

The Level of IgE Produced by a B Cell Is Regulated by Norepinephrine in a p38 MAPK- and CD23-Dependent Manner¹

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Although the causes of asthma vary, the severity of the disease correlates with the level of IgE produced. In this study we show that mice produced less IgE when they were depleted of the neurotransmitter norepinephrine (NE) before the administration of Ag. The suppression was prevented when a β_2 -adrenergic receptor (β_2 AR)-selective agonist was administered, suggesting that NE stimulated the β_2 AR to regulate the level of an IgE response in vivo. Although the cell targeted by NE to produce this effect in vivo is unknown, we show in vitro that the level of IgE increased on a per cell basis without an effect on class switch recombination when NE stimulated the β_2 AR on a B cell directly. The β_2 AR-induced increase in IgE depended on p38 MAPK but not protein kinase A activation, was due to an increased rate of mature IgE mRNA transcription, and was lost when β_2 AR-deficient B cells were used. Also, CD23 transcription was increased in a p38 MAPK-dependent manner and resulted in an increased level of soluble CD23 (sCD23). The β_2 AR-induced increase in sCD23 was associated with IgE up-regulation and possibly interacted with CD21/CD19. Using B cells from respective knockout mice, data showed that the β_2 AR-induced increase in IgE depended on B cell expression of CD23, CD21, and CD19. These findings suggest that at least one mechanism by which endogenous B cell activity in vivo is regulated by NE involves stimulation of the β_2 AR on the B cell alone to increase the level of IgE produced in a p38 MAPK- and sCD23-dependent manner. *The Journal of Immunology*, 2006, 177: 2926–2938.

Immunoglobulin E plays a central role in the pathophysiology of allergic asthma and contributes to the inflammatory process, as indicated by findings in humans and mice that show a strong correlation between the level of IgE detected in the serum and bronchoalveolar lavage (BAL)³ and the severity of the allergic airway inflammation that develops (1–3). Because of this correlation, the use of anti-IgE neutralizing Abs has become a very effective treatment for allergic asthma (4). In contrast to the apparent negative role played by IgE, a positive role is also played in the defense against certain parasites (reviewed in Ref. 5). Also, a minimal level of IgE in the lung seems to be required to effectively clear Ag so that the extent of airway inflammation after Ag exposure is limited (6). Thus, a thin line exists between the normal physiological response to Ag in the lung, which is protective, and the one that leads to the development of asthmatic pathology.

Given the strong association between the level of IgE, which is produced by a B cell, and the development of asthmatic disease, it is apparent that we need to understand not only the mechanisms by which IgE production is initiated in a B cell but also the mechanisms by which the amount of IgE produced by a B cell is increased or decreased on a per cell basis. A germinal center (GC) that forms in a secondary lymphoid organ upon Ag challenge establishes a specialized microenvironment in which activated B cells undergo class switch recombination (CSR) to IgE (reviewed in Ref. 7). During the development of allergic airway inflammation, GCs develop also in bronchus-associated lymphatic tissue (BALT), providing a readily available local source of IgE in the lung (8). A B cell requires a minimum of two signals to initiate CSR to IgE, one signal delivered via CD40 and one by the IL-4R, both of which are provided to the B cell by a CD40L on and an IL-4 secreted by an activated Th2 cell during the GC reaction (reviewed in Ref. 7). However, in contrast to CSR, which initiates the IgE response, the amount of Ab produced by an individual B cell is under the control of a regulatory mechanism that is activated within the B cell itself. This mechanism of regulation involves a change in the activity of the 3'-IgH enhancer, which is located downstream to the IgH region where CSR occurs (9) and is induced by transcription factors that are activated subsequent to B cell activation, e.g., Oct-2 (10) and AP-1 (11).

In addition, another mechanism endogenous to the B cell itself has been proposed to specifically regulate the level of IgE produced by a B cell. This mechanism involves either the stimulation or release of the low affinity receptor for IgE, the Fc ϵ R1, which has been designated CD23. Several different immune cell types express CD23 upon activation (reviewed in Ref. 12), and two forms of CD23 exist, namely, a membrane-bound molecule (mCD23) and a soluble molecule (sCD23) that forms when shed from the cell surface (13). In CD23-deficient mice, serum IgE and

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³ Abbreviations used in this paper: BAL, bronchoalveolar lavage; AR, adrenergic receptor; ATF-2, activating transcription factor 2; BALT, bronchus-associated lymphatic tissue; CSR, class switch recombination; GC, germinal center; i.t., intratracheal; mCD23, membrane-bound CD23; NE, norepinephrine; sCD23, soluble CD23; 6-OHDA, 6-hydroxydopamine; PKA, protein kinase.

bronchial hyperresponsiveness were increased when compared with that in wild-type mice, suggesting a negative regulatory role for mCD23 in regulating the level of IgE produced (14, 15). By contrast, sCD23 has been shown to up-regulate the level of IgE produced by an activated human B cell (16, 17), possibly by interacting with the CD21/CD19 complex (18, 19), suggesting a positive regulatory role for sCD23 in regulating the level of IgE produced (19, 20). Thus, B cells possess endogenous mechanisms that not only initiate the process of CSR to IgE but also self-regulate the level of IgE produced by the cell.

In addition to these well-characterized mechanisms of endogenous B cell regulatory activity, a homeostatic mechanism exists external to the B cell itself by which the level of Ab produced by a B cell is regulated further. This mechanism involves activation of the sympathetic nervous system and the subsequent release of the neurotransmitter norepinephrine (NE) from nerve endings that penetrate the parenchyma of all lymphoid tissues (reviewed in Refs. 21 and 22), including the BALB (23). The nerve endings in these tissues are in close proximity to immune cells that express the β_2 -adrenergic receptor (β_2 AR), which binds NE as it is released from nerve endings shortly after Ag exposure (24). The level of IgE (25–27), IgG1 (24, 27–30), and IgM (24, 29, 30) produced by a B cell in vivo or in vitro is reported as being affected by NE and β_2 AR stimulation. In addition, GC formation in the spleens of NE-depleted *scid* mice that were reconstituted with Ag-specific Th2 and B cells was diminished after Ag exposure in comparison to mice in which NE remained intact (24), likely due to loss of CD86 up-regulation on B cells in the absence of NE (31), a molecule that is critical for GC formation (32). Thus, the level of 3'-IgH enhancer activity seems to be regulated by mechanisms that are endogenous and exogenous to the B cell itself.

At the molecular level, the rate of mature IgG1 mRNA production, as determined by nuclear run-on analysis, was increased when a CD40L/IL-4-activated B cell was cultured in the presence of either NE or a β_2 AR agonist. The mechanism by which the β_2 AR-induced enhancing effect on IgG1 was mediated involved a protein kinase A (PKA)-dependent and phosphorylated CREB-dependent increase in not only the level of expression of the coactivator protein OCA-B, but also the level of binding of the OCA-B/Oct-2 complex to the 3'-IgH enhancer (33). In contrast, the process of CSR was unaffected by β_2 AR stimulation. However, although CD40L and IL-4 also promote the differentiation of B cells to IgE-secreting cells, it remained unknown as to whether or not the same molecular mechanism that regulates the β_2 AR-induced increase in the level of IgG1 produced per B cell was the same mechanism involved in mediating the increase in IgE (27). The importance of understanding whether or not these mechanisms are the same or different becomes evident when one considers that β_2 AR agonist drugs are frequently used in the treatment of allergic asthma to relieve bronchoconstriction but are also often associated with adverse effects after long term use in the absence of immunosuppressive drugs (34). Thus, a β_2 AR agonist-induced increase in the level of IgE produced by a B cell might likely interfere with the therapeutic effectiveness of the agonist when it is used to relieve bronchoconstriction.

Therefore, in the present study we first determined whether the increase in the level of IgE produced by a B cell resulted from β_2 AR stimulation by NE on a B cell directly and whether the mechanism involved a change in either CSR, 3'-IgH enhancer activity, and/or the CD23/sCD23 regulatory mechanism. Because the level of sCD23 released from a human monocytic cell line was reported to increase when the β_2 AR was stimulated (35), and because sCD23 was reported to exert a stimulatory effect on IgE production by a B cell, it was possible that the increase in the level

of IgE produced following β_2 AR stimulation on a B cell involved the CD23/sCD23 regulatory system. The data show that the normal Ag-induced level of IgE in serum and BAL, as well as the severity of allergic airway inflammation, were determined by the presence of NE and stimulation of the β_2 AR. In vitro data indicate one mechanism that may have increased the IgE response in vivo involved NE stimulation of the β_2 AR on a B cell directly to increase the rate of mature IgE mRNA transcription without an effect on CSR. Furthermore, the β_2 AR-induced increase in IgE was mediated by a p38 MAPK-dependent up-regulation in the level of CD23 mRNA expression and CD23 release from the cell, suggesting that β_2 AR stimulation on a B cell targets the CD23/sCD23 regulatory system to control the level of IgE produced by a B cell.

Materials and Methods

Mice

Mice were housed under pathogen-free conditions and used at 9–12 wk of age. Female BALB/c (H-2^d restricted) and female FVB (H-2^q-restricted) mice were purchased from Taconic Farms. β_2 AR^{-/-} mice (H-2^q restricted) were provided by Dr. B. Koblika (Stanford University, Stanford, CA). All mice were bred and housed in a pathogen-free facility until 7–8 wk of age and then housed at Ohio State University (Columbus, OH) in microisolator cages and provided autoclaved food and water ad libitum. CD23^{-/-} and CD23-overexpressing transgenic mice, as well as control mice (BALB/c), were housed at Virginia Commonwealth University (Richmond, VA). Spleens from CD21/CD35^{-/-} mice were provided by Dr. T. Tedder (Duke University, Durham, NC) (36). CD19^{-/-} mice and appropriate control mice were obtained from The Jackson Laboratory. All experiments complied with the Animal Welfare Act and the National Institutes of Health (Bethesda, MD) guidelines for the care and use of animals in biomedical research.

Isolation of CD43-negative B cells and culture conditions

Spleens were removed after mice were euthanized with CO₂. RBC were lysed by treating total splenocytes with 0.4% ammonium chloride. Splenocytes were then incubated with anti-mouse CD43 magnetic beads following the manufacturer's directions (Miltenyi Biotec), and CD43-negative cells were collected using autoMACS (Miltenyi Biotec) and cultured in complete RPMI consisting of RPMI 1640 medium (Mediatech), 10% FBS (Atlas Biologicals), 20 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, and 50 μ M 2-ME for either RNA analysis in 24-well plates (Costar) at 2.5×10^5 cells/ml in a final volume of 2 ml or protein analysis in 96-flat-bottom well plates (Costar) at 2.5×10^5 cells/ml in a final volume of 200 μ l at 37°C and 5% CO₂. B cells were activated with CD40L expressed on Sf9 cells at a ratio of 10:1 and with IL-4 (1 ng/ml) (eBioscience) in the presence or absence of either the β_2 AR agonist terbutaline (10^{-6} M final concentration or as indicated) or norepinephrine ((-)-arterenol at 10^{-6} M final concentration or as indicated) that were purchased from Sigma-Aldrich. The p38 and PKA inhibitors SB203580 (1.0 μ M final concentration), SB202190 (60 nM final concentration), H-89 (0.2 μ M final concentration), and KT5720 (0.1 μ M final concentration) were purchased from BIOMOL. The concentration of Sf9/CD40L cells and IL-4 used was found to be optimal to detect the β_2 AR-induced effect on Ab production (data not shown). Adrenergic receptor blocking studies were performed using selective antagonists to either the β AR (nadolol), β_2 AR (butoxamine), β_1 AR (metoprolol), α_1 AR (prazosin), or α_2 AR (yohimbine) at either a 10^{-5} or a 10^{-6} M final concentration (Sigma-Aldrich). All of the reagents used for B cell isolation, activation, and pharmacologic treatment tested negative for the presence of endotoxin using E-TOXATE (Sigma-Aldrich), a *Limulus* lysate assay with a level of detection of <0.1 U/ml.

Depletion of norepinephrine

Chemical sympathectomy was performed using 6-hydroxydopamine (6-OHDA) as described elsewhere (24). Briefly, BALB/c mice (20–25 g) were injected i.p. with either 6-OHDA or saline in a volume of 200 μ l at a dose of 100 mg/kg on day -6 followed by 200 mg/kg on days -4 and -2 in relation to sensitization on day 0.

Immunization and induction of airway inflammation

Sensitization of animals and elicitation of airway inflammation was performed according to published protocols (37) with slight modifications. Briefly, 2 days after the last 6-OHDA injection (day 0) mice were immunized with 100 μ g/mouse OVA in 200 μ l of alum (Imject Alum; Pierce)

and boosted with OVA/alum on day 21. One week after the booster immunization, animals were anesthetized with halothane and challenged intratracheally (i.t.) using an intrapulmonary aerosolizer (Penn-Century) with 25 μ l of OVA/PBS (2 mg/ml) on three consecutive days. In some experiments mice received terbutaline (5 mg/kg) 12 h following the primary immunization. Blood samples were obtained at indicated time points by retroorbital bleedings, and serum was stored at -80°C until analysis. Five or 6 days after the first i.t. challenge mice were sacrificed, the trachea was cannulated, and BAL was performed twice using 0.3 ml of PBS with 1% FCS. The two fractions were combined, and cell-free supernatant was stored at -80°C until analysis. Total IgE in BAL and serum samples was analyzed by ELISA at the indicated dilutions and time points (Figs. 1–3).

Protein ELISA

B cell culture supernatants were collected at indicated time points after the initial activation. Supernatant was frozen immediately at -80°C until analyzed by ELISA as described in detail previously (38, 39). A standard curve for IgE, IgG1, and sCD23 was prepared using known quantities of IgE (IgE κ ; BD Pharmingen) or sCD23 as described previously (39). Coating Abs used were rat anti-mouse IgE (clone R35-72; BD Pharmingen), rabbit anti-mouse IgG (H + L; Southern Biotechnology Associates), and rat anti-mouse CD23 (clone 2G8). Detecting Abs used were alkaline phosphatase-labeled rat anti-mouse IgE (clone 23G3; Southern Biotechnology Associates), alkaline phosphatase-labeled goat anti-mouse IgG1 (Southern Biotechnology Associates), polyclonal rabbit anti-mouse CD23, and alkaline phosphatase-labeled goat anti-rabbit IgG (H + L; Southern Biotechnology Associates). Plates were developed with *p*-nitrophenyl phosphate substrate (Sigma-Aldrich) in Dulbecco's Eagle's medium buffer. Densitometric analysis was performed on a SpectraMax Plus microplate reader (Molecular Devices) at a wavelength of 405 nm. The lower limit of detection for IgE and IgG1 was <5 ng/ml and <8 ng/ml, respectively, for sCD23. Intra- and inter-assay variation was $<10\%$.

ELISPOT assay

ELISPOT assays were performed as described in detail elsewhere (40, 41). To determine the number of cells that were secreting IgE, plates were developed with a developing solution containing 5-bromo-4-chloro-3-indolyl phosphate (Sigma Fast BCIP/NBT; Sigma-Aldrich). Spot-forming cells were enumerated using a dissecting microscope. For assays following preculture in vitro, 5×10^4 viable B cells for each treatment condition were plated and incubated for 5 h at 37°C with 5% CO_2 . For the detection of the amount of IgE secreted by 5×10^4 viable B cells, plates were developed with a standard soluble ELISA substrate instead of an insoluble ELISPOT substrate, and color development was determined at 405 nm as described above for the IgE ELISA. To determine the number of IgE-secreting cells in the spleen 5 days following immunization with 100 μ g OVA in 200 μ l of alum, 2.5×10^5 splenocytes were plated after RBC lysing, the plates were incubated for 18 h at 37°C with 5% CO_2 , and IgE $^{+}$ spots were developed as described above and counted.

Quantitative real-time PCR

Quantitative real-time PCR was performed as described elsewhere (28). Briefly, total RNA was isolated from B cells using TRIzol (Invitrogen Life Technologies) following the manufacturer's protocol. The concentration and purity of the isolated RNA were determined by measuring the absorbance at 260 and 280 nm. Samples were stored at -80°C until analysis. All RNA samples were treated with 1 μ g of DNase I (Invitrogen Life Technologies) per 1 μ g of RNA preparation. For quantitative real-time PCR, a common master mix (LightCycler-FastStart DNA SYBR Green I (Roche), MgCl_2 (concentration adjusted to specific primer), and 0.5 μM gene-specific primer), and 1.0 μ l of cDNA were used at a final reaction volume of 10 μ l. For each gene the following cycling protocol was used: 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 15 s, a gene-specific annealing temperature for 2 s, and a 72°C extension for 20 s. The gene-specific cDNA expression level was determined by comparison to a standard curve of gene-specific PCR product diluted 1/10 for concentrations ranging from 1 ng/ml to 1 fg/ml and normalization of the results to the expression level of β -actin in the same sample to control for differences in the RT-PCR. The following primers were used: β -actin, 5'-TACAGCTTCACCACCACAGC-3' (top primer) and 5'-AAGGAAGGCTGGAAAA GAGC-3' (bottom primer); CD23b mRNA, 5'-CCCAGAACTGGCTCATTTC-3' (top primer) and 5'-TCCCGTCCGACCATAAACTC-3' (bottom primer); germline IgE transcript, 5'-TGGGCATGAATTAATG GTTACTAGAG-3' (top primer) and 5'-TGGCCAGACTGTTCTTATTC GAA-3' (bottom primer); and mature IgE transcript, 5'-TCAAGGAACC TCAGTCACCGTCTC-3' (top primer) and 5'-TTACAGGGCTTCAAGG GGTAGAGC-3' (bottom primer). All primers were synthesized and

purchased from Integrated DNA Technologies. After the PCR, 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 present. PCR was preformed using the Roto-Gene 2000 real-time cycler (Phoenix Research Products).

Determination of mature IgE mRNA stability

Stability of the mature IgE mRNA transcript was determined using an actinomycin D inhibition assay as described previously (28). Briefly, after 5 days of culture, transcription was stopped by adding 20 $\mu\text{g}/\text{ml}$ actinomycin D (Sigma-Aldrich) to each well. B cells were collected from the cultures at 0, 1, 3, and 6 h following the addition of actinomycin D, and total RNA was isolated. Cell viability was analyzed by trypan blue exclusion and did not significantly change over the course of the experiment. The level of mature IgE transcript was quantified for each time point by quantitative real-time PCR as described above.

Nuclear run-on analysis

The rate of mature IgE transcription was determined by nuclear run-on analysis as described in detail previously (28, 42). Briefly, 5×10^6 B cells were collected on ice at day 5 following activation and washed twice with cold 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 . Proper lysis of the cell membrane was monitored by dark field microscopy. Nuclei were collected by centrifugation at $300 \times g$ for 10 min at 4°C , resuspended in 500 μ l 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 used for nuclear run-on analysis. Nuclear run-on and RNA isolation were performed in the presence of biotin-16-UTP (Roche). Dynabeads M-280 (Invitrogen Life Technologies) were used to capture and purify the biotin-labeled RNA molecules that were produced during the nuclear run-on period. Beads attached to RNA were washed twice with $2 \times \text{SSC}$ plus 15% formamide, once with $2 \times \text{SSC}$, and resuspended in 30 μ l of diethyl pyrocarbonate-treated H_2O before the preparation of random hexamer-primed cDNA. Semiquantitative real-time PCR was preformed from serially diluted cDNA samples for the levels of actin and mature IgE transcripts. The OD values obtained for IgE were normalized to β -actin. Samples that did not contain biotin-16-UTP served as negative controls and were found to be negative for the presence of β -actin and mature IgE transcripts.

Western blot analysis

CD23 total protein was determined in cells lysates by Western blot analysis as described elsewhere (33). Briefly, resting B cells were activated in 24-well plates as described above. At the indicated time points B cells were collected, washed with PBS, lysed on ice in 120–200 μ l of lysis buffer (100 mM Tris-HCl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, and protease inhibitor mixture (1/200) (Sigma-Aldrich)), and stored at -80°C until analysis. Fifteen micrograms of protein sample per lane was run together with a m.w. standard on a 7.5% polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad). After blocking membranes with TBST (140 mM NaCl, 25 mM Tris (pH 7.5), KCl (0.2 mg/ml), and 0.05% Tween 20 plus 5% dry milk) for 1 h at room temperature, membranes were probed with rabbit polyclonal anti-mouse CD23 (in-house production), rabbit polyclonal anti-mouse phosphorylated p38 MAPK (Thr ^{180} /Tyr ^{182} ; Cell Signaling Technology), rabbit anti-mouse p38 MAPK (Cell Signaling Technology), or polyclonal goat anti-actin (C-11; Santa Cruz Biotechnology) overnight at 4°C . After washing with TBST, membranes were probed with corresponding HRP-labeled secondary Abs diluted in TBST at room temperature for 1 h, and HRP-labeled Abs were detected using a LumiGlo detection kit (Cell Signaling Technology). Luminescence of specific bands was visualized on Kodak Biomax MS film using an intensifying screen-enabled film cassette. Densitometry was performed using NIH image 1.61.

p38 MAPK activity assay

A p38 MAPK activity kit (Cell Signaling Technology) was used. Resting B cells (10×10^6) were activated in 6-well plates with CD40L/IL-4 in the presence or absence of terbutaline (10^{-6} M). The kinase assay was performed according to the manufacturer's instructions. At the indicated time points, B cells were collected and total protein was isolated. Phosphorylated p38 (Thr ^{180} /Tyr ^{182}) was immunoprecipitated from 80 μ g of total protein for each sample, followed by an in vitro kinase assay using the provided activating transcription factor 2 (ATF-2 fusion protein 2) as a substrate. ATF-2 phosphorylation was detected by Western blot analysis using the provided phosphorylated ATF-2 (Thr ^{71}) Ab. Densitometry was performed using NIH Image 1.61.

Statistics

Data were analyzed by ANOVA to determine whether an overall statistically significant change existed. Certain *p* values were calculated using either a Bonferroni post hoc test for comparison of more than two treatment groups or a Student *t* test for comparison between two treatment groups. Regression analysis was used as described in the corresponding figure legends. Statistically significant differences were reported when the *p* value was <0.05. Prism 4 for Macintosh V.4.0a (GraphPad Software) was used to prepare graphs and analyze data statistically.

Results

The level of IgE produced by a B cell is increased when NE stimulates the β_2 AR

Previous findings showed that the level of IgG1 (28) and IgE (27, 43) produced in vitro was increased when the β_2 AR was stimulated by a selective agonist and that this increase was prevented when either a β_2 AR-selective antagonist was present in the culture or β_2 AR-deficient B cells were used (27, 28). To determine

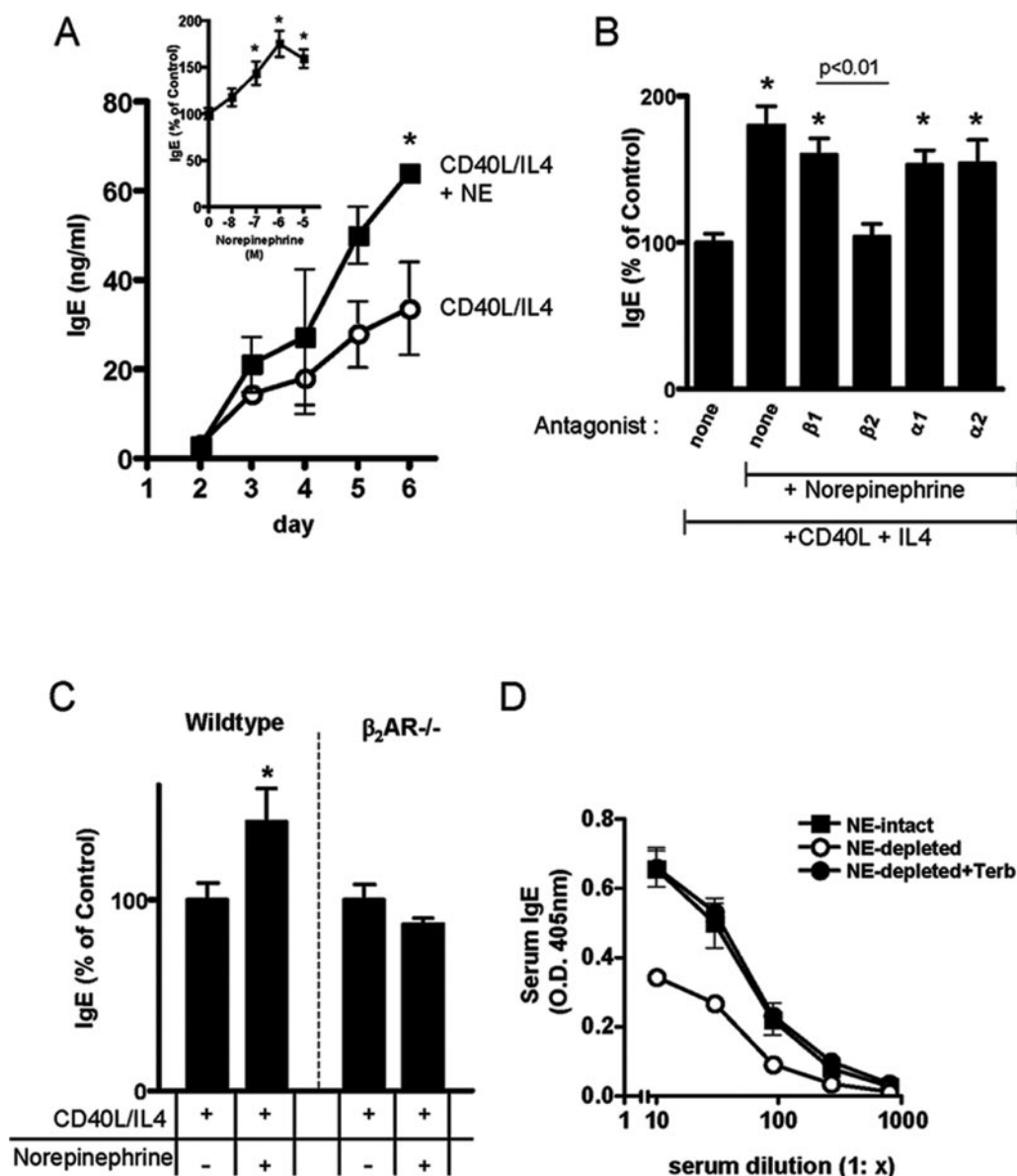


FIGURE 1. IgE production in vitro and in vivo is increased when NE stimulates the β_2 AR on a B cell. *A–C*, Resting B cells were isolated from mouse spleens and activated with CD40L/Sf9 cells (10:1) and IL-4 (1 ng/ml) in the presence or absence of NE (10^{-6} M or indicated concentration). A significant difference was determined by ANOVA with post hoc test; *, *p* < 0.05. *A*, Time course and concentration response using B cells from BALB/c mice. Data are presented as the mean IgE value (ng/ml) $\pm$ SEM from two independent experiments. *B*, Selective pharmacologic antagonists (10^{-6} M) to the following adrenergic receptor subtypes were added with NE: α_1 (prazosin); α_2 (yohimbine); β_1 (metoprolol); or β_2 (butoxamine). Data are presented as the mean percentage $\pm$ of IgE produced by control B cells activated with CD40L/IL-4 alone (control) from two independent experiments. The absolute value for control IgE was 41.3 ± 2.1 ng/ml. *C*, B cells from wild-type FVB or β_2 AR-deficient (β_2 AR^{-/-}) mice. Data are presented as the percentage $\pm$ SEM of IgE produced by control B cells activated with CD40L/IL-4 alone from three independent experiments. The absolute value for control IgE was 40 ± 3.2 ng/ml (FVB) and 60.4 ± 4 ng/ml (β_2 AR^{-/-}). *D*, Serum was obtained from NE-depleted mice (open circles; *n* = 2), NE-intact mice (filled squares; *n* = 3), and NE-depleted mice administered terbutaline (Terb) i.p. at 5 mg/kg (closed circles; *n* = 3) 3 wk following primary immunization i.p. with OVA/alum. Total IgE was determined by ELISA in serial diluted serum, and data are presented as OD values obtained at 405 nm from one representative experiment of three independent experiments.

whether the physiological ligand norepinephrine stimulated the β_2 AR on a B cell alone to induce an equivalent increase in the level of IgE, resting splenic B cells were activated *in vitro* with CD40L and IL-4 in the absence or presence of NE and/or antagonists selective for the α_1 AR, α_2 AR, β_1 AR, and β_2 AR. The level of IgE was increased by NE in a concentration-dependent (Fig. 1A, *inset*) and time-dependent manner (Fig. 1A) that reached a 2-fold maximum, as compared with B cells activated in the absence of NE. The increase was inhibited only when cells were cultured with NE in the presence of a β_2 AR antagonist, indicating that the increase was mediated by NE stimulation of the β_2 AR (Fig. 1B). The latter finding was confirmed when NE was added to cultures of β_2 AR-deficient B cells and failed to produce an increase in IgE (Fig. 1C). The addition of antagonists alone to the activated B cells did not change either total cell number or cell viability, and the addition of either NE or antagonists alone to resting B cells in the absence of CD40L and IL-4 did not induce IgE production (data not shown). These findings suggest that the B cell is one potential target by which NE can increase the level of IgE produced and that the NE-induced increase in B cell activity is direct and mediated by the β_2 AR specifically.

The parenchyma of lymphoid organs is known to be innervated with sympathetic nerve fibers that release NE within hours of Ag administration (31). To determine *in vivo* whether the level of IgE produced after primary immunization was affected by the release of NE, BALB/c mice were depleted of NE with the pharmacologic drug 6-OHDA before administration of OVA/alum. The level of serum IgE was reduced in the NE-depleted mice 3 wk after immunization when compared with control mice in which NE was intact (Fig. 1D). To determine whether the reduction in serum IgE levels was due to a lack of β_2 AR stimulation in the mouse, the β_2 AR agonist terbutaline was administered within hours of the immunization of NE-depleted mice. These mice showed an ~2-fold increase in the level of serum IgE when compared with NE-depleted mice, which was comparable to the level produced by NE-intact controls (Fig. 1D). Thus, the *in vivo* findings suggest that NE stimulates the β_2 AR in OVA-immunized mice to increase the level of serum IgE produced. Although the exact cell type affected directly by NE *in vivo* was unknown, the B cell may represent one potential target cell, as indicated by the *in vitro* data described above.

To determine whether the lung pathophysiology associated with asthma was affected by the presence of NE *in vivo*, NE-depleted and NE-intact mice were immunized *i.p.* with OVA/alum on days 0 and 21 and challenged on day 28 with an *i.t.* administration of OVA/PBS for three consecutive days before BAL was performed. The level of IgE was below the level of detection in mice before Ag administration when the mice are either NE-intact or NE-depleted. The level of serum IgE in the NE-depleted mice at 3 wk after the day 0 immunization was decreased when compared with NE-intact controls (Fig. 2A), as was the level of locally produced IgE in the BAL after the 3 days of challenge (Fig. 2B). Thus, the IgE response to Ag exposure *in vivo*, both systemically and locally in the lung, are increased by NE, suggesting a potential role for NE in modulating the pathophysiology of allergic airway inflammation.

The cellular and molecular basis for the increase in IgE induced by NE and β_2 AR stimulation

To determine the exact mechanism by which IgE production is increased following β_2 AR stimulation, we used the β_2 AR agonist terbutaline instead of NE. This approach was taken because the β_2 AR was defined in Fig. 1 as the sole target for NE on the B cell to increase IgE production. To determine whether the increase in IgE induced by direct β_2 AR stimulation on a B cell was due to an

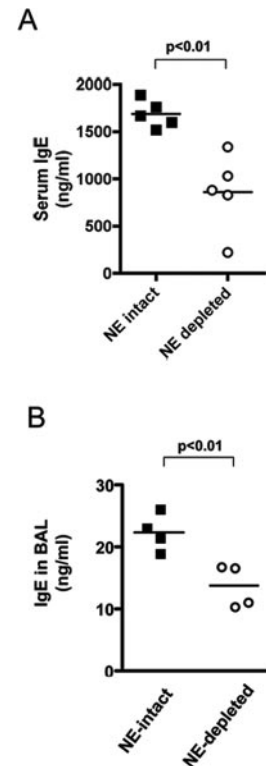


FIGURE 2. The level of serum IgE and BAL fluid IgE, respectively, is decreased by NE depletion *in vivo*. NE-depleted (open circles; $n = 5$) and NE-intact (closed squares; $n = 5$) mice were immunized *i.p.* with OVA/alum. **A**, Three weeks following immunization, total IgE was determined by ELISA in 1/3 diluted serum, and data are presented in nanograms per milliliter from individual mice. Data from one representative experiment of three experiments are shown. Horizontal bars represent the median of each treatment group. Significant differences were determined by ANOVA with a post hoc test. **B**, The same mice as shown in **A** were challenged with OVA/alum at wk 3, and 1 wk later they were challenged three times with OVA administered *i.t.* Five days after the first *i.t.* challenge BAL fluid was collected, with the exception that one mouse was removed from each group to obtain histology without the prior collection of BAL fluid. Total IgE was determined by ELISA in undiluted cell-free BAL, and data are presented from individual mice as the IgE value (ng/ml) from one representative experiment of two independent experiments. Horizontal bars represent the median of each treatment group. Significant differences were determined by ANOVA with a post hoc test.

increase in either the number of B cells secreting IgE and/or the amount secreted per cell, B cells were activated with CD40L/IL-4 in the absence or presence of the β_2 AR agonist terbutaline and evaluated for the number of cells secreting IgE and/or the amount of IgE secreted per cell using ELISPOT. The amount of IgE secreted per B cell was significantly increased by ~65% when B cells were activated in the presence of terbutaline, an effect that was blocked by the addition of nadolol, a β AR antagonist (Fig. 3A, *left*). In contrast, the number of IgE-secreting cells did not differ between treatment groups (Fig. 3A, *right*). Similarly, the level of serum IgE in OVA/alum-immunized mice on day 5 was ~50% less in the NE-depleted mice as compared with NE-intact controls (Fig. 3B, *left*), whereas the number of IgE-secreting cells was equivalent in the spleens from NE-depleted and NE-intact mice 5 days following primary immunization with OVA/alum (Fig. 3B, *right*). Thus, the amount of IgE secreted per B cell is increased by NE and β_2 AR stimulation as opposed to the number of cells secreting IgE.

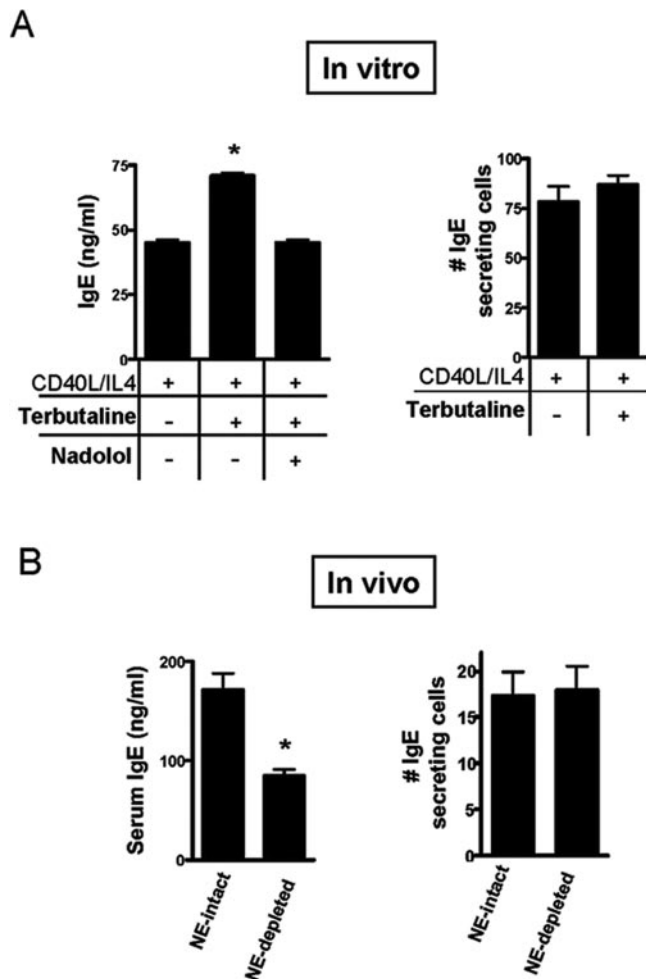


FIGURE 3. The level of IgE produced per cell increases after β_2 AR stimulation on a B cell without a change in the number of IgE-secreting cells. **A**, Resting B cells were isolated from the spleens of BALB/c mice and cultured as described in Fig. 1 in the absence or presence of terbutaline. To some cultures nadolol (10^{-5} M) was added. The amount of total IgE secreted (*left*) and the number of IgE-secreting cells (*right*) were determined by an ELISPOT assay of 5×10^4 viable B cells collected on day 5. Data are presented as either the mean IgE value (ng/ml) $\pm$ SEM or the number of IgE-secreting cells $\pm$ SEM from one representative experiment of three independent experiments. Significant differences were determined by ANOVA with a post hoc test; *, $p < 0.05$. **B**, Five days following immunization with OVA/alum, serum from NE-intact ($n = 3$) or NE-depleted ($n = 4$) BALB/c mice was analyzed for total IgE by ELISA (*left*), and splenocytes were analyzed for the number of IgE-secreting cells by ELISPOT (*right*). Data are presented from a single experiment as either the mean serum IgE value (ng/ml) $\pm$ SEM or the number of IgE-secreting cells $\pm$ SEM per 1×10^6 input cells. Significant differences were determined by ANOVA with a post hoc test; *, $p < 0.05$.

A previous finding obtained *in vitro* using limiting dilution analysis of B cells exposed to Ag and Th₂ cells showed that the frequency of resting B cells that differentiated to produce IgE after β_2 AR stimulation remained the same as that of control B cells (38). This finding indicated that the level of CSR was unaffected by β_2 AR stimulation and supported the present finding that the increase in IgE induced by β_2 AR stimulation was likely due to an increase in the level of IgE produced per cell as opposed to more cells producing IgE. To confirm this finding at the transcriptional level, the expression of germline and mature IgE mRNA transcript was measured at 2 and 5 days of culture, respectively, in CD40L/IL-4-activated B cells cultured in the presence or absence of ter-

butaline. Using quantitative real-time PCR, the level of germline IgE transcript (I_{ϵ}) was equivalent in both sets of B cells (Fig. 4A). In contrast, the level of mature IgE mRNA was increased ~ 2 -fold in B cells exposed to β_2 AR stimulation when compared with control B cells (Fig. 4B), and the increase was prevented when either the β AR antagonist nadolol was added with terbutaline (Fig. 4B) or B cells from β_2 AR-deficient mice were used (Fig. 4C). Thus, the mechanism responsible for the increase in IgE protein per cell involved an increase in the level of mature IgE transcription without an effect on germline IgE.

The two primary mechanisms that regulate the amount of mRNA produced in mammalian cells are transcript stability and/or rate of mRNA transcription. To determine whether either of these mechanisms was involved in regulating the increase in mature IgE mRNA produced by B cells exposed to a β_2 AR agonist, resting B cells were activated with CD40L/IL-4 in the absence or presence of terbutaline, and RNA was collected 5 days later. Using an actinomycin D inhibition assay, the mRNA from B cells exposed to terbutaline appeared to degrade faster but was not statistically significant than the mRNA collected from control cells (Fig. 4D), suggesting that this mechanism was likely not responsible for the increase in IgE per cell. In contrast, nuclear run-on analysis indicated that IgE mRNA transcription was increased from a rate of 1.2 ± 0.2 normalized U/min in control cells to a rate of 2.1 ± 0.1 normalized U/min in B cells exposed to terbutaline (Fig. 4E). Thus, the findings suggest that the increase in IgE induced by β_2 AR stimulation on a B cell is due to an increase in the rate of mature IgE mRNA transcription.

The increase in IgE induced by NE and β_2 AR stimulation is dependent on p38 MAPK activation

IgG1 is another Ab isotype that is regulated by B cell activation with CD40L and IL-4. It was previously reported that the level of IgG1 was increased by NE and β_2 AR stimulation and that this increase also involved increases in the rate of mature IgG1 mRNA transcription and the level of IgG1 produced per cell (28). It was also reported that the β_2 AR-induced increase in IgG1 mRNA involved an increase in PKA activation. To determine whether the increase in IgE induced by β_2 AR stimulation involved the activation of PKA, B cells were activated in the presence or absence of terbutaline and the selective PKA inhibitors H-89 (Fig. 5A) or KT5720 (data not shown). Both of these PKA inhibitors prevented the terbutaline-induced increase in IgG1 production in a concentration-dependent manner with an IC_{50} of $\sim 2 \times 10^{-8}$ M. In contrast, a 10-fold higher concentration of the PKA inhibitors was required to inhibit the terbutaline-induced increase in IgE. Although PKA is the primary signaling intermediate activated by β_2 AR stimulation, MAPKs are also activated by β_2 AR stimulation (44–48) and are also involved in CD23 regulation (49, 50). To determine whether IgE was increased by β_2 AR stimulation in a MAPK-dependent manner, B cells were activated in the absence or presence of the selective p38 MAPK inhibitor SB203580. The inhibitor prevented the terbutaline-induced increase in IgE production in a concentration-dependent manner with an IC_{50} of $\sim 2 \times 10^{-7}$ M, whereas 100-fold higher concentrations were unable to inhibit the β_2 AR-induced increase in IgG1 (Fig. 5B). Pharmacologic inhibitors alone in the absence or presence of terbutaline did not affect either the ability of B cells to expand or cell viability (data not shown). Because CD40 stimulation on a B cell also increases the activity of p38 MAPK (51–53), it was important to determine whether β_2 AR stimulation also augmented the level of CD40-induced p38 MAPK activation. The level of p38 MAPK phosphorylation (Fig. 6A) and the activity of p38 MAPK (Fig. 6B)

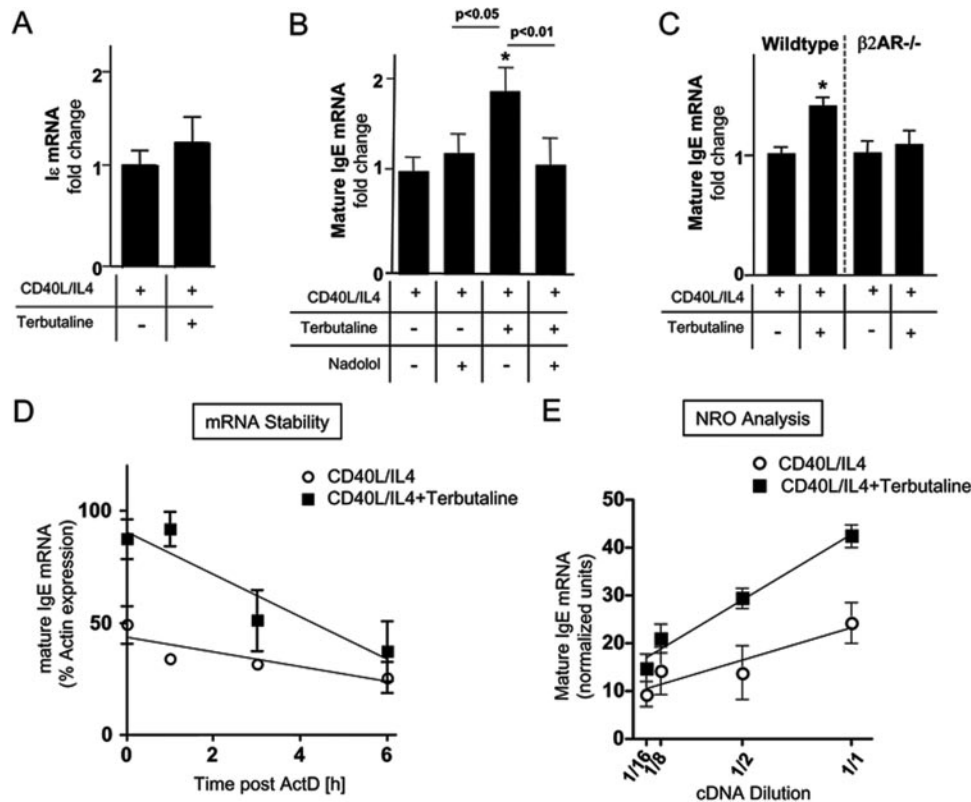


FIGURE 4. The rate of mature IgE mRNA production increases after β_2 AR stimulation on a B cell. *A–C*, Resting B cells were isolated from the spleens of BALB/c (*A* and *B*), FVB (*C*), or β_2 AR^{-/-} (*C*) mice cultured as described in Fig. 1 in the absence or presence of terbutaline. To some cultures nadolol (10^{-5} M) was added. Quantitative real-time PCR analysis for germline (*A*) and mature IgE mRNA (*B* and *C*) was conducted. Data are presented as fold change $\pm$ SEM from control B cells activated with CD40L/IL-4 alone following normalization to β -actin from one representative experiment of two independent experiments. Significant differences were determined by ANOVA with a post hoc test; *, $p < 0.05$ unless otherwise indicated. *D*, At day 5 of culture and in the presence (filled squares) or absence (open circles) of terbutaline (10^{-6} M), actinomycin D (ActD) at $10 \mu\text{g/ml}$ was added, and RNA was isolated at 0, 1, 3, and 6 h. Data are presented as mature IgE mRNA values $\pm$ SEM normalized to β -actin expression in each sample as detected by quantitative real-time PCR from one representative experiment of two independent experiments. Linear regression analysis was performed, regression lines are shown, and degradation rates, i.e. slopes between the regression lines, were not significantly different ($p = 0.17$). *E*, Nuclear run-on (NRO) analysis was performed on nuclei isolated on day 5 and activated in the presence of biotin-16-UTP for 20 min. Biotinylated RNA was collected using streptavidin magnetic beads, and the level of mature IgE mRNA was determined in serially diluted samples by semiquantitative RT-PCR. Data are presented as the mean densitometric units normalized to β -actin $\pm$ SEM from three representative experiments. Significant differences were determined by ANOVA with a post hoc test; *, $p < 0.05$.

were increased by terbutaline in a time-dependent manner to ~ 2 - to 2.5-fold above the level induced by CD40. Taken together, these findings suggest that the level of IgE is increased by β_2 AR stimulation on a B cell in a p38 MAPK-dependent manner and that the level of IgG1 is increased in a PKA-dependent manner.

CD23 expression and release are both increased by β_2 AR stimulation in a p38 MAPK-dependent manner

The low-affinity receptor for IgE (Fc ϵ R2, CD23) was shown to be specifically involved in the regulation of IgE production, and its expression was also shown to be regulated in a p38 MAPK-dependent manner (49, 50). To determine whether the IL4-induced expression of CD23 was increased by β_2 AR stimulation in a p38-dependent manner, B cells were activated in the absence of terbutaline with the selective p38 inhibitors SB203580 or SB202190, and the level of CD23 mRNA was measured by quantitative real-time PCR on day 4. The level of CD23 mRNA was increased ~ 1.5 -fold by terbutaline when compared with control cells. The terbutaline-induced increase was prevented by both p38 MAPK inhibitors, whereas the inhibitors had no effect on the level of CD23 mRNA induced by CD40L/IL-4 alone (Fig. 7A). In the same experiments, the PKA inhibitor H-89 did not prevent the terbutal-

ine-induced increase in CD23 mRNA (Fig. 7A). To determine whether the increase in CD23 mRNA translated to an increase in CD23 protein, Western blot analysis of cell lysates was performed. The level of CD23 protein on days 4 and 5 of culture increased by ~ 2 -fold when the β_2 AR was stimulated as compared with activated B cells alone (Fig. 7B). Thus, the level of CD23 mRNA and protein is increased by β_2 AR stimulation on a B cell in a p38 MAPK-dependent manner.

It has been reported (16, 17) that β_2 AR stimulation augmented the level of sCD23 released from human PBMCs and that the increase in sCD23 induced an increase in the level of IgE produced. To determine whether the increase in CD23 protein induced by β_2 AR stimulation on a B cell translated into an increased level of either sCD23 released by the B cell and/or CD23 expressed on the B cell surface, B cells were activated with CD40L/IL-4 in the absence or presence of terbutaline, and the level of sCD23 and membrane-bound CD23 (mCD23) was determined by ELISA and FACS analysis, respectively. The level of sCD23 was increased through terbutaline exposure by ~ 1.5 -fold above unexposed control cells in a concentration-dependent (Fig. 8A, *inset*) and time-dependent manner, and the increase was prevented by the addition of nadolol (Fig. 8A). In contrast, the level of mCD23 on days 2 and

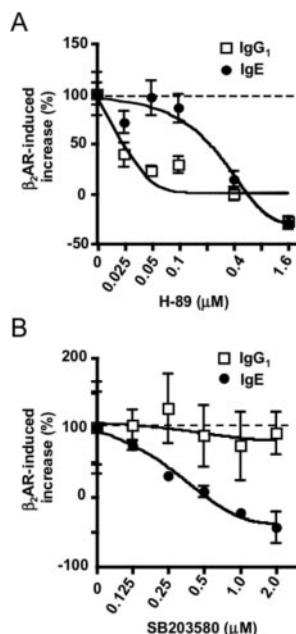


FIGURE 5. The increase in IgE after β_2 AR stimulation on a B cell is mediated by p38 MAPK, whereas PKA mediates the increase in IgG₁. Resting splenic B cells were preincubated with increasing concentrations of either the PKA inhibitor H-89 (A) or the p38 MAPK inhibitor SB203580 (B) for 25 min, washed, and cultured as described in Fig. 1 in the absence or presence of terbutaline (10^{-6} M). On day 6, supernatants were collected and IgE and IgG₁ were measured by ELISA. Data are presented as the mean percentage $\pm$ SEM of the terbutaline-induced increase in IgE (closed circles) and IgG₁ (open squares) in the absence of inhibitors (100%; dashed line). One representative experiment of three independent experiments is shown. Absolute values for IgE produced by CD40L/IL-4-activated cells were 96 ± 2.4 ng/ml (A) and 171.3 ± 13.9 ng/ml (B), and for IgG₁ the absolute values were 76.4 ± 2.4 ng/ml (A) and 93.5 ± 5.5 ng/ml (B).

5 were equivalent on terbutaline-exposed and -unexposed cells (data not shown). Thus, although the level of CD23 mRNA and protein is increased by β_2 AR stimulation on a B cell, the cell surface level remains unchanged due to an increase in the level of sCD23, suggesting that the mechanism responsible for cleaving CD23 from the cell surface may be targeted by β_2 AR stimulation on a B cell, e.g., via metalloproteases (54, 55).

If the level of sCD23 was responsible for regulating the level of an IgE response as reported previously (17), then the present findings suggest that the increase in the IgE response induced in vivo by NE might be due to a β_2 AR-induced increase in the level of sCD23 produced after Ag exposure. To determine whether this was true, serum was collected at 3 wk after day 0 from NE-intact, NE-depleted, and NE-depleted mice that were administered terbutaline 12 h after immunization with OVA/alum and analyzed for the level of sCD23 using an ELISA. The level of sCD23 in the serum was reduced in NE-depleted mice when compared with the level in NE-intact mice, and the decrease in NE-depleted mice was prevented by the administration of terbutaline (Fig. 8B). Thus, a similar CD23 regulatory mechanism may be targeted by β_2 AR stimulation in vitro and in vivo. Taken together, the above findings indicate that the level of sCD23 generated in vitro and in vivo is increased following NE exposure and β_2 AR stimulation through a mechanism that involves a p38 MAPK-dependent increase in CD23, which may also be involved in increasing the level of IgE produced.

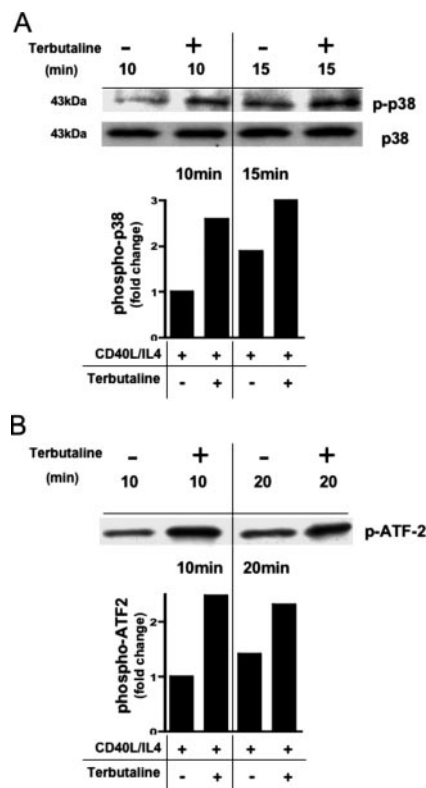


FIGURE 6. Phosphorylation and activity of p38 MAPK is increased after β_2 AR stimulation. Resting B cells were activated as described in Fig. 1 in the absence or presence of terbutaline (10^{-6} M), and cellular protein was isolated at the indicated time points. A, Western blot analysis was performed with specific Abs for phosphorylated p38 MAPK (p-p38) and total p38 MAPK (p38). Top, One representative blot from one of three independent experiments is shown. Bottom, Densitometric analysis of phosphorylated p38 MAPK following normalization to total p38 MAPK. Data are presented as fold change as compared with CD40L/IL-4-activated cells. B, Activity of p38 MAPK was determined following immunoprecipitation of active p38 MAPK. Phosphorylation of ATF-2 fusion protein (p-ATF-2 (top) or phosphorylated ATF2 (bottom)) was detected by Western blot analysis. Top, One representative blot from one of two independent experiments is shown. Bottom, Densitometric analysis of phosphorylated ATF-2. Data are presented as fold change as compared with CD40L/IL-4-activated cells.

The increase in IgE and IgG1 induced by β_2 AR stimulation on a B cell is dependent on CD23 expression by the B cell

To determine whether CD23 was critical for mediating the increase in IgE produced by β_2 AR stimulation on a B cell, B cells from wild-type, CD23-deficient, and CD23 transgenic mice were activated by CD40L/IL-4 in the absence or presence of terbutaline, and supernatants were collected for the analysis of IgE. The level of IgE produced by CD23-deficient B cells exposed to terbutaline did not increase but was similar to the level produced by unexposed cells (Fig. 9). In contrast, the level of IgE produced by wild-type and transgenic B cells was increased $\sim$ 2-fold by terbutaline exposure (Fig. 9), and this increase was prevented by the addition of nadolol (data not shown). This change was not due to a dysregulation of β_2 AR expression or function, because both CD23-deficient and -transgenic B cells expressed equivalent levels of β_2 AR mRNA and surface protein as wild-type B cells as determined by quantitative real-time PCR and Western blot analysis, and the receptor was able to increase the level of intracellular cAMP upon exposure to terbutaline as determined by ELISA (data not shown). Thus, the findings suggest that the increase in IgE

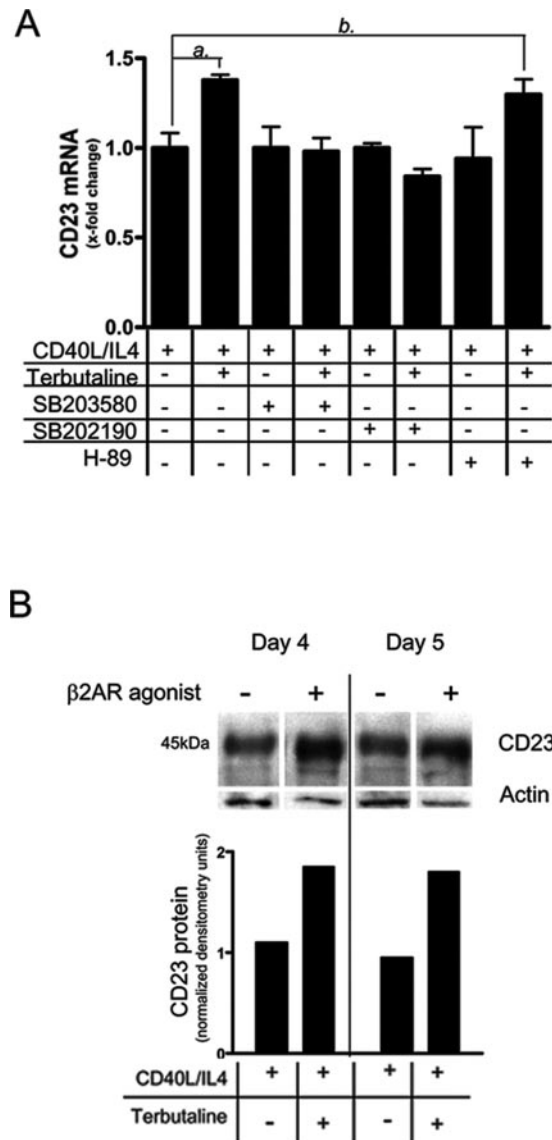


FIGURE 7. CD23 is increased after β_2 AR stimulation in a p38 MAPK-dependent manner. **A**, Resting splenic B cells were preincubated for 25 min with either the p38 MAPK inhibitor SB203580 (1 μ M) or SB202190 (0.6 μ M) or the PKA inhibitor H-89 (0.2 μ M). Cells were then activated as described in Fig. 1 in the absence or presence of terbutaline (10^{-6} M). On day 4 quantitative real-time PCR was performed with primers for CD23 mRNA and β -actin RNA. Data are presented as fold change compared with CD40L/IL-4-activated cells following normalization to β -actin. One representative experiment of two independent experiments is shown. Significance was determined by ANOVA with a post hoc test: *a*, $p = 0.013$; *b*, $p = 0.057$. **B**, resting B cells were activated as described in **A**. On days 4 and 5 CD23 and β -actin total protein was determined by Western blot. *Top*, One representative experiment of two independent experiments is shown. *Bottom*, Densitometric units of CD23 following normalization to β -actin.

induced by β_2 AR stimulation is dependent on the expression of CD23 by a B cell.

The increase in IgE production induced by β_2 AR stimulation requires expression of CD21/35 and CD19

Although controversial, CD23 was reported to interact with CD21 on a human B cell to regulate the level of IgE produced (18). It was also reported that the majority of CD21 molecules on the B cell surface exist in a complex with CD19, which serves as the signaling molecule for CD21 (56). To determine whether the increase in

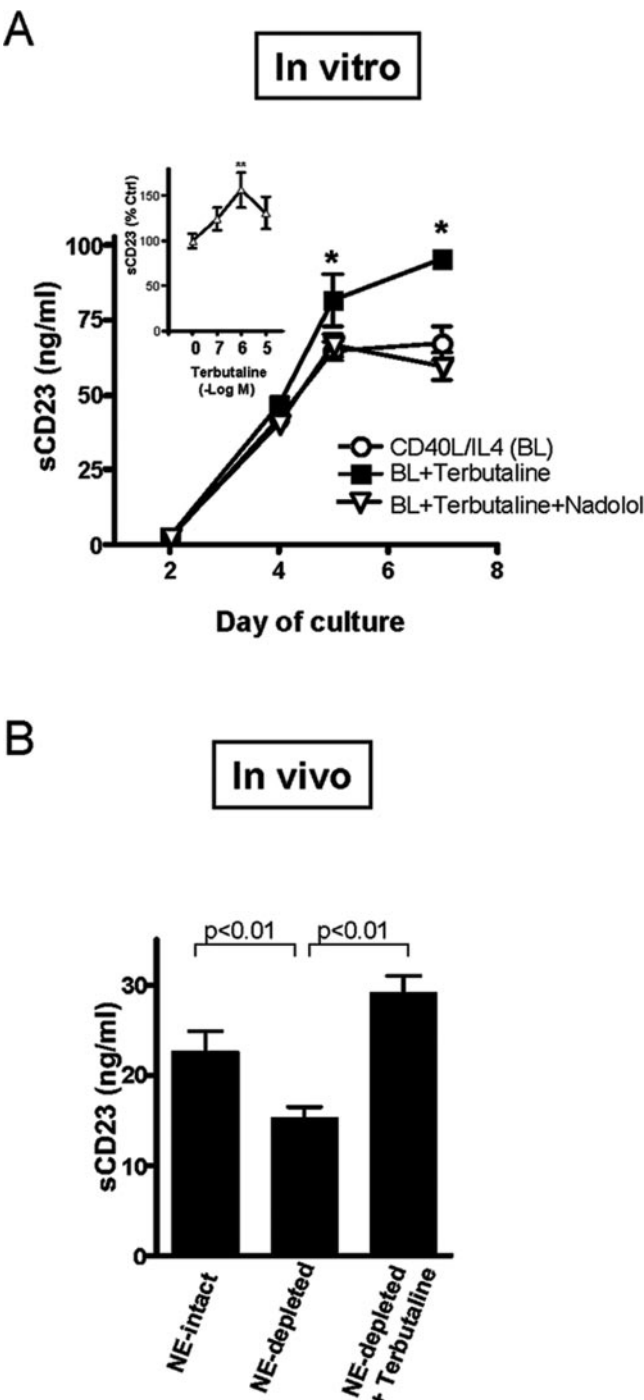


FIGURE 8. The release of sCD23 in vitro and in vivo is increased after β_2 AR stimulation. **A**, resting splenic B cells were activated as described in Fig. 1 in the presence or absence of terbutaline (10^{-6} M). Nadolol was added to some cultures (10^{-5} M). At the indicated time points (**A**) or on day 6 of culture (*inset*) the level of sCD23 was measured by ELISA. *Inset*, Data are presented as the mean percentage change $\pm$ SEM compared with CD40/IL-4-activated control cells (100%). At the indicated time points (**A**) or on day 6 of culture (*inset*) the level of sCD23 was measured by ELISA. *Inset*, Data are presented as the mean percentage change $\pm$ SEM compared with CD40/IL-4-activated control cells (100%), and the absolute values for sCD23 in this group were 64.3 ± 3.8 ng/ml. One representative experiment of two independent experiments is shown. Significance was determined by ANOVA with a post hoc test; *, $p < 0.05$. **B**, sCD23 in undiluted serum was determined by ELISA 3 wk after primary immunization i.p. with OVA/alum. NE-depleted ($n = 3$), NE-intact ($n = 3$), and NE-depleted plus terbutaline i.p. (5 mg/kg) 12 h postimmunization ($n = 3$). Data are presented from one experiment as mean sCD23 value (ng/ml) $\pm$ SEM, and significance was determined by ANOVA with a post hoc test.

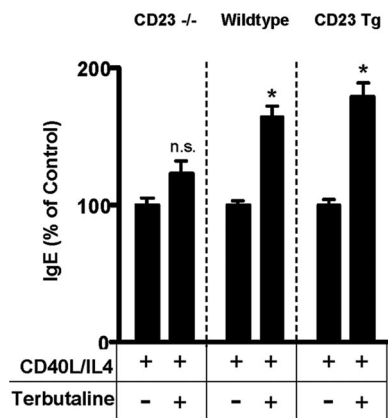


FIGURE 9. CD23 needs to be expressed on a B cell to increase IgE production via stimulation of the β_2 AR. Resting splenic B cells from CD23-deficient (CD23^{-/-}), BALB/c (wild type), and CD23 transgenic (CD23 Tg) mice were activated as described in Fig. 1. IgE was determined at day 6 by ELISA. Data are presented as mean percentage change $\pm$ SEM as compared with cells activated with CD40L/IL-4 alone for each phenotype. Three independent experiments were performed, and the absolute values for IgE in the CD40L/IL-4-alone groups were 48.7 ± 2.6 (CD23^{-/-}), 57.1 ± 3.6 (BALB/c), and 45.3 ± 1.6 (CD23 transgenic). Significance was determined by ANOVA with a post hoc test; *, $p < 0.05$; n.s., not significant.

IgE induced following β_2 AR stimulation was dependent on the expression of either CD21 and/or CD19. B cells from wild-type and CD21/CD35- and CD19-deficient mice were activated with CD40L/IL-4 in the absence or presence of terbutaline, and supernatants were collected for analysis of IgE. The level of IgE produced by wild-type B cells exposed to terbutaline increased 1.5- to 2-fold when compared with unexposed wild-type cells, whereas the level produced by B cells from both CD21/35-deficient (Fig. 10A) and CD19-deficient mice (Fig. 10B) exposed to terbutaline was the same as that produced by unexposed deficient B cells. The lack of effect from terbutaline exposure was not due to a dysregulation of β_2 AR expression or function in the CD21/35-deficient and CD19-deficient B cells (data not shown). Thus, expression of both CD21/35 and CD19 by a B cell appears to be necessary for the level of IgE to be increased by β_2 AR stimulation.

Discussion

It is known that a strong correlation exists in humans and mice between the level of IgE detected in the serum and BAL and the severity of allergic airway inflammation that develops (1–3). In addition, a recent finding in humans indicates that only a small difference exists between the level of serum IgE in healthy individuals and that which is detected in patients with allergic asthma, and that patients with severe asthma have a higher IgE level than those patients with mild or moderate asthma (57). The present murine results in vivo show for the first time that the neurotransmitter NE induces an increase in the level of IgE produced by a B cell in vivo, both systemically and locally in the lung following Ag challenge. Therefore, if it is correct, as suggested in the literature (1–3), that the level of IgE in serum and BAL is correlated with the severity of allergic airway inflammation, then NE plays a potential role in the pathophysiology of allergic asthma by regulating the amount of IgE produced by a B cell. Although most research focuses on understanding the mechanism by which the IgE response is initiated, less is known about the mechanisms by which the amount of IgE produced by an IgE-committed B cell is regulated. The present results show in vitro that the B cell is at least one target for the NE-induced increase in the level of IgE produced per

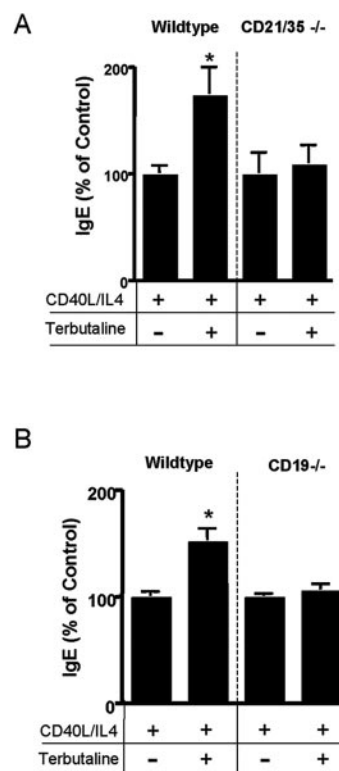


FIGURE 10. Lack of CD21/CD35 or CD19 inhibits the β_2 AR-induced increase in IgE production. Resting B cells from C57BL/6 (wild type; A), CD21/CD35^{-/-} (A), BALB/cJ (wild type; B), and CD19^{-/-} (B) mice were activated as described in Fig. 1. Culture supernatants were analyzed for IgE on days 5 (A) and 6 (B), respectively. Data are presented as the mean percentage change $\pm$ SEM of IgE produced by control B cells activated with CD40L/IL-4 alone from two independent experiments. The absolute values for IgE produced by CD40L/IL-4-activated B cells were 72 ± 5.9 (C57BL/6), 91.3 ± 18.8 (CD21/CD35^{-/-}), 114.4 ± 6.4 (BALB/cJ), and 95.6 ± 2.7 (CD19^{-/-}). Significance was determined by ANOVA with a post hoc test; *, $p < 0.05$.

cell in vivo and that this effect may be mediated via a mechanism that involves a β_2 AR-induced and p38 MAPK-induced synthesis and release of CD23 from the B cell surface.

A major finding in this report is that, in contrast to the CD40L/IL-4-induced IgG1 that is increased via stimulation of the β_2 AR in a PKA-dependent manner, we find that the increase in IgE following β_2 AR stimulation appears to be dependent on an increase in p38 MAPK activity. Although the p38 MAPK inhibitor SB205380 inhibited the β_2 AR-induced increase in IgE, only a minor effect of the inhibitor was observed on the β_2 AR-induced increase in IgG1. This finding strongly suggests that another intracellular pathway is activated by β_2 AR stimulation on a B cell and that this pathway may be more selective for the regulation of IgE vs IgG1. Other examples have been reported in which the level of IgE vs IgG1 produced by a B cell is differentially regulated in the presence of the same stimuli. For example, the IL-4 concentration-response profiles for the amount of IL-4 required for an optimal IgE response, as well as for the length of time that the IL-4 signal is required, differ from those required for an optimal IgG1 response (58). Other parameters that selectively influence the level produced of IgE vs IgG1 are B cell density (59), deletion of the 3'-IgH enhancer (60), dependency on different NF- κ B family members (61, 62), and stimulation of the CD21 molecule with a stimulatory anti-CD21 Ab (18). However, the latter finding is controversial because CD21/CD35-deficient mice show suppressed Ab levels as

compared with wild-type mice for all isotypes, including IgE and IgG1 (63, 64). It has been reported that CD40 stimulation also activates p38 MAPK, which is required for the transcription of germline IgE and CSR to IgE, as indicated from studies using a p38 MAPK inhibitor (53). In contrast, we found that in the presence of a p38 MAPK inhibitor only a minor decline occurred in the baseline level of IgE produced after CD40L- and IL-4-activation alone but that the β_2 AR-induced increase in IgE above baseline was completely prevented. This finding suggests that inhibition of p38 MAPK with the concentrations used in our study, which are 10-fold lower than the concentrations used in previously reported studies, specifically inhibits the increase in IgE following β_2 AR stimulation without affecting the baseline level of IgE produced after CD40L and IL-4 activation alone. Thus, even though IgE and IgG1 are both induced by CD40 and IL-4R stimulation on a B cell, the presence of a ligand for the β_2 AR may up-regulate the level of mature IgE and IgG1, respectively, via distinct mechanisms, with one involving p38 MAPK preferentially and the other involving PKA almost exclusively.

It has been established in the literature that CD23 participates in the regulation of an IgE response (reviewed in Refs. 13 and 65), although the exact mechanism for the regulation remains controversial. We show that CD23 mRNA expression increases after β_2 AR stimulation on a B cell in a p38 MAPK-dependent manner. The mechanism by which p38 MAPK mediates the increase in CD23 expression may involve an enhancement in either the IL-4-induced, STAT6-mediated increase in CD23 transcription (49) or the level of CD23 mRNA translation (50). Our data suggest that p38 MAPK regulates CD23 expression at the transcriptional level because the β_2 AR-induced increase in CD23 mRNA production was specifically prevented by the presence of a highly selective p38 MAPK inhibitor. At present, two mechanistic models exist to explain how CD23 participates in the regulation of an IgE response. First, stimulation of the membrane form of CD23 with the Fc portion of IgE results in negative feedback to the B cell to inhibit IgE production. However, this negative feedback was observed only when cultures contained a mixed cell population, as opposed to one containing a purified population of B cells alone. Therefore, negative feedback on the B cell might potentially be mediated indirectly via other products released by APCs such as follicular dendritic cells, which also express CD23 (66). Second, sCD23 interacts with an unknown ligand expressed by the B cell to increase IgE production, possibly via the stimulation of the CD21/CD19 complex as reported in a human cell model system (17–19, 43). Our data would favor the second model to explain the mechanism by which the level of IgE produced per cell was increased by β_2 AR stimulation. Specifically, β_2 AR stimulation in vitro and the administration of a β_2 AR agonist to NE-depleted mice in vivo increased the level of sCD23 measured in the supernatant or serum, respectively, but did not affect the level of membrane CD23 expressed by the same B cells in vitro, where the possible effect due to other cells was eliminated. Also, the present finding that CD21/CD19-deficient B cells were unable to increase the level of IgE produced after β_2 AR stimulation suggests that a link may exist between β_2 AR stimulation and activation of the CD21/CD19 signaling complex, and this link may be sCD23.

However, the latter conclusion implies that a direct physical interaction occurs between murine sCD23 and CD21, a topic that has been controversial for many years. The level of human and mouse sCD23 in the serum has been reported to correlate directly with the level of serum and BAL IgE produced, as well as the level of cytokine produced by monocytes isolated from the blood (67–70). These findings suggest strongly that the biological function of sCD23, which affects the level of IgE produced in both humans

and mice, is the same. However, although human and murine recombinant sCD23 is available, to our knowledge only the human recombinant sCD23 has been tested to show that it was able to increase the level of IgE produced by human cells in vitro (13, 71). Taken together with other findings from studies using a human cell model system to show that a direct physical interaction occurs between sCD23 and CD21 (18, 19), it becomes more probable that sCD23 binds to CD21 to increase the level of IgE produced by a human B cell. Given that both the human and murine in vivo studies show a direct correlation between sCD23 and the level of IgE in serum, a similar sCD23/CD21 physical interaction in mice may also occur. Our data support this possibility, because the β_2 AR-induced increase in IgE production was dependent on the expression of both CD23 and CD21 on the B cell.

However, a second possible interpretation of our data is that sCD23 increases IgE production independently of CD21. Possible alternative mechanisms might involve the binding of sCD23 to membrane-bound IgE on B cells, which has been reported to up-regulate IgE synthesis (17). Another alternative mechanism might involve a factor or molecule that prevents IgE from interacting with membrane-bound CD23, an interaction that is proposed to inhibit IgE production in vivo (14). However, both alternative mechanisms fail to explain why CD21/CD35- and CD19-deficient B cells showed no increase in the level of IgE produced following stimulation of the β_2 AR, because both alternative mechanisms should still be functional. Thus, our finding suggests that an interaction between murine CD21 and sCD23 may occur, although the possibility remains that sCD23 in the mouse binds to a yet unknown ligand, e.g., CD35, which is found complexed with CD19 on murine but not human B cells (72). Our data also suggest that the CD21/CD19 signaling complex may serve a function other than to lower the threshold of BCR signaling as has been proposed previously (73) and support a previously suggested BCR-independent model of the CD21/19-complex as a “response regulator” that does not influence isotype switching but regulates the magnitude of the Ab response (73, 74).

NE regulates the level of IgE production via stimulation of the β_2 AR on a B cell by increasing the rate of mature IgE mRNA transcription without influencing CSR. This result confirms reports that β_2 AR stimulation on either a murine or a human B cell increases the level of IgE produced in vitro without increasing the number of cells secreting IgE (26, 38). In contrast, another cAMP-elevating agent, PGE₂, is reported to increase the level of IgE produced in vitro by increasing the number of IgE-secreting cells that develop (75), suggesting that the effect on B cell activity differs depending on whether the cAMP was generated by the stimulation of either the β_2 AR or the PGE₂ receptor. This possibility is supported by reports that a β_2 AR agonist, as compared with PGE₂, induces a cAMP signal in a T cell that activates a different PKA isoform (76), which might affect the substrates that are phosphorylated. Also, the spatial localization of the cAMP signal within the cell may differ when either the β_2 AR or PGE₂ is stimulated, possibly causing the phosphorylation of different proteins that localize to different areas within the cell (reviewed in Ref. 77). Another example of how the quality of a cAMP signal that is generated via different receptors might differentially affect cell activity comes from studies with cardiomyocytes. The data showed that β AR stimulation increased intracellular Ca²⁺ concentration to induce a typical ionotropic effect, whereas other ligands that elevated cAMP did not induce this effect (78, 79). Also, it is possible that differences between the mechanism involved with the β_2 AR agonist-induced and PGE₂-induced increase in IgE production are due to the different B cell activation protocols used in the different studies, i.e., LPS vs Ag or APCs vs CD40L. Such differences may

be important, because when B cells were activated by LPS in the presence of PGE₂, the level of IgE increased as compared with that of control cells activated in the absence of PGE₂, whereas the level of IgE remained unchanged from that of the control when the cells were activated via CD40 stimulation in the presence of PGE₂ (80). Thus, even though both PGE₂ and β_2 AR stimulation lead to an increase in cAMP, the mechanism may differ for how the cAMP increases the level of IgE produced by a B cell.

NE appears to affect endogenous B cell activity to increase the level of IgE produced per IgE-secreting cell. These in vitro results suggest that one target cell in vivo that might mediate the NE-induced increase in IgE is the B cell. The fact that the NE-induced increase in IgE is mediated via stimulation of the β_2 AR on the B cell alone provides a possible explanation for the adverse effects of β_2 AR agonist therapy in human asthma, i.e., an increase in mortality among asthmatics was positively correlated with the use of β_2 AR agonists (reviewed in Ref. 34). This phenomenon is particularly observed in patients who were not coadministered anti-inflammatory drugs such as corticosteroids that would have counteracted the possible β_2 AR-mediated increase in IgE production by exerting an immunosuppressive effect on the B cell. The present finding also provides a possible explanation for the observation that stress is often associated with the exacerbation of allergic airway inflammation in humans and mice (81, 82). Stress is known to increase the release of NE from sympathetic nerve terminals (83), an effect that might increase the level of IgE produced by a B cell above the amount that would have been produced normally by the NE released by Ag alone. Thus, if β_2 AR stimulation on a CD40L/IL-4-activated B cell is able to up-regulate the level of IgE produced by a B cell, then long-term β_2 AR agonist therapy and/or stress may raise the level of IgE higher. In this way, it is possible that severe lung pathology would ensue and that conventional β_2 AR agonist therapy would eventually fail to relieve bronchoconstriction due to the swamping of the lung environment with IgE. The present findings may also indicate new targets that can be used for drug design to lower IgE levels in atopic disease, such as p38 MAPK, CD23, CD21, and/or the β_2 AR on a B cell specifically.

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Disclosures

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