cell lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40 (Sigma-Aldrich) for 5 min at 4°C. Nuclei were collected by centrifugation at $300 \times g$ for 10 min at 4°C; resuspended in 500 μl of nuclear freezing buffer containing 50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA; and stored at −80°C until use for nuclear run-on. Nuclear run-on and RNA isolation were preformed in the presence of biotin-16-UTP (Roche). To control for the possibility of non-biotin-labeled RNA contamination, replicate sets of nuclei were used in the nuclear run-on that did not contain biotin-16-UTP. Dynabeads M-280 (Dynal Biotech) were used to capture the biotin-labeled molecules from the purified nuclear RNA, and beads were washed twice with 2× SSC plus 15% formamide and once with 2× SSC and resuspended in 30 μl of diethylpyrocarbonate-treated H_2O before preparing random hexamer-primed cDNA as described in *Quantitative real-time PCR* above.

Semiquantitative RT-PCR was preformed from serially diluted cDNA samples to determine the levels of actin and mature IgG1 transcript. PCR of cDNA was performed with 5 U of AmpliTaq DNA polymerase (PerkinElmer), 25 mM $MgCl_2$, 10× PCR buffer, and 10 μM of each gene-specific primer. cDNA was denatured for 10 min at 95°C and then amplified for 35 cycles (95°C for 45 s, 58°C for 45 s, 72°C for 2 min) for actin and mature IgG1 transcript. PCR reactions were run on a 1.5% agarose gel with ethidium bromide, and densitometry was preformed using NIH Image 1.61 software (National Institutes of Health, Bethesda, MD). Actin served as an internal control to ensure the efficiency of the RT, and the amount of RNA used in each reaction. The OD values obtained for actin were used to normalize the mature IgG1 OD values. All samples that did not contain biotin-16-UTP were found to be negative for the presence of actin and mature IgG1 transcripts.

Statistics

Data were analyzed by one-way ANOVA to determine whether an overall statistically significant change existed before using two-tailed unpaired Student's *t* test. Statistically significant differences were reported at $p < 0.05$.

Results

Level of IgG1 protein produced following CD86 and/or β2AR stimulation

In the present study B cells were activated in a way that mimicked the resting B cell interacting with an activated Th2 cell, i.e., CD40 and the IL-4R on the B cell were stimulated prior to the stimulation of CD86. B cells were also exposed in the present study to a β_2AR agonist during initial activation with CD40L/IL-4, since this may be the period during which the B cell is exposed to the neurotransmitter, norepinephrine, in vivo (28, 45). This exposure protocol differs from that reported previously by our laboratory in which CD40 and IL-4R were stimulated at the same time as CD86 and preceded 24 h earlier by BCR stimulation in the absence or the presence of β_2AR stimulation (16). Using the previously published activation protocol, the BCR-generated signal was found to be essential for CD86 to induce an effect on the level of IgG1 produced by the B cell, whereas the present activation protocol did not appear to be limited by this requirement, as will be shown below.

Resting B cells were activated in the presence of CD40L and IL-4 with or without the β_2AR-selective agonist terbutaline. Sixteen to 24 h after the initial activation, either an anti-CD86 Ab or a species- and isotype-matched control Ab was added, and supernatants were collected on day 7. As shown in Fig. 1, when an anti-CD86 Ab was added to CD40L/IL-4-activated B cells, the level of IgG1 secreted by day 7 increased by 180% above the level produced by cultures receiving CD40L/IL-4 and a species- and isotype-matched Ab alone. Likewise, the addition of the β_2AR-selective agonist terbutaline at the time of CD40L/IL-4 activation increased the level of IgG1 produced 150% above the level produced by the control cultures, a level that was increased to 333% above that produced by the control cultures when CD86 was also stimulated. In contrast, if either CD86 or β_2AR was stimulated in the absence of both CD40L and IL-4, no IgG1 was produced (data not shown).

To determine whether the increase in IgG1 produced by a B cell stimulated through CD86 and/or β_2AR on day 7 was due to an alteration in the kinetics of the B cell response, culture supernatants were analyzed on days 2–7. The stimulation of CD86 and/or β_2AR altered the magnitude, but not the kinetics, of the IgG1 response (Fig. 1, inset). To determine whether the increase in secreted IgG1 protein at the population level was due to an increase in the number of IgG1-secreting cells and/or the amount of IgG1 secreted per cell, ELISPOT and ELISA, respectively, were used to

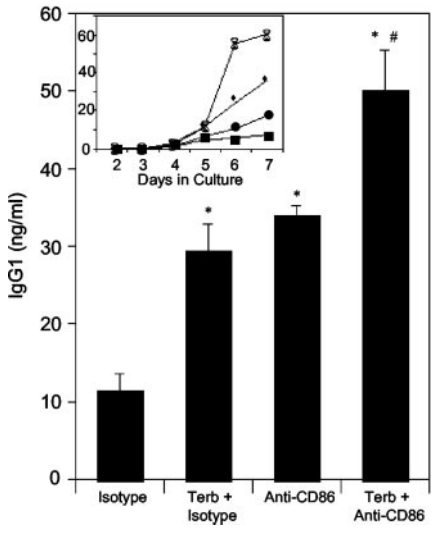

FIGURE 1. IgG1 production following CD86 and/or β_2AR stimulation. Resting B cells (2.5×10^5 cells/ml) were activated in the presence of Sf9/CD40L cells (1/10) and IL-4 (10 ng/ml) in the absence or the presence of the β_2AR-selective agonist terbutaline (Terb; 10^{-6} M) for 16–24 h in triplicate wells. Following the initial activation period, either an anti-CD86 Ab (clone PO3) or a species- and isotype-matched control Ab (1 μg/ml) was added. After 7 days of culture, supernatants were collected and analyzed for IgG1 by ELISA. Data are presented as the mean (nanograms per milliliter of IgG1) ± SE from triplicate determinations. One representative experiment of three independent experiments is shown. Inset, The kinetics of IgG1 production were determined for isotype (■), Terb plus isotype (●), anti-CD86 (▲), and Terb plus anti-CD86 (X) treatment group supernatants collected on days 2–7. One representative experiment of three independent experiments is shown. *, $p < 0.05$ compared with the isotype group; #, $p < 0.05$ compared with the anti-CD86 alone and Terb alone groups.

analyze cells from replicate cultures. While the number of IgG1-secreting cells remained constant between treatment groups, both CD86 and β_2AR stimulation alone of CD40L/IL-4-activated B cells increased the amount of IgG1 secreted per cell compared with cells activated with CD40L and IL-4 alone, and the stimulation of both receptors increased this effect further (data not shown). Therefore, this sequence of activation signals allows CD86 to deliver a signal to a B cell to increase the level of IgG1 secreted and provides an activation protocol that allows for study of the signaling mechanism used by CD86 and β_2AR to enhance the level of IgG1 produced by a B cell in the absence of BCR-induced signals.

The B7 family of costimulatory molecules is made up of three different cell surface proteins (B7-1/CD80, B7-2/CD86, and B7-H) that share a high level of homology and interact with costimulatory molecules expressed on the Th cell (46). To determine whether the anti-CD86 Ab-induced increase in the level of IgG1 protein produced was due specifically to the stimulation of CD86, resting B cells from CD86$^{-/-}$ mice were used. When resting B cells from CD86$^{-/-}$ mice were activated with CD40L/IL-4 in the presence or the absence of terbutaline, the addition of an anti-CD86 Ab did not increase the level of IgG1 produced above that in control cells (Fig. 2*A*). In contrast, the addition of terbutaline alone to CD40L/IL-4-activated CD86$^{-/-}$ B cells induced a 75% increase in the level of IgG1 above the control value, confirming not only that expression of β_2AR was unaffected on CD86$^{-/-}$ cells, but also that the β_2AR-associated signaling pathway remained intact.

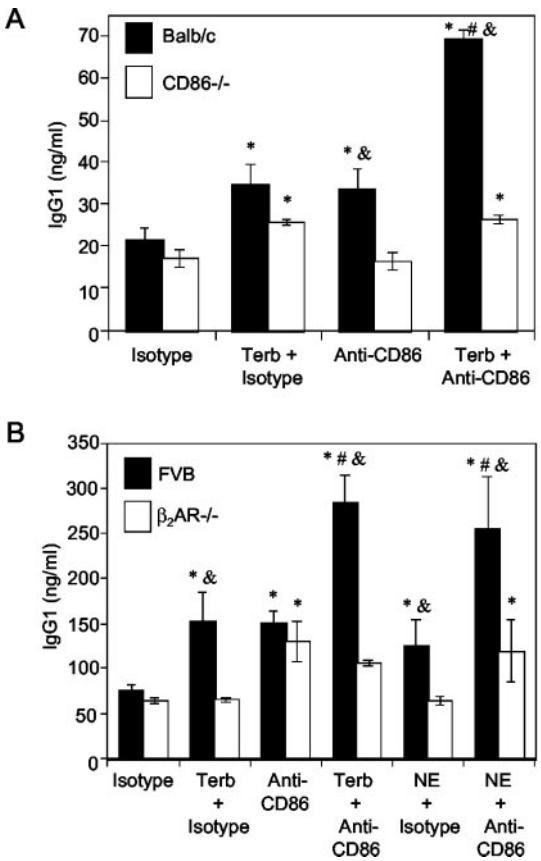

FIGURE 2. IgG1 production following exposure of CD86$^{-/-}$ and β_2AR$^{-/-}$ B cells to respective ligands. Resting B cells were isolated from BALB/c and CD86$^{-/-}$ mice (*A*) or FVB and β_2AR$^{-/-}$ mice (*B*). Cells were activated, cultured, and assayed as described in Fig. 1, except for groups that received norepinephrine (NE; 10^{-6} M). Supernatants were collected on day 7 and analyzed for IgG1 by ELISA. Data are presented as the mean (nanograms per milliliter of IgG1) ± SE from triplicate determinations. One representative experiment of three independent experiments is shown. *, $p < 0.05$ compared with the isotype group; #, $p < 0.05$ compared with the anti-CD86 alone and terbutaline alone groups; &, $p < 0.05$ compared with the wild-type group.

Likewise, ARs are divided into two major subsets, αAR and βAR, both of which are able to bind the physiological ligand norepinephrine to transduce an intracellular signal. Additionally, each AR subset is further divided into multiple subtypes. On normal murine resting B cells, it appears that the β_2AR is expressed exclusively (reviewed in Ref. 47). To determine whether the increase in IgG1 induced by either terbutaline or the physiological ligand norepinephrine resulted from stimulation of β_2AR, as opposed to up-regulation of another AR subtype, resting B cells from wild-type and β_2AR$^{-/-}$ mice were activated with CD40L/IL-4 in the absence or the presence of either terbutaline or norepinephrine, followed by the addition of an anti-CD86 Ab or a species- and isotype-matched control Ab. When β_2AR$^{-/-}$ resting B cells were activated in the presence of either terbutaline or norepinephrine alone, the level of IgG1 produced did not increase above the level produced by control cells (Fig. 2*B*). This finding suggests that the physiological ligand norepinephrine induces an IgG1-enhancing effect by stimulating β_2AR, as opposed to another AR subtype that may be up-regulated on the β_2AR$^{-/-}$ B cell. In contrast, as shown in Fig. 2*B*, the addition of anti-CD86 Ab alone induced a 110% increase in the level of IgG1 produced, and this increase was not enhanced further when terbutaline or norepinephrine was added, confirming the expression of a functional CD86 molecule on B cells from β_2AR$^{-/-}$ mice.

Level of germline γIγ1 and number of B cells expressing surface IgG1 following stimulation of CD86 and/or β_2AR

The increase in the level of IgG1 protein produced by a population of B cells may be due to an increase in the number of B cells that switch to produce IgG1 at the level of gene rearrangement. Previous reports indicate that a correlation exists between the level of Iγ1 and the number of B cells that class-switch to IgG1 (29, 34, 36–38). Therefore, total cellular RNA was isolated from B cells, and the amount of Iγ1 produced was analyzed by real-time PCR. As shown in Fig. 3*A*, the level of Iγ1 did not increase when either CD86 and/or β_2AR was stimulated on CD40L/IL-4-activated B cells. To assure that the ability of signals generated through CD86 and/or β_2AR was not limited by a given concentration of a particular ligand, the Sf9/CD40L to B cell ratio and the IL-4 concentration were titrated in the presence or the absence of various concentrations of anti-CD86 Ab and/or terbutaline. Although the level of Iγ1 increased in a concentration-dependent manner when using various concentrations of Sf9/CD40L and IL-4 alone as expected, the addition of anti-CD86 and/or terbutaline to CD40L/IL-4-activated B cells, alone or together, did not increase the level of Iγ1 further (Fig. 3*B*).

To determine on a cellular level whether the number of B cells expressing IgG1 was altered following the different exposure protocols, FACS analysis was performed on cells collected 6 days following initial activation and stained for the surface expression of B220 and IgG1. As shown in Fig. 3*C*, the percentage of B cells expressing surface IgG1 did not change following the stimulation of CD86 and/or β_2AR. Likewise, the absolute number and percentage of viable cells was not altered following the stimulation of CD86 and/or β_2AR (data not shown). Although the percentage of IgG1-positive B cells appears to be low, it was equal to the percentage of IgG1-secreting cells, as determined by ELISPOT (data not shown). Therefore, the findings taken together suggest that the

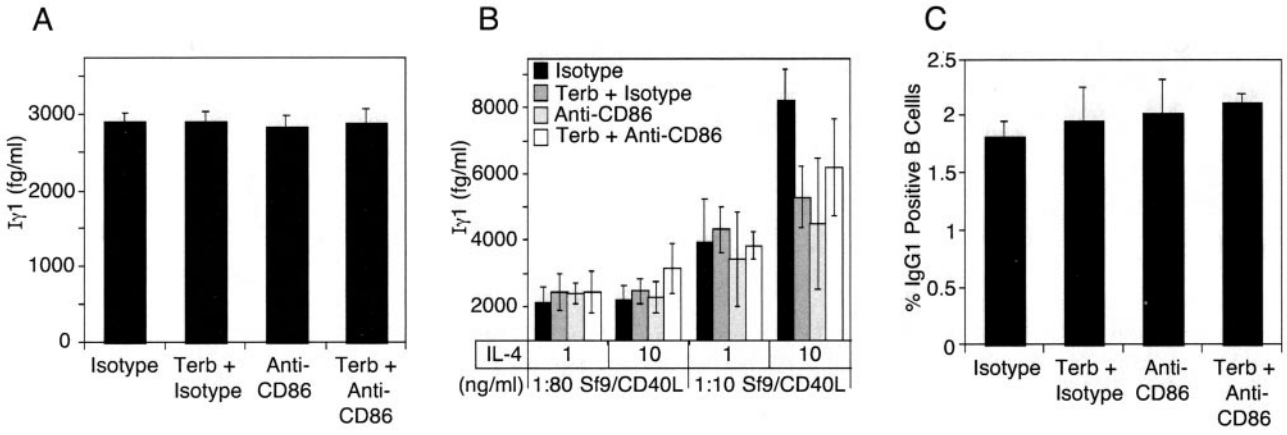

FIGURE 3. Quantitation of Iγ1 by real-time PCR. *A*, Resting B cells (1×10^6 cells/ml) were activated in the presence of CD40L/Sf9 cells (1/10) and IL-4 (10 ng/ml) in the absence or the presence of the β_2AR-selective agonist terbutaline (Terb; 10^{-6} M) for 16 h in triplicate wells. Following the initial activation period, either an anti-CD86 Ab (clone PO3) or a species- and isotype-matched control Ab (1 μg/ml) was added. Twenty-four hours following the initial activation, total cellular RNA was collected. *B*, The level of Iγ1 produced was quantified by real-time PCR. Data are presented as the mean (femtograms per milliliter of Iγ1) ± SE from triplicate determinations. One representative experiment of three independent experiments is shown. Resting B cells were activated as described above, except two different concentrations of CD40L/Sf9 cells (1/80 or 1/10) and IL-4 (1 or 10 ng/ml) were used. *C*, Resting B cells were activated using the activation protocol presented in *A*. After 6 days in culture, the percentage of IgG1-positive cells was analyzed using immunofluorescence and a FACSCalibur flow cytometer. The data presented are the averaged percentage of $B220^+/IgG1^+$ cells measured on day 6. Data are presented as the mean percentage of $B220^+/IgG1^+$ cells ± SE from triplicate determinations. One representative experiment of three independent experiments is shown.

increased level of IgG1 produced by CD40L/IL-4-activated B cells following CD86 and/or β_2AR stimulation is not due to an increase in either Iγ1 or the number of B cells expressing IgG1 on their surface.

Level of mature IgG1 transcript following stimulation of CD86 and/or β_2AR

The level of IgG1 protein produced by a population of B cells may also be regulated by the amount of mature IgG1 transcript produced (31, 48). To determine whether the increase in IgG1 protein produced per cell is controlled transcriptionally, the level of mature IgG1 transcript was analyzed using real-time PCR. As shown in Fig. 4*A*, when an anti-CD86 Ab was added to CD40L/IL-4-activated B cells, the level of mature IgG1 transcript produced by day 6 increased 250% above the level produced by cultures receiving CD40L/IL-4 and a species- and isotype-matched Ab alone. The addition of terbutaline at the time of CD40L/IL-4 activation increased the level of mature IgG1 transcript produced 150% above the level produced by control cultures, a level that was increased to 350% above that produced by control cultures when CD86 was also stimulated. To determine whether the stimulation of CD86 and/or β_2AR altered the kinetics of mature IgG1 transcript production, the level of mature IgG1 transcript was analyzed by real-time PCR on days 2–6. Stimulation of CD86 and/or β_2AR altered the magnitude, but not the kinetics, of mature IgG1 transcript produced on days 2–6 (data not shown). To confirm that the increase in mature IgG1 transcript was indeed mediated through stimulation of CD86 and/or β_2AR, B cells from $CD86^{-/-}$ and $\beta_2AR^{-/-}$ mice were also used. When resting B cells from $CD86^{-/-}$ mice were used, the addition of anti-CD86 Ab was not able to increase the level of mature IgG1 transcript produced above that induced by CD40L/IL-4-activation in the absence or the presence of terbutaline (Fig. 4*A*). Similarly, when resting B cells from $\beta_2AR^{-/-}$ mice were used, the addition of terbutaline was not able to increase the level of mature IgG1 transcript above those in the isotype and anti-CD86 treatment groups, respectively (Fig. 4*B*). Therefore, this finding suggests that the increase in IgG1 protein following CD86 and/or β_2AR stimulation is due to an increase in mature IgG1 transcript.

Stability of mature IgG1 transcript following stimulation of CD86 and/or β_2AR

The level of a specific mRNA transcript within a cell is regulated by either the stability of the transcript and/or the rate at which the transcript is produced (49). To determine whether the stability of mature IgG1 transcript was affected by stimulation of CD86 and/or β_2AR, actinomycin D was added to cultured B cells on day 6 after activation. Cells were collected before and at various times after the addition of actinomycin D. Cell viability was assessed at each of these time points by trypan blue exclusion, and the overall viability of the cells did not change significantly until 8–16 h after the addition of actinomycin D. Since a 2-fold decrease in cell viability was seen at 16 h, these cells were eliminated from analysis. The level of mature IgG1 transcript present at the remaining time points was quantified by real-time PCR. Linear regression was performed for each of the treatment groups, so that the stability of the mature transcript could be determined following the stimulation of CD86 and/or β_2AR in our in vitro model system. As shown in Fig. 5, stimulation of CD86 or β_2AR alone does not affect the stability of mature IgG1 transcript compared with the species- and isotype-matched control Ab group. When CD86 and β_2AR were both stimulated, the stability of mature IgG1 decreased compared with that of the other treatment groups. Therefore, although the stimulation of either CD86 or β_2AR alone on a CD40L/IL-4-activated B cell increases the level of mature IgG1 transcript, it does not affect the stability of mature IgG1 transcript. In contrast, stimulation of both CD86 and β_2AR increases further the level of mature IgG1 transcript produced, but decreases the stability of this transcript. Therefore, the results suggest that the CD86- and β_2AR-induced increase in mature IgG1 transcript produced is not due to an increase in transcript stability, but may be due to an increase in the rate at which mature IgG1 transcript is produced.

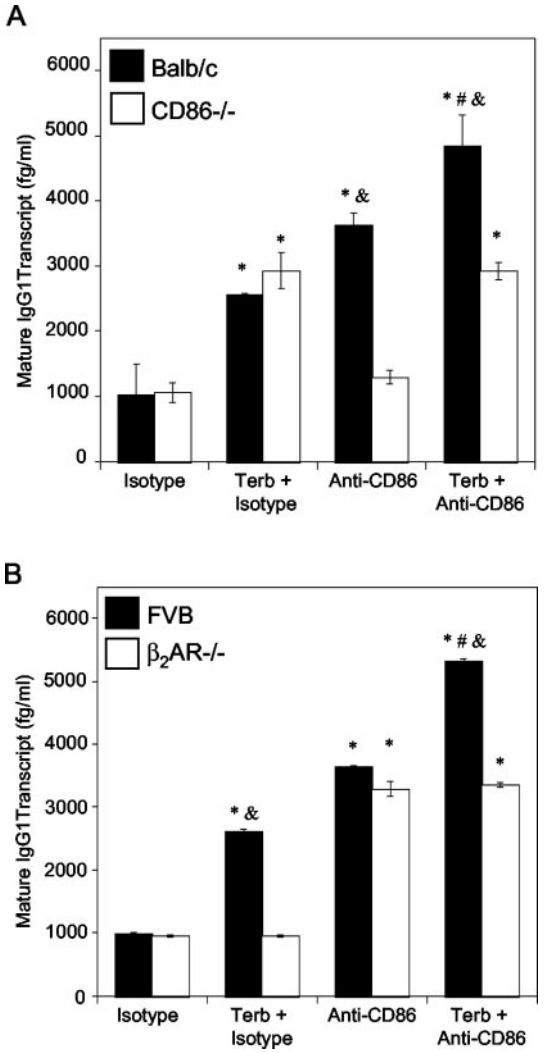

FIGURE 4. Quantitation of mature IgG1 transcript by real-time PCR. Resting B cells were isolated from *A*) BALB/c (■) and CD86$^{-/-}$ (□), or *B*) FVB (■) and β_2AR$^{-/-}$ (□) mice. Cells were activated in culture as described in Fig. 1. After 6 days in culture, total cellular RNA was collected, and the level of mature IgG1 transcript was quantified by real-time PCR. Data are presented as the mean (femtograms per milliliter of mature IgG1 transcript) ± SE from triplicate determinations. One representative experiment of three independent experiments is shown. Data are presented as one representative experiment of three independent experiments. *, $p < 0.05$ compared with the isotype group; #, $p < 0.05$ compared with the anti-CD86 alone and terbutaline alone groups; &, $p < 0.05$ compared with the wild-type group.

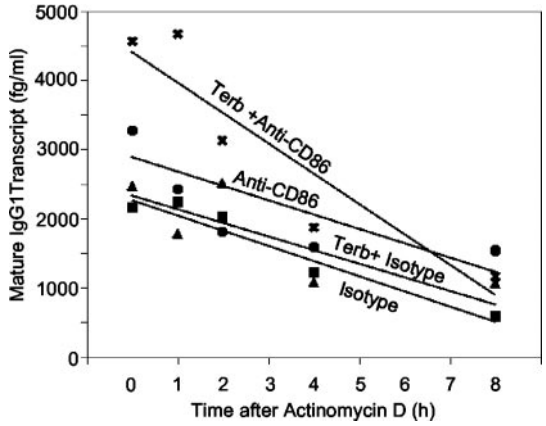

FIGURE 5. Analysis of mature IgG1 transcript stability by real-time PCR. Resting B cells were activated, cultured, and assayed as described in Fig. 1. After 6 days in culture, 1×10^6 cells were collected (time zero), and then actinomycin D (20 μg/ml) was added to the remaining cells in culture. Cells with actinomycin D were collected over an 8-h time course. Cell viability was analyzed by trypan blue exclusion, and total cellular RNA was collected for quantitation of mature IgG1 transcript by real-time PCR. Linear regression was performed to determine the rate of mature IgG1 degradation. One representative experiment of three independent experiments is shown.

Rate of mature IgG1 transcription following stimulation of CD86 and/or β_2AR

To determine whether the rate of mature IgG1 transcription was affected by stimulation of CD86 and/or β_2AR, nuclear run-on using biotin-16-UTP was preformed on nuclei isolated from B cells on day 6 following activation. Because only gene transcripts produced during nuclear run-on incorporate biotin-16-UTP, the amount of biotin-labeled mature IgG1 transcript isolated and quantified by semiquantitative PCR served as an indicator of the DNA polymerase II activity at the IgH locus, which reflected any change that occurred in the rate of mature IgG1 transcription (44). As shown in Fig. 6*A*, when anti-CD86 Ab was added to CD40L/IL-4-activated B cells, the amount of mature IgG1 transcript and, therefore, the rate of mature IgG1 transcription, increased 110% above the level produced by cultures receiving CD40L/IL-4 and a species- and isotype-matched Ab alone. When terbutaline was added at the time of CD40L/IL-4 activation, the amount of mature IgG1 transcript, and therefore the rate of mature IgG1 transcription, increased 130% above the level produced by control cultures. When both anti-CD86 and terbutaline were added, the amount of mature IgG1 transcript increased to 350% above that produced by control cultures. In contrast, when resting B cells from CD86$^{-/-}$ (Fig. 6*B*) or β_2AR$^{-/-}$ (Fig. 6*D*) mice received the same activation signals, the addition of both anti-CD86 Ab and terbutaline, respectively, was not able to increase the level of mature IgG1 transcription above that induced by either ligand alone. Therefore, the results suggest that the increase in mature IgG1 transcript shown in Fig. 4*A* was due specifically to an increase in the rate of mature IgG1 transcription following CD86 and/or β_2AR stimulation.

Discussion

Previous data indicated that the amount of IgG1 produced by CD40L/IL-4-activated B cells changes because of an increase in the level of class switching to IgG1 and the level of mature IgG1 transcript (19, 29). Previous data also indicated that the amount of IgG1 protein produced by CD40L/IL-4-activated B cells is increased further when CD86 (16, 26) and/or β_2AR (16) are costimulated on the B cell. However, the mechanism responsible for mediating the latter enhancing effect was unknown. The data from the present study are the first to show at the molecular level that the CD86- and β_2AR-mediated increase in IgG1 protein produced by a CD40L/IL-4-activated B cell is due to an increase in the rate of mature IgG1 transcript as opposed to either a further increase in the level of class switching to IgG1 or an increase in mature IgG1 transcript stability. The present findings also establish a distinct molecular mechanism by which two independent membrane-associated receptors, other than CD40 and IL-4R, generate intracellular signals to up-regulate the level of IgG1 produced by a CD40L/IL-4-activated B cell independently of the IgG1 class switching mechanism induced by CD40 and IL-4R stimulation.

In the present study the CD86 signal was able to induce an increase in the level of IgG1 protein and mature IgG1 transcript

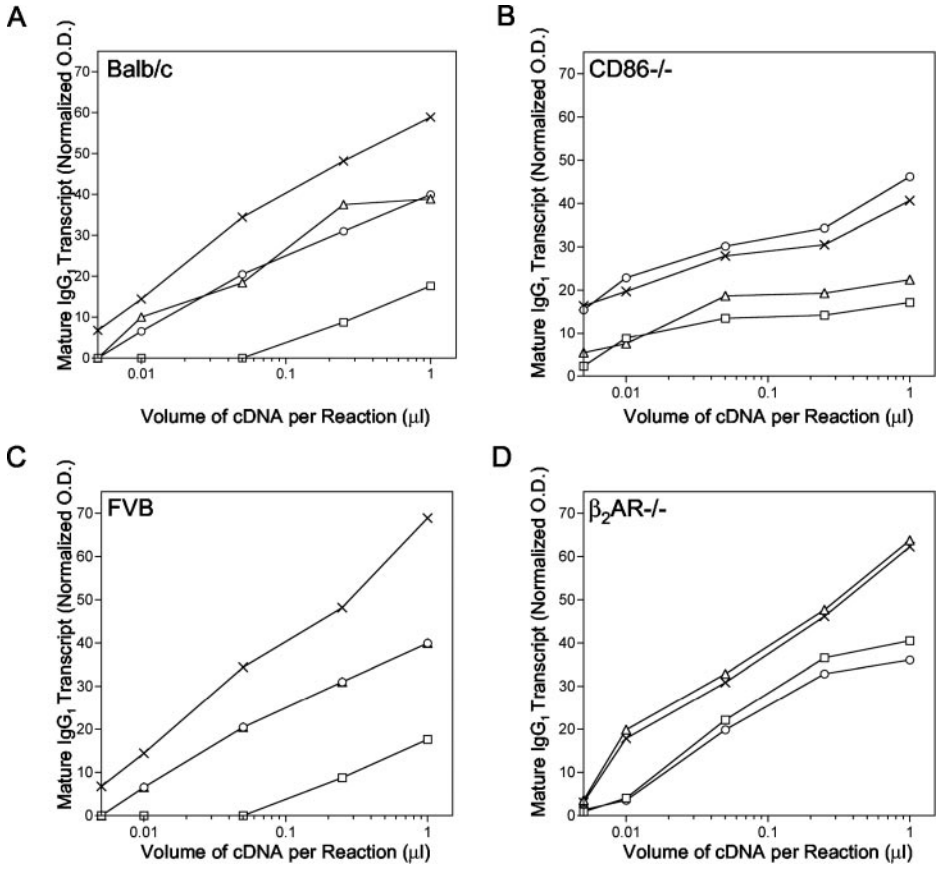

FIGURE 6. Analysis of mature IgG1 transcription rate by nuclear run-on. Resting B cells were isolated from BALB/c (*A*), CD86$^{-/-}$ (*B*), FVB (*C*), or β_2AR$^{-/-}$ (*D*) mice. Cells were activated in culture as described in Fig. 1. After 6 days in culture, nuclei were isolated from 20×10^6 cells, and nuclear run-on was performed in the presence of biotin-16-UTP. Biotin-labeled RNA was incubated with strepavidin-labeled magnetic beads and isolated by magnetic separation. The level of mature IgG1 transcript was analyzed by semiquantitative RT-PCR. The level of mature IgG1 transcript produced was determined for isotype (□), terbutaline (Terb) plus isotype (○), anti-CD86 (△), and Terb plus anti-CD86 (×) treatment groups. Data are presented as the normalized OD of mature IgG1 transcript from one representative of two to four independent experiments.

when the CD86 signal was delivered to the B cell 16–24 h after the CD40 and IL-4R signals. This finding is in contrast to what we reported previously (16), where no change in IgG1 protein was seen when CD86 was stimulated at the same time as CD40 and IL-4R. In the latter exposure protocol, a CD86-mediated increase in IgG1 was seen only if the BCR was stimulated prior to CD40, IL-4R, and CD86 stimulation. Although these two findings appear to be contradictory, they suggest that the ability of CD86 to deliver a signal to a B cell may depend on the order in which activation stimuli, such as Ag, CD40L, and IL-4, are delivered to the B cell in relation to the time of CD86 stimulation. Nonetheless, with the current exposure protocol, we will now be able to use the end point of mature IgG1 transcription to define the signaling pathway responsible for the CD86-mediated enhancing effect on the level of IgG1 protein without the confounding participation of BCR-activated signaling pathways. Future studies will address the role played by BCR stimulation in this response, but only after the signaling pathway induced by CD86 is clearly defined.

Although the present findings do not indicate the transcription factors activated by CD86 stimulation, a few possibilities become less likely in light of the present finding that dissociates the CD40L/IL-4-induced increase in Iγ1 from the CD86/β_2AR-induced increase in the rate of mature IgG1 transcription. For example, the commitment of a B cell to a class switch to IgG1 is dependent on the delivery of specific signals provided by CD40L and IL-4 from a Th2 cell. Experimentally, CD40L and IL-4 induce signals in a B cell that activate NF-κB and STAT-6, respectively (33, 35, 38, 50). Together these two transcription factors activate the germline γ1 promotor to initiate Iγ1 transcription within 24 h, an event that precedes and correlates with the switch recombination event at 48 h (35, 51). The present data show that CD86 stimulation does not increase the level of Iγ1 above that induced by CD40 and IL-4R stimulation alone, suggesting that CD86 is less likely to activate either NF-κB or STAT-6 to induce an increase in mature IgG1 transcript. The finding that Iγ1 did not increase after CD86 stimulation, even when the levels of CD40L stimulation and IL-4 concentration were altered, strengthens this conclusion. On the other hand, because the CD86 signal was generated 16–24 h after the CD40 and IL-4R signals, we cannot rule out the possibility that either NF-κB or STAT-6 may be activated by CD86, but after the time when these transcription factors would have an effect on the level of Iγ1 produced. These possibilities are currently being addressed in our laboratory.

The present finding that both the level and the rate of mature IgG1 transcription, as opposed to Iγ1, are increased by CD86 and/or β_2AR stimulation on CD40L/IL-4-activated B cells strongly suggests that the 3′ IgH enhancer may be activated by an as yet unknown CD86- and/or β_2AR-induced signaling molecule. It is known that transcription of the mature Ig heavy chain gene is tightly regulated during B cell development by primarily three regulatory regions contained within the Ig heavy chain locus, namely the IgH variable promoters, the intronic enhancer (52, 53), and the 3′ IgH enhancer (54). Of these three regulatory regions, the 3′ IgH enhancer is the active regulatory region in mature B cells and plasma cells (55, 56). When the 3′ IgH enhancer is deleted in mature B cells, they maintain the ability to class switch to IgG1, but produce a lower level of secreted IgG1 and mature IgG1 transcript compared with wild-type cells (41). The activity of the 3′ IgH enhancer is known to be regulated by Oct-1/2 and their coactivator OCA-B (40). Since B cells deficient in OCA-B maintain the ability to class switch to IgG1, but the level of IgG1 produced is decreased compared with that in wild-type B cells (57), these published data suggest that activation of the 3′ IgH enhancer in

mature B cells affects mature IgG1 transcript, but not Iγ1. Furthermore, these results suggest Oct-1/2 or OCA-B as possible mediators of the CD86- and/or β_2AR-induced increase in the rate of mature IgG1 transcription. Therefore, since the present data show that the rate of mature IgG1 transcription appears to be affected when CD86 and β_2AR are stimulated, as opposed to the level of Iγ1 or mature IgG1 transcript stability, our proposal that the 3′ IgH enhancer region is targeted by CD86- and β_2AR-induced signals is strengthened by the published data cited above. The present data also indicate that the final signaling intermediates induced by CD86 and β_2AR stimulation may be the same, since the level of mature IgG1 transcript induced when both receptors are stimulated is slightly more than additive. Hence, even though the proximal signaling pathways induced by each receptor may differ, the distal pathways may be the same.

Based on the data presented herein, an end-point response at the gene level has been proposed to explain the mechanism by which CD86 and/or β_2AR stimulation affects the level of IgG1 produced by CD40L/IL-4-activated B cells. This end-point response is distinct from the IgG1 class switching mechanism induced by CD40 and IL-4R stimulation alone and will enable us to study the specific transcription factors and signaling pathways that are activated by CD86 and/or β_2AR signaling to mediate the IgG1 enhancer activity. Therefore, understanding the mechanisms by which IgG1 is regulated by CD86 and β_2AR is important, since these signals are most likely involved in the homeostatic regulation of B cell activity. For example, CD86 is stimulated on a B cell during a Th cell-B cell interaction in either the initial Ag presentation event to a resting Th2 cell (13) or during interaction with a previously activated Th2 cell (58). On the other hand, the β_2AR on a B cell is stimulated by norepinephrine released from nerve terminals residing within the parenchyma of lymphoid tissue in the B cell microenvironment shortly after Ag entry (28). Thus, the stimulation of both CD86 and β_2AR on a B cell may help to regulate the level of normal B cell activity in vivo. Also, the finding that a common transcriptional mechanism may be used by CD86 and β_2AR for the regulation of mature IgG1 transcription is attractive because it may serve as a target for therapeutic intervention to regulate the amount of IgG1 produced by cells that have undergone class switching to IgG1.

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