A STUDY OF THE DISTAL MOLECULAR MECHANISM BY WHICH BETA-2 ADRENERGIC RECEPTOR STIMULATION ON A B CELL REGULATES IgE PRODUCTION

DISSERTATION

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ABSTRACT

The goal of this dissertation was to determine the mechanism by which beta-2 adrenergic receptor (β_2AR) stimulation on a CD40L/IL-4-primed B cell regulates the level of IgE produced. IgE is the mediator of allergic asthma, an inflammatory condition treated with β_2AR agonists to relieve bronchoconstriction. However, our laboratory and others reported previously that β_2AR stimulation *in vivo* or *in vitro* by norepinephrine or an agonist drug increase the amount of IgE produced by a B cell on a per cell basis, without affecting class switch recombination, potentially worsening allergic asthma symptoms due to IgE in the long term. The proximal mechanism responsible for the increase in IgE involves a β_2AR-dependent activation of the cAMP/PKA/HePTP/p38 MAPK signaling pathways. The distal mechanism involves an increase in the generation of soluble CD23 (sCD23), a positive mediator of IgE production, which results from the cleavage of membrane-bound CD23 by A Disintegrin And Metalloprotease 10 (ADAM10). What remained unknown was the mechanism responsible for the β_2AR-mediated increase in sCD23, and if sCD23 was the ultimate mediator of the β_2AR-mediated increase in IgE. Recently, CD23 and ADAM10 were described on B cell-derived vesicles known as exosomes, suggesting a novel pathway by which β_2AR stimulation could regulate CD23 cleavage. Thus, the hypothesis tested in this dissertation

is that β_2AR stimulation on a B cell enhances IgE production by increasing CD23 and ADAM10 expression on B cell-derived exosomes that play a role in mediating the β_2AR-associated increase in IgE production. The present data using an *in vitro* cell culture system are the first to show that β_2AR stimulation on a CD40L/IL-4-primed B cell enhanced the expression of CD23 and ADAM10 in the B cell in a PKA- and p38 MAPK-dependent manner. Using β_2AR-deficient mice and pharmacological agents, we showed that the β_2AR-dependent enhancement of CD23 and ADAM10 expression was detectable on exosomes, rather than on the cell surface. Exosomes from β_2AR agonist-exposed primed B cells were able to transfer the β_2AR-enhancing IgE effect to primed-only B cells, resulting in an increased amount of IgE produced on a per cell basis, rather than an increase in the number of cells that switch to IgE. Thus, our findings suggest that β_2AR stimulation on a B cell modulates the level of IgE production by increasing CD23 and ADAM10 expression on exosomes, which are ultimately responsible for mediating the effect. This is the first study to identify the distal mechanism by which β_2AR stimulation on a primed B cell increases the level of IgE. The knowledge gained from this work contributes to an understanding of the physiologically relevant mechanism by which the level of IgE is regulated both homeostatically by norepinephrine and during agonist therapy with a β_2AR agonist. The latter effect will help to predict the impact that current allergic asthma therapies may have on the level of IgE. In addition, these findings identify novel molecular targets for therapeutic interventions that can be used to selectively regulate the β_2AR-induced effects on IgE, while maintaining β_2AR-induced bronchoconstriction.

DEDICATION

I dedicate my Dissertation to my parents and grandparents for giving me every opportunity possible and for instilling in me a strong work ethic, and to my husband for being my best friend and providing unwavering support.

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LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
7-AAD	7-aminoactinomycin D
ADAM10	A disintegrin and metalloprotease 10
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AP1	Activating protein 1
APC	Allophycocyanin
APCs	Antigen presenting cells
AR	Adrenergic receptor
ATF2	Activating transcription factor 2
β_2AR	Beta-2 adrenergic receptor
BALF	Bronchoalveolar lavage fluid
BBB	Blood brain barrier
BCR	B cell receptor
C	Constant

cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CD40L	CD40 ligand
C_h	Heavy chain constant region
CLPs	Common lymphoid progenitors
CNS	Central nervous system
CREB	cAMP response element binding protein
CSR	Class switch recombination
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ERK	Extracellular-signal regulated kinase
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FcεRII	Low affinity receptor for IgE
FDCs	Follicular DCs
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G-protein	GTP-binding protein
GM1	Ganglioside M1
GPCRs	G-protein coupled receptors

GRKs	GPCR kinases
$G\alpha_s$	Gα stimulatory
H	Histone
HEK293	Human embryonic kidney 293 cells
HePTP	Hematopoietic protein tyrosine phosphatase
HRP	Horseradish peroxidase
HSCs	Hematopoietic stem cells
IFN- γ	Interferon-gamma
IgE	Immunoglobulin E
IgG1	Immunoglobulin Gamma 1
IgH	Immunoglobulin heavy chain
IgM	Immunoglobulin M
IL	Interleukin
IL-4R	IL-4 receptor
IRS	Insulin receptor substrate
Iγ1	Interfering γ1
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPKK	MAPK kinase
MAPKs	Mitogen-activated protein kinases
MEF2	Myocyte enhancing factor-2
MFI	Mean fluorescence intensity

MHC II	Major Histocompatibility Complex II
mRNA	Messenger ribonucleic acid
MSK1	Mitogen and stress-activated protein kinase 1
MVB	Multivesicular bodies
NE	Norepinephrine
NF-kB	Nuclear factor-κB
NH_4Cl	Ammonium chloride
NK	Natural killer cells
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCD6	Programmed cell death 6
PE	Phycoerythrin
PI_3K	Phosphoinositol-3 kinase
PKA	Protein kinase A
PLC γ2	Phospholipase Cγ2
PNPP	Para-nitrophenyl phosphate
PRRs	Pattern recognition receptors
Rag	Recombinase activating gene
RARα	Retinoic acid receptor-α
RPMI	Roswell Park Memorial Institute
S	Switch

sCD23	Soluble CD23
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Shc	Src homologous and collagen
SHM	Somatic hyper-mutation
SNS	Sympathetic nervous system
SP1	Specificity protein 1
SSC	Side scatter
STAT6	Signal transducer and activator of transcription 6
TCR	T cell receptor
TEM	Transmission Electron Microscopy
Tfh	Follicular Th cells
Th1	T helper 1
Th2	T helper 2
TNFR	Tumor necrosis factor receptor family
TRAFs	TNFR-associated factors
USF	Upstream transcription factor
VDJ	Recombined Variable, Diversity, and Joining Segments
WT	Wild type

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Divisions of the immune system

1.1.1 The innate immune system

The immune system protects the body from foreign pathogens through multiple pathways that are mediated by cellular and molecular components. The immune system is divided into two unique arms that are composed of the early, non-specific response attributed to the innate immune system, and the later, antigen-specific response attributed to the adaptive immune system. Defense of the body by the innate immune system [as reviewed by (1,2)] occurs from minutes to hours after initial exposure and is mediated by physical and chemical barriers, phagocytic cells, and soluble proteins found in the plasma. Physical barriers are the first line of defense and include the skin and mucosal surfaces of the gastrointestinal and respiratory tract that physically prevent the entry of pathogens. Chemical barriers to pathogen survival include the low pH on the surface of the skin due to fatty acid-rich glandular secretions such as sweat, saliva, and tears and the low pH of the stomach. Furthermore, chemical barriers such as antimicrobial peptides and lysozyme, as well as physiological barriers, such as high body temperature, prevent

further survival of pathogens in the body. These physical and chemical barriers are further discussed in the following reviews (1,2) and will not be further examined in this dissertation. Macrophages, dendritic cells (DCs), neutrophils, and natural killer (NK) cells are the effector cells (3-5) of the innate immune system. These effector cells are responsible for clearance of microbes from the body and activating the adaptive arm of the immune system. Pattern Recognition Receptors (PRRs) are expressed on the macrophage surface and, upon recognition of Pathogen Associated Molecular Patterns (PAMPs) expressed on the microbe surface, triggers macrophage activation (4-6). Phagocytic cells such as macrophages and DCs are also called antigen presenting cells (APCs) due to their ability to phagocytose, or engulf, an antigen through endocytosis, then degrade, process, and present the antigen via Major Histocompatibility Complex II (MHC II) to activate CD4+ T cells (6,7). In addition to being APCs, phagocytic cells release cytokines and chemokines that are capable of attracting neutrophils to sites of microbial entrance (8,9). Neutrophils are polymorphonuclear leukocytes that are capable of both early phagocytosis of antigens and release of granules containing proteins with antimicrobial properties that are able to prevent pathogen survival (10). Similar to neutrophils, NK cells are recruited to sites of inflammation by cytokines that are released by macrophages (11). NK cells are activated to kill target cells that they interact with when MHC I receptors are expressed at a low level on the target cell due to microbial infection, thus, NK cells are a non-specific sensor of cells that are infected (12,13).

In addition to the physiological barriers and effector cells of innate immunity, there are soluble proteins, including complement, pentraxins, collectins and ficollins, that are found in the plasma that recognize PAMPs and are capable of acting as effector

molecules of the innate immune system (14). Upon recognition of PAMPs on microbes, these soluble proteins initiate the well-characterized complement cascade that allows for opsonization and phagocytosis or lysis of the microbe (15). Furthermore, the complement system also works with a component of the adaptive immune system, antibodies, to fix and activate the complement cascade to eliminate microbes (15). Thus, soluble proteins, cytokines and chemokines that are released as a result of phagocytosis and killing of infected cells by the innate immune system are the first line of defense against microbes and are responsible for triggering the activation of the adaptive arm of the immune system.

1.1.2 The adaptive immune system

The second arm of the immune system, the adaptive immune system, is capable of eliminating microbes in a highly specific manner and is characterized by the ability to generate specific memory (2). Defense of the body by the adaptive immune system occurs over the time course of days to weeks after initial exposure to a microbe and is mediated by effector cells that release cytokines and antibodies (2). The effector cells of the adaptive immune system include T and B lymphocytes, which are immune cells that express antigen-specific receptors, including the T cell receptor (TCR) and the B cell receptor (BCR), on the cell surface. Whereas PAMPs on microbes can be recognized by the innate immune system in a non-specific manner, T and B cells, via their BCR, can recognize epitopes on microbes in a specific manner (16). The types of epitopes that are recognized differ between T and B cells. Whereas T cells recognize processed antigen or a linear epitope (6-8 amino acids) presented in conjunction with MHC II via the TCR

(2,17,18), B cells recognize intact antigen based on the surface epitopes of a folded protein (15-22 amino acids), via the BCR (16,19). The interaction of the BCR and TCR with antigen is of high affinity and specificity, and allows for the clearance of low levels of antigen by a small number of highly specific adaptive immune cells to protect the host. The finely tuned, highly specific nature of the adaptive immune response to remove foreign antigen that is mediated by a small number of cells, therefore, must be tightly regulated in order to prevent reactions to "self" antigens (20-22), or autoimmunity (23,24). The specificity of the adaptive immune response is further promoted by the ability of the cells of the adaptive immune system to develop memory to previously encountered antigens, such that, upon re-exposure to the antigen, the memory cells release antibodies in a time course that is quicker than the initial exposure, and that will clear the antigen in a more robust manner (25,26). Thus, the adaptive immune system is unique due to its ability to adapt a highly specific response to antigens and the development of memory to previously encountered antigens.

1.2 $CD4^+$ T cell activation and differentiation

Naïve $CD4^+$ T cells require both antigen recognition and costimulatory signals for activation and differentiation into T helper 1 (Th1) or Th2 cells (27). For antigen recognition, the TCR of the naïve $CD4^+$ T cell must recognize peptide presented in the context of MHC II on the surface of an APC (28). The peptide is presented in the context of MHC II by APCs only after it has been endocytosed, processed, and packaged with MHC II. Binding of the TCR to antigen allows for Interleukin-2 (IL-2) production by T cells and results in clonal expansion (29). Costimulatory signals are comprised of cell

surface molecules and cytokines that allow for both activation and differentiation of a $CD4^+$ T cell (30). The cytokines that drive the conversion of a $CD4^+$ T cell towards a Th1 or Th2 phenotype are produced by both the T cell and APCs. For example, IL-12 produced by activated macrophages and DCs, as well as Interferon-gamma (IFN-γ) produced by NK cells and activated T cells, are known to drive naïve $CD4^+$ T cells towards a Th1 phenotype (31). IL-4, on the other hand, is produced by activated T cells and is known to drive $CD4^+$ T cells towards a Th2 phenotype, in the absence of Th1-driving conditions (31). Costimulation of CD28 on the activated and differentiated T cell by CD86 expressed on the APC allows for further activation of the T cell. CD28 stimulation on T cells increases the expression of CD40 ligand (CD40L), a cell surface protein that stimulates the APC, and, depending on the phenotype of the activated T cell, increases IL-12 or IL-4 production (6). Mice deficient in CD28 produce less antibody in response to immunization with a T-dependent antigen in comparison to wild type (WT) mice, illustrating the importance of costimulation of CD28 in antibody production (32). In addition to CD28 stimulation, the binding of CD86 to CD28 also sends a signal to the APC expressing CD86, a protein that has been shown to directly regulate the level of IgG_1 produced by a B cell (33-35). This mechanism involves a CD19/Prohibitin1/2/Phosphoinositol-3 kinase (PI_3K)/nuclear factor-kB (NF-kB) pathway mediated by Oct-2 expression and binding to the 3'Immunoglobulin Heavy Chain (IgH) enhancer (33-39). This dissertation focuses on the IL-4 dependent antibody; IgE and further discussion of the involvement of CD86 in antibody production can be found in (36,38,39). Thus, several signals are important for the activation and differentiation of naïve $CD4^+$ T cells, including antigen recognition that activates the T cell and both

cytokines and costimulatory signals that allow for further differentiation of the T cell.

1.3 B cell development, activation and differentiation

1.3.1 B cell development

The role of the B cell in the adaptive immune response is to produce antibodies that are specific for and bind to antigens with high affinity to promote clearance of pathogens from the body (25). Before the B cell can produce antigen-specific antibodies, it must first undergo antigen-independent development in the bone marrow, followed by antigen-dependent development in the lymph node or spleen (40-42). There are two types of B cells with different development programs: B-1 and B-2 B cells. The B-1 B cell develops in the fetal liver from fetal liver-derived hematopoietic stem cells (HSCs) and exhibits a limited ability to generate a diverse repertoire of antigen-specific antibodies (43). B-1 B cells are found in the peritoneum where they produce IgM in a spontaneous (43), rather than a stimulated fashion, and targets microbial polysaccharides and lipids. B-1 B cells are also found at mucosal sites where they secrete IgA in response to microbial antigens (44). Thus, B-1 B cells represent a subset of B cells with limited capabilities to produce diverse antibody repertoires. B-2 B cells develop in the bone marrow from bone marrow-derived HSCs as depicted in **Figure 1** and reviewed in (40-42). Within the bone marrow, bone marrow-derived HSCs mature into common lymphoid progenitors (CLPs) in response to IL-7 secretion by stromal cells. CLP can develop further into B, T, and NK cells. The progression from CLPs to pro-B cells occurs after bone marrow stromal cells release signals that activate the transcription factors EBF and E2A and induce the transcription factor Pax-5, and, all together, induce the

expression of recombinase activating gene (Rag)-1 and Rag-2, surrogate light chains, and proteins important for BCR signaling (45). Pro-B cells are $CD43^+/CD19^+/CD10^+$. Still within the bone marrow, the transition from the pro-B to the pre-B stage of B cell development is characterized by rearrangement of the IgH gene that produces a functional heavy chain. The heavy chain, in conjunction with surrogate light chains and Igα/β heterodimer, forms the pre-BCR complex, a defining feature of pre-B cells. Maturation of a pre-B cell to an immature B cell in the bone marrow requires immunoglobulin light chain gene recombination. Expression of the light and heavy chains in conjunction with Igα/β results in a functional BCR that is of the IgM isotype. However, B cells at the immature B cell stage do not differentiate and proliferate in response to antigen. Indeed, if the BCR expressed on an immature B cell engages the antigen it is specific for in the bone marrow, negative selection occurs wherein the receptor undergoes further editing or the immature B cell is eliminated to prevent self-reactivity. Immature B cells that are not self-reactive can then leave the bone marrow and travel to the spleen to complete their maturation program. Immature B cells are IgM^{low} and CD43-negative. The progression of an immature B cell to a mature B cell within peripheral lymphoid organs, such as the spleen, lymph nodes, and mucosal lymphoid tissues, is characterized by expression of both IgM and IgD. At this point, these B cells are mature, naïve B cells that have not previously encountered antigen. Anatomically, mature B cells are localized to the B cell follicles of lymphoid organs. After antigen entry, they then interact with T cells at the interface of the T/B cell zone, and upon encounter with antigen via the BCR, they will further differentiate into an effector B cell to produce high affinity antibodies or a memory B cell.

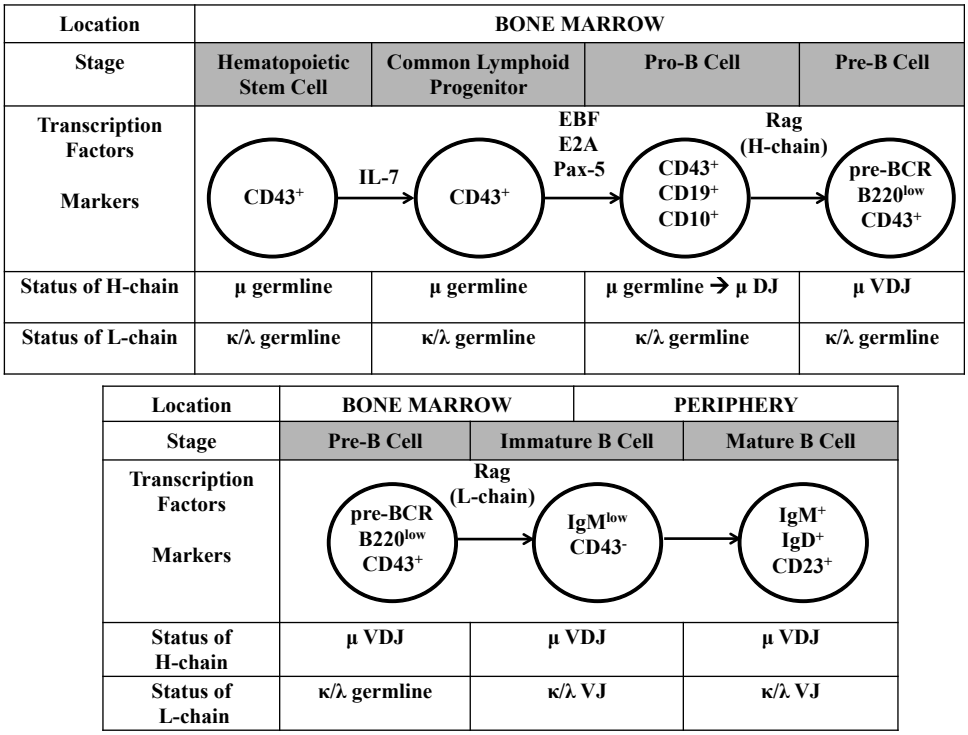

Figure 1. B cell development.

B cells in the bone marrow are characterized at each stage by the status of germline and/or rearranged DNA for Ig heavy and light chains. B cell development begins in the bone marrow when a hematopoietic stem cell responds to IL-7 released from stromal cells by differentiating into a common lymphoid progenitor. If transcription factors that include EBF, E2A, and Pax-5 become active, the common lymphoid progenitor will differentiate into a pro-B cell that is characterized as CD43+/CD19+/CD10+. At the pro-B cell stage, the Heavy chain (H-chain) rearranges. Following H-chain rearrangement, the expression of Rag genes in conjunction with the antigen receptor H-chain, the Igα/Igβ heterodimer, and the surrogate light chain (L-chain) allows for the expression of the pre-BCR and characterizes the Pre-B cell stage of B cell development. Rag-mediated L-chain formation then leads to IgM expression on the now immature B cell surface. Following selection, the cell migrates to the periphery and is characterized by the loss of CD43 expression and has low levels of IgM on the surface. Finally, when the cell is IgM+/IgD+/CD23+, it is considered a mature B cell capable of responding to antigen stimulation.

1.3.2 B cell activation and the germinal center

Upon completion of the maturation program, mature naïve B cells that have migrated to peripheral lymphoid organs will become activated to produce high affinity antibodies, depending on the type of antigen that binds to the BCR, in either a T-independent or T-dependent fashion (20). T-independent antigens consist of antigens that cannot be displayed via MHC II due to the absence of amino acid residues. Examples of T-independent antigens include polysaccharides, like LPS from *E. coli*, membrane glycolipids, such as alpha-galactosylceramide (46), and nucleic acids such as microorganism DNA with low amounts of methylation (47). T-independent antigens are often polyvalent, meaning that they include several repeating antigenic epitopes, thus, crosslinking of the BCR by multiple antigens expressed by the same pathogen can potentially fully activate the B cell independent of T cell help (47). For the purposes of this dissertation, we will focus on the activation of B cells by T-dependent antigens to produce high affinity antibodies (48-50).

B cell activation by a T-dependent antigen occurs within peripheral lymphoid organs in the following manner. Mature, naïve B cells typically reside in and circulate through the follicles of peripheral lymphoid organs (51). Localization to these compartments is governed by the presence of CXCR5, a chemokine receptor for CXCL13, a chemokine released by follicular DCs (FDCs) (52). Upon T-dependent antigen exposure, a B cell will downregulate CXCR5 (52) and migrate toward the T-B cell interface (53-55). "Signal 1" consists of binding of antigen to, and crosslinking of, at least two BCRs, which triggers a series of intracellular events mediated by the Igα/Igβ heterodimer (56).

This results in the activation of a number of transcription factors responsible for inducing clonal proliferation of the B cell, preparation for subsequent interactions with Th cells, and the production of molecules critical for optimal B cell-T cell interaction, including MHC II and the costimulatory molecule, CD86, which modifies the level of antibody produced by the B cell (36,38,39). When antigen binds to the BCR, it is endocytosed and processed into peptide fragments that are displayed in the cleft of an MHC II molecule (**Figure 2**). The MHC II-antigenic peptide complex is displayed on the surface of the activated B cell and is critical for downstream interactions with Th cells that recognize the peptide in association with MHC II (57). A few days after antigen activation of a B cell, the activated B cell has either differentiated to an IgM-secreting cell or will form a germinal center (53-55). The germinal center reaction occurs in the follicles and is divided into three distinct phases as depicted in **Figure 3** and involves both dark and light zones of the peripheral lymphoid organ. Rapidly proliferating activated germinal center B cells, or centroblasts, form the "dark zone" of the germinal center and undergo the process of somatic hyper-mutation (SHM), a process that involves mutation of the immunoglobulin surface receptor that increases it's affinity towards antigen. Following SHM, in the second phase of the germinal center reaction, centrocytes, the non-dividing progeny of centroblasts, migrate towards the "basal light zone", an area that is structurally organized by FDCs (42,58). Whereas FDCs do not present antigen directly via MHC II (59), the abundance of complement receptors and Fc receptors (60) on the extensive processes of FDCs allow for optimal interaction of opsonized antigen with centrocytes and allows for the selection of centrocytes with high affinity for antigen,

while centrocytes with lower affinity undergo apoptosis (61-63). This affinity selection process, together with SHM, is known as affinity maturation. (58,64). The final phase of the germinal center reaction begins upon migration of centrocytes with high affinity immunoglobulin receptors towards the edge of the follicle to the "apical light zone". These high affinity B cells will now express antigen in the context of MHCII for presentation to follicular Th cells (Tfh) (65-70). Since Tfh normally reside in the T cell zone of peripheral lymphoid organs, Tfh must upregulate CXCR5 in order to migrate towards the apical light zone at the edge of the follicle (71-73). Tfh are characterized by their expression of CD40L and CXCR5 and have been found to be essential for germinal center formation and B cell differentiation into antibody-secreting plasma cells or memory cells (67-70). Whereas the antigen recognition that leads to B cell activation is recognized as "Signal 1", the interaction of Th cells that express CD40L and secrete IL-4 is known as "Signal 2" and ultimately leads to antibody production (**Figure 2**). Thus, B cell interaction with FDCs allows for affinity maturation while interaction with Tfh allow for downstream class switch recombination and antibody production.

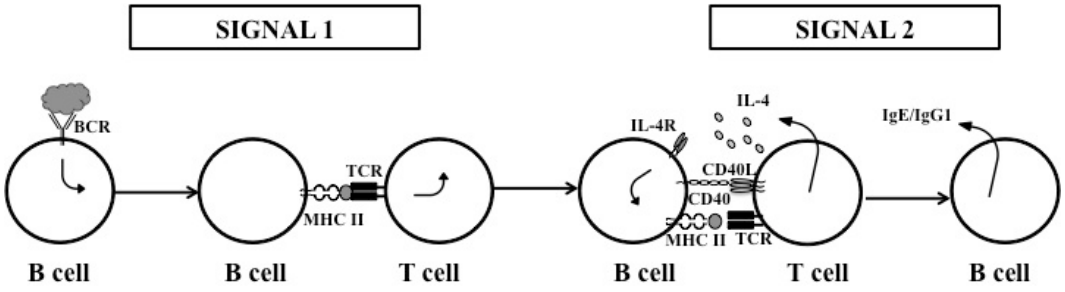

Figure 2. B cell differentiation to an antibody-secreting cell.

Antigen binding to the BCR, or "Signal 1" for the B cell, triggers antigen endocytosis, processing, and display in association with MHC II molecules on the surface of the B cell. The MHC II-antigen complex interacts with the TCR that recognizes the antigen displayed by MHC II and activates the T cell to secrete the cytokine IL-4 and to upregulate surface receptors including CD40L. "Signal 2" occurs when CD40L on, and IL-4 released by, the Th cell bind to CD40 and the IL-4R on the surface of the B cell to fully activate the B cell, induce CSR, and lead to differentiation into an antibody-secreting cell.

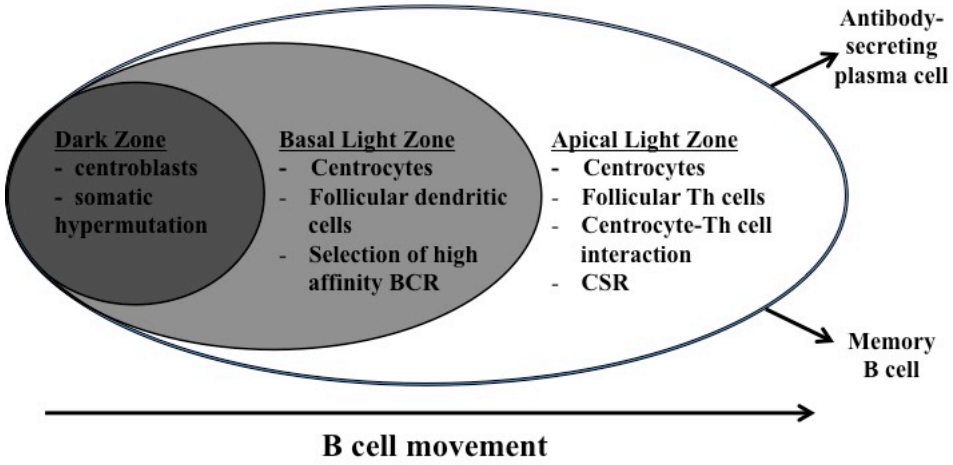

Figure 3. The germinal center reaction.

The germinal center reaction consists of three phases upon antigen recognition within secondary lymphoid organs that include the lymph nodes and spleen. Following engagement of the BCR, the B cell is considered a centroblast and undergoes the processes of clonal proliferation and somatic hypermutation in the "dark zone" of the lymphoid organ. Following these processes, the cell is called a centrocyte and migrates to the "basal light zone" that is populated by FDCs. Within the "basal light zone", selection occurs wherein only centrocytes with high affinity BCRs will receive survival signals. The remaining centrocytes then migrate into the "apical light zone" that is populated by follicular T helper cells (Tfh cells). The binding of Tfh cells to centrocytes promotes CSR that allows the B cell to become a plasma cell and produce antibody critical to combat infections. Alternatively, B cells may become memory B cells capable of long term survival and able to respond quickly with a high magnitude response upon reencounter with the antigen and a T cell.

1.3.3 Immune receptor involvement in antibody production by a B cell

Class switch recombination (CSR), as depicted in **Figure 4** and reviewed in (74) is defined as the process encompassing the rearrangement of the BCR gene segment containing Variable, Diversity, and Joining segments (VDJ) that recombine with a downstream heavy chain constant region (C_H) and leads to deletion of the intervening deoxyribonucleic acid (DNA). CSR to Cε and $C_{\gamma 1}$ has been reviewed previously (74,75). Recombination to Cε and Cγ1 is controlled by a gene segment called the switch region (S) located in the 5' intronic region of each C_H region, and includes Cμ, Cγ, Cε, and Cα, but not Cδ (74). To switch from IgM to IgG_1 or IgE, two signals from a Th2 cell are essential, IL-4 and CD40L (49,50,76,77), and these signals comprise **Signal 2** (Figure 2) of the signals required to activate a B cell. CD40L on the T cell binds to CD40 on the B cell and IL-4 binds to the IL-4 Receptor (IL-4R) to complete activation and trigger CSR. Engagement of CD40 and the IL-4R triggers numerous downstream events that activate several transcription factors including NF-κB and Signal transducer and activator of transcription 6 (STAT6), respectively. Whereas IL-13 is also known to induce CSR to IgE (78), this dissertation will focus on IL-4. As depicted in **Figure 5**, IL-4 binding to the IL-4R activates downstream STAT6 signaling. Dimerization of STAT6, translocation to the nucleus, and binding to STAT6 consensus sites within the germline ε and γ1 promoters induces transcription (79). The importance of STAT6 was demonstrated when STAT6-deficient B cells were unable to produce germline transcripts upon CD40 and IL-4 activation (79). Germline transcription has been proposed to allow recombination machinery access to the Cγ1 and Cε gene segment which promotes interaction of the

switch regions Sμ with Sγ1 or Sε (80) such that switching can occur. Furthermore, the level of germline transcription has been positively correlated with the level of CSR (81,82), suggesting that germline transcription is a prerequisite for CSR to IgG_1 or IgE. Finally, mature antibody transcription of IgG_1 and IgE occurs after switch region binding and the deletion of intervening DNA.

As mentioned above, CSR requires not only IL-4 stimulation, but also CD40L stimulation. The expression of CD40, CD40L, and the downstream signaling events that binding triggers are reviewed extensively (83). CD40 is a member of the Tumor Necrosis Factor Receptor (TNFR) family and is constitutively expressed on the surface of a B cell (83). CD40L is expressed as a homotrimer on the Th2 cell and promotes oligomerization of CD40 on the surface of the B cell, that promotes signaling via the TNFR-associated factors (TRAFs) (84-88). CD40L binding to CD40 promotes TRAF phosphorylation and downstream activation of NF-κB (89,90), p38/extracellular-signal regulated kinase (ERK)/c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) (91-94), phospholipase C γ2 (PLCγ2), and PI_3K (95), as diagrammed in **Figure 6**. The CD40L-induced switch to IgG1 or IgE has been found to be dependent on the NF-κB pathway (96,97). Upon CD40 engagement, TRAF2 associates with CD40 and becomes phosphorylated, activating NF-κB (88). Ultimately, activated NF-κB (p50/RelB) translocates to the nucleus to induce germline γ1 or ε transcription by binding to consensus sites in the germline γ1 or ε promoter regions (96,97). Blockade of the CD40/CD40L interaction results in decreased antibody production *in vivo* (98) and *in vitro* stimulation of naïve B cells with anti-CD40 or CD40L and IL-4 allows for CSR to

IgG_1 or IgE (49,50,76,77), suggesting that B cells can undergo CSR in the absence of BCR stimulation. Additionally, the CD40L/CD40 interaction was shown to be essential for the maintenance of germinal centers (99-101), indicating a requirement for CD40L in CSR resulting from the Th2-B cell interaction. Overall, CD40 ligation and IL-4 stimulation on a naïve B cell are essential for CSR to produce IgG_1 and IgE.

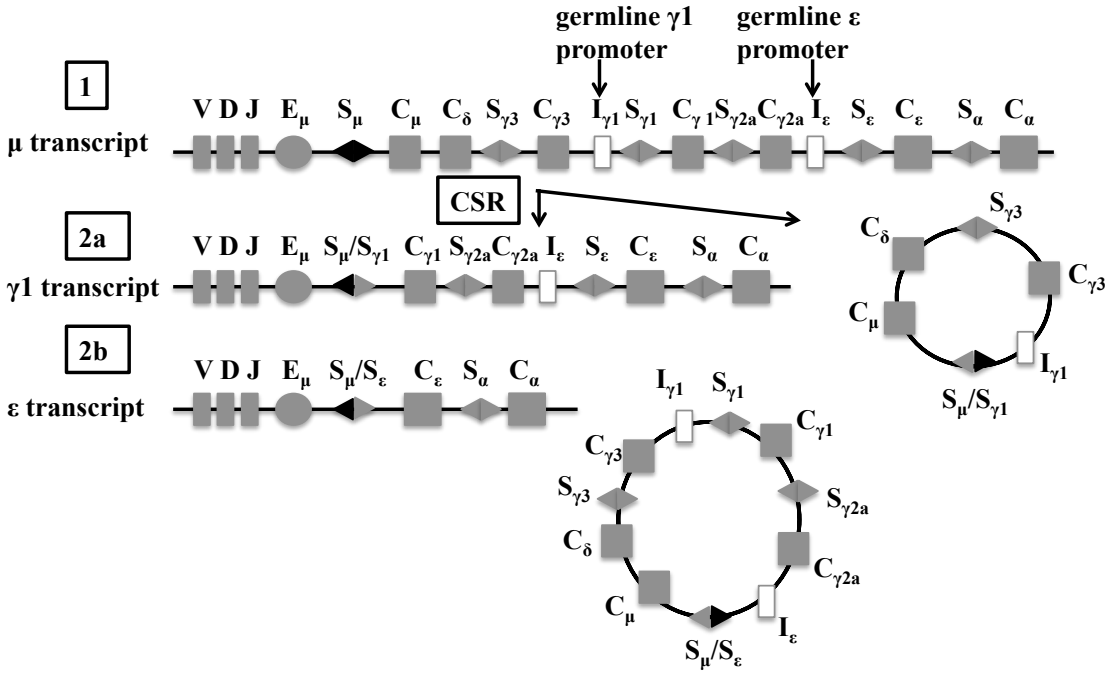

Figure 4. Class Switch Recombination to IgG1 or IgE by a B cell.

(1) Upon engagement of the IL-4R and CD40 on the B cell surface, the activation of downstream signaling networks within the B cell promote germline γ1 or ε transcription, also known as interfering γ1 (Iγ1) or Iε, to allow recombination machinery access to the switch regions (S) μ and Sγ1 or Sε to facilitate their interaction. (2) Once the switch regions fuse, the intervening DNA is removed to form a "switch circle" of DNA. The end product is an IgH gene locus where the constant region has switched from constant (C) Cμ to Cγ1 (2A) or Cε (2B), which is transcribed as the heavy chain region. The "switch circle" of DNA contains portions of Sμ and any regions between Sμ and Sγ1 or Sε, while Iγ1 or Iε is also removed. VDJ represents the variable, diversity, and joining segments of the IgH gene locus, while Eμ represents the intronic enhancer.

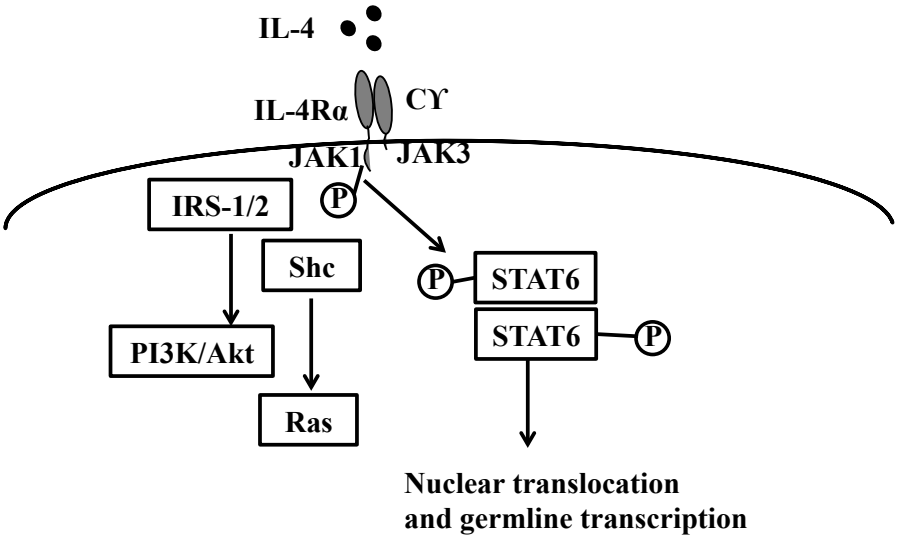

Figure 5. IL-4 receptor signaling networks.

The IL-4 receptor (IL-4R) consists of two different chains, the IL-4Rα chain and the common (C) Cγ chain. Binding of IL-4 to the IL-4 receptor triggers Jak1/3 binding to the IL-4R cytoplasmic domain and phosphorylation of downstream mediators, which ultimately leads to dimerization of phosphorylated STAT6. Dimerized STAT6 translocates to the nucleus and promotes germline transcription. IL-4R engagement also activates the PI3K/Akt pathway, a pathway dependent on Insulin Receptor Substrate (IRS1/2) activation and Src homologous and collagen (Shc)-mediated activation of the Ras pathway.

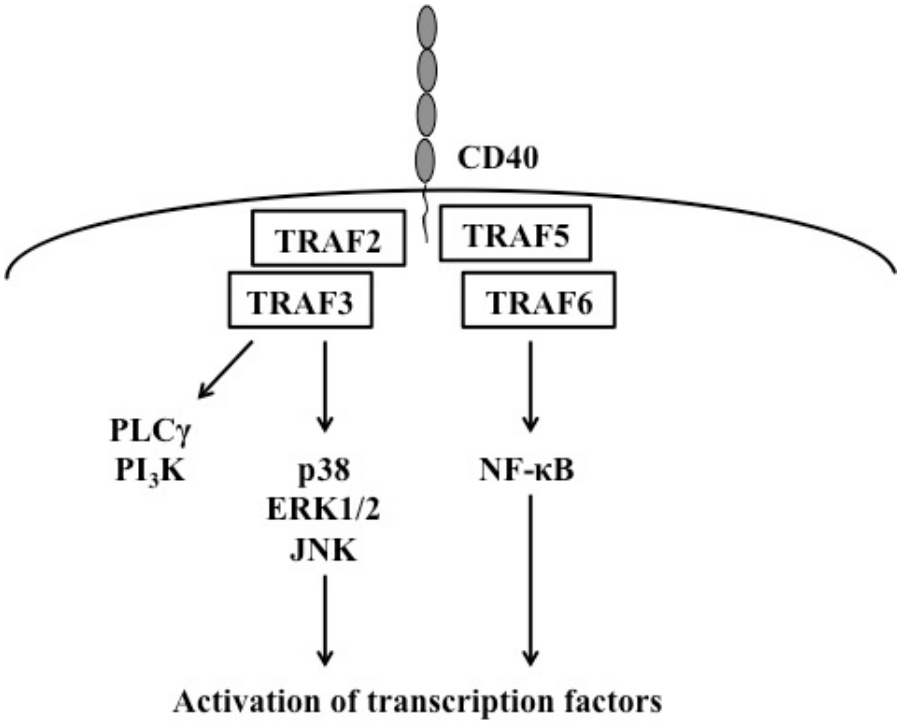

Figure 6. CD40 signaling in a B cell.

Upon interaction with trimerized CD40L on the surface of the Th cell, CD40 on the B cell oligomerizes and recruits TNF-receptor associated factors (TRAFs). The TRAF proteins recruited to CD40 in a B cell are TRAF2, 3, 5, and 6. Phosphorylation of these TRAFs leads to activation of NF-κB, p38/ERK/JNK MAPKs, phospholipase C-gamma (PLCγ), and phosphatidylinositol-3 kinase (PI3K). These factors can activate transcription factors in the B cells and modulate gene expression or cell function.

1.4 Sympathetic nervous system innervation of secondary lymphoid organs

1.4.1 Anatomy of sympathetic innervation of lymphoid organs

Direct neural modulation of immune cell activity by the sympathetic nervous system (SNS) has been demonstrated by neurochemical and neuroanatomical data (102-107). Sympathetic nerve activity is typically associated with the "fight or flight" response by regulation of the cardiovascular and respiratory systems in response to a perceived threat. In addition, norepinephrine (NE), a SNS neurotransmitter, is considered a factor that aids in the maintenance of immune system function in response to the threat of antigen (102). In order for the rest of the immune system to become activated via the nervous system during the fight against an antigen, innate immune cells must release cytokines after their initial encounter with the antigen. These cytokines bind their cognate receptors and stimulate central nervous system (CNS) neurons that are connected to the sympathetic nerve pathway via peripheral nerves with efferent activity, crossing of the cytokines of the blood brain barrier via specific cytokine transporters, or via immune cells crossing the blood brain barrier in times of altered blood brain barrier permeability (108). Sympathetic neurotransmission from the CNS to the periphery occurs via projections to the hypothalamus (109). Axons of the hypothalamic neurons project to the spinal cord and synapse on postganglionic sympathetic neurons located in the sympathetic chain ganglia along the spinal column, and the celiac, superior mesenteric, and inferior mesenteric ganglia located in the abdominal cavity. The axons of the postganglionic neurons then follow the vasculature to innervate target organs, including all primary (thymus and bone marrow) and secondary (spleen and lymph node) lymphoid organs (108,110) (illustrated

in **Figure 7**). Therefore, an anatomical basis for neurotransmission was established that resulted in the release of NE from granules from sympathetic nerve terminals upon sympathetic nerve activation.

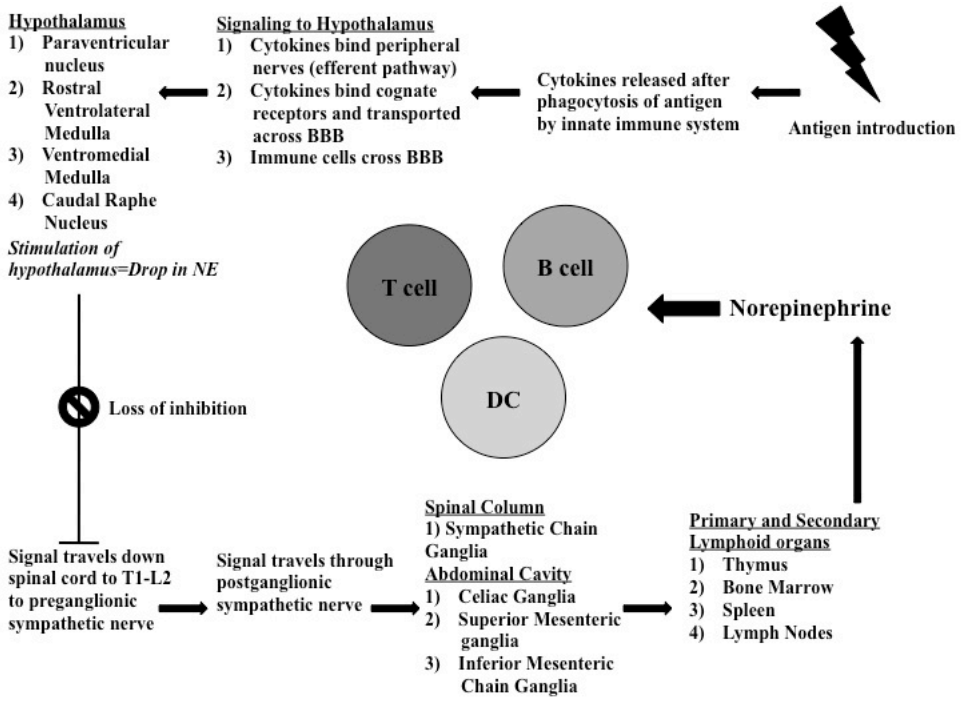

Figure 7. Communication between the nervous and immune system.

Anatomical basis for neurotransmission via the sympathetic pathway upon antigen exposure. Antigen introduction via injury allows for the release of inflammatory cytokines by innate immune cells upon phagocytosis of antigen at the site of injury. Cytokines stimulate the hypothalamus using three different pathways: 1) Binding to cognate receptors on peripheral nerves and sending an efferent signal to the hypothalamus; 2) Binding to cognate receptors and transport across the Blood Brain Barrier (BBB); 3) Immune cell permeation of the BBB that is often compromised due to infection. Stimulation of the hypothalamus inhibits Norepinephrine (NE) release and leads to a loss of inhibition of preganglionic sympathetic neurons within the hypothalamus. Preganglionic sympathetic neurons synapse on postganglionic sympathetic neurons at the level of the spinal cord and into the abdominal cavity. The sympathetic ganglia, celiac ganglia, superior mesenteric ganglia, and inferior mesenteric chain ganglia follow the vasculature and innervate the primary and secondary lymphoid organs: thymus, bone marrow, spleen, and lymph nodes. It is at these junctures where NE is released in the proximity of B cells, T cells, and DCs that express the β_2AR. This is the mechanism that maintains immune homeostasis.

1.4.2 Norepinephrine release in the proximity of immune cells

The synthesis of NE begins with the hydroxylation of tyrosine by tyrosine hydroxylase (111) to become L-DOPA. L-DOPA is then transported via the vesicular monoamine transporter into synaptic vesicles (112). Within the synaptic vesicles, L-DOPA is decarboxylated by DOPA decarboxylase to become dopamine (111). Dopamine is then converted to NE by dopamine β-hydroxylase (111). NE release from sympathetic nerve terminals in the proximity of immune cells was demonstrated by electron microscopic studies of the splenic white pulp that showed that sympathetic nerve terminals are in close proximity to T cells, interdigitating DCs, and B cells (113) and that NE is released within 8-18 hours of antigen administration to mice (104). A recent report shows that, in addition to sympathetic nerve terminals, phagocytes release NE and express the enzymes for NE synthesis and degradation (114), providing an autocrine mechanism of NE release within the immediate vicinity of the immune cells to affect their function.

1.4.3 Adrenergic receptor expression by immune cells

NE binds to two families of adrenergic receptors expressed on immune cells, namely the alpha- (αAR) and beta-adrenergic receptor (βAR), so the pattern of expression established the lymphoid cells that were more likely to respond to NE (115,116). Both families of receptors are expressed on innate immune cells including monocytes/macrophages, DCs, and neutrophils. In contrast to innate immune cells, adaptive immune cells, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells, as well as the innate NK cells, express the β_2AR exclusively (102,115). The dichotomy in adrenergic

receptor expression by these immune cells is interesting given that adaptive immune cells and the innate NK cell develop from lymphoid precursors in the bone marrow, as opposed to all other innate immune cells that develop from myeloid precursors, as was shown using radioligand binding (117). Exclusive β_2AR expression by the B cell was confirmed via flow cytometry, messenger ribonucleic acid (mRNA) analysis, and competitive radioligand binding studies (118,119). The β_2AR expressed on B cells was confirmed to be functional using an agonist and through the measurement of intracellular cyclic adenosine monophosphate (cAMP), an intermediate downstream of the β_2AR (119,120). The level of adrenergic receptor expression varies among the different immune cell types and is regulated by a variety of factors, including the activation state of the cell, cytokines, and neurotransmitters (115). The timing of receptor expression may also play an important role in mediating neurotransmitter regulation of immune cells (102). For example, naïve $CD4^+$ T cells express the β_2AR, but upon differentiation, the Th2 cell loses expression of the β_2AR, whereas the Th1 cell retains expression of the β_2AR (115). Earlier data suggested that expression of the β_2AR was regulated by epigenetic histone methylation patterns within the β_2AR promoter(121). It was later determined that the absence of β_2AR expression by Th2 cells was due to decreased Histone (H) 3/H4 acetylation and decreased H3K4, H3K9, and H3K27 methylation in comparison to Th1 cells which exhibited increased H3/H4 acetylation and increased H3K4 methylation of the β_2AR promoter (122), suggesting that specific epigenetic modifications were responsible for the expression of the β_2AR in different Th cell subsets. Thus, immune cells express an adrenergic receptor that binds to the NE released

from nerve terminals residing within lymphoid tissue and/or the immune cell itself.

1.5 β_2AR Signaling

1.5.1 G-protein mediated β_2AR signaling

Adrenergic receptors are seven-transmembrane spanning receptors that associate with heterotrimeric GTP-binding proteins (G-proteins) and are known as G-protein coupled receptors (GPCRs). Early mechanistic studies of the β_2AR focused on the study of Gα stimulatory ($G\alpha_s$) activation and subsequent elevation in cAMP, a signaling intermediate that was thought at the time to be the exclusive intermediate activated by β_2AR stimulation. [For a comprehensive review of the findings from early studies of cAMP-mediated effects in immune cells, (108)]. More recent studies, as summarized in **Figure 8**, have revealed that the β_2AR is able to associate with either the G-protein $G\alpha_s$ or $G\alpha_i$, that are both capable of activating a distinct molecular pathway within an immune cell to differentially regulate its function (123). Lefkowitz and his colleagues discovered that the β_2AR also associates with GPCR kinases (GRKs) and β-arrestins that activate signaling pathways independently of Gα-proteins (124). Activation of each of these signaling intermediates requires ligand binding to the receptor. This requirement is one reason why early studies using cAMP analogs that bypass Gα-proteins and/or β-arrestin activation, may be misleading.

Classically, agonist binding to the β_2AR on an intact cell induces coupling to the $G\alpha_s$ subunit that activates the enzyme adenylate cyclase to cause a rapid transient increase in cAMP levels (123). cAMP subsequently activates cAMP-dependent protein

kinase A (PKA) (123), a kinase that mediates phosphorylation of either the transcription factor cAMP response element binding protein (CREB) (125) or the phosphatase hematopoietic protein tyrosine phosphatase (HePTP) (126) that is involved in the regulation of ERK1/2 and p38 MAPK (127). These two PKA-induced molecular pathways, CREB *vs.* HePTP, appear to differentially regulate the level of IL-4-dependent IgG_1 and IgE antibody produced by B cells (126), respectively, and will be discussed in detail later.

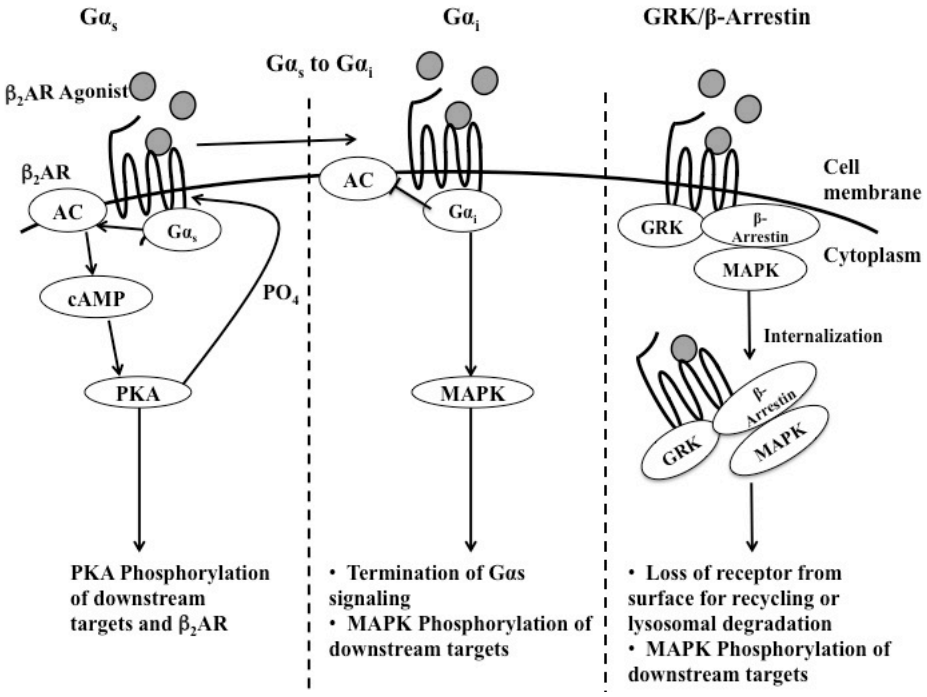

Figure 8. β_2AR signaling in immune cells.

Molecular pathways activated intracellularly by β_2AR engagement on an immune cell. The sympathetic neurotransmitter, norepinephrine (NE), binds to the β_2AR. Ligand binding to the receptor will induce receptor binding to a heterotrimeric G-protein complex comprised of the Gα-protein and the β and γ subunits. GDP will be released from the Gα-protein and replaced by GTP, leading to activation and separation of the heterotrimeric G-protein complex to $G\alpha_s$ and βγ, followed by the following events: **1)** $G\alpha_s$ activates the enzyme adenylate cyclase to subsequently raise the level of cAMP. cAMP activates PKA, a kinase that phosphorylates multiple substrates. PKA also phosphorylates the intracellular tail of the β_2AR to cause a $G\alpha_s$-to-$G\alpha_i$ switch and, subsequently, activates a negative-feedback pathway through $G\alpha_i$; **2)** Activation of $G\alpha_i$ halts β_2AR binding to $G\alpha_s$ and inhibits the $G\alpha_s$ signaling pathway. β_2AR binding to $G\alpha_i$ also leads to activation of molecules of the MAPK family of proteins; and **3)** Ligand binding to the β_2AR activates a G-protein-independent molecular pathway, GRK/β-arrestin2. Receptor stimulation recruits GRK, and GRK binds to and phosphorylates specific sites on the β_2AR to create a binding site for β-arrestin2. This complex will internalize the receptor and inhibit all G-protein-mediated signaling. β-arrestin2 can then serve as a scaffolding protein to bind and activate molecules of the MAPK family of proteins.

1.5.2 β_2AR signaling in a B cell

The β_2AR is expressed on all cell types except Th2 cells, but, of the adrenergic receptors, it is the only one expressed on B cells (118,119). Due to the proximity of B cells to sympathetic neurons releasing NE within lymphoid organs, it is important to understand the role of β_2AR signaling in both homeostatic and proinflammatory conditions. To begin with, the antibody-enhancing effect of β_2AR stimulation requires CD40L/IL-4-priming of a naïve B cell. These two signals allow for CSR and survival of the B cell (17,18,128,129), and, as later discussed, and contribute to the downstream signaling pathways capable of mediating the β_2AR-induced increase in IgE. The timing of β_2AR signaling is essential for the antibody-enhancing effect. The addition of the β_2AR agonists NE or terbutaline must occur within the first six hours after immunization with sheep red blood cells to enhance antibody production five days post-immunization (130,131). The addition of β_2AR agonists after this timepoint did not increase antibody production. Furthermore, the addition of a β_2AR antagonist is only effective in abolishing the β_2AR-enhancing effect within the first six hours post-immunization (130,131). The narrow window of time when β_2AR stimulation is antibody-enhancing precedes a significant time lag between β_2AR stimulation of a primed B cell and the enhancement of antibody production that may partially be explained by the time it takes for the cell to class switch and proliferate. CD40L/IL-4-stimulated CSR and proliferation occurs within the first 2-3 days of stimulation (74,75), and antibody production is first detectable between days 3 through 5 (33,132). Thus, the narrow time after β_2AR stimulation of a

primed B cell when it is capable of enhancing antibody production and the lag between initial stimulation and enhancement indicate the possibility of a single or multiple mediators of the β_2AR-associated enhancement of antibody production.

β_2AR stimulation on B cells is important in the homeostatic response to antigens. *In vivo* depletion of sympathetic nerve terminals that release NE in proximity to the β_2AR on B cells using 6-hydroxydopamine (6-OHDA) results in decreased antibody production in response to antigen. Decreased production of IgM and IgG_1 were associated with reduced germinal center formation and clonal expansion (119). Later studies associated sympathetic nerve terminal depletion with decreased IgE production (132), indicating that β_2AR stimulation is essential for optimal germinal center formation and expansion and for antigen-specific antibody production. Thus, β_2AR signaling is intricately involved with homeostasis and adaptive immunity.

The downstream molecular pathways of β_2AR stimulation of CD40L/IL-4-primed B cells diverge to enhance both IgG1 and IgE production (**Figure 9**). As discussed previously, β_2AR stimulation on a B cell activates $G\alpha_s$ and $G\alpha_s$ activates adenylyl cyclase (123). Adenylyl cyclase converts ATP to cAMP. cAMP then phosphorylates PKA. Specifically, while the β_2AR-induced increase in PKA activation regulates the level produced of both IgG_1 and IgE, the PKA-induced increase in CREB activation regulates IgG_1 alone (126) and the PKA-induced increase in HePTP and p38 MAPK activation regulates IgE (126,132). For IgG_1, PKA-phosphorylated CREB translocates to the nucleus to transcribe the transcriptional co-activator OCA-B, then OCA-B heterodimerizes with the transcription factor Oct-2, to regulate the rate of mature IgG_1

transcription (133). For IgE, PKA phosphorylates HePTP bound to p38 MAPK. Phosphorylation of HePTP allows for a conformational change to occur so that p38 MAPK is released from HePTP, making it free to be phosphorylated by the MAP kinase pathway (126). Phosphorylated HePTP releases bound p38MAPK, and p38 MAPK is phosphorylated by the MAPK kinase (MAPKK) pathway downstream of CD40 ligation (126). Phosphorylated p38 MAPK is capable of phosphorylating various transcription factors including activating protein-1 (AP-1), activating transcription factor-2 (ATF2), STAT1, myocyte enhancer factor-2 (MEF2), and mitogen and stress-activated protein kinase (MSK1) [reviewed by (134,135)]. Blockade of this pathway using pharmacological inhibitors affects IgE production and the IgE regulator, CD23 (132). Thus, the β_2AR-associated enhancement of antibody production in a B cell is time- and transcription factor-dependent and essential for modulation of homeostasis

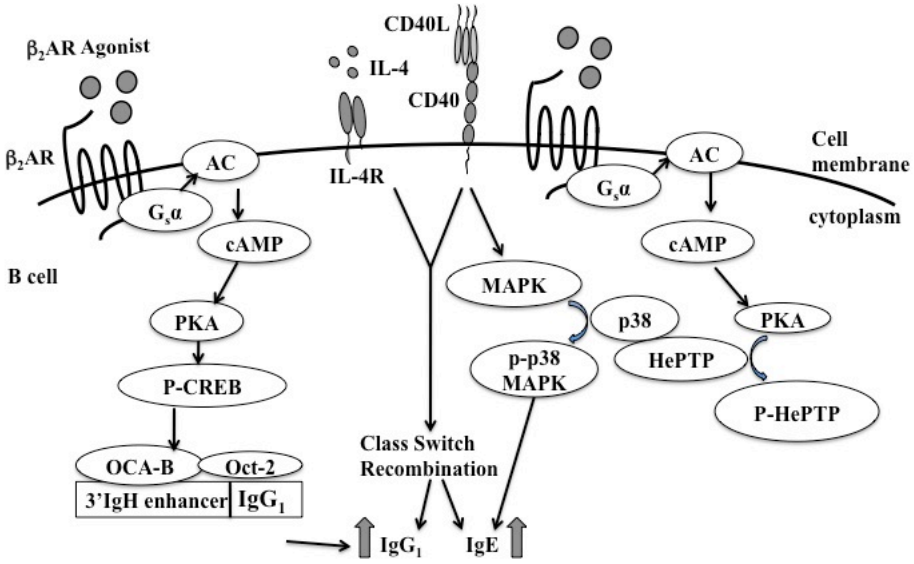

Figure 9. β_2AR and antibody production.

β_2AR stimulation increases IgE and IgG_1 production by primed B cells using two distinct pathways. Stimulation of the β_2AR on a CD40L/IL-4 primed B cell works through two separate pathways to increase production of IgE and IgG_1 without affecting class switch recombination. The increase in IgG_1 production is mediated by the molecular pathway that includes $G\alpha_s$ activation of adenylyl cyclase, cAMP, and PKA. PKA phosphorylates CREB and allows for OCA-B production. OCA-B binds OCT-2 to create a heterodimer that binds to the 3'IgH-enhancer and promotes an increase in the rate of IgG_1 transcription. For IgE, $G\alpha_s$ activation allows for the activation of adenylyl cyclase, cAMP, and PKA. However, the molecular pathway differs from above in that PKA phosphorylates Hematopoietic Protein Tyrosine Phosphatase (HePTP) that is bound to p38 MAPK (p38 MAPK-HePTP), releasing p38 to be phosphorylated by the MAPK pathway that was activated by the engagement of CD40. The phosphorylated p38 MAPK is now responsible for mediating the increase in IgE, involving other signaling intermediates, which will be discussed further.

1.6 Regulation of IgE production

1.6.1 β_2AR stimulation and IgE production

Several studies have demonstrated a solid connection between β_2AR stimulation and IgE production both *in vivo* and *in vitro*. Initial experiments with antigen-specific B cells indicated that β_2AR stimulation on a CD40L/IL-4 primed B cell was capable of enhancing IgE production in a β_2AR-specific manner, as was shown using β_2AR-deficient B cells and a β_2AR antagonist (34) and was confirmed with an antigen-free system (132). The β_2AR-associated enhancement in IgE production was not due to an increased amount of B cells that switched to IgE, as demonstrated by Enzyme-Linked Immunosorbent Spot (ELISPOT) and limiting dilution analysis, but by increasing the level of IgE produced per B cell (34,132). Further studies revealed *in vivo*, that in an allergic asthma model, mice depleted of NE via chemical sympathectomy by 6-OHDA administration exhibited reduced IgE production in both serum and bronchoalveolar lavage fluid (BALF) and was not due to a reduced number of B cells switching to IgE (132). Indeed, it was demonstrated *in vitro* that the β_2AR-associated enhancement in the level of IgE produced by B cells was associated with an increase in the level of mature IgE mRNA and was due to an increased rate of transcription, as determined by nuclear run-on, and not stability, as determined using ActinomycinD (132). The β_2AR-associated increase in IgE was further demonstrated to be PKA- and p38 MAPK-dependent using inhibitors of these pathways (132). Finally, β_2AR stimulation also enhanced IgE production in a CD23 and sCD23-dependent manner both *in vitro* and *in vivo* (132).

Thus, the early pathways that underlie the β_2AR-associated enhancement of IgE include both PKA and p38 MAPK and rely on CD23.

1.6.2 CD23 and the regulation of IgE production

It is known that the level of IgE produced by a B cell is regulated by CD23 (136-144), the low affinity receptor for IgE (FcεRII) that is expressed as a homotrimer on not only the cell surface of B cells (145,146), but also other immune cells, such as macrophages (147). As depicted in **Figure 10**, CD23 negatively regulates the level of IgE produced by a B cell when soluble IgE binds to it (136-138), but positively regulates the level of IgE when CD23 is cleaved to a soluble form (139-144), soluble CD23 (sCD23), that subsequently binds to CD19/CD21 on a human B cell (140,141). As described above, the level of IgE, as well as the level of sCD23, in the serum and BALF of immunized NE-depleted mice was found to be lower than the level produced in NE-intact mice (132), suggesting that β_2AR stimulation may play a physiological role in regulating the IgE response to antigen. Also, the level of IgE produced by primed CD23-deficient B cells that were exposed *in vitro* to an agonist to the β_2AR, is the same as that produced by primed CD23-deficient B cells alone (132), suggesting that the β_2AR on a B cell plays a role in regulating the level of IgE produced by first mediating an effect on CD23. Thus, CD23 is intricately involved in the regulation of IgE production by B cells.

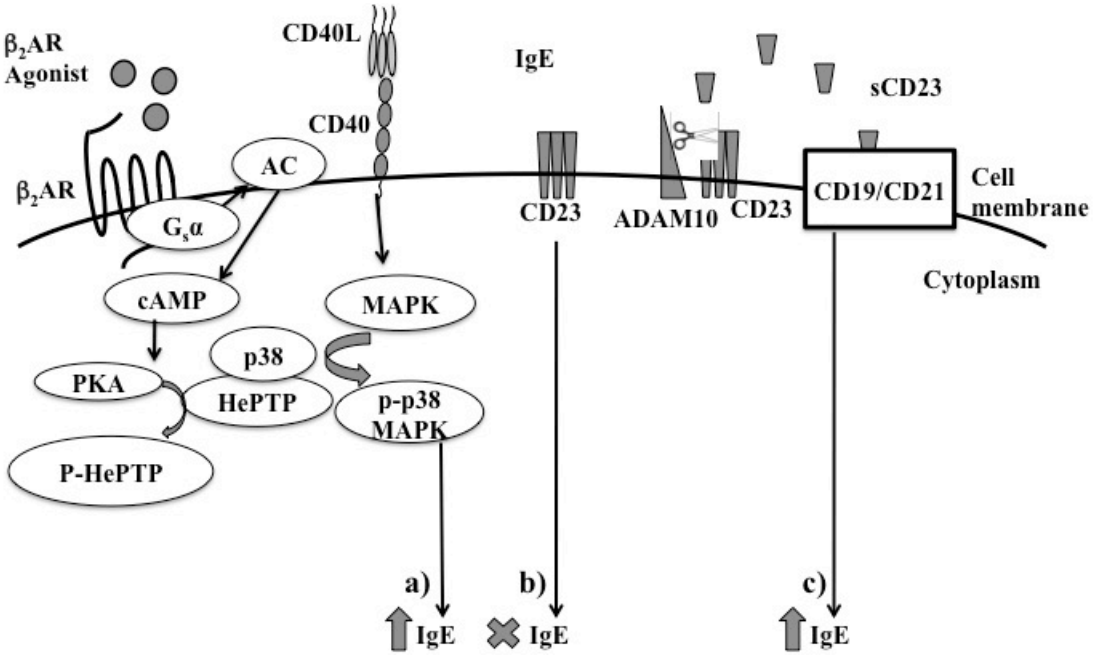

Figure 10. Regulation of IgE production.

IgE production can be regulated by two pathways. A) The proximal pathway governing the β_2AR-associated increase in IgE in a CD40L/IL-4 primed B cell. β_2AR stimulation activates adenylyl cyclase, and adenylyl cyclase activates cAMP. cAMP activates PKA. PKA phosphorylates hematopoietic protein tyrosine phosphatase (HePTP). Phosphorylated HePTP releases bound p38 MAPK, and is then phosphorylated by the CD40-induced MAPK pathway and increases IgE production. B) IgE production is negatively regulated by the binding of the Fc portion of IgE to CD23 expressed on the surface of the B cell. C) CD23 can be cleaved by ADAM10 to produce sCD23. sCD23 binds to the CD21/CD19 co-receptor complex to enhance IgE production.

1.6.3 ADAM10 cleavage of CD23

ADAM10 is a constitutively expressed zinc-dependent metalloprotease capable of cleaving several types of substrates, including CD23 (148). It has been identified as the primary sheddase of CD23 (146,149). CD23 is also cleaved by ADAM8, 15, 28, and 33 (150-152), but ADAM10 was independently confirmed by two laboratories to be the primary sheddase when a B cell is activated (146,149). ADAM10 deletion is embryonic lethal, a complication that has made it difficult to study the functional effects in the absence of ADAM10 (153). Recently, a B cell-specific ADAM10-deficient mouse was made (154). The importance of ADAM10 in Notch-Delta processing, proteins that are important in embryonic development, is underscored by the embryonic lethality of global ADAM10 deficiency, B cell-specific ADAM10 deficiency was associated with severe marginal zone B cell deficiency (154). Marginal zone B cells reside in the marginal zone of the spleen and respond to blood borne antigens (155) in a T-independent fashion, as opposed to follicular B cells, and reside in the follicles of lymphoid organs and respond to antigens in a T-dependent fashion (156). B cell-specific ADAM10 deficiency is also associated with severely reduced cleavage of CD23 (154), confirming the role of ADAM10 as the primary sheddase of CD23. Thus, ADAM10 has been shown to be the primary sheddase of CD23 and β_2AR-associated regulation of ADAM10 may contribute to the β_2AR-associated enhancement in sCD23 and IgE.

1.6.4 Regulation of ADAM10 activity by lipid raft localization

Most studies to date that focused on the regulation of ADAM10 activity towards substrates involve the analysis of the role of lipid rafts, cholesterol rich microdomains of

the cellular membrane that are especially important for organization of dynamic signaling assemblies, especially in BCR-mediated signaling (157). ADAM10-mediated cleavage of substrates other than CD23 from monocytes, neuroblastoma cell lines, and lymphoma cell lines is 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 (158-161). However, there are no studies that have investigated the role that lipid rafts play in the regulation of ADAM10-mediated cleavage of CD23. Thus, the importance of lipid raft localization in the β_2AR-associated enhancement in CD23 cleavage is at present, unexplored.

1.6.5 Expression of CD23 and ADAM10 on exosomes

Recently, the expression of CD23 and ADAM10 on B cell-derived exosomes has been reported (162,163). Exosomes are cell-derived, cholesterol-rich vesicles that are released by cells, including B cells primed with either lipopolysaccharide (LPS) or anti-CD40 in the presence of IL-4 (162-164). B cell-derived exosomes also express other proteins, such as MHCII and CD86 (162,163) and contain microRNAs (165). 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 (166-168). To date, most studies have focused on the regulation of CD23 cleavage on the B cell surface (146,149). However, recent studies demonstrated that both CD23 and ADAM10 form a unique interaction intracellularly that results in their packaging into exosomes that are

subsequently released from the cell (162), and that CD23 cleavage on exosomes is ADAM10-dependent (162). Because cholesterol-rich lipid raft microdomains are plentiful in exosomes (169,170), the ADAM10 expressed on exosomes is in an ideal membrane environment 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.

1.7 Allergic asthma

1.7.1 Allergic asthma, the β_2AR, and IgE

Bronchoconstriction and the respiratory distress that is associated with allergic asthma are treated clinically by the administration of a β_2AR agonist, a drug that binds to the β_2AR expressed on the surface of bronchiolar smooth muscle cells to reduce their contraction around the bronchioles (171). Of relevance to the present study is the clinical evidence that suggests a role for IgE in mediating an exacerbation of allergic asthma in humans and mice (172-175), as well as a role for the β_2AR expressed on B cells in the human lung (176,177) to increase the level of IgE produced during β_2AR agonist administration. An understanding of the various molecular links between β_2AR stimulation on a B cell and the subsequent increase in IgE, if unique, could potentially be blocked during β_2AR agonist inhalation therapy to specifically prevent the increase in IgE that might potentially compromise the beneficial effect of the agonist on bronchiolar smooth muscle relaxation.

1.8 Conclusions and hypothesis

Our lab has reported previously that β_2AR stimulation on a CD40L/IL-4-primed B cell increases the amount of IgE produced *in vivo* and *in vitro* on a per cell basis (34,132). The β_2AR-associated enhancement in IgE could only be blocked with β_2AR-specific antagonists (132). Increased IgE was associated with a pathway that utilized PKA and p38 MAPK and increased expression of CD23 at the mRNA level (132). The β_2AR-associated increase in IgE was also associated with sCD23, and CD23-deficient, as well as CD21- and CD19-deficient B cells were unable to increase IgE upon β_2AR stimulation (132), indicating that sCD23 mediates the β_2AR-associated increase in IgE. Finally, CD23 and the CD23 sheddase, ADAM10, have been shown to be expressed on exosomes (162,163) that contain cholesterol rich lipid raft domains (169,170). Thus, the β_2AR-associated increase in IgE is PKA-, p38 MAPK-, and sCD23-dependent. Notably, clinical findings have shown that small increases in IgE are associated with increased severity of IgE-mediated allergic asthma symptoms (172-175). Furthermore, the most effective therapy for severe allergic asthma is an antibody that targets IgE for removal from the bloodstream, omalizumab (178). An understanding of the mechanism that underlies the β_2AR-associated enhancement of IgE production by a B cell may help identify intermediates that can be targeted in the treatment of allergic asthma to allow for the continued use of β_2AR agonists while preventing the β_2AR-associated increase in IgE. Therefore, the present study was designed to identify the mediators of the β_2AR-mediated increase in IgE production by B cells. Previous studies from our laboratory used an *in vitro* model system to determine how β_2AR stimulation increased IgE production by a

CD40L/IL-4 primed B cell. The *in vitro* system consisted of culturing resting splenic murine primary B cells in the presence of CD40L expressed on the surface of a Sf9 cell to engage CD40 and recombinant IL-4 to engage the IL-4R to induce CSR to IgE in the absence or presence of terbutaline, a β_2AR agonist, for various times prior to the collection of samples for the functional assays. The current study used this system to examine the mechanism that underlies the β_2AR-associated increase in IgE, as depicted in **Figure 11**, using B cells from either wild type (WT) or β_2AR-deficient mice.

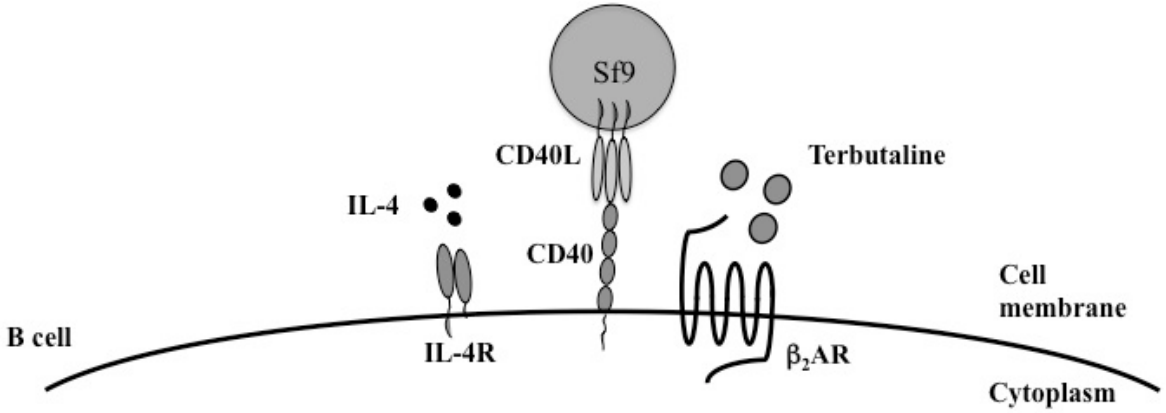

Figure 11. In vitro model system.

Resting naïve splenic B cells (CD43-negative) were activated in the presence of CD40L-expressing Sf9 cells (1 CD40L/Sf9 cell:10 resting B cells) and IL-4 (1 ng/ml). Terbutaline (10^{-6}M), a β_2AR agonist, was added at the time of activation to stimulate the β_2AR.

Although we had elucidated the proximal signaling pathway triggered by β_2AR stimulation and increased IgE production, what remained unknown was the link between sCD23 and the β_2AR-induced enhancement in IgE production. We **hypothesized** that β_2AR stimulation of a CD40L/IL-4-primed B cell enhances IgE production by increasing CD23 and ADAM10 expression on B cell-derived exosomes.

This study is the first to demonstrate that β_2AR stimulation on a CD40L/IL-4-primed B cell enhances both CD23 and ADAM10 expression at the protein level and ADAM10 expression at the mRNA level, and that this enhancement is β_2AR-, PKA-, and p38 MAPK-dependent. The β_2AR-associated enhancement of CD23 and ADAM10 expression does not occur on the surface of the B cell, rather, it occurs on B cell-derived exosomes. We also show for the first time that B cell-derived exosomes can transfer the β_2AR-stimulated enhancing effect on IgE production to B cells that have not been exposed to a β_2AR agonist, suggesting the role of exosomes as a mediator of the β_2AR-associated enhancement of IgE production. Finally, we demonstrate that the transfer of exosomes from B cells exposed to a β_2AR agonist to B cells unexposed to a β_2AR agonist enhances IgE production by increasing the level of IgE produced on a per cell basis rather than increasing the number of B cells that switch to IgE. The **significance** of this dissertation is that it is the first study to identify a novel mechanism that mediates the β_2AR-associated enhancement of IgE production involving B cell-derived exosomes. This unique pathway may provide new targets to allow for the continued use of β_2AR agonists in the treatment of allergic asthma-associated bronchoconstriction but prevent the β_2AR-associated upregulation in IgE production.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Mice were housed under pathogen-free conditions and used between 6-12 weeks of age. Female BALB/c mice were purchased from the pathogen-free facility at Taconic Farms (Hudson, NY) at six weeks of age and then housed at The Ohio State University in microisolator cages with autoclaved food and water ad libitum. Congenic BALB/c β_2AR-deficient mice were derived from FVB β2AR-deficient mice [kindly provided by Brian Kobilka (Stanford University, CA)], after backcrossing for ten generations (126). 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.

2.2 Reagents

CD40L-Sf9 cells were prepared as described previously (77). 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 (St. Louis, MO). INCB3619, which is a selective ADAM10/17 inhibitor (179), was kindly provided by Incyte Corporation (Wilmington, DE). All reagents used for B cell isolation, activation, and pharmacologic treatment tested negative for the presence of endotoxin using E-TOXATE (Sigma).

2.3 Antibodies

Western blot antibodies were rabbit polyclonal anti-mouse CD23 (kindly provided by Dr. D. Conrad, Virginia Commonwealth University), 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 (R5G2, MBL International, Woburn, MA) or rabbit monoclonal anti-actin (13E5, Cell Signaling Technology, Beverly, MA). Secondary polyclonal antibodies were HRP-labeled goat anti-rabbit IgG and horseradish peroxidase (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 B subunit (Sigma) and goat polyclonal affinity purified HRP-labeled anti-biotin (Cell Signaling Technology). ELISA antibodies included rat monoclonal anti-mouse CD23 (coating, clone 2G8, Southern Biotech, Birmingham, AL), rabbit polyclonal anti-mouse CD23 (secondary, kindly provided by D. Conrad, Virginia Commonwealth University), and goat polyclonal alkaline phosphatase-labeled anti-rabbit IgG (detecting, H + L; Southern Biotech). Plates were developed with para-NitroPhenylPhosphate *(PNPP)* substrate (Sigma) in DEM. Antibodies used for

ELISPOT included monoclonal anti-mouse IgE (clone R35-72; BD, Franklin Lakes, NJ) and secondary monoclonal alkaline phosphatase (AP)-labeled rat anti-mouse IgE (clone 23G3; Southern Biotech). Flow cytometry rat monoclonal anti-mouse antibodies and reagents included ADAM10-fluorescein isothiocyanate (FITC) (Clone 139712), ADAM10-phycoerythrin (PE) (Clone 139712), and isotype control rat IgG2a kappa-PE from R&D, CD23-FITC (B3B4), B220-allophycocyanin (APC) (RA3-6B2), 7-Amino-Actinomycin D (7-AAD), isotype rat IgG2a kappa-FITC, and isotype rat IgG2a kappa-APC from eBioscience.

2.4 Resting B cell isolation and cell priming

Resting B cell isolation and priming were performed as described previously (132) using negative selection for CD43-negative B cells using an AutoMACS machine (Miltenyi, Bergisch Gladbach, Germany). B cell purity was greater than 98% as determined by FACS. Purified B cells were primed at a density of .5 x 10^6 B cells/ml with CD40L-Sf9 (1:10) and IL-4 (1 ng/ml) in the absence or presence of terbutaline (10^{-6} M) and cultured in complete RPMI [RPMI 1640 medium (Cellgro, Manassas, VA), 10% FBS (Atlas Biologicals, Fort Collins, CO), 20 mM HEPES (Gibco, Carlsbad, CA), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 100 ug/ml streptomycin (Invitrogen), 2 mM glutamine (Invitrogen), 50 uM 2-mercaptoethanol (Sigma)] at 37°C, 5% CO_2. In cell concentration experiments, CD40L-Sf9 (1:10) and B cells were serially diluted from 400,000 B cells/well to 12,500 B cells/well in 200 ul volume, followed by the addition of equal amounts of IL-4 (1 ng/ml) in the absence or presence of terbutaline (10^{-6} M).

2.5 Western blot

Western blot and analysis was performed on equal amounts of protein as described previously (36,132) to detect ADAM10, CD23, CD63, Actin, and GM1. Blots were scanned and inverted to perform densitometry using NIH ImageJ64 1.47m.

2.6 Quantitative RT-PCR

Quantitative real-time PCR was performed as described previously (132). The following primers were used: beta-actin, 5'-TACAGCT TCACCACCACAGC-3' (forward) and 5'-AAGGAAGGCTGGAAAAGAGC-3'(reverse), annealing temperature, 58°C; ADAM10, 5'-CACCAATATTTGGGAAACGG-3' (forward) and 5'TCCTGAGCTCCTGAGGAAAA-3' (reverse), annealing temperature, 60°C. All primers were synthesized and purchased from Integrated DNA Technologies (Coralville, IA). Polymerase chain reaction (PCR) was performed using the Rotor Gene Q (Qiagen, Germantown, MD) and software used was Rotor Gene Q Series Software (version 2.0.3).

2.7 ELISA

ELISA was performed as described previously for sCD23 detection (132). IgE protein in B cell culture supernatants was measured using BD OptEIA mouse IgE kit (BD) and developed with tetramethylbenzidine (TMB) substrate (BD) and acid stop (2N H_2SO_4, Sigma). 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 to a standard curve where the level of detection for each protein is 1-500 ng for sCD23 and 1-100 ng for IgE.

2.8 Exosome isolation and gradient centrifugation

cRPMI was depleted of exosomes by ultracentrifugation at 150,000 x *g* for fifteen hours at 4°C to pellet exosomes naturally present in FBS in a SW-41 swinging bucket rotor (Beckman Coulter, Brea, CA) in a L-70 ultracentrifuge (Beckman). Ultracentrifuge tubes were sterilized prior to exosome depletion of cRPMI by washing with 10% bleach, 70% ethanol, and a final wash with sterile phosphate buffered saline (PBS) (Gibco). Ultracentrifuged cRPMI was filtered using a 0.2 uM 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 (162) in a SW-41 swinging bucket rotor in a L-70 ultracentrifuge (Beckman). Prior to any experiments, a characterization of the ultracentrifuged culture supernatant was done to confirm the presence of exosomes by layering of isolated exosomes on an iodixanol (Axis-Shield, Dundee, Scotland) 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 one hour 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 (162) and was resuspended in PBS and either frozen at -80°C until analysis by Western blot or stained for flow

cytometry as described below.

2.9 Flow cytometry

B cells were prepared for fluorescence-activated cell sorting (FACS) as described previously (34) for cell surface detection of CD23, ADAM10, B220 and 7-AAD. Appropriate isotype- and species-matched antibodies were used as controls for gating. Stained cells were detected on a FACSCanto II flow cytometer (BD) and results were analyzed using FlowJo (TreeStar, Ashland, OR). 100,000 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.

2.10 Flow cytometry of exosomes

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

2.11 Electron Microscopy of exosomes

Exosomes were prepared as described above, fixed, and then analyzed by Transmission Electron Microscopy (TEM) as described previously (164). Formvar-carbon coated 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).

2.12 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. *Exosome-intact*: Exosomes were isolated under sterile conditions as described above and resuspended in 4 ml of exosome-free media (1X concentration) and added to recipient cultures. *Exosome-depleted*: 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^{-6} M at the time of transfer. *Recipient cultures*: Two days 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% CO_2, for 4 days and then supernatants were removed for analysis of IgE production by ELISA or cells were analyzed by ELISPOT.

2.13 ELISPOT

ELISPOT assay was performed as described previously (132). IgE^+ spots were quantified on an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) using KS ELISPOT software (Zeiss, version 4.2.0).

2.14 Sucrose Density Gradient Isolation of Lipid Rafts

100 x 10^6 B cells were activated as described above, lysed, and then fractionated by sucrose density gradient separation as described previously (180). 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.

2.15 Statistics

Data were analyzed by ANOVA, followed by Bonferroni post test when data were normally distributed, to determine whether an overall statistically significant change existed. A Student's *t* test was used for comparison between two treatment groups. A *p* value of ≤ 0.05 is designated by *, $p \leq 0.01$ by **, and $p \leq 0.001$ by ***.

CHAPTER 3

RESULTS

3.1 ADAM10 mediates the β_2AR-specific enhancing effect on sCD23 and IgE

Previous findings showed that IgE, as well as CD23 and sCD23, were increased after the β_2AR was engaged on a CD40L/IL-4-primed murine B cell, and that these effects failed to occur in the presence of a β_2AR antagonist (132). In the present study, we confirmed that the β_2AR was responsible for mediating the IgE-enhancing effect. B cells from WT or β_2AR-deficient mice were primed with CD40L/IL-4 in the absence or presence of the selective β_2AR agonist, terbutaline, and supernatants were collected on days 2 through 7 for analysis of IgE production by ELISA. The IgE-enhancing effect of β_2AR stimulation on primed B cells, in comparison to primed B cells alone, was evident on day 4 and reached a maximal 2-fold increase on day 6 (**Figure 12A**, **left panel**); whereas, B cells from β_2AR-deficient mice were unable to do so (**Figure 12A**, **middle panel**), indicating that the β_2AR agonist-induced upregulation in the level of IgE production by primed B cells was specifically a result of β_2AR stimulation. It should be noted that we consistently found that β_2AR-deficient B cells function similarly to WT B cells for all immune functions, but appear to do so at a lower

level of response (132,181). To determine if the β_2AR agonist-induced upregulation in the level of IgE production was dependent on cell concentration, as reported previously (182,183), B cells were serially diluted and supernatants were collected on day 6 for analysis of IgE production by ELISA. The IgE-enhancing effect of β_2AR stimulation was evident with an input of between 12,500 and 200,000 B cells per well in 200 ul volume, with the maximum β_2AR-enhancing effect on IgE exhibited between 50,000 and 100,000 B cells per well (**Figure 12A, 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 β_2AR-enhancing effect on IgE production, and was significantly reduced in comparison to wells with fewer input B cells, indicating that the number of B cells required to observe the β_2AR-enhancing effect was limited by the concentration of B cells per well. β_2AR stimulation enhanced CD23 total protein production within 24 hours and continued to increase through day 5 (**Figure 12B**). Likewise, β_2AR stimulation on primed B cells enhanced the level of sCD23 produced, in comparison to 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 (**Figure 12C**); whereas, B cells from β_2AR-deficient mice were unable to increase CD23 or sCD23 in response to the β_2AR agonist at any timepoint (**Figure 12B-C**), also confirming that the agonist-induced increase in CD23 and sCD23 was specifically due to β_2AR stimulation on a B cell. Taken together, these findings confirm a role for the β_2AR on a B cell in mediating an increase in total CD23 expression, as well as the level of sCD23 and IgE produced.

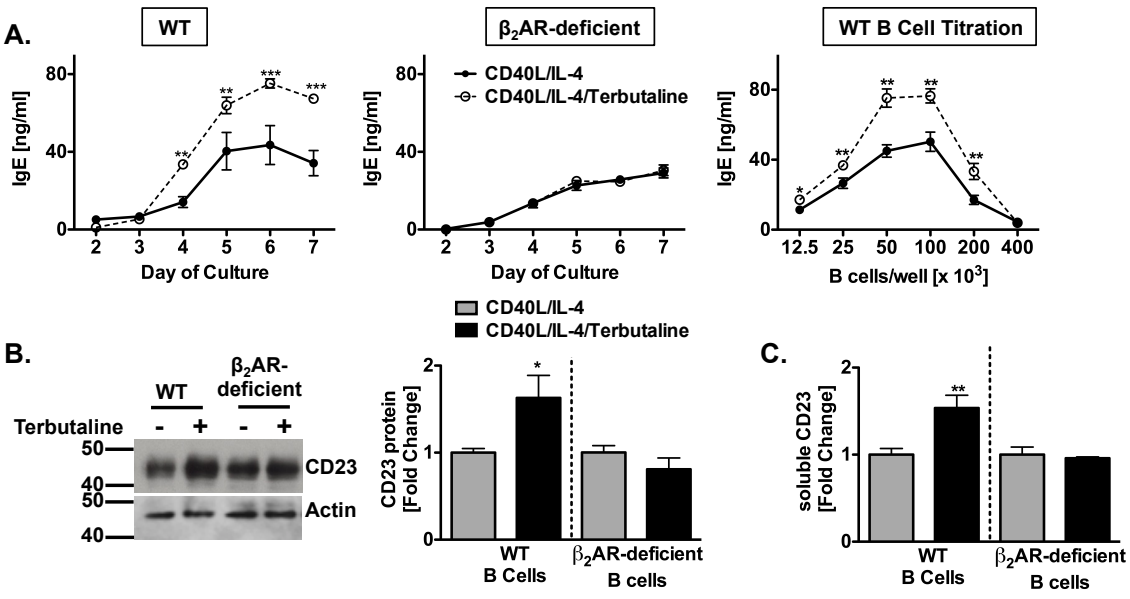

Figure 12. β_2AR stimulation enhances IgE, CD23, and sCD23.

Resting B cells were isolated from Balb/c (WT) and β_2AR-deficient mouse spleens and were primed with CD40L/Sf9 cells (1:10) and IL-4 (1 ng/ml) in the presence (open circles or black bar) or absence (solid circles or grey bar) of the β_2AR agonist, terbutaline (Terb, 10^{-6} 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 β_2AR-deficient (middle) B cells using ELISA. *Right*: Serial dilutions of B cells and CD40L were cultured as above and analyzed for IgE at 6 days using ELISA. Data are presented as mean IgE value (ng/ml) +/- standard error of the mean (SEM) from one representative of two to three independent experiments. (**B**) CD23 total protein expression at 24 hours using Western blot analysis. One representative blot is shown, with Molecular Weight in kDa, and data are presented as the mean fold difference of total CD23 protein expression in total cellular lysates from WT and β_2AR-deficient B cells as standardized to actin +/- SEM from three independent experiments. (**C**) sCD23 expression at 5 days 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 (β_2AR-deficient). Significant differences from priming alone were determined by ANOVA followed by Bonferroni correction or unpaired t-test. *, $p < .05$, **, $p < .01$, ***, $p < .005$.

Based on recent reports that ADAM10 is the primary sheddase of CD23 (146,149,154), it was imperative to determine if the β_2AR-induced increase in sCD23 and IgE production was due to a β_2AR-induced increase in ADAM10-mediated cleavage of CD23. INCB3619 is a dual ADAM10/ADAM17 inhibitor with an IC_{50} for various ADAM10 ligands in the range of 0.2-0.7 uM (179). Because ADAM17 is unable to cleave CD23 (146,148), INCB3619 is an excellent compound for the study of ADAM10-specific cleavage of CD23 (146,148). Primed B cells were cultured in the absence or presence of a β_2AR agonist with increasing concentrations of INCB3619. The concentration of INCB3619 required to reduce both the control level by half, as well as the β_2AR-induced increase of both sCD23 (**Figure 13**, **left panel**) and IgE (**Figure 13, right panel**) was between 0.1 and 0.3 uM and occurred independently of changes in cell morphology or cell viability. Concentrations above 2 uM caused a slight reduction in viability (data not shown). This result suggests, for the first time, that ADAM10 plays a role in the β_2AR-enhancing effect on sCD23, and further supports that a link exists between the β_2AR-mediated increase in sCD23 cleavage and the increase in IgE.

To determine the extent to which β_2AR stimulation affected the level of ADAM10 expression, primed B cells from WT or β_2AR-deficient B cells were cultured as described above and cell lysates were collected at 1 and 24 hours to measure ADAM10 mRNA and protein expression, respectively, using quantitative RT-PCR and Western blot. The level of ADAM10 mRNA (**Figure 14A**) and total protein (**Figure 14B**) were increased 2 and 1.5-fold, respectively, after β_2AR stimulation on primed B

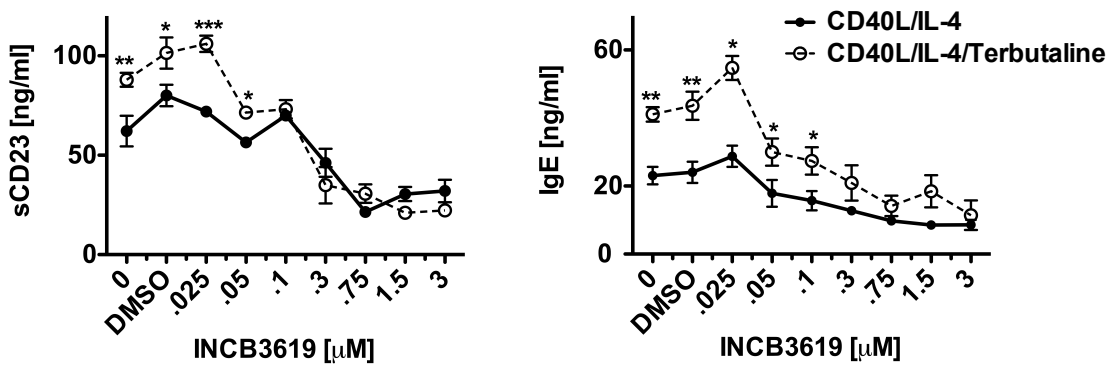

Figure 13. ADAM10 modulates the β_2AR-mediated enhancing effect on sCD23 and IgE.

Resting B cells were isolated from Balb/c (WT) mouse spleens and were primed with CD40L/Sf9 cells (1:10) and IL-4 (1 ng/ml) in the presence (open circles or black bar) or absence (solid circles or grey bar) of the β_2AR agonist, terbutaline (10^{-6} M). Total cellular protein and/or culture supernatants were collected for analysis. sCD23 (**left**) and IgE (**right**) production after five days in the presence of increasing concentrations of the selective ADAM10 inhibitor INCB3619 using ELISA. 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. *, $p < .05$, **, $p < .01$, ***, $p < .005$.

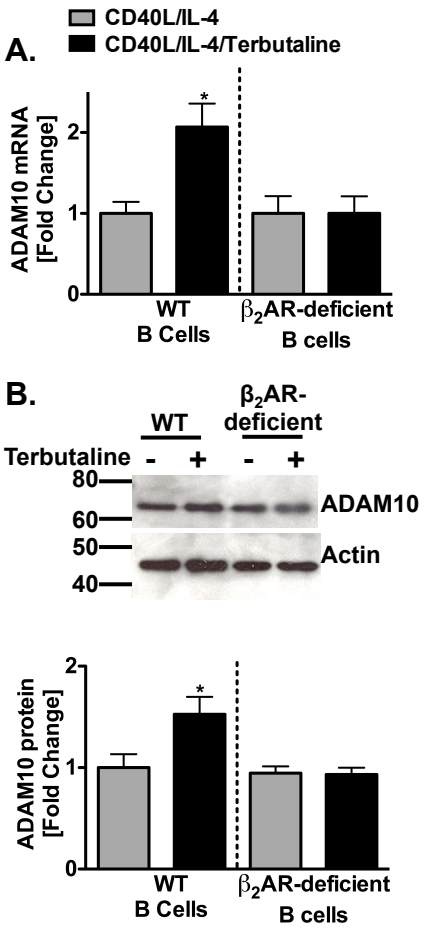

Figure 14. ADAM10 gene and protein expression are increased by β_2AR stimulation.

B cells from WT and β_2AR-deficient mice were cultured as described in Figure 12 in the presence (black bars) or absence (grey bars) of terbutaline (Terb). mRNA and total cellular protein, respectively, were collected at one hour and twenty-four hours. (**A)** Data are presented from three independent experiments as the mean ADAM10 mRNA fold difference as standardized to beta-actin +/- SEM. (**B**) One representative blot is shown, with Molecular Weight in kDa, and data are presented from three independent experiments as the total ADAM10 protein fold difference as standardized to actin +/- SEM. Significant differences from priming alone were determined by unpaired t-test. *, $p < .05$.

cells, as compared to primed B cells alone. Conversely, the β_2AR enhancement did not occur when β_2AR-deficient B cells were used. Taken together with the results from Figure 12-13, β_2AR 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 β_2AR-stimulated and PKA-mediated enhancement of the level of p38 MAPK phosphorylation and IgE (132). To determine if the β_2AR-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 hours, cell lysates were prepared for Western blot analysis. Whereas β_2AR stimulation of primed B cells enhanced the level of CD23 and ADAM10, in comparison to 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 β_2AR agonist in the presence of a PKA or p38 MAPK inhibitor, respectively, were unable to enhance either CD23 (**Figure 15A, 15C**) or ADAM10 (**Figure 15B, 15D**) expression. These results indicate that activation of the β_2AR/PKA/p38 MAPK pathway is essential for the β_2AR-induced enhancement in the level of CD23, ADAM10, and IgE.

3.2 β_2AR 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 (FasL), and Amyloid Precursor Protein, has traditionally been thought to occur on the cell surface

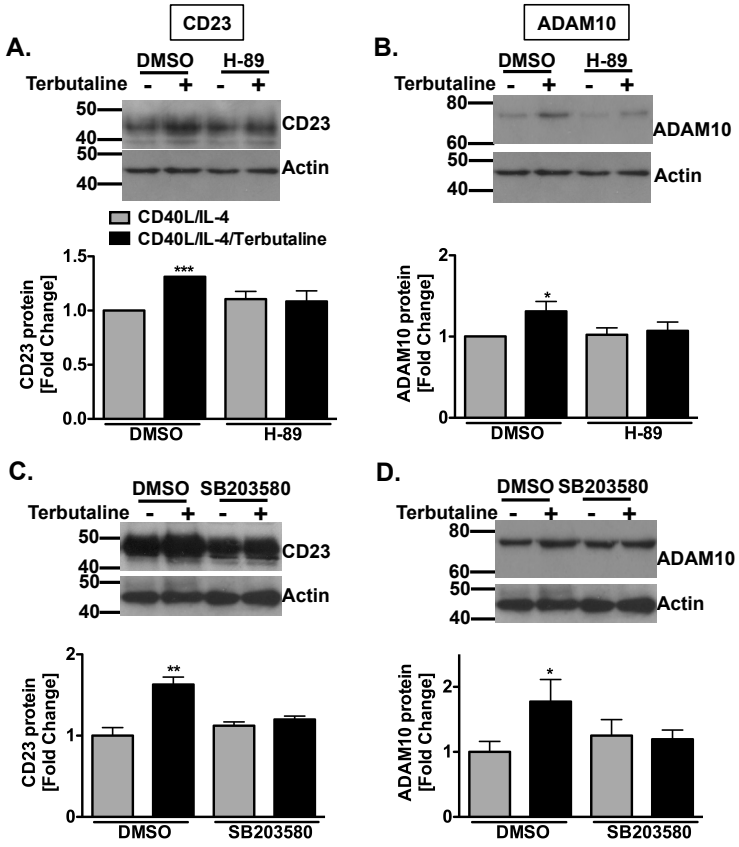

Figure 15. PKA and p38 MAPK inhibition prevents the β_2AR-induced enhancement in CD23 and ADAM10 protein.

B cells were cultured as described in Figure 12 in the presence (black bar) or absence (grey bar) of terbutaline (Terb). Cell lysates were collected after 24 hours and analyzed by Western blot. *A-B*. CD23 (**A**) and ADAM10 (**B**) expression in the presence of H-89 (.5 uM). *C-D*. CD23 (**C**) and ADAM10 (**D**) expression in the presence of SB203580 (1 uM). One representative blot is shown, with Molecular Weight in kDa, and data are presented from three to four independent experiments as the total protein fold difference compared to priming alone as standardized to actin +/- SEM. Significance based on an unpaired t-test. *, $p < .05$, **, $p < .01$, ***, $p < .005$, as compared to priming alone.

plasma membrane (146,148,184). Because we showed above that expression of CD23 and ADAM10 were both increased in response to β_2AR 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 through 5 after priming. B cells were gated with appropriate antibody isotype controls to include only cells that were both $B220^+$ and viable as determined by 7-AAD exclusion. As shown in **Figure 16**, the percent of $ADAM10^+CD23^+$ cells from both primed and β_2AR agonist-exposed conditions increased three-fold on day 2, but went below baseline on day 3 (**Figure 16A**). Representative histograms of all data are shown in **Figure 16**. We also found that within one day of priming, as determined by mean fluorescence intensity (MFI), cell surface CD23 increased four-fold in both primed-only B cells and β_2AR agonist-exposed primed B cells, and remained elevated until day 3 when levels for both groups began to decrease to baseline levels (**Figure 16B**). In contrast, the level of ADAM10 on the cell surface slightly increased for both exposure conditions on day 1 then declined to below baseline from days 2 through 5 after priming (**Figure 16C**). Additionally, almost all B cells were CD23-single positive after CD40L/IL-4 priming, while the same number of B cells that were ADAM10-single positive were also CD23/ADAM10-double positive (**Figure 17**), suggesting that cell surface expression of ADAM10 is linked to CD23 and not vice versa. Also, when using B cells from β_2AR-deficient mice, the MFI of cell surface CD23 and ADAM10 after B cell priming in the presence of β_2AR stimulation was equivalent, as shown in **Figure 18-**

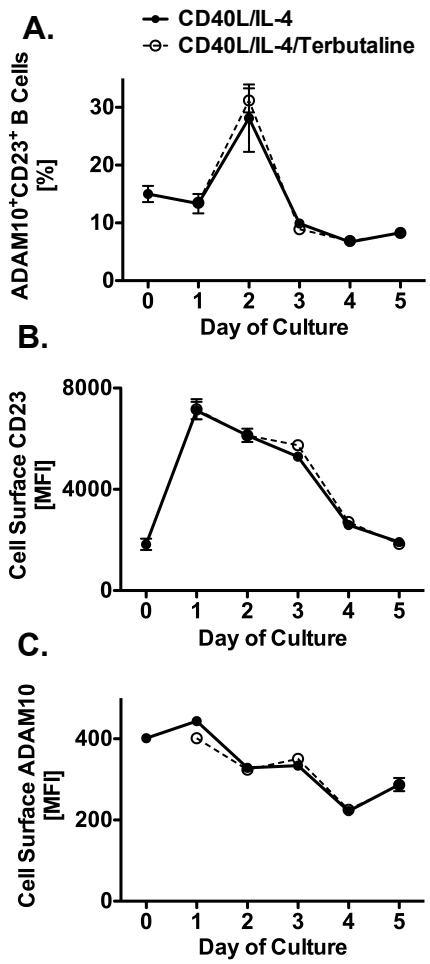

Figure 16. β_2AR stimulation does not enhance expression of CD23 and ADAM10 on the surface of WT B cells.

B cells from WT mice were cultured as described in Figure 12 in the presence (open circle) or absence (solid circle) of terbutaline (Terb). Cells were collected for FACS analysis from days 1 through 5 after priming. 7-AAD$^-$/B220$^+$/ADAM10$^+$CD23$^+$ Cells Percent Positive (**A**), MFI of cell surface CD23 (**B**) and ADAM10 (**C**). Data are presented as mean percent positive cells (%) or MFI +/- SEM from three independent experiments.

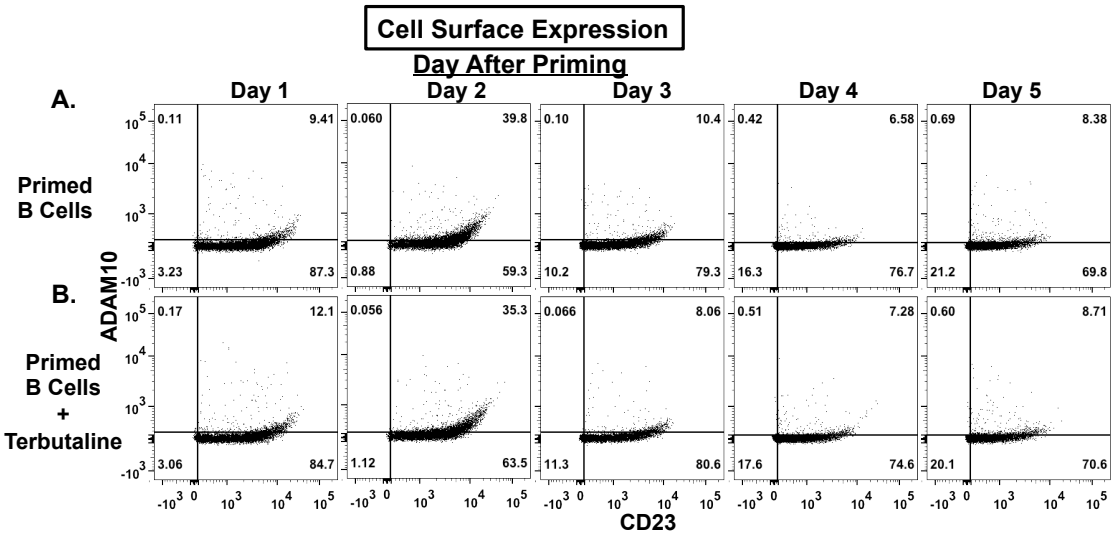

Figure 17. β_2AR stimulation does not enhance expression of CD23 and ADAM10 on the surface of WT B cells.

B cells from WT mice were cultured as described in Figure 12 in the absence (**A**) or presence (**B**) of terbutaline. Cells were collected for FACS analysis from days 1 through 5 after priming. (**A-B**) Cell surface CD23 (**x axis)** and ADAM10 (**y axis**) expression on 7-AAD$^-$/B220$^+$ cells. Data are presented as one representative histogram from three independent experiments.

19. Taken together, β_2AR 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 β_2AR-enhancing effect on IgE production.

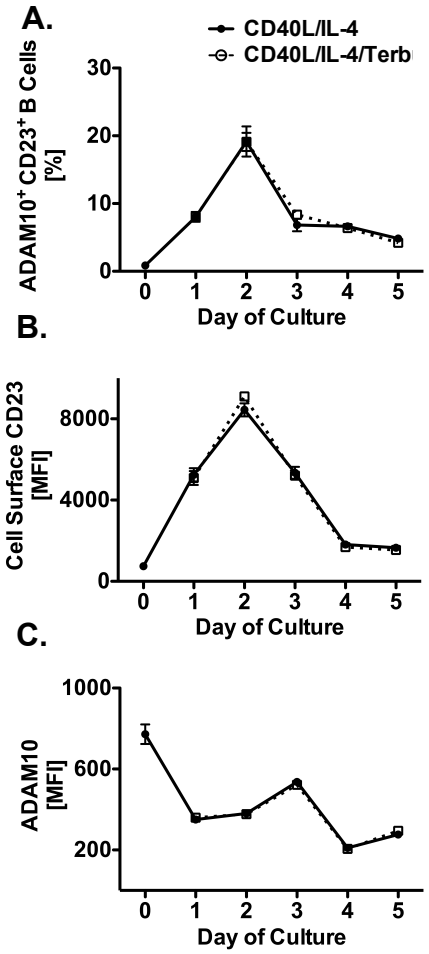

Figure 18. β_2AR stimulation does not enhance expression of CD23 and ADAM10 on the surface of β_2AR-deficient B cells.

B cells from β_2AR-deficient mice were cultured as described in Figure 12 in the presence (open circle) or absence (solid circle) of terbutaline (Terb). Cells were collected for FACS analysis from days 1 through 5 after priming. 7-AAD$^-$/B220$^+$/ADAM10$^+$CD23$^+$ Cells Percent Positive (**A**), MFI of cell surface CD23 (**B**) and ADAM10 (**C**). Data are presented as mean percent positive cells (%) or MFI +/- SEM from three independent experiments.

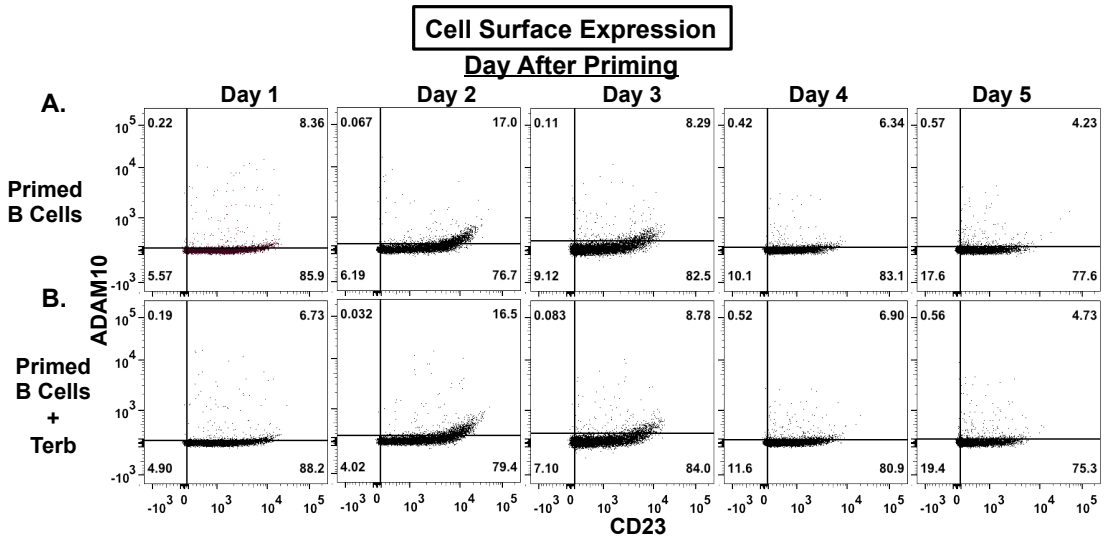

Figure 19. β_2AR stimulation does not enhance expression of CD23 and ADAM10 on the surface of β_2AR-deficient B cells.

B cells from β_2AR-deficient mice were cultured as described in Figure 12 in the absence (**A**) or presence (**B**) of terbutaline. Cells were collected for FACS analysis from days 1 through 5 after priming. (**A-B**) Cell surface CD23 (**x axis)** and ADAM10 (**y axis**) expression on 7-AAD$^-$/B220$^+$ cells. Data are presented as one representative histogram from three independent experiments.

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

Because the β_2AR-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 (162). To determine the extent to which β_2AR 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 (NH_4Cl) to inhibit exosome release, as has been previously described (162,185). In the absence of NH_4Cl, the level of both CD23 (**Figure 20A**) and ADAM10 (**Figure 20B**) protein expression, as determined by Western blot analysis of cell lysates, was augmented by β_2AR stimulation, in comparison to the level expressed by primed B cells alone. In the presence of NH_4Cl, β_2AR stimulation on primed B cells increased both CD23 and ADAM10 protein expression 2.2- and 3-fold, respectively, in comparison to primed B cells (**Figure 20A-B).** These findings suggested that the level of expression of both CD23 and ADAM10 proteins is increased in B cells after β_2AR stimulation and that the increase is localized to primarily exosomes.

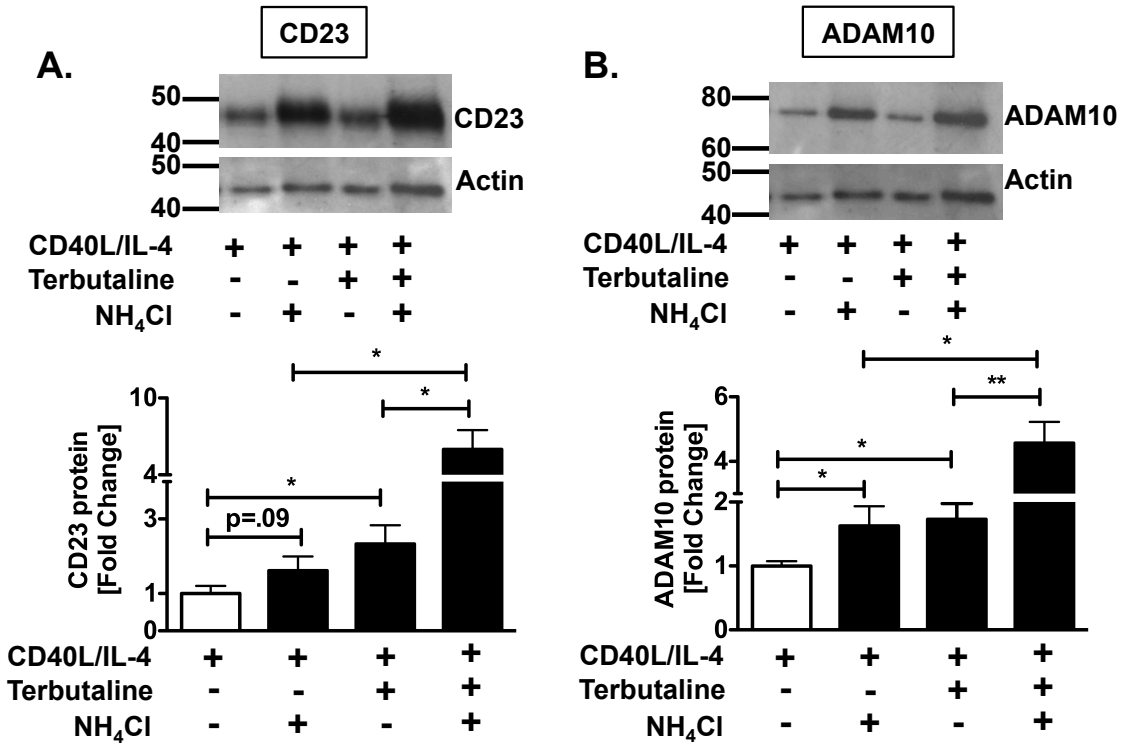

Figure 20. Inhibition of exosome release increases CD23 and ADAM10 protein retention within a primed B cell.

Resting B cells were cultured as described in Figure 12 in the absence or presence of ammonium chloride (NH_4Cl, 10 mM) to inhibit the release of exosomes. Cell lysates were collected after 24 hours and analyzed for CD23 (**A**) and ADAM10 (**B**) protein expression by Western blot analysis. Representative blots are shown, with Molecular Weight in kDa, and data were normalized to actin and are presented as the mean fold difference of CD23 and ADAM10 expression +/- SEM from 3-4 independent experiments. Significance based on an unpaired t-test. *, $p < .05$, **, $p < .01$, compared to priming in the absence of ammonium chloride alone.

Because several types of vesicles are released from cells (186), 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 (163), namely, "cup-like" morphology, size between 40-100 nm, and density between 1.08-1.15 g/ml. To evaluate morphology and size, exosomes were isolated from the supernatant of primed B cells cultured for 3 days and fixed and analyzed via TEM. Vesicles isolated on day 3 of priming showed a characteristic cup-like morphology that was less than 100 nm in size (**Figure 21A**). To confirm size, isolated exosomes were analyzed by flow cytometry. Exosomes measured between 40-100 nm in size (**Figure 21B**) as compared to ultra-small polystyrene beads to control for accurate size gating (**Figure 21B, 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 (**Figure 21C**), which is in agreement with previous reports (163). Additionally, the fractions between 1.05 and 1.15 g/ml were the only fractions that contained exosomes as detected by TEM (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.

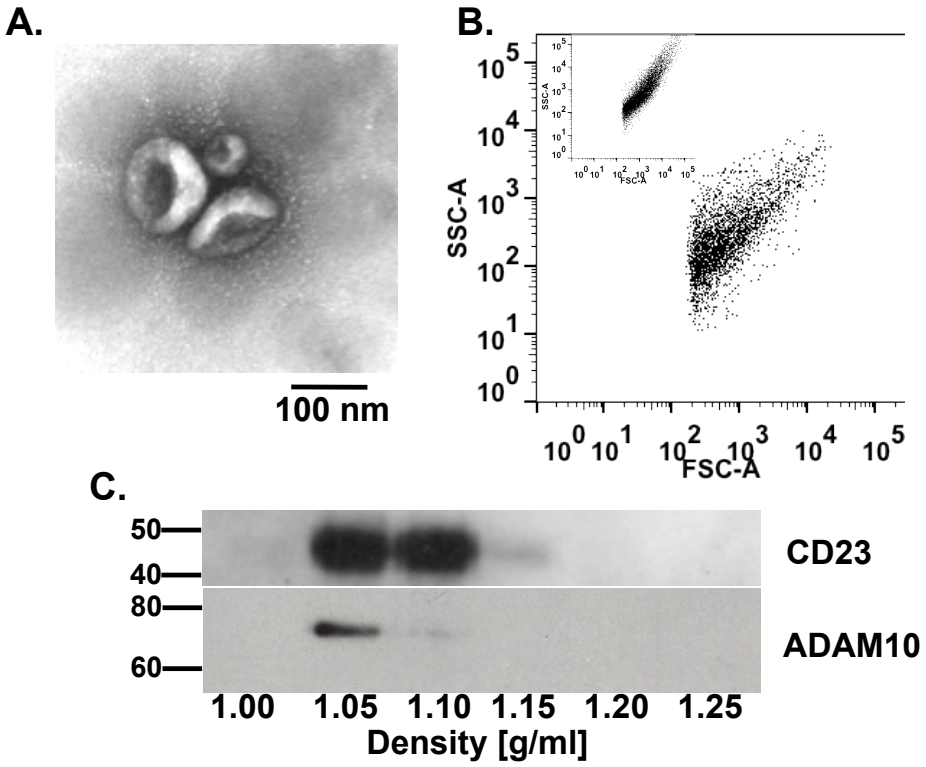

Figure 21. Exosomes are released by primed B cells that express both CD23 and ADAM10.

B cells were cultured as described in Figure 12 and B cell-derived exosomes were isolated after 72 hours. (**A**) Exosomes were fixed and prepared for TEM. Micrograph is representative of three independent experiments. Scale bar represents 100 nm. (**B**) Exosomes were analyzed by FACS and compared to 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 Weight in kDa.

3.4 β_2AR stimulation enhances CD23 and ADAM10 expression on exosomes early after priming

Because inhibition of exosome release with ammonium chloride suggested that the β_2AR-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 if CD23 and ADAM10 were expressed on exosomes released from resting B cells, or if 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 through 2 days. Initial analysis of exosome-associated ADAM10 by Western blot with a polyclonal antibody directed at the C-terminal domain of ADAM10 revealed two bands around 65 kDa that corresponded to mature ADAM10 (**Figure 22, upper panel**), which was in contrast to a single band detected by the same antibody around the same molecular weight in cellular lysates (**Figure 22, lower panel**). To determine if this band was non-specific, both cellular lysates and exosomes were examined using monoclonal antibodies directed at either the C-terminal domain or the ectodomain of ADAM10. In exosomes, we found that both monoclonal antibodies detected a single band of ADAM10 (**Figure 22, upper panel**), which corresponded to the lower band detected by the polyclonal ADAM10 antibody, indicating that the upper band detected by the polyclonal antibody was non-specific. A very faint upper band was detected by the ectodomain-specific ADAM10 monoclonal antibody. Thus, all further analyses of ADAM10 expression of exosomes by Western blot were performed with the monoclonal antibody directed at the C terminal domain of ADAM10. Furthermore, in

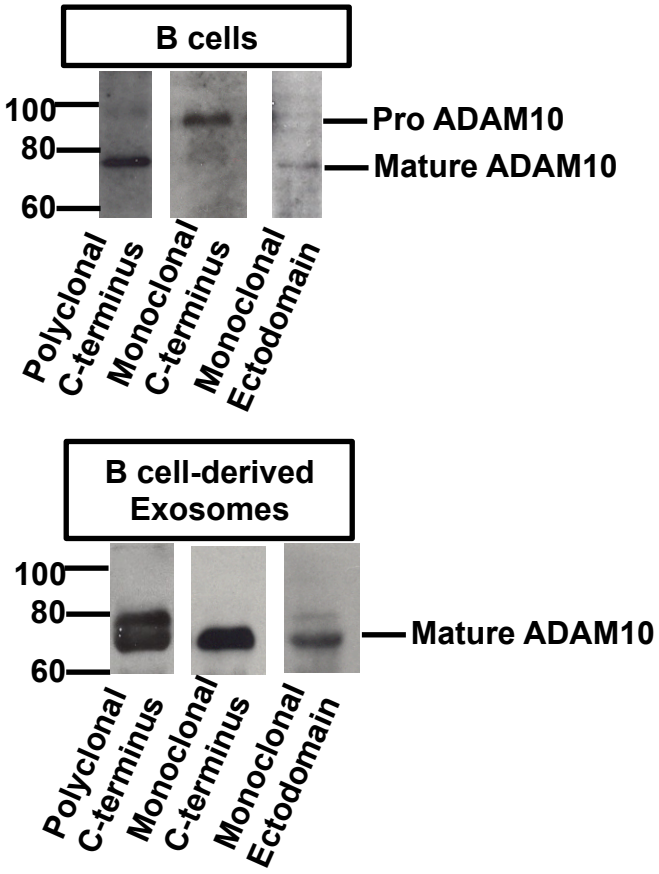

Figure 22. Comparison of antibodies for the detection of ADAM10 in B cell-derived exosomes and B cell lysates.

Exosomes (**top**) and cell lysates (**bottom**) from B cells cultured as described in Figure 12 were isolated and analyzed for ADAM10 expression using a polyclonal ADAM10 antibody directed at the C-terminus, a monoclonal ADAM10 antibody directed at the C-terminus, and a monoclonal ADAM10 antibody directed at the ectodomain. Data are presented as one representative blot, with Molecular Weight in kDa, from two to three independent experiments.

cellular lysates, the C-terminal domain-specific monoclonal ADAM10 antibody detected a higher molecular weight form, around 95 kDa, of ADAM10 (**Figure 22, lower panel**), likely the pro form of ADAM10, a form that was not found in exosomes (**Figure 22, 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, while exosomes derived 2 days after priming alone expressed CD23 and ADAM10, which was enhanced by β_2AR stimulation (**Figure 23**). As a positive control, a marker for exosomes, CD63, was measured (165), 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 β_2AR agonist at 1 day after priming (**Figure 23**), but since 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 days after priming indicates that there is a delay between the synthesis of CD23, which appears in cell lysates and on the cell surface 1 day after priming, and the shuttling of CD23 to exosomes. Furthermore, since ADAM10 is a constitutively expressed protein, the delay in the appearance of ADAM10 in exosomes until 2 days after priming indicates the possibility that CD23 and ADAM10 form an association which allows for shuttling into exosomes, as has been shown previously (162).

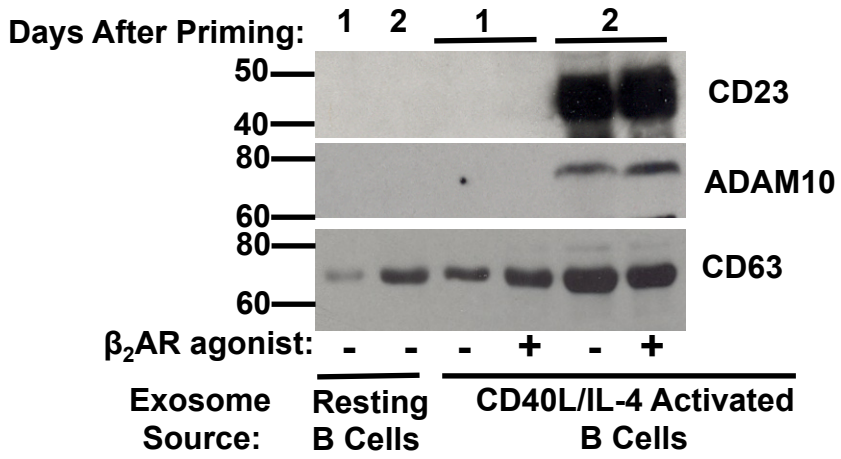

Figure 23. β_2AR stimulation enhances CD23 and ADAM10 expression on exosomes at early timepoints after priming.

Resting B cells and CD40L/IL-4 primed B cells were cultured as described in Figure 12 and B cell-derived exosomes were isolated from supernatants after 1 through 2 days. Equal amounts of protein were analyzed by Western blot for CD23, ADAM10 and CD63 for each timepoint. One representative blot from three independent experiments is shown, with Molecular Weight in kDa.

Because the β_2AR-enhancement for exosome-associated CD23 and ADAM10 occurred within 2 days of priming, it was important to determine if this enhancement occurred at any later timepoints. Therefore, primed B cells were cultured as described above and exosomes were isolated after 2 through 5 days and analyzed by Western blot. The overall protein level of isolated exosomes was enhanced by β_2AR stimulation of primed B cells in comparison to primed alone B cells on day 4, but not on day 2,3, or 5. However, there was an increase over time in the level of protein from isolated exosomes (**Figure 24**).

The positive control for exosomes, CD63, was increased by β_2AR stimulation 3 through 4 days after priming in comparison to exosomes isolated from primed B cells alone. Yet, 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 β_2AR stimulation 2 days after priming, but remained equal 3 through 5 days after priming (**Figure 25A**). The positive control for exosomes, CD63, was increased by β_2AR stimulation 3 through 4 days after priming. Further analysis by densitometry confirmed that CD23 (**Figure 25B**) and ADAM10 (**Figure 25C**) on exosomes released from primed B cells were enhanced by β_2AR stimulation 2 days after priming, but not 3 through 5 days after priming, indicating that there is a tight regulation of CD23 and ADAM10 expression on exosomes. Exosomes derived from primed β_2AR-deficient B cells were

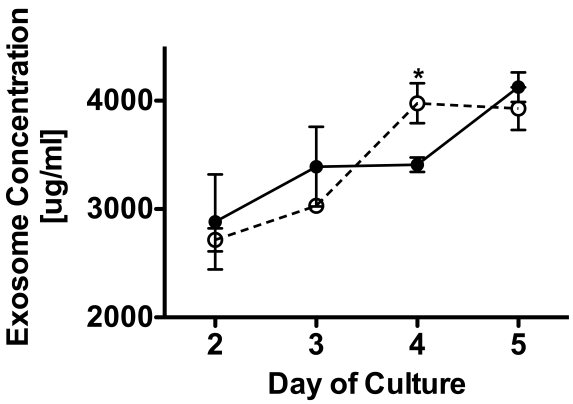

Figure 24. β_2AR stimulation does not change the concentration of exosomes.

CD40L/IL-4 primed B cells were cultured as described in Figure 12 and B cell-derived exosomes were isolated from supernatants after 2 days. Equal amounts of protein were analyzed by BCA analysis of isolated exosomes. Data are presented as mean amount of protein (mg/ml) +/- SEM from 2-3 independent experiments. Significance based on an unpaired t-test. *, $p < .05$, compared to priming alone.

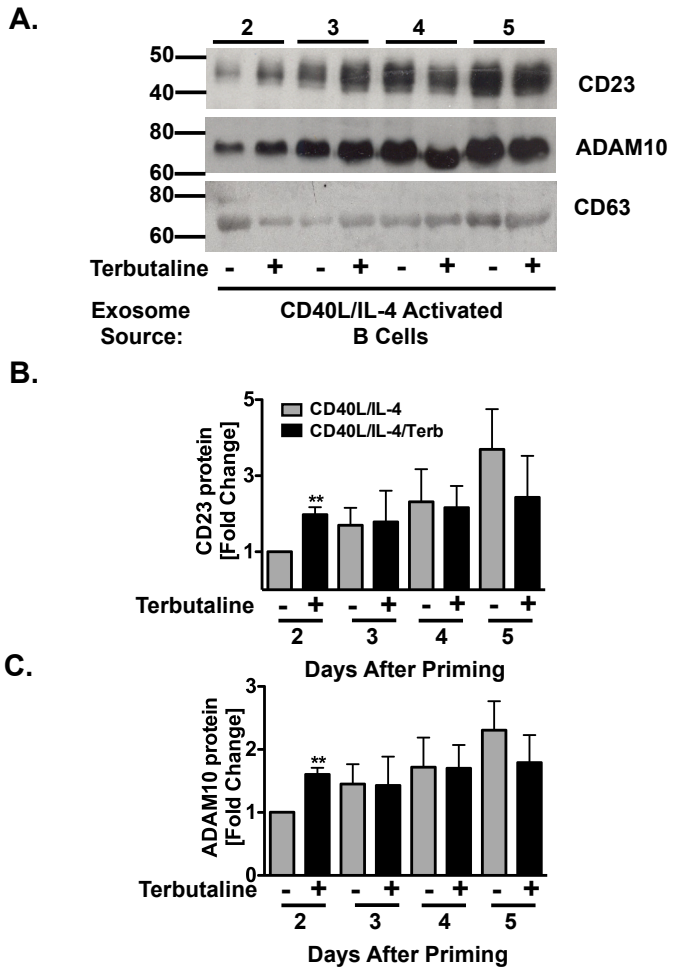

Figure 25. β_2AR stimulation enhances CD23 and ADAM10 expression on exosomes after priming.

WT CD40L/IL-4 primed B cells were cultured as described in Figure 12 and B cell-derived exosomes were isolated from supernatants after 2 through 5 days. Equal amounts of protein were analyzed by Western blot for CD23, ADAM10 and CD63 for each timepoint. *A-C*. **A**) One representative blot from three independent experiments is shown, with Molecular Weight in kDa, and data are presented as the mean fold difference of CD23 (**B**) and ADAM10 (**C**) expression +/- SEM from 3 independent experiments. Significance based on an unpaired t-test. **, p< .01, compared to priming alone.

also analyzed by Western blot. There were no appreciable differences in levels of CD23 or ADAM10 on B cell-derived exosomes in the absence or presence of β_2AR stimulation either by Western blot (**Figure 26A**) or densitometry (**Figure 26B-C**). However, similar to exosomes derived from WT cells, exosomes derived from β_2AR-deficient B cells contained more ADAM10 and CD63 at later timepoints compared to earlier timepoints (**Figure 26**). Collectively, these results indicate for the first time that CD23 and ADAM10 expression appears on exosomes only after 2 days of B cell priming, and that levels expressed of both proteins increases after β_2AR stimulation on primed B cells.

To extend the Western blot findings to a quantitative level, FACS analysis of individually isolated exosomes was performed to indicate the number of exosomes expressing CD23 and ADAM10, as well as the levels expressed. B cells from WT and β_2AR-deficient mice were prepared as described previously and exosomes were isolated on days 2 through 5 after priming. Because the Western blot results in Figure 23 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 antibodies 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 WT and β_2AR-deficient primed B cells alone and those primed B cells exposed to a β_2AR agonist increased from 2 through 5 days after priming, as compared to the previous day (**Figure 27**), and was not increased by β_2AR stimulation on primed B cells, in comparison to primed-only B cells at any time

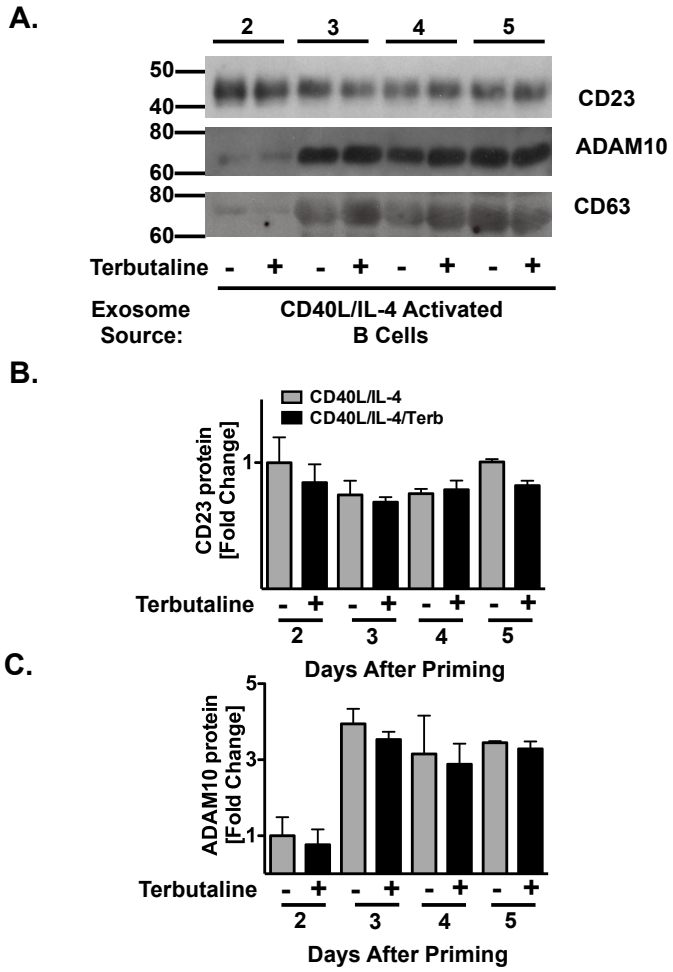

Figure 26. β_2AR stimulation does not enhance CD23 and ADAM10 expression on β_2AR-deficient exosomes after priming.

β_2AR-deficient CD40L/IL-4 primed B cells were cultured as described in Figure 12 and B cell-derived exosomes were isolated from supernatants after 2 through 5 days. Equal amounts of protein were analyzed by Western blot for CD23, ADAM10 and CD63 for each timepoint. *A-C*. **A**) One representative blot from three independent experiments is shown, with Molecular Weight in kDa, and data are presented as the mean fold difference of CD23 (**B**) and ADAM10 (**C**) expression +/- SEM from 3 independent experiments. Significance based on an unpaired t-test. **, p< .01, compared to priming alone.

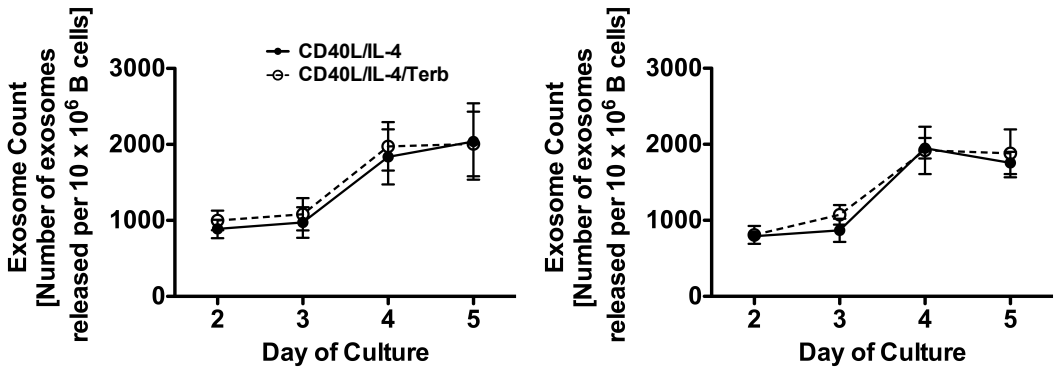

Figure 27. B cell-derived exosomes increase over time.

B cells were cultured as described in Figure 12 in the presence (open circle) or absence (closed circle) of terbutaline (Terb). Exosomes were isolated from WT (left) or β_2AR-deficient (right) B cells and analyzed from 2 through 5 days after priming and analyzed by FACS. Total exosome count is presented as mean count +/- SEM from three to six independent experiments.

point. Likewise, the number of $CD23^+$ exosomes remained constant throughout days 1 through 5 (**Figure 28A**). However, after 2 days 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 β_2AR agonist (**Figure 28A)**. This difference in CD23 expression was not evident on days 3 through 5 after priming, and was not evident on exosomes derived from β_2AR-deficient B cells at any time point (**Figure 29A**). In contrast to CD23, the number of $ADAM10^+$ exosomes was augmented 1.9-fold by β_2AR stimulation 2 days after priming, but returned to a control number on days 3 through 5 after priming (**Figure 28B**) and was not evident on exosomes derived from β_2AR-deficient B cells (**Figure 29B**). Also, in contrast to CD23, the expression of ADAM10 on exosomes, as determined by MFI, was not augmented by β_2AR stimulation after days 2 through 5 of priming, but remained the same as exosomes from primed B cells alone. Together, these data indicate that β_2AR stimulation induced changes in exosome phenotype only after 2 days of priming that included an increase in the level of CD23 expression on, and the number of $ADAM10^+$, exosomes.

3.5 B cell-derived exosomes directly enhance IgE production

What remained unknown was the extent to which the exosomes derived from β_2AR agonist-exposed primed B cells, in comparison to those from primed B cells alone, were able to mediate an increase in IgE. To address this unknown, B cells from WT or β_2AR-deficient mice were primed in the absence or presence of the β_2AR agonist, terbutaline, for 2 days, at which time either the whole supernatant was removed and transferred directly to another culture of 2-day primed recipient B cells, or the

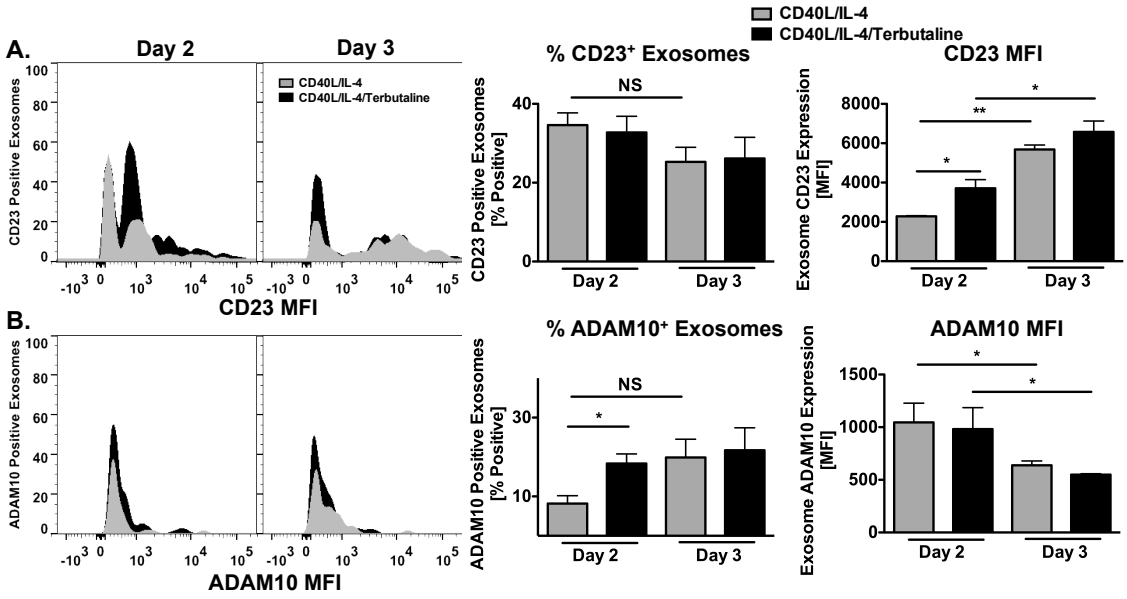

Figure 28. β_2AR stimulation enhances the level of CD23 expression and the percent of ADAM10$^+$ exosomes at early timepoints.

B cells were cultured as described in Figure 12 in the presence (black histogram, bar) or absence (grey histogram, bar) of terbutaline (Terb). Exosomes were isolated between 2 and 3 days and analyzed by FACS for CD23 and ADAM10 expression. Data are presented as one representative histogram, mean Percent, or MFI +/- SEM from 2-3 independent experiments. (**A**) CD23 expression on exosomes. Percent CD23$^+$ and MFI of CD23$^+$ exosomes. (**B**) ADAM10 expression on exosomes. Percent ADAM10$^+$ and MFI of ADAM10$^+$ exosomes. Significant differences were determined by unpaired t-test. *, $p < .05$, **, $p < .01$.

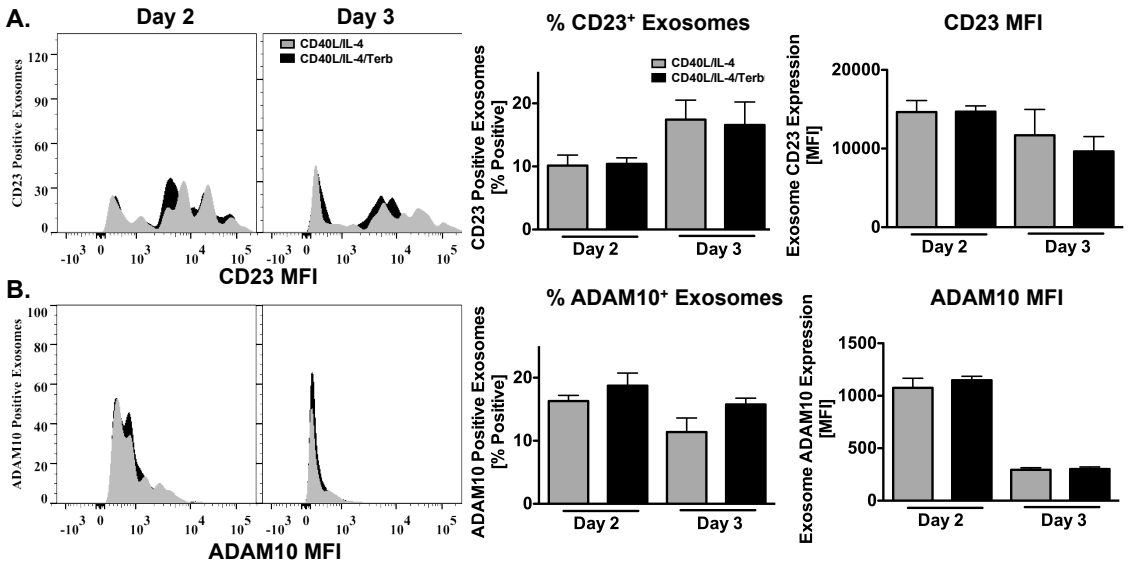

Figure 29. β_2AR stimulation does not enhance CD23 or ADAM10 expression on exosomes derived from β_2AR-deficient B cells at early timepoints.

β_2AR-deficient B cells were cultured as described in Figure 12 in the presence (black histogram, bar) or absence (grey histogram, bar) of terbutaline (Terb). Exosomes were isolated between 2 and 3 days and analyzed by FACS for CD23 and ADAM10 expression. Data are presented as one representative histogram, mean Percent, or MFI +/- SEM from 2-3 independent experiments. (**A**) CD23 expression on exosomes. Percent $CD23^+$ and MFI of $CD23^+$ exosomes. (**B**) ADAM10 expression on exosomes. Percent $ADAM10^+$ and MFI of $ADAM10^+$ exosomes. Significant differences were determined by unpaired t-test. *, $p < .05$, **, $p < .01$.

supernatant was first fractionated by ultracentrifugation into exosome-intact and exosome-depleted components that were then transferred to 2-day primed recipient B cells. The recipient B cells were then cultured for another 4 days, 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 β_2AR agonist-exposed primed B cells, in comparison to those fractions from primed B cells alone, increased the level of IgE produced by recipient cells by 1.2- or 1.3-fold, respectively, while the transfer of the exosome-depleted fraction did not (**Figure 30, left panel**). In contrast, the transfer of exosome fractions from β_2AR-deficient B cells exposed to a β_2AR agonist did not increase IgE (**Figure 30, right panel**). Because previous work from our laboratory indicated that β_2AR stimulation on a B cell did not affect CSR, 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 (132), 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 β_2AR agonist **(Figure 31, left panel)**. As expected, B cell-derived exosomes with a β_2AR-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 (**Figure 31, right panel**). These findings suggest that the exosomes released from primed B cells exposed to a β_2AR agonist not only

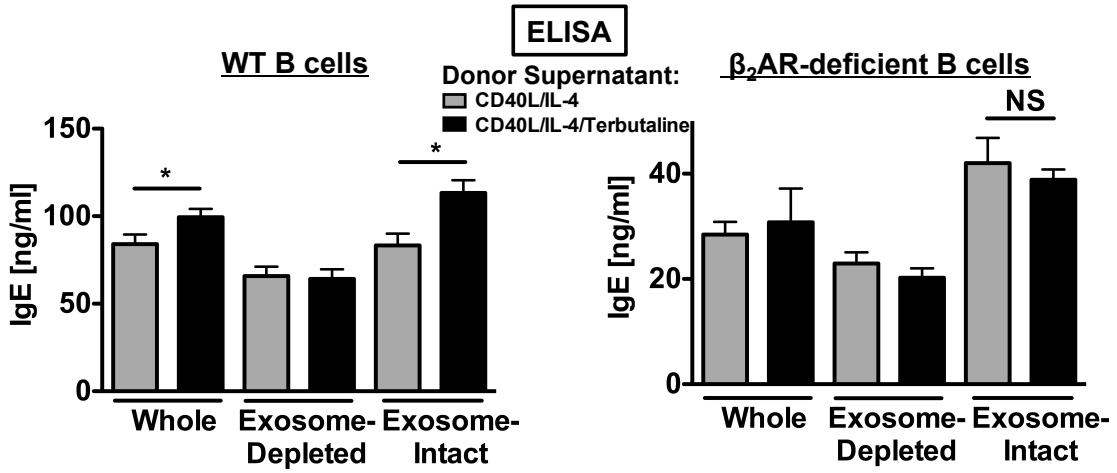

Figure 30. Transfer of exosomes derived from primed B cells exposed to a β_2AR agonist directly enhance IgE production by recipient B cells.

Donor supernatants: resting B cells were cultured as described in Figure 12 in the presence (black) or absence (grey) of terbutaline. Supernatants containing B cell-derived exosomes were removed after two days and the supernatant was fractionated by ultracentrifugation into exosome-intact and exosome-depleted components. Recipient B cells: resting B cells were cultured as described in Figure 12 in the absence of terbutaline. 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 days in the absence of terbutaline and which had supernatants removed prior to donor supernatant transfers. Supernatant fractions were transferred from donor cultures containing either WT (**left panel**) or β_2AR-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. Significance is based on an unpaired t-test. *, $p < .05$, as compared to B cells exposed to fractions obtained from primed B cells alone.

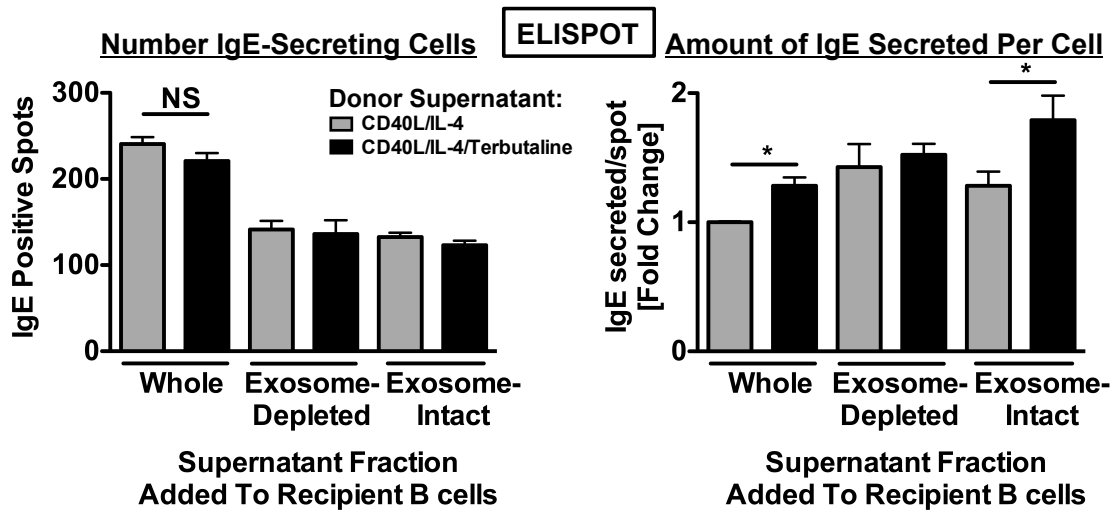

Figure 31. Transfer of exosomes derived from primed B cells exposed to a β_2AR agonist directly enhance IgE production by recipient B cells.

Donor supernatants: resting B cells were cultured as described in Figure 12 in the presence (black) or absence (grey) of terbutaline. Supernatants containing B cell-derived exosomes were removed after two days and the supernatant was fractionated by ultracentrifugation into exosome-intact and exosome-depleted components. Recipient B cells: resting B cells were cultured as described in Figure 12 in the absence of terbutaline. 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 days in the absence of terbutaline and which had supernatants removed prior to donor supernatant transfers. 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-positive spots/well or as the fold change compared to 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. *, $p < .05$, as compared to B cells exposed to fractions obtained from primed B cells alone.

express a higher level of CD23 and a greater percentage of ADAM10^{+} exosomes, as compared to exosomes from primed B cells alone, but also function to directly mediate the β_2AR-induced increase in the level of IgE.

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 to 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 (**Figure 31**) and, significantly, the same fractions from primed B cells exposed to β_2AR stimulation were further able to enhance IgE production (**Figure 31**), indicating that the β_2AR-enhancing effect on IgE could be transferred via exosomes. Second, to neutralize any effect that might have been transferred due to any residual β_2AR agonist, the β_2AR antagonist nadolol was added to recipient cell cultures along with the transferred supernatant fractions. Under this condition, the β_2AR-enhancing effect on the level of IgE produced by the recipient cells remained when the donor supernatants were exosome-intact and whole supernatants (**Figure 32A**), indicating that any residual

terbutaline did not alter the β_2AR-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 to day 2 transfers, and the primed β_2AR-exposed donor fractions were unable to enhance the level of IgE produced, in comparison to fractions from primed alone B cells (**Figure 32B**). 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 1X or 2X concentration prior to transfer to recipient B cells. The 1X and 2X concentrations of exosomes derived from B cells exposed to a β_2AR agonist enhanced IgE production, in comparison to those derived from primed B cells alone **(Figure 32C)**, indicating that the β_2AR-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 β_2AR agonist-induced enhancing effect on IgE, demonstrating that the β_2AR-induced increase in CD23 and ADAM10 on B cell-derived exosomes is functionally relevant.

3.6 ADAM10 and CD23 organize into membrane microdomains

The question remained as to the membrane region into which the increased level of β_2AR-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

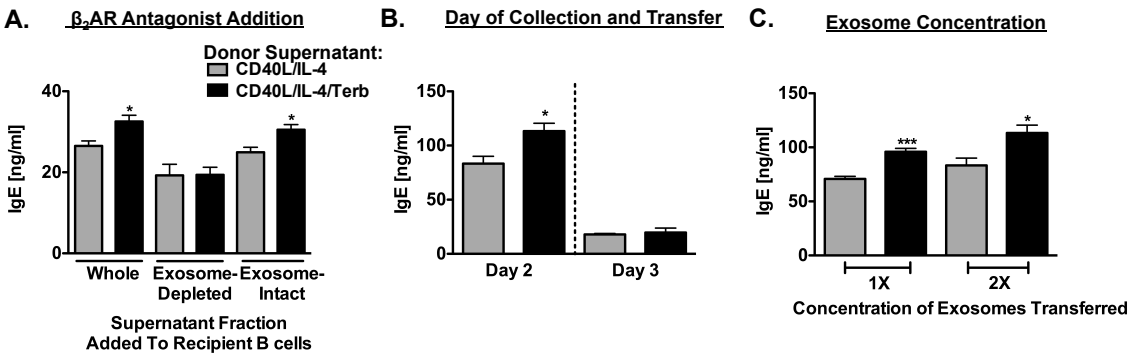

Figure 32. Transfer of exosomes derived from primed B cells exposed to a β_2AR agonist directly enhances IgE production by recipient B cells.

Resting B cells were cultured as described in Figure 12 in the presence (black) or absence (grey) of terbutaline (Terb) (donor condition). Donor supernatant fractions were transferred as described in Figure 30, unless day 3 was analyzed or different concentrations of exosomes were transferred, as indicated in specific graph legend. IgE produced by recipient cells was measured by ELISA on day 6. (**A**) Supernatant fractions were transferred in the presence of nadolol, a β_2AR antagonist. (**B**) Exosomes were transferred on either day 2 or 3 to either day 2 or 3 recipient cultures. (**C**) Exosomes were transferred at either 1X or 2X concentrations. Data are presented as mean IgE values (ng/ml) +/- SEM from one representative independent experiment of three from at least three replicate measurements. Significance is based on an unpaired t-test. *, $p < .05$, ***, $p<.005$ as compared to B cells exposed to supernatant fractions obtained from primed B cells alone.

resulted in a disruption of lipid rafts (158-161), suggesting that ADAM10 activity was greater outside of a cholesterol-rich lipid raft region of a membrane. To determine if membrane localization within B cell membranes was affected by β_2AR stimulation, B cells were primed and cultured as described previously and cell lysates were fractionated on days 0 through 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 (187), and GM1, which is a sphingolipid enriched in membrane lipid raft fractions (188). In resting cells, CD23 and ADAM10 were primarily located in non-lipid raft regions (**Figure 33A**) and, after priming, there was a change in distribution of CD23 and ADAM10 to both fractions. Two days after priming and β_2AR stimulation, an increased localization of CD23, but not ADAM10, to non-lipid raft fractions was observed, as compared to primed B cells alone (**Figure 33B**), 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 to 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 were compared from the lipid and non-lipid raft fractions, fractions 3-4 and 8-9, respectively, in order to determine if 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 **(Figure 33C**). 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 to 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 to resting cells **(**Figure 16**).** Thus, whereas the resting lipid raft fractions contained very little CD23, priming of the B cell enhanced CD23 expression to the extent that a large amount of CD23 was 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 β_2AR stimulation.

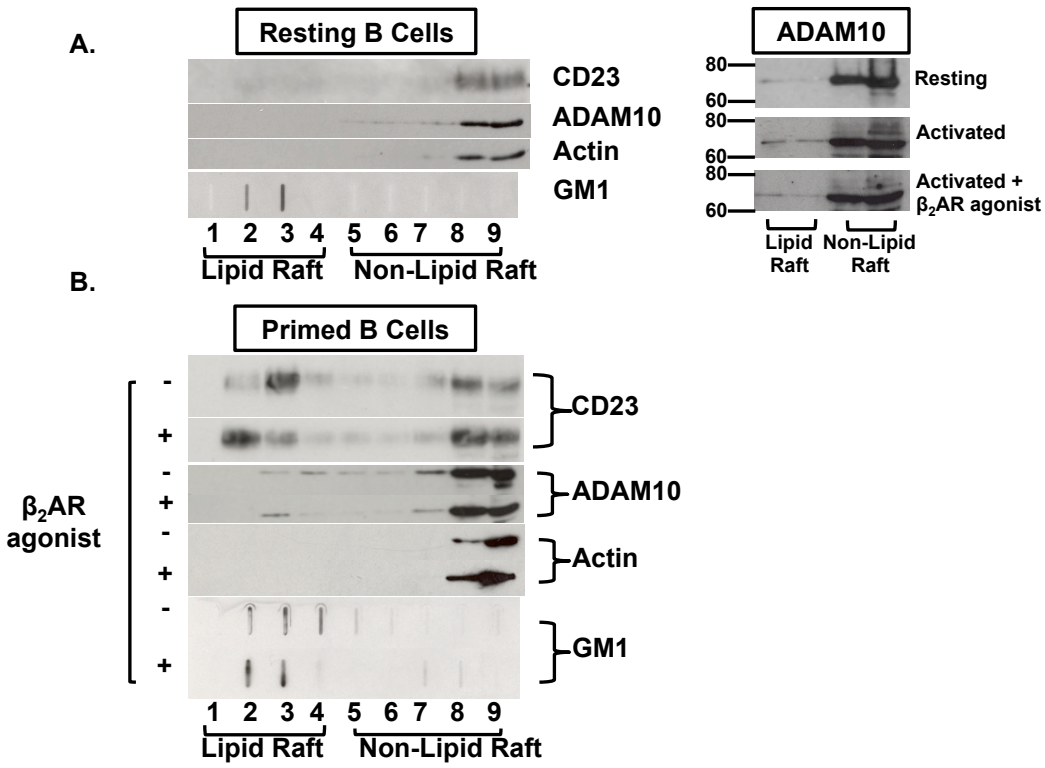

Figure 33. CD23 and ADAM10 localization to non-lipid raft domains within primed B cell membranes increases after β_2AR stimulation.

Resting B cells (**A**) or CD40L/IL-4-primed B cells (**B**) were cultured as described in Figure 12 in the presence (+) or absence (-) of terbutaline (Terb). Whole cell lysate fractions were isolated from a sucrose density gradient after 2 days. Fractions 1-4 represent less dense lipid raft fractions and Fractions 5-9 represent more dense non-lipid raft fractions. Equal volumes of each cell lysate fraction was 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 Ganglioside 1 (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 3-4 (Lipid Raft) and Fractions 8-9 (Non-lipid Raft) and analyzed for ADAM10. One representative blot from three independent experiments is shown, with Molecular Weight in kDa.

CHAPTER 4

DISCUSSION

4.1 Summary of Results

Connections between the nervous and immune system have been the topic of research for many years. The pathway being investigated in this dissertation involves the downstream mechanism triggered by β_2AR stimulation on an activated B cell that results in an enhanced level of IgE production. The *in vivo* correlate to this pathway has been demonstrated neuroanatomically to involve sympathetic innervation of lymphoid organs, such that the neurotransmitter, NE, is released in direct proximity of immune cells that express adrenergic receptors, including the T and B cell, which express the β_2AR exclusively (102-107). Previous work from our laboratory demonstrated that the loss of normal SNS signaling after chemical sympathectomy has immune consequences that include decreased germinal center formation, clonal proliferation, and antibody production (104). Specifically, it has been shown that β_2AR stimulation of immune cells enhances the level of IgE production on a per cell basis (34,132). What remained unknown was the mechanism responsible for the β_2AR-mediated increase in the level of IgE production by B cells.

Previous data *in vitro* showed that stimulation of the β_2AR on a CD40L/IL-4 primed B cell increased the level of IgE by increasing the rate of mature IgE transcription (132). Increased IgE production was linked to PKA and p38 MAPK activation (132). Although not obvious at first, the link between PKA and p38 MAPK was discovered to be HePTP, which normally sequesters p38 MAPK in the cytoplasm, but, upon cell activation and phosphorylation by PKA, releases p38 MAPK to be phosphorylated by the MAPKK pathway triggered by CD40 signaling (126). The β_2AR-associated increase in IgE was also shown to be linked to sCD23, a known positive regulator of IgE. Neither B cells from mice deficient in CD23 nor B cells from mice deficient in CD21 or CD19, which are mediators of the sCD23-associated enhancement of IgE, were able to upregulate the level of IgE production upon β_2AR stimulation (132). Furthermore, *in vivo*, chemical sympathectomy of mice prior to immunization resulted in reduced sCD23 in serum, which was abrogated with the inclusion of a β_2AR agonist at the time of immunization (132), further suggesting that sCD23 and the level of IgE production are linked. Recent work demonstrated that B cell-derived exosomes contain CD23 as well as ADAM10 (162), the metalloprotease that is the primary sheddase of CD23 (146,149). Although we previously reported that β_2AR stimulation increased CD23 mRNA (132), we were unsure of the cellular location of this increase. Thus, it remained unknown where in the cell that CD23 was increased and if β_2AR stimulation regulated ADAM10 expression and ultimately regulated IgE production.

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 β_2AR 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 antigen exposure, and clinically, when a β_2AR agonist is used therapeutically to treat bronchoconstriction. The goal of the present study was to identify the mechanism that links β_2AR engagement on a CD40L/IL-4-primed B cell to an increase in the level of CD23, sCD23 and IgE. We report in the present study that three mechanisms appear to be involved. The first mechanism involves a β_2AR-induced PKA- and p38 MAPK-dependent increase in both CD23 and ADAM10 expression at the mRNA and protein level. The second mechanism involves localization of the β_2AR-induced increase in CD23 and ADAM10 to B cell-derived exosomes, as opposed to the cell surface, and their release from the cell. And finally, the third mechanism involves the ability of the released exosomes to directly mediate the β_2AR-induced increase in IgE. Thus, our findings suggest that β_2AR stimulation of a CD40L/IL-4 primed B cell enhances IgE production by increasing the expression and localization of CD23 and ADAM10 to B cell-derived exosomes that are capable of transferring the β_2AR-enhancing effect by increasing the level of IgE produced on a per cell basis.

4.2 Localization of the β_2AR-associated increase of CD23 and ADAM10 to exosomes

One of the more unexpected findings in the present study was that the β_2AR-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 (146), but instead, 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 (163), and that B cell

priming with LPS and IL-4 induced both CD23 and ADAM10 (162). The enhancement of CD23 and ADAM10 expression on B cell-derived exosomes, as opposed to the cell surface, following β_2AR engagement on a primed B cell, suggested three possible means by which the β_2AR-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 (146) 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 (162), to then fuse with intracellular multivesicular bodies (MVB) (167,189) that would subsequently fuse with the plasma membrane for release from the cell (167,189,190). In support of this possibility is the report that the cell surface expression of ADAM10 on the surface of Human Embryonic Kidney 293 (HEK293) cells is regulated by endocytosis, as evidenced when using an inhibitor to the endocytic protein dynamin (191), suggesting that any ADAM10 expression at the plasma membrane was cleared from the surface via endocytosis. Support for the co-endocytosis 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 (162), 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 β_2AR-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 (184,190). Support for the immediate shuttling of these proteins to exosomes was that specific inhibition of exosome release by ammonium chloride, as defined previously (162,185), 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 ammonium chloride. Thus, the means by which CD23 and ADAM10 localize to exosomes are not mutually exclusive, but the present results suggest that the β_2AR-induced increase in CD23 and ADAM10 is preferentially shuttled to exosomes for release from the cell and subsequent cleavage of CD23, instead of shuttled to the cell surface plasma membrane.

4.3 Transfer of the β_2AR-enhancing effect

We show that exosomes released by donor cells exposed to a β_2AR 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 a recipient primed B cell that had already undergone CSR. We propose that the transfer of the β_2AR-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 β_2AR agonist, which might have affected recipient cells, was unlikely because the addition of a β_2AR antagonist along with the donor exosomes was unable to prevent the enhancing effect. Also, the half-life of terbutaline in culture is 7 hours (130), and exerts an enhancing effect only within the first 24 hours of priming, as reported previously (131), suggesting that any effect on a B cell 48 hours after priming was unlikely. And, finally, because the transfer of the enhancing effect occurred only with exosomes collected on the peak day of the β_2AR-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 level 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 due 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.

4.4 Localization of CD23 and ADAM10 to membrane fractions

Previous work focused on regulation of ADAM10 activity by membrane localization to lipid versus non-lipid raft fractions (158-161). 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 (158-161). 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 β_2AR-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, while 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 by (170)], and exosomes are derived from the lipid-rich lipid raft regions of cellular membranes (192) where the interaction of CD23 and ADAM10 within this region allows for exosome formation within MVB's that are released from the cell (162). The fact that both exosomes and lipid rafts have similar densities when isolated by sucrose and iodixanol density centrifugation (163,193,194), 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 β_2AR-induced increase in ADAM10-mediated cleavage of CD23, and will also identify the extent to which preferential localization occurs on an exosome, as opposed to a plasma membrane.

4.5 Comparison of WT and β_2AR-deficient B cells

Throughout this dissertation, β_2AR-deficient cells were used as a control, and whereas they were very similar to WT cells at all endpoints and they did not respond to β_2AR stimulation, the baseline values from β_2AR-deficient cells were consistently below cells from WT mice. For example, baseline IgE and sCD23 were decreased, along with a lower resting number of $CD23^+/ADAM10^+$ cells coupled with a lower day 2 peak in $CD23^+/ADAM10^+$ cells upon CD40L/IL-4 priming. These differences were also seen in the B cell-derived exosomes: Exosomes derived from β_2AR-deficient B cells had a quicker decrease in CD23 and ADAM10 expression from day 2 to day 3 than seen with exosomes from WT B cells. In addition, exosomes transferred from β_2AR-deficient B cells promoted a lower amount of IgE production by recipient WT B cells, despite the number of exosomes being transferred being equivalent between WT and β_2AR-deficient donor cells. Since ADAM10 was only present on the cell surface if CD23 was present, and β_2AR-deficient B cells had lower amounts of $CD23^+/ADAM10^+$ cells prior to priming and after priming at the peak on day 2, CD23 emerges as a key regulator of the level of IgE production. One explanation for differences in baseline CD23 expression may be due to the intrinsic differences between WT and β_2AR-deficient mice. *In vivo*, these mice respond to antigen and immunization in a very similar manner, however, analysis of surface expression of receptors that regulate CD23 expression may explain differences in CD23 expression. It was shown that splenocytes from β_2AR-deficient mice had lower levels of CD40 expression and slightly lower levels of CD40L expression (181). Since CD40 signaling is known to regulate CD23 expression (195), reduced expression of CD40 may explain the reduced peak in $CD23^+/ADAM10^+$ B cells after

priming with CD40L. IL-4 is another known regulator of CD23 expression (196,197), however, surface expression of the IL-4R has not been examined. Alternatively, B cells derived from WT mice have been exposed to β_2AR stimulation in the environment of the spleen over the course of their lifetime (104). For instance, any stressor that these mice encountered would allow for the release of NE in the proximity of any B cells present in the spleen. Since β_2AR stimulation is capable of inducing the expression of histone demethylases (198), it is possible that the CD23 and ADAM10 promoters in the B cells of WT mice have received epigenetic modifications that make it easier for NE to upregulate the transcription of target genes important for adaptive immunity. Since β_2AR-deficient B cells have never responded to NE, these epigenetic modifications are absent and the consequences of not having these epigenetic modifications is seen in the decreased amount of $CD23^+$/$ADAM10^+$ cells and exosomes. Additionally, the GC-rich nature of the ADAM10 promoter increases the possibility that epigenetic modifications are required to allow for quick induction of ADAM10 expression in response to β_2AR stimulation (199). Future studies should examine the downstream epigenetic modifications conferred by β_2AR stimulation and the accessibility of β_2AR-inducible genes.

4.6 Regulation of CD23 expression

Whereas the signaling intermediates that were responsible for mediating the β_2AR-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 (196,197), anti-CD40 antibody (195), or a Th2 clone in the

presence of antigen (200). We reported previously that β_2AR stimulation on a CD40L/IL-4-primed B cell enhanced CD23 expression beyond the level induced by CD40L/IL-4 alone (132), and in the present report, we confirm that this increase was mediated by the β_2AR specifically and in a PKA- and p38 MAPK-dependent manner. The signaling intermediates responsible for the β_2AR-induced enhancement in CD23 may also be linked to the known role of STAT6 in regulating the IL-4–induced increase in CD23 (201) via p38 MAPK regulation of the transactivation domain of STAT6 (202). Because the pathway by which β_2AR engagement enhances IgE is via a β_2AR-induced, PKA-dependent phosphorylation of HePTP (126), which releases bound p38 MAPK for phosphorylation by the MAPKK pathway activated following CD40 engagement (203), we propose that the β_2AR-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.

4.7 Regulation of ADAM10 expression

In contrast to CD23 regulation, signaling intermediates that upregulate ADAM10 expression are largely unknown. B cells express ADAM10 constitutively (204,205), as was confirmed in the present study, and which is in contrast to CD23 expression that is negligible in resting B cells (197). In the present report, β_2AR 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 (199,206,207).

Promoter analysis of the ADAM10 gene reveals binding sites for the Retinoic Acid Receptor-α (RARα) and NF-κB (199), supporting a previous finding that ADAM10 protein was upregulated by activation of the retinoic acid pathway in a neuroblastoma cell line (206). Additionally, ADAM10 gene and protein expression were also found to be upregulated by activation of the p38 MAPK/ERK1/2 pathway in primary cortical cultures (207), while other reports showed that p38 MAPK was able to phosphorylate both RARα and NF-κB when using Mouse Embryonic Fibroblasts and NIH 3T3 cells (208,209). In the present report, we show that the β_2AR-enhanced expression of ADAM10 is also mediated by p38 MAPK, and is likely mediated by the aforementioned β_2AR-activated PKA/HePTP/p38 MAPK signaling pathway described for CD23. Taken together, both CD23 and ADAM10 expression are regulated by p38 MAPK, and the β_2AR on a B cell further contributes to this regulation through the same signaling intermediate, indicating that a common mechanism exists by which β_2AR engagement increases two proteins important for IgE regulation, which is also linked to increased p38 MAPK activation.

4.8 Cell density and *in vitro* IgE production

Cell density has been previously linked to the regulation of IgE production (182,183). We report in this study that cell density is also important for the β_2AR-associated enhancement of the level of IgE production. Because previous studies were able to reverse the density-dependent inhibition of IgE with apoptosis inhibitors (183), the suppression was attributed to cell-cell contact. However, B cells lacking Fas and FasL were still susceptible to the cell density effect, suggesting that the mediator of IgE suppression might be something still unknown or that it was a different member of the

death receptor family. The identification of exosomes in this study as a mediator of the level of IgE production by B cells raises the possibility that the cell density-associated suppression of IgE is mediated by a molecule expressed on exosomes. Indeed, initial exosome transfer studies indicated that concentrating exosomes by resuspending in a lower volume than what was collected would not transfer the β_2AR-enhancing effect. We found that dilutions 1-2X of the original volume/concentration unmasked the β_2AR-enhancing effect, suggesting that the concentration of exosomes was important for optimal IgE production and observation of the β_2AR-enhancing effect. One explanation for an exosome density-dependent suppression of the β_2AR-enhancing effect independent of the Fas/FasL interaction may be due to glycolipid-induced cell death. Synthesis of exosome-like nanoparticles and treatment of pancreatic tumor cells with nanoparticles of varying lipid content induced cell death (210), that was attributed to nanoparticles with higher levels of cholesterol and sphingomyelin, lipids that are particularly enriched in lipid raft-rich exosomes. Thus, the lipid composition of exosomes may be enough to mediate the cell-density dependent suppression of IgE. There are also many proteins that are expressed on exosomes that participate in apoptosis. FasL (211), apoptosis inducing factor-1, and Programmed Cell Death 6 (PDCD6) (212) have been found in exosomes of hematopoietic origin. Indeed, infection of B cells with oncogenic viruses downregulated the expression of PDCD6 and PDCD6 interacting protein on B cell-derived exosomes (212), suggesting that the absence of these proteins promotes tumor survival. Thus, the presence of pro-apoptotic proteins on exosomes suggests a possible mechanism by which cell and exosome density suppress IgE production by B cells.

4.9 Clinical Relevance

Although we elucidated the kinetics of the β_2AR-induced enhancement of CD23 and ADAM10 expression on exosomes, our ultimate goal was to identify the mechanism by which β_2AR engagement on a primed B cell enhanced the level of IgE produced. The present finding suggested that exosomes might play an essential 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 BALF 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 (168,213), suggesting that exosomes from tolerized mice were able to induce tolerance in response to sensitization to the same antigen. The mechanism of the transfer of an anti-inflammatory phenotype *in vivo*, is suggested by previous findings that B cell-derived exosomes transferred antigen-MHC II complexes to follicular dendritic cells (214), and that $CD23^+$ B cells were required for optimal IgE-antigen presentation to $CD11c^+$ dendritic cells to allow for $CD4^+$ T cell activation (215). Whereas the transfer of a sensitizing phenotype via exosomes offers many intriguing avenues of research, prevention of a potential β_2AR-induced increase in the level of IgE in the treatment of allergic asthma would also be helpful. The present findings suggest a mechanism that can be targeted to prevent a β_2AR-induced increase in the production of IgE. Early intervention after β_2AR agonist inhalation using either ADAM10 inhibitors and/or

molecules that would prevent the biogenesis of exosomes might prevent a β_2AR-induced increase in the level of 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 beta 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 **Figure 34**, the present study demonstrated that β_2AR stimulation on a primed B cell, in comparison to 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 β_2AR-mediated enhancement in CD23 and ADAM10 protein is found on B cell-derived exosomes that are released by the B cell, and which, upon transfer to recipient primed B cells, mediate the β_2AR-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 β_2AR-mediated enhancing effect on IgE, without affecting the β_2AR-mediated dilating effect on bronchiolar smooth muscle.

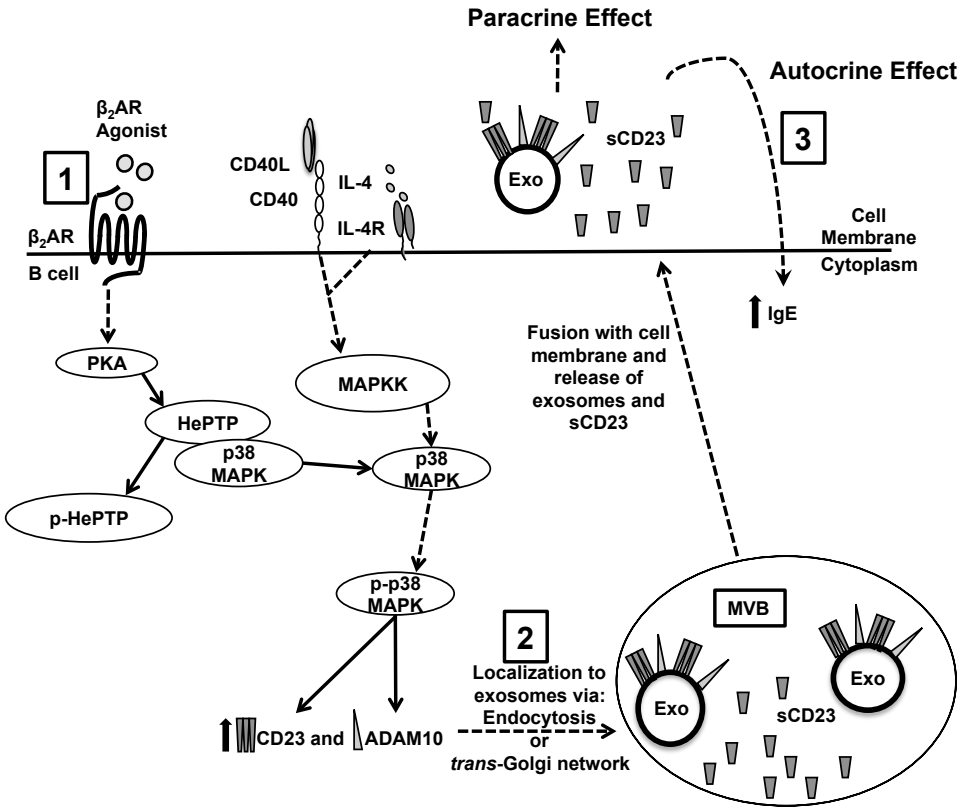

Figure 34. Adrenergic Regulation of IgE Involves Modulation of CD23 and ADAM10 Expression on Exosomes.

(**1**) Wild type B cells primed in the presence of a β_2AR 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**) β_2AR 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 MVB with the plasma membrane. The release of sCD23 from exosomes enhances the level of IgE production by acting in either an autocrine or paracrine manner, as suggested previously (162,214,215). The autocrine mechanism may involve a sCD23-mediated enhancement of the level 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 antigen bound to IgE. This complex will be phagocytosed by dendritic cells so that they present antigen 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 β_2AR-enhanced expression of CD23 and ADAM10 on B cell-derived exosomes may explain the mechanism responsible for mediating the effect of β_2AR stimulation on a B cell to increase the level of IgE produced.

4.10 Future Directions

4.10.1 CD23 and ADAM10 trafficking to exosomes

CD23 and ADAM10 were present on the cell surface and on exosomes. However, the kinetics of their expression in these membranes bring up the question of how and when the β_2AR-mediated increase in these proteins occurred. To begin with, CD23 expression increased dramatically from a resting to an activated state within 24 hours of priming both in cell lysates and on the cell surface. ADAM10, however, was barely increased by priming alone on the cell surface and in the cell lysate. Then, it took another 24 hours to detect CD23 and ADAM10 on exosomes isolated from the supernatant 2 days after priming, suggesting that there was a delay between protein synthesis and shuttling to exosomes. This delay indicates the possibility of an intermediate step wherein the delay is due to expression of the increased CD23 on the surface of the cell, subsequent interaction with ADAM10 and endocytosis of the CD23-ADAM10 complex to form exosomes, which might explain the peak of CD23 expression at 24 hours and the decrease of ADAM10 from that timepoint. Alternatively, the newly synthesized CD23 might be packaged with the constitutively expressed ADAM10 in the trans-Golgi network and released as exosomes.

These ideas could be tested in several ways. One method would utilize ammonium chloride, which was used earlier in this report to inhibit exosome release. If exosome release is inhibited and the β_2AR-associated increase in CD23 and ADAM10 are detected on the cell surface by FACS, this would support the idea that the proteins are expressed on the surface first and not immediately shuttled to exosomes. If there was no change on the surface, differential staining of surface versus intracellular CD23 and

ADAM10 would determine if the enhanced protein was kept internally until exosome release, as the western blot results indicated. Another method would utilize a dynamin inhibitor to inhibit endocytosis, a method that was used to demonstrate how ADAM10 is trafficked (191). One drawback of this method is that it requires the transfection of a dominant negative form of dynamin into a cell line, thus requiring replication of these findings in a B cell line. This is further complicated by the absence of CD23 in most B cell lines (unpublished observations). However, if this method prevented the β_2AR-mediated increase in CD23 and ADAM10 expression in exosomes, it would likely support a mechanism involving initial surface expression followed by endocytosis and exosome release. Finally, to determine if the CD23 and ADAM10 packaged into exosomes were primarily newly synthesized, radioactive leucine would be used for a pulse chase experiment. Based on kinetic studies of mRNA production for CD23, we would pulse at 9 hours post-priming for CD23. For ADAM10 studies, we would pulse within the first hour of priming, as this is the time that we detect the β_2AR-mediated increase in ADAM10 transcription. We would then chase with nonradioactive buffer for 48 hours then isolate exosomes from the supernatant and analyze immunoprecipitated CD23 and ADAM10 for radioactivity. The presence of radioactivity in exosomes would indicate that CD23 and ADAM10 were newly synthesized and exported in exosomes. However, most of the literature suggests that the shuttling of proteins to exosomes occurs after endocytosis (167,189,190). Our FACS data agree with the endocytosis mechanism, especially taking into account the delay between appearance of the CD23 and ADAM10 in the cell lysate then on exosomes.

4.10.2 Regulation of CD23 and ADAM10 expression

The β_2AR-mediated increase in CD23 and ADAM10 was attributed to an increase in p38 MAPK activity that utilized a PKA/HePTP/p38 MAPK-dependent pathway. There are, however many transcription factors downstream of p38 MAPK, and in order to understand the mechanism by which the β_2AR- and p38 MAPK-mediated increase in CD23 and ADAM10 functions, it is imperative to study their promoters. Most studies have pointed to STAT6 as being the central mediator of CD23 expression (201,216). A mechanism by which β_2AR stimulation upregulates CD23 involves the PKA/HePTP/p38 MAPK pathway and transactivation of STAT6 by p38 MAPK. To test the hypothesis that the β_2AR-associated increase in CD23 is due to transactivation of STAT6, STAT6-deficient mice would be utilized. If the β_2AR-associated increase in CD23 is not observed, the upregulation in CD23 is likely STAT6 regulated. Other possible transcription factors that may mediate the increase in STAT6 include AP-1, ATF2, STAT1, MEF2, and MSK1 [reviewed by (134,135)]; however, there are very few reports in the literature of binding sites for these transcription factors in the promoter for CD23. This is one approach to determine the mechanism by which CD23 is upregulated by β_2AR stimulation. One key difference between CD23 and ADAM10 is the availability of reports (199) that note the presence of sites within the promoter of ADAM10 that are downstream of p38 MAPK, thus, providing a different mechanism than STAT6 by which β_2AR stimulation increases ADAM10 expression. The transcription factors that have binding sites in the promoter of ADAM10 that are downstream of p38 MAPK include specificity protein 1 (SP1) (217), upstream transcription factor (USF) (218), and NF-kB through MSK1/2 (219). To test the hypothesis that any of these binding sites are

important for ADAM10 expression, a luciferase construct with the parts of the ADAM10 promoter containing these sites would be used to assess if β_2AR stimulation utilizes these pathways through p38 MAPK to increase ADAM10. Furthermore, knockout mice and inhibitors are other options to test this hypothesis. There are currently knockout mice that have been reported in the literature for USF (220) and MSK1 (221), however, SP1 knockout is embryonic lethal (222), but an inhibitor to SP1 is available, mithroamycin (223). Thus, the elucidation of the pathways downstream of p38 MAPK that upregulate CD23 and ADAM10 upon β_2AR stimulation would be useful to identify targets to prevent their upregulation.

4.10.3 *In vivo* relevance

The importance of the β_2AR on the B cell for allergic asthma, and the ability of exosomes to transfer an inflammatory phenotype in an allergic asthma model are two *in vivo* extensions of the data presented in this dissertation. Previous studies have demonstrated sCD23 is correlated with IgE levels in an allergic asthma model and modulation of IgE levels through SNS depletion reduces sCD23 (132). An additional tool that could be used is the β_2AR-deficient mouse, the mouse from which B cells were used for *in vitro* experiments throughout this dissertation. There is, however, the caveat that previous studies with the β_2AR-deficient mouse on an FVB background exhibited equivalent T-dependent antibody responses (181), in contrast to the effects of chemical sympathectomy on antibody production (132). In order to determine if the background strain of the mouse contributed to a T-dependent antigen response, the β_2AR-deficient mouse on the Friend Virus Type B (FVB) background was backcrossed for ten generations to the Balb/c background. Preliminary *in vivo* experiments with β_2AR-

deficient mice on the Balb/c background indicated that the global absence of the β_2AR does not affect IgE production and airway hyperresponsiveness (AHR), an indicator of airway inflammation, in an allergic asthma model (**Figure 35**). These results contrast with the attenuation of IgE production detected in the serum and BAL upon chemical sympathectomy in an allergic asthma mouse model (132). One important difference between these *in vivo* models is that the immune cells of β_2AR-deficient mice have never responded to NE whereas the immune cells in the chemically sympathectomized mice have had a lifetime of exposure to β_2AR stimulation by NE (104). Prior exposure to NE and subsequent epigenetic modifications may affect the availability of the promoter for IgE transcription and other immune factors that participate in the allergic asthma response. Wherein the loss of a protein via genetic deletion and/or manipulation in mouse models will often elucidate information about the function of that protein, the global absence of the β_2AR may not be the best *in vivo* model to study it's function in the immune system because of the influence of prior signaling on existing transcription of genes downstream of β_2AR stimulation due to epigenetic modification and also because of possible compensatory mechanisms via other adrenergic subtypes on other immune cells. Thus, the chemical sympathectomy model is a better model to study the effects of depletion of β_2AR signaling on B cells because it takes into account prior epigenetic modifications introduced by normal β_2AR signaling. These data, however, do not invalidate the use of β_2AR-deficient mice to study the function of the β_2AR stimulation on B cells, as B cells can still be adoptively transferred into mice deficient of B and T cells. Indeed, in comparison to WT B cells, adoptive transfer of β_2AR-deficient B cells into Rag-2 deficient mice was associated with decreased IgE production in the BAL in an

allergic asthma model (J. McAlees, unpublished observations), suggesting that the β_2AR, specifically on a B cell, was essential for IgE production.

What remains unknown is the effect that β_2AR deficiency has on AHR. This question could be addressed in two ways by using either chemically sympathectomized mice undergoing an allergic asthma protocol or transferring β_2AR-deficient B and T cells to Rag2-deficient mice followed by an allergic asthma protocol, with the primary endpoint including the measurement of IgE in the BAL and AHR. One potential complicating factor is that B cells from β_2AR-deficient mice may be somehow phenotypically different due to the unknown effects of β_2AR deficiency on B and T cell development. Another potential complicating factor is that sympathectomy may unmask unknown effects due to increased populations of sensory neurons, which also innervate the airways (224). Thus, the effect of β_2AR stimulation on B cells in allergic asthma has yet to be determined.

Future studies that would lend *in vivo* relevance to the role of exosomes in IgE production would utilize exosome transfer in an allergic asthma model to determine if increased CD23 and ADAM10 expression are capable of enhancing IgE *in vivo*. A tolerizing effect has been demonstrated previously with exosomes derived from tolerized mice (168,213), suggesting that an inflammatory phenotype is transferable. The *in vitro* model used in this study did not require antigen, suggesting that the mechanism used to upregulate the level of IgE was by globally increasing IgE production. Therefore, to test the hypothesis that exosomes are capable of enhancing the level of IgE production, exosomes would be isolated from the serum and/or BAL of mice in an allergic asthma model that had either previously underwent chemical sympathectomy, or normal mice,

including β_2AR-deficient mice and WT mice. The exosomes would then either be injected intravenously or inhaled by mice that had received immunization. The mice would then be challenged for three days with antigen and at the end of the protocol, analyzed for AHR. Since the exosomes from mice that underwent chemical sympathectomy would have less CD23, they should transfer less of an inflammatory phenotype than those isolated from control mice. While there may be many considerations including timing of the transfer and immunizations, these experiments would be the ultimate proof of principle for the enhancement of the level of IgE production from exosomes derived from cells exposed to β_2AR stimulation.

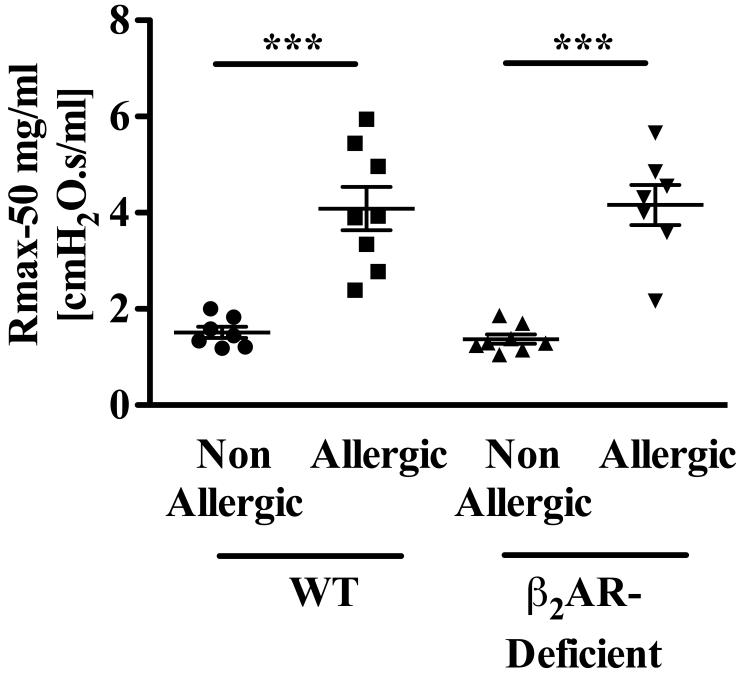

Figure 35. Airway hyperresponsiveness is unchanged in β_2AR-deficient mice.

WT and β_2AR-deficient mice were immunized intraperitoneally with PBS (non-allergic, WT n=6, β_2AR-deficient n=8) or OVA/ alum (allergic, WT n=8, β_2AR-deficient n=7) and reimmunized at week 3. On days 28-32 they were challenged with PBS (non-allergic) or OVA (allergic) administered intranasally. On day 33, airway hyperresponsiveness was performed. Airway resistance was determined with increasing doses of methacholine. The data shown represent the maximum dose of methacholine (Rmax-50 mg/ml) and are presented from individual mice as resistance (cmH2O.s/ml). Horizontal bars represent the SEM of each treatment group. Significant differences were determined by student's t test. ***, $p<.001$.

4.11 Concluding Remarks

At the time these dissertation experiments were initiated, it was known that β_2AR stimulation regulated the level of IgE both *in vivo* and *in vitro* either through NE or a β_2AR agonist. Previous studies showed that β_2AR stimulation on a CD40L/IL-4 primed B cell mediated the increase in the level of IgE through a PKA-, HePTP-, p38 MAPK-, and sCD23-dependent pathway by increasing the level of IgE produced per B cell, rather than increasing the number of B cells that switched to IgE. What remained unknown was the intermediate pathway that increased sCD23 production and mediated the increase in the level of IgE production. The present data show for the first time that β_2AR stimulation on a CD40L/IL-4 primed B cell increases the level of IgE production by first increasing CD23 and ADAM10 expression in a PKA- and p38 MAPK-dependent manner on B cell-derived exosomes as opposed to the surface of the B cells. Furthermore, we show for the first time that the β_2AR-enhancing effect is transferable to B cells that have not been exposed to the β_2AR agonist by increasing IgE production on a per cell basis.

The contribution made by the molecular findings in this dissertation is that they identify a novel mechanism that regulates the level of IgE production. Specifically, this mechanism involves a β_2AR stimulation-dependent enhancement of the expression on exosomes of two molecules important for IgE production. These exosomes are released by the B cell and are capable of mediating an enhancement in the level of IgE production since CD23 serves as the substrate for ADAM10, effectively allowing for cleavage of CD23 to sCD23 in an autocrine or paracrine manner, i.e., at the site of release or at locations further away. The significance of these findings are that they identify signaling intermediates that regulate IgE production, and which can be targeted in the treatment of

IgE-mediated allergy. Because allergic asthma is often treated with β_2AR agonists, these findings identify a target to prevent the β_2AR agonist-associated increase in the level of IgE, while allowing the continued use of life-saving bronchodilation therapy.

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