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Adrenergic Regulation of IgE Involves Modulation of CD23 and ADAM10 Expression on Exosomes

Caroline J. Padro,* Todd M. Shawler, † Matthew G. Gormley, ‡,§ and Virginia M. Sanders‡,§

Soluble CD23 plays a role in the positive regulation of an IgE response. Engagement of the β2 adrenergic receptor (β2AR) on a B cell is known to enhance the level of both soluble CD23 and IgE, although the mechanism by which this occurs is not completely understood. In this study, we report that, in comparison with a CD40 ligand/IL-4–primed murine B cell alone, β2AR engagement on a primed B cell increased gene expression of a disintegrin and metalloproteinase (ADAM)10, which is the primary sheddase of CD23, as well as protein expression of both CD23 and ADAM10, in a protein kinase A– and p38 MAPK–dependent manner, and promoted the localization of these proteins to exosomes as early as 2 d after priming, as determined by both Western blot and flow cytometry and confirmed by electron microscopy. In comparison with isolated exosomes released from primed B cells alone, the transfer of exosomes released from β2AR agonist–exposed primed B cells to cultures of recipient primed B cells resulted in an increase in the level of IgE produced per cell, without affecting the number of cells producing IgE, as determined by ELISPOT. These effects still occurred when a β2AR antagonist was added along with the transfer to block residual agonist, and they failed to occur when exosomes were isolated from β2AR-deficient B cells. These findings suggest that the mechanism responsible for mediating the β2AR-induced increase in IgE involves a shuttling of the β2AR-induced increase in CD23 and ADAM10 proteins to exosomes that subsequently mediate an increase in IgE. The Journal of Immunology, 2013, 191: 5383–5397.

Immunoglobulin E is proposed to play a role in the pathogenesis of allergy and allergic asthma in humans and mice (1). It is known that the level of IgE produced by a B cell is regulated by CD23 (2–10), which is the low-affinity receptor for IgE (FceRII), and which is expressed as a homotrimer on not only the cell surface of B cells (11, 12) but also other immune cells, such as macrophages (13). CD23 negatively regulates the level of IgE produced by a B cell when soluble IgE binds to it (2–4), but it positively regulates the level of IgE when CD23 is cleaved to a soluble form (5–10), soluble CD23 (sCD23), which subsequently binds to CD19/CD21 on a human B cell (6, 7). Recently, the expression of CD23 on B cell–derived exosomes has been reported (14, 15). Exosomes are cell-derived, cholesterol-rich vesicles that are released by cells, including B cells primed with either LPS or anti-CD40 in the presence of IL-4 (14–16). B cell–derived exosomes also express other proteins, such as MHC class II and CD86 (14, 15), and contain microRNAs (17). The importance of these molecules being expressed on exosomes is that released exosomes are able to strategically regulate immune cell activity in either an autocrine or paracrine manner at locations far removed from the exosome source (18–20). To date, most studies have focused on the regulation of CD23 cleavage on the B cell surface plasma membrane (12, 21). However, recent studies demonstrated that both CD23 and a disintegrin and metalloproteinase (ADAM)10, the primary sheddase for CD23 in a primed B cell (12, 22), form a unique interaction intracellularly that results in their packaging into exosomes that are subsequently released from the cell (14), and that CD23 cleavage on exosomes is ADAM10–dependent (14). ADAM10-mediated cleavage of substrates other than CD23 from monocytes, neuroblastoma cell lines, and lymphoma cell lines is also promoted by ADAM10 localization to membrane regions outside of lipid raft domains, as was shown by an increase in ADAM10–mediated cleavage when cholesterol-rich lipid raft microdomains were disrupted by cholesterol depletion or cholesterol-lowering agents (23–26). Because cholesterol-rich lipid raft microdomains are plentiful in exosomes (27, 28), the ADAM10 expressed on exosomes is in an ideal membrane environment in which to regulate the cleavage of CD23. Thus, the mechanisms that are known to regulate the level of IgE produced by a B cell involve well-characterized cellular mechanisms involving CD23, ADAM10, sCD23, and possibly exosomes.

In turn, the levels of CD23, sCD23, and IgE are regulated by other physiological factors exogenous to the immune system itself. One of these physiological factors is the neurotransmitter norepinephrine (NE), which is released after Ag exposure from nerve endings that terminate in the parenchyma of lymphoid tissues (reviewed in Ref. 29), and also is synthesized in, and released by, CD4+ T cells (30). The level of IgE, as well as the level of sCD23, in the serum and bronchoalveolar lavage fluid of immunized NE-depleted mice was found to be lower than the level produced in NE-intact mice (31), suggesting that NE may play a physiological role in regulating the level of IgE and other factors in the immune system.
role in regulating the IgE response to Ag. Also, the level of IgE produced by primed CD23-deficient B cells that were exposed in vitro to an agonist to the β2 adrenergic receptor (β2AR), which is the endogenous receptor for NE, is the same as that produced by primed CD23-deficient B cells alone (31), suggesting that the β2AR on a B cell plays a role in regulating the level of IgE produced by first mediating an effect on CD23. More recently, our laboratory reported that β2AR engagement on a CD40 ligand/IL-4–primed B cell increased the rate of mature IgE transcription and protein production, without affecting class switch recombination (31), through a proximal mechanism that involved a protein kinase A (PKA)–dependent phosphorylation of hematopoietic protein tyrosine phosphatase (HePTP) (32), which is a phosphatase that functions to bind all unphosphorylated p38 MAPK inside the cell (33). When HePTP is phosphorylated by PKA, the bound p38 MAPK is released and contributes to the pool of free p38 MAPK, causing an increase in the level of p38 MAPK that is now available for phosphorylation by MAPKK (31, 32), which is activated by CD40 engagement (34). The β2AR-activated HePTP/p38 MAPK pathway was linked to the regulation of CD23 expression when an inhibitor to p38 MAPK abrogated the β2AR-induced enhancement in CD23 expression at the mRNA level (31). Likewise, the β2AR-induced enhancement in IgE was also linked to p38 MAPK, indicating a common pathway by which β2AR stimulation enhanced CD23 and IgE production by the B cell (31). Taken together, these findings suggested that NE and the β2AR expressed on a B cell play a role in the positive regulation of IgE expression when an inhibitor to p38 MAPK abrogated the β2AR-induced enhancement in CD23 expression at the mRNA level (31). Likewise, the β2AR-induced enhancement in IgE was also linked to p38 MAPK, indicating a common pathway by which β2AR stimulation enhanced CD23 and IgE production by the B cell (31).

Bronchoconstriction and the respiratory distress that is associated with allergic asthma are treated clinically by the administration of a β2AR agonist, which binds to the β2AR expressed on the surface of bronchiolar smooth muscle cells to reduce their contraction around the bronchioles (35). Of relevance to the study is the clinical evidence that suggests a role for IgE in mediating an exacerbation of allergic asthma in humans and mice (36–39), as well as a role for the β2AR expressed on B cells in the human lung (40, 41) to increase the level of IgE produced during β2AR agonist administration. An understanding of the various molecular links well as a role for the is the clinical evidence that suggests a role for IgE in mediating an exacerbation of allergic asthma in humans and mice (36–39), as well as a role for the β2AR expressed on B cells in the human lung (40, 41) to increase the level of IgE produced during β2AR agonist administration. An understanding of the various molecular links well as a role for the

**Materials and Methods**

**Animals**

Mice were housed under pathogen-free conditions and used between 6 and 12 wk of age. Female BALB/c mice were purchased from the pathogen-free facility at Taconic Farms (Hudson, NY) at 6 wk of age and then housed at the Ohio State University in microisolator cages with autoclaved food and water ad libitum. Congenic BALB/c β2AR-deficient mice were derived from FVB β2AR-deficient mice (provided by Brian Kobilka, Stanford University, Stanford, CA), after backcrossing for 10 generations (32). 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.

**Reagents**

CD40L-S9 cells were prepared as described previously (42). Recombinant murine IL-4 was purchased from eBioscience (San Diego, CA). Terbutaline hemisulfate salt, nadolol, the p38 MAPK inhibitor SB203580, and the PKA inhibitor H-89 were purchased from Sigma-Aldrich (St. Louis, MO). INCB3619, which is a selective ADAM10/17 inhibitor (43), was provided by Incyte (Wilmington, DE). All reagents used for B cell isolation, activation, and pharmacologic treatment tested negative for the presence of endotoxin using Luminex (Sigma-Aldrich).

**Abs**

Western blot Abs were rabbit polyclonal anti-mouse CD23 (provided by D. Conrad, Virginia Commonwealth University, Richmond, VA), rabbit polyclonal anti-mouse ADAM10 (Millipore, Billerica, MA) for cell lysates, rabbit monoclonal anti-mouse ADAM10 (EPR5622 [C terminus]; Abcam, Cambridge, MA) for exosomes, rat monoclonal anti-mouse ADAM10 (clone 139712 [ectodomain]; R&D Systems, Minneapolis, MN), rat monoclonal anti-mouse CD63 (R52; MBL, International, Woburn, MA), or rabbit monoclonal anti-actin (13E5; Cell Signaling Technology, Beverly, MA). Secondary polyclonal Abs were HRP-labeled goat anti-rabbit IgG and HRP-labeled chicken anti-rat IgG (Cell Signaling Technology). Proteins were detected using a LumiGlo detection kit (Cell Signaling Technology). Ganglioside M1 (GM1) was detected using biotin-labeled cholera toxin A subunit (Sigma-Aldrich) and goat polyclonal affinity-purified HRP-labeled anti-biotin (Cell Signaling Technology). ELISA Abs included monoclonal anti-mouse IgE (clone R35-72; BD Biosciences, Franklin Lakes, NJ) and secondary monoclonal AP-labeled rat anti-mouse IgE (clone 23G3; SouthernBiotech). Flow cytometry rat monoclonal anti-mouse Abs and reagents included ADAM10-FITC (clone 139712), ADAM10-PE (clone 139712), and isotype control rat IgG2a-κ-PE from R&D Systems, CD23-FITC (B3B4), B220-allophycocyanin (RA3-6B2), 7-aminomethoxy-cin- C (7-AAD), isotype rat IgG2a-κ-FITC, and isotype rat IgG2a-κ-allophycocyanin from eBioscience.

**Resting B cell isolation and cell priming**

Resting B cell isolation and priming were performed as described previously (31) using negative selection for CD43 B cells using an autoMACS machine (Miltenyi Biotec, Bergisch Gladbach, Germany). B cell purity was >98% as determined by FACS. Purified B cells were primed at a density of 0.5 × 10^6 B cells/ml with CD40L-S9 (1:100) and IL-4 (1 ng/ml) in the absence or presence of tertbutamine (10^{-6} M) and cultured in complete RPMI medium (cRPMI; RPMI 1640 medium [Cellgro, Manassas, VA], 10% FBS [Atlas Biologicals, Fort Collins, CO], 20 mM HEPES [Life Technologies, Carlsbad, CA], 100 U/ml penicillin [Invitrogen, Carlsbad, CA], 100 μg/ml streptomycin [Invitrogen], 2 mM glutamine [Invitrogen], 50 μM 2-ME [Sigma-Aldrich]) at 37°C, 5% CO2. In cell proliferation experiments, CD40L-S9 (1:100) and B cells were serially diluted from 400,000 B cells per well to 12,500 B cells per well in 200 μl volume, followed by the addition of equal amounts of IL-4 (1 ng/ml) in the absence or presence of tertbutamine (10^{-6} M).

**Western blot**

Western blot and analysis was performed on equal amounts of protein as described previously (31, 44) to detect ADAM10, CD23, CD63, actin, and GM1. Blots were scanned and inverted to perform densitometry using National Institutes of Health ImageJ64 1.47m.

**Quantitative real-time PCR**

Quantitative real-time PCR was performed as described previously (31). The following primers were used: β2-actin, 5′-TACAGCT TCACCCACACGC-3′ (forward) and 5′-AAGAGGGCTTCGAAAAGACG-3′ (reverse), annealing temperature, 58°C; ADAM10, 5′-CACCAATTTTGGGAAAAACGG-3′ (forward) and 5′-AAGAGGGCTTCGAAAAGACG-3′ (reverse).
for flow cytometry as described below.

Ultracentrifuged culture supernatant that was not separated by a density gradient was ultracentrifuged at 100,000 g at 4˚C with no brake. Pellets were resuspended in PBS and frozen at −80˚C until analysis by Western blot or stained with the maximum expression of the primary antibody. Western blot analysis was performed using an eCLIPSE 800 (Nikon, Melville, NY) and Gene Q series software (version 2.0.3).

ELISA

ELISA was performed as described previously for sCD23 detection (31). IgE protein in B cell culture supernatants was measured using a BD OptEIA mouse IgE kit (BD Biosciences) and developed with tetramethylbenzidine substrate (BD Biosciences) and 0.2% H2O2. Densitometric analysis was performed on a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA) using Softmax Pro (Molecular Devices), version 5.4.1, at a wavelength of 405 nm for sCD23 and 450 nm for IgE, and values were compared with a standard curve where the level of detection for each protein was 1–500 ng for sCD23 and 1–100 ng for IgE.

Exosome isolation and gradient centrifugation

cRPMI was depleted of exosomes by ultracentrifugation at 150,000 x g for 15 h at 4˚C to pellet exosomes naturally present in PBS in a SW-41 swinging bucket rotor (Beckman Coulter, Brea, CA) in an L-70 ultracentrifuge (Beckman Coulter). Ultracentrifuge tubes were sterilized prior to exosome depletion of cRPMI by washing with 10% bleach, 70% ethanol, and a final wash with sterile PBS (Life Technologies). Ultracentrifuged cRPMI was filtered using a 0.2-µm pore filter (Corning, Corning, NY) prior to cell culture. cRPMI was confirmed to be depleted of exosomes by flow cytometry performed as described below. For the isolation of exosomes released by B cells, exosome-containing supernatant from cultured B cells was fractionated as described previously (14) in a SW-41 swinging bucket rotor in an L-70 ultracentrifuge (Beckman Coulter). Prior to any experiments, a characterization of the ultracentrifugation of conditioned media supernatant was done to confirm the presence of exosomes by layering of isolated exosomes on an iodixanol (Axis-Shield, Dundee, U.K.) density gradient with densities of 1.00, 1.05, 1.10, 1.15, 1.20, and 1.25 g/ml. The iodixanol density gradient was ultracentrifuged at 100,000 x g overnight at 4˚C with no brake. The following day, the various density fractions were collected, diluted in PBS, and then each isolated fraction was ultracentrifuged at 100,000 x g for 1 h at 4˚C with no brake. Pellets were resuspended in PBS and frozen at −80˚C until analysis by Western blot as described above. For most experiments, ultracentrifuged culture supernatant that was not separated by a density gradient was fractionated as described previously (14) and was resuspended in PBS and either frozen at −80˚C until analysis by Western blot or stained for flow cytometry as described below.

Flow cytometry

B cells were prepared for FACS as described previously (45) for cell surface detection of CD23, ADAM10, B220, and 7-AAD. Appropriate isotype- and species-matched Abs were used as controls for gating. Stained cells were detected on a FACSCanto II flow cytometer (BD Biosciences) and results were analyzed using FlowJo (Tree Star, Ashland, OR). One hundred thousand events were collected and cells were gated on 7-AAD− and B220+ cells to exclude Sf9-CD40L cells. Additionally, CD23 and ADAM10 staining on Sf9-CD40L cells was below the level of detection.

Flow cytometry of exosomes

Exosomes were isolated as described above, resuspended in PBS, single stained for CD23 or ADAM10, or stained with an appropriate species- and isotype-matched control for gating. Because we found that much less Ab was required for the staining of exosomes, in comparison with intact B cells, we titrated all isotype-specific and molecule-specific Abs to assure that non-specific staining was minimal. Stained exosomes were detected on a FACSCanto II flow cytometer (BD Biosciences). Polystyrene beads 50–100 nm in size (Spherotech, Lake Forest, IL) were used to optimize for exosome size and granularity. Results were analyzed using FlowJo (Tree Star).

Electron microscopy of exosomes

Exosomes were prepared as described above, fixed, and then analyzed by transmission electron microscopy as described previously (16). Formvar/carboncoated grids (Ted Pella, Redding, CA), onto which the fixed exosomes were placed, were then observed under a transmission electron microscope (Tecnai G2 Spirit; FEI, Hillsboro, OR).

Exosome transfer for functional analysis

Two days after priming in the absence or presence of terbutaline, whole supernatant was either transferred to recipient cultures or fractionated into an exosome-depleted or an exosome-intact fraction. For intact exosomes, exosomes were isolated under sterile conditions as described above and resuspended in 4 ml exosome-free media (1X concentration) and added to recipient cultures. For depleted exosomes, the exosome-depleted supernatant that remained after exosome isolation was transferred to a sterile tube and added to recipient cultures. IL-4 was added to all transfer cultures at a concentration of 1 ng/ml. Additionally, some cultures also received natalol at a concentration of 10−5 M at the time of transfer. For recipient cultures, 2 d after priming in the absence of terbutaline, 96-well plates containing cultured cells were centrifuged at 300 x g to pellet cells. Supernatant was removed and whole, exosome-depleted, or exosome-intact fractions were transferred to recipient cells. Cultures receiving transferred fractions were incubated at 37˚C, 5% CO2, for 4 d and then supernatants were removed for analysis of IgE production by ELISA or cells were analyzed by ELISpot.

ELISpot

ELISpot was performed as described previously (31). IgE+ spots were quantified on an Axioptas 2 microscope (Zeiss, Oberkochern, Germany) using KS ELISpot software (Zeiss, version 4.2.0).

Sucrose density gradient isolation of lipid rafts

B cells (100 × 106) were activated as described above, lysed, and then fractionated by sucrose density gradient separation as described previously (46). Fractions were collected from the top of the gradient for analysis by SDS-PAGE and Western blot probing of equal volumes for ADAM10, CD23, actin, and GM1.

Statistical analysis

Data were analyzed by ANOVA, followed by a Bonferroni posttest when data were normally distributed, to determine whether an overall statistically significant change existed. A Student t test was used for comparison between two treatment groups. A p value ≤0.05 is considered statistically significant.

Results

ADAM10 mediates the β2AR-specific enhancing effect on sCD23 and IgE

Previous findings showed that IgE, as well as CD23 and sCD23, were increased after the β2AR was engaged on a CD40L/IL-4–primed murine B cell, and that these effects failed to occur in the absence of a β2AR agonist (31). In the present study, we confirmed that the β2AR was responsible for mediating the IgE-enhancing effect. B cells from wild-type (WT) or β2AR-deficient mice were primed with CD40L/IL-4 in the absence or presence of the selective β2AR agonist terbutaline, and supernatants were collected on days 2–7 for analysis of IgE production by ELISA. The IgE-enhancing effect of β2AR stimulation on primed B cells, in comparison with primed B cells alone, was evident on day 4 and reached a maximal 2-fold increase on day 6 (Fig. 1A, left panel), whereas B cells from β2AR-deficient mice were unable to do so (Fig. 1A, middle panel), indicating that the β2AR agonist–induced upregulation in the level of IgE production by primed B cells was specifically a result of β2AR stimulation. Note that we consistently found that β2AR-deficient B cells function similarly to WT B cells for all immune functions, but they appear to do so at a lower level of response (31, 47). To determine whether the β2AR agonist–induced upregulation in the level of IgE production was dependent on cell concentration, as reported previously (48, 49), agonist–induced upregulation in the level of IgE production was dependent on cell concentration, as reported previously (31, 47). To determine whether the β2AR agonist–induced upregulation in the level of IgE production was dependent on cell concentration, as reported previously (48, 49), B cells were serially diluted and supernatants were collected on day 6 for analysis of IgE production by ELISA. The IgE-enhancing effect of β2AR stimulation on primed B cells was specifically a result of β2AR stimulation. Note that we consistently found that β2AR-deficient B cells function similarly to WT B cells for all immune functions, but they appear to do so at a lower level of response (31, 47). To determine whether the β2AR agonist–induced upregulation in the level of IgE production was dependent on cell concentration, as reported previously (48, 49), B cells were serially diluted and supernatants were collected on day 6 for analysis of IgE production by ELISA. The IgE-enhancing effect of β2AR stimulation on primed B cells was specifically a result of β2AR stimulation. Note that we consistently found that β2AR-deficient B cells function similarly to WT B cells for all immune functions, but they appear to do so at a lower level of response (31, 47). To determine whether the β2AR agonist–induced upregulation in the level of IgE production was dependent on cell concentration, as reported previously (48, 49), B cells were serially diluted and supernatants were collected on day 6 for analysis of IgE production by ELISA. The IgE-enhancing effect of β2AR stimulation on primed B cells was specifically a result of β2AR stimulation. Note that
production, and it was significantly reduced in comparison with wells with fewer input B cells, indicating that the number of B cells required to observe the β2AR-enhancing effect was limited by the concentration of B cells per well. β2AR stimulation enhanced CD23 total protein production within 24 h and continued to increase through day 5 (Fig. 1B). Likewise, β2AR stimulation on primed B cells enhanced the level of sCD23 produced, in comparison with primed B cells alone, and this increase was first evident on day 3 (data not shown) and reached a maximal 1.5-fold increase on day 5 (Fig. 1C), whereas B cells from β2AR-deficient mice were unable to increase CD23 or sCD23 in response to the β2AR agonist at any time point (Fig. 1B, 1C), also confirming that the agonist-induced increase in CD23 and sCD23 was specifically due to β2AR stimulation on a B cell. Taken together, these findings confirm a role for the β2AR on a B cell in mediating an increase in total CD23 expression, as well as the level of sCD23 and IgE produced.

Based on recent reports that ADAM10 is the primary sheddase of CD23 (12, 21, 22), it was imperative to determine whether the β2AR-induced increase in sCD23 and IgE production was due to a β2AR-induced increase in ADAM10-mediated cleavage of CD23. INCB3619 is a dual ADAM10/ADAM17 inhibitor with an IC50 for various ADAM10 ligands in the range of 0.2–0.7 μM (43). Because ADAM17 is unable to cleave CD23 (12, 50), INCB3619 is an excellent compound for the study of ADAM10-specific cleavage of CD23 (12, 50). Primed B cells were cultured in the absence or presence of a β2AR agonist with increasing concentrations of INCB3619. The concentration of INCB3619 required to reduce both the control level by half, as well as the β2AR-induced increase of both sCD23 (Fig. 1D, left panel) and IgE (Fig. 1D, right panel), was between 0.1 and 0.3 μM and occurred independently of changes in cell morphology or cell viability. Concentrations >2 μM caused a slight reduction in viability (data not shown). To our knowledge, this result suggests, for the first time, that ADAM10 plays a role in the β2AR-enhancing effect on sCD23, and it further supports that a link exists between the β2AR-mediated increase in sCD23 cleavage and the increase in IgE.

To determine the extent to which β2AR stimulation affected the level of ADAM10 expression, primed B cells from WT or β2AR-deficient B cells were cultured as described above and cell lysates were collected at 1 and 24 h to measure ADAM10 mRNA and protein expression, respectively, using quantitative RT-PCR and Western blot. The level of ADAM10 mRNA (Fig. 2A) and total protein (Fig. 2B) were increased 2- and 1.5-fold, respectively, after β2AR stimulation on primed B cells, as compared with primed B cells alone. Conversely, the β2AR enhancement did not occur when β2AR-deficient B cells were used. Taken together

FIGURE 1. ADAM10 modulates the β2AR-mediated enhancing effect on sCD23 and IgE. Resting B cells were isolated from BALB/c (WT) and β2AR-deficient mouse spleens and were primed with CD40L-Sf9 cells (1:10) and IL-4 (1 ng/ml) in the presence [open circles (A, D) or gray bar (B, C)] or absence [solid circles (A, D) or gray bar (B, C)] of the β2AR agonist terbutaline (Terb; 10−8 M). Total cellular protein and/or culture supernatants were collected for analysis. (A) Left, middle, Six day time course of secreted IgE by WT (left) or β2AR-deficient (middle) B cells using ELISA. Right, Serial dilutions of B cells and CD40L were cultured as above and analyzed for IgE at 6 d using ELISA. Data are presented as mean IgE values (ng/ml) ± SEM from one representative of two to three independent experiments. (B) CD23 total protein expression at 24 h using Western blot analysis. One representative blot is shown, with molecular mass in kilodaltons, and data are presented as the mean fold difference of total CD23 protein expression in total cellular lysates from WT and β2AR-deficient B cells as standardized to actin ± SEM from three independent experiments. (C) sCD23 expression at 5 d using ELISA. Data are presented as the mean fold difference of sCD23 from supernatants ± SEM from three independent experiments. Absolute values of sCD23 were 84 ± 6 ng/ml (WT) and 61 ± 5.3 ng/ml (β2AR-deficient). (D) sCD23 (left) and IgE (right) production after 5 d in the presence of increasing concentrations of the selective ADAM10 inhibitor INCB3619. Data are presented as the mean IgE or sCD23 (ng/ml) ± SEM from one representative of three independent experiments. Significant differences from priming alone were determined by ANOVA followed by Bonferroni correction or unpaired t test. *p < 0.05, **p < 0.01, ***p < 0.005.
with the results from Fig. 1, β2AR stimulation on a primed B cell appears to play a role in the upregulation of ADAM10 at the gene level and both CD23 and ADAM10 expression at the protein level.

Previous reports had established a proximal link between the β2AR-stimulated and PKA-mediated enhancement of the level of p38 MAPK phosphorylation and IgE (31). To determine whether the β2AR-stimulated enhancement of CD23 and ADAM10 was mediated by PKA and/or p38 MAPK, B cells were cultured as described previously in the absence or presence of H-89, a PKA inhibitor, or SB203580, a p38 MAPK inhibitor. After 24 h, cell lysates were prepared for Western blot analysis. Whereas β2AR stimulation of primed B cells enhanced the level of CD23 and ADAM10, in comparison with primed B cells alone, and, also, the PKA inhibitor had no effect on CD23 and ADAM10 in primed B cells alone, B cells exposed to a β2AR agonist in the presence of a PKA or p38 MAPK inhibitor, respectively, were unable to enhance either CD23 (Fig. 3A, 3C) or ADAM10 (Fig. 3B, 3D) expression. These results indicate that activation of the β2AR/PKA/p38 MAPK pathway is essential for the β2AR-induced enhancement of the level of CD23, ADAM10, and IgE.

### β2AR stimulation does not enhance expression of CD23 and ADAM10 on the B cell surface

Cleavage of various molecules by ADAM10, such as Notch, Fas ligand, and amyloid precursor protein, has traditionally been thought to occur primarily on the cell surface plasma membrane (12, 50, 51). Because we showed above that expressions of CD23 and ADAM10 were both increased in response to β2AR stimulation on a primed B cell, it was important to determine to what extent this enhancement translated to an increase in expression on the B cell surface. Cultures were set up with B cells, as described previously. FACs analysis of CD23 and ADAM10 expression was done using cells collected on days 0–5 after priming. B cells were gated with appropriate Ab isotype controls to include only cells that were both B220+ and viable as determined by 7-AAD exclusion. As shown in Fig. 4, the percentage of ADAM10+CD23+ cells from both primed and β2AR agonist–exposed conditions increased 3-fold on day 2, but went below baseline on day 3 (Fig. 4A). Representative histograms of all data are shown in Supplemental Fig. 1. We also found that within 1 d of priming, as determined by mean fluorescence intensity (MFI), cell surface CD23 increased 4-fold in both primed-only B cells and β2AR agonist–exposed primed B cells and remained elevated until day 3 when levels for both groups began to decrease to baseline levels (Fig. 4B). In contrast, the level of ADAM10 on the cell surface slightly increased for both exposure conditions on day 1 and then declined to below baseline on days 2–5 after priming (Fig. 4C). Additionally, almost all B cells were CD23 single-positive after CD40L/IL-4 priming, whereas the same number of B cells that were ADAM10 single-positive were also CD23/ADAM10 double-positive (Supplemental Fig. 1), suggesting that cell surface expression of ADAM10 is linked to CD23 and not vice versa. Also, when using B cells from β2AR-deficient mice, the MFI of cell surface CD23 and ADAM10 after B cell priming in the presence or absence of β2AR stimulation was equivalent (data not shown). Taken together, β2AR stimulation on a B cell enhanced ADAM10 expression at the gene and total protein level, as well as enhanced CD23 expression and cleavage, despite the lack of a change in expression of both CD23 and ADAM10 on the cell surface. These findings suggest a tight regulation of CD23 cleavage by ADAM10, which may occur in a compartment of the B cell, other than the cell surface, that is involved with mediating the β2AR-enhancing effect on IgE production.

### Exosomes released by primed B cells express both CD23 and ADAM10

Because the β2AR-induced increase in CD23 and ADAM10 protein did not translate to a change in expression at the cell surface, it became necessary to identify into which cellular compartment the proteins localized to promote CD23 cleavage. Our data thus far did not exclude the possibility that they localized to another membrane within the B cell, as opposed to the cell surface. Data from a recent report showed that, in addition to the cell surface plasma membrane, CD23 and ADAM10 were expressed on vesicles derived from B cells that were characterized as exosomes (14). To determine the extent to which β2AR stimulation on a B cell enhanced CD23 and ADAM10 expression on released exosomes, B cells were cultured as described above in the absence or presence of ammonium chloride (NH4Cl) to inhibit exosome release, as has been previously described (14, 52). In the absence of NH4Cl, the level of both CD23 (Fig. 5A) and ADAM10 (Fig. 5B) protein expression, as determined by Western blot analysis of cell lysates, was augmented by β2AR stimulation, in comparison with the level expressed by primed B cells alone. In the presence of NH4Cl, β2AR stimulation on primed B cells increased both CD23 and ADAM10 protein expression 2.2- and 3-fold, respectively, in comparison with primed B cells (Fig. 5). These findings suggested that the level of expression of both CD23 and ADAM10 proteins is increased in B cells after β2AR stimulation and that the increase is localized to primarily exosomes.

Because several types of vesicles are released from cells (53), it was essential to confirm that the vesicles released by primed B cells in our system satisfied the criteria defined by experts in the
field for classifying a microvesicle as an exosome. To this end, B cell–derived vesicles were examined for characteristic features described previously (15), namely, “cup-like” morphology, size between 40 and 100 nm, and density between 1.08 and 1.15 g/ml. To evaluate morphology and size, exosomes were isolated from the supernatant of primed B cells cultured for 3 d and fixed and analyzed via transmission electron microscopy. Vesicles isolated on day 3 of priming showed a characteristic cup-like morphology that was 100 nm in size (Fig. 6A). To confirm size, isolated exosomes were analyzed by flow cytometry. Exosomes measured between 40 and 100 nm in size (Fig. 6B) as compared with ultra-small polystyrene beads to control for accurate size gating (Fig. 6B, inset). To measure the density of released exosomes, an iodixanol density gradient centrifugation was used to fractionate supernatant-derived exosomes on day 3, which was the earliest time point at which a large number of exosomes could be isolated. Exosomes expressed both CD23 and ADAM10 in the fractions isolated between 1.05 and 1.10 g/ml (Fig. 6C), which is in agreement with previous reports (15). Additionally, the fractions between 1.05 and 1.15 g/ml were the only fractions that contained exosomes as detected by transmission electron microscopy (data not shown). Therefore, these data show that the vesicles released from primed B cells in our model system fulfill the accepted definition required to classify them as exosomes.

\[ \beta_2AR \] stimulation enhances CD23 and ADAM10 expression on exosomes early after priming

Because inhibition of exosome release with NH\textsubscript{4}Cl suggested that the \( \beta_2 \)AR-induced enhancement in CD23 and ADAM10 expression might be localized to exosomes, we sought to confirm these findings using Western blot analysis of isolated exosomes. To determine whether CD23 and ADAM10 were expressed on exosomes released from resting B cells, or whether priming was required for CD23 and ADAM10 exosome expression, resting and primed B cells were cultured as described above and exosomes were isolated after 1–2 d. Initial analysis of exosome-associated ADAM10 by Western blot with a polyclonal Ab directed at the C-terminal domain of ADAM10 revealed two bands around 65 kDa that corresponded to mature ADAM10 (Supplemental Fig. 2, upper panel), which was in contrast to a single band detected by the same Ab around the same molecular mass in cellular lysates (Supplemental Fig. 2, lower panel). To determine whether this band was nonspecific, both cellular lysates and exosomes were examined using mAbs directed at either the C-terminal domain or the ectodomain of ADAM10. In exosomes, we found that both mAbs detected a single band of ADAM10 (Supplemental Fig. 2, upper panel), which corresponded to the lower band detected by the polyclonal Ab, indicating that the upper band detected by the polyclonal Ab was nonspecific. A very faint upper band was detected by the ectodomain-specific ADAM10 mAb. Thus, all further analyses of ADAM10 expression of exosomes by Western blot were performed with the mAb directed at the C-terminal domain of ADAM10. In exosomes, we found that both mAbs detected a single band of ADAM10 (Supplemental Fig. 2, upper panel), which corresponded to the lower band detected by the polyclonal ADAM10 Ab, indicating that the upper band detected by the polyclonal Ab was nonspecific. A very faint upper band was detected by the ectodomain-specific ADAM10 mAb. Thus, all further analyses of ADAM10 expression of exosomes by Western blot were performed with the mAb directed at the C-terminal domain of ADAM10. Furthermore, in cellular lysates, the C-terminal domain-specific monoclonal ADAM10 Ab detected a higher molecular mass form, around 95 kDa, of ADAM10 (Supplemental Fig. 2, lower panel), likely the pro form of ADAM10, a form that was not found in exosomes (Supplemental Fig. 2, upper panel), indicating the possibility that only mature ADAM10 is present in exosomes. Exosomes derived from resting B cells did not express CD23 or ADAM10, whereas exosomes derived

![FIGURE 3. PKA and p38 MAPK inhibition prevents the \( \beta_2 \)AR-induced enhancement in CD23 and ADAM10 protein. B cells were cultured as described in Fig. 1 in the presence (black bar) or absence (gray bar) of terbutaline (Terb). Cell lysates were collected after 24 h and analyzed by Western blot. (A and B) CD23 (A) and ADAM10 (B) expression in the presence of H-89 (0.5 \( \mu \)M). (C and D) CD23 (C) and ADAM10 (D) expression in the presence of SB203580 (1 \( \mu \)M). One representative blot is shown, with molecular mass in kilodaltons, and data are presented from three to four independent experiments as the total protein fold difference compared with priming alone as standardized to actin ± SEM. Significance is based on an unpaired t test, *\( p \) < 0.05, **\( p \) < 0.01, ***\( p \) < 0.005, as compared with priming alone.](http://www.jimmunol.org/)

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2 d after priming alone expressed CD23 and ADAM10, which was enhanced by β2AR stimulation (Fig. 7). As a positive control, a marker for exosomes, CD63, was measured (17), even though CD63 is not a housekeeping gene or loading control, but serves only as an exosome marker. The level of CD63 expression was increased on exosomes derived from B cells exposed to a β2AR agonist at 1 d after priming (Fig. 7), but because the function of CD63 is unknown, it was used in this study as purely an exosome marker. The absence of CD23 on released exosomes until 2 d after priming indicates that there is a delay between the synthesis of CD23, which appears in cell lysates and on the cell surface 1 d after priming, and the shuttling of CD23 to exosomes. Furthermore, because ADAM10 is a constitutively expressed protein, the delay in the appearance of ADAM10 in exosomes until 2 d after priming indicates the possibility that CD23 and ADAM10 form an association that allows for shuttling into exosomes, as has been shown previously (14).

Because the β2AR enhancement for exosome-associated CD23 and ADAM10 occurred within 2 d of priming, it was important to determine whether this enhancement occurred at any later time points. Therefore, primed B cells were cultured as described above and exosomes were isolated after 2–5 d and analyzed by Western blot. The overall protein level of isolated exosomes was enhanced by β2AR stimulation of primed B cells in comparison with primed alone B cells on day 4, but not on days 2, 3, or 5. However, there was an increase over time in the level of protein from isolated exosomes (Fig. 8A). The positive control for exosomes, CD63, was increased by β2AR stimulation 3–4 d after priming in comparison with exosomes isolated from primed B cells alone. However, because the level of protein did not correlate with CD63 expression, we concluded that CD63 is not a proper loading control, although it is routinely used currently in exosome analyses. Until a better loading control is determined, CD63 continues to be used, but the caveat associated with its use is noted. The level of both CD23 and ADAM10 released on exosomes by primed B cells was enhanced by β2AR stimulation 2 d after priming, but remained equal 3–5 d after priming (Fig. 8B). The positive control for exosomes, CD63, was increased by β2AR stimulation 3–4 d after priming. Further analysis by densitometry confirmed that CD23 (Fig. 8C) and ADAM10 (Fig. 8D) on exosomes released from primed B cells were enhanced by β2AR stimulation 2 d after priming, but not 3–5 d after priming, indicating that there is a tight regulation of CD23 and ADAM10 expression on exosomes. Collectively, to our knowledge, these results indicate for the first time that CD23 and ADAM10 expression appears on exosomes only after 2 d of B cell priming, and that levels expressed of both proteins increases after β2AR stimulation on primed B cells.

To extend the Western blot findings to a quantitative level, FACS analysis of individually isolated exosomes was performed to in-

FIGURE 4. β2AR stimulation does not enhance expression of CD23 and ADAM10 on the B cell surface. B cells from WT mice were cultured as described in Fig. 1 in the presence (open circle) or absence (filled circle) of terbutaline (Terb). Cells were collected for FACS analysis from days 1 to 5 after priming. The percentage of positive 7-AAD− B220+ ADAM10+CD23+ cells (A), MFI of cell surface CD23 (B), and ADAM10 (C) are shown. Data are presented as mean percentage positive cells or MFI ± SEM from three independent experiments.

FIGURE 5. Inhibition of exosome release increases CD23 and ADAM10 protein retention within a primed B cell. Resting B cells were cultured as described in Fig. 1 in the absence or presence of NH4Cl (10 mM) to inhibit the release of exosomes. Cell lysates were collected after 24 h and analyzed for CD23 (A) and ADAM10 (B) protein expression by Western blot analysis. Representative blots are shown, with molecular mass in kilodaltons, and data were normalized to actin and are presented as the mean fold difference of CD23 and ADAM10 expression ± SEM from three to four independent experiments. Significance is based on an unpaired t test. *p < 0.05, ***p < 0.005, compared with priming in the absence of NH4Cl alone.
expression was not evident on days 3–5 after priming, and was not increased by a β2AR agonist (Fig. 9A). This difference in CD23 expression between β2AR-deficient and WT B cells was evident on exosomes derived from primed B cells exposed to a β2AR agonist (Fig. 9A). These findings suggest that the exosomes released from primed B cells exposed to a β2AR agonist not only express a higher level of CD23 and a greater percentage of ADAM10+ exosomes, as compared with exosomes from primed...
A number of parameters were tested to establish the effectiveness and specificity of the exosome-mediated effect. First, we found that recipient B cells produced a very low level of IgE unless IL-4 was added to the recipient culture along with the transferred fractions (data not shown). We also observed that the exosome-depleted supernatant was capable of enhancing the level of IgE produced in recipient B cells alone, in comparison with the transfer of medium alone with IL-4 (data not shown), indicating that some soluble factor present in the exosome-depleted supernatant obtained from the primed B cells alone was able to transfer a slight enhancement themselves to the recipient B cells above baseline. Also, a further 1.3-fold enhancement of IgE was observed in the level of IgE produced by recipient B cells with the transfer of either exosome-intact or whole supernatant fractions that was above that produced by exosome-depleted supernatant fractions (Fig. 10A) and, significantly, the same fractions from primed B cells exposed to β2AR stimulation were further able to enhance IgE production (Fig. 10A), indicating that the β2AR-enhancing effect on IgE could be transferred via exosomes. Second, to neutralize any effect that might have been transferred owing to any residual β2AR agonist, the β2AR antagonist nadolol was added to recipient cell cultures along with the transferred supernatant fractions. Under this condition, the β2AR-enhancing effect on the level of IgE produced by the recipient cells remained when the donor supernatants were exosome-intact and whole supernatants (Supplemental Fig. 4A), indicating that any residual terbutaline did not alter the β2AR-enhanced exosome effect on IgE. Third, to determine the extent to which the timing of exosome transfer affected the transfer of the enhancing effect, exosome-intact supernatant fractions from day 3 donor cultures were transferred onto day 3 primed–only recipient B cells. The level of IgE produced by recipient B cells was 4-fold less, in comparison with day 2 transfers, and the primed β2AR-exposed donor fractions were unable to enhance the level of IgE produced, in comparison with fractions from primed alone B cells (Supplemental Fig. 4B). This finding suggested that exosomes released by day 2 from a primed B cell are able to function maximally. Finally, to determine the extent to which the concentration of exosomes transferred affected the level of the enhancing effect, day 2 exosomes were resuspended to a 1× or 2× concentration prior to transfer to recipient B cells. The 1× and 2× concentrations of exosomes derived from B cells exposed to a β2AR agonist enhanced IgE production, in comparison with those derived from primed B cells alone (Supplemental Fig. 4C), indicating that the β2AR-enhancing effect was unaffected by exosome concentration. Taken together, these findings established the conditions under which the exosome transfer conditions were optimal for them to mediate the β2AR agonist–induced enhancing effect on IgE, demonstrating that the β2AR-induced increase in CD23 and ADAM10 on B cell–derived exosomes is functionally relevant.

**ADAM10 and CD23 organize into membrane microdomains**

The question remained as to the membrane region into which the increased level of β2AR-induced CD23 and ADAM10 was compartmentalized, which would influence where CD23 cleavage occurred. Recent reports showed that ADAM10-mediated cleavage of various substrates within a membrane was enhanced upon cholesterol depletion, which resulted in a disruption of lipid rafts (23–26), suggesting that ADAM10 activity was greater outside of a cholesterol-rich lipid raft region of a membrane. To determine whether membrane localization within B cell membranes was affected by β2AR stimulation, B cells were primed and cultured as described previously and cell lysates were fractionated on days 0–5 after priming via sucrose density gradient centrifugation. Equal

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**FIGURE 8.** β2AR stimulation enhances CD23 and ADAM10 expression on exosomes after priming. CD40L/IL-4–primed B cells were cultured as described in Fig. 1 and B cell–derived exosomes were isolated from supernatants after 2–5 d. Equal amounts of protein were analyzed by Western blot for CD23, ADAM10, and CD63 for each time point. (A) Bicinchoninic acid analysis of isolated exosomes. Data are presented as mean amount of protein (mg/ml) ± SEM from two to three independent experiments. (B–D) One representative blot from three independent experiments is shown (B), with molecular mass in kilodaltons, and data are presented as the mean fold difference of CD23 (C) and ADAM10 (D) expression ± SEM from three independent experiments. Significance is based on an unpaired t test. **p < 0.01, compared with priming alone.

B cells alone, but they also function to directly mediate the β2AR-induced increase in the level of IgE.
volumes of each fraction were analyzed by Western blot for the presence of CD23, ADAM10, actin, which is a protein found in non-lipid raft fractions (54), and GM1, which is a sphingolipid enriched in membrane lipid raft fractions (55). In resting cells, CD23 and ADAM10 were primarily located in non–lipid raft regions (Fig. 11A) and, after priming, there was a change in distribution of CD23 and ADAM10 to both fractions. Two days after priming and β2AR stimulation, an increased localization of CD23, but not ADAM10, to non–lipid raft fractions was observed, as compared with primed B cells alone (Fig. 11B), and persisted throughout day 3 (data not shown). Because the resting level of ADAM10 differed in non–lipid raft fractions derived from resting as compared with primed B cells, equal volumes from resting fractions that were three times the volume used for activated fractions and corresponded to equal protein in the non–lipid raft fractions, fractions three/four and eight/nine, respectively, to determine whether ADAM10 was present in lipid raft fractions of resting cells. Low amounts of ADAM10 were detected in lipid raft fractions from resting B cells, and ADAM10 expression in these fractions increased marginally upon priming of the B cell (Fig. 11C). The relatively small amount of change in localization of ADAM10 versus CD23 may be explained by the effect that priming has on the expression of CD23 in comparison with ADAM10. Whereas ADAM10 is constitutively expressed and its surface expression is relatively unaffected by B cell priming, priming of the B cell substantially enhances the surface expression of CD23 in comparison with ADAM10. The small amount of CD23 detected in both fractions. In comparison, the absence of an upregulation of ADAM10 expression in response to priming alone translated to a very subtle increase in ADAM10 expression in the lipid raft fractions. These findings suggested that B cell membranes, be it on the cell surface or released exosomes, express CD23 and ADAM10 in regions where cleavage is optimal, and that this pattern of localization is the same in primed B cells alone and after β2AR stimulation.

Discussion

A critical role for CD23 and sCD23 in regulating the level of IgE produced by a B cell is well known. However, the role of the β2AR expressed on a B cell in regulating the level of CD23, sCD23, and IgE is less well studied, even though this form of regulation may be relevant both physiologically, when NE is released in the vicinity of B cells after Ag exposure, and clinically, when a β2AR agonist is used therapeutically to treat bronchoconstriction. The goal of the present study was to identify the mechanism that links β2AR engagement on a CD40L/IL-4–primed B cell to an increase in the level of CD23, sCD23, and IgE. In this study, we report in that three mechanisms appear to be involved. The first mechanism involves a β2AR-induced increase in both CD23 and ADAM10 expression at the mRNA and protein levels. The second mechanism involves localization of the β2AR-induced increase in CD23 and ADAM10 to B cell–derived exosomes and their release from the cell. Finally, the third mechanism involves the ability of the released exosomes to directly mediate the β2AR-induced increase in IgE.

One of the more unexpected findings in the present study was that the β2AR-induced increase in CD23 and ADAM10 expression in a B cell did not translate to an increase in expression on the plasma membrane, which is where the cleavage of CD23 was originally proposed to occur (12), but instead it preferentially increased on B cell–derived exosomes. The presence of CD23 and ADAM10 on exosomes confirmed recent reports using Western blot analysis showing that B cell priming with anti-CD40 and IL-4 alone induced the expression of CD23 on B cell–derived exosomes (15), and that B cell priming with LPS and IL-4 induced
The enhancement of CD23 and ADAM10 expression on B cell–derived exosomes, as opposed to the cell surface, following β2AR engagement on a primed B cell, suggested three possible means by which the β2AR-induced increase in CD23 and ADAM10 protein expression might occur. First, an increase in cell surface expression failed to occur because CD23 was cleaved at the cell surface and released immediately by ADAM10 to produce sCD23, as was proposed previously (12), and, thus, a transient increase at the cell surface might occur but would be undetectable using FACS analysis. Second, any increase of CD23 and ADAM10 proteins at the B cell surface might preferentially endocytose together, as was proposed previously (14), to then fuse with intracellular multivesicular bodies (MVBs) (19, 56) that would subsequently fuse with the plasma membrane for release from the cell (19, 56, 57). In support of this possibility is the report that the cell surface expression of ADAM10 on the surface of HEK293 cells is regulated by endocytosis, as evidenced when using an inhibitor to the endocytic protein dynamin (58), suggesting that any ADAM10 expression at the plasma membrane was cleared from the surface via endocytosis. Support for the coendocytosis of CD23 with ADAM10 is provided by a previous report that the stalk region of CD23 binds to ADAM10 in a protease-independent manner, and that ADAM10 is essential for the inclusion of CD23 on exosomes (14), indicating that a unique interaction occurs between CD23 and ADAM10 that directs their preferential shuttling into exosomes. Furthermore, the present findings showed that all ADAM10−B cells were also CD23−, but not vice versa, suggesting that CD23 may be required for cell surface expression of ADAM10 on B cells. Finally, the β2AR-induced increase in newly synthesized CD23 and ADAM10 proteins may shuttle directly from the Golgi into MVBs for fusion with the plasma membrane and subsequent release from the cell, as shown for ADAM10 in breast tumor cell lines (51, 57). Support for the immediate shuttling of these proteins to exosomes was that specific inhibition of exosome release by NH4Cl, as defined previously (14, 52), caused retention of exosomes intracellularly, which was measured as an increase in the CD23 and ADAM10 content of cellular lysates. This finding indicated that the proteins were preferentially localized to exosomes that were unable to leave the cell in the presence of NH4Cl. Thus, the means by which CD23 and ADAM10 localize to exosomes are not mutually exclusive, but the present results suggest that the β2AR-induced increase in CD23 and ADAM10 is preferentially shuttled to exosomes for release from the cell and subsequent cleavage of CD23, instead of being shuttled to the cell surface plasma membrane.

We show that exosomes released by donor cells exposed to a β2AR agonist expressed a higher level of both CD23 and ADAM10, suggesting that the exosomes provided a source for a higher level of sCD23 to positively regulate the IgE response of recipient B cells. For donor supernatants, resting B cells were cultured as described in Fig. 1 in the presence (black) or absence (gray) of terbutaline (Terb). Supernatants containing B cell–derived exosomes were removed after 2 d and the supernatant was fractionated by ultracentrifugation into exosome-intact and exosome-depleted components. For recipient B cells, resting B cells were cultured as described in Fig. 1 in the absence of Terb. Whole, exosome-intact, or exosome-depleted supernatants were transferred in the presence of IL-4 (1 ng/ml) to recipient cells that were primed for 2 d in the absence of Terb. Whole, exosome-intact, or exosome-depleted supernatants were transferred in the presence of IL-4 (1 ng/ml) to recipient cells that were primed for 2 d in the absence of Terb and that had supernatants removed prior to donor supernatant transfers. (A) Supernatant fractions were transferred from donor cultures containing either WT (left panel) or β2AR-deficient (right panel) B cells. IgE produced by recipient cells was measured by ELISA. Data are presented as mean IgE values (ng/ml) ± SEM from one representative independent experiment of three from at least three replicate measurements. (B) Comparison of the number of recipient IgE-secreting cells (left panel) and the amount of IgE secreted by these cells (right panel) using the ELISPOT assay. Data are presented as the number of IgE+ spots per well or as the fold change compared with whole supernatant transfer in IgE secreted on a per spot basis ± SEM from one representative independent experiment of three from at least three replicate measurements. Significance is based on an unpaired t test. *p < 0.05, as compared with B cells exposed to fractions obtained from primed B cells alone.
a recipient primed B cell that had already undergone class switch recombination. We propose that the transfer of the $\beta_2$AR-enhancing effect on IgE was due to the exosomes released from the donor cells themselves, and not due to something inherent to the recipient B cells, for the following reasons. First, the recipient B cells received equal numbers of exosomes from both the agonist-exposed and -unexposed donor cells, ruling out any effect due to a difference in exosome number, and suggesting that the effect was mediated by the higher levels of CD23 and ADAM10 expressed on the transferred exosomes. Second, the transfer of any residual $\beta_2$AR agonist, which might have affected recipient cells, was unlikely because the addition of a $\beta_2$AR antagonist along with non–lipid raft regions in a resting cell, to both non–lipid and lipid raft regions. It is possible that the presence of CD23 and ADAM10 in non–lipid raft regions and the apparent $\beta_2$AR-induced increase in CD23 localization to non–lipid raft regions indicates that non–lipid raft fractions are the site of CD23 cleavage to produce enhanced sCD23. This finding also suggests that redistribution to lipid raft regions is a means by which the cell controls the extent of ADAM10-mediated cleavage. Thus, the localization within non–lipid raft regions assures that cleavage of CD23 will occur, whereas the localization to lipid raft regions assures that ADAM10 activity will be controlled. The presence of lipid rafts within exosomes has previously been noted (reviewed in Ref. 28), and exosomes are derived from the lipid-rich lipid raft regions of cellular membranes (61) where the interaction of CD23 and ADAM10 within this region allows for exosome formation within MVBs that are released from the cell (14). The fact that both exosomes and lipid rafts have similar densities when isolated by sucrose and iodoxanol density centrifugation (15, 62, 63) suggests that the lipid raft measurements made in the present study detect both exosome and plasma membrane lipid raft–associated CD23 and ADAM10. Because we were able to isolate lipid raft and non–lipid raft fractions expressing CD23 and ADAM10 from specifically the exosome fraction of an iodoxanol density gradient (data not shown), the possibility exists that ADAM10-mediated cleavage of CD23 also occurs on lipid-rich exosomes that is also regulated by localization to non–lipid raft domains. Future studies will focus on the role that lipid raft and non–lipid raft domains play in the $\beta_2$AR-induced increase in ADAM10-mediated cleavage of CD23, and they will also identify the extent to which preferential localization occurs on an exosome, as opposed to a plasma membrane.

Whereas the signaling intermediates that were responsible for mediating the $\beta_2$AR-induced increase in CD23 and ADAM10 are mostly unknown, a number of reports that address CD23 and ADAM10 regulation have provided some clues. The upregulation of CD23 expression on a B cell occurs in both human and mouse B cells following exposure to either IL-4 (64, 65), anti-CD40 Ab (66), or a Th2 clone in the presence of Ag (67). We reported previously that $\beta_2$AR stimulation on a CD40L/IL-4–primed B cell enhanced CD23 expression beyond the level induced by CD40L/IL-4 alone (31), and in the present study, we confirm that this increase was mediated by the $\beta_2$AR specifically and in a PKA- and p38 MAPK–dependent manner. The signaling intermediates responsible for the $\beta_2$AR-induced enhancement in CD23 may also

![Image](http://www.jimmunol.org/content/181/7/5394/F1.large.jpg)
be linked to the known role of STAT6 in regulating the IL-4–induced increase in CD23 (68) via p38 MAPK regulation of the transactivation domain of STAT6 (69). Because the pathway by which β2AR engagement enhances IgE is via a β2AR-induced, PKA-dependent phosphorylation of HePTP (32), which releases bound p38 MAPK for phosphorylation by the MAPKK pathway activated following CD40 engagement (34), we propose that the β2AR-induced increase in CD23 expression is likely due to the p38 MAPK–dependent enhancement of IL-4–mediated STAT6 activation via interaction with the transactivation domain, which ultimately leads to an increase in the level of CD23 expression.

In contrast to CD23 regulation, signaling intermediates that upregulate ADAM10 expression are largely unknown. B cells express ADAM10 constitutively (70, 71), as was confirmed in the present study, and which is in contrast to CD23 expression that is negligible in resting B cells (65). In the present study, β2AR engagement on a primed B cell induced an increase in ADAM10 expression at both the gene and protein level, which was associated with an increase in CD23 cleavage, and which was blocked with an ADAM10 inhibitor. The reported signaling intermediates that regulate ADAM10 expression appear to function through the activation of p38 MAPK (72–74). Promoter analysis of the ADAM10 gene reveals binding sites for the retinoic acid receptor α (30) and NF-κB (34), supporting a previous finding that ADAM10 protein was upregulated by activation of the retinoic acid pathway in a neuroblastoma cell line (73). Additionally, ADAM10 gene and protein expressions were also found to be upregulated by activation of the p38 MAPK/ERK1/2 pathway in primary cortical cultures (74), whereas other reports showed that p38 MAPK was able to phosphorylate both retinoic acid receptor α and NF-κB when using mouse embryonic fibroblasts and NIH 3T3 cells (75, 76). In the present study, we show that the β2AR-enhanced expression of ADAM10 is also mediated by p38 MAPK and is likely mediated by the aforementioned β2AR-activated PKA/HePTP/p38 MAPK signaling pathway described for CD23. Taken together, both CD23 and ADAM10 expression can be regulated by p38 MAPK, and the β2AR on a B cell can further contribute to this regulation through the same signaling intermediate, indicating that a common mechanism exists by which β2AR engagement increases two proteins important for IgE regulation, which is also linked to increased p38 MAPK activation.

Although we elucidated the kinetics of the β2AR-induced enhancement of CD23 and ADAM10 expression on exosomes, our ultimate goal was to identify the mechanism by which β2AR engagement on a primed B cell enhanced the level of IgE produced. The present finding suggested that exosomes might play an essential role in the regulation of IgE production.

FIGURE 12. Adrenergic regulation of IgE involves modulation of CD23 and ADAM10 expression on exosomes. (1) WT B cells primed in the presence of a β2AR agonist enhance CD23 and ADAM10 production through a PKA-dependent phosphorylation of HePTP, which subsequently releases bound p38 MAPK for phosphorylation by the CD40-triggered MAPKK pathway. (2) β2AR stimulation augments both CD23 and ADAM10 expression on exosomes, but not on the cell surface, either through an endocytic pathway starting at the cell surface or through fusion of vesicles containing CD23 and ADAM10 that are derived from the trans-Golgi network with MVBs. (3) Exosomes and sCD23 generated in MVBs are released from the cell upon fusion of the MVBs with the plasma membrane. The release of sCD23 from exosomes enhances IgE production by acting in either an autocrine or paracrine manner, as suggested previously (14, 78, 79). The autocrine mechanism may involve a sCD23-mediated enhancement of IgE production by the cell from which the sCD23 was released. The paracrine mechanism may involve the exosomes acting at distal sites where the CD23 on the exosomes will complex with Ag bound to IgE. This complex will be phagocytosed by dendritic cells so that they can present Ag to T cells to activate them to help B cells that have endocytosed the exosome complexes for processing and presentation to the dendritic cell–activated T cell. Taken together, the β2AR-enhanced expression of CD23 and ADAM10 on B cell–derived exosomes may explain the mechanism responsible for mediating the effect of β2AR stimulation on a B cell to increase the level of IgE produced.
role in mediating this effect on IgE and potentially play a role in mediating a physiological effect that influences conditions such as allergy and asthma. However, to our knowledge, few studies have addressed the pathological relevance of exosomes derived from mice undergoing an allergic sensitization protocol to promote allergic asthma. Two recent studies measured the tolerizing potential of exosomes derived from the serum and bronchoalveolar lavage fluid of mice undergoing a tolerizing protocol. Exosomes, also called tolerosomes in these studies, were used prophylactically to prevent the IgE response in a murine allergic asthma model (20, 77), suggesting that exosomes from tolerized mice were able to induce tolerance in response to sensitization to the same Ag. The mechanism of the transfer of an anti-inflammatory phenotype in vivo is suggested by previous findings that B cell-derived exosomes transferred Ag/MHC class II complexes to follicular dendritic cells (78), and that CD23 + B cells were required for optimal IgE-Ag presentation to CD11c + dendritic cells to allow for CD4 + T cell activation (79). Whereas the transfer of bexosomes or the formation of exosomes may provide a therapeutic avenue of any of the mechanisms that induce the increased expression on B cell–derived exosomes that are released by the B cell, and mediated enhancement in CD23 and ADAM10 protein is found on the PKA/HePTP/p38 MAPK signaling pathway. The b2AR alone, mediates an increase in the level of IgE first by enhancing biogenesis of exosomes might prevent a b2AR-induced increase in IgE production. Additionally, in patients, the expression of CD23 or ADAM10 on exosomes in the blood might be used as a biomarker to predict outcomes for b agonist therapy in patients, with high levels of these proteins indicating that higher IgE might be produced to exacerbate the clinical condition. In conclusion, and as depicted in Fig. 12, the present study demonstrated that b2AR stimulation on a primed B cell, in comparison with primed B cells alone, mediates an increase in the level of IgE first by enhancing CD23 and ADAM10 gene and protein expression via activation of the PKA/HePTP/p38 MAPK signaling pathway. The β2AR-mediated enhancement in CD23 and ADAM10 protein is found on B cell–derived exosomes that are released by the B cell, and that, upon transfer to recipient primed B cells, mediate the β2AR-enhancing effect on the level of IgE produced per cell. Targeting of any of the mechanisms that induce the increased expression on exosomes or the formation of exosomes may provide a therapeutic approach that can be used to prevent the β2AR-mediated enhancing effect on IgE, without affecting the β2AR-mediated dilating effect on bronchial smooth muscle.

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Disclosures

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