The role of ADAM10, ADAM17, and Spag6 in humoral immunity and secondary lymphoid tissue architecture

Lauren Folgosa Cooley

Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/3808

This Thesis is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
The role of ADAM10, ADAM17, and Spag6 in humoral immunity and secondary lymphoid tissue architecture

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

by

Lauren Folgosa Cooley
B.S., University of Richmond 2010

Director: Daniel H. Conrad, PhD
Professor
Department of Microbiology and Immunology
School of Medicine
Virginia Commonwealth University
Richmond, VA
March 17, 2015
Dedication

This dissertation is dedicated to my parents, Dr. Mauro and Justine Folgosa, who always challenged me to reach for dreams beyond what I thought possible. Thank you for providing me with every opportunity to make my dreams a reality.
Acknowledgements

First, I wish to thank my mentor and friend, Dr. Daniel H. Conrad. He welcomed me into his lab in 2012 and I have loved working in his lab. He challenged me to think critically, independently and creatively. He gave me the intellectual freedom to explore new ideas and create my projects. I valued his open door policy and felt I could always walk in to discuss my research, ideas, or even do a data dance. Thank you for financially supporting my work and my travel to both national and international meetings. Thank you for inspiring me to continue in research and reinforcing my desire to do academic medicine.

Second, I wish to thank Dr. Mohey Eldin El Shikh who served as a co-mentor to me during my PhD. He was a constant source of support and knowledge and helped guide my projects. His immunohistochemistry expertise is unparalleled and helped me to confirm many of my hypotheses. It was a pleasure to finally meet him at the Germinal Centre Conference in Sweden and I look forward to collaborating with him in the future.

Third, I wish to thank past and present Conrad Lab members who have helped me over the years. First, I wish to thank Hannah Zellner, who helped me tremendously during my PhD. From tail clipping parties to early morning flexivent days, she was a constant source of support and I could always rely on her. Dr. Rebecca Martin for her friendship and help during my PhD. She coached me through countless protocols and is an endless source of lab knowledge. I also wish to thank Sheela Damle and Andrea Elkovich for their friendship and support. Lee Dean for guiding me through molecular techniques and continuing to answer questions even after he moved on from VCU. Dr.
Jamie Sturgill, Dr. Natalia Chaimowitz, Dr. Sheinei Saleem, and Keith Brooks for their support during their time in the Conrad lab. To my rotation students and friends, Joseph Lownik and Chelsea Cockburn, I wish you both great success during your careers.

Fourth, I wish to thank each of my committee members: Dr. Christopher Wise, Dr. Lawrence Schwartz, Dr. Kathleen McCoy, and Dr. John Ryan. Each challenged me to think critically about my research and supported me with their time and expertise in discovering new avenues for my projects. I wish to thank Dr. Wise for allowing me to work in clinic with him every month during my PhD to help keep my research relevant to patient needs and also maintain my skills. I wish to thank Dr. Zhibing Zhang and Dr. Jerome Strauss for reaching out to us for collaboration on the Spag6 project. It has been a very interesting and novel project from which I have learned a lot. I wish to thank Dr. Gordon Archer for giving me the opportunity to be part of the MD/PhD program and for his support throughout medical school and my PhD. I also wish to thank Dr. Anne-Marie Irani and her nursing staff for their help recruiting and consenting patients for the allergic patient study. Without their hard work, my human studies project would not have been possible. Lastly, I wish to thank Dr. Joyce Lloyd and Dr. Devanand Sarkar for inviting me to the Clinical and Translational Research (CCTR) program and helping to provide support for my research and travel throughout my PhD.

Next, I wish to thank the American Association of Immunologists for a travel award to present my research at the 2013 AAI meeting in Honolulu, Hawaii. I also wish to thank the Gordon Research Conference for granting me a travel award and oral presentation at the 2014 GRC meeting on proteolysis of cell surface proteins. I lastly
wish to thank VCU and the CCTR for their support of my research through travel awards and poster presentation opportunities.

Lastly, I wish to thank my family and my husband, Casey Cooley, for their unconditional love and support of my career goals.
# Table of Contents

List of Tables........................................................................................................................................... xiv

Table of Figures .......................................................................................................................................... xv

List of Abbreviations .................................................................................................................................... xix

Abstract ........................................................................................................................................................ xxvii

**Section 1: The role of B cell ADAM10, ADAM17, and TNF in secondary lymphoid tissue architecture and antibody responses** .......................................................................................................................... 1

Chapter 1: Introduction to ADAM10, ADAM17, and their respective ligands in humoral immunity and secondary lymphoid tissue architecture .......................................................... 1

1.1.1 Introduction to ADAM10 and ADAM17................................................................................. 1

   **ADAMs Overview**................................................................................................................................. 1

   **ADAM10**.............................................................................................................................................. 2

   **ADAM17**............................................................................................................................................ 4

1.1.2 Introduction to secondary lymphoid tissues........................................................................ 8

1.1.3 B cell activation ......................................................................................................................... 10

   **T dependent responses**...................................................................................................................... 11

   **T independent responses**................................................................................................................ 11

1.1.4 Germinal Centers ....................................................................................................................... 12

   **Structure, formation, and maintenance**........................................................................................ 12

   **Somatic hypermutation and class switch recombination**......................................................... 14
Affinity maturation and high affinity B cell selection.................................14

1.1.5 Th differentiation and associated antibodies............................................15

1.1.6 ADAMs 10 and 17 ligands in maintaining secondary lymphoid tissue architecture and humoral responses.........................................................17

TNF Family Cytokines .....................................................................................17

ICOSL................................................................................................................19

CD23, IgE and asthma.....................................................................................20

1.1.7 T regulatory cells and their role in allergic airway disease.....................23

1.1.8 B regulatory cells: A unique B cell subset............................................25

1.1.9 Section 1 aims..........................................................................................28

Chapter 2: Materials and Methods .................................................................29

1.2.1 Mice.........................................................................................................29

1.2.2 Cell Isolation..........................................................................................29

   Tissue preparation.........................................................................................29

   Magnetic bead isolation................................................................................30

1.2.3 In vitro activation ..................................................................................30

   B cells: Anti-CD40, IL-4, LPS.................................................................30

   B cells: PMA..............................................................................................31
In vitro generation of CD4+CD25+ T cells.............................................................31

1.2.4 [3H] thymidine proliferation ...........................................................................32

1.2.5 Immunization ..................................................................................................32

NP-KLH ..................................................................................................................32

1.2.6 ELISA ..............................................................................................................32

Total antibody .........................................................................................................33

NP-specific antibody .............................................................................................33

TNF ..........................................................................................................................33

HDM specific IgE ...................................................................................................34

MUC5AC ....................................................................................................................34

1.2.7 PCR and Quantitative PCR ............................................................................34

PCR.........................................................................................................................34

Quantitative PCR ..................................................................................................35

1.2.8 Western ..........................................................................................................37

1.2.9 Flow cytometry and Immunohistochemistry ...............................................39

Flow cytometry – Surface, total (intracellular and extracellular), and nuclear
staining ....................................................................................................................39

Flow cytometry - Tyramide Signal Amplification ..................................................39

Flow cytometry – ICOSL cleavage kinetics ............................................................40
Chapter 3: Th1 vs. Th2 background does not affect antigen specific antibody production following B cell ADAM10 deletion; however, B cell ADAM17 and TNF as well as secondary lymphoid tissue architecture are greatly altered.

1.3.1 Generation of B cell specific ADAM10 deficient mice

1.3.2 Humoral immune responses in C57-ADAM10^{B/-} deficient mice

\textit{ADAM10 is highly expressed in GC B cells}

Reduced total and antigen specific humoral immune responses in C57-ADAM10^{B/-}^{/-} mice

1.3.3 Aberrant secondary lymphoid tissue architecture, TNF, and ADAM17 in C57-ADAM10^{B/-} mice

\textit{Reduced GC B cells in C57-ADAM10^{B/-} spleen}
C57-ADAM10\textsuperscript{B/-} mice exhibit abnormal lymph node architecture and excessive TNF in B cell regions.................................................................54

B cells from C57-ADAM10\textsuperscript{B/-} mice exhibit increased expression, stability, and production of TNF.................................................................55

C57-ADAM10\textsuperscript{B/-} B cells exhibit higher ADAM17 gene and protein expression...56

Reconstitution of irradiated C57Bl/6 WT with combination C57-ADAM10\textsuperscript{B/-} + TNFKO bone marrow rectifies lymph node follicular abnormalities ..............63

1.3.4 Balb-ADAM10\textsuperscript{B/-} exhibit reduced antigen specific antibody production and GC B cells similar to C57-ADAM10\textsuperscript{B/-} mice .................................................................68

1.3.5 Th1 and Th2 strain dependent differences in B cell ADAM17 and TNF as well as secondary lymphoid tissue architecture following B cell ADAM10 deletion........68

Strain dependent differences in B cell TNF and ADAM17..............................................68

Unlike C57-ADAM10\textsuperscript{B/-}, Balb-ADAM10\textsuperscript{B/-} exhibit normal secondary lymphoid tissue architecture ........................................................................69

Chapter 4: Th1 and Th2 prone mouse strains and humans exhibit differential B cell ADAM10, ADAM17, and TNF levels.................................................................75

1.4.1. Th2 prone strains (Balb/c, A/J) B cells exhibit increased ADAM10 and decreased ADAM17 and TNF compared to those of Th1 prone strains (C57Bl/6, SJL/J) .............................................................................................................75

1.4.2 High B cell ADAM10 predicts increased susceptibility to Th2 disease............79
High B-ADAM10 level in the context of a Th2 environment allows optimal
induction of allergic airway disease symptoms………………………………………………..79

Allergic patients exhibit increased B cell ADAM10, CD23 cleavage, and IgE yet
reduced ADAM17 and TNF………………………………………………………………………………81

Chapter 5: C57-ADAM10^{B-/} B cells exhibit a B10 phenotype with enhanced ICOSL
expression and induction of T regulatory cells in secondary lymphoid tissues…………..88

1.5.1 Increased B10 cells in C57-ADAM10^{B-/} mice………………………………………………..88

1.5.2 Increased T regulatory cells in ADAM10^{B-/} mice………………………………………………..89

1.5.3 Increased ICOSL expression on C57-ADAM10^{B-/} B cells……………………………………..93

1.5.4 Conditioned C57-ADAM10^{B-/} B cell supernatants cause enhanced Treg
induction in vitro ..........................................................................................................................93

1.5.5. In vivo Treg depletion studies……………………………………………………………………….99

HDM acute airway hypersensitivity studies with Treg depletion……………………………99

1.5.6 Other unique features of ADAM10^{B-/} B cells………………………………………………….105

Chapter 6: Discussion………………………………………………………………………………………….108

1.6.1 Increased ADAM17 and excessive B cell sTNF production provides the
mechanism for aberrant secondary lymphoid tissue architecture in C57-ADAM10^{B-/}
mice……………………………………………………………………………………………………………….108
1.6.2 Increased B cell ADAM10 and reduced ADAM17 and TNF is predictive of increased susceptibility to Th2 responsiveness

1.6.3 C57-ADAM10^B/-^ mice exhibit decreased antibody production, which may be explained by an increased percentage of B10 cells and increased Tregs.

Section 2. Impaired immunological synapse in sperm associated antigen 6 (SPAG6) deficient mice

Chapter 1: Introduction to SPAG6 and its role in microtubule rearrangement

2.1.1 SPAG6

2.1.2 SPAG6 and its role in the MTOC

Chapter 2: Materials and Methods

2.2.1 Mice

2.2.2 Bone Marrow Reconstitution

2.2.3 Reagents and T cell culture

2.2.4 ELISA

\textit{NP-specific antibody}

\textit{IFN\gamma}

2.2.5 Flow cytometry

2.2.6 Cytotoxic assay
2.2.7 CTL induction and slide set up for synapse staining ........................................ 125

2.2.8 Immunohisto-, cyto-chemistry, and confocal microscopy ........................... 126

2.2.9 Centrosome staining .................................................................................. 127

2.2.10 RT-PCR .................................................................................................... 128

2.2.11 Statistical analysis .................................................................................... 128

Chapter 3: Results and Discussion ........................................................................ 129

2.3.1 SPAG6 is expressed in primary and secondary lymphoid tissues and co-
localizes with the centrosome ............................................................................ 129

2.3.2 SPAG6 is required for centrosome polarization and actin clearance at the
immunological synapse ..................................................................................... 130

2.3.3 Reduced T cell cytotoxicity in Spag6KO .................................................... 131

2.3.4 WT mice reconstituted with SPAG6 deficient bone marrow exhibit impaired GC
formation, diminished follicular CD4 T cells, reduced class-switched antibody
production, and expansion of B1 B cells ......................................................... 138

List of References .............................................................................................. 142

VITA ..................................................................................................................... 164
List of Tables

Table 1. PCR and qPCR primers and probes.................................................................36
Table 2. Western Antibodies.......................................................................................38
Table 3. Flow Cytometry and Immunohistochemistry Antibodies..............................42
Table 4. ADAM10 expression in Naïve and Stimulated WT B cells by qPCR............78
Table 5. Increased HDM specific IgE correlates with increased B cell ADAM10
expression.......................................................................................................................87
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic of ADAM10 and ADAM17</td>
</tr>
<tr>
<td>2</td>
<td>B cell specific ADAM10 deficient mouse model</td>
</tr>
<tr>
<td>3</td>
<td>ADAM10 expression on GC B cells</td>
</tr>
<tr>
<td>4</td>
<td>ADAM10^{B−/−} mice have impaired humoral responses</td>
</tr>
<tr>
<td>5</td>
<td>Decreased NP-specific IgG secretion is not IgG subset dependent and cannot be overcome by high antigen dose</td>
</tr>
<tr>
<td>6</td>
<td>GC formation after T-dependent immunization</td>
</tr>
<tr>
<td>7</td>
<td>Naïve C57-ADAM10^{B−/−} lymph nodes (LN) display abnormal follicular architecture</td>
</tr>
<tr>
<td>8</td>
<td>Increased TNF surface expression and production in C57-ADAM10^{B−/−} cells</td>
</tr>
<tr>
<td>9</td>
<td>Increased TNFα gene expression and message stability in C57-ADAM10^{B−/−} B cells</td>
</tr>
<tr>
<td>10</td>
<td>Increased ADAM17 message and protein levels in C57-ADAM10^{B−/−} cells</td>
</tr>
<tr>
<td>11</td>
<td>Confirmation of successful bone marrow reconstitution</td>
</tr>
</tbody>
</table>
Figure 12. Reconstitution of irradiated WT naïve LNs with ADAM10\textsuperscript{B/-} + TNFKO bone marrow restores normal follicular architecture and rectifies structural abnormalities induced by ADAM10\textsuperscript{B/-} reconstitution alone……………………………………………………67

Figure 13. Balb-ADAM10\textsuperscript{B/-} mice exhibit reduced NP-specific antibody production but a normal percentage GC B cells……………………………………………………………………………71

Figure 14. Differential TNF production and expression in C57Bl/6 and Balb/c ADAM10\textsuperscript{B/-} (A10K0) and WT B cells……………………………………………………………………………………72

Figure 15. Increased ADAM17 in C57Bl/6 WT and A10KO over Balb/c WT and A10KO, respectively……………………………………………………………………………………73

Figure 16. Balb-ADAM10\textsuperscript{B/-} lymph nodes exhibit WT architecture unlike C57-ADAM10\textsuperscript{B/-} nodes………………………………………………………………………………………………74

Figure 17. Increased B cell ADAM10 and decreased ADAM17 and TNF in Th2 biased strains………………………………………………………………………………………………77

Figure 18. HDM lung inflammation protocol………………………………………………83

Figure 19. B cell ADAM10 deletion attenuates bronchoconstriction and HDM specific IgE………………………………………………………………………………………………84

Figure 20. B-ADAM10 deletion reduces cellular infiltration, goblet cell metaplasia, and mucus production in a strain dependent manner……………………………………………………85

Figure 21. Allergic patient B cells exhibit increased ADAM10 and sCD23 but decreased ADAM17 and TNF…………………………………………………………………………………86
Figure 22. C57-ADAM10^{B/-} B cells have a B regulatory or B10 cell phenotype and C57-ADAM10^{B/-} mice exhibit increased IL10 in circulation.................................................90

Figure 23. Increased FoxP3+ T cells in 14 day immunized C57-ADAM10^{B/-} spleen...91

Figure 24. Increased Tregs in C57-ADAM10^{B/-} naïve and immunized spleen.........92

Figure 25. Increased ICOSL expression and cleavage on C57-ADAM10^{B/-} B cells.....96

Figure 26. C57-ADAM10^{B/-} conditioned B cell supernatants induce enhanced FoxP3 expression in CD4+CD25- T cells compared to WT B cell supernatants.......................97

Figure 27. ICOSL neutralization reduces FoxP3 expression in co-cultures of CD4+CD25- T cells with PMA-conditioned C57-ADAM10^{B/-} B cell supernatants.......98

Figure 28. Treg depletion in C57-ADAM10^{B/-} prior to HDM challenge increases AHR, BALF cellularity, eosinophil infiltration, and HDM-specific IgE.................................102

Figure 29. Treg depletion increases peribronchiolar and perivascular inflammatory cellular infiltration in WT and C57-ADAM10^{B/-}.................................................................103

Figure 30. Anti-CD25 treatment increases mucin in WT and C57-ADAM10^{B/-} airways................................................................................................................104

Figure 31. Increased CD43 expression on C57-ADAM10^{B/-} B cells......................106

Figure 32. C57-ADAM10^{B/-} B cells fail to reconstitute secondary lymphoid tissues as effectively as WT B cells.................................................................107

Figure 33. SPAG6 expression in lymphoid cells and tissues...............................133
Figure 34. Presence of SPAG6 protein in the centrosome…………………………………….134

Figure 35. Defective actin clearance and centrosome polarization at the immunological synapse in Spag6KO mice………………………………………………………………………………..135

Figure 36. Reduced CTL function in Spag6KO mice……………………………………………….137

Figure 37. Defective humoral immune response in SPAG6KO mice………………………..141
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-/-</td>
<td>Homozygous deletion of a gene</td>
</tr>
<tr>
<td>2.4G2</td>
<td>mAb recognizing the stalk region of murine CD23</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ADAM-TS</td>
<td>ADAM-thrombospondins</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway resistance</td>
</tr>
<tr>
<td>AID</td>
<td>Activation induced cytosine deaminase</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AT</td>
<td>Adoptive transfer</td>
</tr>
<tr>
<td>B-ADAM10</td>
<td>B cell ADAM10</td>
</tr>
<tr>
<td>B10</td>
<td>IL10 producing B cell</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchiolar lavage fluid</td>
</tr>
<tr>
<td>BALT</td>
<td>Bronchiolar-associated lymphoid tissue</td>
</tr>
<tr>
<td>BBS</td>
<td>Borate buffered saline</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone marrow derived mast cell</td>
</tr>
<tr>
<td>BMP</td>
<td>Bovine metalloprotease</td>
</tr>
<tr>
<td>Breg</td>
<td>B regulatory cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
<tr>
<td>C/EBP-β</td>
<td>CAAT/enhancer binding protein β</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen induced arthritis</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CMEP</td>
<td>Common myelo-erythroid progenitor</td>
</tr>
<tr>
<td>CMLP</td>
<td>Common myelo-lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>CoA</td>
<td>Co-Activators</td>
</tr>
<tr>
<td>CoR</td>
<td>Co-Repressors</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CYP</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DLL</td>
<td>Delta-like Notch ligand</td>
</tr>
<tr>
<td>DLN</td>
<td>Draining lymph node</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EPG</td>
<td>Eggs per gram of feces</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence active cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FcεRI</td>
<td>The high affinity IgE receptor</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FO</td>
<td>Follicular</td>
</tr>
<tr>
<td>f.p.</td>
<td>Footpad</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA-binding protein 3</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte macrophage progenitor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venule</td>
</tr>
<tr>
<td>HR</td>
<td>Histamine receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iTreg</td>
<td>Inducible T regulatory cell</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>kuz</td>
<td>Kuzbanian</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LM</td>
<td>Littermate</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage Sca&lt;sup&gt;+&lt;/sup&gt;-c-Kit&lt;sup&gt;+&lt;/sup&gt; bone marrow cells</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>M1</td>
<td>Classically activated macrophages</td>
</tr>
<tr>
<td>M2</td>
<td>Alternatively activated macrophages</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic cell sorting</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MC</td>
<td>Mast cell</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein 1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Myeloid derived suppressor cells</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MEP</td>
<td>Myeloid erythroid progenitor</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric Lymph node</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent progenitor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>mTNF</td>
<td>Membrane bound TNF</td>
</tr>
<tr>
<td>sTNF</td>
<td>Soluble TNF</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing center</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural T regulatory cell</td>
</tr>
<tr>
<td>ND</td>
<td>No difference</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NP-KLH</td>
<td>4-Hydroxy,3-Nitrophenylacetyl Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature medium</td>
</tr>
<tr>
<td>OVA</td>
<td>Ova albumin</td>
</tr>
<tr>
<td>PALS</td>
<td>Peri-arteriolar lymphoid sheath</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyl protein</td>
</tr>
<tr>
<td>PL</td>
<td>Peritoneal lavage</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut agglutinin</td>
</tr>
<tr>
<td>RAG1</td>
<td>Recombination activation gene 1</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RBP-Jk</td>
<td>Canonical Notch transcription factor</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor intramembrane proteolysis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SHP-1</td>
<td>SH2-homology-containing protein-tyrosine phosphatase-1</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>sICOSL</td>
<td>Soluble ICOSL</td>
</tr>
<tr>
<td>SPAG6</td>
<td>Sperm associated antigen 6</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>sTNF</td>
<td>Soluble TNF</td>
</tr>
<tr>
<td>SVR</td>
<td>Snake venom reprolysins</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-α converting enzyme</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>T cell or thymus dependent</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor Beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell type 2</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper cell type 17</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloprotease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNP</td>
<td>Trinitrophenol</td>
</tr>
<tr>
<td>Treg(s)</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TSPAN</td>
<td>Tetraspanin</td>
</tr>
<tr>
<td>TTP</td>
<td>Tristetraprolin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
Abstract

THE ROLE OF ADAM10, ADAM17, AND SPAG6 IN HUMORAL IMMUNITY AND SECONDARY LYMPHOID TISSUE ARCHITECTURE

By Lauren Folgosa Cooley, Ph.D.

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

Virginia Commonwealth University, 2015

Director: Daniel H. Conrad, Ph.D., Professor
Department of Microbiology and Immunology School of Medicine

ADAM10, ADAM17, and SPAG6 contribute significantly to humoral immunity and secondary lymphoid tissue architecture. ADAM10 and ADAM17 are two closely related zinc-metalloproteinases. Through cleavage of their ligands CD23 and TNF, respectively, they greatly influence IgE production and secondary lymphoid tissue architecture maintenance. Th1 prone WT strains initially exhibit increased ADAM17 and TNF yet reduced ADAM10 relative to Th2 prone WT strains. In the absence of B cell ADAM10, a compensatory increase in ADAM17 and TNF cleavage is noted only in Th1 prone C57Bl/6, not Th2 prone Balb/c. B cell TNF homeostasis is important for maintaining secondary lymphoid tissue architecture. We show for the first time that
excessive B cell TNF production in C57-ADAM10^{B/-} lymph nodes contributes to loss of B/T segregation, increased HEV number and size, fibrosis, loss of FDC networks, and impaired germinal center formation. Furthermore, B cell ADAM10, which enhances IgE production through CD23 cleavage, is shown to be a marker of Th2 susceptibility. B cell ADAM10 is elevated in Th2 prone mouse strains and allergic patients compared to Th1 prone controls and as B cell ADAM10 level increases, so does IgE production. Lastly, the B cell profile of allergic patients is determined to be B cell ADAM10^{high}·ADAM17^{low}·TNF^{low}.

Furthermore, the mechanism underlying reduced class-switched antibody production in C57-ADAM10^{B/-} mice is explored. C57-ADAM10^{B/-} B cells exhibit a B10, or IL-10 producing, phenotype, which is linked to reduced antibody production. Furthermore, increased Tregs noted in C57-ADAM10^{B/-} mice contributed to reduced class switched IgE production and disease parameters following a house dust mite airway inflammation challenge.

SPAG6, a component of the central apparatus of the “9+2” axoneme, plays a central role in flagellar stability and motility. Immune cells lack cilia, but the immunological synapse is a surrogate cilium as it utilizes the same machinery as ciliogenesis including the nucleation of microtubules at the centrosome. We demonstrate that Spag6 localizes in the centrosome and is critical for centrosome polarization at and actin clearance away from the synapse between CTL and target cells. Furthermore, improper synapse formation and function likely explains reduced CTL function and class-switched antibody production in Spag6KO mice.
Section 1: The role of B cell ADAM10, ADAM17, and TNF in secondary lymphoid tissue architecture and antibody responses.

Chapter 1: Introduction to ADAM10, ADAM17, and their respective ligands in humoral immunity and secondary lymphoid tissue architecture

1.1.1 Introduction to ADAM10 and ADAM17

ADAMs Overview

A disintegrin and metalloproteinases (ADAMs) belong to the metzincin superfamily of zinc dependent proteases and specifically to the M12B adamalysin protease subfamily along with structurally related snake venom reprolysins (SVR) and ADAM-thrombospondins (ADAMTS) [1,2]. All known ADAM proteins contain the following structural domains starting at the N terminus: (1) A pro-domain, which behaves as a chaperone for protein folding [3], and is thought to be cleaved intracellularly by pro-protein convertases during Golgi transit [4]; (2) A zinc dependent metalloprotease domain, which participates in substrate cleavage; (3) A 14 amino acid disintegrin loop binding domain, which confers substrate specificity [5]; (4) A cysteine rich ligand binding domain; (5) An EGF-like domain, which is absent in ADAM10 and ADAM17; (6) A transmembrane domain, which differentiates ADAMs from other metzincin family members; and (6) A cytoplasmic tail domain that varies widely in length and sequence between ADAM members (Figure 1).

Unlike other metzincin family proteases such as SVR, ADAMTS, and matrix metalloproteases (MMPs), ADAMs are unique in that they are catalytically active while membrane bound. Because they are membrane bound, ADAMs mediate ectodomain
shedding of mainly type 1 and type 2 transmembrane proteins. This ectodomain shedding can lead to downregulation of signaling events and/or effect autocrine and paracrine signaling through release of soluble mediators [6]. Furthermore, they participate in regulated intramembrane proteolysis (RIP), which requires receptor-ligand interaction [7]. ADAM-mediated proteolysis is both constitutive and inducible and can be modulated by many factors including: intracellular calcium levels, membrane lipid composition, ease of removal of the inhibitory pro-domain, and post-translational modification of the cytoplasmic tail domain [8]. Lastly, the number and function of each ADAM protein varies between species. For instance, the human genome contains 25 ADAM genes whereas the mouse has 37. Furthermore, which ADAMs are catalytically active may vary between species and some ADAMs, specifically in humans, are thought to play a role in protein folding rather than catalysis because the critical zinc binding active site is absent [1]. Overall, catalytically active ADAMs, termed sheddases, share the following zinc-binding consensus sequence: (HEXGHXXGXXHD)[9].

**ADAM10**

ADAM10 was initially characterized in 1996 through study of its homolog in *Drosophila, Kuzbanian (kuz)*. Studies of *kuz* deficient embryos revealed its critical importance in the development of the peripheral and central nervous system (CNS) [10]. Cloning and sequencing of *kuz* demonstrated disintegrin and metalloprotease domains homologous with bovine metalloprotease (BMP), which would later be called ADAM10 [10]. Subsequent studies regarding nervous system defects in the absence of ADAM10 revealed the key role of ADAM10 in Notch signaling [11]. Given the importance of Notch in early development, ADAM10 deficient embryos experience early death at
embryonic day 9.5 due to defects in CNS and cardiovascular development [12]. Therefore, conditional knockout models have typically been used to study ADAM10 in vivo.

Regulation and trafficking of ADAM10 to its sites of action is still poorly understood although some players have been identified. ADAM10 is ubiquitously expressed and is synthesized in the endoplasmic reticulum (ER) in an inactive pro-form. Tetraspanin 15 (TSPAN15) helps to accelerate ER exit to the Golgi [13]. Tetraspanins are termed “molecular facilitators” or chaperones that modulate the formation and stabilization of signaling complexes [14,15]. During Golgi transit, furin and pro-protein convertase (PC7) remove the inhibitory, pro-domain from ADAM10 [16]. TSPAN15 remains with ADAM10 throughout this process and helps with the integration and stabilization of ADAM10 into the membrane and further helps ADAM10 gain access to substrates. Furthermore, ADAM10 is known to bind TSPAN12, which has been shown to aid in ADAM10 maturation. siRNA ablation of TSPAN12 reduced ADAM10 maturation and its shedding of amyloid precursor protein (APP) [17]. While it is well known ADAM10 cleaves membrane bound substrates from the plasma membrane, there is also evidence that ADAM10 cleaves in the endosomal pathway and is more catalytically active at acidic pH [18]. Following internalization of ADAM10 and its substrate, they are trafficked through the endosome into multivesicular bodies where shedding occurs and both are then sorted into exosomes [19]. Exosomes are 30-100nm, membrane-containing vesicles that are released from cells, express MHC and integrins, and may be able to present antigen to T cells [19,20]. Furthermore, Tousseyn et al. showed that ADAM10 itself can be processed by ADAM9, ADAM15, and γ-
secretase, releasing the ADAM10 intracellular domain, which can modulate gene transcription [21]. One direct consequence of ADAM10 processing was described by Arima et al. in human prostate cancer progression. ADAM10 translocation to the nucleus occurred more frequently in prostate cancer cells compared to benign prostatic hyperplasia (BPH), which expressed predominantly membrane ADAM10 [22].

Little is known about ADAM10 regulation following pro-domain cleavage. Following ADAM10 activation, in vitro ADAM10 activity can be inhibited by tissue inhibitors of metalloproteases (TIMP)-1 and TIMP-3 [23]. Additionally, its proteolytic activity is enhanced by calcium influx, retinoic acid receptor signaling, PKC signaling, cholesterol depletion, kainite receptor signaling and N-glycosylation [24–29]. Regulation of ADAM10 activity is critical for maintaining a homeostatic release of substrates. A critical ADAM10 substrate for both organ system development as well as lymphocyte development is Notch receptor(s). While ADAM10 has many substrates several important ones for this discussion will be CD23 and TNF. The low affinity IgE receptor, CD23, regulates IgE production [30]; and, TNF is a pro-inflammatory cytokine that is also important for maintaining secondary lymphoid tissue architecture (section 1.1.6) [31–34]. Lastly, ADAM10 participates predominately in cis-cleavage but has been shown in certain instances to cleave substrates in trans [35].

**ADAM17**

TNF is a key pro-inflammatory cytokine, which exists as a 26kDa transmembrane protein (mTNF) and a 17kDa soluble molecule (sTNF) [36]. Given its critical importance, many groups aimed to discover the protease responsible for its cleavage. In 1997, two groups simultaneously described a new ADAM protease, ADAM17, which
was responsible for the cleavage of pro-TNF [37,38]. Its gene is located on chromosome 2p25 in humans and chromosome 12 in mice. Similar to ADAM10, it is ubiquitously expressed in many cells and tissues and is critical during development [37]. Although ADAM17 is most closely related in structure to ADAM10, as both lack the EGF-like domain found in other ADAMs, they still only share 30% homology [39].

Data on the overall regulation and trafficking of ADAM17 is sparse and often contradictory. ADAM17 has an inhibitory pro-domain that is cleaved similar to ADAM10 by furin in the trans-Golgi network [40]. While pro-domain removal is critical for enzymatic activity, it is not required for ADAM17 transport to the plasma membrane [41]. Furthermore, immunohistochemistry evidence suggests that active ADAM17 exists in the perinuclear region with very little on the plasma membrane [40], but many still believe cleavage is mostly restricted to the plasma membrane. Whereas tetraspanins are important for ADAM10 trafficking through the ER and Golgi, it is a rhomboid protein, iRhom2, which is critical for ADAM17 trafficking [42,43]. iRhom2 escorts ADAM17 from the ER, through the trans-Golgi network, and ultimately to the site of ligand cleavage. In the absence of iRhom2, furin-mediated proteolysis of ADAM17 and trafficking to the cell surface does not occur [42,43]. Post-translational modifications of ADAM17 protein include phosphorylation of ADAM17 residues, specifically serine and threonine. Currently, there is conflicting evidence about which residues are critical for ADAM17’s activity and whether phosphorylation or de-phosphorylation of those residues enhance activity. One example is ADAM17 modification following phorbol ester (PMA) stimulation. PMA is known to stimulate ADAM17 activity through phosphorylation events, but conflicting reports have named
both threonine 735 and serine 819 as the critical phosphorylation site [44,45]. In
general, however, it is accepted that various stimuli may cause a differential
phosphorylation or de-phosphorylation pattern but exact mechanisms are unknown.
Lastly, ADAM17 activity is inhibited endogenously by TIMP-3 and has been shown to
have several other binding partners, including α5β1 integrin, whose roles in modulating
ADAM17 activity are still under investigation [39].
Figure 1. Schematic of ADAM10 and ADAM17.

ADAM10 and ADAM17 are membrane bound proteases and are composed of the following domains: (1) zinc dependent metalloproteinase (yellow); (2) disintegrin (red); (3) cysteine-rich (green); (4) transmembrane (orange); (5) short cytoplasmic tail (black). ADAM10 and 17 mediate cleavage of cytokines and growth factors as well as initiate signaling events through receptor cleavage.
1.1.2 Introduction to secondary lymphoid tissues

Secondary lymphoid tissues are primary sites of adaptive immune responses and consist of lymph nodes (LN), spleen, tonsils, Peyer’s patches (PP), bronchiolar-associated lymphoid tissues (BALT), gut-associated lymphoid tissues (GALT), and mucosa-associated lymphoid tissues (MALT). They are located strategically throughout the body in order to efficiently alert immune cells to the presence of foreign antigen and allow a structured framework for their encounter [46–49]. While lymphocytes and other immune cells are mobile and migrate in and out of secondary lymphoid tissues, their architecture is tightly regulated and organized. During embryogenesis, secondary lymphoid tissues begin to develop when hematopoietic lymphoid tissue inducer cells (LTi), expressing lymphotoxin αβ (LT), and mesenchymal cells, expressing LTβ receptor, interact. Induction of LT signaling in mesenchymal cells triggers their differentiation into stromal organizer cells and induces homeostatic chemokine expression, such as CXCL13, CCL19, and CCL21, that aid in migration of naïve lymphocytes [47,50,51]. The critical importance of LT is demonstrated in LT-deficient mice, which lack peripheral LN, PP, and demonstrate severely disrupted splenic architecture.

Secondary lymphoid tissues are highly organized tissues, which is thought to aid in efficient response to foreign antigen. LNs, for instance, have an afferent vasculature supply, which brings antigen presenting cells (APCs) and foreign antigen into the LN marginal sinus. Lymphocytes and APCs can also access the LN through high endothelial venules (HEVs) found in the paracortex. The LN is divided into a cortex and medulla. The cortical region consists of (1) the outer cortex, or B cell zone, which
contains resting, primary B cell follicles; and, (2) the paracortex, where T cells reside. The medulla is divided into medullary chords containing macrophages and plasma cells and medullary sinuses, which drain immune cells into efferent lymphatics. Splenic structure, while reminiscent of LN structure, varies in several ways [47,51]. The spleen is divided into red and white pulp. The red pulp is responsible for the filtration of red blood cells (RBCs) and removal of damaged or aged RBCs from circulation [47]. The white pulp consists of periarteriolar lymphoid sheaths (PALS), which are comprised of a central arteriole surrounded by a T cell rich compartment encircled by B cell follicles containing FDCs [47,49]. The red and white pulp is separated by the marginal zone (MZ), which holds MZ macrophages and MZ B cells (MZB). MZB development and maintenance requires CD19, Notch2, and ADAM10 protein expression and signaling [52–54]. Briefly, B cell specific Notch2-/- mice fail to develop MZB as do B cell specific ADAM10 deficient mice.

B and T cells home to their respective zones due to chemokine gradients. B cells express CXCR5, which binds CXCL13 causing migration to the follicle. It is thought that follicular dendritic cells (FDCs), which reside in the B cell follicle, release CXCL13 [55]. B cells typically enter the LN through HEVs located in the T cell zone before migrating to the B cell follicle; however, they may also enter through the afferent lymphatic system. Fibroblastic reticular cells (FRC) reside in the T cell zone and release CCL19 and CCL20, which bind CCR7 on naïve T cells and dendritic cells (DC) [56]. Thus, CCL19/20 interaction with CCR7 is important for both T cell homing to the paracortex as well as DC migration to lymphoid tissues. When dendritic cells encounter antigen in the periphery, the antigen is processed and presented in major
histocompatibility complex class II (MHCII) and co-stimulatory molecules, CD80/86, are upregulated. When the DC enters the paracortex, MHCII plus antigen is recognized by the TCR on antigen-specific CD4$^+$ T cells and CD80/86 interacts with T cell CD28 to provide the second signal for activation. Furthermore, the cytokine environment will cause CD4$^+$ T cell differentiation into various T helper (Th) cell classes (see section 1.1.5) [57,58].

1.1.3 B cell activation

B cells are responsible for the humoral arm of the adaptive immune system and produce high affinity antibody against foreign antigens. B cells initially express membrane bound immunoglobulin, also known as the B cell receptor (BCR), which confers specificity to a particular antigen. The BCR complex is comprised of cell surface immunoglobulin made of a heavy and light chain as well as antigen-nonspecific, invariant proteins Ig$\alpha$ and Ig$\beta$, which express immunoreceptor tyrosine-based activation motifs (ITAMs) [59,60]. Activation of B cells requires cross-linking of the BCR, thus complex, multivalent antigens are stronger B cell activators because they allow extensive receptor crosslinking. Following crosslinking, ITAMs are phosphorylated by Src family tyrosine kinases (Lyn, Fyn, and Blk) followed by activation of Syk tyrosine kinase. Syk then phosphorylates its targets including BLNK, CD19, and PLC-$\gamma$, which activates Ca$^{2+}$ and MAP kinase signaling. Ultimately, NFkB, NFAT, and AP-1 transcription factors are activated and lead to downstream activation of genes required for B cell proliferation and differentiation. BCR signaling is further modulated by a B cell co-receptor complex comprised of CD21, CD19, and CD81 [59–61]. In addition to antigen induced signaling through the BCR, B cells process and present antigen in
MHCII for CD4+ T cell recognition and receive cytokine signals that further affect their activation.

**T dependent responses**

Protein antigens are considered T cell or thymus dependent (TD) antigens as B cells require T cell help for activation. Antigen-bound B cells encounter T cells at the T-B border in secondary lymphoid tissues. A cognate interaction between antigen-specific B and T cells occurs as B cells process and present antigen in MHCII to CD4+ T helper cells. Additionally, CD40 receptor on B cells engages CD40 ligand (CD40L) on T cells and T cells deliver cytokines signals such as IL-4, IL-5, IL-6, and IL-21, which help shape humoral responses. Following this cognate interaction, B cells proliferate or clonally expand in a primary, extra-follicular focus at the T-B border and form antibody-synthesizing plasmablasts, which have both membrane bound and secretory antibody (Ig). At this point, B cells commit to an extra-follicular or germinal center (GC) pathway. It is not clear what causes commitment to either pathway. Paus et al. proposed that B cells with highest antigen affinity initially will differentiate along the extra-follicular pathway into plasma cells [62]. These extra-follicular origin plasma cells tend to be short-lived, non-migratory, and primarily secrete IgM [63,64]. The germinal center pathway is discussed in section 1.1.4.

**T independent responses**

B cell responses to T-independent (TI) antigens do not require T cell help and is why antibody responses exist in humans and mice with T cell deficiencies. TI antigens are subdivided into TI type 1 (TI-1) and TI type 2 (TI-2) antigens [65]. TI-1 antigens,
also called B cell mitogens, can activate immature or mature B cells and intrinsically induce B cell division. They typically cause polyclonal B cell activation, especially at high concentration. A classic TI-1 antigen is lipopolysaccharide (LPS) [66]. TI-2 antigens can only activate mature B cells to make IgM and are thought to do so by cross-linking many BCRs on mature, antigen-specific B cells. B cells responding to TI-2 antigens further require a cytokine signal called B cell activating factor (BAFF) in order to induce class-switching to IgG [67,68]. TI-2 antigens are highly repetitive polysaccharides and help confer immunity to encapsulated bacteria [65]. B1b B cells (B220+CD23-CD11b+CD5+) and MZB are thought to make antibody by this mechanism [69]. In general, both TI-1 and TI-2 antigens are thought to be poor at generating B cell memory responses; but; it has been reported that LPS and polysaccharide antigens can induce a memory response in rats [66].

1.1.4 Germinal Centers

Structure, formation, and maintenance

Once B cells are activated at the T-B border, some will migrate to the follicle via CXCL13/CXCR5 interactions and form a germinal center (GC) [55,57]. A GC is a specialized microenvironment within the follicle where B cells rapidly divide and undergo class switch recombination, somatic hypermutation, and affinity maturation to strengthen antibody binding. The GC can be divided into the mantle zone, dark zone and light zone [57,58]. The mantle zone includes resting B cells that are pushed to the periphery by the rapidly proliferating B cell population as well as recirculating B cells. The dark zone consists of rapidly dividing B cells, or centroblasts, that undergo somatic
hypermutation and class switch recombination [70]. The light zone contains FDCs, T follicular helper cells (Tfh), and B cells (centrocytes) undergoing affinity maturation [58,71]. B cells can proliferate albeit to a lesser extent in the light zone; but, this is considered a site of massive apoptosis for B cells that lose antigen specificity. Furthermore, while B cells classically move from dark to light zone, they can also move from light to dark [72]. GCs are present for 3-4 weeks after antigen exposure with their peak response occurring around day 15.

Other factors that are critical for GC formation and maintenance are Bcl6 and IL-21 [73–76]. Bcl6 is a transcriptional repressor found in both B cells and Tfh cells [73–75]. Bcl6 is required to prevent apoptosis induced by DNA damage that occurs during somatic hypermutation and class switch recombination [77]. Furthermore, Bcl6 is thought to prevent differentiation of B cells into plasma cells until sufficient antibody affinity is achieved [78]. Within the germinal center activated FDC produce IL-6, which is known to induce Bcl-6 expression [79,80]. Lastly, IL-21 is known to play a critical role in GC formation and antibody responses as IL-21 or IL-21 receptor deficient mice exhibit significantly reduced antigen-specific IgG [81] and decreased GC persistence [76]. IL-21 is not required, however, for memory B cell formation [76]. IL-21 induces STAT3 signaling, which is essential for generation of IgG1 secreting cells [82].

Lastly, sphingosine 1-phosphate receptor 2 (S1P$_2$) is critical for homing and retention of cells in the germinal center. First, B cells require S1P$_2$ to stay within the GC niche [83]. Furthermore, Moriyama et al. described that Tfh cells within the GC express high levels of S1P$_2$ [84]. S1P$_2$ deficient mice had reduced Tfh retention in GCs and it is
thought that S1P₂ and CXCR5 work cooperatively to traffic Tfh cells and retain them in the GC [84].

Somatic hypermutation and class switch recombination

Both somatic hypermutation (SHM) and class switch recombination (CSR) occur within the dark zone of the GC. The overall goal of SHM is to introduce mutations into V regions of the immunoglobulin gene in the hopes to increasing antibody specificity. While antigen specificity is not altered during class switch recombination, the class of antibody (e.g. IgM, IgG) changes, which determines its function as well its distribution throughout the body. Activation-induced cytidine deaminase (AID) is expressed only in B cells and drives both SHM and CSR [71]. AID deaminates cytidine to form uridine in switch regions of the immunoglobulin gene. This event triggers recruitment of base excision repair machinery (BER) including uracil-DNA glycosylase (UNG), which removes uridine. These mutations alter the amino acid sequence and may (1) disrupt antibody structure; (2) cause reduction or loss of antibody specificity; or (3) improve antibody specificity [65,71]. Because SHM can increase antibody specificity, it is said to drive affinity maturation. After AID and UNG steps, CSR further requires apurinic/apyrimidinic site endonuclease 1 (APE1) to introduce single strand nicks on both DNA strands, which leads to staggered double strand nicks. Double strand break repair machinery (DSBR) is then recruited and removes the damaged DNA region, ultimately driving recombination of the VDJ with a C region (e.g. Cµ, Cγ₁, Cε), which determines the isotype [85].

Affinity maturation and high affinity B cell selection
B cell selection contributing to affinity maturation is still a very controversial topic. Many mechanisms have been proposed for B cell selection post GC including (1) competition for FDC bound antigen; and, (2) competition for Tfh help [86]. The ultimate goal of B cell selection is to purge the germinal center of low affinity B cells and only allow B cells with high affinity Ig to survive. In the classical model, B cells compete for binding to FDC, which have retained antigen. B cell fate is dependent on the strength of interactions with the antigen. If BCR signaling is too weak, apoptosis occurs [87]. If BCR signaling is sufficient, the B cell survives and presents antigen to Tfh cells, which stimulate B cell differentiation into plasma cells or memory B cells. Critics of this theory state that this theory alone does not account for the robustness of affinity maturation that is observed [88]. An alternate theory states that Tfh cells do not only provide differentiation signals at the end but also play an important role in the selection process [57,88]. While the topic is still controversial, high affinity B cells ultimately leave the GC following selection and differentiate into plasma cells or memory B cells.

1.1.5 Th differentiation and associated antibodies

When naïve CD4+ T cells leave the thymus, they are capable of differentiating into several different Th subtypes depending on the cytokine environment, the antigen to which the immune system is responding, and cell signaling events that upregulate transcription factors characteristic of a particular subtype [89]. For the purposes of work described herein, Th1 and Th2 differentiation are highlighted.

Th1 differentiation occurs during infection with intracellular pathogens and when activated inappropriately, can result in autoimmunity. IFNγ and IL-27 (IL-12 family
cytokine) initiate STAT1 and STAT1/4 signaling, respectively, leading to *T-bet* (Th1 transcription factor) upregulation, which together with *Runx3* increases transcription of more IFNγ [90]. Furthermore, *T-bet* and *Runx3* silence the IL-4 gene and *GATA3*, thus inhibiting Th2 differentiation [89]. IgG2a/c and IgG2b are considered “Th1” isotypes and are increased by Th1 promoting cytokines such as IFNγ and IL-12 [91,92]. Th2 cells respond to extracellular parasite infection and are required for the induction and persistence of asthma and allergy [93,94]. Th2 differentiation requires IL-4 and IL-2 signaling. IL-4 induces STAT6, which drives *GATA3* transcription factor expression. *GATA3* has three critical actions. First, it suppresses Th1 differentiation by downregulating STAT4. Second, it enhances Th2 cytokine production (e.g. IL4, IL5, IL9, IL13, IL10, IL25). Third, it aids in the proliferation of Th2 cells [95,96]. IL-2 activates STAT5. STAT5 and *GATA3* bind different sites of the IL-4 gene and their coordinated activity is required for IL-4 production [97]. IgG1 and IgE are considered “Th2” isotypes as they are increased by Th2 cytokines such as IL-4 [91–93].

The Th1/Th2 paradigm is classically attributed to differences in CD4+ T cell response, with B cells considered bystanders waiting for the T cell cytokine signal. Allergic diseases, such as asthma and allergic rhinitis, are skewed towards a Th2 phenotype whereas rheumatoid arthritis and other autoimmune disease are skewed toward Th1 [98]. Furthermore, different mouse strains are considered Th1 or Th2-prone based on their propensity to make “Th1” or “Th2” classes of antibody post immunization. Classic Th1 (such as C57Bl/6 and SJL/J) and Th2-prone (such as Balb/c and A/J) strains were characterized as high (Balb/c, A/J), intermediate (C57Bl/6), and low (SJL/J) IgE responders following immunization [99]. Differences in their antibody production
were attributed to differences in CD4+ T cell signal. Whether B cells from Th1 or Th2-biased mouse strains have intrinsic differences that may affect IgE production, such as varied ADAM10 and ADAM17 levels, has never been elucidated and is discussed herein.

1.1.6 ADAMs 10 and 17 ligands in maintaining secondary lymphoid tissue architecture and humoral responses

TNF Family Cytokines

TNF and its closely related family members, lymphotoxin alpha (LTα) and beta (LTβ), have been extensively studied in the development and maintenance of secondary lymphoid tissue architecture. Lymphotoxin is a trimeric cytokine and typically exists as a secreted homotrimer, LTα3, or a membrane bound heterotrimer, LTα1β2, on activated B, T, and NK cells [100]. LTα deficient mice lack lymph nodes and Peyer’s patches and exhibit abnormal splenic architecture including loss of B cell/T cell segregation and a complete absence of FDC networks, germinal centers, and MZB [101,102]. LTα as a soluble homotrimer (LTα3) is also known to play an integral role in lymphoid organization including B cell/T cell segregation by binding to the TNF receptor, p55TNFR-1 [103]. LTβ deficient mice, however, experience more mild disruption as they retain mesenteric and cervical lymph node development and maintain B cell/T cell segregation, FDC networks, and germinal center formation in spleen [104]. There is currently only one report that states that membrane bound LTα1β2 heterotrimer on T cells is cleaved by ADAM17 in vitro and this cleavage is blocked by the ADAM17 inhibitor, TAPI-1[100]. They specifically were looking for a mechanism underlying increased LTα1β2 in the serum and synovial fluid of rheumatoid arthritis patients [100].
To date, there are no studies analyzing the relationship between ADAM17 and LTα1β2 in maintaining secondary lymphoid tissue architecture.

ADAM10 and ADAM17 are closely related ADAMs; therefore, many questions have been raised about their functional redundancy under physiologic conditions as well as in various genetically manipulated animal models. While ADAM17 is the principle sheddase of TNF under physiologic conditions, ADAM10 is known to cleave TNF, especially in the absence of ADAM17 [105]. Mezyk-Kopec et al. further demonstrated that in ADAM17 deficient mouse embryonic fibroblasts, ADAM10 increases and ADAM10 dependent cleavage of TNF is seen [106]. With regards to TNF and secondary lymphoid tissue architecture, global TNF deficient mice exhibit disorganized follicular dendritic cell (FDC) networks, aberrant germinal centers, and lack of splenic B cell follicles [32]. Furthermore, in a B cell specific mutant model, which expresses a non-cleavable form of mTNF, abnormal B cell/T cell localization with loss of a proper cortico-medullary junction, reduced germinal center formation, impaired FDC network development, and reduced IgG production against T dependent antigens was seen [34]. Thus, adequate levels of B cell produced sTNF is critical for maintaining secondary architecture in the lymph node, spleen and Peyer’s patches and for optimal antibody responses [34]. Interestingly, B cell specific ADAM10 deficient mice exhibit similar alterations in immunized and naïve lymph nodes [31,107], which is described herein. While lack of B cell sTNF is known to lead to phenotypic alterations, work herein demonstrated for the first time that excessive B cell sTNF also leads to defects in secondary lymphoid tissue architecture.
Lastly, B cell TNF production can affect downstream antibody production. First, given that proper secondary lymphoid tissue architecture is critical for optimal germinal center formation and antibody production, B cell TNF homeostasis is critical for proper antibody responses [32–34]. Furthermore, Frasca et al. demonstrated that increased B cell TNF production is a key factor implicated in B cell aging, which ultimately curtails class-switched antibody production [108]. Therefore, both excessive and too little B cell TNF has negative consequences on optimal antibody production.

**ICOSL**

ICOS is inducible costimulator molecule, which is a member of the CD28/CTLA-4/B7 immunoglobulin superfamily. It is found on activated T cells and binds ICOSL, which is found on B cells, DCs, as well as non-immune cells [109]. While not classically discussed as a co-stimulation signal needed for B cell activation, it is known that ICOS-/- [110] and ICOSL-/- [111] mice have defects in germinal center formation and antigen-specific antibody production. Marczynska et al. demonstrated that ADAM17 regulates ICOSL cleavage in vitro [112]. Treatment of WT B cells with PMA resulted in cleavage of ICOSL. B cells with significantly reduced ADAM17 expression (ADAM17\textsuperscript{ex/ex} B cells), however, exhibited reduced ICOSL cleavage following PMA stimulation [112]. To determine if ADAM17 directly cleaved ICOSL, they performed purified recombinant enzyme studies and found that ADAM17 does not directly cleave ICOSL, but instead is indirectly involved in the downregulation of surface ICOSL [112]. Following in vivo immunization, furthermore, ADAM17\textsuperscript{ex/ex} mice have increased germinal center B cells and plasma cells in draining LN, increased total and antigen specific IgG1 and IgG2a,
and draining LN hyperplasia with normal architecture [112]. Therefore, decreased ADAM17 and increased surface bound ICOSL lends to increased antibody production.

Furthermore, ICOS-ICOSL interactions are critical for the generation and maintenance of T regulatory cells (described in 1.1.7). Zheng et al. noted that blockade of ICOS-ICOSL interactions in vitro impaired Treg induction as well as expression of CTLA-4 and other mechanisms characteristic of Treg suppression [113]. Furthermore, Redpath et al. described that ICOS deficiency led to FoxP3+ Treg apoptosis, impaired IL-10 production, and increased Th2 responses in the intestine during helminth infection [114]. It is not clear, however, if ICOSL must be membrane-bound or soluble to induce Tregs through ICOS receptor. Overall, given the importance of ADAM17 in regulating ICOSL levels, ADAM17 may indirectly affect Treg induction and function by controlling ICOSL levels. Specifically, increased B cell ADAM17 would lead to increased soluble ICOSL, which may cause increased Treg induction through binding to ICOS receptor.

CD23, IgE, and asthma

CD23 is a unique Fc receptor as it is a type 2 transmembrane protein and a member of the calcium dependent (C type) lectin family. It exists in two isoforms, CD23a and CD23b, and has three domains: (1) IgE interacting carboxy terminal domain, (2) stalk regions, and (3) a short cytoplasmic tail [115]. Surface CD23 levels can be increased in an IL-4 or IL-13 dependent manner [115]. In humans, peripheral blood mononuclear cells stimulated with IL-4 exhibit increased CD23 shedding and IgE production whereas treatment with anti-CD23, which prevents its cleavage, inhibits IgE production [116]. Several enzymes had been implicated in the cleavage of CD23 including ADAM8 [117] and other hydroxymate sensitive metalloproteinases [118], but
ADAM10 was determined by Weskamp et al. in a series of loss and gain of ADAM protease function experiments to be the principle sheddase of CD23 in B cells [30]. Once cleaved from the surface, sCD23 enhances IgE production by an unknown mechanism. Several models exist that attempt to explain this relationship including: (1) High levels of IgE stabilize mCD23 and reduce further IgE production while allergen proteases and anti-CD23 stalk monoclonal antibodies destabilize CD23, increase its proteolysis, and increase IgE production [115]; and, (2) sCD23 crosslinks membrane IgE and CD21 resulting in increased IgE production [119]. This relationship between ADAM10, CD23, and IgE has since ignited much interest in the asthma and allergy fields regarding the use of ADAM10 or CD23 cleavage inhibitors as a mechanism to prevent IgE synthesis.

Asthma is a chronic airway disease characterized by wheezing, cough, shortness of breath, chest tightness, and “asthma attacks” caused by obstruction in airflow. In the United States, prevalence has increased since 2001 with children and African Americans having the highest incidence [120]. Unfortunately, this report did not distinguish between the incidence in allergic and non-allergic asthma. Another community based clinical study of Danish adolescents and adults, reported that allergic asthma represents three out of five asthma cases. Furthermore, they reported that allergic asthma patients had greater AHR compared to non-allergic asthma patients [121]. Allergy and allergic asthma are considered classic Th2 diseases with IgE as the predominant antibody class coordinating the response. IgE levels are tightly regulated and of all Ig classes have the lowest levels in vivo [122]. Interestingly, IgE can detect very low levels of antigen and is often the first to detect foreign particles in areas of
interface with the environment. When these foreign particles are innocuous, such as pollen, cat dander or peanut proteins, IgE moves from beneficial to potentially life threatening. IgE mediates allergic responses from mild to severe reactions, such as allergic rhinitis, atopic dermatitis, urticaria, asthma, and anaphylactic shock [123].

IgE participates in the immune response in one of three ways. First, antigen specific IgE binds the high affinity IgE receptor, FcεRI, on mast cells (MCs) or basophils where it can persist for up to 21 days waiting to bind to antigen, cross-link the FcεRI and degranulate the MC [124,125]. Degranulation results in the release of many mediators including (1) histamine, which increases vascular permeability and smooth muscle contraction; (2) MC enzymes, such as tryptase and chymase, which cause connective tissue and matrix remodeling; (3) cytokines, such as IL-4, IL-13, IL-5, and TNF, which amplify the Th2 response, recruit eosinophils, and promote inflammation, respectively; and (4) lipid mediators, such as leukotrienes, which increase vascular permeability, stimulate mucus secretion, and cause smooth muscle contraction [126]. While histamine, MC enzymes, and some TNF are stored as preformed mediators and released immediately, the others are synthesized following IgE/ FcεRI cross-linking and are important for the late-phase reaction [123,127]. Second, circulating IgE can bind to its antigen creating an IgE-immune complex. IgE immune complexes are picked up by circulating follicular (FO) B cells by binding to CD23, the low affinity IgE receptor (FcεRII) [128]. These CD23+ B cells then traffic to the splenic follicles where antigen transfer occurs followed by rapid increases in antigen-specific CD4+ T cell proliferation and IgG responses [128,129]. Third, circulating IgE can bind to mCD23 on B cells and shut down excessive IgE synthesis [115].
1.1.7 T regulatory cells and their role in allergic airway disease

T regulatory cells (Tregs) are critical for maintaining peripheral tolerance, immune homeostasis, and preventing autoimmunity. Furthermore, they are important for suppressing allergy and asthma as well as inducing tolerance to dietary food antigens and maintaining maternal tolerance to the fetus [130]. They are divided into two sub-populations, natural Tregs (nTregs) and inducible Tregs (iTregs) [131]. nTregs are derived centrally in the thymus through MHCII/TCR dependent interactions resulting in high-avidity selection and express a T cell receptor (TCR) that has a diverse repertoire for both self (although controversial) and foreign antigens [130,132]. Because self-reactive T cells are eliminated during negative selection, it has been proposed that FoxP3+ T cells may resist negative selection [132]. Others believe, however, that while a small subset of Tregs recognize self antigen weakly, this is “the exception not the rule” [130]. Following differentiation in the thymus, nTregs migrate to the periphery to prevent autoimmune reactions by raising the threshold of activation for all immune responses and suppressing autoreactive T cells. nTregs require CD28 as a costimulatory signal and IL-2 or IL-15 for activation [94]. iTregs, which have been more recently defined, are generated in the periphery in secondary lymphoid or inflamed tissues from CD4+FoxP3- T cells following TCR stimulation in the presence of TGFβ and IL-2 leading to FoxP3 expression [94]. While not described herein, there are also 2 subclasses of FoxP3+ iTregs that produce either IL-10 (Tr1 cells) or TGFβ (Th3 cells) [94].

In mice, both nTreg and iTregs are defined by expression of the transcription factor, forkhead box 3 (FoxP3) and express the following markers: CD4+CD25+FoxP3+
The difference is that nTregs leave the thymus already expressing FoxP3 whereas FoxP3 expression is induced in iTregs following TCR engagement with TGFβ and IL-2 signaling [94]. While the exact function of FoxP3 is unknown, genome analysis found that it binds to about 1000 genes, some of which are important for TCR signaling, and therefore may act as both a transcriptional activator and repressor [130]. Other markers that are also characteristic of Treg cells include ICOS, PD-1, GITR, LAG3, CTLA-4, neuropilin-1, and Helios. Huang et al. compared these surface markers on iTregs and nTregs and found that iTregs had increased ICOS and CTLA-4 compared to nTregs but equal levels of PD-1, GITR, LAG3, neuropilin-1 and reduced Helios [134]. A different report, however, states that nTregs exhibit higher expression of PD-1, neuropilin 1, and Helios compared with iTregs [135]. It is still difficult, therefore, to differentiate between these two populations in vivo as there is still controversy over their surface markers; and, a unique marker has not been discovered to differentiate between the two groups.

Functionally, both nTregs and iTregs act synergistically to induce tolerance and suppress immune cell proliferation and function. Bilate et al. proposed that the collaboration between nTregs and iTregs is due to their non-overlapping TCR repertoires [131]. Both are able to suppress (1) T cell proliferation in addition to other immune cell types; (2) Tbet and GATA3 upregulation, (3) cytokine production; (4) CD8+ T cell cytotoxic activity; and (5) antibody production [136]. Suppression mechanisms used include secretion of inhibitory cytokines (IL-10, TGFβ, and IL-35), competition for growth factors such as IL-2, and CTLA-4 mediated downregulation of T cell responses.
However, the exact mechanisms by which nTregs and iTregs induce tolerance are still under investigation.

Given that Tregs inhibit T cell proliferation, Th2 differentiation, Th2 cytokine production, and antibody production, it is obvious that Tregs would be beneficial in reducing or preventing induction of allergic diseases and asthma [136,137]. In a chronic OVA-induced lung inflammation model, adoptive transfer of CD4⁺CD25⁺ Tregs after airway disease was already established led to a reduction in eosinophils, Th2 cytokines, peribronchiolar lung collagen, and mucus. Airway resistance (AHR) in this study, however, was not affected by Treg transfer after disease was already established [138]. Other acute and chronic murine airway hypersensitivity studies, however, clearly demonstrated that Tregs reduce AHR [139–141]. Furthermore, the presence of Tregs appears to be protective in healthy individuals. Healthy children or those diagnosed with chronic cough had increased CD4⁺CD25⁺ Tregs in BALF compared to children with asthma [142]. With regards to B cell IgE production, Meiler et al. demonstrated that Tregs exert their negative influence on allergy induction by suppressing IgE production and instead inducing IgG4 class-switching [143]. Furthermore, Xu et al. [141] reported that adoptive transfer of in vitro generated iTregs reduced OVA-specific IgE levels following OVA sensitization and challenge. Overall, both nTregs and iTregs are thought to cooperatively limit allergic disease induction by (1) suppression of eosinophil and mast cell migration and activation, (2) isotype switching from IgE to IgG4, (3) suppression of inflammatory cell migration into lung tissue, (4) reducing AHR, and (5) suppressing Th2 differentiation and cytokine production (IL-4, IL-5, IL-13) [144].

1.1.8 B regulatory cells: A unique B cell subset
Similar to T helper cell subsets, B cells can also be classified based on cytokines they produce. A subset of B cells, termed B regulatory cells, dampen or suppress immune responses [145–147]. Thomas Tedder, a pioneer in the B regulatory cell field, initially described B regulatory cells as IL-10 producing B cells. However, in his recent review, he acknowledges that IL-10 producing B cells (B10 cells) represent a specific subset of B regulatory cells [148]. Mizoguchi et al. describes other mechanisms B regulatory cells may use, in addition to IL-10 production, to mediate immunosuppression including (1) TGFβ1 production, (2) IgG and IgA production to mediate neutralization of harmful factors, and (3) induction of apoptosis in effector T cells [147]. Non-IL10 producing B regulatory subsets, however, are far less defined. B10 cells, specifically, are known to suppress aspects of both the innate and adaptive immune response. B10 cells are defined functionally by intracellular IL-10 staining and unfortunately a unique set of surface markers has not been identified [145,146]. In mice, they are known to share overlapping markers with marginal zone B cells and B1 B cells including CD1d+CD5−IgDloIgMhi but intracellular IL-10 staining is still required for identification [145].

B10 cells develop from a B10 progenitor cell (B10pro) defined as CD1d−CD5−MHCII−IgM+CD40+ and represent 1-2% of total splenic B cells [145,149]. The initiation of IL-10 secretion by B10 cells requires several factors including BCR ligation with antigen [150,151], CD40 ligation [152,153], and IL-21 signaling [154]. Interestingly, T cells are not required for B10 development but cognate interactions are important for eventual IL-10 secretion [154]. TLR-4 (LPS ligand) and TLR-9 (CpG oligonucleotide ligand) signaling augments IL-10 secretion but is not essential for B10 development.
For instance, MyD88-/- mice have equal frequencies of B10pro and B10 cells compared to WT but not optimal IL-10 expression [151].

Functionally, B10 cells suppress aspects of both innate and adaptive immunity through IL-10 production [155]. IL-10 can suppress proinflammatory cytokine production [155], MHCII and costimulatory molecule expression [156], T cell activation, and antigen uptake by dendritic cells [157]. Currently, there is controversy over whether or not B10 cells enhance Treg development and function. Various studies using an experimental autoimmune encephalomyelitis (EAE) model to study multiple sclerosis (MS) have concluded that IL-10 made by B10 cells was both involved [158] and not involved [159,160] in Treg generation and function. However, another study concluded that the supportive effect of B10 cells on Tregs in EAE is IL-10 independent and therefore another regulatory mechanism may be involved [161]. It is known, however, that CD19-deficient NZB/W mice, which have significantly reduced B10 cells, have reduced Tregs. Furthermore, adoptive transfer of B10 cells into CD19-deficient NZB/W mice induced Treg expansion [162]. With regards to murine models of airway hypersensitivity, Amu et al. demonstrated that adoptive transfer of B10 cells induced nTreg recruitment into lungs, which reduced airway inflammation [163]. Overall, many studies have demonstrated a link between B10 cells and Treg expansion, but this relationship needs to be further explored.

A central question regarding B10 function is whether B10 cells differentiate into plasma cells and secrete antibody. Maseda et al. described B10 cells as transient IL-10 producing B cells of which a subset will become antibody secreting plasma cells [164]. Following IL-10 secretion, expression of blimp1 and irf4 transcription factors increases
while *bcl6* decreases, which is characteristic of plasma cell differentiation. Furthermore, Maseda et al. found B10 derived IgM was reactive with both self and foreign antigen; and, B10 cells made antigen specific IgG1 post immunization [164]. Therefore, B10 cells are a unique B cell subset of which some retain the ability to make antibody but are by definition immunosuppressive in nature.

### 1.1.9 Section 1 aims

Overall, the aim of section 1 is first to define immune system characteristics of B cell specific ADAM10 deficient mice on two backgrounds, Th1 prone C57Bl/6 and Th2 prone Balb/c. Specifically, the mechanisms underlying aberrant secondary tissue architecture and defects in antigen-specific, class-switched antibody production are revealed. Furthermore, the regulation of ADAM10, ADAM17, and TNF in Th1 and Th2 prone mouse strains and in humans is explored with an emphasis on how B cell ADAM10 level affects susceptibility to Th2 diseases and IgE production potential. Lastly, the benefit of ADAM10 inhibitor therapy and insight into its mechanism of action in airway inflammation models is described.
Chapter 2: Materials and Methods

1.2.1 Mice

C57Bl/6 ADAM10$^{B-/-}$ (CD19-cre$^{+/}$) mice (C57-ADAM10$^{-/-}$) were generated as previously described [52] (see section 1.3.1) and backcrossed to Balb/c (Balb-ADAM10$^{B/-}$) for 8 generations and compared to respective littermate controls (CD19-cre$^{-/-}$). TNF knockout (TNF-αKO) mice used for bone marrow reconstitution studies were purchased from Jackson Laboratory (no. 005540, B6.129S-Tnf). B6-Ly5.2/Cr (CD45.1) congenic mice were purchased from National Cancer Institute/National Institutes of Health. For B cell reconstitution studies, C57Bl/6 B cell deficient mice (B6.129S2-Ighmtm1cgn/J, Jackson 002288) were used. A/J, SJL/J, C57Bl/6, and Balb/c WT were from Jackson Laboratories. Healthy male and female mice aged 6–12 weeks were used in all experimentation except bone marrow reconstitution where only WT female mice ages 6–8 weeks were reconstituted with sex-matched bone marrow cells. The Virginia Commonwealth University Institutional Animal Care and Use Committee approved all animal care and experimental protocols. For experimentation, mice were euthanized by isoflurane inhalation and cervical dislocation.

1.2.2 Cell Isolation

Tissue preparation

Single cell suspensions of spleen or peripheral lymph node was prepared by disrupting tissue between frosted glass slides. For peritoneal lavage, 1 mL of 2%FBS in PBS was injected into the peritoneal cavity and removed. Suspensions were filtered
through 40-µm Nylon Mesh (Fisherbrand) and RBCs lysed with ACK Lysing Buffer (Quality Biological).

*Magnetic bead isolation*

Specific cell isolation was performed with magnetic bead isolation (Miltenyi Biotec). Briefly, a single cell suspension was generated as described above. Cells were washed and resuspended in MACS buffer (PBS pH 7.2, 0.5% BSA, and 2mM EDTA) and incubated with the corresponding magnetic beads for 20 minutes on ice. Cells were then washed and passed through a pre-washed magnetic column according to manufacturer’s protocol (Miltenyi Biotec). For positive selection, LS columns were used. Following addition of cells, columns were washed three times and then flushed with MACS buffer to elute cells for experimentation. For negative selection, LS columns were used as described previously but flow through from column was collected. For B cell isolation, total B cells were isolated using B220+ positive selection or naïve B cells were isolated by CD43 depletion (CD43- cells collected from flow through). For T regulatory cell induction experiments, CD4+CD25- cells were isolated. First, total splenocytes were incubated with CD25-PE for 10 minutes in the dark, washed, incubated with anti-PE beads, and CD25 negative cells collected in flow through. CD25- cells were then incubated with CD4 (L3T4) beads and positive selection performed for CD4+CD25- T cells.

1.2.3 *In vitro activation*

*B cells: Anti-CD40, IL-4, LPS*
B cells were grown in complete RPMI 1640 medium containing 10% heat-inactivated (56°C, 30 min) FBS (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate, 50 µM 2-ME, 1× nonessential amino acids, and 20 mM HEPES buffer (all from Invitrogen, Carlsbad, CA), and stimulated in vitro for 1, 3, or 5 d with 1000 U IL-4 (NIH) and either 50 µg/ml LPS (from *Escherichia coli* 0111:B4; Sigma) or 1.25 µg/ml purified anti-mouse CD40 (no. 102902; Biolegend). Supernatants were harvested at day 1, 3, or 5 for ELISA and cells analyzed by flow cytometry, western, and qPCR.

**B cells: PMA**

B220+ B cells were isolated from total splenocytes and were left untreated (media alone) or were treated with 250ng/mL PMA (Sigma). Cells were grown in complete RPMI at 37°C. Protocol adapted from [112].

**In vitro generation of CD4+CD25+ T cells**

Plates were coated with anti-CD3 (1µg/mL, Biolegend) for 2 hours at 37°C then washed with PBS before plating. 1mL of 2 x 10^6 CD4+CD25- cells (isolation described above) was resuspended in X-Vivo15 serum-free medium supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA) (without antibiotics) and added to anti-CD3 coated plates. Immediately, in vitro stimulating condition was added with all groups having anti-CD28 antibody (2 mg/mL, Biolegend) and IL-2 (100U/mL, Peprotech) in supplemented X-Vivo15 media [165]. Groups included media alone (negative control), rTGF-β1 (5 ng/mL) alone (positive control), and conditioned supernatants from 5 day anti-CD40/IL-4 treated B cells (WT or ADAM10^Bv-/−) with or without ICOSL neutralizing
antibody (Biolegend, isotype control Rat IgG2a). All groups were cultured for 4 days. Percent purity of CD4+CD25- cells was determined by flow cytometry before culture (>97%). After 4 days, percent induction of FoxP3 expression in CD4+CD25+ cells was determined by flow cytometry or qPCR.

1.2.4 $[^{3}H]$ thymidine Proliferation

B cell and T cell proliferation was assessed after 72 h of growth, and a 24-h pulse of $[^{3}H]$thymidine, 1 µCi/well (Perkin Elmer, Waltham, MA), was used. Plates were then harvested using a Filtermate cell harvester onto UniFilter-96 GF/C microplates (Perkin Elmer), dried overnight, and analyzed using a Topcount Plate Counter (Perkin Elmer).

1.2.5 Immunization

NP-KLH

Mice pre-bled the day before immunization and were immunized in the footpad (two ipsilateral paws) and/or intraperitoneal with 10 µg 4-hydroxy-3-nitrophenylacetyl coupled to keyhole limpet hemocyanin at a ratio of 27:1 (NP$_{27}$KLH or NP-KLH herein) (Biosearch Technologies) in 4 mg alum. For footpad injections, mice were given 10µg NP-KLH (1µg/mL stock) in 9µL of PBS and 15µL of alum for a total of 25µL per paw. For intraperitoneal injections, mice were given 10µg of NP-KLH (1µg/mL stock) in 90µL of PBS and 100µL of alum (4 mg) for a total of 200 µL per injection. Mice were bled at day 7, 14, and/or 21 post immunization and serum used for ELISA.

1.2.6 ELISA
**Total antibody**

ELISA plates were coated with 5µg/mL of goat-anti Ig (SouthernBiotech) in BBS (0.15M sodium chloride, 0.01 M borate buffer, pH=8.5) and incubated at 37°C for an hour or at 4°C overnight. Plates were then washed three times with ELISA wash (PBS with 0.02% Tween20). Blocking solution (PBS with 0.02% Tween20 and 2% FBS) was then added and incubated at 37°C for an hour or at 4°C overnight. Plates were then washed three times with ELISA wash and samples were added, diluted in block. For standard curve, normal mouse Ig (Southern Biotech) was used and incubated at 37°C for an hour. Plates were washed three times with ELISA wash and incubated with goat-anti Ig-AP diluted 1:400 in block (Southern Biotech) for 1 hour at 37°C. Plates were washed five times and developed with phosphate tablets (Sigma-Aldrich) dissolved in substrate buffer (0.1g MgCl2.6H20, 0.2 NaN3, 50mL diethanolamine, pH to 9.8 per 500mL). Absorbance at 405nm was measured.

**NP-specific antibody**

*ELISA plates were coated with 15µg/mL NP₄BSA (high affinity) or NP₂₅BSA (low affinity) in PBS for sample wells and with 5 µg/mL of goat-anti-Ig (Southern Biotech) in BBS for standards. The remaining steps were carried out as described above in ‘total antibody’ section.*

**TNF**

Soluble TNF from B cell supernatants was determined by mouse quantitative ELISA kit (88-7324-88; eBioscience) according to manufacturers’ protocol.
**HDM specific IgE**

HDM specific IgE ELISA was performed as described [31,166]. Briefly, plates were coated with 20µg/mL HDM extract in 50mM carbonate buffer, blocked with SuperBlock (Thermo Scientific), detected using goat anti-mouse IgE (Abcam), followed by addition of Streptavidin-HRP (Southern Biotechnologies), and color developed with tetramethylbenzidine + substrate (BD Biosciences). Reaction was stopped with 1N H₂SO₄ and absorbance read at 450nm.

**MUC5AC**

BALF supernatants were analyzed for MUC5AC protein by ELISA as described [167]. Briefly, BALF was diluted and 75µL was incubated with 75µL carbonate buffer. Samples were incubated overnight without a lid at 37°C then washed three times with PBS. Plates were blocked, detected with anti-Muc5AC mouse monoclonal antibody (Pierce, 1:100), followed by addition of goat anti-mouse IgG-HRP (Southern Biotechnologies,1:10,000), developed with tetramethylbenzidine + substrate (BD Biosciences), stopped with 1N H₂SO₄, and absorbance read at 450nm.

**1.2.7 PCR and Quantitative PCR**

**PCR**

For genotyping, DNA was isolated using Direct Tail PCR Solution as directed by the manufacturer (Viagen). Mangomix (Bioline) was used for amplification of CD19 cre-lines, ADAM-10 floxed alleles, YFP-Rosa and ADAM17. Cycling conditions were as follows, 95°C for 3 minutes then 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C
for 2 minutes, and a final extension at 72°C for 10 minutes. Sequences of all primers described above are listed in Table 1.

**Quantitative PCR**

Total RNA was extracted from naive and stimulated total B cells using TRIzol reagent (Invitrogen) according to manufacturer’s protocol and RNA concentration quantified by a ND-100 NanoDrop spectrophotometer. RNA (400 ng/µl) was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative PCR (qPCR) was performed with a real-time PCR machine (iQ5; Bio-Rad Laboratories). Primers and probes used for TaqMan qPCR assay (all from Applied Biosystems) are listed in Table 1. Fold variation was determined using the ΔΔCt method of analysis [168].
Table 1: PCR and qPCR primers and probes

<table>
<thead>
<tr>
<th>PCR</th>
<th>5’&gt;3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10Flox (75302-75324) Exon 9</td>
<td>GTTGGACATAACTTTGGATCTCC</td>
</tr>
<tr>
<td>A10Flox (75536-75516) Intron 9</td>
<td>CGTATCTCAAAAACTACCCCTCC</td>
</tr>
<tr>
<td>A10Flox (74490-74513) Intron 8</td>
<td>TGTGTGAATAGTGCAAGTGACGCC</td>
</tr>
<tr>
<td>A17Flox F</td>
<td>TCCCCCAAGCTAGATTGTTTG</td>
</tr>
<tr>
<td>A17Flox R</td>
<td>AGGACCCAGGTTCCGTTCCT</td>
</tr>
<tr>
<td>CD19 sense</td>
<td>TCGCGATTATCTTCATATCTTCATTCAG</td>
</tr>
<tr>
<td>CD19 antisense</td>
<td>GCTCGAACCAGTTACCACTCC</td>
</tr>
<tr>
<td>YFP1 (oMIR 316)</td>
<td>GGAAGCAGGAAGAAATGGATATG</td>
</tr>
<tr>
<td>YFP2 (oMIR 833)</td>
<td>AAAGTCGCTCTGAGTTGTTAT</td>
</tr>
<tr>
<td>YFP3 (Ro 4982)</td>
<td>AAGACCGAGGAGATTTCTCTCC</td>
</tr>
<tr>
<td>TNF F1</td>
<td>TACACAGAAGTTCACAAATG</td>
</tr>
<tr>
<td>TNF F2</td>
<td>TCCCTCTCATACATTCTATG</td>
</tr>
<tr>
<td>TNF R1</td>
<td>GAAATCTTACACGACGCTGT</td>
</tr>
<tr>
<td>TNF R2</td>
<td>AAGCATCTATGCACATTGAC</td>
</tr>
</tbody>
</table>

**PCR: For detecting CRE-mediated deletion of the STOP cassette at the ROSA/EYFP locus**

| ROSA26 (3937-3958)          | TGGAGGCAGGAAGACCTTGCT                   |
| EYFP (29-8)                 | GTGAACAGCTCCTCCTCCCTTCC                |

**Quantitative PCR**

| 18s                         | Mm03928990                                 |
| ADAM10                     | Mm00545742                                 |
| ADAM17                     | Mm00456428                                 |
| FoxP3                      | Mm00475162                                 |
| GAPDH                      | Mm99999915                                 |
| Matrix metalloproteinase 13 (MMP13) | Mm00439491                              |
| TNF-α                      | Mm00443258                                 |
| TTP/Zfp36                  | Mm00457144                                 |
| Human adam10               | Hs00153853                                 |
| Human adam17               | Hs01041915                                 |
| Human TNF                  | Hs01113624                                 |
| Human GAPDH                | Hs02758991                                 |
1.2.8 Western

Protein lysates were made according to the manufacturer’s protocol using Cell Lysis Buffer (no.9803; Cell Signaling). Equal amounts of protein determined by Bradford Assay were loaded onto Novex NuPAGE 10% Bis-Tris gels (Invitrogen), run for 35 min at 200 V at RT, transferred to nitrocellulose membrane at 90 V for 2 hours on ice, and equal transfer verified by Ponceau S (Sigma) staining. Blots were blocked (PBS, 0.02% Tween-20, 5% dry milk) for 1 hour at RT or overnight at 4°C. Blots were then washed 3 times for 10 minutes (PBS, 0.02% Tween-20). Primary antibodies listed in Table 2 were diluted in block and incubated overnight. Blots were again washed 3 times and incubated with respective secondary antibodies or anti–β-actin peroxidase (Table 2) for 2 hours at RT. Plates were washed 3 times and signal detected with 1:1 mix of SuperSignal West Pico Chemiluminescent Substrate (no. 34080; Thermo Scientific).
Table 2: Western Antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Catalog Number</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti–β-actin peroxidase</td>
<td>Sigma</td>
<td>A3854</td>
<td>1:25,000</td>
</tr>
<tr>
<td>anti-ADAM17</td>
<td>Abcam</td>
<td>2051</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG, HRP conjugate secondary</td>
<td>Southern Biotechnologies</td>
<td>4030-05</td>
<td>1:5,000</td>
</tr>
</tbody>
</table>
1.2.9 Flow cytometry and immunohistochemistry

For all flow staining procedures, cells were isolated as in 1.2.2.

Flow cytometry – Surface, total (intracellular and extracellular), and nuclear staining

0.5-3 x 10^6 cells were washed in FACS buffer (2% FBS in PBS), decanted, and incubated on ice with 1-5µg of Fc block (2.4G2) for 10 minutes to prevent non-specific staining. Subsequently, cells were surface stained by incubating 0.25-0.5µg of anti-mouse antibodies (Table 3) for 30 minutes on ice. Cells were then washed with FACS buffer. If further performing total staining, cells were fixed (FCM Fixation Buffer, Santa Cruz Biotechnology) at RT for ten minutes, washed, permeabilized for 10 minutes at RT, washed, Fc-blocked for 10 minutes on ice, and stained for 30 minutes on ice with anti-mouse or anti-human antibodies. For secondary staining, cells were washed with FACS buffer after primary staining and then stained with secondary antibodies (Table 3). If performing nuclear staining, cells were fixed (Biolegend Nuclear Fixation Buffer) for 20 minutes at RT, washed, permeabilized (Biolegend Nuclear Permeabilization Buffer), Fc Blocked, and stained.

Flow cytometry - Tyramide Signal Amplification

0.5-3 x 10^6 cells were stained using tyramide signal amplification (TSA) Kit #26 with HRP streptavidin (no. T20936; Molecular Probes) and an anti-mouse B220 antibody (Table 3). Kit reagents were prepared according to manufacturer’s protocol, and tyramide amplification using the “Peroxidase Labeling assay” was performed with the following modifications: cells were incubated with blocking reagent (10 µg anti-mouse unlabeled CD16/32 [2.4G2]) for 15 min; stained with biotin anti-mouse TNF-α
primary Ab (Biolegend); and after tyramide labeling, cells were washed twice and stained with anti-mouse B220 (see earlier) for 30 minutes.

Flow cytometry – ICOSL cleavage kinetics

B220+ B cells were stained at time 0 for baseline ICOSL expression following Fc blocking with 10µg anti-mouse unlabeled CD16/32 [2.4G2] for 15 minutes. B cells treated with PMA were cultured for 5, 10, 20, 30, 45, 60, or 120 minutes at 37°C. At each time point, cells were blocked and then stained for biotin anti-mouse ICOSL followed by PE-Streptavidin.

Flow Analysis

For analysis, all samples were washed and resuspended in 500µL PBS. Flow cytometry analysis was performed using a Canto or Fortessa (BD Biosciences) using DIVA software at the Flow Cytometry Core at Virginia Commonwealth University. Data analysis was performed with FCS Express, v. 4.

Immunohistochemistry

Ten-micrometer-thick frozen sections were cut from the excised mouse LNs, fixed in absolute acetone, air-dried, and blocked with serum-free protein block (X0909; Dako). The sections were dual- and triple-labeled for FDCs (CD21/CD35), B cells (CD45R/B220), high endothelial venules (HEVs; anti-mouse/human peripheral node addressin), T cells (rat anti-mouse CD90.2/Thy-1.2-PE), collagen type 1, and TNF-α (Table 3). Sections were mounted with antifade mounting medium, Vectashield (Vector Laboratories), coverslipped, and examined with a Leica TCS-SP2 AOBS confocal laser-
scanning microscope. Three lasers were used: argon (488 nm), HeNe (543 nm), and HeNe (633 nm; far red emission is shown as pseudoblue). Parameters were adjusted to scan at 1024 x 1024 pixel density and 8-bit pixel depth. Emissions were recorded in two or three separate channels, and digital images were captured and processed with Leica Confocal, LCS Lite software, and ImageJ for color separation and quantitative assessment of immunohistochemistry.

**Immunohistochemistry – Tyramide Signal Amplification**

TNF labeling was enhanced using fluorescein-TSA (TSA Plus Fluorescein System; NEL741001KT; Perkin Elmer). In brief, after quenching endogenous peroxidase using 1% H₂O₂, anti–TNF-α Ig was applied for 2 h, washed, then HRP-conjugated secondary Ig was added for 1 h. After washing, HRP was allowed to catalyze the deposition of fluorescein-labeled tyramide for 10 min, then was washed, mounted, and examined.
### Table 3. Flow Cytometry and Immunohistochemistry Antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Label</th>
<th>Source</th>
<th>Concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flow Cytometry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAM10</td>
<td>PE</td>
<td>R&amp;D</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-human ADAM10</td>
<td>FITC</td>
<td>R&amp;D</td>
<td></td>
</tr>
<tr>
<td>Anti- mouse and human ADAM17</td>
<td>Unconjugated</td>
<td>Abcam</td>
<td>N/A</td>
</tr>
<tr>
<td>B220/CD45R</td>
<td>APC, FITC, PE</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>CD4</td>
<td>PECy7</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>CD25</td>
<td>PE</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Alexa 647</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>TNF</td>
<td>Biotin</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>DyLight-649 anti-rabbit IgG (secondary for ADAM17)</td>
<td>APC</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>Mouse IgG2b</td>
<td>FITC</td>
<td>R&amp;D</td>
<td>N/A</td>
</tr>
<tr>
<td>Rat IgG2a</td>
<td>PE</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Unconjugated</td>
<td>In house</td>
<td>N/A</td>
</tr>
<tr>
<td>ICOSL</td>
<td>Biotin</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>PE</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>IL-10</td>
<td>APC</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-human CD19</td>
<td>PE</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-human CD14</td>
<td>APC</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-human CD3</td>
<td>PE</td>
<td>Biolegend</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD21/CD35</td>
<td>PE</td>
<td>Biolegend</td>
<td>5-10 µg/mL</td>
</tr>
<tr>
<td>CD45R/B220</td>
<td>Alexa Fluor 647</td>
<td>Biolegend</td>
<td>5-10 µg/mL</td>
</tr>
<tr>
<td>CD90.2/Thy-1.2</td>
<td>PE</td>
<td>Southern Biotechnologies</td>
<td>5-10 µg/mL</td>
</tr>
<tr>
<td>Collagen Type 1</td>
<td>Unconjugated</td>
<td>Abcam</td>
<td>5-10 µg/mL</td>
</tr>
<tr>
<td>Peripheral node addressin (anti-mouse/human)</td>
<td>Unconjugated</td>
<td>Biolegend</td>
<td>5-10 µg/mL</td>
</tr>
<tr>
<td>TNF</td>
<td>TSA</td>
<td>Abcam</td>
<td>5-10 µg/mL</td>
</tr>
</tbody>
</table>

*All antibodies are anti-mouse unless otherwise specified.
1.2.10 Bone marrow reconstitution

Bone marrow cells were isolated as previously described with the following modifications [169]. In brief, two femurs and two tibias from each mouse (WT [CD45.2], ADAM10<sup>−/−</sup>[CD45.2], or TNF-αKO) were centrifuged, RBCs lysed with ACK Lysing Buffer (Quality Biological), bone marrow cells counted, and 5 million cells were i.v. injected. For 50/50 mixtures, such as ADAM10<sup>−/−</sup> + TNFKO, 100 µl/2.5 million cells from each were used to prepare the final injection mixture.

B6-Ly5.2/Cr (CD45.1) congenic mice from National Cancer Institute/National Institutes of Health were pretreated 5 days before irradiation with 100mg/L (concentration 0.01%) enrofloxacin (Baytril) in sterile water. CD45.1 mice were anesthetized using a 100 µL i.p. injection of ketamine/xylazine in PBS at a dose of 80 and 8 mg/kg, respectively. This was followed by two doses of 550 cGy irradiation, separated by a 2-hour rest period, using an MDS Nordion Gammacell 40 research irradiator with a [<sup>137</sup>Cs] source. After irradiation, mice were reconstituted by i.v. injection with the indicated bone marrow cells as described earlier. After 6 weeks of reconstitution, mice were footpad immunized in two ipsilateral paws with 10 µg 4-hydroxy-3-nitrophenylacetyl coupled to keyhole limpet hemocyanin at a ratio of 27:1 (Biosearch Technologies) in 4 mg alum. Draining and non-draining popliteal and axillary LNs were dissected at 14 days post-immunization and analyzed by immunohistochemistry.

1.2.11 Human Studies

The Virginia Commonwealth University IRB approved all human studies. Sixteen symptomatic allergic rhinitis patients and 16 controls were recruited for the study by Dr.
Anne-Marie Irani at VCU. Inclusion criteria included active allergic rhinitis symptoms and a documented positive skin test or ImmunoCAP to an antigen as consented in IRB #00870. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood using Ficoll, stained, and remaining PBMC underwent CD19 B cell selection using magnetic beads (Miltenyi Biotec). B cells were cultured for 2 days (or 5 days for CD23 ELISA) with 200 ng/mL human IL-21 (ATCC), 1 µg/mL anti-CD40 (Clone G28-5, ATCC), and 10 ng/mL rhIL-4 (R&D). Stimulated B cells were harvested, stained for flow cytometry, analyzed by qPCR, and sCD23 determined by ELISA [170]. HDM *Dermatophagoides pteronyssinus* IgE was determined by ImmunoCAP (Phadia, 14-4107-01).

1.2.12 Murine HDM Studies

C57Bl/6 WT, C57-ADAM10°/°, Balb/c WT, and Balb-ADAM10°/° mice were intranasally exposed to 25µL saline or 15µg/25µL HDM extract (Greer Laboratories) as described in Figure 18 [171]. For Treg depletion studies, 0.5mg/mL anti-CD25 (BioXCell, PC-61.5.3), 0.5mg/mL COH2 (control rat IgG1), or PBS was given i.p. 3 days before first intranasal immunization and again 3 days after the first intranasal immunization. Bronchoconstriction was assessed using Flexivent (Scireq, Montreal, QC, Canada) as previously described [167]. Airway resistance (Rrs or AHR) was determined at increasing doses of methacholine (5, 10, 25 mg/ml) and presented as (1) percent increase from PBS baseline or (2) resistance value for each dose shown.

Total BALF volume and cell count was assessed and total cell count normalized to 100µL. BALF supernatants were analyzed for MUC5AC protein by ELISA as described [167] (see Section 1.2.6).
1.2.13 Murine Lung Histology

Five µm sections of formalin-fixed, paraffin-embedded murine lung tissue were stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) (AML Laboratories, Inc. Baltimore, MD) and photographed using Olympus-DP70 camera on Olympus-BX41 microscope. Histopathologic evaluation of H&E stained lung sections from at least 4 mice per group was performed by a pathologist in a blinded fashion using a semi-quantitative scoring system on a Nikon Eclipse microscope. Peribronchiolar and perivascular inflammatory cellular infiltration were scored separately as follows: 0, no or occasional cells; 1, few or loosely arranged cells; 2, focal involvement of lung parenchyma with majority of airways or vessels having rings (partial or complete) of inflammatory cells one cell layer deep; 3, patchy involvement of lung parenchyma with majority of airways or vessels having rings (partial or complete) of inflammatory cells two to four cell layers deep; 4, extensive involvement of lung parenchyma with majority of airways or vessels having rings (partial or complete) of inflammatory cells more than four cell layers deep. Individual scores for peribronchiolar and perivascular inflammation were added together for a total maximum score of 8.

1.2.14 BALF by flow cytometry

BALF cells were stained with APC-B220, APC-CD3, BV421-1A/1E, BV605-CD11c, and PE-CCR3 (Biolegend) after FcR blockade with anti-mouse CD16/32 (2.4G2). Samples were examined on a BD Fortessa and analyzed with FCS Express, v. 4. using the gating strategy described [172].

1.2.15 Statistical Analysis

45
Normal distribution of data sets was determined using the Shapiro-Wilk normality test with SigmaPlot 12.5. Furthermore, the mean and medians were close for data sets indicative of normal distribution. When dealing with two groups, p-values were calculated using unpaired two-tailed Student's t-tests in GraphPad Prism. In the case of non-parametric populations, p-values were calculated using two-tailed Mann-Whitney test. When more than two were compared, p-values were calculated using a one-way analysis of variance (ANOVA) with a Tukey post-test. Error bars represent the standard error of the mean (SEM), standard deviation, or 95% confidence interval between samples. p<0.05 is considered significant.
Chapter 3: Th1 vs. Th2 background does not affect antigen specific antibody production following B cell ADAM10 deletion; however, Th context greatly affects B cell ADAM17 and TNF regulation and thus secondary lymphoid tissue architecture

1.3.1 Generation of B cell specific ADAM10 deficient mice on two backgrounds

Our lab previously generated a B cell specific ADAM10 deficient mouse model (ADAM10^{B/-}) on both a C57Bl/6 and Balb/c background. C57Bl/6 ADAM10^{B/-} (C57-ADAM10^{B/-}) mice were initially generated by crossing homozygous floxed ADAM10 (exon 9) mice with CD19-cre knockin mice [52]. Exon 9 in the ADAM10 gene encodes the zinc binding domain of the protease active site and cre-mediated deletion causes a frameshift mutation, which disrupts transcription. CD19 is expressed starting at the pro-B cell stage, but CD19-cre recombination is limited to the immature B cell or pre-B cell [173]. To confirm B cell specific ADAM10 deletion, PCR analysis for exon 9 was performed on B220^{+} B cells and B220^{-} splenocytes [52]. C57- ADAM10^{B/-} mice were then crossed with YFP reporter mice, which have a floxed stop codon upstream of a YFP reporter gene. Successful recombination defined by YFP expression using flow cytometry analysis indicated 87% of splenic B cells and 95% of peripheral lymph node B cells in C57-ADAM10^{B/-} were YFP positive [52]. Therefore, this mouse only expresses YFP after cre-mediated recombination in B cells as outlined in Figure 2. Balb/c ADAM10^{B/-} (Balb-ADAM10^{B/-}) mice were generated by crossing C57-ADAM10^{B/-} to Balb/c WT (Jackson) for 8 generations.
Figures 2. B cell specific ADAM10 deficient mouse model.

Cre-recombinase (cre) is under the control of the CD19 promoter (P) on one CD19 allele. CD19 expression occurs in B cells leading to cre expression. Note, that this allele is kept heterozygous with one WT CD19 allele to avoid disruption in CD19 expression. Cre cleaves at loxP sites that flank exon 9 of both ADAM10 alleles, which renders ADAM10 ineffective. Additionally, these mice express a YFP reporter gene to track successful ADAM10 deletion. Cre cleaves at loxP sites flanking a STOP sequence upstream of the YFP-Rosa gene. With the excision of the STOP sequence, the YFP-Rosa gene is transcribed and expressed on ADAM10 deficient B cells. (A) WT genotype. Cre negative mice exhibit WT ADAM10 expression and no YFP expression. (B) B cell ADAM10 deficient genotype. Cre positive mice express cre when CD19 is transcribed. Cre deletes ADAM10 and the STOP sequence, thus YFP is expressed.
1.3.2 Humoral immune responses in C57-ADAM10\textsuperscript{B/-} deficient mice

Figures 3-6 are presented as background work performed by Dr. Joanna Cichy and Dr. Natalia Chaimowitz prior to my project.

**ADAM10 is highly expressed on GC B cells**

Because ADAM10 cleaves many substrates potentially involved in B cell humoral responses including Notch 1, Notch 2, TNF, and CD23 [1,174,175], our lab in collaboration with Joanna Cichy from Jagiellonian University, Krakow, Poland investigated the expression of ADAM10 on GC B cells. Peyer’s patches (PPs) are a secondary lymphoid tissue located in the intestine and exhibit a high level of GC activity. Flow cytometric analysis of WT PPs demonstrated that 2% of naïve B cells express ADAM10 while 80% of GC B cells are ADAM10\textsuperscript{+} (Figure 3A, B). Increased ADAM10 expression on GC B cells was confirmed by immunoflourescent staining of sectioned PPs (Figure 3C). Taken together, ADAM10 is expressed highly in GC B cells.

**Reduced total and antigen specific humoral responses in C57-ADAM10\textsuperscript{B/-} mice**

Given that ADAM10 is expressed highly on GC B cells, both total and antigen specific antibody responses were next examined. Basal levels of total IgM, IgG1, IgG2a, and IgG2b from naïve mice were reduced in C57-ADAM10\textsuperscript{B/-}, suggesting a defect in antibody production (Figure 4A). Next, mice were immunized i.p. with NP-KLH (NP) in alum and antigen specific antibody production was assessed. NP-specific IgM was reduced in C57-ADAM10\textsuperscript{B/-} mice 7 days post immunization, but reached WT levels by day 14 and remained comparable at day 21 and 28 (Figure 4B). Both total and high affinity NP-specific IgG were significantly reduced C57-ADAM10\textsuperscript{B/-} mice at day
7, 14, 21, and 28 post immunization (Figure 4B). When boosted with NP-KLH at day 42 post immunization, total and high affinity IgG antibody titers still failed to recover to WT levels (Figure 4C). NP-specific IgG1, IgG2a, and IgG2b were assessed at day 28 and were reduced in C57-ADAM10^{B/-} compared to WT (Figure 5A). Furthermore, immunization with 1mg of NP-KLH in alum still revealed reduced NP-specific IgG responses in C57-ADAM10^{B/-} demonstrating that increased antigen dose cannot overcome the antibody defect (Figure 5B).
Figure 3. ADAM10 expression on GC B cells.
(A and B) Expression of ADAM10 on gated GC PNA$^{hi}$IgD$^{lo}$ (blue) and naive PNA$^{lo}$IgD$^{hi}$ (red) B cells (CD19$^+$) isolated from PPs. Flow cytometry gates used to define the GC and naive B cell populations are indicated on the left. To demonstrate specificity of staining, we used isotype-matched monoclonal antibody (mAb). (A) Gating protocol is shown. (B) Percentage of ADAM10$^+$ cells in naive and GC B cells. (C) Frozen serial sections of PPs were stained to detect B cell follicles (B220$^+$, blue), GCs (PNA$^+$, green), and ADAM10 (red) (right panel) or isotype matched mAb (left panel). Original magnification 20x. The mucosal epithelium (E) (left panel) also reacts with PNA and anti-ADAM10. Figure courtesy of Joanna Cichy, from Jagiellonian University, Krakow, Poland.
Figure 4. (A) Serum total IgM, IgG1, IgG2a, and IgG2b were measured by ELISA from naive 8- to 12-wk-old mice and WT littermate controls were immunized with 10 μg NP-KLH emulsified in alum. At the indicated times, serum samples were collected and total and specific IgM, IgG1, IgG2a, and IgG2b were determined by ELISA, with NP-16–BSA as capture Ag for total and NP-4–BSA as capture Ag for high-affinity ELISA. (B) Mice were immunized with NP-KLH emulsified in alum, rested for 42 d, and boosted with 10 μg NP-KLH for 5 d. Mice were bled weekly throughout the course of the experiment. Total and high-affinity Ag-specific IgG levels were measured by ELISA at each time point. The relative unit (RU) values for alum-injected mice were <0.001. WT (white bars) and ADAM10 B−/− (black bars) and B+/− mice were measured. Bars represent the mean ± SEM of five to nine mice per group (*p < 0.05, **p < 0.01, ***p < 0.001). Data represent results obtained in at least two independent experiments. Figure courtesy of Dr. Natalia Chaimowitz.
Figure 5. Decreased NP-specific IgG secretion is not IgG subset dependent and cannot be overcome by high antigen dose.

(A) ADAM10^{B/-} mice and WT controls were immunized with 10µg NP-KLH emulsified in alum. Twenty-eight days after primary immunization, samples were collected and NP-specific IgG1, IgG2a and IgG2b antibody titers were determined by ELISA with NP_{14}BSA as capture antigen. (B) ADAM10^{B/-} and WT controls were immunized i.p. with 1mg of NP-KLH emulsified in alum. Samples were collected at the indicated time post immunization, and NP-specific antibodies were measured. The relative unit (RU) values for alum-injected mice were <0.001. WT (white bars) and ADAM10^{B/-} (black bars). Bars represent the mean ± SEM of 5 mice per group. Data represents results obtained in two independent experiments. (*p<0.05, **p<0.01, ***p<0.001). Figure courtesy of Dr. Natalia Chaimowitz.
1.3.3 Aberrant secondary lymphoid tissue architecture, TNF, and ADAM17 in C57-ADAM10^{B/-} mice

*Reduced GC B cells in C57-ADAM10^{B/-} spleen*

Figures 4 and 5 demonstrated that C57-ADAM10^{B/-} mice exhibit impaired antibody production and most impressively impaired class-switched antibody production. Because class switching occurs in germinal centers, we next enumerated GC B cells (IgM\textsuperscript{lo}IgD\textsuperscript{lo}B220\textsuperscript{+}IgG1\textsuperscript{+}CD38\textsuperscript{lo}) by flow cytometry (Figure 6A). Following 14 or 21 days post NP-KLH/alum immunization, splenic GC B cells were reduced by both percentage (Figure 6B) and total number (Figure 6C) in C57-ADAM10^{B/-} mice compared to WT.

*C57-ADAM10^{B/-} mice exhibit abnormal lymph node architecture and excessive TNF in B cell regions*

In addition to reduced GC B cell numbers, Chaimowitz et al. described secondary lymphoid tissue architecture defects in immunized LNs isolated from C57-ADAM10^{B/-} mice, which supported the reduced antibody production noted in these mice [107]. These defects included improper localization of B and T cells, reduced germinal center formation, and a decrease in follicular dendritic cell (FDC) networks [107]. These architecture aberrancies bore some similarity with secondary lymphoid tissue defects noted in early studies of global TNF deficient mice. Specifically, lack of splenic B cell follicles, disorganized FDC networks, and aberrant germinal centers [32]. Recent studies using B cells only capable of expressing mTNF showed that sTNF produced by B cells is required for maintaining secondary architecture and for IgG production against...
T dependent antigens [34]. Taken together, B cell ADAM10 and TNF are both critical in maintaining proper secondary lymphoid architecture and class switched antibody production, but a link between them is still unclear.

**Figure 7** shows immunohistochemistry analysis of lymph nodes from naïve (non-immunized) WT and C57-ADAM10<sup>B/-</sup> mice. While initial studies had indicated a relatively normal architecture in C57-ADAM10<sup>B/-</sup> nodes in the absence of immunization [107], additional analysis revealed that not only are lymph node FDC networks largely absent and B/T boundaries intermingled (**Figure 7A, 7B**), but other abnormalities can also be seen. These changes include excessive collagen deposition as well as an increase in high endothelial venules (HEVs), especially within B cell cortical regions (**Figure 7B, C**). Most striking, however, was the dramatic increase in TNF within the B cell regions of C57-ADAM10<sup>B/-</sup> nodes (**Figure 7D**). Given the data in **Figure 7** and previous reports of secondary lymphoid tissue architecture abnormalities in TNF knockouts and those only capable of expressing mTNF, it appears that not only subnormal but also excessive TNF levels in the lymph node cortices may lead to disruption of normal follicular architecture.

* B cells from C57-ADAM10<sup>B/-</sup> mice exhibit increased expression, stability and production of TNF

Given the excessive TNF staining in B cell regions of C57-ADAM10<sup>B/-</sup> lymph nodes (**Figure 7**), we further analyzed differences in TNF expression and production in B cells purified from both C57-ADAM10<sup>B/-</sup> and WT mice. As can be seen in **Figures 8A** and **8B**, flow analysis for mTNF revealed that both naïve and stimulated C57-ADAM10<sup>B/-</sup>
B cells exhibit increased mTNF. Furthermore, ELISA analysis of supernatants from C57-ADAM10^{B/-} B cells cultured with LPS/IL-4 (Figure 8C) or anti-CD40/IL-4 (Figure 8D) for 1, 3, or 5 days all showed significantly higher sTNF production compared to WT.

Relative (Figure 9A) and absolute (Figure 9B) TNF message analysis by qPCR indicated a strong increase in TNF message in C57-ADAM10^{B/-} compared to WT B cells post stimulation in vitro. Furthermore, Tristetraprolin (TTP), which promotes TNF mRNA degradation [176,177], was reduced 2 fold in C57-ADAM10^{B/-} B cells (Figure 9C), which have increased sTNF and mTNF (Figure 8) compared WT B cells. The combined results of increased TNF message as well as membrane and soluble protein, suggests a possible feedback mechanism in which increased TNF shedding upregulates further TNF production. In any case, it is clear that TNF is increased at both the message and protein level in C57-ADAM10^{B/-} B cells.

*C57-ADAM10^{B/-} B cells exhibit higher ADAM17 gene and protein expression*

ADAM17 is known to be the principle sheddase of membrane bound, pro-TNF. Since, C57-ADAM10^{B/-} B cells exhibit higher expression and production of TNF, we next examined ADAM17 expression and function by analyzing ADAM17 message (Figure 10A, 10B) and protein levels (Figure 10C, 10D) in WT compared to C57-ADAM10^{B/-} B cells. While both naïve and stimulated C57-ADAM10^{B/-} B cells express significantly more ADAM17 message (Figure 10A, 10B), relative gene expression analysis showed that in the naïve state, C57-ADAM10^{B/-} B cells express 2 times higher ADAM17 compared to WT, which increased to 5 fold higher expression upon stimulation (Figure 10A). Similarly, absolute RNA quantification revealed that naïve C57-ADAM10^{B/-} B
cells exhibit significantly increased ADAM17 mRNA expression, which increases further upon stimulation (Figure 10B). Western blot analysis, furthermore, showed a 2.3 fold increase in ADAM17 protein levels in naïve C57-ADAM10^{B/-} B cells over WT, which too increased upon stimulation to 5 fold (Figure 10C, 10D). Upon overexposure of the blots, both the precursor and glycosylated forms of ADAM17 were seen (data not shown).

In addition, Vandenbroucke et al. recently established that matrix metalloproteinase MMP13 also cleaves TNF at least in intestinal epithelium [178]. qPCR analysis of MMP13 in naïve B cells, however, showed there was no difference between WT and C57-ADAM10^{B/-} B cells (\(\Delta\Delta C_t = 1.04\)); fold change ADAM10^{B/-} over WT). Western blot, furthermore, failed to show MMP13 protein in naïve WT and ADAM10^{B/-} B cells compared to positive control, RAW 264.7 macrophages (data not shown). This finding is in agreement with the report that significant levels of MMP13 are not found in B cells [179]. Taken together, Figures 6-10 demonstrate that ADAM10 deletion from C57Bl/6 B cells results in a compensatory increase in ADAM17 expression and activity leading to excessive TNF cleavage. The aberrant signaling environment created by this compensatory effect is an excellent candidate to explain the abnormal lymphoid tissue architecture in our C57-ADAM10^{B/-} model and was thus further explored.
Figure 6. GC formation after T-dependent immunization.

ADAM10^{B−/−} and WT mice were immunized with 10µg NP-KLH emulsified in alum. Fourteen and 21 days post-immunization, flow cytometry was carried out and the presence of GC B cells (IgM^{lo}IgD^{lo}B220^{+}IgG1^{+}CD38^{−}) in the spleen of WT and ADAM10^{B−/−} mice was assessed. Staining protocol is depicted (A). Both percentage (B) and total number (C) of GCs were enumerated. WT (white bars) and ADAM10^{B−/−} (black bars). Bars represent the mean ± SEM of eight mice per group. *p < 0.05, **p < 0.01. Data is representative of three independent experiments. Figure courtesy of Dr. Natalia Chaimowitz.
Figure 7. Naïve C57-ADAM10$^{B/-}$ lymph nodes (LN)s display abnormal follicular architecture.

Compared to WT, ADAM10$^{B/-}$ mice (A) lack well developed FDC reticula (red, CR1/2) in the B cell follicle (blue, B220), (B) lack B cell/T cell (red, CD3) segregation with more HEVs (green, pNAD) in the LN cortex, (C) display more collagen (green) deposition in the B cell follicle, and (D) express higher levels of TNF$\alpha$ than WT mice. Scale bar = 50 mm, and micrographs are representatives of at least 3 LNs.
Figure 8. Increased TNF surface expression and production in C57-ADAM10<sup>B-/-</sup> B cells.

Naïve (A) and 5 day stimulated (B) (LPS + IL-4) WT (black thin line) and ADAM10<sup>B-/-</sup> (black bold line, A10KO) live B cells were analyzed for co-expression of TNF using tyramide signal amplification. Black bars (overlay plots) indicate B cells staining high in mTNF, represented in bar graphs (A,B). N=9 per group, 3 independent studies. (C,D) Supernatants were harvested on day 1, 3, or 5 from WT (white) or ADAM10<sup>B-/-</sup> (A10KO, black) B cell cultures stimulated with LPS + IL-4 (C) or anti-CD40 + IL-4 (D) and sTNF determined by ELISA. N=9 per group, 3 independent studies. *indicates p<0.05, ** indicates p<0.005.
Figure 9. Increased TNF gene expression and message stability in C57-ADAM10^{B/-} B cells.

Naïve (3A [checkered], 3B [left], 3C [checkered]) or 5 day stimulated (LPS + IL-4) (3A [stripe], 3B [right], 3C [stripe]) B cells were analyzed by qPCR for TNFα (A, B) and TTP (C) message normalized to 18s. Data presented as fold change of ADAM10^{B/-} over WT (A) or fold change of WT over ADAM10^{B/-} (C) using the (ΔΔ)Ct method of analysis. Absolute RNA quantification for TNFα normalized to 18s in WT (white) and ADAM10^{B/-} (black) B cells appears in (B). N=9 per group, 3 independent studies. *indicates p<0.05, ***indicates p<0.0005.
Figure 10. Increased ADAM17 message and protein levels in C57- ADAM10<sup>B<sub>−/−</sub></sup> B cells.

Naïve (4A [checkered], 4B [left], 4C) or 5 day stimulated (4A [stripe], 4B [right], 4D) (LPS + IL-4) B cells were analyzed by qPCR and western blotting. (A) Relative ADAM17 expression normalized to 18s presented as fold change of ADAM10<sup>B<sub>−/−</sub></sup> (A10KO) over WT using the (∆∆)Ct method of analysis. (B) Absolute quantification of ADAM17 RNA normalized to 18s. N=9 mice per group, 3 independent studies. Band densitometry of naïve (C) and stimulated (D) B cells represents ADAM17 (~93kDa, mature form) normalized to actin (~42kDa). N=6 per group total, 4 independent studies. *indicates p<0.05.
Reconstitution of irradiated C57Bl/6 WT with combination C57-ADAM10^{B/-} + TNFKO bone marrow rectifies lymph node follicular abnormalities

In order to assess whether TNF is involved in the mechanism underlying lymph node tissue abnormalities in C57-ADAM10^{B/-} mice, we performed a bone marrow chimera experiment in which irradiated CD45.1 WT C57Bl/6 mice were reconstituted with one of the following bone marrow combinations: (1) WT (CD45.2) alone; (2) C57-ADAM10^{B/-} (CD45.2) alone; (3) TNF deficient (TNFKO) alone; (4) 50/50 mix C57-ADAM10^{B/-} + WT (CD45.2); or (5) 50/50 mix C57-ADAM10^{B/-} + TNFKO. Following 6 weeks of reconstitution, mice were bled and analyzed for successful reconstitution and CD45.2 cells predominated (Figure 11). The mice were then footpad immunized with NP-KLH, and draining lymph nodes were assessed by immunohistochemistry 14 days post-immunization (Figure 12A-H). As expected, lymph nodes from WT mice reconstituted with WT (CD45.2) bone marrow exhibited normal lymph node architecture (Figure 12D) and TNF levels (Figure 12G), which was comparable to non-irradiated WT nodes (Figure 12A, 12G). Those reconstituted with C57-ADAM10^{B/-} bone marrow (Figure 12B, 12G), however, had a similar phenotype to those of our C57-ADAM10^{B/-} mice (Figure 7): loss of B cell/T cell segregation, decreased FDC networks, and increased cortical TNF. Those reconstituted with TNFKO bone marrow alone (Figure 12C) exhibit no TNF staining but do still demonstrate FDC networks as these are resistant to irradiation. Furthermore, reconstitution with 50/50 mix C57-ADAM10^{B/-} + WT CD45.1 still yielded abnormal lymph node architecture (Figure 12E) and high TNF staining (Figure 12G) similar to C57-ADAM10^{B/-}. Thus, the amount of TNF made by B cells from this combination is still too high. Interestingly, when WT mice were
reconstituted with combination C57-ADAM10^{B/-} + TNFKO bone marrow (Figure 12F, 12G), lymph node tissue architecture and TNF staining returned to WT levels. TNF staining is further compared and quantified by mean gray values in multiple equal areas (at least 6) in Figure 12H. Here, mice reconstituted with C57-ADAM10^{B/-} alone as well as C57-ADAM10^{B/-} + WT CD45.1 exhibit significantly more TNF staining compared to WT and C57-ADAM10^{B/-} + TNFKO (Figure 12H). We reason that while B cells from C57-ADAM10^{B/-} mice produce excessive TNF, those from the TNFKO mice produce none, thus averaging to a normal, WT range allowing proper TNF signaling to occur.
Figure 11. Confirmation of successful bone marrow reconstitution.

Irradiated CD45.1 WT mice were reconstituted with CD45.2 (A) or C57-ADAM10<sup>B/-</sup> (CD45.2) (B) bone marrow and peripheral blood sample collected at 6 weeks post reconstitution. Percentage of CD45.2<sup>+</sup> live peripheral blood lymphocytes is shown.
Figure 12. Reconstitution of irradiated WT naïve LNs with ADAM10\(^{B/-}\) + TNFKO bone marrow restores normal follicular architecture and rectifies structural abnormalities induced by ADAM10\(^{B/-}\) reconstitution alone.

Draining lymph node sections from non-irradiated WT (A, G (CD45.1 WT)) and irradiated CD45.1 WT reconstituted with ADAM10\(^{B/-}\) (B, G (Reconstitution with ADAM10KO)), TNFKO (C), WT CD45.2 (D, G (Reconstitution with CD45.2 WT)), ADAM10\(^{B/-}\) + WT CD45.1 (E, G (Reconstitution with ADAM10KO and CD45.1 WT)), ADAM10\(^{B/-}\) + TNFKO (F, G (Reconstitution with ADAM10KO and TNFKO)) were compared in (A-F) with regards to B/T compartmentalization (left column), FDC reticular development (middle column), and TNF\(\alpha\) staining (right column). In (A-G) the following stains were used: B cells (blue, B220), T cells (red, Thy1.2), Collagen 1 (green), FDCs (red, CR1/2), and TNF\(\alpha\) (green). White boxes in (A-F, right column) are magnified in (G, left column), with separation of the TNF\(\alpha\) labelling (middle column) and measurements (histograms, right column). (H) TNF\(\alpha\) expression (mean gray values) in multiple equal areas (at least 6) have been calculated and the results expressed as mean ± SD. Using unpaired T test, the p value between CD45.1 WT and reconstitution with CD45.2 WT is 0.33; CD45.1 WT and reconstitution with ADAM10KO and TNFKO is 0.36; and reconstitution with ADAM10KO and ADAM10KO and CD45.1 WT is 0.2. Micrographs are representative of at least 3 LNs from 3 independent experiments. Scale bar in A-F = 50 mm, in G = 20 mm.
Figure 12. Reconstitution of irradiated WT naïve LNs with ADAM10<sup>B/-</sup> + TNFKO bone marrow restores normal follicular architecture and rectifies structural abnormalities induced by ADAM10<sup>B/-</sup> reconstitution alone.
1.3.4 Balb-ADAM10^{B/-} exhibit reduced antigen specific antibody production and GC B cells similar to C57-ADAM10^{B/-} mice

Similar to C57-ADAM10^{B/-} mice, we characterized antibody production by Balb-ADAM10^{B/-} mice following NP-KLH immunization in alum. At day 7 post immunization, NP-specific IgM was reduced in Balb-ADAM10^{B/-} serum and IgG1 was very lowly detected (Figure 13A, 13B). At day 14, NP-specific IgM and IgG1 were significantly reduced in Balb-ADAM10^{B/-} compared to Balb-WT as expected given our results in C57Bl/6 mice (Figure 13A,13B). Given the reduction in class-switched antibody production, we next wanted to assess the percentage of GC B cells in the draining LN of Balb-ADAM10^{B/-} compared to WT. Unlike C57-ADAM10^{B/-} (Figure 6B), the Balb-ADAM10^{B/-} lymph nodes exhibited a normal percentage of GC B cells compared to Balb-WT (Figure 13C). This finding was unexpected given our results in C57-ADAM10^{B/-} and thus prompted an architecture analysis of Balb-ADAM10^{B/-} lymph nodes as well as characterization of ADAM17 and TNF production by Balb-ADAM10^{B/-} B cells.

1.3.5 Th1 and Th2 strain dependent differences in B cell ADAM17 and TNF as well as secondary lymphoid tissue architecture following B cell ADAM10 deletion

Strain dependent differences in B cell TNF and ADAM17

C57-ADAM10^{B/-} B cells exhibit a compensatory increase in ADAM17 expression and function resulting in excessive TNF cleavage, which provided the mechanism for the aberrant secondary lymphoid tissue architecture noted in these mice [31,107] (Figure 7-12). We, therefore, analyzed both TNF and ADAM17 expression and function.
by Th2 prone Balb/c WT (Balb-WT) and Balb-ADAM10^{B/-} compared to C57Bl/6 WT (C57-WT) and C57-ADAM10^{B/-} B cells to see if differences in B cell TNF and ADAM17 exist between strains. First, sTNF production by Balb-WT and Balb-ADAM10^{B/-} B cells was comparable following in vitro stimulation unlike our previous report in C57Bl/6 (Figure 14A). Furthermore, mTNF in stimulated B cells is comparable between Balb-WT and Balb-ADAM10^{B/-} but reduced compared to C57-WT and even further reduced compared to C57-ADAM10^{B/-} (Figure 14B). Similarly to C57, Balb-WT and Balb-ADAM10^{B/-} express comparable TNF message in the naïve state (Figure 14C). Upon stimulation, however, Balb-ADAM10^{B/-} B cells fail to significantly increase TNF message above Balb-WT levels unlike C57-ADAM10^{B/-} B cells (Figure 14C). Furthermore, C57-ADAM10^{B/-} B cells exhibit increased TNF message compared to Balb-ADAM10^{B/-} (Figure 14C).

Given that TNF levels were reduced in Balb-ADAM10^{B/-} compared to C57-ADAM10^{B/-} B cells, we next compared the levels of ADAM17 protein and message. C57-ADAM10^{B/-} B cells exhibited increased ADAM17 protein compared to C57-WT, but also compared to Balb-WT and most interestingly, Balb-ADAM10^{B/-} (Figure 15A). Furthermore, ADAM17 protein was comparable between Balb-WT and Balb-ADAM10^{B/-} (Figure 15A), which is consistent with TNF production in Figure 14A. Unlike C57Bl/6, ADAM17 message in naïve Balb-WT and Balb-ADAM10^{B/-} B cells was comparable and increased to 2-fold higher expression upon stimulation (Figure 15B).

Unlike C57-ADAM10^{B/-}, Balb-ADAM10^{B/-} exhibit normal secondary lymphoid tissue architecture.
We previously noted the defects in secondary lymphoid tissue architecture in C57-ADAM10^{B-/} lymph nodes (Figure 7), including aberrant B cell/T cell localization, decreased FDC development, excessive collagen deposition, decreased germinal center formation, and increased HEV formation, was attributed to increased B cell ADAM17 resulting in excessive B cell TNF production and signaling within the follicle [31]. Given the marginal changes in TNF (Figure 14) and ADAM17 (Figure 15) in Balb-ADAM10^{B-/}, however, compared to C57-ADAM10^{B-/} [31] (Figures 7-12), we hypothesized Balb-ADAM10^{B-/} lymph nodes would maintain WT architecture. Indeed, Balb-ADAM10^{B-/} exhibited comparable primary follicular characteristics to C57-WT and Balb-WT, including normal B cell/T cell compartmentalization (Figure 16A), number and size of HEVs (Figure 16A), FDC reticular (Figure 16B), and collagen deposition (Figure 16B). Again, C57-ADAM10^{B-/} lymph node sections are provided as a reference as they exhibit a lack of B/T compartmentalization, increased HEVs, minimal CD21+ FDC reticula, and excess collagen deposition. Furthermore, TNF labeling (boxed regions of Figure 16C) demonstrated that C57-ADAM10^{B-/} follicles displayed the highest TNF intensity compared to C57-WT, Balb-WT, and Balb-ADAM10^{B-/} (Figure 16C), with the latter groups all having comparable TNF staining (Figure 16C), which is quantified in Figure 16D. These results provide further support that excess B cell sTNF underlies the aberrant lymphoid tissue architecture in C57-ADAM10^{B-/} mice and demonstrates the inherent difference in B cell ADAM10 and ADAM17 regulation in Th1 vs. Th2 prone mice.
Figure 13. Balb-ADAM10<sup>B<sub>−</sub>−</sup> mice exhibit reduced NP-specific antibody production but a normal percentage GC B cells.

Balb-WT (WT) (white bars) and Balb-ADAM10<sup>B<sub>−</sub>−</sup> (Balb-A10KO) (black bars) were immunized i.p. and f.p. with NP-KLH in alum and bled at day 7 and 14 post immunization. Serum levels of NP-specific IgM (A) and IgG1 (B) were determined at day 7 and day 14 post immunization. (C) At day 14, draining axillary LNs were analyzed for GC (B220<sup>+</sup>GL7<sup>+</sup>) B cells and percentage compared to total B220<sup>+</sup> B cell population. Error bars represent SD; *p<0.05, **p<0.005, ***p<0.0005.
Figure 14. Differential TNF production and expression in C57Bl/6 and Balb/c ADAM10^{B/-} (A10KO) and WT B cells.

(A) Supernatants were harvested on day 3 from C57Bl/6 (left) or Balb/c (right) WT (white) or A10KO (black) B cell cultures stimulated with LPS + IL-4 and sTNF determined by ELISA. (B) Balb/c WT (black), Balb/c A10KO (red), C57Bl/6 WT (green), and C57Bl/6 A10KO (blue) B cells stimulated 3 days with LPS + IL4 were analyzed for coexpression of TNF using TSA. (C) Naïve (white) or 3 day stimulated (black) B cells were analyzed by qPCR for TNF message normalized to 18s. Data presented as fold change of A10KO over WT for C57Bl/6 (left) and Balb/c (right) using the ΔΔCt method of analysis. n = 9 per group, three independent studies. *p < 0.05, **p < 0.005.
Figure 15. Increased ADAM17 in C57Bl/6 WT and A10KO over Balb/c WT and A10KO, respectively.

(A) 5 day stimulated B cells were analyzed by western blot (left) for ADAM17 (~93kDa) relative to actin (~42kDa) and band densitometry shown (right). (B) Relative ADAM17 expression normalized to 18s for naïve (white) or 3 day stimulated (black) B cells presented as fold change of ADAM10KO over WT using the ∆∆Ct method of analysis. n=9 per group, three independent studies. *p < 0.05, **p < 0.005.
Naïve lymph node sections from C57Bl/6 and Balb/c WT (right) and ADAM10<sup>B<sup>-/-</sup></sup> (left) were analyzed for (A) B cell (blue, B220)/T cell (red, Thy1.2) localization and HEV formation (green, pNAD); (B) FDC reticula (red, CR1/2) development, collagen (green) deposition, and presence of a distinct cortico-medullary junction (dotted line in inset box, Balb-ADAM10<sup>B<sup>-/-</sup></sup> only); (C) TNF staining (green) within B cell (blue, B220) follicle regions. (D) Average TNF staining intensity from 12 follicle sections (represented by box in (C)), **p<0.005. Scale bar is 50 µm. Micrographs are representative of at least 3 LNs.

Figure 16. Balb-ADAM10<sup>B<sup>-/-</sup></sup> lymph nodes exhibit WT architecture unlike C57-ADAM10<sup>B<sup>-/-</sup></sup> nodes.
Chapter 4: Th1 and Th2 prone mouse strains and humans exhibit differential B cell ADAM10, ADAM17, and TNF levels

1.4.1. Th2 prone strains (Balb/c, A/J) B cells exhibit increased ADAM10 and decreased ADAM17 and TNF compared to those of Th1 prone strains (C57Bl/6, SJL/J)

Based on results described in Figures 14-16, clear differences in TNF and ADAM17 exist between C57-WT and Balb-WT B cells. Therefore, we aimed to confirm if differences in TNF, ADAM10, and ADAM17 exist between other Th1 and Th2 prone strains. Because Th2 prone mouse strains are more susceptible to models of airway hyper-reactivity and increased B cell ADAM10 correlated with exacerbated airway symptoms and immunopathology in mice, we hypothesized that Th2 prone mouse strains would exhibit increased B cell ADAM10 and reduced ADAM17 and TNF compared to Th1 counterparts [180]. In fact, naïve B cells from Th2 prone Balb/c and A/J expressed higher total ADAM10 (Figure 17A) and decreased total ADAM17 (Figure 17B) compared to Th1 prone C57Bl/6 and SJL/J. Upon stimulation in vitro, B cells from the chosen Th1 prone strains expressed increased mTNF compared to the chosen Th2 prone strains (Figure 17C). Furthermore, TNF message was lower in Th2 prone A/J and Balb/c mice compared to C57Bl/6 WT by ≥2 fold (Figure 17D). C57Bl/6 WT TNF expression was not significantly different from SJL/J (Figure 17D). B cell ADAM10 in both Th1 prone-C57Bl/6 and Th2 prone-Balb/c strains increased following in vitro stimulation (Table 4). However, Th2 prone Balb/c maintained a consistently higher level of B cell ADAM10 even after equal stimulation (Table 4). This finding
demonstrates that T cell help, in the form of anti-CD40/IL-4 or anti-CD40 alone, augments ADAM10 expression but does not provide the entire explanation for why B cell ADAM10 is initially increased in Th2 prone over Th1 prone strains.
Figure 17. Increased B cell ADAM10 and decreased ADAM17 and TNF in Th2 biased strains.

Naïve B220⁺ B cells (A, B) stained for total ADAM10 (A) or ADAM17 (B) expression by flow cytometry. (C) Surface TNF expression on 3 day stimulated (anti-CD40+IL4) B220⁺ B cell by TSA. (A,B,C) Bar graph represents percent total B cells staining high in ADAM10 (A), ADAM17 (B), or TNF (C). (A,B,C) Balb/c (-----), A/J (-----), C57Bl/6 (-----), SJL (*****), respective isotype control (shaded grey) in representative overlay (left); grey gate demonstrates high stained population. n = 8 per group, 3 independent studies. *p<0.05, **p<0.005, ***p<0.0005. (D) TNF message from 3 day stimulated (anti-CD40 + IL4) B220⁺ B cells. Presented as fold change from C57Bl/6 WT. * signifies ≥2 fold change between groups.
Table 4. ADAM10 expression in Naïve and Stimulated WT B cells by qPCR.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Average Fold Change in ADAM10 mRNA expression (±SD) using ∆∆Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb naïve / C57 naïve</td>
<td>2.35 ± 1.00</td>
</tr>
<tr>
<td>Balb A+I / Balb naïve</td>
<td>2.02 ± 0.66</td>
</tr>
<tr>
<td>C57 A+I / C57 naïve</td>
<td>2.40 ± 1.34</td>
</tr>
<tr>
<td>Balb A / Balb naïve</td>
<td>2.88 ± 1.45</td>
</tr>
<tr>
<td>C57 A / C57 naïve</td>
<td>1.91 ± 0.30</td>
</tr>
<tr>
<td>Balb A+I / C57 A+I</td>
<td>5.34 ± 1.84</td>
</tr>
<tr>
<td>Balb A / C57 A</td>
<td>5.70 ± 1.45</td>
</tr>
</tbody>
</table>

A+I (3 days stimulation with anti-CD40+IL4); A (3 day stimulation with anti-CD40 alone). ADAM10 expression normalized to 18s. Fold change in gene expression of 2 or more between groups considered significant.
1.4.2 High B cell ADAM10 predicts increased susceptibility to Th2 disease

*High B-ADAM10 level in the context of a Th2 environment allows optimal induction of allergic airway disease symptoms*

C57Bl/6 WT exhibit a decreased response to mouse lung inflammation models compared to Balb/c including airway bronchoconstriction and IgE production, however, these findings have never been linked to B cell ADAM10 levels, specifically [31,181,182]. Our results thus far indicate that Th2 prone mice, whose B cells are known to produce excess IgE post immunization, exhibit increased B cell ADAM10 even in a naïve, non-stimulated state. After stimulation with anti-CD40/IL-4 (Table 4), B cell ADAM10 levels increased more dramatically in Th2 prone strain B cells as well. Therefore, we next sought to compare the consequences of B cell ADAM10 level in the context of a Th1 or Th2 immune-environment on acute airway hypersensitivity induction following house dust mite (HDM) challenge (Figure 18), specifically with regards to IgE production. The groups considered included: B cell ADAM10 high, Th2 prone (Balb/c WT); B cell ADAM10 low, Th1 prone (C57 WT); B cell ADAM10 deleted, Th2 prone (Balb-ADAM10\(^{-/-}\)); and B cell ADAM10 deleted, Th1 prone (C57-ADAM10\(^{B^{-/-}}\)). When each group was treated intranasally with saline, all disease parameters were similar (not statistically different). For clarity, saline treated Balb WT was chosen as the representative saline group in Figures 19 and 20.

Th2 prone, B cell ADAM10 high Balb WT exhibit the most severe induction of lung inflammation following HDM challenge, including increased bronchoconstriction (Figure 19A) and inflammatory, cellular infiltrate in lung tissue (boxed regions at 10x
magnification in Figure 20A, 20C). Similarly, Balb WT exhibit the highest goblet cell metaplasia as indicated by the intense pink staining mucin (arrows in Figure 20B) and mucus (MUC5AC protein) production (Figure 20D). Furthermore, the thickness of the alveolar epithelium (Figure 20B) is greatest in the Balb/c compared to other groups with the C57-ADAM10\(^{B/-}\) being reflective of saline controls. In accordance with our main hypothesis that increased B cell ADAM10 lends to increased IgE production, Balb WT exhibited the highest HDM-specific IgE (Figure 19C) as well. When ADAM10 is deleted from B cells but remains in the context of a Th2 environment in the Balb-ADAM10\(^{B/-}\), bronchoconstriction (Figure 19A), goblet cell hyperplasia (Figure 20B), and mucus production (Figure 20D) are reduced significantly from Balb WT and are quite similar to the Th1 prone, B cell ADAM10 low C57-WT. Furthermore, HDM-specific IgE (Figure 19C) is reduced from Balb WT levels demonstrating the critical importance of B cell ADAM10 for IgE production even in a Th2 prone environment. Lastly, B cell ADAM10 deletion in the context of a Th1 environment (C57-ADAM10\(^{B/-}\)) provides the least suitable context for allergic airway disease induction. Thus, as indicated previously, C57-ADAM10\(^{B/-}\) mice exhibited bronchoconstriction (Figure 19A), cellular infiltration of lung tissue (Figure 20A, 20C), goblet cell metaplasia (Figure 20B), and mucus production (Figure 20D) similar to saline control. While the HDM-specific IgE ELISA was not sensitive enough to detect differences between saline, C57-WT, and C57 and Balb-ADAM10\(^{B/-}\), it does indicate the importance of both ADAM10 and strain background in IgE production as both the Balb-ADAM10\(^{B/-}\) and C57-WT demonstrated significantly reduced HDM-specific IgE production compared to Balb WT (Figure 19C). Although anticipated, this is the first time reduced IgE production has been
demonstrated in both C57 and Balb-ADAM10<sup>B−/−</sup> mice. Furthermore, B cell ADAM10 deletion from Balb or C57 fails to reduce eosinophil recruitment in BALF, but it appears to be a strain dependent phenomena as Balb/c mice regardless of B cell ADAM10 presence had increased eosinophils compared to C57 WT and C57-ADAM10<sup>B−/−</sup> (Figure 19B).

Allergic patients exhibit increased B cell ADAM10, CD23 cleavage, and IgE yet reduced ADAM17 and TNF

Our results thus far indicate that mouse strains with higher B cell ADAM10 have increased susceptibility to airway hyperreactivity induction, especially when in a Th2 context. Furthermore, B cells from Th2 prone strains exhibit increased B cell ADAM10 and reduced ADAM17 and TNF. In humans, we define “Th2 context” as patients actively suffering from allergic rhinitis, a Th2 disease. Our goal was to characterize the B cell phenotype of active allergic rhinitis patients and compare it to patients without a history of atopic disease (controls). Naïve peripheral B cells from 16 allergic patients exhibited significantly increased B cell ADAM10 compared to 16 controls (Figure 21A) by flow cytometric analysis. Contrastingly, peripheral blood T cells (Figure 21B) and monocytes (Figure 21C) from allergic and control patients revealed comparable ADAM10 expression. Thus, only B cell ADAM10 expression served as an allergy-associated differential indicator. Following 5 days of stimulation in vitro with IL-21/IL-4/anti-CD40, allergic-B cell supernatants demonstrated increased sCD23 indicating higher ADAM10 activity in allergic patient B cells compared to controls (Figure 21D). This finding supports a previous report that demonstrated increased circulating sCD23 in allergic patient’s sera [183]. Furthermore, B cell ADAM10 message in allergic
patients was increased nearly 5-fold, while ADAM17 and TNF message were decreased 2-fold compared to controls (Figure 21E).

Given that increased B-ADAM10 leads to increased IgE production through CD23 cleavage [30], we next compared serum antigen-specific IgE between allergic and control patients. We were reliant on each patient self-reporting a specific antigen to which he/she was allergic in order to perform an antigen specific ImmunoCAP [184]. Five of 16 allergic patients self-reported HDM sensitivity and ImmunoCAP demonstrated positive HDM specific IgE levels (>0.35 kuA/l) (Figure 21F). Five control patients, who are also quite likely exposed to HDM daily but do not develop allergy, demonstrated negative ImmunoCAP results (all <0.35 kuA/l) or very low levels (0.41 kuA/l) (Figure 21F). We next compared each individual patient’s HDM-specific IgE result to his/her respective B cell ADAM10 expression (Table 1). In general, all control patients exhibited very low or negative HDM specific IgE and low ADAM10 expression (< 25%) (Table 5). As HDM specific IgE levels increased, B cell ADAM10 expression also increased in allergic patients. One allergic patient, however, had a lower HDM specific IgE result at 5.4 kuA/l but demonstrated a high B cell ADAM10 level (69%) (Table 5). Overall, Figure 21 and Table 5 demonstrates a correlation between increased serum antigen-specific IgE and increased B cell ADAM10 in allergic patients.
Figure 18. HDM lung inflammation protocol.

Mice were intranasally (I.N) exposed to HDM or saline (black arrow) and on day 15 analyzed for AHR, BALF cell distribution, and lung tissue was collected for sectioning.
Figure 19. B cell ADAM10 deletion attenuates bronchoconstriction and HDM specific IgE.

Airway resistance (cmH20.s/mL) with increasing doses of methacholine (left); Balb-WT (---, ○), Balb-ADAM10B-/- (----, ●), C57-WT (-----, □), C57-ADAM10B-/- (-----, ■), Saline (-----, ●) presented as fold increase from saline control. All mice immunized with saline demonstrated comparable results. For simplicity, saline represents Balb WT mice given saline. Bar graph (right) represents 25 mg/mL methacholine dose. (B) Percent macrophages (left) and eosinophils (right) from total BALF determined by flow cytometry; Saline control (white), Balb WT (checkered), Balb-ADAM10B-/- (dotted), C57 WT (slash), and C57-ADAM10B-/- (black). (C) HDM specific IgE production. n=7-9 per group, 3 independent experiments. *p<0.05, **p<0.005, ***p<0.005.
Figure 20. B-ADAM10 deletion reduces cellular infiltration, goblet cell metaplasia, and mucus production in a strain dependent manner.

(A) H&E stain for lung tissue demonstrates inflammatory cellular infiltration (red box); 10x magnification, scale bar 200 µm. (B) Goblet cell metaplasia determined by PAS stain; note dark pink mucin producing cells (black arrow) and alveolar epithelium thickness; 20x magnification, scale bar 100 µm. (C) Lung pathology score representing quantitation of inflammatory cellular infiltration on H&E stained lung sections; 2 sections per mouse and at least 4 mice per group were assessed. (D) MUC5AC protein. n=7-9 per group, 3 independent experiments. All mice immunized with saline demonstrated comparable results. For simplicity, saline represents Balb WT mice given saline. *p<0.05, **p<0.005.
Figure 21: Allergic patient B cells exhibit increased ADAM10 and sCD23 but decreased ADAM17 and TNF.

(A) Total ADAM10 in naïve CD19+ B cells from control (thin line, open dot) and allergic (bold line, black dot) patients; dot plot (right) shows percent of total B cells staining high in ADAM10 (grey gate). Isotype control is shaded grey in histogram. (B,C) Total ADAM10 on naïve T cells (B) and monocytes (C). (D) sCD23 from control (open dot) or allergic (black dot) supernatants. (E) Naïve B cells from 4 allergic and 4 control patients analyzed for ADAM10, ADAM17, and TNF message normalized to GAPDH. Significance (*) indicates ≥2 fold change between groups. (F) HDM specific IgE levels in sera of control (open dot) or allergic (black dot) patients determined by ImmunoCAP. <0.35kuA/l considered a negative result. *p<0.05, **p<0.005, ***p<0.0005.
Table 5. Increased HDM specific IgE correlates with increased B cell ADAM10 expression.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Allergic</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDM Specific IgE (kuA/l)</td>
<td>Percent CD19&lt;sup&gt;*&lt;/sup&gt;ADAM10&lt;sub&gt;high&lt;/sub&gt;</td>
<td>HDM Specific IgE (kuA/l)</td>
</tr>
<tr>
<td>&lt;0.35</td>
<td>13</td>
<td>5.4</td>
</tr>
<tr>
<td>&lt;0.35</td>
<td>18</td>
<td>12.8</td>
</tr>
<tr>
<td>&lt;0.35</td>
<td>22</td>
<td>15.0</td>
</tr>
<tr>
<td>&lt;0.35</td>
<td>25</td>
<td>18.0</td>
</tr>
<tr>
<td>0.41</td>
<td>15</td>
<td>22.0</td>
</tr>
</tbody>
</table>

Direct comparison of individual patient’s HDM specific IgE determined by ImmunoCAP and B cell ADAM10 expression determined by flow cytometry.
Chapter 5: C57-ADAM10<sup>B/-</sup> B cells exhibit a B10 phenotype with enhanced ICOSL expression and induction of T regulatory cells in secondary lymphoid tissues.

While excessive B cell TNF explained the mechanism underlying aberrant secondary lymphoid tissue architecture in C57-ADAM10<sup>B/-</sup>, it does not explain the deficit in class-switched antibody production post immunization because both C57 and Balb-ADAM10<sup>B/-</sup> mice demonstrated reduced antibody levels (Figures 4 and 13). Therefore, we next sought to explain the mechanism by which loss of B cell ADAM10 causes reduced antigen-specific antibody production.

1.5.1 Increased B10 cells in C57-ADAM10<sup>B/-</sup> mice

B10 cells are a unique subset of B cells that produce the anti-inflammatory cytokine, IL-10. Classically, their surface markers are defined as CD1<sup>d+</sup>CD5<sup>-</sup>IgD<sup>lo</sup>IgM<sup>hi</sup> or B220<sup>+</sup>IL-10<sup>+</sup> [145]. Maseda et al. described that of the B10 cell population, only a subset will become antibody secreting plasma cells [164]. Given the reduction in antibody production in C57-ADAM10<sup>B/-</sup> mice, we hypothesized that a larger portion of their B cells may be B10 cells. We used flow cytometry to assess the percentage of B220<sup>+</sup>IL-10<sup>+</sup> B cells present following 3 days of stimulation in vitro with anti-CD40/IL-4. Of the total B220<sup>+</sup> B cell population, 30% of C57-ADAM10<sup>B/-</sup> B cells expressed IL-10 compared to only 10% of WT B cells (Figure 22A). We chose not to define B10 cells as CD1<sup>d+</sup>CD5<sup>-</sup>IgD<sup>lo</sup>IgM<sup>hi</sup> because these markers overlap with MZ B cell markers and the C57-ADAM10<sup>B/-</sup> mice lack MZ B cells [52], which may affect interpretation of the data. Furthermore, soluble IL-10 production by 3 day stimulated (anti-CD40/IL-4) C57-ADAM10<sup>B/-</sup> B cells was 3 fold higher compared to WT (Figure 22B). While B cell IL-10
message was comparable in naïve WT and C57-ADAM10^{B-/} B cells, following in vitro stimulation (anti-CD40/IL-4) for 5 days, IL-10 message increased to 6.37 fold higher in C57-ADAM10^{B-/} B cells over WT (Figure 22C). We next assessed the presence of IL-10 in circulation. In naïve mice, IL-10 was below levels of detection in both WT and C57-ADAM10^{B-/} mice (Figure 22D). 14 days post immunization with NP-KLH in alum, however, IL-10 levels rose to ~ 60 ng/mL in C57-ADAM10^{B-/} serum, which is 6 fold higher than WT (Figure 22E).

1.5.2 Increased T regulatory cells in ADAM10^{B-/} mice

B cells alone are not normally thought to produce such high levels of IL-10 as noted in Figure 22E. Treg cells, however, are a well-known IL-10 producing cell type that may contribute to the very high levels of IL-10 seen. Furthermore, some reports state that B10 cells enhance Treg development and function [161–163]. For these reasons, we hypothesized that C57-ADAM10^{B-/} mice have increased Treg cells in secondary lymphoid tissues, which may be explained at least in part by more B10 cells. Immunohistochemistry revealed an increase in CD3^{+}FoxP3^{+} cells in C57-ADAM10^{B-/} spleen 14 days post immunization with NP-KLH in alum (Figure 23A and 23B). While we are still working to optimize our staining of FoxP3+ Tregs by immunohistochemistry, the single color sections (Figure 23A, bottom) clearly demonstrate an increase in FoxP3+ staining in C57-ADAM10^{B-/} spleen. This finding was confirmed by flow cytometry, which demonstrated increased Tregs using two conventional sets of markers (CD4^{+}CD25^{+} and CD4^{+}FoxP3^{+}) in both naïve (Figure 24A and 24B) and 14 day immunized (Figure 24C) C57-ADAM10^{B-/} spleen.
Figure 22. C57-ADAM10^{B/-} B cells have a B regulatory or B10 cell phenotype and C57-ADAM10^{B/-} mice exhibit increased IL10 in circulation.

(A, B) 3 day stimulated (anti-CD40/IL-4) B cells have increased (A) IL10 expression by flow cytometry and (B) IL10 secretion by ELISA. (C) IL-10 message in naïve (white) and 3 day stimulated (anti-CD40/IL-4) (black) B cells. Serum collected from (D) naïve mice or (E) 14 days post immunization with NP-KLH for determination of circulating IL-10 by ELISA. (A, B, E) *p<0.05; (C) * signifies ≥ 2 fold change between WT and A10KO.
Figure 23. Increased FoxP3+ T cells in 14 day immunized C57-ADAM10^B^−/^− spleen.

(A) C57-WT (left) and A10KO (right) spleens stained for B cells (B220), T cells (CD3) and FoxP3 (green). Threshold single color sections with outline (cell) counting shown below. (B) Percent CD3+FoxP3+ compared to CD3+ alone shown in bar graph. Representative of 3 mice/group. *p<0.02
Figure 24. Increased Tregs in C57-ADAM10^B-^ naïve and immunized spleen.

(A, B) Naive and (C) 14 day immunized spleen from WT (white, thin black line) or A10KO (black, thin red line) were analyzed for Tregs using two surface marker combinations: CD4+CD25+ (A,C) and CD4+FoxP3+ (B). (C) A representative overlay of CD4+CD25+ Tregs; isotype control shaded grey; marker gate demonstrates high staining CD25+ population. *p<0.05, **p<0.005.
1.5.3 Increased ICOSL expression on C57-ADAM10<sup>B/-</sup> B cells

Given that we are using a B cell specific knockout model characterized by increased B cell ADAM17 activity, we hypothesized that a B cell associated ADAM17 substrate or ADAM17-regulated ligand may be involved in increasing Treg development and persistence. It is known that blockade of ICOS-ICOSL interactions in vitro impairs Treg induction [113] and that ICOS deficiency leads to FoxP3+ Treg apoptosis and impaired IL-10 production [114]. Furthermore, Marczynska et al. demonstrated that ADAM17 indirectly decreases surface ICOSL and increases sICOSL production [112]. Given that C57-ADAM10<sup>B/-</sup> B cells exhibit increased ADAM17 activity, we analyzed ICOSL surface expression. We found that naïve C57-ADAM10<sup>B/-</sup> B cells have significantly increased ICOSL expression compared to naïve WT B cells (Figure 25A). Even though C57-ADAM10<sup>B/-</sup> B cells have increased ADAM17, this ADAM17 is not yet active until the B cell is stimulated. Following PMA stimulation in vitro to activate ADAM17 activity, we tracked ICOSL cleavage kinetically and found that ICOSL levels decrease more dramatically in C57-ADAM10<sup>B/-</sup> B cells at each time point compared to WT (Figure 25B). Furthermore, while C57-ADAM10<sup>B/-</sup> B cell ICOSL levels remained very low at later time points, it appears that WT B cell ICOSL expression begins to increase slightly starting at 60 minutes. Therefore, C57-ADAM10<sup>B/-</sup> B cells may have a deficient recovery of ICOSL surface levels (Figure 25B). Figure 22A demonstrated that 30% of stimulated C57-ADAM10<sup>B/-</sup> B cells are IL-10 producing, B10 cells. Given that ICOSL is increased on C57-ADAM10<sup>B/-</sup> B cells and 30% of these are B10 cells, ICOSL may be a potential marker for B10 cells. To verify this point, we plan to stain
B220+ cells for IL-10 and ICOSL concomitantly to verify if IL-10 producing cells express more ICOSL than non-IL-10 producing B cells.

**1.5.4 Conditioned C57-ADAM10^{B/-} B cell supernatants cause enhanced Treg induction in vitro**

The next central question is what the C57-ADAM10^{B/-} B cell is expressing or producing that is causing an induction of Tregs. We used an in vitro Treg induction protocol [165] to see if soluble factors released during 5 days of B cell culture with anti-CD40 and IL-4 would induce FoxP3 expression in CD4+CD25- T cells. We isolated CD4+CD25- T cells and cultured them in one of the following conditions: (1) media alone (with IL-2/anti-CD3/CD28 co-stimulation) (negative control), (2) + recombinant TGFβ alone (positive control), (3) + conditioned WT B cell supernatant, or (4) + conditioned C57-ADAM10^{B/-} B cell supernatant. While recombinant TGFβ caused ~72% of T cells to become FoxP3+, conditioned C57-ADAM10^{B/-} supernatants resulted in ~50% and WT supernatant ~12% (similar to media alone) induction of FoxP3 expression (**Figure 26**). We are currently testing anti-ICOSL and anti-IL10 neutralizing antibodies in vitro to see if neutralization of either or both together will block FoxP3 induction in CD4+CD25- T cells. Furthermore, we are testing PMA stimulated WT and C57-ADAM10^{B/-} B cell supernatants to see if PMA stimulation of B cells results in more FoxP3 induction when the supernatants are cultured with CD4+CD25- T cells. We predict there will be more induction since PMA is far better at activating ADAM17, which we believe is controlling release of a soluble molecule (e.g. ICOSL) that is ultimately responsible for induction of FoxP3 expression.
As a preliminary study, we analyzed FoxP3 expression by qPCR in CD4+CD25- T cells cultured with 2 hour PMA-stimulated WT or C57-ADAM10^{B/-} B cell supernatant with and without ICOSL neutralizing antibody, which prevents ICOSL binding to the ICOSR (Figure 27). Compared to CD4+CD25- T cells treated with media alone, those cultured with C57-ADAM10^{B/-} B cell supernatant increased FoxP3 expression by 12 fold, which was reduced to 7 fold by the addition of ICOSL neutralizing antibody (Figure 27). Interestingly, however, WT supernatant alone still induced FoxP3 expression 7 fold, which we believe may be due to PMA remaining in the B cell supernatant (Figure 27). Therefore, we must perform a PMA only control to see if PMA alone induces FoxP3 expression in CD4+CD25- T cells. According to Majowicz et al., CD4+CD25- T cells cultured with PMA and anti-CD3 increased FoxP3 and CD25 expression [185]. Therefore, we may need to remove the PMA with dextrin labeled charcoal before the co-culture. We can confirm PMA is removed by performing a CD62 ligand kinetic shedding assay in which the supernatants with and without PMA removed are assessed for their ability to induce CD62 ligand shedding from WT B cells. Furthermore, we will culture WT or C57-ADAM10^{B/-} supernatants with an isotype control antibody to validate our ICOSL neutralizing antibody results.
Figure 25. Increased ICOSL expression and cleavage on C57-ADAM10<sup>B/-</sup> B cells.

(A) Representative overlay of baseline ICOSL expression in naïve WT (black solid) and C57-ADAM10<sup>B/-</sup> splenic B cells (red). Unstained (thin dashed) and isotype control (thick black dashed) shown. (B) ICOSL expression on WT (open dot) and C57-ADAM10<sup>B/-</sup> B cells (black dot) following PMA stimulation vitro.
Figure 26. C57-ADAM10<sup>B/-</sup> conditioned B cell supernatants induce enhanced FoxP3 expression in CD4<sup>+</sup>CD25<sup>-</sup> T cells compared to WT B cell supernatants.

4 day stimulated CD4<sup>+</sup>CD25<sup>-</sup> T cells with cultured with IL-2/CD3/CD28 alone (green) or IL-2/CD3/C28 plus 5 day conditioned (anti-CD40/IL-4) WT B cell supernatant (black), 5 day conditioned (anti-CD40/IL-4) A10KO B cell supernatant (red), or rTGFβ (purple). Representative overlay of changing FoxP3 expression (left) and dot plots (right) demonstrate percent CD4<sup>+</sup>FoxP3<sup>+</sup> T cells following culture with rTGFβ (purple) or WT (black) or A10KO (red) 5 day conditioned B cell supernatants.
Figure 27. ICOSL neutralization reduces FoxP3 expression in co-cultures of CD4+CD25- T cells with PMA-conditioned C57-ADAM10^{B/-} B cell supernatants.

WT (white) or C57-ADAM10^{B/-} (grey) B cells were cultured for 2 hours with PMA. Conditioned supernatants with (striped) or without (solid) anti-ICOSL neutralizing antibody (aICOS) or media alone (containing IL-2) were collected and cultured with CD4+CD25- T cells for 4 days with plate-bound CD3 and CD28. FoxP3 expression relative to GAPDH was assessed and data presented as fold change from FoxP3 expression when cultured with media alone. A fold change of ≥2 is considered significant. Fold change calculated using ΔΔCt equation.
1.5.5. In vivo Treg depletion studies

Our next goal was to see if increased Treg cells played a functional role in the C57-ADAM10^{B/-} phenotype. Specifically, is increased Tregs the mechanism underlying decreased antigen-specific antibody production and reduced disease parameters following HDM immunization?

**HDM acute airway hypersensitivity studies with Treg depletion**

In vivo depletion of CD4+CD25+FoxP3+ Treg cells can be achieved using anti-CD25 (PC61mAb). Setiady et al. described that Treg depletion with anti-CD25 is FcγRIII dependent as FcγRIII deficient mice were resistant to Treg depletion with anti-CD25 [186]. Furthermore, it was FcγRIII+ macrophages, but not FcγRIII+ NK cells, that were critical for antibody-mediated phagocytosis [186]. WT or C57-ADAM10^{B/-} mice were injected i.p. 3 days before and 3 days after the first intranasal HDM challenge to effectively deplete Tregs. The acute HDM challenge protocol was performed exactly as described in Figure 18. All mice treated with saline regardless of i.p. injection (PBS, COH2, anti-CD25) had statistically comparable disease parameters and are shown in Figures 27 and 28 as a representative saline group. Similar to our previous results in Figure 19, C57-ADAM10^{B/-} mice treated with COH2/HDM exhibit AHR comparable to saline treated mice (Figure 28A). Furthermore, COH2/HDM treated WT mice have increased AHR compared to both C57-ADAM10^{B/-} COH2/HDM and saline control (Figure 28A). Interestingly, anti-CD25/HDM treated C57-ADAM10^{B/-} mice have increased AHR compared to COH2/HDM treated C57-ADAM10^{B/-} mice and it is comparable to COH2/HDM treated WT mice (Figure 28A). Anti-CD25/HDM treated WT
mice exhibit the highest AHR, which is consistent with previous reports that Tregs are important for regulating AHR in murine HDM models [139–141]. Overall, depletion of Tregs in C57-ADAM10^{B/-} mice increased AHR.

Total cell number in BALF increased with anti-CD25 treatment compared to COH2 and PBS. While this increase was significant in WT, it trended upward in C57-ADAM10^{B/-} mice (Figure 28B). When comparing the BALF cell differential, specifically eosinophils, varied between COH2 and anti-CD25 treated groups (Figure 28C). Treatment with anti-CD25/HDM increased eosinophils in both WT and C57-ADAM10^{B/-} mice compared to COH2/HDM treatment (Figure 28C). In Figure 19B, there was not a statistical difference between HDM treated WT and C57-ADAM10^{B/-} mice, however there was a trend toward reduced eosinophils as is shown in Figure 28C.

Furthermore, H&E stained lung sections were assessed from each group for both perivascular and peribronchiolar inflammatory cellular infiltration. Intraperitoneal injection of PBS, COH2, or anti-CD25 did not result in inflammatory cellular infiltration as all saline treated groups lacked peribronchiolar or perivascular cell infiltration (Figure 29, top row). Anti-CD25/HDM treatment increased both perivascular and peribronchilar inflammatory cellular infiltration in both WT and C57-ADAM10^{B/-} mice compared to COH2/HDM treatment (Figure 29). Therefore, Treg depletion results in increased peribronchiolar and perivascular inflammatory cellular infiltration. PAS stained lung sections were analyzed for mucin production, which stains bright pink when positive. Saline treated mice were negative for mucin staining as was COH2/HDM treated C57-ADAM10^{B/-} lung sections (Figure 30). Similar to Figure 20, COH2/HDM treated WT mice had increased mucin compared to COH2/HDM treated C57-ADAM10^{B/-} (Figure
Interestingly, mucin staining increased in both WT and C57-ADAM10\textsuperscript{B-/} treated with anti-CD25/HDM (Figure 30, bottom row). Furthermore, the number or extent of bronchi staining positive for mucin increased as well. Currently, we are in the process quantifying mucin production.

Lastly, HDM-specific IgE was assessed with and without Treg depletion. Consistent with previous results (Figure 19C), COH2/HDM treated C57-ADAM10\textsuperscript{B-/} mice produce significantly less HDM-specific IgE compared to COH2/HDM treated WT. Interestingly, anti-CD25 treatment increased HDM-specific IgE in C57-ADAM10\textsuperscript{B-/} mice to WT levels (Figure 28D). HDM-specific IgE levels in anti-CD25 treated WT mice also trended upward compared to WT treated with COH2. Figure 28D is critically important for understanding the phenotype of C57-ADAM10\textsuperscript{B-/} mice. It demonstrates that increased Tregs in C57-ADAM10\textsuperscript{B-/} mice plays a central role in reducing antigen specific Ig production. It is likely, however, that increased Tregs along with other factors provide a complete explanation for the phenotype. One additional factor perhaps is that a greater percentage of B220+ B cells are IL-10 producing B10 cells, which are less adept at producing class switched antibody.
Figure 28. Treg depletion in C57-ADAM10^{B-/} prior to HDM challenge increases AHR, BALF cellularity, eosinophil infiltration, and HDM-specific IgE.

(A) Airway resistance following increasing doses of methacholine. Bar graph (right) demonstrates airway resistance at 25 mg/mL methacholine dose. (A) Total BALF cells normalized to 100 \( \mu \)L volume. Anti-CD25/HDM treated (black) and COH2/HDM treated (checkered). (C) BALF cell differential; eosinophils (black), PMN (white), lymphocytes (horizontal striped), and mononuclear cells (vertical striped). (D) HDM-specific IgE determined by ELISA. *p<0.05, **p<0.005.
**Figure 29.** Treg depletion increases peribronchiolar and perivascular inflammatory cellular infiltration in WT and C57-ADAM10^B^-.

Representative H&E stained lung sections demonstrating peribronchiolar (thin arrow) and perivascular (thick arrow) inflammatory cellular infiltration. Note the differences in the thickness of rings of inflammatory cell as well. All 10x magnification, scale bar 200 µm. Representative of 2 lung sections per mouse and 3 mice per group.
**Figure 30.** Anti-CD25 treatment increases mucin in WT and C57-ADAM10<sup>B/-</sup> airways.

Representative PAS stained lung sections demonstrating the presence of dark pink staining mucin (arrow). All at 20x magnification, scale bar 100 µm. Representative of 2 lung sections per mouse and 3 mice per group.
1.5.6 Other unique features of ADAM10<sup>B-/−</sup> B cells

C57-ADAM10<sup>B-/−</sup> B cells have other unique properties that may also affect their ability to effectively produce antibody. Naïve B cells should be CD43 negative and upon activation CD43 expression increases. When trying to isolate CD43 negative B cells by magnetic bead isolation (CD43 depletion), significantly fewer B cells were recovered from C57-ADAM10<sup>B-/−</sup> compared to WT. This led us to investigate CD43 expression levels on C57-ADAM10<sup>B-/−</sup> B cells. Figure 31 demonstrates that naïve B cells isolated from C57-ADAM10<sup>B-/−</sup> spleen and lymph node have increased CD43 expression in the naïve state. Increased B cell activation markers suggests that C57-ADAM10<sup>B-/−</sup> B cells are in a pre-activated state and may be refractory to subsequent stimulation. If so, this finding may help explain reduced antibody production and other B cell defects seen in these mice.

Furthermore, C57- ADAM10<sup>B-/−</sup> B cells fail to reconstitute the secondary lymphoid organs of B cell deficient mice as effectively as WT B cells (Figure 32A and 32B). C57Bl/6 B cell deficient mice were reconstituted i.v. with WT or C57- ADAM10<sup>B-/−</sup> B cells and subsequent B cell reconstitution of secondary lymphoid organs was assessed 2 weeks post immunization. Figure 32 demonstrates that B cell reconstitution of both LN and spleen was reduced nearly 3 fold in C57- ADAM10<sup>B-/−</sup>. While the mechanism underlying this phenomena is still under investigation, some potential options are (1) C57- ADAM10<sup>B-/−</sup> B cells simply take longer to reconstitute lymphoid tissues than WT; (2) Differential signaling or lack of chemokine signaling slows or prevents their reconstitution; or (3) C57- ADAM10<sup>B-/−</sup> are pre-activated (Figure 31) and may exhibit a shorter half-life causing high B cell turnover before our 2 week assessment.
Figure 31. Increased CD43 expression on C57-ADAM10^{B/-} B cells.

CD43 expression in naïve, B220+ B cells isolated from LN (left) and spleen (right) of WT (black) and C57-A10KO (red) mice. Gate represents population staining negative and low in surface CD43.
Figure 32. C57-ADAM10$^{B-/-}$ B cells fail to reconstitute secondary lymphoid tissues as effectively as WT B cells.

Following 2 weeks of reconstitution with either WT (white) or A10KO (black) B cells, percent B220$^+$ B cells in LN (A) and spleen (B) of B cell deficient mice was determined by flow cytometry.
Chapter 6: Discussion

1.6.1 Increased ADAM17 and excessive B cell sTNF production provides the mechanism for aberrant secondary lymphoid tissue architecture in C57-ADAM10^B^-/- mice

Chaimowitz et al. noted two key immune system alterations in C57-ADAM10^B^-/- mice; however, no mechanism was described [107]. First was that lymph node tissue architecture is disrupted 14 days post immunization with T-dependent antigen including aberrant B cell/ T cell localization, reduced GC formation, and reduced FDC network development [107]. Architecture studies herein, further, describe secondary lymphoid tissue alterations in naïve lymph nodes from C57-ADAM10^B^-/- mice as well (Figure 7). Their naïve lymph nodes exhibited aberrant B cell/ T cell localization, reduced FDC network development, reduced GC formation, increased HEV size and number, fibrosis, and increased TNF staining in B cell follicle regions [31]. Furthermore, we demonstrate herein key phenotypic characteristics of C57Bl/6 ADAM10 deficient B cells including increased ADAM17 expression and function (Figure 15), which resulted in enhanced TNF message, surface expression, and soluble production (Figure 14).

Three TNF family cytokines, TNF, LTα, and LTβ, are classically studied in secondary lymphoid tissue architecture maintenance. While ADAM17 is most notably studied as the TNF sheddase, one study demonstrated that ADAM17 can cleave membrane bound LTα1β2 heterotrimer on T cells in vitro and this cleavage is blocked by the ADAM17 inhibitor, TAPI-1 [100]. These structurally homologous and genetically linked cytokines have been studied individually and as double deficient mouse models, in an attempt to tease out the contributions of each to secondary lymphoid tissue
microarchitecture development and maintenance [187]. LTα deficient mice lack lymph nodes and Peyer’s patches and exhibit abnormal splenic architecture including loss of B/T segregation and a complete absence of FDC networks, germinal center formation, and marginal zone B cells [101,102]. LTα as a soluble homotrimer (LTα3) is also known to play an integral role in lymphoid organization including B/T segregation by binding to the TNF receptor, p55TNFR-1, suggesting that blockade of this interaction would result in a similar phenotype to C57-ADAM10^B/-^ lymph nodes [103]. LTβ deficient mice, however, experience more mild disruption as they retain mesenteric and cervical lymph node development and maintain B/T segregation, FDC networks, and germinal center formation in spleen [104]. Thus far, no reports have been made about the contribution of B cell specific LTα or LTβ to secondary lymphoid tissue architecture. More similar to LTα deficient mice, global TNF knockouts lack FDC networks in B cell follicles and fail to form germinal centers post immunization [32,188,189]. Furthermore, it is known that without TNF or its receptor p55TNFR-1, B cell follicles and FDC networks fail to form in peripheral lymph nodes, Peyer’s patches, and spleen; however, the effects of B cell TNF overexpression had not been previously reported [32,33]. The role of B cell TNF, specifically, in architecture maintenance has been elucidated using a B cell mutant model only capable of expressing mTNF. Without B cell sTNF production, there is loss of a proper cortico-medullary junction, reduced germinal center formation, impaired FDC network development, and reduced IgG production against T dependent antigens [34]. While it is known that lack of B cell TNF causes aberrant architecture, this study is the first to describe that excessive B cell TNF also leads to defects in secondary lymphoid tissue architecture.
Because p55TNFR-1 binds both TNF and LTα3, it is reasonable to conclude that excessive TNF could outcompete LTα3 for binding to this receptor, resulting in noted defects in B/T segregation. Furthermore, it has been shown that increased sTNF contributes to HEV neogenesis [190]. Excessive B cell TNF, therefore, could explain the induction of increased cortical HEV neogenesis resulting in increased T cell recruitment via CCL21/CCR7 interactions and ultimate aberrancies in B/T segregation (Figure 7). Furthermore, Weskamp et al. recently demonstrated that ADAM17 is critical for neovascularization using a conditional knockout model where ADAM17 was selectively deleted from endothelial cells and pericytes [191]. Therefore, ADAM17 overexpression by C57-ADAM10^{B-/-} B cells may be directly involved in increased follicular HEVs (Figure 7). Furthermore, in a Notch-dependent pathway, ADAM17 overactivation has been implicated in fibroblast activation, excessive collagen formation, and fibrosis [192]. Increased ADAM17 in C57-ADAM10^{B-/-} B cells could lead to aberrant Notch signaling and increased fibrosis within the lymph node (Figure 7). Given the profibrotic properties of ADAM17, our result that ADAM17 overcompensates for ADAM10 deficiency must be well-considered prior to attempting ADAM10 therapeutic neutralization.

There are two key pieces of data furthering the mechanistic contribution of TNF over other ligands. First, is the recovery of WT architecture in irradiated WT mice reconstituted with a 50/50 mix of C57-ADAM10^{B-/-} + TNFKO bone marrow (Figure 12F 12G). Compared to high TNF levels noted in WT mice reconstituted with C57-ADAM10^{B-/-} bone marrow alone (Figure 12B and 12G) or a 50/50 mix of C57-ADAM10^{B-/-} + WT CD45.1 (Figure 12E and 12G), those reconstituted with a 50/50 mix of C57-
ADAM10\(^{B-/}\) + TNFKO bone marrow produce an appropriate level of TNF considering ADAM10\(^{B-/}\) B cells make too much and TNFKO B cells make none; thus, further supporting the mechanistic contribution of TNF. With the combination C57-ADAM10\(^{B-/}\) + TNFKO model, it is important to note that all cells in the TNFKO do not make TNF, which may contribute to the effect seen. Second, our findings in Balb-ADAM10\(^{B-/}\) B cells and lymph nodes indicate that Balb-ADAM10\(^{B-/}\) B cells exhibit only a marginal increase in ADAM17 expression (Figure 15) and activity leading to WT levels of TNF production, surface expression, and mRNA message levels (Figure 14). This coincided with maintenance of WT lymph node tissue architecture and normal TNF staining within B cell follicle regions (Figure 16).

To conclude, this part of our study demonstrates that compensatory changes in ADAM17 expression and function in the absence of ADAM10 is strain dependent and more exaggerated in Th1 prone C57Bl/6 mice (Figure 15). This finding has substantial implications in therapeutic design where specific targeting of one ADAM may lead to changes in other closely related ADAMs depending on genetic background. Furthermore, it is important to study the interaction between ADAM10 and ADAM17 on other cell types as well to further elucidate other potential complications. Lastly, this part of our study describes the mechanism underling aberrant secondary lymphoid tissue architecture in C57-ADAM10\(^{B-/}\) LNs. The compensatory increase in B cell ADAM17 leads to increased production of B cell TNF, which ultimately underlies architectural aberrancies noted in C57-ADAM10\(^{B-/}\) LNs. Balb-ADAM10\(^{B-/}\) B cells fail to increase ADAM17 and TNF production and retain WT architecture characteristics. This finding lends new insight to the discussion regarding how B cell TNF homeostasis is
critical for secondary lymphoid tissue organization and how a proper B cell ADAM10/ADAM17 ratio is needed to ultimately control TNF signaling.

1.6.2. Increased B cell ADAM10 and reduced ADAM17 and TNF is predictive of increased susceptibility to Th2 responsiveness

Compelling evidence, herein, demonstrates that B cell ADAM10, ADAM17, and TNF are differentially regulated in Th1 and Th2-dominated immune responses and directly influences host susceptibility to allergy and IgE production. We demonstrate that Th2 prone mouse strains (Figure 17) and humans (Figure 21, Table 5) exhibit increased B cell ADAM10 and IgE, while concomitantly having reduced ADAM17 and TNF (Figure 17 and 21). With regards to mouse strains, we described above that Th environment is critical for ADAM17 and TNF regulation in the absence of ADAM10 as Balb-ADAM10\(^{B-/-}\) are less adept at increasing ADAM17 and thus, TNF cleavage (Figure 14 and 15) [31].

A critical question is whether the B cell ADAM10 level is strictly determined by T cell help or is an inherent characteristic of B cells from Th1 or Th2 prone strains. The answer is both. T cell help as demonstrated by in vitro culture with equal concentrations of anti-CD40/IL-4 shows that B cell ADAM10 increases in both C57 and Balb WT B cells following stimulation (Table 4, Figure 17). Thus, T cell help augments ADAM10 expression. Importantly, however, Balb WT B cells exhibit increased ADAM10 in the naïve state and even more dramatically increased ADAM10 after anti-CD40/IL4 stimulation compared C57-WT B cells (Table 4). This finding indicates an innate
difference between their B cells that affects their sensitivity to T cell mediated ADAM10 induction.

Next, regulation of ADAM17 and its ligand, TNF, in different Th contexts was analyzed. C57-WT B cells demonstrated increased ADAM17 in naïve B cells and increased TNF expression following in vitro stimulation compared to Balb-WT B cells (Figure 14). These B cell differences in the classic Th1 and Th2-biased strains extended to other strains of mice as well. For our studies, we used SJL/J and C57Bl/6 as our Th1 prone strains and A/J and Balb/c as our Th2 prone strains (Figure 17). Furthermore, Th context is critical for understanding the difference in ADAM17 and TNF regulation following B cell ADAM10 deletion (Figures 14 and 15), as Balb-ADAM10^{B/-} B cells fail to increase ADAM17 and TNF production, thus permitting WT architecture maintenance (Figure 16). Our evidence suggests that regulation of B cell ADAM10 and ADAM17 is determined by both Th context (e.g. T cell help) and inherent differences in B cell responsiveness.

We next described how both Th context and B cell ADAM10 level are critical when determining susceptibility to airway hypersensitivity induction in mice. Our lab had previously used an OVA-induced airway hypersensitivity model, which showed a reduction in disease parameters in C57-ADAM10^{B/-} mice including airway resistance, OVA- specific IgE, mucus production, and eosinophil infiltration [180]. Using a more clinically relevant HDM model, herein, we directly compare C57 and Balb ADAM10^{B/-} mice and both experienced significantly less bronchoconstriction, goblet cell metaplasia, mucus, and HDM-specific IgE production (in Balb/c) compared to their respective WT strain (Figures 19 and 20). Importantly, however, Balb- ADAM10^{B/-} mice had lung
inflammation parameters comparable to WT-C57 mice, while C57-ADAM10<sup>B-/-</sup> had the lowest disease induction. Furthermore, while clinically relevant features of human asthma including antigen-specific IgE, mucus, and airway resistance were clearly enhanced in an ADAM10 dependent manner, eosinophil infiltration was strain dependent only as Balb/c mice regardless of ADAM10 level had increased eosinophil counts compared to C57 WT and C57-ADAM10<sup>B-/-</sup> mice (Figure 19). This finding does not detract from the potential use of ADAM10 inhibitor therapy, however, because blockade of eosinophil infiltration by anti-IL-5 monoclonal antibodies alone failed to reduce asthma related symptoms in most allergic patients [193,194]. Overall, the data indicates that B cell ADAM10 plays an important role in this Th2 disease model with the highest ADAM10 expression indicating worst symptomology. While the mechanism by which ADAM10 levels influence Th2 disease is unknown, a candidate mechanism could be that ADAM10-mediated CD23 shedding increases IgE production, thus enhancing IgE cross-linking and mast cell degranulation [30]. If further validated in a larger human cohort study, ADAM10 level could serve as a potential indicator of the directionality or strength of a Th1 or Th2 response, with high ADAM10 level indicating a propensity toward enhanced Th2 responsiveness with excess IgE production.

Our data in mouse strains was validated by our findings in active allergic rhinitis patients (Th2 prone) who demonstrated increased B cell ADAM10 and reduced B cell ADAM17 and TNF production (Figure 21). Furthermore, increasing B cell ADAM10 level correlated with increased antigen-specific IgE production (Table 5). Together this suggests that B cell ADAM10 screening could effectively stratify allergic from non-allergic patients and potentially for their susceptibility to more severe disease. An
appropriate, cost-effective means to perform this task is used herein: flow cytometric analysis of ADAM10 levels on peripheral blood CD19+ B cells obtained from a minimally invasive venous puncture. Furthermore, while ADAM10 screening could be a critical diagnostic tool, ADAM10 inhibition therapy is an attractive alternative to other commonly used therapies as it would inhibit the initial step in the allergic cascade: IgE production. We demonstrate, herein, that B cell ADAM10 deletion reduces antigen-specific IgE production (Figure 19). Consequently, ADAM10 inhibitors have the potential to provide anti-allergic prophylaxis when locally administered (e.g. inhaler) particularly to atopic patients with high B cell ADAM10.

Allergic patients with increased B cell ADAM10 concomitantly had reduced B cell ADAM17 and TNF, which could also provide mechanistic insight into their enhanced IgE production. Increased B cell TNF production, as seen in aging B cells, limits antibody production, suggesting a potentially protective role of ADAM17 and TNF from induction of an allergic phenotype [108]. Our findings, overall, suggest that allergy-prone B cells display an ADAM10$^{hi}$/ADAM17$^{lo}$/TNF$^{lo}$ phenotype, with greater risk of atopic manifestations in Th2-biased environments. Our results are an advance in elucidating the key role of ADAM10 in allergy pathogenesis, and provide a novel approach to the diagnosis, prognosis, and treatment of atopic disorders.

1.6.3. C57-ADAM10$^{B/-}$ mice exhibit decreased antibody production, which may be explained by an increased percentage of B10 cells and increased Tregs

The second immune system alteration in C57-ADAM10$^{B/-}$ mice described by Chaimowitz et al. was a reduction in antigen-specific class switched antibody production
post immunization with T dependent antigen. Unlike the difference in secondary lymphoid tissue architecture, both C57 and Balb-ADAM10^{B/-} mice have reduced antigen specific antibody production post NP-KLH immunization (Figures 4, 5, and 13). Therefore, increased B cell TNF production cannot be the mechanism underlying this alteration because Balb-ADAM10^{B/-} mice do not exhibit increased TNF. We, therefore, looked into other possible factors that may affect antibody production including a regulatory B cell phenotype and Treg influence.

B10 cells are a unique subset of B cells that have only recently been described [145,146]. B10 cells are IL-10 producing B cells, of which only a subset becomes antibody-secreting cells [164]. Herein, we demonstrate that following in vitro activation, 30% of C57-ADAM10^{B/-} B cells become IL-10 expressing B cells compared to only 10% of WT B cells (Figure 22A), which corresponded to increased soluble IL-10 production as well (Figure 22B). Furthermore, IL-10 message was increased 6 fold in C57-ADAM10^{B/-} B cells following in vitro stimulation (Figure 22C). While the topic is controversial, some reports state that B10 cells enhance Treg development and maintenance [161–163]. Our finding of increased IL-10 in the circulation of C57-ADAM10^{B/-} mice (Figure 22E) further supported a potential induction of Tregs, given that B cells alone are not thought to make that much IL-10.

Tregs were in fact increased in C57-ADAM10^{B/-} spleens (Figure 23 and 24). Candidate mechanisms for this Treg enhancement include increased B cell production of ICOSL and/or IL-10. ICOS-ICOSL interaction is critical for the development and persistence of Tregs [113,114]. ADAM17 is known to downregulate membrane-bound ICOSL and increase soluble ICOSL (sICOSL) production by an indirect mechanism.
Given that C57-ADAM10\(^{B/-}\) B cells have increased ADAM17, this suggests that increased sICOSL production may enhance ICOS receptor signaling on T cells, thus inducing FoxP3 expression in a larger subset of T cells. ICOSL was increased in naïve C57-ADAM10\(^{B/-}\) B cells and thus there is more to be cleaved following ADAM17 activation (Figure 25). Preliminary data suggested that neutralization of ICOSL in co-cultures of CD4+CD25- T cells and C57-ADAM10\(^{B/-}\) B cell supernatants reduced FoxP3 expression compared to treatment with C57-ADAM10\(^{B/-}\) B cell supernatant alone (Figures 27 and 28). Further studies are needed to confirm that neutralization of ICOSL signaling in vitro inhibits FoxP3 induction in CD4+CD25- cells. While IL-10 is not thought to induce FoxP3 expression, it is known to be critical for maintaining FoxP3 expression [195]. Therefore, it is likely that increased IL-10 production by C57-ADAM10\(^{B/-}\) B cells supports FoxP3 expression, which was increased initially by an alternate source (e.g. ICOSL). IL-10 neutralization experiments should demonstrate a reduction, even if slight, in FoxP3 expression following stimulation with WT or C57-ADAM10\(^{B/-}\) B cell supernatants. Furthermore, inhibition of both ICOSL and IL-10 together should cause the largest suppression of FoxP3 induction in CD4+CD25-/B cell supernatant co-cultures.

While studies in Balb-ADAM10\(^{B/-}\) still need to be performed, increased Tregs in C57-ADAM10\(^{B/-}\) mice contributed to decreased class-switched antibody production and reduced lung inflammation parameters following HDM challenge. Treg depletion in vivo led to enhanced HDM-specific IgE production in both WT and C57-ADAM10\(^{B/-}\) mice as well as increased AHR, BALF cellularity, inflammatory cellular infiltration in airways, and mucus (Figure 28, 29, and 30). Therefore, Tregs do play a mechanistic role in limiting
class-switched antibody production and lung inflammation in C57-ADAM10^B-/^ mice. To validate our findings as a Treg-specific phenomenon, we must still confirm Treg depletion by flow cytometry. In Setiady et al. anti-CD25 treatment decreased CD25+FoxP3+ cells from 6.5% to 0.7%, although FoxP3+ cells only decreased from 3.3% to 2.3% [186]. Furthermore, control Rat IgG1 treatment failed to reduce CD25+FoxP3+ cells from WT levels [186]. We expect to demonstrate similar results.

Furthermore, our findings herein may further the understanding of B10 cells including their relationship to Tregs and other, un-reported surface markers. Our data suggests that ICOSL and CD43 may be potential markers of B10 cells. To prove this point, co-staining with B220, IL-10, and ICOSL or CD43 is needed to show if IL-10 producing B cells specifically upregulate these markers compared to non-IL-10 producing B cells.

Lastly, the overarching goal of section 1 was to define the immune system alterations in B cell specific ADAM10 deficient mice and determine how differential ADAM10 levels affects Th2 disease. Ultimately, we aimed to determine if ADAM10 inhibitor therapy would be beneficial in treating allergic diseases and if so, by what potential mechanism. Previously, we mentioned that ADAM10 inhibitor therapy for Th2 disease was a unique way to regulate IgE production. Our recent findings suggest that a potential mechanism by which ADAM10 inhibitors may reduce IgE is through enhancement of Treg cells downstream of enhanced B cell ICOSL or IL-10 production. It is important to note that ADAM10 inhibitor therapy, however, would not be B cell specific and could cause off-target effects. An i.v administered dual ADAM10/17 inhibitor has been evaluated in human clinical trials for its anti-neoplastic effects and
was found to be well tolerated [196]. While it lacked efficacy in blocking tumor growth, our data suggests that locally administered ADAM10 inhibitor therapy (e.g. inhaler) to the airway may be preventative against important disease parameters such as bronchoconstriction and mucus production and should be further explored.
Section 2: Impaired immunological synapse in sperm associated antigen 6 (SPAG6) deficient mice

Chapter 1: Introduction to SPAG6 and its role in microtubule rearrangement

2.1.1 SPAG6

Mammalian sperm associated antigen 6 (SPAG6), or its orthologue, PF16 protein, from *Chlamydomonas reinhardtii*, is classically known as a component of the central apparatus of the “9+2” axoneme, which consists of a central pair of microtubules (C1 and C2) surrounded by nine outer microtubule doublets with associated dynein arms [175,197–199]. Mutagenesis of *Chlamydomonas* PF16 results in flagellar paralysis and disturbance of C1 central microtubule stability revealing its central role in flagellar stability and motility [199]. In mammals, SPAG6 is widely expressed, mainly in tissues with cilia-bearing cells including lung, nervous system, inner ear, bone marrow, and particularly, testicular germ cells where SPAG6 resides in the sperm flagella [197,200]. Many of the noted abnormalities associated with SPAG6 deficiency are related to dysfunctional ciliary or flagellar appendages in ciliated cells and tissues. In humans, SPAG6 in the sperm tail is targeted by a class of anti-sperm autoantibodies associated with immune-mediated infertility in males [200]. Global SPAG6-deficient mice (*Spag6*KO) exhibit significant defects including a high percentage of neonatal deaths, hydrocephalus, and infertility in surviving male mice [198]. They also experience a significant reduction in axoneme numbers and the two central microtubules exhibit a random orientation compared to WT mice [201]. Furthermore, *Spag6*KO tracheal epithelial cells lose their polarized morphology and polarized distribution of mucin; and these phenotypes appear to be related to a disrupted microtubule system [201].
addition, *Spag6KO* mice develop otitis media more readily than WT given abnormalities in the ciliated columnar epithelium in the middle ear and Eustachian tube [202]. While these examples demonstrate the importance of SPAG6 in ciliated tissues, the role of SPAG6 in cells lacking classic cilia and/or flagella, such as lymphocytes or other immune cells, remains unreported. Another protein critical for ciliogenesis, IFT20, has been shown to be important for microtubule organization and T cell receptor (TCR) recycling at the immunological synapse [203,204]. Therefore, given the contribution of SPAG6 protein to microtubule organization, and the critical role of microtubule organization in cognate immune cell interaction, we sought to analyze the role of SPAG6 in the formation of the immunological synapse as well as the impact of SPAG6 deficiency on the immune response.

### 2.1.2 SPAG6 and its role in the MTOC

The microtubule organizing center (MTOC) is a subcellular apparatus that functions to form and organize microtubules, which in eukaryotic somatic cells is the centrosome, composed of two orthogonally arranged centrioles (mother centriole and daughter centriole) [205,206]. During an immune response, upon cognate recognition between an antigen presenting cell and effector cell, reorientation of the centrosome, actin, the Golgi, and secretory vesicles occurs in the effector cell at the immunological synapse, allowing receptor/ligand interactions and targeted release of cytokines [207]. Furthermore, during targeted killing by an effector cell, the same reorientation occurs with docking of the centrosome at the synapse membrane of the effector cell, effectively creating the synaptic cleft by which cytolytic enzymes are released for targeted destruction [205,208]. While SPAG6 is known to be a microtubule-binding protein [197]
and co-localizes with polymerized microtubules [209], we demonstrate, herein, the expression of SPAG6 protein in primary and secondary lymphoid tissues, its association with the centrosome, and the lack of centrosome polarization and actin clearance at the synaptic cleft of SPAG6 deficient mice. In addition, we show that these synaptic malformations are associated with reduced CTL cytotoxicity, diminished follicular CD4 T cell retention in germinal centers, defective germinal center reactions, low production of class-switched antibodies, and expansion of the B1 B cell subset.
Chapter 2: Materials and Methods

2.2.1 Mice

Mice were housed in the Virginia Commonwealth University mouse vivarium in accordance with NIH guidelines. All animal care and experimental protocols were approved by IACUC. Spag6KO mice were generated as previously described [198] and compared to WT littermate controls. C57/SV129 WT male mice age 6-8 weeks were used for reconstitution.

2.2.2 Bone Marrow Reconstitution

C57/SV129 WT were pretreated with 100 mg/L enrofloxacin for 5 days prior to irradiation. C57/SV129 WT were anesthetized using 80 mg/kg ketamine and 8 mg/kg xylazine in PBS and given two doses of 550 cGy irradiation, with a 2-h rest period, using a MDS Nordion Gammacell 40 research irradiator with a $^{137}$Cs source. Bone marrow cells were isolated as previously described [169] (see section 1.2.10) and 5 million cells i.v. injected into irradiated WT. After 6 weeks, mice were footpad and i.p immunized with 10 µg 4-hydroxy-3-nitrophenylacetyl coupled to keyhole limpet hemocyanin at a ratio of 27:1 (Bioresearch Technologies) in 4 mg alum. Mice were bled at day 7 and organs harvested at day 14.

2.2.3 Reagents and T cell culture

Spleen was crushed, filtered, RBCs lysed with ACK Lysing Buffer (Quality Biological), and cytotoxic T cells isolated from splenocytes by PE-positive selection using CD8-PE (Biolegend) and EasySep kit (Stemcell Technologies). T cells were grown in complete
RPMI [31] with 100 Units/mL IL-2 (Peprotech) on overnight anti-CD3ε (1µg/mL, Biolegend) treated plates with anti-CD28 (2µg/mL, Biolegend) and supernatants collected for IFN-y ELISA (eBioscience) after 72 h of growth. Proliferation was assessed after 72 h with a 24-h pulse of [³H] thymidine, 1µCi/well (Perkin Elmer). using Filtermate cell harvester onto UniFilter-96 GF/C microplates and analyzed by TopCount Plate Counter (Perkin Elmer).

**2.2.4 ELISA**

*NP-specific antibody*

NP-specific IgM and IgG1 was determined by NP-specific ELISA using NP-14–BSA (15 µg/ml; Biosearch Technologies) as previously described in section 1.2.6.

*IFNγ*

Soluble IFN-y from T cell supernatants was determined by mouse quantitative ELISA kit (88-7314-77, eBioscience) according to manufacturer’s protocol.

**2.2.5 Flow cytometry**

Single cell suspension of splenocytes, lymph node, and peritoneal lavage cells were incubated with Fc blocking reagent (10 µg anti-mouse unlabeled CD16/32 (2.4G2)); stained with B220-APC or PeCy5, CD3-PE or PECy7, CD11b-FITC, or GL7-FITC (Biolegend), washed, examined on a BD Canto Flow analyzer, and data analyzed with FCS Express, v. 4.

**2.2.6 Cytotoxic assay**
CD8+ T cells effector cells were isolated from spleen using EasySep mouse PE selection Kit (Stemcell Technologies) with CD8-PE (Biolegend). CD8 T cell purity was verified (95-98%) and cultured with platebound anti-CD3ε and anti-CD28 as described above in T cell media for 2 days and then seeded into fresh media daily and used 6 days later. P815 (ATCC) target cells were grown in complete RPMI. Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega) was used to first determine P815 optimal cell concentration (1.2 x 10⁵ cells/mL) and then cytotoxicity assay was performed in quadruplicate.

2.2.7 CTL induction and slide set up for synapse staining

Balb/c WT spleen was collected, crushed, RBC lysed, resuspended to 10⁷/mL, and irradiated at 3000rad. Cells were then counted, washed in T cells media (RPMI supplemented with 10% FBS, 50µM β-mercaptoethanol, L-glutamine, sodium pyruvate, and 50U/mL penicillin and streptomycin), resuspended to 1.25x10⁶/mL, and 1mL of Balb/c stimulator cells cultured with 1mL (at 1.25x10⁶/mL) of WT or Spag6KO responder cells for 5 days. Live cells were collected, washed, and resuspended to 1x10⁶/mL in IL-2 (100U/mL, Peprotech) supplemented T cell media. Cells were cultured 5 days and seeded into fresh IL-2 supplemented media daily.

Chamber slides (Thermo Scientific, no. 154534) were coated with ICAM-Fc (1µg/mL) in sterile BBS overnight at 4°C. WT or Spag6 (three per group) CTLs and P815 were resuspended to 4x10⁶/mL. 125µL of CTL and P815 were incubated separately at 37°C for 5 minutes, mixed, incubated again for 5 minutes, then diluted to 1x10⁶/mL in warm T cell media. Cells were incubated on slides for 10 minutes at 37°C,
media removed, ice cold 100% methanol added, incubated for at least 5 minutes, methanol removed, and slides dried for 30 minutes at 4°C. Slides were set up as CTL alone, P815 alone, CTL+ P815, or PBS only.

2.2.8 Immunohisto-, cyto-chemistry, and confocal microscopy

Ten um OCT-embedded frozen sections of WT and Spag6KO lymph nodes were fixed in ice-cold acetone for 10 mins then left to air-dry. The sections were re-hydrated and blocked with 2% horse serum, then labelled with 10 \( \mu \)g/ml FITC-conjugated T- and B-Cell Activation Antigen (clone GL7, BD Biosciences 553666), PE-conjugated Rat Anti-Mouse CD4 (clone GK1.5, southern biotech 1540-09L), and Alexa Flour 647-conjugated rat anti mouse CD21/35 (clone 7E9, Invitrogen Molecular Probes, RMCD213521) for 45 mins. Sections were thoroughly washed, mounted with anti-fade mounting medium, Vectashield (Vector Laboratories), and cover-slipped. The sections were examined with Leica TCS-SP2 AOBS confocal laser scanning microscope fitted with an oil plan-apochromat \( \times 40 \) objective. Three lasers were used: Argon (488 nm) for FITC, HeNe (543 nm) for PE, and HeNe (633 nm) for Alexa Flour 647 (shown as pseudo-color blue). Parameters were adjusted to scan at 1024 \times 1024 pixel density and 8-bit pixel depth. Emissions were recorded in three separate channels, and digital images were captured and processed with Leica Confocal and LCS Lite software. The number, size and CD4 content of GCs in the LNs of WT and Spag6KO mice were counted and measured, and the average was presented \( \pm \) SD.

For immuno-cyto-chemistry, WT or Spag6KO splenocytes were co-cultured with irradiated Balb/c splenocytes for 5 days, remaining live cells seeded into fresh IL-2 supplemented cRPMI for 3 days, and then allogeneic CTLs were cultured briefly with
P815 target cells. Cells were transferred to pre-coated (ICAM-Fc, 1µg/mL, R&D) Nunc chamber slides (Thermo Scientific) and fixed in cold methanol, left to dry then blocked with 2% horse serum. The cells were labeled with rabbit anti α-actin antibody (Sigma-Aldrich A2066-.2ML) or mouse anti gamma-tubulin (clone GTU-88, Sigma-Aldrich T6557-.2ML) for 45 mins then washed. Alexa Flour 488-conjugated Fab’2 fragment donkey anti-Rabbit IgG (H+L) (Jackson Immuno-Research 711-546-152) or anti Mouse IgG (H+L) (Jackson Immuno-Research 715-546-150) [with minimal cross reactivity to horse, mouse, rabbit, goat, human and rat proteins] secondary antibodies were then added together with rat anti-mouse CD8a-PE (southern Biotech, 1550-09L) and NucRed 647 nuclear stain (Invitrogen, R37113). After 45 mins, the cells were thoroughly washed, mounted with anti-fade mounting medium, Vectashield (Vector Laboratories), and cover-slipped. The cells were examined with Zeiss Laser Scanning Microscope LSM 710 fitted with an oil plan-apochromat ×40, x63, and x100 objectives. Three lasers were used: Argon (488 nm) for FITC, HeNe (543 nm) for PE, and HeNe (633 nm) for NucRed 647 (shown as pseudo-color blue). Parameters were adjusted to scan at 1024 × 1024 pixel density and 8-bit pixel depth. Emissions were recorded in three separate channels, and digital images were captured. Z stacks running through the immunological synapse with 0.40 µm step-size were generated and re-structured into 3-dimensional snap shots and movies using Zeiss Zen 2012 (Blue Edition) software.

2.2.9 Centrosome staining

HEK293-T cells were transfected with SPAG6/pcDNA3 plasmid and 48 hours post transfection cells were permeabilized with 1% Triton X-100, blocked, incubated overnight at 4°C with a polyclonal anti-SPAG6 and monoclonal anti-γ-tubulin, washed,
and incubated for 1 h at room temperature with Alexa 488-anti-mouse IgG (Jackson Immuno Research Laboratories) and Cy3-anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Finally slides were washed and sealed using VectaMount with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Images were taken by confocal laser-scanning microscopy (Leica TCS-SP2 AOBS).

2.2.10 RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen), reverse transcribed using RETROscript® (Ambion, AM1710), and PCR performed to examine Spag6 mRNA expression compared to Gapdh control. The primer set used in our previous study was applied [210].

2.2.11 Statistical analysis

Normal distribution of data was determined using Shapiro-Wilk normality test in SigmaPlot 12.5. The p values were calculated using unpaired two-tailed Student t tests in GraphPad Prism. Error bars represent the SEM between samples. A p value <0.05 is considered significant.
Chapter 3: Results and Discussion

2.3.1 SPAG6 is expressed in primary and secondary lymphoid tissues and co-localizes with the centrosome

The expression and role of SPAG6 in immune system development and adaptive functions is unknown. While the murine immune system fails to mature until about six weeks of age, the majority of Spag6KO mice survive less than three weeks due to numerous developmental defects. Therefore, we first confirmed expression of Spag6 in various immune cells and tissues of adult WT mice including spleen, thymus, bone marrow, lymph node and naïve T cells by RT-PCR (Figure 33). In order to study SPAG6 loss in a mature immune system, adult WT mice were irradiated and reconstituted with 7-14 day old WT or Spag6KO bone marrow. SPAG6 is known to be critical for ciliogenesis and microtubule-based motility [201]; and despite the fact that lymphocytes do not have cilia, their engagement with antigen presenting cells and target cells involves extensive remodeling of cytoskeletal and motor proteins. De la Roche et al. proposed that the T cell immunological synapse at the interface between T cells and antigen presenting / target cells is a “surrogate cilium” because it utilizes the same machinery as ciliogenesis including the nucleation of microtubules at the MTOC or centrosome [207,211]. De la Roche et al. also described how Hedgehog signaling, originally known for its role in primary cilia formation, is also critical for CTL function and immunological synapse formation [207]. Consequently, we sought to determine if SPAG6 is present in the MTOC or centrosome and, if so, could SPAG6 be required for proper immunological synapse formation and function. We previously reported that SPAG6 decorated and appeared to organize the microtubules in transfected CHO cells.
[209], however, whether SPAG6 protein is a structural component of the MTOC or centrosome is not known. To explore the SPAG6-centrosome association, HEK293 cells were transfected with SPAG6/pcDNA3 plasmid and then the cells were double labeled with a polyclonal antibody against SPAG6 and a monoclonal antibody against γ-tubulin, a centrosome component. As shown in Figure 34, SPAG6 co-localized with γ-tubulin indicating that SPAG6 protein is structurally associated with the MTOC/centrosome apparatus.

2.3.2 SPAG6 is required for centrosome polarization and actin clearance at the immunological synapse

Given that SPAG6 is structurally associated with the centrosome (Figure 34), and the centrosome is crucially involved in synapse organization, we predicted that SPAG6 plays a critical role in immunological synapse formation. Two hallmarks of adequate synapse formation are centrosome polarization to the synapse and actin clearance from the synapse [207]. At the central supra-molecular activation cluster of the immunological synapse, the centrosome moves to and contacts with the plasma membrane, whereas actin is cleared away from the synapse. It has been proposed that centrosome polarization might be driven by the reorganization of the actin cytoskeleton, clearing the plus ends of microtubules from the area of contact and pulling the centrosome towards the plasma membrane [212]. To analyze the role of SPAG6 in synapse formation, allogeneic CTLs were cultured briefly with P815 target cells, then labeled for α-actin and γ-tubulin to visualize the synaptic cleft at the interface between CTLs and their targets. Figure 35 A, C demonstrates that SPAG6 is required for actin clearance at the synapse as lack of SPAG6 in Spag6KO CD8 T cells was associated
with inefficient actin clearance and persistence of actin staining at the synapse. On the other hand, WT CD8 T cells efficiently cleared actin at the synapse and labeling showed an actin ring distal to the synapse. Movies constructed from z stacks passing through the whole depth of the synapses confirmed lack of actin clearance in Spag6KO mice and efficient regression in the WT mice (data not shown). Actin reorganization is thought to play a role in centrosome polarization [212], consequently, lack of SPAG6 also resulted in improper polarization of the centrosome and lack of positioning at the synapse (Figure 35 B, C). Specifically, in WT CTL cells, centrosome docking at the synapse occurred in 50% of CTL/target cell synapses with only 10% positioned distal to the synapse; whereas, Spag6KO CTLs exhibited centrosome docking in only 30% of synapses (Figure 35C). Furthermore, the majority of centrosomes were docked or proximal to the synapse in WT CD8 T cells compared to Spag6KO CD8 T cells where the majority were distal or proximal to the synapse, with fewer docked (Figure 35C).

2.3.3 Reduced T cell cytotoxicity in Spag6KO

Thus far, we have determined that SPAG6 is expressed in primary and secondary lymphoid tissues (Figure 33), co-localizes with the centrosome (Figure 34), and its deficiency is associated with defective centrosome polarization and actin clearance (Figure 35) suggesting that these structural abnormalities will obstruct proper functional communication at the synapse site, which can be assessed by analyzing cytotoxic T cell (CTL) killing of target cells and T cell-dependent class-switched antibody production in WT and Spag6KO mice.
CTLs destroy target cells following T cell receptor activation through release of secretory lysosomes at the immunological synapse. T cell receptor activation triggers migration of the MTOC to the contact site, causing the generated microtubule network to polarize towards the target [208]. Secretory lysosomes then travel in a minus end direction toward the MTOC [208] followed by transient fusion of the centrosome with the plasma membrane to mediate secretion into the synapse [212]. Given the presence of SPAG6 in the centrosome, we reasoned that in the absence of SPAG6, reduced CTL-induced cytotoxicity would occur. We performed an allogeneic cytotoxic T cell assay and demonstrated that Spag6KO T cells had significantly reduced capacity to induce P815 cell death compared to WT CD8 T cells (Figure 36A). Furthermore, stimulated Spag6KO CD8 T cells had reduced secretion of soluble IFNγ (Figure 36B). These findings are not due to reduced T cell number (Figure 36C) or their ability to proliferate (Figure 36D). In fact, Spag6KO CD8 T cells cultured with anti-CD3/CD28 exhibit enhanced proliferation compared to WT CD8 T cells (Figure 36D), suggesting an even greater reduction in IFNγ secretion per cell than is suggested in Figure 36B.
Figure 33. SPAG6 expression in lymphoid cells and tissues.

*Spag6* expression in adult WT mice compared to testis (positive control) and negative control (water). *Spag6* is expressed in primary (thymus, bone marrow) and secondary (spleen, lymph node) lymphoid tissues and T cells.
Figure 34. Presence of SPAG6 protein in the centrosome.

HEK293 cells were transfected with SPAG6/pcDNA3 expressing plasmid, and the cells were stained for DAPI (blue), SPAG6 (red) and γ-tubulin (green), a component of centrosome. Co-localization of SPAG6 and γ-tubulin is indicated by white arrows. Figure courtesy of Dr. Zhibing Zhang.
Figure 35. Defective actin clearance and centrosome polarization at the immunological synapse in Spag6KO mice.

(A, B) The immunological synapse between WT or Spag6KO CD8+ CTLs (red) and P815 target cells was stained for (A) α-actin (green, white arrows, upper panel) or (B) γ-tubulin (green dots, white arrows, lower panel). (C) Quantification of actin clearance (left) and centrosome (γ-tubulin) polarization and docking distance from the synapse in WT and Spag6KO CD8+ cells shown. P values shown.
Figure 35. Defective actin clearance and centrosome polarization at the immunological synapse in Spag6KO mice.
Figure 36. Reduced CTL function in Spag6KO mice.

(A) Allogeneic cytotoxicity assay with decreasing ratios of WT (solid) or Spag6KO (dashed) CD8+ T cells to P815 target cells; (B) Soluble IFNγ production by WT or Spag6KO CD8+ T cells (C) CD3+ T cell percentage of total splenocytes; gated from live singlets. (D) WT or Spag6KO (KO) CD8+ T cell proliferation in media alone (white) or with CD3/CD28 stimulation (stripes). N=3-7 per group; **p<0.005, ***p<0.0005.
2.3.4 WT mice reconstituted with SPAG6 deficient bone marrow exhibit impaired GC formation, diminished follicular CD4 T cells, reduced class-switched antibody production, and expansion of B1 B cells

Establishment of a proper immunological synapse is required for optimal antibody production as well. A series of synapses is generated to mount an effective humoral immune response. First, a synapse between cognate antigen-specific B and T cells occurs at the B/T border to trigger downstream GC formation. Furthermore, synapse formation and signaling between B cells and Tfh cells is required for class switch recombination and affinity maturation [213]. Moreover, BCR engagement with FDC-retained antigens is critical for GC persistence and selection of high affinity B cell clones [214]. We, therefore, hypothesized that Spag6KO mice, who have a comparable number of B cells to WT (data not shown), would exhibit impaired GC formation and reduced class-switched antibody production post immunization with T cell-dependent antigens. Following immunization with the T cell-dependent NP-KLH antigen, the germinal center reactions and CD4+ T cells in the draining LNs were assessed in WT or Spag6KO reconstituted mice. Following 14 days of immunization, the number and size of GCs in the follicle were significantly reduced in Spag6KO (Figure 37A). Furthermore, the number of GC CD4+ T cells were significantly diminished, suggesting reduced migration of Tfh cells to the GC perhaps due to lack of initial signaling/interaction with the B cell, or due to impaired retention in the GC due to lack of effective GCB cell/T cell synapse (Figure 37A). In fact, stable GC-B cell/T cell interaction is critical for selection of antigen-specific B cells, B cell activation and plasma cell differentiation, and this interaction is associated with reduction in both GCB and Tfh
cell velocities, along with an increase in the duration and size of the T-B contacts [84,215–218].

The reduction in GC formation and CD4+ T cells in Spag6KO mice corresponded with significantly reduced NP-specific IgG1 production on day 14 (Figure 37B, right). While NP-specific IgM and IgG1 levels were comparable at day 7 post immunization (data not shown), NP-specific IgM is increased in Spag6KO serum at day 14 (Figure 37B, left). This result could be attributed to increased extrafollicular antibody production in Spag6KO B2 cells or perhaps due to the increase in peritoneal B1 B cells (Figure 37C). B1 B cells are innate, self-renewing, long lived B cells, which typically reside in the peritoneal and pleural cavities as well as spleen [219]. They fail to enter germinal centers, are thought to make natural antibodies, and do not require cognate T cell interaction for IgM production [219]. Our noted increase in B1 B cells in the absence of SPAG6 suggests that SPAG6 is involved in attenuating the B1 response and may be an interesting target for limiting autoimmune reactions, as increased B1 B cells are related to predisposition to autoimmunity [220,221].

In conclusion, while classically studied in ciliary and flagellar motility, SPAG6 is expressed in primary and secondary lymphoid tissues and its role in immune system development and function stems from its critical role in proper immunological synapse formation. We provide novel and substantial evidence that SPAG6 is present in the centrosome and is required for optimal centrosome polarization and actin clearance during synapse formation. SPAG6 deficiency is associated with inadequate synapse formation manifested by significant functional consequences including reduced CD8 cytotoxicity, CD8 T cell IFNγ secretion, GC formation, GC CD4+ T cell retention, and
class-switched antibody production. These structural and functional abnormalities were associated with an increase in B1 B cell subset numbers. Finally, original data presented in this study paves the way for new investigations on the role of SPAG6 in synapse-dependent immunological interactions and the impact of SPAG6 targeting by autoantibodies in immune infertility on cellular and humoral immune responses.
Figure 37. Defective humoral immune response in SPAG6 KO mice.

(A) Reduced GC formation and GC CD4+ T cells in draining LN (day 14) of WT (left) and Spag6KO (right) reconstituted mice. GCs (GL7+, green), FDCs (CD21+, blue), and CD4 T cells (red) are shown. On the top, triple overlay (left) and separate channel recordings (right) shown for WT and Spag6KO. Below, GC morphometric analysis of at least 6 sections per group showed reduced number and size of GCs and CD4+ T cells per GC in Spag6KO. Day 14 NP-KLH specific (B, left) IgM and (B, right) IgG1 levels in sera. (C) Percentage of peritoneal B1 B cells (B220+CD11b+) collected at day 14. N=9 per group, 3 independent experiments. *p<0.05, **p<0.005.
List of References


31. Folgosa L, Zellner HB, El Shikh ME, Conrad DH (2013) Disturbed follicular architecture in B cell A disintegrin and metalloproteinase (ADAM)10 knockouts is mediated by compensatory increases in ADAM17 and TNF-
alpha shedding. J Immunol 191: 5951-5958. jimmunol.1302042 [pii];10.4049/jimmunol.1302042 [doi].


128. Martin RK, Brooks KB (2014) Antigen transfer from exosomes to dendritic cells as an explanation for the immune enhancement seen by IgE immune complexes.


of tolerance to an environmental antigen. J Immunol 191: 1136-1143. jimmunol.1201899 [pii];10.4049/jimmunol.1201899 [doi].


146. Matsushita T, Tedder TF (2011) Identifying regulatory B cells (B10 cells) that produce IL-10 in mice. Methods Mol Biol 677: 99-111. 10.1007/978-1-60761-869-0_7 [doi].


151. Yanaba K, Bouaziz JD, Matsushita T, Tsubata T, Tedder TF (2009) The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. J Immunol 182: 7459-7472. 182/12/7459 [pii];10.4049/jimmunol.0900270 [doi].


157. Matsushita T, Horikawa M, Iwata Y, Tedder TF (2010) Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. J Immunol 185: 2240-2252. jimmunol.1001307 [pii];10.4049/jimmunol.1001307 [doi].


necrosis factor alpha. Mol Biol Cell 20: 1785-1794. E08-11-1135 [pii];10.1091/mbc.E08-11-1135 [doi].


176. Frasca D, Landin AM, Alvarez JP, Blackshear PJ, Riley RL, Blomberg BB (2007) Tristetraprolin, a negative regulator of mRNA stability, is increased in old B cells and is involved in the degradation of E47 mRNA. J Immunol 179: 918-927. 179/2/918 [pii].


serum IgE levels and eosinophil cationic protein during and out of the pollen season. Allergy Asthma Proc 20: 119-125.


211. Le BM, Shaw AS (2013) Immunology. Do T cells have a cilium? Science 342: 1177-1178. 342/6163/1177 [pii];10.1126/science.1248078 [doi].


VITA

Lauren Folgosa Cooley was born on June 2, 1988 and grew up in Fayetteville, Georgia. She graduated high school from Woodward Academy in College Park, Georgia in 2006. She attended University of Richmond in Richmond, VA on a dual merit and athletic golf scholarship and graduated summa cum laude with a Bachelor of Science in Honors Biochemistry and Molecular Biology with minors in History and Medical Humanities in 2010. She is currently pursuing her MD/PhD degree at Virginia Commonwealth University in Richmond, Virginia. She entered the MD/PhD program in 2010 and the Center for Clinical and Translational Research program as a graduate student in 2012.