ADAM10 is a critical regulator of B cell development, antibody production, and myeloid-derived suppressor cell expansion: Effects of B cell-specific ADAM10 deletion and overexpression in vivo.

David Gibb

Virginia Commonwealth University

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ADAM10 is a critical regulator of B cell development, antibody production, and myeloid-derived suppressor cell expansion: Effects of B cell-specific ADAM10 deletion and overexpression in vivo.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

David Randolph Gibb
B.S., University of Virginia, 2004

Director: Daniel H. Conrad, Professor, Department of Microbiology and Immunology

Virginia Commonwealth University
Richmond, Virginia
August, 2010
DEDICATION

This dissertation is dedicated to my family for their sincere support of my academic pursuits. My parents, Dr. C. Ernest Gibb, Jr. and Ann Major Gibb have supported each stage of my education and continue to encourage my goal of making an impact on human well being through science. My sister, Emily Gibb Beamer, has directed my pursuits by encouraging me to take advantage of opportunities that help me develop as a person and a scientist. Additionally, this dissertation is dedicated to my first-cousins once-removed, Susan Shaffer and Daniel Harrison, for their interest and support of me as a medical and graduate student. Finally, this dissertation is dedicated to the loving memory of my grandparents, Clyde Ernest Gibb and Nancy Harrison Gibb.
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I especially thank Mohey El Shikh and Rania El Sayed for performing immunohistochemistry of spleen follicles that was invaluable to the B cell knockout publication. I thank the VCU knockout/transgenic mouse core facility, especially Mark Subler, for generating the ADAM10 transgenic mice. I also thank VCU collaborators for their technical help and advice regarding cancer immunotherapy, including Harry D. Bear, M.D., Ph.D., Masoud Manjili, Ph.D., Maciej Kmiecik, Ph.D., and Laura Graham.
I am truly appreciative of all the valuable feedback from my graduate committee members, including Suzanne Barbour, Ph.D. and John Tew, Ph.D. for reviewing my manuscripts, David Straus, Ph.D. for reviewing my pre-doctoral grant, and Lawrence Schwartz, M.D., Ph.D. for his advice and interest in my pursuit of an M.D., Ph.D. degree. I especially thank Gordon Archer, M.D. and the M.D./Ph.D. program for giving me the opportunity to pursue my degree and invaluable advice throughout the medical and graduate phases of the program. I thank the members of my M.D./Ph.D. class for all their support, especially Omar Mian for his advice and enthusiasm for research.

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I thank my family for all their loving support and encouragement. Finally, I thank my undergraduate research advisor, Klaus Ley, M.D., and his post-doctoral fellow, Margaret M. Fears, for inspiring me to pursue research.
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<tr>
<td>+/-</td>
<td>Heterozygous deletion of a gene</td>
</tr>
<tr>
<td>-/-</td>
<td>Homozygous deletion of a gene</td>
</tr>
<tr>
<td>19G5</td>
<td>mAb recognizing the stalk region of murine CD23</td>
</tr>
<tr>
<td>2.4G2</td>
<td>mAb recognizing the murine FcγRII and FcγRIII</td>
</tr>
<tr>
<td>a.a.</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>A10Tg</td>
<td>ADAM10 transgenic mouse</td>
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<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
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<td>ADAM10Δ/Δ</td>
<td>Floxed ADAM10 mice</td>
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<td>ADAM-TS</td>
<td>A disintegrin and metalloprotease-thromospondins</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>ALPS</td>
<td>Autoimmune lymphoproliferative syndrome</td>
</tr>
<tr>
<td>Alum</td>
<td>Aluminum hydroxide</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid plaque precursor</td>
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<tr>
<td>B220</td>
<td>mAb recognizing the murine CD45R; mouse B cell marker</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>B3B4</td>
<td>mAb recognizing the lectin domain of murine CD23</td>
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<td>Balb/c</td>
<td>Inbred mouse strain</td>
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<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<td>BMP</td>
<td>Bovine metalloprotease</td>
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<tr>
<td>C57BL/6</td>
<td>Inbred mouse strain</td>
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<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CD23Tg</td>
<td>CD23 transgenic</td>
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<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
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<td>CIA</td>
<td>Collagen-induced arthritis</td>
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<tr>
<td>CLP</td>
<td>Common lymphocyte progenitor</td>
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<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DII</td>
<td>Delta-like Notch ligand</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative or double negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dnKuz</td>
<td>Dominant negative form of ADAM10</td>
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<tr>
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<td>Definition</td>
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<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>ECD</td>
<td>Electrochemical detector or PE/Texas-Red</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ES</td>
<td>Embyronic stem cells</td>
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<td>EYFP</td>
<td>Enahanced yellow fluorescent protein</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
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<tr>
<td>FcεRI</td>
<td>The high affinity IgE receptor</td>
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<tr>
<td>FcεRII</td>
<td>The low affinity IgE receptor (CD23)</td>
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<td>FDC</td>
<td>Follicular dendritic cell</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FO</td>
<td>Follicular</td>
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<td>GI254023X</td>
<td>ADAM10 selective inhibitor</td>
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<tr>
<td>GM-CFU</td>
<td>Granulocyte monocyte- colony forming unit</td>
</tr>
<tr>
<td>GSI</td>
<td>γ-secretase inhibitor</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>Hes</td>
<td>Hairy enhancer of split</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>i.v.</td>
<td>Intravenous</td>
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<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>kuz</td>
<td>Kuzbanian</td>
</tr>
<tr>
<td>Lag-3</td>
<td>Lymphocyte activated gene 3</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LZ-CD23</td>
<td>Chimeric CD23 composed of a leucine zipper attached to the extracellular domain of CD23</td>
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<tr>
<td>M15</td>
<td>Anti-leucine zipper mAb</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic cell sorting</td>
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<td>MDSCs</td>
<td>Myeloid-derived suppressor cells</td>
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<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MZB</td>
<td>Marginal zone B cell</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
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<td>NP40</td>
<td>Nonidet-P40</td>
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<tr>
<td>NRR</td>
<td>Negative regulatory region</td>
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<td>OVA</td>
<td>Ovalbumin</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PerCP</td>
<td>Peridinin Chlorophyll Protein</td>
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<tr>
<td>PKC(\Theta)</td>
<td>Protein kinase C theta chain</td>
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<tr>
<td>PMZB</td>
<td>Marginal zone B cell precursor</td>
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<tr>
<td>pre-B</td>
<td>B cell precursor</td>
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<td>pro-B</td>
<td>B cell progenitor</td>
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<td>PS</td>
<td>Presenilin</td>
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<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RBP-J(\kappa)</td>
<td>Canonical Notch transcription factor</td>
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<tr>
<td>RIP</td>
<td>Receptor intramembrane proteolysis</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<tr>
<td>sCD23</td>
<td>Soluble CD23</td>
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<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>T1</td>
<td>Transitional type 1 B cell</td>
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<tr>
<td>T2</td>
<td>Transitional type 2 B cell</td>
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<tr>
<td>TACE</td>
<td>TNF-α converting enzyme</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T cell acute lymphocytic leukemia</td>
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<tr>
<td>TCRβ</td>
<td>T cell receptor β chain</td>
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<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
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<tr>
<td>Th2</td>
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<tr>
<td>Th17</td>
<td>T helper cells that secrete IL-17</td>
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<tr>
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<td>Tumor necrosis factor</td>
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<tr>
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<td>wild-type</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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ABSTRACT

ADAM10 IS A CRITICAL REGULATOR OF B CELL DEVELOPMENT, ANTIBODY PRODUCTION, AND MDSC EXPANSION: EFFECTS OF B CELL-SPECIFIC ADAM10 DELETION AND OVEREXPRESSION IN VIVO

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Proteolytic processing of transmembrane receptors and ligands can have dramatic effects on cell signaling and subsequent cellular responses. Previous studies demonstrated that a disintegrin and metalloproteinase 10 (ADAM10) may cleave numerous B cell-expressed receptors, including the low affinity IgE receptor (CD23). However, lethality of ADAM10-deficient embryos has limited examination of these cleavage events in lymphocytes. To investigate their role in B cell development and function, we generated B cell-specific ADAM10 knockout mice. Intriguingly, deletion prevented development of the entire marginal zone B cell (MZB) lineage. Further analysis revealed that ADAM10 is required for S2 cleavage of the Notch2 receptor and initiation of Notch2 signaling, which is required for MZB development. Additionally,
cleavage of CD23 was dramatically impaired in ADAM10-deficient B cells. This finding and results of *ex vivo* cleavage assays demonstrated that ADAM10 is the principal *in vivo* sheddase of CD23. Previous studies have demonstrated that Notch signaling and CD23 cleavage regulate antibody production. Accordingly, deletion of ADAM10 profoundly inhibited germinal center formation, and T-dependent and T-independent antibody responses to immunization, implicating ADAM10 as a novel regulator of adaptive immunity.

Additionally, to determine the role of ADAM10 activity in hematopoiesis, we generated transgenic mice (A10Tg) that overexpress the protease on lymphoid and myeloid progenitors. Surprisingly, this markedly suppressed B2 cell development and promoted dramatic expansion of myeloid-derived suppressor cells (MDSCs) via a cell intrinsic mechanism. A10Tg MDSCs inhibited T cell proliferation and adoptive immunotherapy of B16 melanoma, resulting in exacerbated metastatic progression that was prevented by MDSC depletion. Thus, A10Tg mice represent a novel model for the examination of MDSC development and MDSC-mediated immune suppression in a tumor-free environment. Finally, hematopoietic stem cell cultures revealed that ADAM10 overexpression directs myeloid development by dysregulating Notch signaling via uncoupling the highly regulated proteolysis of Notch receptors. Collectively, these findings demonstrate that ADAM10 is a critical regulator of Notch signaling, B cell development, and MDSC expansion. Moreover, they have important implications for the treatment of numerous CD23 and Notch mediated pathologies, ranging from allergy to cancer.
INTRODUCTION

I. A disintegrin and metalloproteinases

Given that receptor:ligand binding events are critical for intercellular signaling, proteolytic processing of transmembrane receptors and ligands can have a dramatic impact on signaling-mediated cellular responses. Proteolysis of eukaryotic proteins is performed by members of the metzincin superfamily, including matrix metalloproteinases (MMPs), disintegrin and metalloproteinases (ADAMs), and ADAM-thromospondins (ADAM-TS). Although all active metzincin proteases contain zinc-binding motifs within their protease domains, the presence of a transmembrane domain is characteristic of the ADAMs family. Thus, studies examining the processing of receptors and ligands expressed on cell surfaces have focused on ADAMs, which perform ectodomain shedding as well as regulated intramembrane proteolysis (RIP) of transmembrane proteins. Shedding extracellular domains of membrane-anchored proteins releases soluble fragments into extracellular space. This event can down-regulate signaling events that require transmembrane receptor expression or activate paracrine signaling by soluble products derived from ADAM substrates, such as soluble CD23 (sCD23) and tumor necrosis factor-α (TNF-α). Although ectodomain shedding is thought to occur constitutively, RIP requires the binding of ligands expressed on adjacent cells. Numerous receptors including Notch and CD44 require regulated proteolysis of the
receptor:ligand complex to initiate the release of receptor intracellular domains (ICD) that translocate to the nucleus and alter gene expression. Mutations in the negative regulatory region (NRR) of ADAM substrates can cause ligand-independent intramembrane proteolysis, resulting in excessive ICD signaling and disease states, discussed below\(^1\text{-}^3\).

II. The emergence of ADAM10 as a regulator of lymphocyte development and activation.

The prototypical ADAM contains an inhibitory pro-domain, a highly conserved metalloprotease domain, a disintegrin domain conferring substrate specificity, a cysteine-rich region, a transmembrane portion, and a cytoplasmic tail capable of binding SH3 domains\(^3\). Although 38 ADAMs have been identified to date, a subset of members, including ADAMs 8, 9, 10, 12, 15, 17, and 33, contain the conserved zinc-binding consensus motif that infers proteolytic activity to the protease domain. As a result of in\(^\text{vitro}\)-based assays utilizing pharmacologic inhibitors and various ADAM-deficient murine embryonic fibroblasts (MEFs), ADAM10 has emerged as an important mediator of ectodomain shedding and RIP of numerous substrates, including amyloid plaque precursor (APP), Ephrins, cadherins, chemokines, Notch receptors, Delta-1, CD23, Lag-3, FasL, and CD44. Proteolytic processing of many of these substrates contributes to the pathogenesis of multiple disease states, including cancer and inflammation\(^1,^2\). In particular, the role of ADAM10-mediated APP processing and its effect on Alzheimer’s disease have been extensively studied and are reviewed elsewhere\(^4\). Here, we discuss
recent studies that highlight the physiologic role of ADAM10-mediated cleavage events in lymphocyte development and inflammation related to autoimmunity. Additionally, we review reports describing novel functions of ADAM10, and the regulation of proteolytic activity.

A. The rate-limiting protease in Notch RIP.

1. A historical perspective. Since its discovery, it was evident that ADAM10 and its drosophila homolog, Kuzbanian (kuz), perform critical roles in developmental pathways. Kuz was initially identified by Rooke et al. in 1996, who generated kuz− embryos that revealed its essential role in lateral inhibition required for development of peripheral and central nervous systems. Cloning and sequence analysis of kuz demonstrated the presence of disintegrin and metalloproteinase domains. Data base searches identified a mammalian homolog (43% amino acid identity), bovine metalloprotease (BMP, later named ADAM10) that was isolated from brain myelin and shown to cleave myelin basic protein in vitro. By overexpressing dominate negative (DN) mutants of kuz that lack the protease domain in drosophila and Xenopus, Pan et al. demonstrated that the defect in lateral inhibition is due to the requirement for kuz to initiate RIP-mediated signaling through the Notch receptor. This finding was supported by Sotillos et al. However, they acknowledged phenotypic differences between kuz and Notch mutant flies and concluded that Notch could also be processed in a kuz-independent manner.

In mammalian cells, signaling through Notch receptors critically regulates cell fate processes of numerous cell types, including murine embryos. Following receptor
ligation of Notch ligands, including Delta-like (Dll)1-4 or Jagged 1-2, expressed on adjacent cells, Notch signaling is initiated by ADAM-mediated proteolysis of the extracellular domain of the Notch receptor that is endocytosed by the ligand-expressing cell. This cleavage event, termed the S2 cleavage, produces a substrate for the γ-secretase complex to perform S3 cleavage and release the Notch intracellular domain (NICD) from the cell membrane. This results in NICD translocation to the nucleus, where it complexes with the transcription factor RBP-Jκ, and induces transcription of Notch target genes, including Hairy enhancer of split (Hes1), Hes5, and Deltex-1 (Figure 1).

Although numerous regulators of Notch signaling have been identified, the identity of the metalloprotease that initiates RIP-mediated Notch signaling in mammalian cells was highly controversial\(^\text{10}\). Initially, two independent groups concluded that ADAM17 (TNF-α converting enzyme, TACE) initiates signaling by performing S2 cleavage of the Notch1 receptor\(^\text{11, 12}\). Loading cell membrane fragments from Notch-transfected HeLa cells onto RED-TSK columns resulted in co-elution of Notch receptor cleavage products with ADAM17, rather than ADAM10. Additionally, results of an in vitro cleavage assay and a monocytic cell differentiation experiment demonstrated ADAM17’s ability to cleave truncated forms of Notch1 and direct Notch-dependent monocyte differentiation\(^\text{11}\). Mumm et al. supported this finding by demonstrating Notch cleavage in transfected MEFs derived from ADAM10-null embryos\(^\text{12}\). As a result, ADAM17 is often referenced in the literature as the relevant proteinase that initiates Notch signaling\(^\text{13}\). Although these findings were in direct contrast to studies of kuz in *drosophila*, the authors could not rule out a role for ADAM10 in Notch cleavage, and
suggested that ADAM10 and ADAM17 may be functionally redundant in vivo. This conclusion was supported by later in vitro studies demonstrating that ADAM10 can cleave many ADAM17 substrates from ADAM17+/− MEFs, while ADAM17 can also cleave numerous ADAM10 substrates. However, a critical role for ADAM10 in Notch activation re-emerged following the generation of ADAM10-deficient mouse embryos that displayed many features also observed in nonviable Notch1−/− embryos. In contrast, embryonic loss of ADAM17 did not result in a Notch phenotype. Following these observations, two groups utilizing ADAM10−/− MEFs recently reported that while multiple proteases can perform ligand-independent proteolysis of Notch1, ADAM10 is required for ligand-dependent cleavage. Thus, ADAM10 may play a more critical role in Notch signaling than earlier in vitro studies predicted. However, in utero lethality of ADAM10-null embryos at day E9.5 limited the examination of ADAM10-mediated cleavage events in the development of other cell types, including lymphocytes.

2. T cell development and activation. The impact of Notch signaling in T cell development has been thoroughly examined, and is reviewed elsewhere. Briefly, Notch1 signaling is essential for the development of thymocyte precursors. This is best illustrated by the presence of thymic B cells in mice that lack Notch1 expression in common lymphoid progenitors (CLPs). Additionally, enforced Notch1 signaling in bone marrow progenitors expressing the constitutively active NICD promotes T cell fate. In fact, human mutations in the NRR surrounding the S2 cleavage site of Notch1 results in ligand-independent proteolysis and excessive Notch1 activation, ultimately causing T cell acute lymphocytic leukemia (T-ALL). This mutation accounts for
approximately 50 percent of all T-ALL cases\textsuperscript{10}. Multiple groups have reported effective use of $\gamma$-secretase inhibitors (GSIs) in limiting T cell development \textit{in vitro} and in mouse models of T-ALL\textsuperscript{25, 26}. However, GSIs cause gastrointestinal disease and must be coupled with potent anti-inflammatory drugs, such as dexamethasone, in T-ALL mouse models\textsuperscript{25}. Thus, there has been great interest in elucidating the role of ADAMs in Notch1-mediated thymocyte development.

Manilay et al. circumvented the limitation of ADAM10\textsuperscript{-/-} embryo lethality by generating transgenic mice that overexpress the dominant negative form of ADAM10 (dnKuz) under control of the T cell-specific promoter, lck\textsuperscript{27}. dnKuz expression caused a partial block in thymocyte development between the double negative (DN) and double positive (DP) stages. This corresponded to decreased TCR$\beta$ expression and pre-mature down-regulation of CD25. Although these findings were also observed in Notch1-deficient thymocytes\textsuperscript{28}, there were discrete differences in thymocyte development and gene expression between these mice. dnKuz mice have reduced levels of DN thymocytes and $\gamma\delta$ T cells, whereas conditional Notch1-deficient mice do not, suggesting that ADAM10 may regulate early thymocyte development by processing other substrates, in addition to Notch1. Additionally, expression of Notch target genes, Hes1 and Deltex-1, was only moderately decreased compared to non-transgenic mice. However, this could be due to the persistence of endogenous ADAM10 expression in dnKuz mice.

Interestingly, enforced expression of the predominant Notch ligand,Dll1, rescued thymocyte development in dnKuz mice in a non-cell autonomous manner. This indicated that ADAM10 may regulate thymocyte development by processing Dll1 expressed on
adjacent cells. This possibility is supported by studies demonstrating that ADAM10 also
cleaves Dll1 in transfected MEFs\textsuperscript{29}. However, another study demonstrated that Dll1 is a
substrate of multiple other ADAMs\textsuperscript{30}. Nevertheless, the impaired development of dnKuz
thymocytes is also consistent with the conclusion that dnKuz impairs ADAM10-
dependent processing of the Notch1 receptor.

Further examination of ADAM10’s role in thymocyte development awaited the
production of ADAM10-floxed mice, which has resulted in significant progress toward
elucidating the physiologic impacts of ADAM10-mediated cleavage events. By utilizing
lck-cre transgenic mice, Tian et al. reported impaired development and suppressed
Notch1 signaling in ADAM10-deficient thymocytes\textsuperscript{31}. ADAM10-deficient thymocyte
development resembled that of dnKuz and lck-directed Notch1-deficient mice.
Additionally, Tian et al. demonstrated that production of the NICD was not detectable in
ADAM10-deficient thymocytes. However, in contrast to the previous reports, TCR\textsubscript{β}
expression was not altered by ADAM10 deletion. Thus, the authors concluded that
ADAM17 may perform a compensatory role in Notch activation during early stages of
DN thymocyte development. However, generation of ADAM17 mutant and conditional
knockout mice has not resulted in Notch related phenotypes in lymphocytes\textsuperscript{14, 32}.
Interestingly, although the effects of dnKuz and ADAM10 deletion indicate an important
Notch-mediated role for ADAM10 in thymocyte development, neither demonstrated
altered absolute numbers of T cells in the periphery. Moreover, Tian et al., in addition to
Manilay et al., acknowledged that the modest ADAM10-mediated alterations of
thymocyte development do not approach the complete loss of T cell development in mice
lacking Notch1 expression in CLPs. They hypothesize that this is dependent on the promoter driving dnKuz or cre expression. Lck-cre did not completely prevent ADAM10 expression in early DN thymocytes. Thus, examining the role of ADAM10 in CLP-commitment to the T cell lineage and the development of early thymocyte precursors must await the generation of other conditional knockout or transgenic mice.

Notch signaling has also been implicated in multiple T cell-mediated autoimmune diseases, including autoimmune lymphoproliferative syndrome (ALPS) and systemic lupus erythematosus (SLE)33. Additionally, very recent studies have demonstrated a role for Notch ligands, including Dll4, in regulating the differentiation of Th2 and Th17 cells34, 35. The role of ADAM10 in mature T cell differentiation and activation has been examined. A recent study demonstrated T cell activation was dependent upon ADAM10 cleavage of the lymphocyte activated gene, LAG-336. In early stages of T cell activation, effector molecules are upregulated, while inhibitory proteins are inactivated. Lag-3 is an inhibitory transmembrane protein expressed by activated T cells and NK cells that binds MHC class II molecules with a higher affinity than CD4. It regulates the suppressive activity of regulatory T cells and controls activation-induced effector T cell expansion37, 38. Following T cell activation in vivo, soluble Lag-3 accumulates in mouse serum. Li et al. reported that Lag-3 cleavage is required for antigen-specific T cell activation, as non-cleavable mutants prevent proliferation and cytokine production36. The authors also demonstrate that cleavage is mediated by ADAM10 and ADAM17. ADAM10 is responsible for constitutive and activation-induced cleavage, while ADAM17 mediates PKCθ-dependent cleavage. Additionally, ADAM10 siRNA suppressed T cell
proliferation in a Lag-3 dependent manner. One report also described Lag-3 expression on activated B cells. Thus, Lag-3 cleavage may also regulate B cell proliferation. Li et al. have provided the most direct evidence describing a role for ADAM10 activity in T cell activation, and have raised intriguing questions about ADAM10’s role in adaptive immune responses. However, because ADAM10 also regulates Notch-mediated thymocyte development and, in vivo analysis of Lag-3 cleavage may require the use of conditional knockout mice with unaltered thymocyte development.

3. B cell development is marginal. Four Notch receptors, Notch1-4, are expressed in humans and rodents. However, prior studies have only addressed Notch1 activation. It is unclear whether conclusions drawn from studies on Notch1 cleavage can be generalized to other Notch receptors. Specifically, the Notch1 cleavage site recognized by ADAMs is not present in murine Notch2. Additionally, the phenotype of ADAM10−/− embryos was strikingly similar to that of Notch1,4 double knockout embryos, but quite distinct from features of non-viable Notch2 embryos. To date, the role of ADAMs in Notch2 cleavage has not been examined.

Notch2 signaling regulates B cell development. In contrast to T cells, which express Notch1 during development, B lymphocytes preferentially express Notch2, and express minimal levels of Notch1,3, and 4. B2 lymphocytes, which comprise the majority of circulating B cells, develop from CLPs and differentiate into pro-, then pre-, and ultimately immature B cells prior to exiting the bone marrow. Just as Notch signaling promotes CLP commitment to the T cell lineage, it prevents B lineage fate. Thus, multiple studies have demonstrated that enforced expression of active NICD
in bone marrow progenitors completely abrogates B2 cell development\textsuperscript{24,44}. Following exit from the bone marrow, B2 cells enter a transitional stage and undergo further maturation in the spleen, where the majority differentiate into follicular B cells, while a subset develop into cells of the marginal zone B (MZB) cell lineage, including pre-MZBs and MZB cells. By generating B-cell specific Notch2 knockout mice, Saito et al. demonstrated that Notch2 signaling is required for development of the MZB cell lineage\textsuperscript{41}, which initiates immune responses to blood-borne infections and transports antigen into the spleen follicles\textsuperscript{45}. Additionally, deletion of Dll1 from non-hematopoietic cells\textsuperscript{46}, or RBP-Jk from B cells profoundly inhibits MZB development\textsuperscript{41,47}. Many other regulators of Notch2 signaling have been identified. However, the metalloproteinase necessary for initiating signaling is unknown, and a role for ADAM10 in B cell development or Notch2 signaling has not been examined.

4. **Antibody production and germinal center formation.** Since Notch signaling performs an integral role in cell-fate decisions in developing embryos and lymphocyte precursors, it may also contribute to mature lymphocyte differentiation during active immune responses. The initial report of B cell-specific RBP-Jk deficient mice demonstrated that loss of the canonical Notch transcription factor did not influence antibody production in response to T-dependent and T-independent immunizations\textsuperscript{47}. Thus, it appeared that Notch signaling did not influence B cell activation. However, recent studies have challenged this conclusion. Thomas et al. demonstrated that Notch signaling promotes B cell activation by enhancing B cell receptor and CD40 signaling\textsuperscript{48}. Synergy also exists between Notch and NF-κB signaling, which influences multiple B
cell functions\textsuperscript{49}. Additionally, Santos et al. demonstrated that Notch1 activity, which is induced in LPS-stimulated B cells, promotes the differentiation of antibody-secreting B cells\textsuperscript{42}. Furthermore, Yoon et al. reported that the Notch signaling provided by Dll1 expression on follicular dendritic cells (FDCs) is important for the survival of germinal center B cells\textsuperscript{50}. Moreover, CD21/35, which is upregulated by Notch2 signaling, also regulates antibody production\textsuperscript{51,52}. Thus, the role of ADAM10 in adaptive immune responses may be extensive and warrants further study.

5. Myeloid cell development and MDSC expansion. Myeloid cells, including monocytes and neutrophils, develop from HSCs, common myeloid progenitors (CMPs), and CD11b\textsuperscript{+}Gr-1\textsuperscript{+} immature myeloid cells in bone marrow. Immature myeloid cells differentiate into mature cell lineages, including CD11b\textsuperscript{+} monocytes and Gr-1\textsuperscript{+} neutrophils, prior to exiting the bone marrow. However in disease states such as graft-vs-host disease and cancer, CD11b\textsuperscript{+}Gr-1\textsuperscript{+} cells accumulate in the spleen and peripheral blood and suppress anti-graft or anti-tumor T cell responses. Thus, they are termed myeloid-derived suppressor cells (MDSCs). In tumor-bearing mice, their expansion promotes immune escape and tumor progression. Thus, a basic understanding of the mechanisms that cause MDSC expansion is central to improving immune-based therapy for cancer patients\textsuperscript{53}. Even though multiple tumor-derived factors have been implicated in MDSC recruitment from the bone marrow, the role of basic cell-fate signaling pathways, including Notch signaling, has not been thoroughly examined. Although the role of Notch signaling in B and T cell lineage commitment has been thoroughly described, its role in myeloid development is controversial. Several studies indicate that
Notch signaling promotes myeloid cell development. Kawamata et al. reported that enforced expression of the Notch1 intracellular domain or the Notch target genes, Hes 1 or Hes5, by adoptively transferred BM cells caused non-cell autonomous expansion of Mac-1/CD11b+Gr-1+ myeloid cells\(^{44,54}\). This finding was supported by Schroeder et al.\(^{55}\), who also reported impaired myeloid cell differentiation in mice deficient in downstream products of Notch signaling\(^{56}\). However, Bigas et al. reported that both Notch1 and Notch signaling inhibit myeloid differentiation in response to different cytokines\(^{57}\). This is supported by the finding that diminished presenilin (PS) dependent \(\gamma\)-secretase activity in PS1\(^{+/-}\)/PS2\(^{-/-}\) mice results in myeloproliferative disease, characterized by accumulation of Mac-1/CD11b\(^+\)/Gr-1\(^+\) cells in the BM and spleen, causing splenomegaly. Additionally, Qyang et al. showed that \(\gamma\)-secretase inhibition in WT splenocytes resulted in a 2.5 fold increase in GM-CFU formation\(^{58}\). Furthermore, two independent groups reported that constitutive Notch signaling in bone marrow progenitors had no effect on myeloid development\(^{24,59}\). To date, the role of ADAM10 and ADAM10-mediated Notch signaling in myeloid development and MDSC expansion has not been examined. Analysis of mouse models with altered levels of ADAM10 activity in bone marrow progenitors may help clarify these seemingly contradictory results.

**B. Regulation of ADAM10 activity**

Endogenous regulation of ADAM10 activity has not been thoroughly examined. ADAM10 is expressed as a zymogen. Cleavage of the pro-domain by a furin protease results in ADAM10 activation\(^{60}\). Following pro-domain removal, regulation of ADAM10-mediated cleavage is dependent on the structural conformation of the
substrate. If the cleavage site is exposed, cleavage is thought to occur constitutively. However, several reports have indicated that proteolytic activity is enhanced by retinoic acid receptor signaling, PKC signaling, nardilysin expression, cholesterol depletion, N-glycosylation, and calcium influx\textsuperscript{61-66}. Thus, ionomycin is routinely used to enhance ADAM10-mediated cleavage\textsuperscript{14}. Additionally, inhibition of proteolytic activity by tissue inhibitors of metalloproteinases 1 (TIMP1), TIMP3, and the reversion-inducing cysteine-rich protein with Kazal motifs (RECK) has been reported\textsuperscript{67, 68}. These regulatory mechanisms may represent opportunities for pharmacologic intervention of autoimmune disease states in which ADAM10 substrates contribute to pathogenesis or disease progression.

**C. IgE regulation.** Immunoglobulin E (IgE) is the principal mediator of most type I allergic reactions\textsuperscript{69}. A role for IgE in allergic and asthmatic disease is well established. Upon exposure to an inhaled allergen, activated B cells produce allergen-specific IgE antibodies that bind to high affinity IgE receptors (FcεR1) on mast cells and basophils. Subsequent exposure to the allergen results in crosslinking of armed IgE receptors, mast cell and basophil activation and degranulation, and the release of inflammatory mediators that cause or exacerbate a chronic inflammatory process. In allergic asthma, this airway inflammation leads to pathologic responses including eosinophil infiltration, goblet cell hyperplasia, smooth muscle and basement membrane thickening, elevated mucus secretion, bronchoconstriction, and increased airway responsiveness characteristic of asthma\textsuperscript{70}. 
Many of the therapies utilized in managing asthma, including leukotriene receptor antagonists, alleviate symptoms by blocking actions of IgE or downstream mediators without addressing an underlying cause, IgE production. The newest clinically used treatment for asthma is omalizumab (xolair), a humanized anti-IgE monoclonal antibody that binds the constant fraction (Fc) of IgE and prevents binding of IgE to FcεRI\textsuperscript{71}. However, because omalizumab does not inhibit IgE production, it must be injected repeatedly to compensate for continuous IgE production in asthmatic patients. This characteristic and its high financial burden limit its therapeutic value. Inhaled corticosteroids may suppress IgE synthesis, but the utility of this strategy is limited by the lack of target specificity. Hence, blocking synthesis of IgE in a global fashion remains an important research goal.

1. CD23 as a natural regulator of IgE synthesis. The low affinity IgE receptor, CD23, expressed on mouse B cells and FDCs, is a known regulator of allergic and inflammatory responses. Numerous studies have implicated CD23 as a natural regulator of IgE production and IgE-mediated Type 1 hypersensitivity reactions, including allergic airway inflammation. Yu et al. initially reported that immunization of CD23\textsuperscript{-/-} mice created on a low responder background, C57BL/6, resulted in elevated antigen-specific IgE serum levels, compared to wildtype controls\textsuperscript{72}. Utilizing a mouse asthma model, further studies showed that sensitization and challenges of knockout mice resulted in increased eosinophilia, airway hyperresponsiveness, and antigen specific IgE compared to wildtypes\textsuperscript{73, 74}. Additionally, overexpression of CD23 in two independently generated transgenic mice was shown to suppress IgE production \textit{in vivo}\textsuperscript{51, 75}. These
studies led to the hypothesis that binding of IgE to CD23 on B cells transduces an inhibitory signal that suppresses further IgE synthesis (Figure 3). However, cleavage of CD23 from the cell surface may interrupt this negative feedback mechanism, resulting in elevated IgE levels\textsuperscript{76}. This hypothesis was initially supported by the observation that treatment of immunized mice with the metalloprotease inhibitor, batimistat, not only inhibited CD23 cleavage, but also markedly reduced IgE production\textsuperscript{77}.

Additionally, the soluble cleaved product, sCD23, is a contributing mediator in rheumatoid arthritis. CD23\textsuperscript{+-} mice have a delayed onset and reduced severity of collagen-induced arthritis (CIA), and treatment of wildtype mice with anti-CD23 antibodies markedly attenuates disease signs\textsuperscript{78, 79}. Moreover, sCD23 is elevated in synovial fluids of patients with rheumatoid arthritis, and is associated with erosive status\textsuperscript{80}. sCD23 may contribute to rheumatic disease by activating monocytes via interactions with CD11c-CD18\textsuperscript{81}. Because of these findings, inhibiting cleavage by the endogenous CD23 sheddase has been proposed as a novel therapy for controlling allergic and rheumatic disease\textsuperscript{76}.

2. CD23 cleavage. CD23 is the only Fc receptor that is not a member of the immunoglobulin superfamily. CD23 is a calcium-dependent (C-type) type II receptor consisting of a short cytoplasmic tail, a transmembrane portion, an extracellular stalk region forming a coiled-coil structure, and a lectin head domain that binds IgE. CD23 monomers form a homo-trimeric tertiary structure, which is stabilized by IgE binding\textsuperscript{76}. This stabilization can be mimicked by antibody binding to the lectin heads. Anti-lectin antibodies have been shown to inhibit CD23 cleavage and airway inflammation in a
mouse asthma model\textsuperscript{82, 83}. However, in the absence of ligand, monomers are highly susceptible to proteolytic cleavage within the stalk domain. Demonstration that CD23 cleavage is sensitive to hydroxamic acid inhibitors, which inhibit the proteolytic activity of ADAMs, stimulated significant progress toward identifying the sheddase\textsuperscript{76}. Although early studies implicated the proteolytic activity of ADAMs 8 and 15\textsuperscript{84}, subsequent studies ruled out a role for ADAMs 8, 9, 12, 15, and 17 \textit{in vivo} \textsuperscript{14, 85}. Two independent groups utilized ADAM10-null MEFs and pharmacologic inhibitors to demonstrate that ADAM10 activity is responsible for CD23 cleavage\textsuperscript{85, 86}. However, Jackson et al. later reported that MMP-9 is the principal sheddase of CD23 in LPS-treated mice \textsuperscript{87}, calling into question the physiologic relevance of ADAM10-mediated CD23 cleavage. Thus, examination of ADAM10-null B cells from B cell-specific knockout mice should clarify these seemingly contradictory reports.
III. Dissertation Objective.

Two independent groups utilizing ADAM10-deficient MEFs and pharmacologic inhibitors of ADAM10 activity identified ADAM10 as the primary sheddase of CD23.\textsuperscript{85, 86} However, in utero lethality ADAM10\textsuperscript{-/-} embryos prevented examination of ADAM10’s role in IgE production and allergic airway inflammation. Thus, the initial objective of this dissertation was to determine whether ADAM10 is responsible for CD23 shedding in vivo, and to elucidate the physiologic impact of ADAM10-mediated CD23 cleavage on IgE synthesis and allergic airway inflammation.

However, given that previous reports demonstrated an important role for ADAM10 in embryonic and thymocyte development, we also tested the hypothesis that ADAM10-mediated cleavage events also regulate B cell development. To address these objectives, we generated B-cell specific ADAM10 knockout mice and transgenic mice that overexpress the protease on B cell precursors.
Figure 1- ADAM-mediated RIP of Notch2 is required for Marginal Zone B cell development. Signaling through the Notch2 receptor expressed on transitional B cells directs marginal zone B cell differentiation. The Notch2 heterodimer on B cells binds ligands, Jagged 1-2 and Delta-like 1, present on stromal and antigen presenting cells. Binding initiates sequential cleavage events by an unidentified ADAM and a γ-secretase complex. Cleavage releases the Notch2 intracellular domain (N2ICD), containing RAM, nuclear localization, transactivating (TAD), EGF-repeat, and PEST domains. Transport of the N2ICD to the nucleus followed by binding to the transcription factor, RBP-Jκ, allows the release of co-repressors (CoR), and attraction of co-activators (CoA) to the transcriptional complex. The activated complex transcribes Notch target genes, including CD21/35, Deltex-1, Hes 1, and Hes 5, that promote development of marginal zone B cells. Deletion of Notch2 and RBP-Jκ from B cells, or Delta-like 1 from stromal cells prevents marginal zone B cell development.
Figure 1

ADAM

S

γ-secretase

Deltex-1

Hes 1

Hes 5

RBP-J

CoR

Dendritic cell
Macrophage
Stromal cell
FDC

Jagged

Notch 2

γ-secretase

B cell membrane

NICTD

Marginal Zone

B cell development

CD21
Deltex-1
Hes 1
Hes 5

CoA
**Figure 2- Notch signaling regulates B cell development.** Precursors to the B cell lineage develop from common lymphoid progenitors (CLPs) in the bone marrow. Constitutive expression of the Notch intracellular domain (NICD), Hes 1 or Hes 5 in CLPs prevents B lineage commitment, while promoting T cell development. Development of the marginal zone B cell (MZB) lineage is regulated by Notch2 signaling. The majority of transitional cells differentiate into follicular B cells, which constitute the majority of circulating B cells. However, ADAM-mediated Notch2 signaling is thought to promote MZB lineage development. The role of Notch signaling in myeloid development is highly controversial. HSC-hematopoietic stem cell, CMP-common myeloid progenitor, Im-Immature. Solid arrows and dashed arrows indicate confirmed and hypothetical differentiation events, respectively.
Figure 2

- **HSC**
- **Transitional I**
  - CD23-, CD21-, IgMhi
- **Transitional 2**
  - CD23+, CD21int, AA4.1+
- **Marginal Zone**
  - CD23-, CD21hi, IgMhi
- **Follicular**
  - CD23+, CD21int, IgMint
- **Pre-MZB**
  - CD23+, CD21hi, IgMhi
- **Bone Marrow**
  - B220+, IgM-
  - B220+ IgM+
- **Spleen**
  - T lin.
  - B220+, IgM+B220+, IgM-
  - Notch 1
  - Notch 2

**B cell Follicle**
- **Follicular**
  - CD23+, CD21int, IgMint

**Notch**
**Figure 3- Ectodomain shedding of CD23, a regulator of IgE-mediated inflammation.** CD23 monomers, consisting of a short cytoplasmic tail, a transmembrane portion, an extracellular stalk region forming a coiled-coil structure, and a lectin head domain that binds IgE, form a homo-trimeric tertiary structure, which is stabilized by IgE binding. In the absence of IgE or the presence of anti-stalk antibodies, ADAM10 constitutively cleaves CD23 ectodomains and releases soluble CD23 (sCD23). Cleavage prevents IgE:CD23 interaction at the B cell surface, and results in elevated IgE synthesis. This figure was modified from Conrad et al\textsuperscript{76}.
Figure 3

IgE

ADAM10

CD23

sCD23

promoter

X

C"
MATERIALS AND METHODS

I. Mice

A. B cell-specific ADAM10 knockout mice. Generation of ADAM10^{flox/flox} mice on the C57BL/6J (B6) background was performed by inGenious Targeting Laboratories and is illustrated in Figure 4. Briefly, a targeting vector containing intron8, exon9, and intron9 of mouse Adam10 was inserted in the Adam10 gene via homologous recombination. The inserted sequence contains a Neomycin (Neo) cassette flanked by FRT and loxP sites for selection of ES cell clones. Once ADAM10^{flox/+} founders with germline transmission were established, the Neo cassette was removed from the germline by mating progeny with ACT-FLPe^{+/+} transgenic mice expressing the FLP recombinase under control of the human ACTB promoter. This generated ADAM10^{Δ/+} mice, containing exon9 flanked by loxP sites in intron8 and intron9. ACT-FLPe^{+/+} transgenic mice were obtained from Jackson Laboratories. Further detail will be described elsewhere (PJD and HCC, in preparation). CD19cre^{+/+} knock-in and R26R-EYFP^{+} transgenic mice on a C57BL/6 background were obtained from Jackson Laboratories. Crossing ADAM10^{Δ/Δ} and CD19cre^{+} mice generated ADAM10^{Δ/+}CD19cre^{+/−} heterozygotes. ADAM10^{Δ/Δ}CD19cre^{+/−} mice were generated by crossing heterozygotes with ADAM10^{Δ/Δ} mice. R26R-EYFP^{+} mice crossed with ADAM10^{Δ/Δ}CD19cre^{+/−} mice produced ADAM10^{Δ/Δ}CD19cre^{+/−}R26R-EYFP^{+} mice.
B. ADAM10 transgenic mice and Southern blot analysis. ADAM10 transgenic (A10Tg) mice were generated with the ADAM10-pHSE3’ transgene construct. ADAM10-pHSE3’ was produced by subcloning the murine ADAM10-HA cDNA from mADAM10-pcDNA3.1/Zeo into the previously described pHSE3’ vector, containing the murine H-2Kb promoter and IgH enhancer regulatory elements. Briefly, the ADAM10-HA cDNA was excised using BamHI/Sall and ligated into BamHI/XhoI cut pHSE3’. ADAM10-pHSE3’ was amplified and analyzed by restriction endonuclease digestion and sequence analysis. A 7.5-kb XhoI fragment containing both cDNA and regulatory elements (Figure 18A) was excised from ADAM10-pHSE3’ and injected into C57BL/6 (A10Tg line 240) or C57BL/6 x Balb/c (A10Tg line 258) embryos by the Virginia Commonwealth University Transgenic/Knockout Mouse Core. The resulting offspring were screened for the presence of the ADAM10-HA cDNA by PCR analysis of genomic tail DNA using ADAM10 cDNA sense and anti-sense primers (Table 1). Amplification of DNA from A10Tg founders generated a 652-bp PCR product. Transgene integrity was verified and transgene copy number determined for both A10Tg lines by Southern blot analysis (Figure 18B). Briefly, genomic tail DNA from both founders and their F2 progeny was digested with AccI and electrophoresed on a 0.9% agarose gel, and the injection fragment from ADAM10-pHSE3’ was used as both probe and copy number control. A10Tg line 258 was backcrossed with C57BL/6 mice for at least five generations.

C. Additional mice. ADAM10^AA^CD21/35cre^+/−^ mice were generated by crossing ADAM10^AA^ mice with transgenic mice carrying the BAC CD21/35 transgene. CD23
transgenic and CD23\textsuperscript{+/-} mice were previously described\textsuperscript{51, 72}. C57BL/6 mice were purchased from the National Cancer Institute or wild-type littermates of CD23 transgenics. Congenic CD45.1\textsuperscript{+} (B6-Ly5.2) mice were also purchased from the National Cancer Institute. Pmel-1 mice were progeny of breeding pairs purchased from Jackson Laboratories. All mouse protocols were approved by the Virginia Commonwealth University Animal Use and Care Committee.

II. Flow Cytometry, cell sorting, and antibody biotinylation

A. Cell acquisition and labeling. Single cell suspensions of peripheral lymph node cells, splenocytes, and thymocytes were created by disrupting inguinal, brachial, axillary lymph nodes and spleens between glass slides. Additionally, peritoneal fluid cells were obtained by flushing the peritoneal cavity with PBS (5mL). BM cells for cytometric analysis were isolated by flushing excised tibias and femurs with complete RPMI. Following red blood cell lysis, filtered cells were resuspended in residual buffer and treated with unlabeled 2.4G2 (1 µg/10\textsuperscript{6} cells) for 10 min to block Fc receptor binding. Subsequently, cells were labeled with antibodies (0.25 µg/10\textsuperscript{6} cells) including anti-mouse FITC-conjugated CD62L (MEL-14), IL7R (A7R34), B220 (RA3-6B2), and Gr-1 (RB6-8C5); PE-conjugated B220, CD8 (53-6.7), Gr-1, Ter-119, Thy1.2, (30-H12) CD11b (M1/70), CD3ε (2C11), IgD-PE (11-26c.2a), and CD9-PE (MZ3); APC-labeled B220, CD4 (RM4-5), CD5 (53-7.3), CD45.2 (104), IgM (RMM-1) and c-kit (2B8); PE/Cy7-conjugated CD11b, CD23 (B3B4) and sca-1 (D7); APC/Cy7-conjugated CD19 (6D5) and CD45.1 (A20), PerCP/Cy5.5-conjugated IgM (RMM-1), CD1d (1B1) and
IL7R; and biotinylated CD21/35 (7E9) from Biolegend. Additional antibodies included Ly6G-FITC (1A8) and CD21/35-PE (7G6) from BD Biosciences, AA4.1-APC from eBiosciences, and ADAM10-FITC (FAB946) from R&D Systems. Biotinylated HMN2-35 was provided by Hideo Yagita (Juntendo University; Tokyo, Japan) and used at 0.5 µg/10^6 cells for 30 min on ice to label mouse Notch2.

Cells labeled with biotinylated antibodies were washed twice and stained with streptavidin-ECD (Beckman Coulter) for 30 min. All fluorescently labeled cells were washed and resuspended in 0.5 mL PBS containing 2% FBS or fixed with FCM fixation buffer (Santa Cruz) according to manufacturer’s directions. Flow cytometric analysis was performed using an FC500 (Beckman Coulter), Canto or AriaII (BD Biosciences). Data analysis was conducted with CXP analysis (Beckman Coulter) or FCS Express V3 software. Overlays of histograms were generated in SigmaPlot 10.0. as line plots, smoothed using the SMOOTH transform.

**B. Antibody biotinylation.** Anti-mouse Ly6D (49-H4) (BD Biosciences) was biotinylated with EZ-Link Sulfo-NHS-biotin (Pierce). Antibody and biotin were mixed at a molar ratio of 1:500 for 24 hr at 4°C. The mixture was dialyzed with PBS overnight to remove free NHS-biotin.

**C. FACS.** For fluorescence activated cell sorting (FACS) of spleen B cell subsets, T cells were depleted with CD90.2-conjugated magnetic beads (MACS; Miltenyi Biotec). Remaining B220+ or EYFP+ B cell subsets were sorted with an Aria II according to the following criteria; FO: CD23^{int/hi}CD21^{int}IgM^{int}, PMZB: CD23^{int/hi}CD21^{hi}IgM^{hi}, T1: CD23^{low/hi}CD21^{low/hi}IgM^{hi}, and MZB: CD23^{low/hi}CD21^{hi}IgM^{hi}. For HSC isolation, lineage
positive bone marrow cells were depleted with a lineage cell depletion kit (MACS; Miltenyi Biotec). Remaining HSCs (Lin^−IL-7R^−ckit^hi^sca-1^hi^) were sorted with an Aria II. Lineage positive cells included CD3ε, Gr-1, CD11b, B220, and Ter119 positive cells. Mononuclear (CD11b^+^Ly6G^−^), polynuclear (CD11b^+^Ly6G^+^) and total (CD11b^+^Gr-1^+^) MDSCs and T cells (Th1.2^+^) were sorted for suppression assays. Simultaneous flow analysis confirmed that sorted mononuclear and polynuclear MDSCs were Gr-1^+^. Purity of sorted cells exceeded 95%.

III. PCR, RT-PCR, and Quantitative PCR

A. Quantitative PCR. Total RNA was extracted and purified from sorted B cells from mouse spleen using TRIzol reagent (Invitrogen). Samples were treated with DNase (Takara Bio), mixed with Phenol:Chloroform: Isoamyl Alcohol solution, 25:24:1 (USB), and precipitated with ethyl alcohol. The purity of RNA was quantified by NanoDrop Spectrophotometer ND-100. 1 µg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems, ABI). Real-time quantitative PCR (qPCR) was performed with an iQ5 realtime PCR machine (Biorad). All reagents including primers and probes for running a TaqMan qPCR assay were purchased from ABI. Taqman gene expression assays included Hes1:MA00468601_A1, Hes5:MA00439311_g1, Rbp-Jκ:MA00770450_A1, and Deltex-1:MA00492297_A1. PCR products, labeled with 6-FAM-conjugated probes, were amplified with 18S as an internal control. Reaction parameters were as follows, hold at 48°C for 30 min, hold at
95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Results were analyzed with iQ5 real-time PCR software Ver. 2.0.

Quantitative PCR of CD23 mRNA in MEFs was performed by the Nucleic Acids Research Facility (NARF) of Virginia Commonwealth University with Taq-man probes and primers designed by NARF personnel.

**B. RT-PCR.** For RT-PCR analysis, RNA was reverse transcribed as described above. cDNA was amplified using 2X PCR Master Mix (Promega), forward and reverse primers (4 µM), cDNA template (100 ng) and nuclease-free water. RT-PCR was performed using a Mycycler (Biorad) with the following conditions, initial incubation at 95°C for 3 min, 35 cycles of 94 °C for 1 min, 62°C for 45 s, and 72°C extension for 2 min, and final extension at 72°C for 10 min. The PCR products with expected molecular weights were confirmed by electrophoresis using 1.5% agarose gels. β-actin was used as a reference control.

**C. DNA PCR.** For analysis of cre-mediated recombination of ADAM10-exon9, DNA was isolated using Direct PCR Solution (Viagen). PCR amplification was performed using the PCR Master Mix (Promega) as described for cDNA amplification. MangoMix (Bioline) was used for amplification of the ADAM10-HA transgene in transgenic mice. Cycling conditions were as follows, 95°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and final extension at 72°C for 10 min. Sequences of all primers and probes except those included in purchased Taqman gene expression assays described above are listed in **Table 1.**
**Table 1- Primer and probe sequences.** DN- dominant negative, TG-transgenic, S-sense, AS-anti-sense.

<table>
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<td>18S AS</td>
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IV. Immunizations and ELISAs

A. sCD23 and soluble CD21/35 measurement. ELISAs for measuring sCD23 and soluble CD21/35 have been described \(^8\), \(^9\). Mouse serum was collected via tail vein nick and separated from cells following centrifugation.

B. Total Immunoglobulin levels. Serum immunoglobulin levels were measured by ELISAs, previously described\(^{51}\). Briefly, for total IgM, IgG1, IgG2b, and IgG3 measurement, Immulon 96 well plates were coated with 50 µL of 5 µg/mL unlabeled goat ant-mouse Ig in borate buffered saline (BBS), subsequently blocked with 75 µL of Ig block (PBS containing 2% fetal bovine serum (FBS), 1% HEPES, and 0.02% Tween 20). Samples and standards diluted in Ig block were added and serially diluted. Standards beginning at 1 µg/mL were serially diluted 1:2. Ig was detected with goat-anti Ig conjugated to alkaline phosphatase (AP) (1:400 dilution). Chemiluminescence, generated with AP substrate tablets (1 tablet/5 mL substrate buffer, Sigma), was read at wavelengths of 405/650 with a Bectormax 250 plate reader (Molecular Devices). Plates were washed between each step with water (2x), PBS containing 0.02% Tween 20 (2x), and with water again (2x). Antibodies were purchased from Southern Biotech.

For IgE levels, plates were coated with 10 µg/mL rat-anti mouse IgE (B1E3) in BBS and blocked with Ig block. IgE was detected with biotinylated rat anti-mouse IgE (R1E4), followed by Streptavidin-AP (1:400, Southern Biotech).

C. T-dependent antibody production. 6-9 weeks mice were immunized subcutaneously (s.c.) and boosted i.p. 2 weeks later with 100 µg ovalbumin (OVA) in 4 mg of Alum. Peripheral blood was collected via tail-vein nice on days 7, 12, 18, 21, 25,
and 28; and serum was used for ELISAs following centrifugation. For OVA-specific IgG1 antibody detection, Immulon plates were coated with 100 µL of 100 µg/mL OVA (Sigma) and blocked with 5% milk, prior to detection with goat anti-mouse IgG1-AP (1:1000, Southern Biotech). Total levels of immunoglobulin were measured as described above.

**D. T-independent antibody production.** 6-9 week-old mice were immunized i.p. with the T-independent antigens, NIP-LPS (Type 1, 50 µg), or DNP-Ficoll (Type 2, 25 µg, Biosearch Technologies) in PBS. Anti-NIP and anti-DNP titers were measured by coating Immulon plates with 100 µL of 100 µg/mL of NIP-BSA, or DNP-OVA, respectively. Goat anti-mouse IgG1, IgM, IgG2b, and IgG3 antibodies conjugated to AP were used as detection antibodies at a 1:1000 dilution. Serum was collected as described above 7 and 14 days following immunization.

**E. Cytokine Measurement.** For measurement of VEGF, IL-1β, and GM-CSF levels in A10Tg and LM mice, supernatants were collected from cultures of whole splenocytes and bone marrow cells (10×10⁶/mL, 30 min, 37°C) in complete-RPMI. Levels in serum and supernatants were measured using a Biorad multiplex assay and a Bioplex instrument, according to manufacturer’s direction.

**V. Stimulation of CD23 cleavage and Notch signaling in primary B cells.**

For CD23 cleavage, spleen FO B cells isolated by FACS were cultured (1×10⁶/ml) in the presence of IL-4 (10,000 Units/ml), CD40 ligand trimer (CD40L, 25 ng/ml), and 8:A3 (0.7 µg/ml) for 24 h. Cells were washed and restimulated with cytokines in the presence
of 100 µg/ml C0H2 or 19G5 for 17 h. Levels of sCD23 in cell supernatants and mCD23 on B cells were determined by ELISA and flow cytometry, respectively. Trimeric CD40L containing a leucine zipper (LZ) motif was obtained from Amgen. Generation and purification of 19G5 and C0H2 was described. 8:A3 is a monoclonal rat IgG2a antibody directed against the LZ motif of recombinant LZ-CD23, produced by injecting recombinant LZ-CD23 into rats and fusing rat splenocytes with the IR983 cell line as described. Purification was the same as described for 19G5. Baclovirus expressed IL-4 was a gift from W.E. Paul.

For stimulated Notch signaling, spleen FO B cells isolated via FACS were cultured in the presence of CD40L and 8:A3, as described above, and plate bound Fc-DLL1 (10 µg/ml, R&D Systems) or mouse IgG (10 µg/ml, Southern Biotech) for 36, 60, or 84 hrs. Expression of Dtx1, Hes1, and Hes5 were determined by quantitative-PCR.

VI. Immunofluorescence and confocal microscopy

Spleens were frozen on dry ice in Optimum Temperature Compound (Tissue-Tec). Serial 10 µm sections were cut from frozen blocks using a Jung Frigocut 2800E Cryostat, fixed in absolute acetone, and air-dried. Sections were blocked with 10% BSA in PBS to prevent background staining and then washed and incubated for 60 min with different combinations of 2 mg/ml anti-mouse CD169-FITC (MOMA-1, Serotec Inc), GL-7-FITC (BD Biosciences), IgD-PE (11-26), CD23-PE (2G8, Southern Biotech), IgG-Rhodamine-Red-X, goat anti-mouse IgM-AMCA, goat anti-mouse IgG-AMCA (Jackson Immunoresearch), or CD21/35-PE (7G6, BD Biosciences). Sections were washed,
mounted with anti-fade mounting medium, Vectashield, cover-slipped, and examined with a Leica TCS-SP2 AOBS confocal laser scanning microscope fitted with an oil plan-Apochromat 40X objective. Three lasers were used, blue diode (405 nm) for AMCA, Argon (488 nm) for FITC, HeNe (543 nm) for PE and Rhodamine. Parameters were adjusted to scan at 512 X 512 pixel density and 8-bit pixel depth. Emissions were recorded in 3 separate channels. Digital images were captured, overlaid and processed with Leica Confocal and LCS Lite softwares.

**VII. Western blot analysis.** For analysis of ADAM10 expression in transgenic mice, whole cell lysates (30µg) of BM and spleen cells generated with lysis buffer containing 0.5% NP40 and protease inhibitors (Roche) were applied to SDS-PAGE and blotted on nitrocellulose membranes. Blots were probed with HRP-conjugated anti-mouse β-actin (Sigma-Aldrich) or unlabeled rabbit anti-ADAM10 (AnaSpec Inc.) followed by HRP-conjugated anti-rabbit IgG (Southern Biotech). Chemiluminescence was visualized with the Pico chemiluminescent kit (Pierce). For western analysis of CHO cell lines, 1-5x10^6 cells were lysed with 50uL of 0.5% NP-40 containing protease inhibitors. Nuclei free lysates were denatured with NuPage Reducing reagent and SDS, heated at 70°C for 10 minutes and ran on a NuPage 10% Bis-Tris gel (Invitrogen). Protein was blotted onto nitrocellulose membranes (Biorad) and probed for hemaglutin or ADAM10 with HRP-conjugated anti-HA (Bethyl) or unlabeled rabbit anti-ADAM10 antibodies (AnaSpec Inc.) as described above.
VIII. Wright staining.
For photomicrographs of sorted spleen MDSCs, cells were cytospun on glass slides and stained with the HEMA 3 stain set (Fisher Scientific). Photographs were taken with a BIOQUANT NOVA camera attached to an Olympus BH-2 microscope.

IX. Adoptive Transfer and BM chimeras
For adoptive transfer studies, recipient CD45.2+ A10Tg (F240) and CD45.1+ WT mice were irradiated with 950 rads using a 137Cs source (Mark I, Model 68-0146; JL Shepherd & Associates). Donor Lin^- BM cells were isolated from mouse tibia, femur, and humerus by magnetic cell sorting with a lineage cell depletion kit. (MACS; Miltenyi Biotec). 24 hours after irradiation, recipient CD45.2+ A10Tg and CD45.1+ WT mice were injected i.v. with 2.5x10^6 CD45.1+ WT and 5x10^6 A10Tg Lin^- BM cells, respectively. For generation of mixed BM chimeras, a mixture of Lin^- BM cells from CD45.2+ A10Tg (2.5x10^6) and CD45.1+ WT (1.25x10^6) mice were injected i.v. into irradiated CD45.1+ WT mice. Cell populations were analyzed 42 and 63 days after reconstitution.

X. T cell suppression assays
For polyclonal T cell activation, CD90.2+ T cells (6.67x10^4) sorted from spleen via FACS were activated with immobilized anti-CD3 (2C11, 10µg/mL) and soluble anti-CD28 (37.51, 1µg/mL) in 96-well plates. For suppression assays, sorted CD11b+ MDSCs (Ly6G+, Gr-1+, and Ly6G^-) were added at decreasing T cell:MDSC ratios. Control CD90.2^- LM splenocytes were added to achieve a total cell number of 2x10^5/well. For
antigen-specific T cell suppression assays, soluble gp100 (1µg/mL) was added to defined ratios of pmel-1 transgenic splenocytes (6.67x10⁴) and A10Tg MDSCs. After 54hrs of culture, 1µCi of ³H-thymidine was added to each well for an additional 18 hrs. ³H incorporation was measured as previously described.⁹³

XI. HSC cultures
Murine BM-derived HSCs isolated via magnetic cell sorting and FACS were cultured in the presence of IL-7 (1 ng/mL, Peprotech) and Flt3L (5ng/mL, R&D Systems) as previously described⁹⁴. HSC differentiation was examined via flow cytometric analysis, and HSCs were passed onto freshly plated OP9 cells with additional cytokines every 4-5 days. OP9-GFP and OP9-DL1 cells were kindly provided by J.C. Zuniga-Pflucker (University of Toronto). Compound E (100nM, Alexis Biochemicals) and GI254023X (5µM, Glaxo Smith Kline) were used for blockade of γ-secretase and ADAM10 activity, respectively.

XII. Adoptive immunotherapy of B16-melanoma
AIT subsequent to B16-melanoma challenge was performed as previously described⁹⁵. Briefly, donor LM or pmel-1 mice were sensitized in the left footpad with 1x10⁶ GM-CSF-B16 melanoma cells. Ten days later, popliteal draining lymph nodes were harvested, dispersed into a single cell suspension in complete RPMI at 1x10⁶ cells/mL, and activated with bryostatin 1 (5 nM, provided by the National Cancer Institute), ionomycin (10nM, Calbiochem) (B/I), and rIL-2 (80U/mL, Chiron) at 37°C for 18 h.
Cells were washed 3x with complete RPMI, resuspended at 1-2x10^6 cells/mL in the presence of IL-7 and IL-15 (10 ng/mL each, Peprotech), cultured for 5 days and maintained at 1-2x10^6 cells/mL in the presence of fresh cytokines. Recipient mice were injected *i.v.* with 0.25x10^6 B16-melanoma cells. 2 days after B16 inoculation and 1 day prior to AIT treatment, mice were pre-treated *i.p.* with 2 mg cyclophosphamide (CYP, Mead Johnson). For gemcitabine treatment, mice were injected *i.p.* with 1.2 mg 3 days prior to B16 challenge and every 5 days thereafter. For AIT, 2x10^7 expanded donor cells were washed (2x) in PBS, filtered through a 70-µm nylon mesh strainer (Invitrogen), and injected *i.v.* into recipient B16-challenged mice. No systemic cytokines or vaccinations were administered to these tumor-bearing mice. After 14 days, lungs were harvested and lung metastases were enumerated ± SE of 3 mice per group. If the number of metastases (mets) exceeded countable levels, a count of 250 mets was assigned. B16-melanoma and GM-CSF-B16 cells were kindly provided by R. Prell (Cell Genesys, Inc.) and R. Dutton (Trudeau Institute), respectively.

**XIII. Transient and stable transfections.**

DN-ADAM10 was generated by removing the metalloprotease domain and a portion of the prodomain from mouse ADAM10 as described ⁹⁶ and subsequently cloned into pEF4. For transient expression, CD23-CHO cells previously described ⁹⁷ were transfected with DN-ADAM10 in pEF4, empty vectors, or GFP via electroporation (Amaxa) with Nucleofector Kit T in accordance to the manufacturer’s directions. To create stable cell lines, CD23-CHO cells were transfected via nucleofection with DN-ADAM10 pEF or
ADAM10-HA pCDNA3.1. Positive clones were selected with 250 μg/mL Zeocin (Invitrogen) and screened for protein expression via Western blot analysis. ADAM10−/− and heterozygous MEFs provided by C. Blobel were transfected via electroporation with Nucleofector Kit MEF2 and CD23 contained in pTracerEF-blastA (Invitrogen). Positive ADAM10−/−CD23+ and ADAM10+/−CD23+ clones were selected with 3 and 5 μg/mL blasticidin respectively and screened via flow cytometric analysis for CD23 surface expression. Further selection resulted from quantitative PCR analysis of clones with comparable CD23 message expression.

XIV. Cell line CD23 cleavage Assays

CD23-CHO cells were incubated at 5×10^5 cells per mL with C-RPMI (Gibco) containing ionomycin (5μM in DMSO), I9G5 (100μg/mL), DMSO, and/or an isotype control rat IgG2a antibody for 2 hrs. Supernatants were collected to quantify sCD23 release via ELISA, and cells were stained with B3B4-PE, a CD23 mAb (BD Biosciences), to evaluate surface levels of CD23 via flow cytometry. CD23+ MEFs were cultured at 1×10^6 cells per mL in C-DMEM (Gibco) for 24 hours. Supernatants were immunoprecipitated via an Affi gel 10 (Biorad) containing the polyclonal rabbit anti-mouse CD23 antibody. Protein containing gel was incubated at 70°C in SDS for 10 min to release bound protein, which was subsequently analyzed by Western blot analysis.
XV. Co-cultures of MEFs and CHO cells with primary B cells.

CHO cells were co-cultured with primary mouse B cells as previously described 97. MEFs used for co-cultures were plated on gelatin at 3-12x10^3 cells/well in a 96 well plate. Following a 24 hr culture, cells were irradiated with 1x10^4 rads via a Cesium source and subsequently co-cultured with 1x10^4 CD23−/− B cells in the presence of IL-4, CD40L and M15 in B cell media for 8 days as previously described 97.

XVI. Statistical Analysis

P-values were calculated using unpaired two-tailed Student’s 𝑡-tests. Error bars represent the standard error of the mean between samples. * indicates p <0.05.
RESULTS

I. ADAM10 is essential for Notch2-dependent marginal zone B cell development and CD23 cleavage \textit{in vivo}.

Catabolic shedding of CD23 from the cell surface is an important processing event that regulates its function\textsuperscript{76, 98}. Two independent groups have demonstrated the ability of ADAM10 to cleave CD23 \textit{in vitro}\textsuperscript{85, 86}. These studies generated intriguing questions about the role of ADAM10 in the regulation of CD23-mediated immune responses. However, earlier \textit{in vitro} experiments demonstrated the ability of ADAM8 and ADAM15 to cleave CD23\textsuperscript{84}. In addition, a recent study determined that MMP-9 is the principal sheddase of CD23 in LPS treated mice\textsuperscript{87}. Given that many ADAM substrates, including Notch1 and CD23, can be cleaved by multiple proteases \textit{in vitro}, the physiologic relevance of ADAM10-mediated cleavage in B cells is unclear. This requires examination of ADAM10 null B cells from B cell-specific knockout mice.

Additionally, given the importance of ADAM10 in embryonic development and its potential role in thymocyte development, we also formally tested the hypothesis that ADAM10 regulates B cell development. Generation of B cell-specific knockout mice revealed that ADAM10 is a critical regulator of Notch2 signaling; and as a result, it is essential for the development of the entire marginal zone B cell lineage. Additionally, we report that ADAM10 is the primary sheddase of CD23 \textit{in vivo}. Moreover, despite reports
of compensatory roles for ADAMs in vitro and in thymocytes, other proteases did not compensate for the lack of ADAM10 activity in B cells.

A. Generation of B cell-specific ADAM10 knockout mice. To determine the role of ADAM10 in B cell development and CD23 cleavage, we generated B cell-specific ADAM10 knockouts by crossing mice containing loxP sites surrounding exon9 of ADAM10 (ADAM10Δ/Δ) with CD19cre knock-in animals \(^9\) (Figure 4). PCR analysis of DNA isolated from B220\(^+\) and B220\(^-\) splenocytes indicated that cre-mediated recombination of exon9 occurs in an efficient B cell-specific manner in ADAM10Δ/ΔCD19cre\(^+/−\) (ADAM10Δ/Δcre\(^+/−\)) mice (Figure 5A). To better assess the sensitivity and cell-specificity of recombination, we generated ADAM10Δ/ΔCD19cre\(^+/−\)R26R-EYFP\(^+\) (ADAM10Δ/Δcre\(^+/−\)/EYFP\(^+\)) mice that express the EYFP transgene following cre-mediated recombination \(^10\). Deletion of exon9, which encodes the Zn binding domain of the protease active site, produces a frame shift mutation that interrupts transcription. Thus, recombination of ADAM10 in ADAM10Δ/Δcre\(^+/−\)/EYFP\(^+\) mice resulted in the loss of ADAM10 mRNA and protein expression in EYFP\(^+\) cells, including follicular (FO) and transitional type I (T1) spleen B cells (Figures 5B and 5C). Since ADAM10 surface expression is only detectable on EYFP\(^−\) cells (data not shown), EYFP is a reliable reporter for ADAM10 deletion. Recombination detected by EYFP expression occurs in approximately 87 and 95 percent of B220 B cells in the spleen and peripheral lymph nodes (PLN), respectively (Figure 6A). In addition, approximately 96 and 98 percent of EYFP\(^+\) cells from spleen and PLN express B220. This data combined with the
lack of EYFP expression by B220− cells and Thy1+ (CD90) T cells indicates that recombination occurs exclusively in B lymphocytes (Figure 6B).

B. ADAM10 is indispensable for marginal zone B cell development. Because reductions in ADAM10 activity modestly impair thymocyte development 27, 31, we examined the role of ADAM10 in B cell development. Early development was not altered in ADAM10Δ/Δ cre+/− mice. The percentage of pro-B and pre-B cells (B220+IgM−, and immature B cells (B220+IgM+) in the bone marrow did not differ between ADAM10Δ/Δ and ADAM10Δ/Δ cre+/− mice (Figure 7A). Additionally, deletion of ADAM10 did not alter the level of B1 cells in the peritoneum (Figure 8). In contrast, further B cell development in the spleen was drastically altered. Most interestingly, MZB cells (CD23low/−CD21/35hiIgMhi) were not present in ADAM10Δ/Δ cre+/−/EYFP+ mice (Figures 9B and 9D). Immunohistochemical staining of spleen cryosections revealed a complete absence of IgMhi expressing MZB cells surrounding the marginal sinus, labeled with the metallophilic macrophage marker 1 (MOMA-1) (Figure 9E). Additionally, development of precursors to MZB cells, PMZB cells, (CD23int/hiCD21/35hiIgMhi, Figures 9A and 9D) was severely abrogated. Thus, there was also a dramatic decrease in CD1dhiCD9hi B cells, which include PMZB and MZB populations (Figure 9C).

The development of transitional B cells was moderately altered by ADAM10 deletion. The level of type 2 transitional B cells (T2, CD23+CD21/35intAA4.1+) was significantly decreased in knockout mice (Figure 9D and 10), whereas the percentage of type 1 transitional cells (T1, CD23low/−CD21/35lowIgMhi) was modestly elevated compared to heterozygotes (Figure 9B and 9D). Finally, although the percentage of total
B cells in the spleen was not altered (Figure 7B), levels of FO B cells (CD23\(^{\text{int/hi}}\)CD21/35\(^{\text{int}}\)IgM\(^{\text{int}}\)) were significantly elevated in ADAM10\(^{\Lambda/\Lambda}\)cre\(^{+/−}\)EYFP\(^{+}\) mice compared to controls (Figures 9A and 9D). The increase in FO B cells and the reduction in PMZB cells indicate a developmental defect rather than impaired maintenance of the MZB population. Thus, ADAM10 is essential for development of the entire marginal zone B cell lineage.

C. ADAM10 is the primary sheddase of CD23 in vivo. Precursors of MZBs downregulate surface expression of CD23 as they differentiate.\(^{45}\) Given that in vitro studies have demonstrated the ability of ADAM10 to cleave CD23\(^{85,86}\), we considered that ADAM10 may be required for in vivo CD23 cleavage, which may regulate MZB development. To determine whether ADAM10 cleaves CD23 in vivo, we examined the levels of membrane-bound CD23 (mCD23) on B cells and soluble CD23 (sCD23) in serum of ADAM10\(^{\Lambda/\Lambda}\)cre\(^{+/−}\) and control mice. Flow cytometric and immunohistochemical analysis revealed that deletion of ADAM10 resulted in a dramatic increase in mCD23 (Figures 11 and 12). mCD23 was significantly elevated on FO B cells, total spleen B cells and PLN B cells (Figures 11A, 11B, 12A, and 12B). Immunohistochemical labeling illustrates this profound increase on B cells within the spleen follicle, surrounded by the marginal sinus, labeled with MOMA-1 (Figure 11C). Deletion of ADAM10 from B cells also significantly reduced the amount of sCD23 detected in serum of ADAM10\(^{\Lambda/\Lambda}\)cre\(^{+/−}\) mice by 69.1% compared to ADAM10\(^{\Lambda/\Lambda}\) controls (Figure 11D). Importantly, deletion of ADAM10 did not alter CD23 mRNA levels (Figure 12C).
Thus, ADAM10 clearly regulates CD23 surface expression through post-translational proteolysis. These data indicate that ADAM10 is the primary sheddase of CD23 \textit{in vivo}.

Although this result demonstrates that other proteases do not compensate for the absence of ADAM10, a low level of sCD23 was measured in serum of ADAM10^{Δ/Δ}cre^{+/-} mice. This may have been the result of incomplete cre-mediated recombination in B cells (\textit{Figure 6A}), \textit{trans} cleavage of CD23 by secreted proteases or ADAM10-expressing non-B cells, or cleavage of mCD23 from FDCs. To evaluate cleavage in the complete absence of ADAM10, we purified EYFP FO B cells from spleens of ADAM10^{Δ/Δ}cre^{+/-}EYFP^{+} mice. These isolated cells and FO B cells from control mice were stimulated with CD40-ligand (CD40L) and interleukin-4 (IL-4) to promote mCD23 expression prior to the addition of the anti-CD23 stalk antibody, 19G5, or an isotype control antibody, C0H2. Antibody binding to the coiled-coil stalk region of CD23 disrupts the homo-trimeric structure of mCD23 and promotes mCD23 cleavage. As expected, 19G5 reduced mCD23 levels on FO B cells isolated from ADAM10^{Δ/Δ} and ADAM10^{Δ/+}cre^{+/-} mice (\textit{Figure 11E}) and increased sCD23 levels in culture supernatants (\textit{Figure 11F}). In contrast, 19G5 did not influence the level of mCD23 on purified ADAM10-null B cells, and did not enhance release of sCD23 into supernatants. This result further demonstrates that cleavage of CD23 from ADAM10-null B cells is severely impaired.

\textbf{D. CD23 expression does not regulate marginal zone B cell development.}

Deletion of ADAM10 had profound effects on CD23 cleavage and MZB lineage development. Thus, we examined the role of CD23 in MZB cell development. Levels of MZB cells in wild-type C57BL/6, CD23 transgenic (Tg) \textsuperscript{51}, CD23^{+/-} \textsuperscript{72}, and
ADAM10^Δ/Δ^cre^+/−/EYFP^+^ mice were determined by labeling IgM, IgD, and CD21/35 on splenocytes. IgM^hi^IgD^low^ spleen B cells are a heterogeneous population including T1 and MZB cells. However, T1 and MZB cells are CD21/35^low^ and CD21/35^hi^, respectively. Flow cytometric analysis demonstrated that the percent of IgM^hi^IgD^low^CD21/35^hi^ MZB cells in CD23 Tg and CD23^−/−^ spleens did not differ from C57BL/6 mice. In contrast, similarly defined cells were not present in ADAM10^Δ/Δ^cre^+/−/EYFP^+^ spleens (Figure 13). Thus, ADAM10 mediates MZB cell development by a mechanism independent of CD23 cleavage.

E. ADAM10 initiates Notch2 signaling. Previous studies demonstrated that ADAM10-deficient mouse embryos resemble Notch1,4-deficient embryos \cite{17, 18}, and Notch1 signaling is altered in ADAM10-null thymocytes \cite{31}. Although Notch1, Notch3, and Notch4 are minimally expressed by naive murine B cells, signaling through the Notch2 receptor is critical for MZB cell development \cite{41, 42, 43}. Thus, we considered the possibility that ADAM10 is required for Notch2 signaling. Interestingly, ADAM10 and Notch2 are both preferentially expressed on cells of the MZB cell lineage, including PMZB and MZB cells (Figures 14A and 14B). ADAM-mediated cleavage of Notch receptors initiates the release of the Notch intracellular domain (NICD) that mediates downstream signaling. However, rapid degradation prevents detection of physiologic levels of NICD \cite{10}. Thus, to determine if ADAM10 is required for Notch2 signaling in B cells, we examined the expression of Notch2 target genes, including CD21/35, Hes1, Hes5, and Dtx1.
Multiple reports have demonstrated that Notch signaling enhances expression of CD21/35. By generating Notch2fl/CD19cre+/- mice, Saito et al. demonstrated that Notch2 signaling may regulate CD21/35 expression in B cells. Deletion of Notch2 resulted in a significant decrease in CD21/35 surface expression. Our analysis of ADAM10∆∆cre+/- B cells revealed that ADAM10 regulates CD21/35 expression in a remarkably similar manner. Deletion of ADAM10 resulted in a 61% and 67% reduction in CD21/35 expression on the surface of spleen FO B cells (Figure 15A and 15B) and PLN B cells, respectively (Figure 16). Immunohistochemical staining of spleen follicles verified that the reduced expression was specific to B cells. Although CD21/35 labeling of ADAM10∆∆cre+/- follicles was generally diminished, we observed concentrated areas of intense staining (Figure 15E). Labeling of these areas with anti-IgG to identify immune complexes on FDCs revealed that CD21/35 expression on FDCs was intact in ADAM10∆∆cre+/- mice (Figure 15F). Additionally, the levels of soluble CD21/35 in serum were also significantly lower than controls (Figure 15C), demonstrating that all forms of B cell CD21/35 were reduced. Reductions in CD21/35 mRNA levels in sorted spleen B cells established that the suppressed protein expression was the result of diminished transcriptional activation or mRNA stability in ADAM10∆∆cre+/- B cells (Figure 15D).

The absence of MZB development and the diminished expression of complement receptors in ADAM10∆∆cre+/-EYFP+ mice indicate that ADAM10 may be required for Notch2 signaling. To further investigate this hypothesis, we measured the expression of the canonical Notch targets, Dtx1, Hes1, and Hes5 in sorted spleen B cells. Real-time
PCR analysis revealed that expression levels of the Notch2 selective target, Dtx1, and general Notch receptor targets, Hes1 and Hes5, were all significantly reduced in EYFP+ spleen FO B cells from ADAM10^Δ/Δ^cre^+/−^EYFP+ mice compared to ADAM10^Δ/Δ^ and ADAM10^Δ/+^cre^+/−^ controls (Figures 17A-C). Additionally, Dtx1 and Hes1 expression in T1 B cells was significantly suppressed in the absence of ADAM10. Expression levels of Notch targets in B cells from ADAM10^Δ/+^cre^+/−^ heterozygous mice formed a consistent trend of lower expression compared to ADAM10^Δ/Δ^ mice. ADAM10^Δ/+^cre^+/−^ T1 and MZB cells expressed significantly less Hes1 and Dtx1, respectively. These results clearly demonstrate that deletion of ADAM10 profoundly impairs Notch signaling in B cells.

Although signaling through multiple Notch receptors can induce expression of Hes1 and Hes5, expression of Dtx1 and CD21/35 are tightly regulated by Notch2 signaling 41. Thus, ADAM10 regulates signaling through the Notch2 receptor.

We considered the possibility that ADAM10 may be required for the expression of critical Notch2 signaling components, including the Notch2 receptor or the transcription factor, RBP-Jκ. However, Notch2 and RBP-Jκ expression were not altered in ADAM10-null B cells (Figure 17C and data not shown). In addition, engagement of the Notch2 ligand, DLL1, expressed on spleen stromal, antigen presenting cells, and FDCs 50 is also required for Notch2 signal activation and MZB cell development 46. Impaired interaction between ADAM10-null B cells and DLL1 expressing cells in the spleen could also result in decreased expression of Notch2 targets in primary B cells. To directly determine whether ADAM10-null B cells are capable of responding to DLL1, purified FO B cells were stimulated in the presence of a Fc-DLL1 chimera or control
mouse IgG. Fc-DLL1 stimulated expression of Deltex-1, Hes1, and Hes5 in control B cells cultured for 36, 60, or 84 hrs with peak expression observed at 60 hrs. In contrast, ADAM10-null B cells clearly failed to respond to Fc-DLL1 stimulation at any time point. (Figure 17D and data not shown). Thus, B cells lacking ADAM10 are unable to respond to the primary Notch2 ligand that induces MZB cell development. Collectively, these data confirm that ADAM10 is required for initiating Notch2 signaling in B cells.
**Figure 4- Generation of B cell-specific ADAM10 knockout mice.** A targeting vector containing intron8, exon9, and intron9 of mouse *Adam10*, and loxP (gray triangle) and FRT (white triangle) sites was inserted into the *Adam10* gene via homologous recombination to generate an ADAM10Δ/Neo allele. Removal of the Neo cassette via Flp1-mediated recombination generated ADAM10Δ, containing exon9 flanked by loxP sites. In B cells expressing Cre under control of the CD19 promoter (CD19cre+), exon9 of *Adam10* is removed via Cre-mediated recombination. Primers, P1 and P2, amplify a 955 bp sequence of non-recombined ADAM10Δ or a 217 bp sequence of the recombined allele. The schematic was adopted from H. Crawford (Stonybrook University).
**Figure 5- ADAM10 \(^{AA} CD19\text{cre}^{+/-}\) mice are B cell-specific ADAM10 knockouts.** (A) PCR products for exon9 of ADAM10 performed on isolated DNA, using primers P1 and P2 (see Figure 4). Spleen B220\(^+\) B cells and B220\(^-\) non-B cells from indicated mice were sorted via FACS. 955 bp and 217 bp bands represent full length and recombined ADAM10, respectively. (B) RT-PCR for ADAM10 mRNA isolated from follicular (FO) and type I transitional (TI) spleen B cells. FO B cells identified as B220\(^+\)CD23\(^{int/hi}\)CD21/35\(^{int}\)IgM\(^{int}\) and T1 B cells identified as B220\(^+\)CD23\(^{low/-}\)CD21/35\(^{low}\)IgM\(^{hi}\) were isolated via FACS. (C) Flow cytometric analysis of ADAM10 expression on the surface of FO and T1 spleen B cells from indicated mice. Sorted and analyzed cells from EYFPmice were identified as EYFP\(^+\) instead of B220. Representative of 3 independent experiments. EYFP, enhanced yellow fluorescent protein.
A  Purified B cell PCR P1/ P2

B  Purified B cell RT-PCR

C  Spleen Follicular B cells

B220+ CD23int/hi CD21/35int IgMint

Iso  A10ΔΔ  A10ΔΔ cre+/-- EYFP+  A10ΔΔ cre-- EYFP--

Spleen Transitional 1 B cells

B220+ CD23low/hi CD21/35low IgMhi

ADAM10
Figure 6- CD19cre-mediated recombination is B cell-specific. (A) Representative flow cytometric plots of spleen (SPL), and peripheral lymph node (PLN) cells isolated from indicated mice; numbers on plots indicate percent of B220 cells in box. (B) EYFP$^+$-gated cells from ADAM10$^{Δ/Δ}$CD19cre$^{+/−}$EYFP$^+$ mice labeled for B220 and Thy1; representative of 3 independent experiments. (C) Schematic of ADAM10 and EYFP recombination.
Figure 7- B cell development in bone marrow is not altered in ADAM10Δ/Δ CD19cre+/− mice. Flow cytometric analysis of (A) bone marrow (BM) cells stained for B220 and IgM; ProB/PreB: B220^lowIgM^−, Immature B: B220^int^IgM^+; and (B) splenocytes (SPL) stained for B220 and CD3 in the spleen; B cells: B220^+, T cells: CD3^+. Numbers on plot indicate percent of gated cells in quadrant; representative of 4 independent experiments.
Figure 8- ADAM10 deletion does not alter B1 cell development. Flow cytometric analysis of peritoneal cells stained for (A) B220 and CD11b, and (B) B220 and CD5; B1 B cells: B220^+CD11b^{int}, B1a B cells: B220^+CD5^+; numbers on plot indicate percent of gated cells in box; representative of 4 independent experiments.
Figure 9- ADAM10 is essential for marginal zone B lineage development. (A,B) Flow cytometric analysis of spleen B cells labeled for CD21/35 and IgM expression, gated on (A) B220^+CD23^{int/hi} and (B) B220^+CD23^{low/-} cells. (C) Analysis of pre-MZBs and MZBs labeled for CD1d and CD9 expression, (A-C) flow cytometric plots are representative of 6 experiments. (D) Levels of B cell subsets expressed as percent of total spleen B cells; n=6, except n=3 for T2 cells; data is cumulated from 6 independent experiments for all subsets, except for T2 cells, cumulated from 3 independent experiments. (A-D) FO and T1 cells were identified as in Fig. 5. MZB, T2, and pre-MZB cells were identified as follows: T2: B220^+CD23^+CD21/35^{int}AA4.1^+, MZB: B220^+CD23^{low/-}CD21^{hi}IgM^{hi}, pre-MZB: B220^+CD23^{int/hi}CD21/35^{hi}IgM^{hi}. Numbers on plots indicate the percent of B220^+ cells in boxes. Cells from ADAM10^{−/−}cre^{+/−}/EYFP^+ mice were identified as EYFP^+ instead of B220^+. (E) Immunohistochemical labeling of spleen follicles for MOMA-1, IgM, and IgD expression; representative of 3 independent experiments. Scale bar = 100 µm. Immunohistochemistry performed by Mohey El Shikh.
Figure 10- Deletion of ADAM10 modestly impairs type 2 transitional B cell development. Flow cytometric analysis of type 2 transitional B cells (T2) from indicated mice. T2: B220<sup>+</sup>CD23<sup>+</sup>CD21/35<sup>int</sup>AA4.1<sup>+</sup>; numbers on plot indicate percent of B220<sup>+</sup> cells in box. Cells from ADAM10<sup>Δ/Δ</sup>cre<sup>+/</sup>EYFP<sup>+</sup> mice were identified as EYFP<sup>+</sup> instead of B220<sup>+</sup>; representative of 3 independent experiments.
Figure 11- ADAM10 is the primary in vivo sheddase of CD23. (A) Cell surface expression of CD23 on spleen FO B cells from indicated mice; representative of 6 independent experiments. (B) Mean fluorescent intensity (MFI) of mCD23 expression by FO B cells, B220^+CD21^intIgM^int, n=6. (C) Immunohistochemical staining of spleen cryosections from indicated mice; CD23-PE and MOMA-1-FITC, labeling metallophilic macrophages in the marginal sinus surrounding spleen follicles; representative of 3 independent experiments. Scale bar = 100 µm. Immunohistochemistry performed by Mohey El Shikh. (D) Serum sCD23 levels measured by ELISA, n=4. (E) Cell surface expression of CD23 on FO B cells treated with 19G5 or C0H2 ex vivo. FO B cells isolated from spleens as in Fig. 5b were cultured for 24 h with CD40L, IL-4, and 8:A3 to elevate mCD23 levels prior to washing and culture with 100 µg 19G5 or C0H2 with fresh cytokines. Cells and supernatants were collected 17 h later and analyzed for mCD23 expression via flow cytometry (E) and sCD23 via ELISA (F), respectively, n=4. (D-F) are representative and cumulative data of 4 independent experiments.
Figure 12- Total spleen and peripheral lymph node B cells express elevated CD23 on the cell surface. (A) Cell surface expression of CD23 on total spleen and peripheral lymph node (PLN) B cells from indicated mice. (B) Mean fluorescent intensity (MFI) of histograms in (a) and mCD23 expression by EYFP+ cells isolated from ADAM10^{+/+}CD19^{cre+}EYFP+ mice, n=6 for spleen, n=3 for PLN; (A-B) representative and cumulative data from 6 and 3 independent experiments, respectively. (C) Quantitative real-time PCR for CD23 mRNA from B220+ sorted spleen B cells, n=3, cumulative data of 3 independent experiments.
**Figure 13- CD23 expression does not regulate marginal zone B cell development.**

Flow cytometric analysis of spleen B cells from C57BL/6, CD23−/−, CD23 transgenic (TG), and ADAM10Δ/Δcre+/−/EYFP+ mice labeled for surface expression of IgM, IgD, and CD21/35. (A) Numbers indicate percent of B220− or EYFP cells in box. (B) CD21/35 expression of IgMhiIgDlow B cells boxed in (A), numbers indicate percent of B220−IgMhiIgDlow cells within gate. T1: B220+IgMhiIgDlowCD21/35low, MZB: B220+IgMhiIgDlowCD21/35hi. Cells from ADAM10Δ/Δcre+/−/EYFP+ mice were identified as EYFP+ instead of B220−; representative of 3 independent experiments.
Figure 14- ADAM10 and Notch2 are preferentially expressed by MZB lineage cells.

Flow cytometric analysis of (A) ADAM10 and (B) Notch2 surface expression on spleen B cell subsets from ADAM10^+/Δ control mice. Cells were labeled for expression of B220, CD23, IgM, CD21/35, and either Notch2 (HMN2-35) or ADAM10. (C) Notch2 surface expression on transitional 1 (T1) and follicular (FO) spleen B cells from indicated mice. B cell subsets were identified as described in Figure 9; representative of 3 independent experiments.
Figure 15- CD21/35 expression is reduced in ADAM10^Δ/Δ^CD19cre^+/− mice. (A) Cell surface expression of CD21/35 on spleen FO B cells, representative of 4 independent experiments. (B) Quantified MFI of (A). (C) Serum soluble CD21/35 measured by ELISA; n=5; cumulated data from 5 independent experiments. (D) Quantitative PCR of CD21/35 mRNA expression in B220^+^ spleen cells, relative to 18S expression; n=4, cumulated data from 4 independent experiments (E,F) Immunohistochemistry of spleen follicles labeled with (E) IgM-AMCA and CD21/35-PE. Arrows indicate MZB cells with overlay staining. Scale bar = 100 µm. (F) Higher magnification of intense CD21/35 labeling in ADAM10^Δ/Δ^cre^+/−^ follicles, shown in (E). FDC immune complexes are labeled with anti-IgG-AMCA. Scale bar = 50 µm. Immunohistochemistry is representative of 3 independent experiments. Immunohistochemistry performed by Mohey El Shikh.
Figure 16- CD21/35 expression is reduced in peripheral lymph nodes of
ADAM10^{ΔΔ}CD19cre^{+/-} mice. (A) Flow cytometric analysis of cell surface expression of
CD21/35 on PLN B cells, representative of 3 independent experiments. (B) Quantified
MFI of (A) and CD21/35 expression by EYFP^{+} cells from ADAM10^{ΔΔ}CD19cre^{+/-}EYFP^{+}
mice; n=3, except n=6 for ADAM10^{Δ+}CD19cre^{+/-} cumulated data of at least 3
independent experiments.
Figure 17- ADAM10 initiates Notch2 signaling. (A-C) Gene expression of targets of Notch2 signaling in primary B cells determined by quantitative real-time PCR. Cumulated data of at least 4 independent experiments. Transitional 1 (T1), follicular (FO), pre-marginal zone (PMZB), and marginal zone (MZB) B cells identified as in Figure 9 were sorted via FACS. (a) Deltex-1; n=7, (b) Hes1; n=4 and (c) Hes5; n=4. Expression by PMZB and MZB cells from ADAM10\(^{\Delta\Delta}\)cre\(^{+/−}\)/EYFP mice was not determined (n.d.), because of cell loss (see Figure 9). (D) Expression of Deltex-1 in FO B cells stimulated with CD40L and Fc-DLL1 or control mouse IgG for 60 hrs, expression relative to 18S expression; n=3, cumulated data of 3 independent experiments.
II. Hematopoietic dysregulation in ADAM10 transgenics causes MDSC expansion and enhances metastatic progression of B16-melanoma

Generation of B-cell specific ADAM10 knockout mice utilizing CD19-cre knockin animals allowed for examination of ADAM10-mediated cleavage events in mature B cells. However, cre-mediated recombination was not efficient in B cell progenitors. Thus, the physiologic effects of ADAM10-mediated cleavage events on early B cell development have not been addressed. Although the production of conditional knockout mice has improved the understanding of ADAM10’s role in development, many disease states are characterized by excessive production of ADAM10 cleavage products, rather than a lack of ADAM10 activity. Thus, to elucidate the role of ADAM10-mediated cleavage events in B cell precursors, we generated ADAM10 transgenic mice (A10Tg) that overexpress ADAM10 at early stages of lymphoid and myeloid development. We initiated this study to assess the effect of excessive ADAM10 activity on CD23 cleavage and allergic disease. However, because of ADAM10’s role in Notch signaling, we hypothesized that elevated ADAM10 activity would have a fundamental impact on hematopoiesis and dysregulate the development of multiple cell lineages. Here, we demonstrate that ADAM10 activity dramatically impairs B2 cell development, while promoting the striking expansion of MDSCs via a cell-intrinsic mechanism. Examination of Notch signaling in hematopoietic stem cell (HSC) cultures revealed differential effects of S2 and S3 cleavage blockade, and indicated that ADAM10 overexpression alters hematopoiesis by dysregulating RIP-dependent Notch signaling. Moreover, A10Tg MDSCs inhibited T cell activation and caused exacerbated
metastatic progression of B16-melanoma that was prevented by MDSC depletion. Thus, A10Tg mice represent a novel model for the examination of MDSC expansion and MDSC-mediated immune suppression in a tumor-free environment.

A. Overexpression of ADAM10 inhibits thymocyte development.

To examine the role of ADAM10 in early lymphocyte development, we generated A10Tg mice that overexpress ADAM10 cDNA under control of the pHSE3’ vector, containing the H-2Kb promoter and the IgH enhancer region (Figure 18A). The combination of these transcriptional regulatory units was previously utilized to generate multiple transgenic mice, including TCR, CD23, and bkl transgenics, that express proteins of interest in early lymphocyte progenitors. Although use of pHSE3’ can result in T cell expression in some founder lines, inclusion of the IgH enhancer results in preferential expression on B lineage cells. Two founder lines, F240 and F258, carrying the injection fragment in germline cells were generated. Southern blot analysis of genomic DNA from F2 progeny demonstrated that both lines contain similar copy number of the transgene (Figure 18B). Because progeny of both lines have nearly identical phenotypes, the following data are presented from line F240, unless otherwise stated.

Western blot analysis demonstrated that transgene expression resulted in elevated levels of ADAM10 in bone marrow (BM) cells of A10Tg mice (Figure 18E). Flow cytometric analysis of B and T cell progenitors in BM and thymus, respectively, confirmed that pro/pre B cells (B220intIgM-) from both founder lines and a proportion of CD4 single positive thymocytes from F240 mice expressed elevated levels of ADAM10.
(Figures 18C-D, 19A-B). However, other thymocyte populations and peripheral T cells from transgenic mice did not overexpress ADAM10 (Figures 19A-B, 20). Interestingly, although the relative percentage of thymocyte subsets was not significantly altered in A10Tg mice, the amount of total, double negative, and double positive thymocytes was significantly decreased, resulting in a small thymus size (Figures 19C-E). However, this did not affect the number of peripheral T cells in the spleen or peripheral lymph nodes (PLN) (Figure 21 B,D).

B. ADAM10 overexpression prevents development of B2 lymphocytes.

Unexpectedly, overexpression of ADAM10 markedly reduced the levels of pro/pre B cells and immature B cells (B220\(^{hi}\)IgM\(^{+}\)) in the BM (Figure 18C). This led to a near complete loss of peripheral B cells in peripheral blood (PBL), PLN, and the spleen (Figure 22A). Analysis of B cells from the peritoneal fluid revealed that the levels of B1a (B220\(^{int}\)CD11b\(^{+}\)CD5\(^{-}\)) and B1b cells (B220\(^{int}\)CD11b\(^{+}\)CD5\(^{-}\)) in A10Tg mice were not significantly altered compared to littermate (LM) controls, whereas B2 cells (B220\(^{hi}\)CD11b\(^{-}\)CD5\(^{-}\)) were nearly absent (Figure 22B,C). Thus, the ADAM10-mediated block in B cell development is specific to bone marrow-derived B2 cells. This effect was reflected in the serum immunoglobulin (Ig) levels of A10Tg mice, which had significantly decreased levels of IgG subsets, including IgG1, IgG2b, and IgG3, which are predominantly produced by B2 lymphocytes. B1 cells primarily produce IgM\(^{105}\), which was slightly elevated in A10Tg serum (Figure 22D). The enhanced IgM levels may be the result of a compensatory mechanism for the lack of B2 cells and other Ig isotypes.
C. MDSC accumulation in ADAM10Tg mice. Because of the low level of peripheral B cells, the amount of A10Tg splenocytes was expected to be lower than LM controls. However, surprisingly, the spleens of A10Tg mice were enlarged, weighing an average of 2.5 fold more than LM spleens (Figure 23A), and containing twice as many nucleated cells (Figure 21A). The forward and side scatter pattern of A10Tg splenocytes indicated that A10Tg spleens contain a high level of large granular myeloid cells (Figure 23B). Further analysis, as shown in Figure 2C, revealed that approximately 63% of transgenic splenocytes are CD11b+Gr-1+, compared to 5.3% of LM splenocytes. While the majority of wildtype (WT) CD11b+Gr-1+ cells generated in the BM differentiate into mature myeloid cells, including neutrophils (Gr-1+) and monocytes (CD11b+) prior to exiting the BM, A10Tg CD11b+Gr-1+ cells expand in BM, constituting 93.5% of BM cells, and enter the spleen and PBL at dramatically high levels (Figure 23C). CD11b+Gr-1+ cells outside the bone marrow are classified as MDSCs, consisting of monocytic (CD11b+Gr-1intLy6G-) and granulocytic subsets (CD11b+Gr-1hiLy6G+)106. As illustrated in Figure 2E and 2F, both MDSC subsets are present at elevated levels in peripheral blood and spleen, respectively. Light micrographs of sorted A10Tg CD11b+Gr-1+ splenocytes confirmed that A10Tg MDSCs contain both monocytic and granulocytic subsets (Figure 23D).

D. ADAM10 prevents commitment of CLPs to the B cell lineage. The expansion of myeloid cells in conjunction with the blockade of B2 cell development in A10Tg mice indicates that ADAM10 may regulate the commitment of BM progenitors to myeloid or lymphoid lineages. Thus, we attempted to determine the developmental stage
at which this occurs. According to the classical model of hematopoiesis, hematopoietic stem cells (HSCs) in the BM develop into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs), which give rise to early thymocyte precursors or pro-B cells\(^{107}\). Figure 18C illustrates that a small percentage of B220\(^-\) cells was present in A10Tg BM. However, further analysis revealed that the few B220\(^-\)CD19\(^+\) BM cells in A10Tg mice also expressed CD11b and Gr-1 (Figure 24A,B), indicating that alterations in hematopoiesis occur prior to the pro-B cell stage. Thus, we examined the levels of HSCs, CLPs, and CMPs in A10Tg mice. Analysis of BM lineage positive cells (Ter119, CD3\(\varepsilon\), CD11b, Gr-1) and CD19\(^+\) cells demonstrated the near absence of Lin\(^-\)CD19\(^+\) B cells in A10Tg mice. However, the percentage of Lin\(^-\)CD19\(^-\) cells was similar to LM levels (Figure 25A). Lin\(^-\)CD19\(^-\) LM cells contain B220\(^-\) early B cell precursors, which were absent from A10Tg BM (Figure 25B). Lin\(^-\)CD19\(^-\)B220\(^-\) cells can be further divided based upon IL-7 receptor (IL-7R) expression. Gating on IL-7R\(^-\) cells demonstrated that the percentage of HSCs (Lin\(^-\)CD19\(^-\)B220\(^-\)IL-7R\(^-\)c-kit\(^{hi}\)sca\(^{hi}\)) was slightly lower in A10Tg BM, while the percentage of CMPs (Lin\(^-\)CD19\(^-\)B220\(^-\)IL-7R\(^-\)c-kit\(^{hi}\)sca\(^{hi}\)) was 2-fold higher than the level of LM CMPs (Figure 25C). In contrast, analysis of IL7R\(^+\) cells revealed a 2-fold decrease in CLP (Lin CD19\(^-\)B220\(^+\)IL-7R\(^+\)c-kit\(^{int}\)sca\(^{int}\)) levels in A10Tg BM (Figure 25D). Additionally, Inlay et al. recently demonstrated that Ly6D\(^+\) CLPs are committed to the B cell lineage, whereas Ly6D\(^-\) CLPs are uncommitted lymphoid progenitors\(^{108}\). In accordance with their report, 89% of LM B220\(^+\) cells and 30% of LM CLPs expressed high levels of Ly6D (Figure 25E,F). However, only 0.16% of A10Tg CLPs were Ly6D\(^+\). Finally, A10Tg HSCs and CMPs do not overexpress ADAM10 on
the cell surface. However, 55.3% of A10Tg CLPs express elevated levels of ADAM10 compared to 28.4% of LM CLPs (Figure 24C). These findings indicate that hematopoietic alterations in A10Tg mice occur prior to the commitment of CLPs to the B cell lineage.

**E. Hematopoietic expression of ADAM10 alters development.** To confirm that transgene expression on hematopoietic cells was responsible for the observed phenotype, we reconstituted irradiated CD45.2+ A10Tg hosts with Lin B220− BM cells from WT CD45.1+ congenic mice. Reconstitution was very efficient 42 and 63 days after cell transfer (Table 2). The only substantial fraction of hematopoietic A10Tg cells remaining consisted of Ter119+ red blood cell (RBC) precursors in the BM (data not shown). Analysis of CD45.1+ donor cells showed that reconstitution with WT BM cells reversed the hematopoietic alterations in A10Tg mice, resulting in efficient B cell development and a low level of MDSCs present in peripheral blood (Figure 26B). Transfer of CD45.2+ A10Tg Lin B220− BM cells into irradiated WT CD45.1+ congenic hosts also resulted in efficient reconstitution (Table 3). Nearly all leukocytes developed from CD45.2+ A10Tg bone marrow, except for a fraction of PLN, PBL, and splenic mature T cells, which may be resistant to irradiation (data not shown). This adoptive transfer of A10Tg BM into WT hosts recapitulated the altered hematopoiesis in A10Tg mice (Figure 26A). These results demonstrate that enforced expression of ADAM10 in hematopoietic cells alters hematopoiesis, irrespective of ADAM10 expression by host stromal cells.
F. Altered hematopoiesis is the result of a cell autonomous mechanism.

Interestingly, MDSCs expand in A10Tg mice, despite the lack of ADAM10 overexpression on CMPs, most CD11b<sup>+</sup>Gr-1<sup>+</sup> BM cells, and splenic MDSCs (Figures 24C, 27). Therefore, we hypothesized that MDSCs may expand via an indirect mechanism as a result of abrogated B cell development, ADAM10-mediated cleavage of substrates from BM stromal cells \textit{in trans}, or cytokine secretion by developing lymphocytes causing paracrine expansion of myeloid precursors. To address these issues, we performed mixed BM chimera experiments by reconstituting irradiated CD45.1<sup>+</sup> WT hosts with a mixture of Lin<sup>−</sup>B220<sup>−</sup> BM cells from CD45.2<sup>+</sup> A10Tg and CD45.1<sup>+</sup> WT donors. Because the percentage of A10Tg HSCs in Lin<sup>−</sup>B220<sup>−</sup> BM is approximately 50% lower than the percentage of WT HSCs (Figure 25C), a 2:1 ratio of A10Tg:WT Lin<sup>−</sup>B220<sup>−</sup> BM cells was used. This resulted in similar reconstitution of host BM by A10Tg and WT donor cells 42 (40.7:57.7%) and 63 (45.7:53.5 %) days after cell transfer. However, development from BM into peripheral lymphoid organs was less efficient in A10Tg cells, as 65.9% and 81.4% of host spleen and PLN cells, respectively, originated from WT BM at Day 42. The selective development of WT thymocytes was most striking, as 98.1% of thymocytes at day 63 were of WT origin (Table 4). This result supports the diminished thymocyte development in A10Tg mice (Figure 19). However, despite these differences, hematopoietic development of CD45.1<sup>+</sup> WT cells was similar to development of LM control cells, and development of CD45.2<sup>+</sup> A10Tg cells closely mimicked development in A10Tg mice. MDSCs only expanded from A10Tg BM cells, and B lineage cells only differentiated from WT BM (Figure 28A,C,D). The only change
in development was the accumulation of A10Tg Ter119⁺ RBC precursors in the spleen (Figure 28B). These results demonstrate that ADAM10-mediated MDSC expansion is not the result of abrogated B cell development, trans-cleavage of BM stromal cell ligands, nor cytokine secretion, which would cause WT MDSC expansion. Rather, they expand via an intrinsic cell autonomous mechanism.

G. ADAM10 overexpression alters hematopoiesis by dysregulating Notch signaling. Recent studies have demonstrated a critical role for ADAM10 in embryonic, thymocyte, and marginal zone B cell development. All of these effects appear to be mediated by the requirement for ADAM10 to initiate Notch signaling. Therefore, to determine the effect of ADAM10 overexpression on Notch signaling in HSC development, we cultured purified HSCs from LM and A10Tg mice on OP9 stromal cells that either do not express Notch ligands (OP9-GFP) or express a primary Notch ligand, Delta-1, (OP9-DL1). The addition of IL-7 and Flt3L promote WT HSC differentiation into T cells on OP9-DL1 cells and differentiation of B cells on OP9-GFP cells. Examination of OP9-GFP cultures showed that while LM HSCs developed into B cells, A10Tg HSCs only generated CD11b⁺Gr-1⁺ myeloid cells (Figure 29A). Analysis of OP9-DL1 cultures demonstrated that A10Tg HSCs are capable of T cell differentiation in the presence of ligand (Figure 29B). However, their development was delayed compared to LM HSCs. This further supports the adverse effect of ADAM10 overexpression on early thymocyte development. Interestingly, high expression of Delta-1 on OP9-DL1 cells prevented myeloid expansion of A10Tg HSCs (Figure 30A). This result suggests
that ADAM10 overexpression may cause myeloid development by dysregulating Notch signaling in the absence of sufficient ligand.

**H. Differential effects of S2 and S3 cleavage of Notch.** In order to further test the hypothesis that ADAM10 overexpression deregulates Notch signaling, we examined the effects of dysregulating Notch signaling in LM and A10Tg HSCs. Inhibition of Notch signaling with the addition of a γ-secretase inhibitor (GSI) or the ADAM10 inhibitor (GI254023X) to OP9-DL1 cultures prevented LM and A10Tg T cell development (Figure 29C). In accordance with reports of ADAM10’s critical role in Notch S2 cleavage in other cell types, this result demonstrates that ADAM10 also mediates S2 cleavage in developing hematopoietic precursors. Most interestingly, although both inhibitors equally blocked Notch-dependent T cell development, their effects on the development of myeloid and B lineage cells were distinct. ADAM10 inhibition in OP9-DL1 cultures resulted in B cell development of LM and A10Tg HSCs, whereas GSI inhibition caused myeloid development (79.4% of LM and 76.7% of A10Tg cells) that was strikingly similar to the myeloid differentiation of A10Tg cells in OP9-GFP cultures (88.5%) (Figure 30). Additionally, ADAM10 inhibition in OP9-GFP cultures not only resulted in enhanced LM B cell development, but it also rescued B cell development of A10Tg cells and prevented myeloid differentiation. This further supports our finding that ADAM10 activity inhibits B cell development. Finally, GSI treatment of LM OP9-GFP cultures had no effect on HSC development. This demonstrates that GSI treatment only directs myeloid development following Notch ligand binding and ADAM-mediated S2 cleavage, resulting in accumulation of the S2 product. Accordingly, A10Tg
HSCs only developed into Gr-1\(^+\) myeloid cells in the absence of ligand or following GSI treatment. Ligand engagement and GSI activity prevent S2 accumulation and myeloid development in LM and A10Tg cultures. Thus, these results indicate that ADAM10 overexpression may also direct myeloid development by generating excessive S2 product that is not processed in the absence of ligand-induced \(\gamma\)-secretase activity.

I. ADAM10-derived MDSCs are functionally analogous to tumor-derived MDSCs. Because ADAM10 overexpression causes expansion of CD11b\(^+\)Gr-1\(^+\) MDSCs in PBL and spleen, A10Tg mice may represent a novel model for examining MDSC development and MDSC-mediated immune suppression in the absence of confounding tumor-derived factors that also alter the anti-tumor response. It has been demonstrated that tumor-derived MDSCs, particularly of the monocytic fraction, impair the T cell proliferative response\(^{106}\). To test whether A10Tg-derived MDSCs are functional and thus capable of blocking T cell activation, suppression assays were conducted. A10Tg and LM purified T cells were stimulated under polyclonal (Figure 31A) and antigen-specific (Figure 31B) stimulations in the presence of increasing MDSC concentrations. Parallel to tumor-derived MDSCs, A10Tg MDSCs inhibited T cell proliferation, with the monocytic subset (Ly6G\(^-\)) being more suppressive irrespective of the stimulatory condition. Interestingly, A10Tg T cells were more sensitive to suppression than LM T cells. This may be due to their \textit{in vivo} exposure to MDSCs. MDSCs also promote angiogenesis and tumor expansion by secreting VEGF\(^{53}\). Accordingly, the level of VEGF was significantly higher in A10Tg mice, compared to LM controls (Figure 32). Additionally, MDSCs limit T cell migration by downregulating L-selectin (CD62L), a
receptor required for homing of naive CD4+ and CD8+ T cells to lymph nodes. We also observed a reduction in CD62L expression in both CD4+ and CD8+ A10Tg T cell populations (Figure 31C). These initial studies suggest that MDSCs present in tumor-free A10Tg animals are phenotypically and functionally analogous to tumor-derived MDSCs.

**J. Effective adoptive immunotherapy for enhanced metastatic progression in A10Tg mice requires MDSC depletion.** To further test the functionality of A10Tg MDSCs and analyze their physiological significance in the anti-tumor response, we utilized B16 melanoma as a model for tumor establishment and adoptive immunotherapy (AIT). T cells from tumor-sensitized mice were expanded *ex vivo* with common γ-chain cytokines, IL-2, IL-7, and IL-15, and utilized for AIT in tumor-bearing hosts. Figure 33A,B, demonstrates that A10Tg mice had greatly increased B16 metastasis compared to LM controls. The treatment of A10Tg mice with AIT had a minimal effect, suggesting that the presence of MDSCs diminished the activity of adoptively transferred T cells. AIT treatment of LM controls significantly inhibited metastatic progression. However, AIT treatment alone did not completely eliminate metastases (Figure 33A,B). This may be attributed to the lack of lymphodepleting chemotherapy, indicating the importance of lymphodepletion needed to optimize the efficacy of activated T cell infusion in immunotherapy. Several groups have shown the chemotherapeutic anti-metabolite, gemcitabine (GEM) to preferentially decrease MDSC levels in tumor-bearing mice. We tested the response of A10Tg animals to GEM by administrating 1.2mg of GEM every five days for 22 days. GEM administration preferentially reduced the MDSC
population without influencing lymphocyte levels (Figure 33F). Thus, we modified the AIT protocol by incorporating GEM and cyclophosphamide (CYP). CYP causes lymphodepletion in the recipient mice, allowing homeostatic proliferation of transferred T cells\textsuperscript{114}. B16 metastases were elevated in untreated A10Tg mice as compared to the LM (Figure 33C,D). However, GEM treatment normalized metastatic progression in A10Tg animals to that of LM controls. LM and A10Tg mice had minimal responses to the AIT + CYP treatment. However, the addition of GEM resulted in complete regression of metastasis in both groups, reflecting the important role of MDSCs in the anti-tumor response. A photographic representation of lungs from A10Tg mice demonstrates a complete regression of metastases in A10Tg mice receiving tri-therapy (Figure 33D).

The main consequence of MDSC accumulation is the suppression of antigen-specific T cells, rendering them incapable of responding to tumor antigens. The most effective immunotherapy treatment would utilize antigen-specific T cells in addition to chemotherapy. Thus, we used pmel-1 TCR transgenic T cells that are specific for the melanoma peptide gp100 while maintaining the same protocol as described above. Figure 33E illustrates the improvement in the efficacy of AIT using adoptively transferred pmel-1 T cells. A10Tg mice had much higher numbers of metastases than the LM without treatment and responded less favorably to chemotherapy alone. However, A10Tg mice as well as their LM counterparts demonstrated significant reduction in metastases with the AIT + CYP treatment and were able to completely reject the tumor with the addition of GEM. The incorporation of GEM with CYP and AIT allowed for a complete regression of B16 metastases in A10Tg animals (Figure 33E), again implicating the significant
contribution of MDSCs in the anti-tumor immune response. Together, these results demonstrate the physiologic significance of altered hematopoiesis and immune regulation as a result of ADAM10 overexpression.
Figure 18- Generation of ADAM10 transgenic mice. (A) Schematic of the 7.5-kb XhoI injection fragment containing the murine ADAM10-HA cDNA and the murine H-2Kb promoter and IgH enhancer regulatory elements. (B) Southern blot analysis of genomic tail DNA from both founders (F) and their F2 progeny (F2a and F2b) digested with AccI and electrophoresed on a 0.9% agarose gel along with 1 kb DNA Ladder markers (M). The injection fragment shown in (A) was used as both probe and copy number control (5x and 25x). A10Tg lines 240 and 258 possess greater than 25 copies of the transgene, generating 1797 bp junction fragments (1612 bp + 185 bp) indicative of head-to-tail arrays, as well as 1270 bp and 4464 bp internal fragments. The founder of line 258 appears to be mosaic, identifiable as a transgenic by PCR only. Southern blot performed by Mark Subler. (C) Flow cytometric analysis of pro/pre B cells (B220⁺IgM⁻) and immature B cells (B220²IgM⁺) in BM of littermate (LM) and A10Tg progeny. (D) ADAM10 surface expression by B220⁺IgM⁻ cells and B220⁻IgM⁻ cells shown in (C). Dot plots and histograms in (C,D) are representative of 6 independent experiments. (E) Western blot analysis of ADAM10 and β-actin protein levels in whole cell lysates of BM cells from indicated mice; ADAM10 pro-form (80 kDa) and ADAM10 mature form (60 kDa), representative of 3 independent experiments.
Figure 19- Overexpression of ADAM10 inhibits generation of early thymocyte precursors. (A,B) Flow cytometric analysis of thymocyte populations and ADAM10 surface expression (B) by double negative (DN, CD4-CD8-), double positive (DP, CD4+CD8+), CD4+ single positive (SP), and CD8+ SP thymocytes; representative of 4 independent experiments; numbers indicate percentage of gated cells in quadrant. (C,D) Percentage and total number of thymocyte subsets shown in (A), n=4, p<0.05. (D) Thymus from indicated mice, representative of 3 mice per group.
Figure 20- Peripheral A10Tg T cells do not overexpress ADAM10. (A) Flow cytometric analysis of CD4⁺ and CD8⁺ T cells in PLNs. Numbers indicated percent of gated cells within box. (B) ADAM10 surface expression by T cells gated in (A); representative of 4 independent experiments.
Figure 21- ADAM10 overexpression does not alter the level of peripheral T cells.

Cumulative data of the total cell number in indicated organs (A), the number of lymphocytes in PLN (B), the percentage of B cell and myeloid subsets in BM (C), and the amount of lymphocyte and myeloid subsets in spleen (D). n>4, p<0.05.
**Figure 22- ADAM10 overexpression prevents B2 cell development.** (A,B) Flow cytometric analysis of (A) T cells (CD3+) and B cells (B220+) in spleen (SPL), peripheral lymph nodes (PLN) and peripheral blood (PBL); and (B) B cell subsets in peritoneal fluid. Lower panels are gated on B220+ cells in upper panels. B2 cells: B220hiCD11b-CD5-, B1a cells: B220intCD11b’CD5+, B1b cells: B220intCD11b’CD5-. C) Cumulative data of peritoneal fluid B cell subsets from (B); n=4. (D) Serum immunoglobulin (Ig) levels measured via ELISA; n=4. Dot plots and histograms are representative of 6 (A) and 4 (B) independent experiments. Numbers on plots indicated percent of gated cells within box. p<0.05.
Figure 23- ADAM10 overexpression causes the expansion of myeloid-derived suppressor cells. (A) Representative spleens and average spleen weight of indicated mice, n=4. Flow cytometric analysis of (B) forward scatter (FS) vs. side scatter (SS) of splenocytes, the percentage of (C) CD11b^{+}Gr-1^{+} MDSCs present in the bone marrow (BM), PBL, and spleen (SPL), and the percentage of (E,F) CD11b^{+}Ly6-G^{+} and CD11b^{+}Ly6-G^{-} MDSCs in (E) PBL and (F) spleen of indicated mice. (D) 20X and 100X photo micrographs of sorted CD11b^{+}Gr-1^{+} splenocytes from A10Tg mice. Flow cytometry plots and photomicrographs are representative of 4 independent experiments. Numbers on plots indicate the percentage of cells in the indicated quadrant.
A

B

D

E

C

F
Figure 24- A10Tg B220⁺CD19⁺ bone marrow cells express myeloid markers. (A,B) Flow cytometric analysis of (A) CD19 and B220 labeled cells and (B) Gr-1 and CD11b labeled cells, gated on B220⁺CD19⁺ cells shown in (A). (C) ADAM10 surface expression by hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), and common lymphoid progenitors (CLPs) from BM. Numbers on dot plots and histograms indicate percent of gated cells shown in box or linear markers, respectively; representative of 3 independent experiments.
Figure 25- Overexpression of ADAM10 alters hematopoiesis prior to CLP commitment to the B cell lineage. Flow cytometric analysis of (A) Lineage negative (Lin⁻) and CD19 labeled BM cells, (B) B220 and IL-7 receptor (IL7R) expression by Lin⁻ CD19⁻ gated cells, (C,D) c-kit and sca-1 expression of (C) Lin⁻CD19⁻B220⁻IL7R⁻ cells and (D)Lin⁻CD19⁻B220⁻IL7R⁺ cells. (E,F) Expression of Ly6D by (E) CLPs of LM and A10Tg mice, and (F) Lin⁻CD19⁻B220⁺ BM cells from LM mice. Numbers on dot plots and histograms indicate the percentage of BM cells (percentage of gated cells) in box or linear marker, respectively. The cell lineage includes Ter-119, CD11b, Gr-1, and CD3ε positive cells. Plots are representative of 3 independent experiments. Lin⁻ populations are defined as follows, HSCs: Lin⁻CD19⁻B220⁻IL7R⁻c-kit⁻sca-1⁻; CMPs: Lin⁻CD19⁻B220⁻IL7R⁻c-kit⁺sca-1 hi; CLPs: Lin⁻CD19⁻B220⁻IL7R⁺c-kit int sca-1 int.
Lineage
Gr-1
CD11b
Ter-119
CD3ε
Table 2- Efficient reconstitution of A10Tg mice with congenic WT bone marrow cells. The percentage of cells (± standard error) in indicated organs that developed from adoptively transferred WT CD45.1+ Lin BM or host A10Tg CD45.2+ cells 42 and 63 days after irradiation and cell transfer; n=2, except n=4 for PBL data at day 42.

WT (CD45.1+) → A10TG (CD45.2+)

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<th>Spleen</th>
<th>PLN</th>
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Table 3- Efficient reconstitution of congenic WT mice with A10Tg bone marrow cells. The percentage of cells (± standard error) in indicated organs that developed from adoptively transferred A10Tg CD45.2+ Lin BM or host WT CD45.1+ cells 42 and 63 days after irradiation and cell transfer; n=2, except n=4 for PBL data at day 42.

\[
\text{A10TG (CD45.2+) → WT (CD45.1+)}
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Figure 26- Overexpression of ADAM10 in hematopoietic cells alters hematopoiesis in A10Tg mice. (A,B) Flow cytometric analysis of BM and PBL cells gated on (A) CD45.2+ cells developing from adoptively transferred A10Tg Lin− BM cells in CD45.1+ congenic WT mice, and cells gated on (B) CD45.1+ cells developing from adoptively transferred WT Lin− BM cells in A10Tg mice, 6 weeks after transfer. BM and PBL plots are representative of 2 and 4 independent experiments, respectively. Numbers on plots indicate the percentage of gated cells within box.
A10TG (CD45.2+) → WT (CD45.1+)

A

CD45.2+

BM

Gr1

PBL

CD11b

B20

CD3ε

0.51

88.1

0.14

42.2

2.2

B

CD45.1+

WT (CD45.1+) → A10TG (CD45.2+)

B220

Gr1

IgM

CD11b

B20

CD3ε

58.5

20.7

16.6

2.78

58.7
Figure 27- A10Tg MDSCs do not overexpress ADAM10. ADAM10 expression by (A,B) CD11b^+Gr-1^- immature myeloid cells in BM, and (C,D) CD11b^+Gr-1^- MDSCs in the spleen. (C-E) Mouse mammary carcinoma cells (MMC) were injected into LM controls to generate spleen MDSCs. (E) Ly6-G expression by A10Tg and MMC-induced LM MDSCs. (F) Western blot analysis of ADAM10 and β-actin protein levels in whole cell lysates of splenocytes from indicated mice; ADAM10 pro-form (80 kDa) and ADAM10 mature form (60 kDa). Numbers on plots indicate the percentage of gated cells in box (A,C) or quadrant (E). Representative of 3 independent experiments.
Table 4- Development of WT HSCs is more efficient than A10Tg HSCs in mixed bone marrow chimeras. The percentage of cells (± standard error) in indicated organs that developed from adoptively transferred WT CD45.1+ or A10Tg CD45.2+ Lin− BM cells 42 and 63 days after cell transfer into irradiated WT CD45.1+ hosts; n=3, except n=6 for PBL data at day 42.

WT (CD45.1+) + A10Tg (CD45.2+) → WT (CD45.1+)

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<td>17.1 ± 6.4</td>
<td>17.3 ± 1.5</td>
</tr>
<tr>
<td>63</td>
<td>WT-CD45.1</td>
<td>45.7 ± 1.1</td>
<td>98.2 ± 1.0</td>
<td>88.9 ± 0.1</td>
<td>91.6 ± 0.9</td>
<td>84.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>TG-CD45.2</td>
<td>53.5 ± 1.0</td>
<td>1.55 ± 1.1</td>
<td>9.85 ± 0.2</td>
<td>6.76 ± 0.1</td>
<td>13.8 ± 2.2</td>
</tr>
</tbody>
</table>
**Figure 28- ADAM10 alters hematopoiesis by a cell intrinsic mechanism.** Flow cytometric analysis of (A) myeloid, (B) erythrocyte, (C) lymphocyte, and (D) B cell progenitor differentiation in mixed BM chimeras generated as described in the *Methods* 6 weeks after cell transfer. CD45.1+ and CD45.2+ gated cells differentiated from WT and A10Tg Lin⁻ BM, respectively. Ter-119⁺ cells indicate erythrocyte precursors; representative of 3 independent experiments, except PBL data is representative of 6 independent experiments; numbers on dot plots indicate the percent of CD45.1 or CD45.2 gated cells within boxes.
Figure 29- Inhibition of γ-secretase or ADAM10 activity prevents Notch-dependent T cell development. (A,B) Flow cytometric analysis of differentiated HSCs co-cultured with (A) OP9-GFP or (B) OP9-DL1 stromal cells for 8, 17, and 29 days; representative of 4 independent experiments. (C) T cell development of HSCs co-cultured with OP9-DL1 cells in the presence of a γ-secretase inhibitor, Compound E, or an ADAM10 inhibitor, GI254023X; representative of 2 independent experiments.
Figure 30- γ-secretase and ADAM10 inhibition have differential effects on Notch-mediated HSC differentiation. Flow cytometric analysis of HSC differentiation after 29 days of HSC co-culture with (A) OP9-DL1 or (B) OP9-GFP stromal cells as described in the Methods. Compound E, GI254023X, or control DMSO was added to media to inhibit γ-secretase or ADAM10 activity, respectively. DMSO-treated plots are representative of 4 independent experiments; plots of inhibitor-treated cultures are representative of 2 independent experiments. Numbers on plots indicate the percentage of gated cells within boxes.
Figure 31- A10Tg MDSCs inhibit polyclonal and antigen-specific T cell proliferation. (A) Proliferation of LM and A10Tg T cells or (B) pmel-1 TCR transgenic splenocytes in the presence of increasing amounts of CD11b+ MDSCs (Ly6G+, Ly6G−, or Gr-1+). LM and A10Tg T cells were stimulated with immobilized anti-CD3 and soluble anti-CD23. Pmel-1 splenocytes were stimulated with soluble gp100. Ratios indicate the ratio of T cells or splenocytes: MDSCs; representative of (A) 4 and (B) 2 independent experiments; p<0.05. (C) Cell surface expression of L-selectin (CD62L) by CD4+ and CD8+ gated T cells from PLN; representative of 3 independent experiments.
Figure 32- Elevated VEGF production in A10Tg mice. IL-1β, GM-CSF, and VEGF levels in serum and supernatants of whole bone marrow and splenocyte cell cultures. Levels measured by a multiplex cytokine assay are expressed as the ratio of concentrations recovered from transgenic mice to litter mates; n=4, *, p<0.05.
Figure 33- MDSC-depletion restores the anti-tumor response and prevents metastatic progression of B16 melanoma in A10Tg mice. (A) Number of lung metastases in LM and A10Tg animals challenged with B16-melanoma with or without adoptive immunotherapy (AIT) as described in the Methods. (B) Representative lungs of AIT treated mice from (A). (C) Number of lung metastases of LM and A10Tg mice treated as in (A) with the addition of cyclophosphamide (CYP) and gemcitabine (GEM). (D) Representative lungs of A10Tg mice treated with CYP + AIT ± GEM. (E) Number of metastases in mice treated with AIT comprised of pmel-1 transgenic T cells and chemotherapeutics as described in (C). (F) Peripheral blood leukocyte levels in A10Tg mice following i.p. injections with gemcitabine (↑). Tumor studies were completed by Sheinei Saleem.
A. LM vs A10Tg bar graphs showing average number of mets.

B. AIT Treated Lungs images:
- LM
- A10Tg

C. Bar graphs showing average number of mets for different treatments:
- Untreated
- GEM Only
- AIT + CYP
- AIT + CYP + GEM

D. A10Tg Treated Lungs images:
- AIT + CYP
- AIT + CYP + GEM

E. LM vs A10Tg bar graphs showing average number of mets for different treatments.

F. Line graph showing total cell number (10^6) over days:
- MDSCs
- B Cells
- T Cells

*p<0.05 in comparison to respective untreated controls

#p<0.05 in comparison to respective AIT + CYP treatment
III. ADAM10 deletion inhibits antibody production.

Although studies of ADAM10 deletion and overexpression revealed the importance of the protease in B cell developmental pathways, ADAM10 may also contribute to mature B cell functions, including antibody production. The requirement for ADAM10 in Notch2 signaling and CD23 cleavage has generated numerous questions about the role of ADAM10 in immune responses. MZB cells respond quickly to T-independent antigens by secreting IgM, facilitate the deposition of immune complexes on FDCs by shuttling between the marginal zone and the spleen follicle, and perform a protective role in models of sepsis\textsuperscript{45,115}. Thus, it is likely that these responses are impaired in ADAM10\textsuperscript{Δ/Δ}\textsuperscript{cre+/-} mice. Notwithstanding MZB cells, Thomas et al. demonstrated that Notch signaling promotes B cell activation by enhancing B cell receptor and CD40 signaling\textsuperscript{48}. Synergy also exists between Notch and NF-κB signaling, which influences multiple B cell functions\textsuperscript{49}. Additionally, Santos et al. demonstrated that Notch1 activity, which is induced in LPS-stimulated B cells, promotes the differentiation of antibody-secreting B cells\textsuperscript{42}. This has stimulated interest in ADAM10’s role in regulating Notch1 signaling in activated B cells. ADAM10-null B cells also express elevated CD23 and reduced CD21/35, which also regulate antibody production\textsuperscript{51,52}. Thus, the role of ADAM10 in adaptive immune responses warrants further study.

A. ADAM10 deletion inhibits basal Immunoglobulin production. To determine if antibody production is dependent on ADAM10 activity, we measured the level of various immunoglobulin (Ig) isotypes in serum of B cell-specific ADAM10
knockout mice. Basal levels of IgM, IgG1, IgG2b, and IgG3 were significantly decreased in naïve ADAM10^ΔΔcre^+/− mice, compared to control ADAM10^ΔΔ^ mice (Figure 34).

Additionally, levels in ADAM10^ΔΔcre^+/− mice were also significantly less than levels in heterozygous ADAM10^Δ/+cre^+/− mice. This indicates that the observed difference is not due to a reduction in CD19 expression, which is a characteristic of all CD19-cre knockin mice. In contrast to other isotypes, basal IgE production was not affected by ADAM10 deletion. However, the concentration of IgE in mouse serum is profoundly lower than all other isotypes.

**B. T cell-dependent antibody responses are suppressed in B cell-specific ADAM10 knockout mice.** Since basal Ig levels were reduced in sera of ADAM10^ΔΔcre^+/− mice, we examined responses to immunizations with a T cell dependent antigen, OVA, absorbed in the adjuvant, aluminum hydroxide (Alum).

Measurement of total Ig production 7 and 12 days after subcutaneous immunization demonstrated a significant reduction in IgG2b and IgE levels at both time points. Although levels of IgM and IgG1 were not significantly different than levels in control mice, there was a trend of lower production in ADAM10^ΔΔcre^+/− mice. To assess the secondary immune response, mice were boosted i.p. with OVA-alum 14 days after the initial immunization and levels were measured on days 18, 21, 25, and 28. Total IgG1 and IgG2b levels in ADAM10^ΔΔcre^+/− serum were significantly decreased at all time points, except IgG1 at day 21. Additionally, IgE levels at day 18 were significantly decreased. Although variability prevented statistically significant differences in IgE levels at other time points and IgM levels throughout immunization, trends of lower Ig
production in ADAM10<sup>Δ/Δ</sup>cre<sup>+/−</sup> mice existed for all tested isotypes at all time points. Additionally, measurement of OVA-specific IgG1 antibody levels revealed a dramatic reduction in antigen-specific IgG1 production at all time points following the second immunization, and trends of lower production during the primary response (Figure 35).

C. ADAM10 deletion in B cells inhibits T-independent antibody responses.

We considered the possibility that ADAM10 is necessary for the B cell response to T cell-mediated cytokine production or B:T cell interaction during the adaptive immune response. Thus, we examined the role of B cell expressed ADAM10 in T-independent responses. ADAM10<sup>Δ/Δ</sup>cre<sup>+/−</sup> and control ADAM10<sup>Δ/Δ</sup> mice were immunized with the T-independent Type 1 antigen, NIP-LPS, which activates B cells through Toll-like receptor 4 signaling. NIP-specific IgM, IgG2b, and IgG3 antibody production was significantly reduced in ADAM10<sup>Δ/Δ</sup>cre<sup>+/−</sup> mice at either 7 or 14 days following immunization. However, NIP-specific IgG1 production was not significantly different (Figure 36A).

B cells also respond to Type 2 T-independent antigens, including Ficoll, which is a large polysaccharide that crosslinks B cell receptors. To test the role of ADAM10 in Type 2 T-independent responses, mice were immunized with DNP-Ficoll, and DNP-specific antibody production was measured 7 and 14 days following immunization. Antigen-specific IgG2b production was significantly diminished in ADAM10<sup>Δ/Δ</sup>cre<sup>+/−</sup> mice at day 14. Although levels of DNP-specific IgM, IgG1, and IgG3 were not statistically lower than levels in immunized- ADAM10<sup>Δ/Δ</sup> control mice, there were consistent trends of lower production of all isotypes at both time points in ADAM10<sup>Δ/Δ</sup>cre<sup>+/−</sup> mice (Figure 36B).
D. Germinal center formation is markedly suppressed in ADAM10^Δ/Δ cre^{+/−} mice. To determine whether diminished antibody production in ADAM10^Δ/Δ cre^{+/−} mice is due alterations in germinal center formation, spleens from mice immunized with OVA-alum for 28 days were analyzed by immunohistochemistry. An average of 11.67 ± 3.2 (standard deviation) GL7+ germinal centers were observed in spleen cross-sections of ADAM10^Δ/Δ mice. In contrast only 4.5 ± 1.0 germinal centers were present in spleens of immunized ADAM10^Δ/Δ cre^{+/−} mice (p < .001). Additionally, the size of germinal centers and the amount of antigen retained on FDC reticulum was also diminished in ADAM10^Δ/Δ cre^{+/−} mice (Figure 37). Collectively, these data demonstrate that B cell-expressed ADAM10 contributes to germinal center formation and antibody production in response to T-dependent and T-independent antigens.
Figure 34- Suppression of basal immunoglobulin production in B cell-specific ADAM10 knockout mice. Total IgM, IgG1, IgG2b, IgG3 and IgE levels in serum of naïve 6-9 week old ADAM10Δ/Δ, ADAM10Δ/Δcre+/- and heterozygous ADAM10Δ+/cre+/- mice; p<0.05.
Figure 35- T-dependent antibody responses are diminished in the absence of ADAM10. Total IgM, IgG1, IgG2b, IgE, and antigen-specific IgG1 levels in serum of 6-9 week old ADAM10^ΔΔ and ADAM10^ΔΔcre^+/− mice immunized with OVA absorbed in Alum on day 0 (s.c.) and day 14 (i.p.); p<0.05, ↑immunization time point.
ADAM10 floxed/floxed
ADAM10 floxed/floxed Cre+/−

Total IgM (μg/mL)

Days post immunization

Total IgG2b (μg/mL)

Days post immunization

IgE (ng/mL)

Days post immunization
**Figure 36-** ADAM10 mediates antibody production in response to T-independent **antigens.** Antigen-specific IgM, IgG1, IgG2b, and IgG3 antibody levels in serum of 6-9 week old ADAM10^{Δ/Δ} and ADAM10^{Δ/Δ}cre^{+/−} mice immunized with (A) NIP-LPS or (B) DNP-Ficoll dissolved in PBS on day 0 (i.p.); p<0.05; n.d., not detectable.
A

- ADAM10 fl/fl
- ADAM10 fl/fl Cre<sup>+</sup>/

![Graphs showing NIP-specific IgM, IgG2b, IgG3, and IgG1 levels over days 7 and 14 for ADAM10 fl/fl and ADAM10 fl/fl Cre<sup>+</sup>/ samples.](image)

n.d. (not detectable)
B

ADAM10^fl/fl

ADAM10^fl/fl Cre^+/−

![Graphs showing DNP-specific IgM, IgG2b, IgG3, and IgG1 levels over days 7 and 14 for ADAM10^fl/fl and ADAM10^fl/fl Cre^+/− mice.](image)
**Figure 37- ADAM10 contributes to germinal center formation.** Immunofluorescent images of spleen cross sections from ADAM10^{Δ/Δ} and ADAM10^{Δ/Δ}cre^{+/-} mice 28 days following immunization with OVA-alum. Germinal center B cells are labeled with FITC anti-mouse GL-7 (green). Antigen retained on FDC reticulum is labeled with Rhodamine-Red-X anti-mouse IgG (red). All images were taken at 40X magnification on a confocal microscope. Immunohistochemistry provided by Mohey El Shikh.
Antigen Retaining Reticulum [FDCs, anti-mouse IgG]

ADAM10Δ/Δ

ADAM10Δ/Δ cre+/-

Germinal Centers [GC B cells, GL7]

Overlay

40X
IV. ADAM10-mediated CD23 cleavage from non-lymphoid cells regulates IgE production.

Generation of two CD23 transgenic mouse lines by independent groups demonstrated that CD23 overexpression markedly inhibits IgE production. However, results of adoptive transfer studies indicated that non-lymphoid cells conferred suppression of IgE production via CD23 expression, whereas lymphocyte CD23 expression did not play a role. Immunohistochemical staining of germinal centers from CD23Tg mice indicated overexpression of CD23 on FDCs. In vitro co-cultures of isolated FDCs from CD23Tg mice with wildtype B cells resulted in decreased IgE production compared to LM FDCs\textsuperscript{116}. Additionally, an earlier in vitro study demonstrated that IgE production was suppressed in co-cultures of Chinese hamster ovarian (CHO) cells overexpressing CD23 and activated B cells\textsuperscript{97}. These studies suggest that B cell interaction with high levels of membrane-bound CD23 expressed on adjacent non-B cells, namely FDCs, results in signals that inhibit isotype class switching and IgE synthesis. They also indicate that induction of high CD23 expression on FDCs is a potential strategy for controlling reactions to environmental allergens.

A. Elevated ADAM10-mediated cleavage of CD23 from non-lymphocytes enhances IgE production by surrounding B cells. Given that CD23 on surrounding non-B cells may be responsible for suppressing IgE production\textsuperscript{97,116}, we wanted to investigate the effects of non-B cell ADAM10 activity and CD23 cleavage on IgE production. We initially examined whether a dominant negative form of ADAM10 (DN-ADAM10), which lacks the protease domain, could inhibit CD23 cleavage. Chinese
hamster ovarian (CHO) cells that constitutively overexpress murine CD23 (CD23-CHO) on the cell surface were transiently transfected via electroporation with green fluorescent protein (GFP) and DN-ADAM10 or GFP alone. Following transfection, cells were cultured in media containing an anti-stalk CD23 destabilizing antibody (19G5) to promote cleavage or media alone for 24, 48, or 96 hours. Cleavage of CD23 was assessed by analyzing culture supernatants for the presence of sCD23 via ELISA. Results indicated that expression of DN-ADAM10 inhibits cleavage of CD23 by at least 50% (Figure 38).

Although transient transfection with DN-ADAM10 effectively inhibited CD23 cleavage, adaptive immune responses producing IgE require approximately 8 days to produce detectable antibody levels ex vivo. Thus, stable cell expression of DN-ADAM10 or full length ADAM10 was needed. Therefore, CD23-CHO cells were stably transfected with linear constructs either containing DN-ADAM10 or mouse ADAM10 fused to a hemagglutinin tag (ADAM10-HA). Expression of positive clones selected with zeocin was determined by western blot analysis utilizing anti-ADAM10 and anti-HA antibodies. Blots revealed prominent overexpression of both proteins (Figure 39A,B). In order to determine the effect of ADAM10 or DN-ADAM10 overexpression on levels of membrane-anchored CD23, basal cell surface CD23 expression of the generated cell lines was compared to that of the parental cell line, CD23-CHO. Quantitative PCR and flow cytometric analysis revealed that ADAM10-HA overexpressing cells (ADAM10-CHO) had dramatically reduced CD23 surface expression (Figure 39C), even though message levels of CD23 were comparable to that of parental and DN-ADAM10
expressing cells (data not shown). This difference in cell surface expression is probably
due to a higher basal level of CD23 cleavage in ADAM10-CHO cells. Conversely,
surface levels of DN-ADAM10 expressing cells were not significantly increased. It is
likely that levels on CD23-CHO cells were already maximal. Thus, inhibition of
ADAM10 could not result in further increases. In order to better examine the activity of
ADAM10 in DN-ADAM10 and ADAM10-CHO cells, shedding assays similar to those
reflected in Figure 38 were conducted. However, given that a previous study
demonstrated that the calcium ionophore, ionomycin, can stimulate ADAM10 activity, we
added either ionomycin, 19G5, or both to cell cultures of CD23-CHO, DN-ADAM10
or ADAM10-CHO cells for two hours and subsequently measured release of sCD23 via
ELISA and cell surface expression via flow cytometry. Importantly, results demonstrated
that shedding is accelerated by ADAM10 overexpression and inhibited by DN-ADAM10
expression (Figure 39D-F). Additionally, they demonstrated that 19G5 and ionomycin
stimulate ADAM10 dependent shedding of CD23 by independent mechanisms. Once
shedding of CD23 in the various cell lines was characterized, we examined the effects of
ADAM10-dependent cleavage of non-B cell CD23 on IgE production by utilizing an ex
vivo co-culture model previously established in our laboratory. B cells and various
CHO cells were co-cultured for 8 days in the presence of CD40 ligand and IL-4 to
stimulate B cell activation and IgE production. Overexpression of CD23 by CD23-CHO
cells significantly suppressed IgE production by co-cultured B cells (Figure 39G).
However, ADAM10 overexpression in ADAM10-CHO cells partially restored IgE
production by presumably allowing accelerated cleavage of CD23. IgE levels produced

140
in the presence of DN-ADAM10 cells were not significantly different from levels in CD23-CHO co-cultures. This suggests that inhibition of cleavage by DN-ADAM10 is not sufficient to suppress IgE production.

**B. Suppressed ADAM10-mediated CD23 cleavage from non-lymphocytes inhibits IgE synthesis by surrounding B cells.** To determine whether a more complete block in CD23 cleavage can inhibit IgE production by co-cultured B cells, we minimized cleavage by utilizing ADAM10<sup>−/−</sup> MEFs<sup>85</sup>. ADAM10<sup>−/−</sup> and ADAM10<sup>+/−</sup> MEFs were stably transfected with mouse CD23. This resulted in differential resting cell surface expression of surface CD23 in the two cell lines, with considerably higher expression on ADAM10<sup>−/−</sup>CD23<sup>+</sup> MEFs than heterozygous controls (Figure 40A). Quantitative PCR established that the difference in surface levels was not due to differences in CD23 mRNA levels (Figure 40B). Thus, as shown in CHO cell lines, differences in cell surface expression are likely due to differences in CD23 cleavage. In fact, sCD23 is not detectable in supernatants of ADAM10<sup>−/−</sup>CD23<sup>+</sup> MEFs cultured for 24 hrs, but is detectable from ADAM10<sup>+/−</sup>CD23<sup>+</sup> supernatants (Figure 40C). To determine whether inhibition of cleavage in ADAM10-null MEFs could suppress IgE production, ADAM10-deficient and heterozygous controls MEFs expressing CD23 were co-cultured with CD23<sup>−/−</sup> B cells in the presence of CD40L and IL-4 for 8 days. CD23<sup>−/−</sup> mouse B cells were used to isolate CD23 cleavage to non-B cells, as trans-cleavage of B cell CD23 might have introduced a confounding variable. Co-culture revealed that expression of CD23 in ADAM10<sup>+/−</sup>CD23<sup>+</sup> MEFs did not significantly influence IgE production. However, CD23 expression by ADAM10<sup>−/−</sup>CD23<sup>+</sup> clearly suppressed IgE synthesis.
(Figure 40D). This result demonstrates that inhibiting ADAM10-dependent CD23 cleavage from surrounding non-B cells can suppress IgE production \textit{ex vivo}.

C. Deletion of ADAM10 from FDCs. Payet-Jamroz et al. concluded that suppression of IgE production in CD23 transgenics was due to overexpression of CD23 on FDCs\textsuperscript{116}. To determine whether inhibition of ADAM10-mediated cleavage of CD23 from FDCs also regulates IgE synthesis by surrounding B cells \textit{in vivo}, we attempted to generate FDC-specific ADAM10-deficient mice, utilizing the bacterial artificial chromosome (BAC) CD21/35cre transgene. CD21/35 is expressed by murine B cells and FDCs\textsuperscript{52}. Adoptive transfer studies with ADAM10\textsuperscript{Δ/Δ}CD21/35cre\textsuperscript{+/−} mice would allow for isolation of ADAM10-deletion to FDCs only. However, crossing ADAM10\textsuperscript{Δ/Δ} mice with CD21/35cre mice resulted in an unexpected phenotype. Breeding yielded small litters with fewer ADAM10\textsuperscript{Δ/Δ}CD21/35cre\textsuperscript{+/−} mice than predicted by Mendelian genetics. Approximately 50\% of ADAM10\textsuperscript{Δ/Δ}CD21/35cre\textsuperscript{+/−} mice died prior to 6 weeks of age, and the development of those that survived was markedly impaired. They were smaller in size compared to ADAM10\textsuperscript{Δ/Δ} mice, and had a large scab between the eyes. Other areas of the skin, especially that covering the feet, contained numerous scabs and was noticeably dry. Additionally, these mice had profound splenomegaly (Figure 41).

Analysis of Cre-mediated recombination of exon 9 of ADAM10 revealed that recombination was very efficient in numerous organs, including the liver, spleen, heart and brain (data not shown). Thus, the BAC CD21/35cre transgene exhibits Cre-mediated infidelity, and the developmental delay was due to the nearly global deletion of ADAM10. BAC CD21/35cre infidelity was reported by Schmidt-Suppria and Rajewsky
during the generation of ADAM10^{ΔΔ/Δ} CD21/35cre^{+/−} mice. Although this project was not pursued further, this result demonstrates the critical role of ADAM10 in the development of numerous cell types.
Figure 38- A dominant negative form of ADAM10 inhibits CD23 cleavage. CD23-CHO cells were transiently transfected via electroporation with either GFP alone (□) or DN-ADAM10 and GFP (■) containing constructs. After 24 hrs of culture, cells were washed and cultured in media alone or in media supplemented with 19G5 (100 µg/ml). Flow cytometry analysis of GFP expression demonstrated a transfection efficiency of 85%. After 48 hrs, cell free supernatants were analyzed by ELISA for sCD23. DN expression resulted in a 70% and 52% inhibition of sCD23 production with and without 19G5 respectively.
Figure 39- ADAM10 overexpression on non-lymphoid cells enhances IgE production by cleaving CD23. A) An anti-HA blot illustrating the presence of ADAM10-HA in transfected CHO cells. B) An anti-ADAM10 blot revealing stable expression of DN-ADAM10. C) CD23 expression of resting CHO cell lines overexpressing ADAM10-HA or DN-ADAM10. D) CD23 expression by ADAM10-CHO cells after cleavage is stimulated with 19G5, ionomycin, or both. E) Expression by parental CD23-CHO and DN-ADAM10 cells following stimulation of cleavage by 19G5 and ionomycin. F) Levels of CD23 expressed on the membrane of the parental cell line or stable cell lines overexpressing ADAM10-HA or DN-ADAM10 in the presence of 19G5 and/or ionomycin were measured by flow cytometry. G) Stable cell lines were co-cultured with primary naïve B cells activated with IL-4, CD40L, and M15 for 8 days. Total IgE levels were detected via ELISA. Representative of 3 independent experiments.
Effects of ADAM10 activity on IgE production in vitro

Cell Line
- B cells only
- CHO
- CD23-CHO
- ADAM10
- DN-ADAM10

IgE (ng)
- 0
- 100
- 200
- 300
- 400
- 500
- 600

A
B

CD23-CHO  A10-HA

39 kDa
84 kDa

CD23

Cell Number

CD23

Cell Number

CD23

Cell Number

CD23

ADAM10 dependent cleavage in vitro

Treatment (2 hrs)
- Ionomycin
- 19G5
- Ionomycin + 19G5

% membrane CD23 remaining
- 0
- 20
- 40
- 60
- 80
- 100
- 120

CD23-CHO
- ADAM10-HA
- DN-ADAM10

F
G

Effects of ADAM10 activity on IgE production in vitro

Cell Line
- B cells only
- CHO
- CD23-CHO
- ADAM10
- DN-ADAM10

IgE (ng)
- 0
- 100
- 200
- 300
- 400
- 500
- 600

CD23-CHO  A10-HA  CD23-CHO  ADAM10  DN-ADAM10

A10-19G5
- ADAM10-Isotype
Figure 40- ADAM10-deletion inhibits CD23 cleavage in MEFs and suppresses IgE production by surrounding B cells. A) Cell surface and B) mRNA expression of CD23 in ADAM10−/−CD23+ MEFs and heterozygous controls. C) Western blots of immunoprecipitated sCD23 from supernatants of MEFs cultured for 24 hours. D) MEFs were irradiated with 10⁵ rads to prevent MEF overgrowth and co-cultured with 10³ primary mouse B cells isolated from a Balb/c spleen. IL-4 (10³ Units/mL), CD40L (25ng/mL), and M15 (100ng/mL) were added to induce IgE production. Total IgE present in supernatants was measured by ELISA 7 days later. Representative of 3 independent experiments.
MEFs (x10³)

IgE (ng/mL)

ADAM10 +/-
ADAM10 +/- CD23+
ADAM10 -/- CD23+

D

MEFs (x10³)
Figure 41- Cre-mediated infidelity by the BAC CD21/35 Cre transgene results in developmental delay in ADAM10^{ΔΔ}CD21/35cre^{+/-} mice. A) Anesthetized ADAM10^{ΔΔ}CD21/35cre^{+/-} and ADAM10^{ΔΔ} littermate mice. B) Spleens of indicated mice.
Discussion

I. ADAM10 is essential for Notch2-dependent marginal zone B cell development and CD23 cleavage in vivo.

In this study, we formally tested the hypothesis that ADAM10 regulates B cell development. Generation and analysis of B cell-specific ADAM10 knockout mice revealed that ADAM10 critically regulates development of the entire MZB lineage by initiating Notch2 signaling.

The rate limiting step in Notch2 signaling is cleavage within the receptor’s negative regulatory region (NRR) located in the membrane proximal portion of the extracellular domain. The structure of the NRR prevents ligand-independent Notch cleavage. Mutations in the NRR can allow cleavage in the absence of ligand, leading to constitutive Notch signaling. In the case of Notch1, this leads to the formation of T cell acute lymphocytic leukemia (T-ALL) \(^{10}\). Brou et al. and Mumm et al. identified the Notch1 cleavage site in the NRR between Ala-1710 and Val-1711 just 13 amino acids upstream of the transmembrane domain. These studies in combination with more recent reports have demonstrated that ADAM10, ADAM17, and possibly other proteases recognize this cleavage site \(^{11,12,20,21}\). Thus, in vivo inactivation of only ADAM10 or ADAM17 may result in a milder phenotype than Notch1 inactivation. For example, thymocyte development was altered to a lesser degree in ADAM10-deficient thymocytes
compared to Notch1-deficient thymocytes generated with the same cre-expressing transgene \textsuperscript{28,31}. In contrast, the phenotype of B cell specific ADAM10 knockout mice described in this study is strikingly similar to the phenotype reported for Notch2 B cell knockouts. Although the thymocyte-specific knockout mice were generated with a different ADAM10 loxP/loxP allele, the stronger B cell phenotype may indicate that ADAM10 is the only protease that recognizes the Notch2 cleavage site, which is distinct from the Notch1 site previously described \textsuperscript{11}. The effects of ADAM10 deletion on MZB development, CD21/35 expression, Dtx1 expression, and DLL1 stimulated Notch signaling clearly demonstrate ADAM10’s role in Notch2 signaling. However, we do not rule out the possibility that ADAM10 may also be required for signaling through other minimally expressed Notch receptors on B cells. In fact, quantification of Hes1 and Hes5 expression revealed a more significant impairment of Notch signaling in ADAM10-null B cells than reported for Notch2-null B cells \textsuperscript{41}. This may reflect a greater purity of EYFP\textsuperscript{+} ADAM10-null B cells or the ability of other minimally expressed Notch receptors to signal in the absence of Notch2, but not in the absence of ADAM10. Nevertheless, the effect on MZB cell development is clearly caused by impaired Notch2 signaling; and contrary to studies in thymocytes, this study describes an absolute requirement for ADAM10 activity in the development of a lymphocyte subset.

Our finding that ADAM10 initiates Notch2 signaling has important implications for the treatment of Notch-related diseases. Disregulated Notch signaling underlies numerous human pathologies ranging from developmental impairments to
cancer. Specifically, unregulated Notch2 signaling is a defining characteristic of B cell chronic lymphocytic leukemia (B-CLL), diffuse large B cell lymphoma, and marginal zone lymphoma. Although many regulators of Notch2 signaling have been described, progress in utilizing these findings for therapeutic purposes has been restricted by a lack of understanding of signal activation. Certainly, identifying the proteinase responsible for initiating the irreversible signaling cascade represents significant progress for attempts to control Notch2 related diseases, including B-CLL.

Our finding that ADAM10 is the principal sheddase of CD23 in vivo resolves earlier data describing CD23 cleavage by multiple ADAMs in vitro. Demonstration that CD23 cleavage is sensitive to hydroxamic acid inhibitors, which inhibit the proteolytic activity of ADAMs, stimulated significant progress toward identifying the sheddase. Various studies have ruled out a role for ADAMs 8, 9, 12, 15, and 17 in vivo. A recent study determined that MMP-9 cleaves CD23 in LPS-treated mice. However, MMP-9 dependent cleavage was not examined in naive mice, and a role for ADAM10 was not excluded. Here, we demonstrate that deletion of ADAM10 from B cells profoundly impairs CD23 cleavage in vivo, resulting in a dramatic increase in the level of CD23 expressed on the B cell surface, and a profound reduction in sCD23.

This finding has important implications for the treatment of allergic and inflammatory reactions. Binding of IgE to CD23 on the B cell surface promotes transport and presentation of antigen associated with IgE immune complexes. In addition, mCD23 has been proposed as a natural repressor of IgE synthesis. However, cleavage of CD23 from the cell surface may interrupt this negative feedback mechanism and result
in elevated IgE synthesis. Additionally, the cleaved product, sCD23, is elevated in synovial fluids of patients with rheumatoid arthritis and may contribute to disease by activating monocytes \(^{80,81}\). Thus, inhibiting cleavage by the endogenous CD23 sheddase has been proposed as a novel therapy for controlling allergic and rheumatic disease \(^{76}\).

Moreover, an increased sCD23 level in serum of patients with B-CLL is a negative prognostic indicator for survival \(^{122}\). Given that constitutive Notch2 signaling and CD23 cleavage in B-CLL cells are well established \(^{119,122}\), this study indicates that ADAM10 is a potential target for B-CLL therapy.

Given the structural similarity of many ADAMs and the highly conserved nature of sequences within proteinase domains, we were surprised to find that deletion of ADAM10 had such profound effects on CD23 cleavage and Notch2 signaling. ADAM10 shares the greatest sequence similarity with ADAM17 \(^{123}\). Previous studies utilizing ADAM10\(^{-/-}\) MEFs have indicated considerable overlap in substrate recognition between multiple ADAMs, and especially between ADAMs 10 and 17 \(^{14-16}\). Although B cells also express ADAM17 \(^{124}\), we found no evidence of significant compensatory mechanisms while examining primary ADAM10-null B cells or cells stimulated to enhance CD23 cleavage or Notch2 signaling. Although we did not examine ADAM17-null B cells, Li et al demonstrated that reconstitution of RAG1\(^{-/-}\) mice with bone marrow cells lacking the Zn binding domain of ADAM17 resulted in unaltered B cell development \(^{32}\). Additionally, CD23 cleavage is not altered in B cell specific ADAM17 knockout mice \(^{14}\). Thus, this study reveals that substrate recognition by specific ADAMs may be more
stringent in vivo than in vitro studies would predict. This indicates that development of specific ADAM inhibitors may be more efficacious than previously thought.

In conclusion, examination of CD23 and Notch2 cleavage in B cell-specific ADAM10 knockout mice has shown that other proteases do not compensate for a lack of ADAM10 activity in vivo. This study demonstrates that ADAM10 is the primary sheddase of CD23 in vivo. Moreover, it reveals that ADAM10 critically regulates MZB lineage development by initiating Notch2 signaling.

II. Hematopoietic dysregulation in ADAM10 transgenics causes MDSC expansion and enhances metastatic progression of B16-melanoma.

Because ADAM10-mediated cleavage events regulate the development of numerous cell types, we hypothesized that ADAM10 activity would also regulate differentiation of early lymphoid progenitors. Here, we demonstrate that ADAM10 overexpression in hematopoietic precursors attenuates the development of thymocytes, severely abrogates B2 cell development from mouse BM, and promotes the expansion of functional MDSCs via a cell-intrinsic mechanism. The suppressive properties of A10Tg MDSCs are highlighted by enhanced metastatic progression in A10Tg mice that is prevented by MDSC depletion. This report also indicates that ADAM10 overexpression and blockade of γ-secretase activity may both direct myeloid cell development by dysregulating RIP-mediated Notch signaling.

By generating B cell-specific ADAM10-deficient mice with CD19-cre knockin animals, we recently demonstrated the necessity for ADAM10 activity in the
development of marginal zone B cells. However, since CD19-cre only deletes floxed exons from mature B cells, the role of ADAM10 in early B lineage commitment had not been examined. Here, overexpression of ADAM10 in early lymphoid precursors prevented B2 cell commitment from CLPs. Interestingly, this was specific to BM-derived B2 cells, while development of B1 cells which reside in peritoneal and pleural cavities was not affected. This difference may be the result of unique developmental pathways of B1 and B2 cells. B1 cells largely originate from fetal liver and the molecular pathways that direct B1 cell development are quite distinct from those critical to B2 cell commitment. The sparing of B1 cell development in A10Tg mice is consistent with the finding that B cell-specific deletion of ADAM10 and other regulators of Notch signaling did not affect B1 cell development. In addition to the lack of B2 cell development in A10Tg mice, the demonstration that ADAM10 inhibitors in OP9 cultures enhanced B cell development of LM and A10Tg HSC cultures further illustrates the adverse effect of ADAM10 activity on B2 lineage commitment. Although ADAM10 may influence B cell development by cleaving multiple substrates, its role in Notch signaling is certainly involved, since ADAM10 inhibition in OP9-DL1 cultures prevented Notch-dependent T cell development, while promoting B cell development. This finding is consistent with the report demonstrating that deletion of Notch1 from CLPs results in the development of B cells in the thymus.

Although the effects of Notch signaling in B and T cell lineage commitment have been thoroughly described, its role in myeloid development is controversial. Kawamata et al. reported that enforced expression of the Notch1 intracellular domain or the Notch
target genes, Hes 1 or Hes5, by adoptively transferred BM cells caused non-cell autonomous expansion of Mac-1/CD11b^+Gr-1^+ myeloid cells.\textsuperscript{44, 54} Surprisingly, overexpression of ADAM10 also caused expansion of CD11b^+Gr-1^+ myeloid cells in BM, resulting in high levels of MDSCs in peripheral blood and spleen. The elevated levels of CMPs in A10Tg BM and the lack of ADAM10 overexpression by CMPs, CD11b^+Gr-1^+ BM cells, and splenic MDSCs indicate that HSC development is altered at an early stage prior to mature cell lineage commitment. Additionally, mixed BM chimeras revealed that myeloid expansion in A10Tg mice occurs by a cell autonomous mechanism. Thus, enforced expression of NICD and ADAM10 may direct myeloid development by independent mechanisms. Nevertheless, these studies indicate that perturbation of Notch signaling in HSC development can induce myeloid expansion.

The development of thymocytes from BM precursors in A10Tg mice was also suppressed. This was demonstrated by the diminished thymus size, the relative inability of A10Tg thymocyte precursors to develop in mixed BM chimeras, and the delayed development of A10Tg T cells in OP9-DL1 cultures. The combination of suppressed thymocyte and B cell development indicates that ADAM10 overexpression does not result in excessive nor abrogated Notch signaling, as excessive signaling would enhance T cell commitment and abrogated signaling would promote B cell development. Rather, ADAM10 likely dysregulates Notch signaling via a novel mechanism. Inhibition of γ-secretase activity in LM and A10Tg OP9-DL1 cultures mimicked myeloid development of A10Tg HSCs on OP9-GFP cells. This suggests that ADAM10 overexpression and γ-secretase blockade alter hematopoiesis by similar mechanisms. Following ligand
engagement and ADAM-mediated S2 cleavage of the receptor, γ-secretase blockade results in accumulation of the S2 product\textsuperscript{20, 21}, which could direct myeloid development. This is supported by the finding that diminished presenilin (PS) dependent γ-secretase activity in PS1\textsuperscript{1/2}PS2\textsuperscript{-/-} mice results in myeloproliferative disease, characterized by accumulation of Mac-1/CD11b\textsuperscript{+}Gr-1\textsuperscript{-} cells in the BM and spleen, causing splenomegaly\textsuperscript{58}. Excessive ADAM10 activity could also lead to accumulation of the S2 cleavage product. In the absence of ligand, the γ-secretase complex may not be recruited to Notch receptors at the cell membrane. Thus, in the event of ligand-independent cleavage by ADAM10, which has been reported in disease states\textsuperscript{10, 21}, the γ-secretase complex would not process the S2 cleavage product, which may accumulate at the cell membrane. The presence of ligand and recruitment of γ-secretase would prevent S2 product accumulation and myeloid development. This is consistent with our finding that ligand-expression on OP9-DL1s prevents myeloid development of A10Tg HSCs. Although the findings reported here are consistent with this proposed mechanism, we were unable to directly demonstrate S2 accumulation in A10Tg cells. However, S2 accumulation following GSI treatment has only been demonstrated in cell lines that overexpress Notch receptors\textsuperscript{20, 21}. Because of rapid proteolytic processing, identification of Notch cleavage products from primary cells has been a significant challenge\textsuperscript{10}. Additionally, although GSI treatment and ADAM10 overexpression clearly direct myeloid development, their effects may be independent of S2 accumulation of Notch. Thus, this study should stimulate interest in the developmental effects of Notch mutants that are resistant to S3 cleavage.
Nevertheless, our finding that S2 and S3 blockade of WT Notch signaling have differential effects on hematopoietic development is novel and has significant implications for the treatment of Notch-related diseases. Although numerous reports have proposed the use of GSIs for the treatment of T-ALL and B cell lymphoma\textsuperscript{25, 126}, our findings indicate that GSI treatment may cause MDSC expansion that would promote tumor growth and metastasis. Thus, studies of GSI treatment in mice and clinical trials should include careful monitoring of myeloid cell development. This study indicates that pharmacologic blockade of S2 cleavage with ADAM10 inhibitors may be a more advantageous strategy.

Additionally, this study may help clarify how HSCs develop into a wide array of mature cell types. The classical model of hematopoiesis describes the initial dichotomous differentiation of HSCs into CLPs or CMPs. However, two recent studies demonstrating that early thymocyte progenitors possess myeloid potential have challenged the classical model\textsuperscript{127, 128}. Additional studies have demonstrated that B cell progenitors, including CLPs, also retain myeloid potential\textsuperscript{129, 130}, while other progenitors that lack T cell potential can develop into B cells or macrophages\textsuperscript{131}. Thus, a myeloid-based model describing HSC development into common myeloid-erythroid progenitors or common myelo-lymphoid progenitors has been proposed by multiple groups\textsuperscript{107, 132}. In A10Tg mice, the moderate effects on thymocyte development in combination with the more pronounced effects on B lineage commitment and myeloid expansion indicate that B2 cells and the expanded myeloid cells develop from common progenitors, whereas thymocytes may develop from a unique precursor. The myeloid-B cell precursors in
A10Tg mice may consist of B220$^+$ cells or CLPs, which both express elevated levels of ADAM10, or a unique unidentified progenitor. Nevertheless, the differential effects of ADAM10 overexpression on T cell, B cell, and myeloid development support the myeloid-based model.

Because A10Tg MDSCs expand in the absence of tumor burden, we considered that they may not be suppressive in a tumor-free environment. However, A10Tg MDSCs suppressed T cell proliferation similarly to reported tumor-derived MDSCs$^{106}$. Consistent with this observation, A10Tg mice also have increased VEGF levels in serum, BM and spleen cultures, as well as decreased L-selectin expression on T cells. These initial studies indicate that A10Tg MDSCs are phenotypically and functionally similar to MDSCs isolated from the tumor microenvironment. Previous studies have indicated that splenic MDSCs from tumor bearing mice only inhibit antigen-specific T cell responses, while MDSCs from the tumor milieu inhibit both polyclonal and antigen-specific T cell proliferation$^{133-135}$. Interestingly, A10Tg splenic MDSCs suppress T cell proliferation following both antigen-specific and polyclonal activation. This may be explained by the elevated level of suppressive monocytic MDSCs that reside in the A10Tg spleen. Nevertheless, this indicates that MDSCs are immunosuppressive independent of the tumor microenvironment. However, T cell suppression by A10Tg MDSCs might be multifaceted and warrants a subsequent comprehensive study, including direct comparisons to tumor-derived MDSCs and examination of suppressive mechanisms.

Initial challenge of A10Tg mice with B16 melanoma demonstrated a dramatic increase in metastatic progression and resistance to AIT, compared to LM controls.
Although this result is likely due to the increased levels of MDSCs in A10Tg animals, it is possible that the lower level of B2 cells and the presence of A10Tg T cells may contribute to the suppressed anti-tumor response in transgenics. However, subsequent experiments utilized CYP to deplete host lymphocytes prior to infusion with sensitized LM T cells or Ag-specific pmel-1 T cells. Results of these experiments also demonstrated increased tumor burden similar to levels prior to CYP treatment. This indicates that the increased metastatic progression is due to MDSC-mediated T cell suppression rather than intrinsic defects in A10Tg T cells or deficient B2 cell development. Depletion of MDSCs with tritherapy (GEM + CYP + AIT) restored the anti-tumor immune response and prevented metastatic progression to the lungs. Although the anti-metabolic property of GEM may directly inhibit tumor progression, treatment with GEM alone only suppressed metastasis in A10Tg mice to levels similar to those in untreated LM controls. This demonstrates that GEM improved the anti-tumor immune response rather than directly suppressing tumor growth. This finding also indicates that GEM-mediated MDSC depletion can restore the anti-tumor response of A10Tg T cells to a response comparable to that of LM T cells. Together, these results demonstrate that increased metastatic progression in A10Tg mice is due to ADAM10-mediated expansion of functional MDSCs.

A10Tg mice provide a model for the study of MDSC-mediated immune suppression in the absence of confounding tumor-derived factors that can also regulate immune responses. The A10Tg model may provide considerable advantages over previously described models of myeloid cell accumulation, including, MUC1−/− and
SHIP-/- mice. In studies of mice deficient in MUC1, a tumor associated mucin, MDSCs are only generated in vitro in the presence of IL-4 and GM-CSF\textsuperscript{137}. In contrast, the SHIP-/- model does generate elevated levels of endogenous MDSCs comparable to that of A10Tg mice\textsuperscript{138}. However, unlike A10Tg MDSCs, SHIP-deficient MDSCs fail to alter antigen-specific T cell responses.

Regardless of the mechanisms responsible for altering myeloid development, MDSC accumulation results in significant immune suppression. Such a model as the A10Tg mice could make a considerable contribution to the study of MDSCs in the context of cancer therapy. Additionally, it could have important implications for the examination of other disease states in which MDSCs have been implicated such as graft-vs-host disease, sepsis and autoimmunity.

In conclusion, this study demonstrates that the proteolytic activity of ADAM10 regulates the lineage commitment of B2 cells and the expansion of functional MDSCs in a cell-intrinsic manner. Additionally, it illustrates the requirement for MDSC depletion in effective adoptive immunotherapy of metastatic melanoma. Moreover, it describes an in vivo model for further examination of MDSC expansion and MDSC-mediated immune suppression in the absence of tumor-derived factors. Finally, it proposes a novel mechanism for the differential effects of S2 and S3 blockade of Notch signaling in hematopoietic progenitors.
III. ADAM10 deletion inhibits antibody production.

Because ADAM10-deletion from B cells altered expression of multiple regulators of antibody production, we hypothesized that ADAM10 activity contributes to the humoral immune response. Here, we demonstrate that ADAM10 deficiency in B cell-specific ADAM10-deficient mice impairs germinal center formation and antibody-production in response to T cell-dependent and independent antigens.

These findings could be the product of abrogated development of the marginal zone B cell lineage in ADAM10$\Delta^+/\Delta^+$ cre$^{+/+}$ mice. MZBs are thought to quickly respond to T-independent antigens, including lipids and polysaccharides, and produce high levels of low affinity IgM in response to systemic antigen. MZBs also contribute to T-dependent responses by processing and presenting antigen to T cells in the context of highly expressed MHC class II$^{45}$. However, the lack of MZB:T cell interaction in the marginal zone challenged the physiologic relevance of MZB-mediated antigen presentation and transport, until Cinamon et al. revealed that MZBs constitutively shuttle between the marginal zone and lymphoid follicles. Consequently, MZBs facilitate the transport of antigen from the splenic red pulp to FDCs for immune complex formation and retention$^{115}$. Shuttling also may allow for MZB-mediated antigen presentation to T-helper cells.

These findings suggest that all mouse models lacking MZB cells would have impaired antibody production. However, Tanigaki et al. demonstrated that RBP-Jκ B cell-specific deletion, which prevents MZB development, did not alter antibody production to the same immunizations used in this study$^{47}$. Additionally, analysis of
Notch2-deficient mice, which also lack MZBs, did not reveal aborations in antibody production\textsuperscript{41}. Furthermore, MZB cell loss is not thought to impair adaptive immune responses in peripheral lymph nodes, or other secondary lymphoid tissues. Thus, the role of ADAM10 in antibody production extends beyond its role in MZB development.

Recent studies have implicated a role for Notch signaling in antibody production. Santos et al. demonstrated that Dll1 expression on stromal cells enhances B cell differentiation into antibody secreting cells. Additionally, Cre-mediated deletion of Notch1 significantly inhibited the Dll1-induced enhancement in differentiation. Although naïve B cells express minimal levels of Notch1, the authors illustrated marked elevation in Notch1 expression following LPS-stimulation\textsuperscript{42}. These findings were supported by Thomas et al., who described synergism of Notch signaling with BCR and CD40 signaling during B cell activation\textsuperscript{48}. Although studies of ADAM10-deletion in MZB development focused on Notch2 signaling, numerous other studies have illustrated a requirement for ADAM10 in the initiating Notch1 signaling on other cell types\textsuperscript{20, 21, 31}. Thus, ADAM10 deletion may impair differentiation of antibody secreting cells by suppressing Notch1 signaling in activated B cells. Additionally, Notch signaling has recently been implicated in the survival of germinal center B cells that engage Notch ligands on FDCs\textsuperscript{50}. This may explain the reduced number and size of germinal centers in immunized ADAM10\textsuperscript{Δ/Δ}cre\textsuperscript{+/−} mice. However, as mentioned above, B cell deletion of RBP-Jk, the primary transcription factor downstream of NICD activation had no effect on antibody production \textit{in vivo}\textsuperscript{47}. This indicates that ADAM10’s role in Notch signaling is not responsible for impaired antibody production. However, Notch signaling may
influence gene expression independent of RBP-Jκ. Thus, the effects of ADAM10-mediated Notch signaling and MZB development on antibody production should be directly examined by restoring Notch signaling to ADAM10^{ΔΔ}cre^{+/−} B cells. Analysis of ADAM10^{ΔΔ}cre^{+/−} mice that express the Cre-regulated NICD transgene should determine whether the diminished antibody production is due to a lack of Notch signaling.

Notch signaling also enhances cell surface expression of the complement receptors, CD21/35. Thus, ADAM10^{ΔΔ}cre^{+/−} B cells express significantly lower levels of CD21/35. By generating CD21/35 knockout mice, two independent groups demonstrated that B cell expression of CD21/35 is required for the production of antibodies against T-dependent antigens^{52,139}. Croix et al. hypothesized that this requirement is due to the role of CD21/35 in BCR signaling, endocytosis of antigen, protection from apoptosis, or recruitment to germinal centers^{52}. Additionally, Fischer et al. demonstrated the dependence of germinal center B cell survival on the expression of CD21/35^{140}. However, unlike ADAM10^{ΔΔ}cre^{+/−} mice, basal immunoglobulin levels in serum of CD21/35 knockout mice were not different from those of control mice^{139}. Thus, alterations in CD21/35 expression are unlikely to completely explain the diminished antibody production by ADAM10-null B cells.

This report demonstrated that ADAM10 is the principal protease that cleaves CD23 in vivo. Thus, ADAM10-null B cells express dramatically elevated levels of CD23 on the cell surface. Analysis of two CD23 transgenic mice, generated with distinct transcriptional regulatory units, demonstrated that CD23 overexpression markedly suppressed IgE production and attenuated IgG1 production in response to T-dependent
antigens in vivo\textsuperscript{51, 75}. Additionally, Payet et al. described lower basal levels of IgM, IgG2b, and IgG3 in CD23 transgenic serum. Following immunization with a T-dependent antigen absorbed in alum, there was a consistent trend of lower levels in CD23 transgenic mice, compared to littermate (LM) controls\textsuperscript{51}. These findings were also observed in ADAM10\textsuperscript{Δ/Δ}cre\textsuperscript{+/−} mice, and suggest that ADAM10-mediated cleavage of CD23 from B cells may regulate antibody production. However, a subsequent study by Payet-Jamroz et al. revealed that humoral suppression in the same CD23 transgenics was due to CD23 overexpression on non-lymphoid cells. Immunization of reconstituted irradiated LM mice with CD23 transgenic or LM splenocytes resulted in comparable antibody production, irrespective of donor cell CD23 expression levels\textsuperscript{116}. This indicates that elevated CD23 surface expression on ADAM10-null B cells does not account for their impaired humoral responses reported here. However, subsequent studies to confirm this finding have not been completed. Thus, analysis of antibody production in ADAM10\textsuperscript{Δ/Δ}cre\textsuperscript{+/−} mice on a CD23-null background would determine whether ADAM10-mediated CD23 cleavage from B cells may contribute to the phenotype presented here.

Although the previously discussed alterations in protein expression and signaling in ADAM10\textsuperscript{Δ/Δ}cre\textsuperscript{+/−} mice are the most thoroughly examined pathways that could contribute to diminished antibody production, other ADAM10 substrates may also play a role. Inhibition of ADAM10-mediated cleavage of Lag-3 markedly suppresses T cell activation and proliferation\textsuperscript{36}. Cell surface Lag-3 is also expressed by \textit{ex vivo} activated B cells, and may also inhibit B cell activation. Additionally, a lack of FasL cleavage in T cells treated with an ADAM10 inhibitor enhances FasL:Fas mediated cell-death\textsuperscript{141}. Thus,
a lack of ADAM10-mediated FasL cleavage might also account for diminished germinal centers and proliferation of antibody-producing cells. Collectively, all of these ADAM10-mediated cleavage events may contribute to antibody production at various stages of the humoral immune response. Conversely, ADAM10 may mediate antibody production via processing a completely novel substrate, or even directly regulate germinal center formation and subsequent antibody secretion independent of substrates. Further analysis of various stages of B cell activation, germinal center formation, and plasma cell differentiation should help clarify ADAM10’s role in humoral responses.

IV. ADAM10-mediated CD23 cleavage from non-lymphoid cells regulates IgE production.

The identification of ADAM10 as the CD23 ‘sheddase’ has raised intriguing questions about the role of ADAM10 in regulating IgE production and allergic disease. However, because deletion of ADAM10 from embryos causes lethality, and deletion from B cells alters development, addressing these questions has been challenging. This has forced the use of alternative in vivo and ex vivo models. This study utilized a coculture model to illustrate a clear role for ADAM10 in modulating IgE production ex vivo. ADAM10 activity and cleavage of CD23 negated the receptor’s role in suppressing IgE synthesis. In accordance with previous studies attributing IgE suppression to CD23 on non-lymphocytes^{97,116}, this study focused on ADAM10-dependent cleavage of non-B cell CD23 and its influence on IgE production. Cho et al. demonstrated that expression of CD23 on surrounding CHO cells significantly suppressed IgE synthesis^{97}. However,
inducing cleavage of CD23 by overexpressing ADAM10 in CD23-CHO cells reversed the IgE suppression. This finding highlights an important role for membrane bound CD23, as IgE production is inversely proportional to CD23 membrane levels. IgE production is suppressed in CD23 transgenic mice, which have increased levels of membrane and sCD23. This has led to controversy about the critical mediator, membranous or sCD23 51. By modulating cleavage, this study presents models where membrane and sCD23 levels move in opposite directions, allowing investigation of this issue.

Surprisingly, inhibiting cleavage with a dominant negative version of ADAM10 did not influence IgE production in this model. Although DN-ADAM10 specifically inhibited ADAM10 and subsequent CD23 cleavage, control CD23-CHO cells may already express maximal levels of CD23. Thus, inhibiting cleavage could not further increase levels of membrane CD23. Thus, DN-CHO cells suppressed IgE to a similar degree as control cells. Additionally, DN-ADAM10 only reduced sCD23 release from transiently transfected CHO cells by ~50%. This level of inhibition may not be sufficient to influence IgE production. To investigate this possibility, cleavage was minimized by utilizing ADAM10−/−CD23+ MEFs in co-cultures. Complete deletion of ADAM10 profoundly inhibited cleavage as well as IgE production. Interestingly, CD23 on ADAM10−/− MEFs did not affect IgE production. Because cleavage occurs constitutively, membrane CD23 levels on heterozygous cells were much lower than levels on ADAM10−/− MEFs and may not have been sufficient to influence IgE synthesis. Although cleavage also readily occurred in CD23-CHO cells that significantly suppress IgE
production, CD23 surface expression was substantially higher in CHO cells, allowing for IgE suppression in the presence of ADAM10.

Previous studies have indicated that induction of high CD23 expression on non-lymphocytes that have in vivo contact with B cells, principally FDCs, is a potential strategy for controlling IgE productive reactions to environmental allergens\textsuperscript{97,116}. Such an induction was investigated in a recent study. Quantitative pcr, immunohistochemistry, and flow cytometric analysis revealed that the Th-2 cytokine interleukin-4 (IL-4) in combination with the CD40 ligand (CD40L) upregulated both existing isotypes of CD23, CD23a and CD23b, on B cells, but only increased the CD23a isoform on FDCs. In contrast, CD40L plus the Th-1 cytokine interferon-γ (INF-γ) inhibited CD23 expression by B cells, but upregulated both isoforms on FDCs\textsuperscript{142}. This induction pattern of CD23 on FDCs in response to T cell cytokine production further suggests that the role of FDC CD23 is not only important for regulating adaptive immune responses, but also clearly unique from that expressed on B cells. CD23 on B cells has been implicated in aiding IgE-associated antigen processing and presentation\textsuperscript{143}. Since the receptor was identified on FDCs, it has been proposed to regulate isotype switching to IgE by trapping IgE-antigen complexes\textsuperscript{144}. Given that recent evidence demonstrates a critical role for FDCs and immune complexes in isotype switching and affinity maturation\textsuperscript{145}, trapping of IgE immune complexes represents a possible mechanism by which CD23 on FDCs suppresses antigen specific IgE responses. However, a direct interaction between IgE on B cells and CD23 on FDCs that leads to an inhibitory signal to suppress IgE class switching is an alternative hypothesis. Even though the mechanism of CD23’s role in IgE
suppression remains to be elucidated, cleavage of CD23 by ADAM10 would modulate CD23’s ability to control IgE production.

To test the role of ADAM10-mediated cleavage of FDC CD23 in vivo, we attempted to generate FDC-specific ADAM10-null mice. However, non-specific Cre-mediated recombination in other cell types prevented analysis of FDC-ADAM10. The BAC CD21/35cre transgene has been successfully utilized to generate other FDC-specific knockout mice, including p55TNFR and IKK2-deficient mice. However, p55TNFR and IKK2 are thought to be specifically expressed by FDCs and do not regulate developmental pathways. Thus, CD21/35cre transgenic mice are appropriate for deletion of proteins that share these characteristics. However, production of ADAM10Δ/ΔCD21/35cre+/− mice did result in the interesting observation of severe splenomegaly. This suggests that ADAM10 may regulate the development of multiple hematopoietic cell types. Thus, deletion of ADAM10 from specific hematopoietic precursors may generate interesting phenotypes that further the understanding of ADAM10-mediated cleavage events in hematopoiesis.

Results of this study indicate that ADAM10 plays a critical role in regulating the levels of non-B cell membrane-bound CD23, which is responsible for suppressing IgE responses in ex vivo cultures. They also suggest that this regulation may influence IgE production and resulting pathologies, such as allergic airway inflammation and asthma in vivo. Evidence to support this claim awaits further investigation of in vivo models.
V. Conclusions and Significance

The initial goal of this dissertation project was to determine the physiologic significance of ADAM10-mediated CD23 cleavage on Type 1 allergic disease. However, we quickly learned that the critical role of ADAM10 in developmental pathways, including Notch signaling, would present numerous challenges for the study of CD23 and allergy. Nonetheless, examination of developmental deficits illustrated in B-cell specific ADAM10 knockout and ADAM10 transgenic mice answered numerous questions surrounding the roles of ADAM10 and Notch signaling during immune cell development.

The requirement for ADAM10 in MZB cell development was the first illustration of ADAM10’s role in initiating signaling through the Notch2 receptor. Previous studies had established that ADAM10 contributed to Notch1 activation in murine embryos, MEFs, and thymocytes\textsuperscript{17,21,31}. However, because an earlier study demonstrated that the S2 cleavage sites of Notch1 and Notch2 were distinct, it was uncertain whether ADAM10 also contributed to signaling through other Notch receptors in other cell types\textsuperscript{147}. Demonstration of ADAM10-dependent Notch2 signaling \textit{in vivo} suggests that ADAM10 may also regulate Notch3 and Notch4 activation.

Demonstration of the clear dependence of MZB development and CD23 cleavage on ADAM10 expression indicated the lack of compensatory functions of other proteases, including ADAM17, in ADAM10-deficient B cells. This clarified previous \textit{in vitro} studies demonstrating functional overlap of ADAM10 and ADAM17 in the cleavage of multiple substrates\textsuperscript{14-16}. Thus, various stimuli applied \textit{in vitro} may induce cleavage of
some substrates by multiple proteases. However, these stimuli are either not present in vivo, or do not induce cleavage of CD23 or Notch2 by proteases other than ADAM10 in naïve mice. Additionally, studies of ADAM10-mediated Notch1 signaling in thymocytes indicated that other proteases perform partial compensatory roles in the absence of ADAM1031. Thus, the lack of compensatory roles in B cells may reflect a more stringent dependence on ADAM10 activity for Notch2 or CD23 cleavage. However, differences in ADAM10-floxed alleles or Cre-driven promoters utilized or the use of reporters of Cre-mediated recombination could also account for the differences between thymocyte and B cell studies.

The description of expanded myeloid cells in ADAM10 transgenic mice indicated that the impact of regulated ADAM10 activity is not limited to lymphocyte development. The combined effects on myelopoiesis and B2 cell development supported models of hematopoiesis describing increased plasticity of hematopoietic cell differentiation during lymphocyte development107, 132. These findings suggest an important role for ADAM10 in homeostatic regulation of myeloid development, and should prompt further investigation of the dependence of myeloid cell development on ADAM10 activity in conditional knockout mice.

These effects on immune cell development may also be reflected by the reduced humoral responses of ADAM10-null B cells to immunization. Naïve B cell differentiation into plasma cells, memory B cells, or germinal center B cells may also require ADAM10-mediated Notch signaling. Interestingly, studies of Nippostrongylus brasiliensis infections did not reveal a dependence of ADAM10 activity on IgE
production (data not shown). Thus, ADAM10 activity may only enhance humoral responses to antigens administered at lower doses or rapidly cleared from peripheral circulation, as higher doses and longer exposure seem to overcome ADAM10-mediated events. Further investigation of ADAM10-mediated Notch signaling in distinct phases or B cell activation should help clarify the role of ADAM10-mediated cleavage events in humoral responses.

Of course the dependence of ADAM10 activity on antibody production could also be related to CD23 cleavage. Production of double knockout mice lacking ADAM10 and CD23 expression in B cells should address this possibility. The one study demonstrating that suppressed humoral responses in CD23 transgenic mice is due to FDC CD23 expression, rather than B cell expression, would suggest that B cell-expressed ADAM10 regulates antibody production independent of CD23 processing. However, the importance of FDC CD23-mediated contributions to humoral responses has yet to be supported in other animal models. Additionally, mechanisms underlying these contributions have not been directly examined. Thus, given that production of ADAM10<sup>Δ/Δ</sup>CD21/35cre<sup>+/−</sup> mice did not allow examination of ADAM10 activity in FDCs, this issue warrants more thorough investigations utilizing alternate methods to delete CD23 or ADAM10 from FDCs.

Finally, a growing body of evidence has demonstrated the role of ADAM10 activity in initiating RIP-mediated signaling through numerous receptors. However, signaling events resulting from CD23 binding to IgE or other ligands are poorly understood, and attempts to identify secondary messengers or linker molecules
associated with the intracellular domain of CD23 have not been successful. Thus, a potential role for ADAM10-mediated RIP of CD23 and effects of CD23 ICD on transcriptional activation should be explored.

Generally, altering expression of proteins that were discovered as critical mediators of cell fate pathways, such as ADAM10, is likely to result in developmental phenotypes. Elucidating mechanisms underlying these phenotypes may have significant impacts on approaches to treating developmental and proliferative diseases, including immunodeficiencies and cancer. However, the same phenotypes present challenges for the examination of diseases related to mature cell function, including adaptive immune responses. Thus, future investigations of ADAM10’s role in these processes should take advantage of models that allow inducible alteration of protein expression and activity. Finally, findings presented here are markedly distinct from anticipated results of our initial studies. Thus, this dissertation explifies the fact that ‘scientific investigations should be hypothesis-based, but scientific progress is truly driven my experimental results.’
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