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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.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; ADAM, a disintegrin and metalloproteinase; βAR, β-adrenergic receptor; CD40L, CD40 ligand; cRPMI, complete RPMI medium; GM1, ganglioside M1; HePTP, hematopoietic protein tyrosine phosphatase; MFI, mean fluorescence intensity; MVB, multivesicular body; NE, norepinephrine; NH4Cl, ammonium chloride; PKA, protein kinase A; sCD23, soluble CD23; WT, wild-type.

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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 through a PKA-, HePTP-, and p38 MAPK–dependent mechanism that appears to regulate the levels of CD23 and sCD23. However, the mechanism that links these changes following β2AR engagement on a primed B cell to the increase in IgE remains unknown.

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 on a primed B cell to the increase in IgE remains unknown.

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). INCBl3619, 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 E-Toxate (Sigma-Aldrich).

Abs

Western blot Abs were rabbit polyclonal anti-mouse CD23 (provided by D. Conrad, Virginia Commonwealth University, Richmond, VA), rabbit polyclonal anti-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 (RSG2; MBL, 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). Ganglisonide M1 (GM1) was detected using toxin-labeled cholera toxin B subunit (Sigma-Aldrich) and goat polyclonal affinity-purified HRP-labeled anti-biotin (Cell Signaling Technology). ELISA Abs included rat monoclonal anti-mouse CD23 (coating, clone 2G8; SouthernBiotech, Birmingham, AL), rabbit polyclonal anti-mouse CD23 (secondary, provided by D. Conrad; Virginia Commonwealth University), and goat polyclonal alkaline phosphatase-labeled anti-rabbit IgG (detecting, H+L; SouthernBiotech). Plates were developed with para-nitrophenyl phosphate substrate (Sigma-Aldrich) in diethanolamine. Abs used for ELISPOT 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-IFTC (clone 139712), ADAM10-PE (clone 139712), and isotype control rat IgG2a-PE from R&D Systems; CD23-ITC (B3B4), B220-allophyocyanin (RA3-6B2), 7-aminomethycoumycin D (7-AAD); isotype rat IgG2a-k-IFTC, and isotype rat IgG2a-k-allophyocyanin 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 (1:10) and IL-4 (1 ng/ml) in the absence or presence of tertbutane (10^-6 M) and cultured in complete RPMI medium (CRPMI; RPMI 1640 medium [Cellogr, 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 (1:10) 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 tertbutane (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-arctin, 5′-TACAGCT TCACCCACACGC-3′ (forward) and 5′-AAGGAGGCTCGTGGAAAAGGC-3′ (reverse), annealing temperature, 58°C; ADAM10, 5′-CAACCAATTGTTGGAAACCCG-3′.

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...
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 acid stop (2 N H₂SO₄, Sigma-Aldrich). 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

CPRIM was depleted of exosomes by ultracentrifugation at 150,000 × 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 CPRIM by washing with 10% bleach, 70% ethanol, and a final wash with sterile PBS (Life Technologies). Ultracentrifuged CPRIM was filtered using a 0.2-μm pore filter (Corning, Corning, NY) prior to cell culture CPRIM 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 ultracentrifuged exosome exosupernatant 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 × g overnight at 4°C with no brake. The following day, the various density fractions were collected, diluted in PBS, and each isolated fraction was ultracentrifuged at 100,000 × 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 exosome supernatant that was not separated by a density gradient was fractionated into sterile PBS and a final wash with sterile PBS (Life Technologies). Ultracentrifuge tubes were sterilized prior to usage for exosome depletion. Pellets were resuspended in PBS and frozen at −80°C until analysis by Western blot as described above.

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 + to exclude S9-CD40L cells. Additionally, CD23 and ADAM10 staining on S9-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/carbon-coated grids (Ted Pella, Redding, CA), onto which the fixed exosomes were placed, were then observed under a transmission electron microscope (Technai 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 (1× 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 nadolol 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 × 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 Axioskan 2 microscope (Zeiss, Oberkochen, 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 presence of a β₂AR antagonist (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), 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 was evident with an input of between 12,500 and 200,000 B cells per well in 200 μl volume, with the maximum β₂AR-enhancing effect on IgE exhibited between 50,000 and 100,000 B cells per well (Fig. 1A, right panel). We confirmed the cell concentration dependency of IgE production by B cells reported previously, as the addition of 400,000 B cells per well did not exhibit the β₂AR-enhancing effect on IgE
FIGURE 1. ADAM10 modulates the β2-AR-mediated enhancing effect on sCD23 and IgE. Resting B cells were isolated from BALB/c (WT) and β2-AR-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 β2-AR agonist terbutaline (Terb; 10^{-6} M). Total cellular protein and/or culture supernatants were collected for analysis. (A) Left, middle, 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 β2-AR-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 (β2-AR-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.
FIGURE 2. ADAM10 gene and protein expression are increased by β2AR stimulation. B cells from WT and β2AR-deficient mice were cultured as described in Fig. 1 in the presence (black bars) or absence (gray bars) of terbutaline (Terb). mRNA and total cellular protein, respectively, were collected at 1 and 24 h. (A) Data are presented from three independent experiments as the mean ADAM10 mRNA fold difference as standardized to β-actin ± SEM. Significant differences from priming alone were determined by an unpaired t test. *p < 0.05.

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 both CD23 (Fig. 3A) and ADAM10 (Fig. 3B, 3D) 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 presence of NH4Cl, 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.

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.

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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.

**β₂AR stimulation enhances CD23 and ADAM10 expression on exosomes early after priming**

Because inhibition of exosome release with NH₄Cl suggested that the β₂AR-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 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 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 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 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 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 or the ectodomain 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 from primed B cells expressed both CD23 and ADAM10.

**FIGURE 3.** PKA and p38 MAPK inhibition prevents the β₂AR-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 μM). (C and D) CD23 (C) and ADAM10 (D) expression in the presence of SB203580 (1 μM). 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.
2 d after priming alone expressed CD23 and ADAM10, which was enhanced by \( \beta_2 \)AR 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 \( \beta_2 \)AR 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 \( \beta_2 \)AR 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 \( \beta_2 \)AR 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 \( \beta_2 \)AR 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 \( \beta_2 \)AR stimulation 2 d after priming, but remained equal 3–5 d after priming (Fig. 8B). The positive control for exosomes, CD63, was increased by \( \beta_2 \)AR 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 \( \beta_2 \)AR 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 \( \beta_2 \)AR 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-
Exosomes are released by primed B cells that express both CD23 and ADAM10. B cells were cultured as described in Fig. 1, and B cell–derived exosomes were isolated after 72 h. (A) Exosomes were fixed and prepared for transmission electron microscopy. Micrograph is representative of three independent experiments. Scale bar, 100 nm. (B) Exosomes were analyzed by FACS and compared with polystyrene beads of similar size (50–100 nm, inset). Data are presented as forward scatter (FSC) versus side scatter (SSC) and are representative of three independent experiments. (C) Isolated exosomes were separated by iodixanol density gradient, and equal volumes of fractions of 1.00, 1.05, 1.10, 1.15, 1.20, and 1.25 g/ml density were analyzed by Western blot for the expression of CD23 and ADAM10. One representative blot from three independent experiments is shown, with molecular mass in kilodaltons.

dicate the number of exosomes expressing CD23 and ADAM10, as well as the levels expressed. B cells from WT and β2AR-deficient mice were prepared as described previously and exosomes were isolated on days 2–5 after priming. Because the Western blot results in Fig. 7 indicated an absence of CD23 and ADAM10 expression on exosomes prior to day 2, FACS analysis was performed starting on day 2. CD23 and ADAM10 were analyzed individually on exosomes because there was a concern that the use of both detecting Abs at the same time might provide an inaccurate profile due to the small size of exosomes, and because the number of samples available for gating controls was limited. The total number of exosomes isolated from primed B cells alone and those primed B cells exposed to a β2AR agonist increased from 2 through 5 d after priming, as compared with the previous day (Supplemental Fig. 3), and was not increased by β2AR stimulation on primed B cells, in comparison with primed-only B cells at any time point. Likewise, the number of CD23+ exosomes remained constant throughout days 1–5 (Fig. 9A). However, after 2 d of priming, a 1.8-fold increase in the level of CD23, as determined by MFI, was evident on exosomes derived from primed B cells exposed to a β2AR agonist (Fig. 9A). This difference in CD23 expression was not evident on days 3–5 after priming, and was not evident on exosomes derived from β2AR-deficient B cells at any time point (data not shown). In contrast to CD23, the number of ADAM10+ exosomes was augmented 1.9-fold by β2AR stimulation 2 d after priming, but returned to a control number on days 3–5 after priming and was not evident on exosomes derived from β2AR-deficient B cells (Fig. 9B). Also, in contrast to CD23, the expression of ADAM10 on exosomes, as determined by MFI, was not augmented by β2AR stimulation after days 2–5 of priming, but remained the same as exosomes from primed B cells alone. Taken together, these data indicate that β2AR stimulation induced changes in exosome phenotype only after 2 d of priming that included an increase in the level of CD23 expression on, and the number of ADAM10+ exosomes.

B cell–derived exosomes directly enhance IgE production

What remained unknown was the extent to which the exosomes derived from β2AR agonist–exposed primed B cells, in comparison with those from primed B cells alone, were able to mediate an increase in IgE. To address this unknown, B cells from WT or β2AR-deficient mice were primed in the absence or presence of the β2AR agonist, terbutaline, for 2 d, at which time either the whole supernatant was removed and transferred directly to another culture of 2-d-primed recipient B cells, or the supernatant was first fractionated by ultracentrifugation into exosome-intact and exosome-depleted components that were then transferred to 2-d-primed recipient B cells. The recipient B cells were then cultured for another 4 d, at which time supernatants were removed and analyzed for the level of IgE. The transfer of either the whole unfractionated supernatant or the isolated exosome-intact fraction from β2AR agonist-exposed primed B cells, in comparison with those fractions from primed B cells alone, increased the level of IgE produced by recipient cells by 1.2- or 1.3-fold, respectively, whereas the transfer of the exosome-depleted fraction did not (Fig 10A, left panel). In contrast, the transfer of exosome fractions from β2AR-deficient B cells exposed to a β2AR agonist did not increase IgE (Fig. 10A, right panel). Because previous work from our laboratory indicated that β2AR stimulation on a B cell did not affect class switch recombination, but did induce an increase in the amount of IgE secreted per cell, as opposed to an increase in the number of B cells secreting IgE (31), we tested to what extent the transfer of exosomes induced the same effect. The number of IgE-secreting cells, as determined by ELISPOT, was similar in the recipient cultures, regardless of which condition the supernatant fraction was transferred from, although the number was higher when recipient B cells were exposed to whole supernatant fractions from either primed B cells alone or those primed B cells exposed to a β2AR agonist (Fig. 10B, left panel). As expected, B cell–derived exosomes with a β2AR-induced increase in CD23 and ADAM10 expression mediated an increase in the level of IgE produced on a per cell basis in the recipient cultures by 1.3- to 1.4-fold (Fig. 10B, right panel). 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

FIGURE 7. β2AR stimulation enhances CD23 and ADAM10 expression on exosomes at early time points after priming. Resting B cells and CD40L/IL-4–primed B cells were cultured as described in Fig. 1, and B cell–derived exosomes were isolated from supernatants after 1–2 d. Equal amounts of protein were analyzed by Western blot for CD23, ADAM10, and CD63 for each time point. One representative blot from three independent experiments is shown, with molecular mass in kilodaltons.
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 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× 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
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 B2AR 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 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 goal of the present study was to identify the mechanism that links B2AR 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 B2AR-induced increase in both CD23 and ADAM10 expression at the mRNA and protein levels. The second mechanism involves localization of the B2AR-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 B2AR-induced increase in IgE.

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 B2AR 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 B2AR agonist is used therapeutically to treat bronchoconstriction. The role of the present study was to identify the mechanism that links B2AR 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 B2AR-induced increase in both CD23 and ADAM10 expression at the mRNA and protein levels. The second mechanism involves localization of the B2AR-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 B2AR-induced increase in IgE.

One of the more unexpected findings in the present study was that the B2AR-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...
both CD23 and ADAM10 (14). 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

![FIGURE 10](http://www.jimmunol.org/) Transfer of exosomes derived from primed B cells exposed to a β2AR agonist directly enhance IgE production by 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 exposed to a β2AR agonist, as depicted in Fig. 1, in the presence or absence of terbutaline (Terb). 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 β2AR-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 β2AR agonist, which might have affected recipient cells, was unlikely because the addition of a β2AR antagonist along with the donor exosomes was unable to prevent the enhancing effect. Also, the half-life of terbutaline in culture is 7 h (59) and exerts an enhancing effect only within the first 24 h of priming, as reported previously (60), suggesting that any effect on a B cell 48 h after priming was unlikely. Finally, because the transfer of the enhancing effect occurred only with exosomes collected on the peak day of the β2AR-induced increase in CD23 and ADAM10 expression (day 2), we propose that day 2 might also be the peak time of sCD23 production to exert a positive regulation of the IgE response. To confirm this proposal, future experiments will be done to confirm the level of sCD23 produced by the transferred exosomes, although such measurements may be difficult to perform owing to the extensive ultracentrifugation steps required to isolate exosomes, which may result in a loss of sCD23 during processing of samples, as well as the difficulty in differentiating between sCD23 and the CD23 expressed on exosomes by ELISA.

Previous work focused on regulation of ADAM10 activity by membrane localization to lipid versus non–lipid raft fractions (23–26). It has been reported that lipid rafts inhibit the ability of ADAM10 to cleave substrates, such as amyloid precursor protein, IL-6 receptor, and CD30, and that the disruption of lipid rafts permits ADAM10 to exert sheddase activity on these substrates (23–26). In the present study, CD40L/IL-4 priming of the B cell promoted the localization of CD23 and ADAM10 from exclusively 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 β2AR-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 iodixanol 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 iodixanol 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 β2AR-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 β2AR-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 β2AR 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 β2AR specifically and in a PKA- and p38 MAPK–dependent manner. The signaling intermediates responsible for the β2AR-induced enhancement in CD23 may also

**FIGURE 11.** CD23 and ADAM10 localization to non–lipid raft domains within primed B cell membranes increases after β2AR stimulation. Resting B cells (A) or CD40L/IL-4–primed B cells (B) were cultured as described in Fig. 1 in the presence (+) or absence (−) of terbutaline (Terb). Whole-cell lysate fractions were isolated from a sucrose density gradient after 2 d. Fractions one through four represent less dense lipid raft fractions, and fractions five through nine represent more dense non–lipid raft fractions. Equal volumes of each cell lysate fraction were analyzed by Western blot for the presence of CD23, ADAM10, and actin. Equal volumes were also loaded via slot blot and analyzed for the presence of GM1, a sphingolipid that is enriched in lipid raft fractions. (C) Equal volumes corresponding to equal amounts of protein in the non–lipid raft fractions were loaded from fractions three/four (lipid raft) and fractions eight/nine (non–lipid raft) and analyzed for ADAM10. One representative blot from three independent experiments is shown, with molecular mass in kilodaltons.
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 and NF-κB (72), 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 IgE regulation.

**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 physiological 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 an approach that can be used to prevent the effect on bronchiolar smooth muscle.

Disclosures
The authors have no financial conflicts of interest.

References


