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The role of Fyn and B-cell expressed ADAM10 in early B cell development,
germinal center formation and terminal B cell differentiation

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

By

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June 2012

DEDICATION

This dissertation is dedicated to my family. My husband, Eric, has always supported my educational pursuits, I am thankful for his love, encouragement and support. My mother, Edith S. Messing Ginzburg, and my parents-in-law, Mindy B. Chaimowitz and Steven Chaimowitz have shared my struggles and successes and encouraged me to continue to work hard to reach my goals. I would also like to thank my brothers, Brian and Kevin Ginzburg for the love and support. Finally, this dissertation is dedicated to the loving memory of my father, Gabriel H. Ginzburg who always inspired me to work hard and whom I miss deeply.

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LIST OF ABBREVIATIONS

-/-	Homozygous deletion of a gene
+/-	Heterozygous deletion of a gene
2.4G2	mAb recognizing the murine FcγRII and FcγRIII (CD16/CD32)
ADAM	A disintegrin and metalloproteinases
ADAM10 ^{Δ/Δ}	Floxed ADAM10 mice
AID	Activation induced cytosine deaminase
Alum	Aluminum hydroxide
AP	Alkaline phosphatase
APC	Allophycocyanin
APP	Amyloid plaque precursor
ASC	Antibody Secreting Cell
ASC	Antibody secreting cell
B220	mAb recognizing murine CD45R, pan-B cell marker
Balb/c	Inbred mouse strain
Bcl6	B cell lymphoma 6
BCR	B cell receptor
BEC	Blood endothelial cell
Blimp-1	B lymphocyte induced maturation protein 1
BM	Bone marrow
BSA	Bovine serum albumin
C57BL/6	Inbred mouse strain
CCR	C Chemokine Receptor
CD	Cluster of differentiation
CD23	FcεRII, low affinity IgE receptor
CD23Tg	CD23 Transgenic
CD40L	CD40 ligand

cDNA	Complementary DNA
CHO	Chinese hamster ovary cell line
CLP	Common lymphocyte progenitor
CPM	Counts per minute
CSR	Class switch recombination
CXCR	CX Chemokine Receptor
Cy	Cyanine
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSBs	Double stranded break
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FDC	Follicular Dendritic Cell
FRC	Fibroblastic reticular cell
GC	Germinal Center
i.p.	Intraperitoneal
IC	Immune complex
ICD	Intracellular domain
IFN γ	Interferon γ
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL	Interleukin
IL-6R	IL-6 Receptor
IRF4	Interferon response factor 4
KLH	Keyhole limpet
Kuz	Kuzbanian
LEC	Lymph endothelial cell

LN	Lymph node
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTi	Lymphoid tissue inducer cell
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MZ	Marginal Zone
NICD	Notch intracellular domain
NP	4-Hydroxy-3-nitrophenylacetyl
OVA	Ovalbumin
PBS	Phosphate buffer saline
PC	Plasma Cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
Pre-B	B cell precursor
Pro-B	B cell progenitor
PTK	Protein tyrosine kinase
qPCR	Quantitative PCR
RBP-Jk	Canonical Notch transcription factor
RIP	Receptor intramembrane proteolysis
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RU	Relative units
s.c.	Subcutaneous
SEM	Standard error of the mean

SH	Src homology
SHM	Somatic hypermutation
SSBs	Single stranded break
STAT	Signal transduction and activator of transcription
T _{FH}	Follicular helper T cells
Th1	T helper Type 1
Th17	T helper Type17
Th2	T helper Type 2
Th9	T helper Type 9
TNF- α	Tumor necrosis factor alpha
T _{reg}	T regulatory cell
WT	Wild type
XPB-1	X-box binding protein 1
YFP	Yellow fluorescent protein

ABSTRACT

THE ROLE OF FYN AND B-CELL EXPRESSED ADAM10 IN EARLY B CELL DEVELOPMENT, GERMINAL CENTER FORMATION AND TERMINAL B CELL DIFFERENTIATION

By Natalia Sol Chaimowitz, Ph.D.

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

Virginia Commonwealth University, 2012

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

In these studies we sought to determine the role of Fyn kinase and ADAM10 in B cell biology. A disintegrin and metalloproteinase 10 (ADAM10) is a zinc dependent proteinase related to matrix metalloproteinases. ADAM10 has emerged as a key regulator of cellular processes by cleaving and shedding extracellular domains of multiple transmembrane receptors and ligands. In particular, ADAM10 has been identified as a key regulator of lymphocyte development. Here we report that ADAM10 is dispensable for early B cell development within the bone marrow. However, deletion of ADAM10 from all peripheral B cells or in post-switch cells leads to severe impairments in humoral responses. When ADAM10 was deleted from all peripheral B cells a decrease in antigen specific IgG production was seen both with respect to serum levels and IgG ASCs, indicating that plasma cell (PC) differentiation is influenced. Cells producing high affinity antigen specific antibodies were particularly affected, consistent with defects in germinal center (GC) reactions. Moreover, changes in

lymphoid architecture were also observed. Consistent with these findings, follicular dendritic cell (FDC)-reticula was undetectable following immunization. On the other hand, when ADAM10 was deleted in post-switch B cells, GC formation and lymphoid architecture were not impaired. Despite normal architecture, however, antibody production was still affected, likely due to abnormal gene expression in ADAM10-deficient PCs. Consistent with this hypothesis, PCs isolated from ADAM10^{Δ/Δ}IgG1-cre^{+/-} showed decreased expression of genes that facilitate plasma cell differentiation and function and increased expression of Bcl6, an inhibitor of PC differentiation.

Fyn kinase is a member of the Src protein tyrosine kinase. Fyn is widely expressed in many cell types, including lymphocytes. Fyn has been shown to interact with both the B cell and T cell receptor (BCR and TCR, respectively). While Fyn-deletion did not impair the development of immature T cells and B cells, TCR signaling was altered in mature T cells. Our results demonstrate that Fyn-KO mice have significantly low basal levels of IgG1 and IgG2a. Additionally, these mice displayed delayed kinetics in the production of NP-specific IgG1 and IgG2b, and significantly low NP-specific IgG2a after a T-dependent immunization protocol. Defects in antibody production correlated with significantly reduced numbers of GC B cells, T_{FH} cells and splenic PCs. Moreover, Fyn-KO B cells showed decreased production antibody following *in vitro* activation. Our results thus demonstrate that Fyn-mediated signaling and B cell ADAM10 expression are necessary for optimal humoral responses.

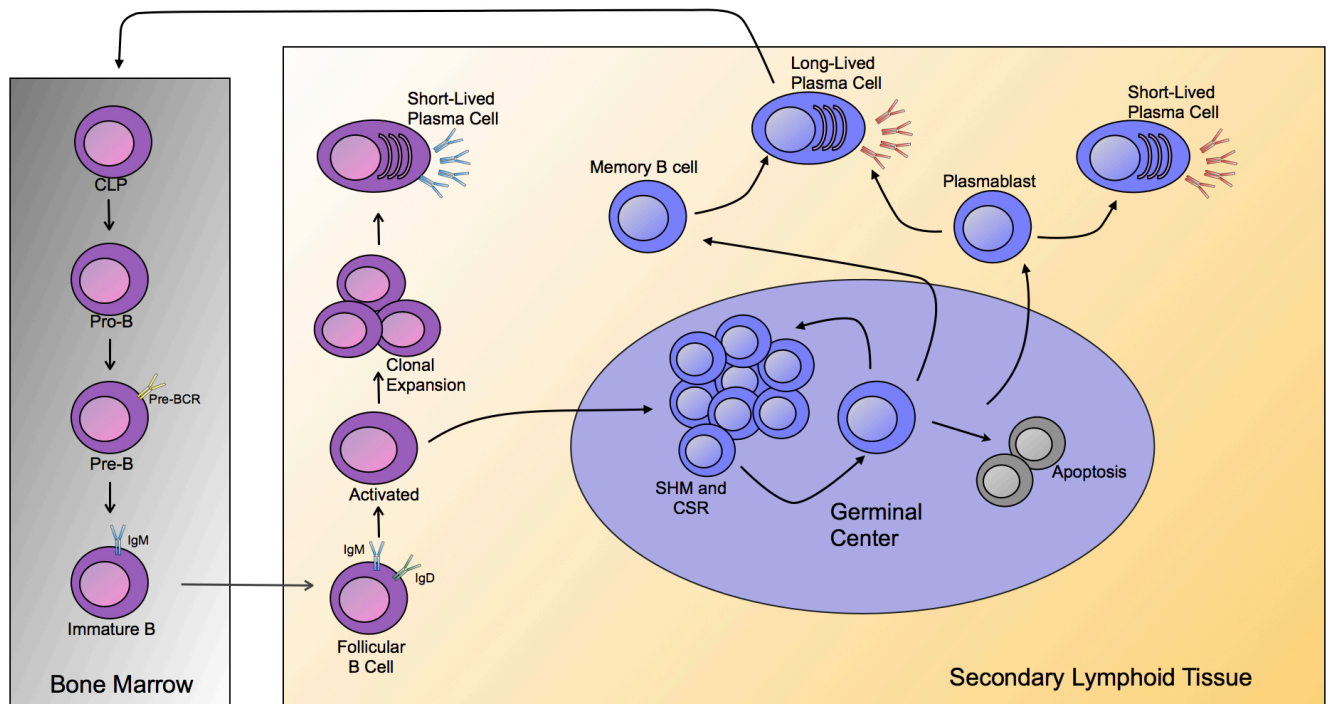
CHAPTER 1: INTRODUCTION TO B CELL DEVELOPMENT AND TERMINAL DIFFERENTIATION

B-lymphocytes (B cells) are the key mediators of humoral immunity. There are two main populations, referred to as B-1 and B-2 cells. B-1 cells are a minor subpopulation of B cells commonly found in peritoneal and pleural cavities and are mediators of innate immunity. B-2 B cells, on the other hand, are the predominant B cell population and can be found in spleen and lymph nodes (LN). Here we will discuss B-2 cells. B-1 B cell development and function is reviewed elsewhere¹.

B-2 cell development can be divided into several stages. The different stages of B cell development are depicted in Figure 1 and will be further described here.

Figure 1. B cell development and differentiation.

B cell development is initiated within the bone marrow (BM). Common lymphoid progenitor cells (CLPs) commit to a B-cell pathway. Pro- and pre- B cells undergo a series of steps in order to generate cells that express a functional surface IgM. Once surface IgM is expressed, immature B cells exit the BM and home to secondary lymphoid tissues, such as lymph nodes and spleen, where they are considered mature B cells. Upon antigen activation, B cells proliferate and differentiate. B cells can rapidly differentiate into PCs; these cells typically secrete IgM and are short-lived. Activated B cells can also enter germinal centers, where they undergo somatic hypermutation (SHM) and class switch recombination (CSR). Cells that have low affinity for antigen undergo apoptosis, while those B cells that express high affinity for antigen are selected for survival and can further differentiate to memory B cells and PCs. Long-lived PCs typically home to the bone marrow, where they can remain for years.



1.1 Early B cell development

B cell development is initiated within primary lymphoid organs - the fetal liver during gestation and the bone marrow (BM) throughout the remainder of life. B cell development is carried out such that B cells with a diverse repertoire of antigen specificities are generated. This diversity is achieved by rearrangement of the immunoglobulin heavy-chain (IgH) and immunoglobulin light-chain (IgL) segments of the B-cell receptor (BCR) genes. Moreover, recombination events often lead to insertions or deletions in coding regions of immunoglobulin genes. This junctional diversity also contributes to the generation of cells with very varied antigen specificities. The recombination activating genes (RAGs) encode enzymes essential for the rearrangement and recombination of immunoglobulin genes. These enzymes are known as RAG-1 and RAG-2². These genes are also involved in T cell receptor (TCR) rearrangement, as described elsewhere³. The importance of these enzymes is highlighted by the fact that RAG-1 or RAG-2 deficiency causes severe combined immunodeficiency (SCID) due to a loss/absence of B cells and T cells⁴.

Gene rearrangement starts with recombination of the variable (V), diversity (D), and joining (J) gene segments of the IgH gene within pro-B cells, commonly termed VDJ rearrangement. The pre-BCR is then assembled. It is composed of the rearranged immunoglobulin M (IgM) heavy chain, a surrogate light chain, composed of VpreB and $\lambda 5$, and accompanying signaling molecules. Productive IgM heavy-chain recombination leads to progression to the pre-B cell stage. The pre-BCR is a key checkpoint for the regulation of B cell development,

since pre-BCR expression and ligand-dependent signaling are prerequisites for further differentiation. Defects in the components of the pre-BCR or signaling molecules, such as Bruton's tyrosine kinase (BTK), result in an early B cell developmental block⁵.

CD19, a B cell specific member of the immunoglobulin superfamily, is a co-receptor molecule that modulates pre-BCR and BCR signaling. CD19 is expressed starting at the pro-B cell stage. It has been proposed that CD19 signaling is important for heavy-chain rearrangement by modulation of RAG-1 and/or RAG-2 expression⁶. Moreover, it has been suggested that CD19 signaling can augment signals through the pre-BCR and aid in the transition from pro to pre-B cell stage⁷. Consistent with this idea, CD19-deficient mice have impaired B-cell proliferation at the pre-B cell stage⁸. Moreover, CD19 deficiency leads to decreased numbers of peripheral B cells. CD19^{+/-} mice (heterozygous), however, had normal B cell development⁹.

Following heavy-chain rearrangement, rearrangement of the V and J segments of the light chain locus (either κ or λ) occurs. This rearrangement occurs at the pre-B cell stage. The light chain protein then associates with the previously rearranged and synthesized IgM heavy chain. Surface IgM expression marks the transition from pre-B cell to immature B cells. The immature, IgM-expressing B cell then leaves the BM and completes its maturation, becoming a mature or naïve B cell, marked by the co-expression of IgM and IgD. Mature B cells home to secondary lymphoid organs due to their expression of CX chemokine receptor 5 (CXCR5) (see section 1.2)⁵.

1.2 Secondary Tissue Structure

The development of secondary lymphoid tissues is initiated during embryogenesis by the interaction between hematopoietic lymphoid tissue inducer cells (LTi) and mesenchymal cells present at the site of future lymphoid organ development. LTi cells express lymphotoxin $\alpha\beta$ (LT) while mesenchymal cells express LT β receptor. LT signaling is essential for the development of secondary lymphoid organs because it triggers mesenchymal cell differentiation to stromal organizer cells and induces homeostatic chemokine expression, such as CXCL13, CCL19, and CCL21¹⁰. Interaction of stromal organizer cells with LTi leads to the recruitment of additional hematopoietic cells and subsequent accumulation of naïve lymphocytes¹¹.

Consistent with LT's key role in lymphoid development, LT-deficient mice lack peripheral LN and have severely disrupted splenic architecture^{12,13}. TNF- α deficient mice also have disrupted splenic architecture and abnormal lymph node follicle formation¹⁴. However, TNF- α involvement in lymphoid tissue development is poorly understood. It has been proposed that TNF- α is involved in the differentiation of stromal cells precursors into follicular dendritic cells (FDCs) and fibroblastic reticular cells (FRCs) (see section 3.3)¹⁵.

Secondary lymphoid organs are highly organized structure containing regions enriched for B cells or T cells. The segregation of B cell areas (follicles), and T cell areas (paracortex in lymph nodes and periarteriolar lymphoid sheath (PALS) in spleen) is dependent on specific chemokine secretion^{16,17}. The

chemokine important for B cell homing is CXCL13. This chemokine is thought to be secreted by follicular dendritic cells (FDCs) within the follicles; its receptor, CXCR5 is constitutively expressed by follicular B cells¹⁸. CCL19 and CCL21 are secreted in T cell areas by fibroblastic reticular cells (FRC)¹⁹. Both of these chemokines are ligands for CCR7, a receptor expressed by naïve T cells and mature dendritic cells (DC) and are thus important for T cell homing and DC migration into lymphoid tissues^{16,20,21}.

Along with B cell and T cell regions, spleens also have a marginal zone (MZ), a structure that separates the lymphoid areas (white pulp) from the areas where arterial to venous exchange occurs (red pulp). The MZ surrounds the splenic follicles and it is made up of MZ macrophages and MZ B cells. MZ B cells are characterized by high expression of CD21, IgM, and CD1, as well as low IgD and CD23 expression^{22,23}. Studies have demonstrated that MZ B cell development requires CD19, Notch2, and ADAM10 protein expression and signaling²⁴⁻²⁶.

Lymphocytes and DCs may enter LN from the blood by exiting the circulation via high endothelial venules (HEV) in the paracortex or through afferent lymphatics. Regardless of how they reach the tissue, B and T cells segregate according to their expressed chemokine receptors²⁷. As mentioned earlier, the correct positioning of cells is dictated by the local production of chemokines by stromal cells as well as the coordinated expression of chemokine receptors by migrating cells¹⁸. The positioning of B cells and T cells, as well as their migration during an immune response, are critical for adequate B cell-T cells

interaction and for optimal immune responses²⁸.

1.3 B cell activation

There are several ways B cells can encounter antigen. B cells can acquire soluble antigen that diffuses freely into lymphoid follicles²⁹ or that is transported through the lymphoid conduits system¹⁵. Moreover, B cells can be presented antigen by DC³⁰ or subcapsular sinus macrophages²⁷. B cells can respond to several kinds of antigens³¹. After challenges with most types of antigens, B cells rapidly differentiate into plasma cells (PCs) and plasmablasts and appear between red and white pulp in the spleen and the outer T-cell areas of the lymph nodes³². Extra-follicular responses are not thought to lead to the generation of long-lived PCs³². It should be noted that extra-follicular responses to T-dependent antigens also require cognate T-cell help³³.

T-cell independent antigens

Certain antigens have the capacity to induce the differentiation of B cells into antibody-secreting cells (ASCs) in the absence of cognate T-cell help. These antigens are commonly referred as T-independent antigens and can be subdivided into two groups. Type-1 antigens can induce B cell proliferation and differentiation to ASCs independently of BCR signaling. LPS is the prototypical Type-1 antigen. Type 2 antigens, on the other hand, require signaling through BCR and are typified by polysaccharides from bacterial cell walls. Common Type 2 antigens are haptens coupled to ficoll, dextran, or acrylamide. Type 2 antigens lead to BCR aggregation; moreover, they may activate non-B cells to produce

cytokines and modulate the immune response³¹.

All B cell subsets can be recruited to extra-follicular responses to T-independent antigens, although MZ B cells and B-1b cells are the only ones capable of responding to T-independent Type 2 antigens³⁴. Low affinity IgM is the most common isotype seen in T-independent responses, although other isotypes can also be detected³⁵. The process of class switching will be further described in Section 1.4.

T-cell dependent antigens

Most antigens are T-dependent, meaning that T cell help is required for optimal antibody responses. Helper T cells aid in the activation of cognate B cells via cell-to-cell interactions (such as CD40 and CD40L) as well as through the release of cytokines that shape the B cell responses, such as IL-4 and IL-21. A hallmark of T-dependent antigens is that they are protein antigens. Like T-independent antigens, they induce extra-follicular responses. Moreover, they also induce germinal center (GC) formation (see Section 1.4)^{36,37}.

1.4 Germinal Centers

GCs are structures observed in the follicular region of secondary lymphoid tissues. As mentioned earlier, GC formation is dependent on the interactions of cognate B cells and follicular helper T (T_{FH}) cells and FDCs³⁸. Within GCs, FDCs and T_{FH} cells promote antigen-specific clonal expansion, class switch recombination (CSR) and somatic hypermutation (SHM) with selection of high

affinity BCR variants³⁹. Clonal expansion refers to the rapid proliferation of antigen-specific B cells.

Early responses to T-dependent antigens

Initial activation of naïve B cells through the BCR leads to increased expression of CC-chemokine receptor 7 (CCR7) and enhanced responsiveness to CD40L, thus promoting more effective cognate T cell help⁴⁰. CCR7 expression allows B cells to migrate toward the T cell zone. Simultaneously, CD4⁺ T cells, activated by exposure to a peptide derived from the same antigen in the context of MHC Class II, upregulate CXCR5 and migrate toward the B cell follicles^{41,42}.

The original Th1/Th2 paradigm subdivides T cell immunity into two subsets specialized to regulate cell-mediated immunity (Th1) versus those that mediate B cell antibody production (Th2)⁴³. Th1 cells mediate immune responses to intracellular pathogens through their secretion of IL-12 and IFN- γ ^{44,45}. On the other hand, Th2 cells mediate responses to extracellular parasites through the secretion of IL-4, IL-5, and IL-13⁴³. Moreover, T cell help for antibody production had been considered a property of Th2 cells^{46,47}. Interestingly, mice deficient in signal transduction and activator of transcription 6 (STAT6), a Th2 transcription factor important for IL-4 signaling, were still capable of mounting T-cell dependent antibody responses, although IgE was not produced⁴⁸. These results demonstrate that antibody responses can occur despite impaired Th2 responses.

More recently, several non-Th1, non-Th2 T cell subsets have been identified. These subsets include T regulatory cells (T_{regs}), IL-17 secreting T cells

(Th17), IL-9 secreting T cells (Th9), and follicular helper T cells (T_{FH})⁴⁹⁻⁵⁴ T_{FH} were first described as a unique CXCR5⁺ T cell population. CXCR5 expression allows T_{FH} cells to home to B cell follicular areas^{53,54}. Moreover, T_{FH} cells are capable of providing B cell help for antibody production^{53,54}.

The discovery of T_{FH} cells raises several questions: (1) How do T_{FH} cells fit within the Th1/Th2 paradigm? (2) How do we explain all the previous data implicating Th2 cells as helper cells for antibody production? A key question is whether T_{FH} represent a unique lineage or whether they represent a CXCR5⁺ subset of T helper cells suggesting plasticity in T cell subsets and the cytokine mode. Moreover, even if they are a distinct lineage, there is a body of evidence of T cell plasticity. Therefore, one could hypothesize that Th2 cells could become T_{FH} cells, and vice versa⁵⁵⁻⁶².

Studies have demonstrated that T_{FH} development requires antigen-specific priming by DCs involving ICOS-ICOSL interaction⁶³⁻⁶⁵. Moreover, full commitment to a T_{FH} phenotype requires B cell and T cell interaction⁶³. The expression of the adaptor molecule SAP (SLAM-associated protein) can regulate B cell– T_{FH} cell contact duration and is thus essential for T_{FH} and GC formation^{66,67}. It has been proposed that IL-2 signaling, through STAT5, leads to effector T cell differentiation, while low IL-2 signaling allows for Bcl6 expression⁶⁸⁻⁷⁰. Bcl6 commits T cells to a T_{FH} phenotype while repressing Prdm1 gene expression and preventing T cell effector differentiations⁷¹. In this context, T_{FH} cells are referred to as helpers, while other CD4⁺ T cell subsets are considered effectors^{69,71,72}.

It has also been proposed that T_{FH} cells are not a distinct helper T cell subset and that T_{FH} development occurs subsequent to Th1, Th2, Th17, and T_{reg} commitment⁶⁰. In agreement with this model, it has been reported that T_{FH} cells differentiate from Th2 cells in response to helminth-derived antigens⁷³. The main issue with this model is that it would predict that T_{FH} differentiation would not antagonize other $CD4^+$ T cell lineages. However, Bcl6, the main T_{FH} transcription factor, is capable of inhibiting other T cell differentiation pathways by blocking Blimp-1 expression^{71,74-76}.

The models proposed above, however, might not be mutually exclusive. $CD4^+$ T cells might differentiate into T_{FH} cells directly, via DC priming and interaction with cognate B cells. At the same time, other Th subsets (ex. Th2) might be capable of differentiating into T_{FH} cells. Although Bcl6 can inhibit the differentiation of other Th subsets, it is not clear whether Bcl6 can inhibit the Th program of already differentiated T cells. Moreover, if both these models were correct, they would explain how Th2 cells were previously considered the key providers of B cell help, as Th2 cells and T_{FH} cells could represent that same cellular subset.

Regardless of how they differentiate, it is well accepted that within the B-T border B cells get help from cognate T_{FH} cells⁶⁰. The interaction between cognate and T and B cells in the periphery of the B-cell follicle leads to the initiation of proliferation. While some cells enter the follicle and form GCs, others continue to proliferate and migrate toward the outer T-cell areas of lymph nodes or bridge channels of the spleen white pulp and initiate extra-follicular responses^{41,77}.

Extra-follicular PC responses to T-dependent antigens seem to also require short B cell and T_{FH} interaction^{33,66}. Recent evidence has also showed that early memory B cells can develop in a GC-independent manner^{78,79}.

Class switch recombination and somatic hypermutation

As previously mentioned, one of the events that occurs within GCs is class switch recombination. Class switch recombination (CSR), also termed antibody class switching, is an irreversible genetic recombination event that changes antibody from one class to another, for example, from IgM to IgG. While CSR does not change antigen specificity, it changes the function of the antibody, as the constant region determines most antibody functions. Briefly, activation-induced cytidine deaminase (AID; also known as AICDA) deaminates cytosine residues within the antibody switch (S) regions following germline transcription⁸⁰. Deamination then triggers the recruitment of the base excision repair (BER) machinery, leading to uracil removal and single-strand breaks (SSBs)⁸⁰. UNG, an enzyme involved in the BER pathway, is particularly important for CSR. Indeed, UNG deficient mice and patients with UNG mutations have a 95% reduction in CSR^{81,82}. If the SSBs are near each other on opposite DNA strands, they destabilize the DNA, forming double stranded breaks (DSBs). However, if they do not destabilize the DNA, they are repaired or converted to DSBs by mismatch repair activity, another DNA repair pathway. DNA breaks then lead to recombinations/translocations via non-homologous end joining (NHEJ)⁸⁰.

Cytokines determine the isotype to which a B cell will switch through the

induction of germline transcripts. While germline transcripts are sterile RNAs, meaning that they do not encode proteins, they are thought to direct AID to a specific S region. Germline promoters have cytokine-responsive elements, therefore, they can be induced by cytokines⁸⁰. It is in this way that different cytokines drive commitment to different antibody classes. For example, IL-4 promotes IgG1 (IgG4 in human) and IgE class switching; interferon- γ (IFN γ) induces IgG2a (in mice only); and transforming growth factor- β (TGF β) leads to IgA in both mice and humans^{40-42,80}. While cytokines and innate stimuli alone can drive B cell class switching, especially *in vitro*, typical responses require cognate T cell help. Studies have demonstrated that antibody class switching can occur in both GC and extra-follicular pathways⁸⁰. Class switch recombination, however, is likely to occur more efficiently within GCs given the cytokine milieu.

AID is not only required for CSR, but it also drives somatic hypermutation⁸³. Much like during CSR, AID is required for cytosine deamination generating uracils and recruiting the somatic hypermutation machinery. These changes target the variable regions of antibody genes. Uracil DNA glycosylase (UNG) can then remove the uracil residues. DNA is subsequently processed by error-prone DNA replication leading to the introduction of point mutations in the actively transcribed immunoglobulin locus⁸⁴. These mutations are likely to change the binding of immunoglobulin to antigen, thus producing clones that with increased or decreased affinity for antigen. Those clones that bind antigen with high affinity will be selected. Therefore, it is said that somatic hypermutation drives affinity maturation. While somatic hypermutation (SHM) occurs mostly

within GCs, SHM has also been reported to occur outside of GCs as evidence by the fact that some mice deficient in GCs exhibit affinity maturation with certain types of immunizations⁸⁵⁻⁸⁸. DNA damage can trigger apoptosis. In order to allow for rapid proliferation and SHM, GC B cells express anti-apoptotic proteins, such as Bcl6 and myeloid cell leukemia sequence 1 (mcl-1)^{89,90}.

As previously mentioned, AID is essential for SHM and CSR to occur. Given that AID activity leads to mutations and changes in chromosomal sequence, it is important for these mutations to be targeted to a specific area instead of occurring throughout the genome. Studies have demonstrated that AID preferentially deaminates the cytosine in WRC (W=A or T, R=purine), the AID hotspot target. Variable and switch regions contain a large number of AID hotspots^{80,91}. However, it has been reported that AID is also able to deaminate cytosines not present within hotspots. Moreover, not all hotspots are targeted⁸⁰. These findings demonstrate that there is another mechanism underlying AID specificity that has yet to be described. Moreover, what prevents AID from being expressed in cells other than B cells is poorly understood.

Affinity maturation

Studies have demonstrated that GC B cells continuously sample FDC networks that are laden with immune complexes that form when several antibodies bind soluble antigens. This B cell – FDC interaction is important for B cell survival⁹²⁻⁹⁴. Following FDC scanning, a few GC B cells make stable contacts with GC T_{FH} cells⁹⁴. While high affinity clones are selected within GCs, B cells

expressing BCRs with low affinity for the antigen undergo apoptosis⁹⁵. How B cells with high affinity for antigen are selected is not fully understood. There are currently two competing models describing selection of B cell expressing high affinity BCRs: (1) BCR signal-based selection (classical model) and (2) BCR signal and T cell help-based selection³⁷.

In the classical model, B cells compete for antigen binding in the form of immune complexes displayed on the surface of FDCs. GC B cell fate is dependent on the degree of interactions with antigen. If BCR signaling is too weak or too brief, the cell will undergo apoptosis – ‘death by neglect’⁹⁶. Apoptotic bodies are cleared by tingible body macrophages. FDCs are thought to control the engulfment of apoptotic bodies⁹⁷. If BCR signal is of sufficient strength, the GC B cell will be selected. These selected cells could then present antigen to T_{FH} cells and this interaction could enhance GC B cell survival and/or stimulate the differentiation into ASC or memory B cell^{37,96}. In this model, GC B cell selection is dependent on BCR signaling strength and the interaction between GC B cells and FDCs. The small number of surviving cells would then interact with T_{FH} cells and further differentiate.

Another model has been recently proposed. This model suggests that B cell- T_{FH} interactions are important for selection of GC B cells expressing BCRs with high affinity for the antigen. As in the classical model, GC B cells compete for antigen binding on the surface FDCs. Those cells that receive an adequate BCR signal survive, while those with inadequate BCR signal undergo apoptosis. Contrary to the classical model, however, the T-cell based selection model

argues that surviving GC B cells compete for T-cell help. Only those cells with high affinity BCRs are capable of receiving enough T cell help to survive and differentiate³⁷. It has been proposed that the interaction between GC B cells and T_{FH} cells is the limiting factor in the selection of clones with high-affinity BCRs³⁷. It is, therefore, likely that both GC B cell – FDC and GC B cell – T_{FH} interactions are important for the selection of B cells expressing high affinity BCRs. Moreover, recent studies have suggested that T_{FH} cells and FDCs also interact, however, whether these interaction occurs and its role in humoral responses has yet to be determined⁹⁸.

Other factors involved in GC formation and maintenance

There are many molecules that have been associated with GC formation. Bcl6 expression, in both B cells and T_{FH} cells, is required for GC development⁹⁹⁻¹⁰¹. Bcl6 is a transcriptional repressor. Within GC B cells, Bcl6 is required to prevent apoptosis in response to DNA damage that occurs during CSR and SHM¹⁰². It has also been suggested that Bcl6 is required to halt plasma cell differentiation until a cell has acquired sufficient affinity³¹. Studies have demonstrated that IL-6 signaling can induce Bcl6 expression^{103,104}. In the context of a GC, IL-6 is produced by activated FDCs and promotes SHM and affinity maturation¹⁰⁵.

IL-21, the main cytokine produced by T_{FH} cells, plays a key role in GC formation and plasma cell differentiation^{78,106}. Initially, mice deficient in either IL-21 or IL-21R were reported to have profound defects in antigen-specific IgG

production despite normal GC formation¹⁰⁷. Detailed analysis demonstrated that IL-21 deficient mice show decreased GC persistence^{78,106}. Interestingly, normal memory B cell were produced in IL-21 deficient mice¹⁰⁶. STAT3, which acts downstream of IL-21, as well as IL-6, IL-2, and IL-10, is essential for the generation of IgG1 secreting cells¹⁰⁸. IL-21, through STAT3 is able to trigger Blimp-1 expression^{109,110}, and could be involved in plasma cell (PC) differentiation (see section 1.4).

Germinal Center Structure

Given the key role cell-cell interactions play in GC formation, affinity maturation, and plasma cell differentiation, the organization of the GC is essential for humoral responses. GCs have two zones, called the dark and light zone, based on their initial histological appearance¹¹¹. FDC, T_{FH} and few GC B cells occupy the light zone, while the dark zone is composed of closely packed GC B cells. The chemokine receptor CXCR4 is required for GC B cell positioning within the dark zone. Its ligand, CXCL12, is more abundant in this zone than in the light zone³⁷. CXCL13 is highly expressed within the light zone, where it accumulates in the processes of FDCs¹⁷. CXCL13-mediated CXCR5 signaling seems to be important for GC B cell positioning within the light zone. Previously, GC B cells have been thought to move preferentially from the dark to the light zone. However, recent studies have demonstrated GC B cells can also migrate from dark zone to light zone. Moreover, B cells are also capable of intra-zonal movement, for example, dark zone B cells can move within the dark zone⁹³.

It has been recently described that sphingosine-1-phosphate receptor 2 (S1P₂) is required for GC B cells to stay within GC niche¹¹². It has been proposed that a gradient of sphingosine-1-phosphate (S1P), with highest concentrations outside of GC and at the GC boundary, prevent GC B cell migration by mediating S1P₂-mediated inhibition of migration¹¹³.

Extra-follicular vs. GC pathway

Antigen-activated B cells that do not give rise to antibody-secreting cells in an extra-follicular fashion can initiate GCs^{36,37}. What commits a B cell to the extra-follicular or GC-pathway is unclear, but it has been proposed that BCR affinity for antigen, the amount of antigen-BCR interaction, and co-stimulatory signals received from T cells might all play a role¹¹⁴⁻¹¹⁸. It has been proposed that B cells with highest affinity differentiate into PCs in an extra-follicular manner, while lower affinity B cells enter GCs where they mature and undergo affinity maturation¹¹⁷. Whether FDCs play a role in the selection of the B cells that will enter GCs is unknown.

Extra-follicular PC differentiation occurs early in an immune response. As extra-follicular responses begin to decrease, long-lived PCs and memory B cells begin to appear⁹⁶. PCs of extra-follicular origin tend to be short-lived and are non-migratory, while long-lived PCs reside in the BM where they secrete antibody for several weeks or longer^{119,120}. Interestingly, most long-lived PCs show large numbers of somatic mutations, suggesting that they are GC-derived^{39,96,121}..

Post-GC cells

The outcome of GCs are antibody secreting PCs and non-immunoglobulin-secreting memory B cells. While PCs secrete large quantities of antigen-specific antibodies, memory B cells undergo rapid proliferation and differentiation to high affinity PCs upon secondary antigen encounter. It has been suggested that the increased affinity of the BCR on memory cells leads to high sensitivity to low-dose soluble antigens that do not induce primary responses. Moreover, recent studies have suggested that memory B cells can re-initiate GC reactions following antigen challenge¹²², although it is unclear whether this secondary GC is a re-expansion of a primary GC or a newly developed GC. This is consistent with the concept of original antigenic sin. It describes that secondary antigen exposures preferentially induce a memory response, while primary responses are downregulated/inhibited¹²³. The molecular changes involved in memory B cell differentiation are not well characterized. Moreover, what determines whether a GC B cell will become a PC or a memory B cell is not well understood.

1.4 Plasma cell differentiation

One of the hallmarks of PC differentiation is a switch from transcription factors that control mature B-cell gene expression programs, such as Pax5 and Bcl6, to those that dictate PC program, such as B lymphocyte induced maturation protein 1 (Blimp-1), XBP-1, and interferon regulatory factor 4 (irf4)³¹. While it is

well established that these sets of transcription factors repress each other, it remains unclear how B cells switch from a mature B cell program to a PC one.

Pax5 is a transcription factor that is expressed throughout the B cell lineage and in all mature B cell subsets⁵⁵. It is essential for both the establishment and maintenance of B-cell identity. It can activate genes involved in immunoglobulin gene rearrangement, BCR signaling, affinity maturation, and SHM. Moreover, Pax5 represses the transcription of genes that are important for PC development and function, such as Xbp-1¹²⁴.

Blimp-1 triggers terminal B cell differentiation by repressing genes required for GC reactions and inducing genes involved in Ig secretion⁷⁶. It is, therefore, important for Blimp-1 not to be expressed prematurely. Bcl6 has been shown to repress *prdm1*, the gene encoding for Blimp-1, in both a direct and indirect fashion^{68,70}. Therefore, timely downregulation of Bcl6 is critical for PC differentiation. Studies have demonstrated that BCR cross-linking induces Bcl6 phosphorylation and subsequent degradation¹²⁵. This de-repression of Blimp-1, as well as STAT3 activation via cytokines secreted by T_{FH} cells, such as IL-21, could lead to Blimp-1 expression¹⁰⁹.

Blimp-1 is a transcriptional repressor often referred to as the “master regulator” of PC differentiation. Studies have demonstrated that Blimp-1 is sufficient to induce plasmacytic differentiation *in vitro*⁷². When Blimp-1-deficient mice were immunized with either a T- dependent or T-independent antigen, the number of Ig secreting cells and serum Ig levels were dramatically decreased compared to control mice^{126,127}. Moreover, deletion of Blimp-1 in mature PCs

resulted in plasma cell apoptosis¹²⁷. Blimp-1 represses mature and GC B-cell genes, such as Pax5, Bcl6, and AID. Moreover, Blimp-1 leads to the expression of genes important for plasma cell development and function, such as Ig genes and XBP-1. Furthermore, Blimp-1 leads to the upregulation of CXCR4, a chemokine receptor important for homing and maintenance of PCs *in vivo*⁷⁵. Interestingly, studies have demonstrated that Blimp-1 is not necessary to initiate the process of plasma cell differentiation, as 'pre-plasmablasts' could be generated in the absence of Blimp-1^{76,128,129}. Plasmablast are PC precursors that express mature B cell markers, such as B220 and CD19, although to a lower degree than naïve B cells¹³⁰.

Irf4 is highly expressed in all PCs and it is essential for PC differentiation^{131,132}. While Irf4 is not required in B cells for GC formation, it promotes isotype switching within GCs¹³². Irf4 is upregulated following B cell activation. At low levels, it promotes AID expression and CSR and at higher levels, it promotes Blimp-1 expression. Consequently, Irf4-deficient B cells fail to induce Blimp-1¹³¹. It is likely that Irf4 plays a role in the initiation phase of PC differentiation.

Blimp-1 and IRF4 lead to cell cycle arrest and promote PC development¹³³. Moreover, they permit XBP-1 (X box binding protein-1) expression^{132,134}. XBP-1 is thought to be expressed following Blimp-1, since XBP-1 deficient B cells express normal Blimp-1 levels following activation^{124,135}. XBP-1 plays an important role in mammalian unfolded protein response (UPR), a series of events that allow for endoplasmic reticulum (ER) expansion and allow cells to handle

extensive protein production¹³⁶ as in the case of PCs. While the number of PCs was normal in XBP-1 deficient mice, serum antibody levels were severely impaired following antigen challenge. Phenotypically, however, these plasma cells showed reduced ER, consistent with the decrease in protein production^{124,135}. These results demonstrate that XBP-1 is not needed for plasma cell development but is essential for antibody secretion.

The survival and differentiation of plasmablasts and PCs are dependent on B-cell activation factor (BAFF) and a proliferation inducing ligand (APRIL), members of the tumor necrosis factor (TNF) family¹³⁷. Indeed, mice deficient in BAFF, APRIL, or B cell maturation antigen (BCMA), one of the receptors for these cytokines, demonstrated the key roles of these factors in the development and maintenance of PCs, as PC numbers were severely impaired in these mice compared to controls¹³⁸⁻¹⁴⁰. Studies have demonstrated that within the murine bone marrow, eosinophils are the main source of these cytokines. Moreover, they have demonstrated that eosinophils are essential for PC survival¹⁴¹.

Many of the mechanisms underlying B cell development, GC formation and PC differentiation are still unknown. Here, we aimed to identify the role of two distinct proteins, ADAM10 and Fyn, in B cell biology. ADAM10 is a membrane of the family of disintegrin and metalloproteinases, and as such, it mediates the cleavage of transmembrane proteins. Previous studies have demonstrated a role for ADAM10 in B cell development²⁴. We, therefore, hypothesized that ADAM10 was involved in humoral responses. Here we demonstrate that B cell expressed ADAM10 regulates lymphoid architecture, GC

formation and PC development (See chapter 3). Fyn is a member of the Src-family kinase. Previous studies have implicated Fyn in antibody responses, but the role of Fyn in GC formation had yet to be determined^{142,143}. Here we demonstrate that Fyn is required for optimal GC formation. Fyn-deficient mice show impaired antibody responses. Moreover, Fyn-deficient B cells produce less antibodies than their wild type counterparts when activated *in vitro*, suggesting a B-cell intrinsic defect (See chapter 4).

CHAPTER 2: MATERIAL AND METHODS

2.1 Mice

ADAM10^{B-/-} mice were previously described²⁴. To generate ADAM10^{B-/-} N2ICD-Tg^{B+}, ADAM10^{B-/-} mice were mated with N2ICD-Tg^{flox/flox} mice¹⁴⁴. CD19-cre⁻ mice were wild type littermates of ADAM10^{B-/-} and ADAM10^{B-/-} N2ICD-Tg^{B+} mice. CD23Tg mice have been previously described¹⁴⁵. Balb/CJ mice were used as littermate controls of CD23Tg mice. Fyn-KO mice were previously described, and B6.129 mice were used as WT controls¹⁴⁶. In order to generate ADAM10^{Δ/Δ} IgG1-cre^{+/-} YFP⁺ mice, ADAM10^{Δ/Δ} YFP⁺ mice were mated with IgG1-cre^{+/-}¹²⁸ mice that were purchased from Jackson mice. ADAM10^{+/+} IgG1-cre^{+/-} YFP⁺ mice were used as controls. To generate ADAM10^{Δ/Δ} mb1-cre^{+/-} YFP⁺ mice, ADAM10^{Δ/Δ} YFP⁺ mice were mated with mb1-cre⁺ mice, kindly provided by Michael Reth through the laboratory of David Allman¹⁴⁷. CX3CR1-deficient mice were purchased from Jackson Laboratory¹⁴⁸. All mouse protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

2.2 PCR and Quantitative PCR

PCR

For genotyping, DNA was isolated using Direct PCR Solution as directed by the manufacturer (Viagen). MangoMix (Bioline) was used for amplification of

mb1, IgG1, and CD19 cre-lines, ADAM10-floxed alleles, and YFP and N2ICD transgenes. Cycling conditions were as follows, 95°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and final extension at 72°C for 10 min. Sequences of all primers described above are listed in **Table 1**.

Quantitative PCR

Total RNA was extracted and purified from draining and non-draining mouse lymph nodes (LNs) using TRIzol reagent (Invitrogen). Samples were treated with DNase (Takara Bio), mixed with phenol/chloroform/isoamyl alcohol solution (25:24:1; USB), and precipitated with ethyl alcohol. The purity of RNA was quantified by a spectrophotometer (ND-100; NanoDrop). One microgram of RNA was reverse transcribed using the High Capacity cDNA RT kit (Applied Biosystems). Real-time quantitative PCR was performed with a real-time PCR machine (iQ5; Bio-Rad Laboratories). Primers and probes for running a TaqMan quantitative PCR assay were purchased from Applied Biosystems. TaqMan gene expression assays used included: CCL19: Mm00839967_g1, IL-21: Mm00517640_m1, Aidca: Mm01184115_m1; Xbp1: Mm00457357_m1, Prdm1: Mm00476128_m1, Bcl6: Mm00477633_m1; and Irf4: Mm00516431_m1. Primers for CCL21 were synthesized by IDT as previously described¹⁴⁹. PCR products, labeled with 6-FAM-conjugated probes, were amplified with 18S as an internal control. Reaction parameters were as follows: hold at 48 °C for 30 min and hold at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60s. Results were analyzed with iQ5 real-time PCR software (version 2.0). Sequences of all

primers and probes, except those included in purchased Taqman gene expression assays described above, are listed in **Table 1**.

Table 1. Primer and probe sequences.

S- sense, AS- anti-sense

PCR	5'→3'
ADAM10: Intron8	CAGTGTAATGTGAACTCACCC
ADAM10: Intron9	CGTATCTCAAACTACCCTCCC
N2ICD: N2AD1	CCTTATGATGTTCCCGATTATGC
N2ICD: N2AD2	AGTACACCCATCTGGGCCTCG
YFP: oIMR4982	AAGACCGCGAAGAGTTTGTC
YFP: oIMR8545	AAAGTCGCTCTGAGTTGTTAT
YFP: oIMR8546	GGAGCGGGAGAAATGGATATG
CD19-cre: oIMR1084	GCGGTCTGGCAGTAAAACTATC
CD19-cre: oIMR1085	GTGAAACAGCATTGCTGTCACTT
IgG1-cre: 9264	TGTTGGGACAAACGAGCAATC
IgG1-cre: 9265	GTCATGGCAATGCCAAGGTCGCTAG
IgG1-cre: 9266	GGTGGCTGGACCAATGTAAATA
Mb1-cre: hCre dir	CCCTGTGGATGCCACCTC
Mb1-cre: hCre rev	GTCCTGGCATCTGTCAGAG
Quantitative PCR	
18S S	AAAATTAGAGTGTTCAAAGCAGGC
18S probe	CGAGCCGCCTGGATACCGCAGC
18S AS	CCTCAGTTCCGAAAACCAACAA
CCL21 S	AGACTCAGGAGCCCAAAGCA
CCL21 AS	GTTGAAGCAGGGCAAGGGT
CCL21 probe	CCACCTCATGCTGGCCTCCGT

2.3 Immunization.

For all immunization protocols, mice were of 6-12 weeks of age, unless otherwise stated. When experiments were terminated, mice were sacrificed via isofluorane inhalation and cervical dislocation. All mouse protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

T-dependent immunizations.

For T-dependent immunization protocol, mice were immunized with a single 10 µg 4-Hydroxy-3-nitrophenylacetyl injection coupled to keyhole limpet hemocyanin at a ratio of 27:1 (NP₂₇KLH – from this point called NP-KLH)(Biosearch Technologies). For memory protocols, mice were then given a second immunization of 10 µg of NP-KLH 42-49 days after primary immunization. For i.p. injections, mice were given 200µL of immunization mixture, made as follows: 10µg of NP-KLH in 100µL of PBS and 100µL of alum (4mg). For footpad injections, mice were given 25µL of immunization mixture, made as follows: 10µg of NP-KLH in 10µL of PBS and 15µL of alum (0.6mg). Mice were bled weekly via tail vein nick and cardiac punctured when the experiment was terminated.

T-independent immunizations.

Mice were immunized with 100 µg of NP_{0.6}LPS (Biosearch Technologies) i.p. in 100µL of PBS. Mice were bled weekly via tail vein nick and cardiac punctured when the experiment was terminated.

Immune Complexes.

Mice were passively immunized subcutaneously (s.c.) in the nape of the neck with 6mg of rabbit polyclonal anti-OVA antibodies in 400µL of PBS (Meridian Life Sciences). Twelve hours later, mice were immunized with 100µg NP₂₄OVA s.c. in alum. Immunization mixture consisted of 100µg of NP₂₄OVA in PBS and 100µL of alum (4mg). Six days following immunization mice were sacrificed and cardiac punctured.

The different NP-conjugates used are summarized in Table 2.

Table 2. NP-conjugated antigens

Antigen	Use
NP ₂₇ KLH	T-dependent immunization
NP ₄ BSA	High affinity NP-specific ELISA/ELISPOT
NP ₁₄ BSA	Total NP-specific ELISA/ELISPOT
NP _{0.6} LPS	T-independent Immunization
NP ₂₄ OVA	Immune complexes studies

2.4 Cell isolation

Tissue Preparation

Single cell suspensions of peripheral lymph node (LN) cells and splenocytes were generated by disrupting tissue between glass slides. BM cells were isolated by flushing excised tibias and femurs with complete RPMI. Red blood cells were then lysed by using ACK lysis buffer. Cells were then plated or stained depending on the experimental design.

B cell and Plasma Cell isolation

Cellular isolations were carried out using magnetic bead (Miltenyi Biotec). Briefly, single cell suspension was generated as previously described (see tissue preparation). Cells were resuspended in MACS buffer (PBS pH 7.2, 0.5% BSA and 2mM EDTA) and incubated with the corresponding magnetic beads for 15 minutes at 4°C. Cells were then washed and passed through a magnetic column. When cells were isolated via negative selection, LD columns were used and flow-through was harvested. On the other hand, when cells were isolated via positive selection, LS columns were used. In this case, after cells were passed through the column, columns were washed and flushed with MACS buffer and the eluted cells were utilized for experiments. When a small number of cells were isolated via positive selection, MS columns were used. For B cell isolation, cells were isolated via positive selection (B220⁺ cells) for all cells or via negative selection (CD43⁻) for isolation of naïve or untouched B cells. PCs were isolated via

negative selection (B220⁻DX5⁻ cells) and subsequent positive selection (CD138⁺ cells).

Stromal Cell Isolation

In order to isolate LN stromal cells, lymph nodes were processed by enzymatic digestions. Protocol was carried out as previously described, with minor modifications¹⁵⁰ LN were isolated, cut in small pieces with micro-scissors and place in RPMI-1640 on ice. After all LN were dissected, media was removed and replaced with 2mL of freshly made enzyme mix. Enzyme mix consists of 0.8mg/mL Dispase (Roche), 0.8mg/mL collagenase IV (Invitrogen) and 0.1mg/mL DNase I (Invitrogen) in RPMI-1640. Cells were incubated at 37°C in a water bath for 20 minutes; gently inverting at 5 minute intervals to ensure the content were well mixed. The LN were then gently aspirated and expired to disrupt the capsule. Large fragments were allowed to settle and the enzyme mix was then removed and added to 10mL of ice-cold FACS buffer (PBS with 2% FBS). 2mL of fresh enzyme mix was added to the digestions tube and gently mixed. Tubes were then incubated for 10 min at 37°C. Cells were then mixed vigorously for 30 seconds. As previously, large fragments were allowed to settle and enzyme mixed was removed and transferred to the tube containing 10mL of ice-cold FACS buffer. 2mL of fresh enzyme mix was then added to digestions tube. Tube was incubated at 37°C and mixed vigorously every 5 minutes until all lymph node fragments were completely digested. All supernatants were then

combined and spun down 1200 RPM for 5 minutes. Cells were then analyzed via flow cytometry (see section 2.6).

2.5 Enzyme Linked Immunosorbant Assay (ELISA) and Enzyme Linked Immunosorbant Spot (ELISPOT)

Total antibody levels.

For total Ig ELISAs, ELISA plates were coated with 5 µg/mL of goat-anti Ig (Southern Biotech) in BBS (0.15 M sodium chloride, 0.01 M borate buffer, pH=8.5). Plates were then 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% Tween-20). Blocking solution (PBS with 0.02% Tween-20 and 2% FBS) was added to plates. Plates were then 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. Plates were then incubated at 37°C for an hour or at 4°C overnight. Plates were then washed three times with ELISA wash and goat-anti-Ig-alkaline phosphatase (AP) was added to plates. Goat-anti-Ig-AP was diluted in block (1:400 dilution) (Southern Biotech). After 1 hour incubation at 37°C, plates were washed and developed with phosphate tablets (sigma) dissolved in substrate buffer (0.1g MgCl₂.6H₂O, 0.2g NaN₃, 50mL diethanolamine, pH to 9.8 per 500mL). Absorbance at 405nm was measured.

NP-specific ELISA.

For NP-specific ELISAs, ELISA was carried out as previously described with minor modification. Plates were coated with NP₁₄BSA (Biosearch Technologies)(15 µg/mL in PBS) for samples and with 5 µg/mL of goat-anti Ig (Southern Biotech) in BBS for standard. The remaining steps were carried out as previously described (see total antibody levels). For measurement of high affinity antibodies, protocol was carried out as described except that plates were coated with NP₄BSA (Biosearch Technologies; 15 µg/mL). While only high affinity antibodies can efficiently bind NP₄BSA, both low and high affinity antibodies bind NP₁₄BSA. The described NP-specific ELISA protocol allows for the relative quantification of high vs. low affinity antibodies¹⁵¹.

TNF-α ELISA

TNF-α levels were measured by ELISA using a TNF-α ELISA assay kit (Peprotech) and following manufacturer's instructions.

ELISPOT.

For ELISPOT, 96-well MultiScreen filtration plates (Millipore) were coated with 15 µg/mL of NP₄BSA or NP₁₄BSA in PBS. Plates were then incubated at 4°C overnight. Plates were then washed with PBS three times and complete RPMI was added to the plates. Plates were then incubated with media for at least 3 hours at 37°C. Meanwhile, splenic, LN, and BM cells were made into a single cell

suspension, as previously described (see section 2.4). Cells were seeded into the plate in duplicate, with at least three serial dilutions, and cultured for 18 hours at 37 °C in 100µL of complete RPMI. Plates were developed as previously described¹⁵². Briefly, following incubation, plates were washed three times with 0.02% Tween-20 in PBS and incubated for 2 hours at 37°C with biotinylated anti-Ig (1:1000 dilution in PBS) (Southern Biotech). During the incubation, APC solution was prepared following manufacturer instructions (VECTASTAIN Elite ABC system, Vector Laboratories). APC solution was incubated for at least 30 minutes prior to addition to ELISPOT plates. Following incubation, plates were then washed as before. Plates were then incubated with APC solution for 1 hour at room temperature. During the incubation, AEC Solution was prepared. AEC solution consists of one 3-Amino-9-ethylcarbazol (AEC) tablet dissolved in 2.5mL of dimethylformamide (DMF). After tablet dissolved, 47.5mL of acetate buffer (0.1N acetic acid and 0.1M sodium acetate) and 25µL of H₂O₂ were added. Following incubation with APC solution, plates were washed and AEC solution was added. Following a 4-minute incubation at room temperature, the reaction was stopped by rinsing the plates with water. Plates were then allowed to dry overnight, and spots were counted under dissecting a microscope. It should be noted that the number of cells plated and time of incubation were optimized. These parameters are affected by immunization protocol and immunoglobulin isotype of interest. Generally, total antigen-specific IgG and IgM are readily detected when cells are incubated for 18 hours at concentration of 0.5×10^6 cells/well, 21 days post-immunization.

2.6 Flow cytometry, immunofluorescence and confocal microscopy.

Flow cytometry

Cell isolation was conducted as described previously (see section 2.4 Cell Isolation). Cells were labeled following RBC lysis and filtration through 40µm cell strainers. For surface staining, cells were washed in FACS buffer (2% FBS in PBS). $1-3 \times 10^6$ cells were re-suspended in 50-100µL. Cells were then incubated on ice with 1-5µg of Fc block (2.4G2) for 10 minutes to prevent non-specific staining. Subsequently, cells were incubated for 30 min on ice with different combinations of 0.25-0.5µg of anti-mouse antibodies. Cells were then washed with FACS buffer and analyzed. For intracellular staining, staining was carried out using PhosphoFlow kit (BD). Briefly, cells were stained extracellularly as described. Cells were then washed with FACS buffer and fixed by the addition of 2mL of pre-warmed Lyse/Fix buffer (1X) and incubation at 37° C for 10 minutes. Cells were then washed with FACS buffer and permeabilized with 1mL of Perm/Wash buffer for 15-30 minutes at room temperature. Following permeabilization, cells were washed and resuspended in 50-100µL of Perm/Wash buffer. Cells were incubated with 1-5µg of Fc block (2.4G2) in Perm/Wash buffer for 5 minutes at room temperature. Subsequently, cells were incubated for 45 minutes with anti-mouse antibodies. Cells were then washed with Perm/Wash buffer, resuspended in FACS buffer, and analyzed. Flow cytometry analysis was performed using a Canto or Arianal (BD Biosciences) using Diva at the Flow Cytometry Core at Virginia Commonwealth University. Expert technical

assistance by Julie Farnsworth is acknowledged. Flow cytometry data analysis was conducted with FlowJo v8.8.7 (Tree Star).

Immunofluorescence and confocal microscopy.

Spleen and LN were frozen on dry ice in OCT compound (Tissue-Tek; Sakura). Serial 10µm sections were cut from frozen blocks using a cryostat (Frigocut 2800E; Jung), fixed in absolute acetone, and air-dried. Sections were blocked with 10% BSA in PBS to prevent background staining and then washed and incubated for 60 min with different combinations of 2-5 µg/ml anti-mouse antibodies. Sections were washed, mounted with anti-fade mounting medium (Vectashield; Vector Laboratories), cover slipped, and examined with a confocal laser scanning microscope (TCS-SP2 AOBS; Leica) fitted with an oil Plan-Apochromat 40× objective. Two lasers were used: argon (488 nm) for FITC, and HeNe (543 nm) for PE and APC. Parameters were adjusted to scan at a 512 × 512 pixel density and an 8-bit pixel depth. Emissions were recorded in three separate channels. Digital images were captured, overlaid, and processed with the Confocal and LCS Lite programs (Leica).

Antibodies.

All antibodies are anti-mouse, unless otherwise noted.

Table 3. Flow cytometry and Immunohistochemistry antibodies

Specificity	Clone	Label	Source
ADAM10		PE, FITC	R&D
B220/CD45R	RA3-6B2	FITC, PE, APC, PE-Cy7, Alexa 674, Alexa 700, PerCP/Cy5.5	Biolegend
BCL6*	K112-91	PE	BD Bioscience
CCL21		Unlabeled	R&D
CD16/32	2.4G2	Unlabeled	Conrad Lab
CD19	6D5	PE, APC, PerCP/Cy5.5	Biolegend
CD21/CD35	7G6	PE	Biolegend
CD31	MEC13.3	APC	Biolegend
CD38	90	PE-Cy7	Biolegend
CD4	RM4-5	APC	Biolegend
CD4	H129.19	PE	Biolegend
CD43	1B11	PE-Cy7	Biolegend
CD45	30-F11	FITC	Biolegend
CXCR5	RF8B2	Biotin	BD Biosciences
Fas (CD95)	Jo2	PE	BD Bioscience
GL7 (B and T activation marker)	GL7	FITC, APC	eBioscience, BD Bioscience
gp38 (podoplanin)	8.1.1	PE	Biolegend
IgD	11-26c.2a	PE, PerCP/Cy5.5	BD Biosciences
IgG1	A85-1	APC, PE	BD Bioscience
IgM	RMM-1	PE, APC, APC-Cy7, PerCP/Cy5.5	Biolegend
Thy1.2	30-H12	FITC	Southern Biotech

*Anti-human, cross-reactivity with mouse Bcl6.

2.7 In vitro activation

In vitro activation of antibody production

For IgG1 and IgE production, B cells or splenocytes were cultured with CD40L-transfected Chinese hamster ovary (CHO) cells as a B cell stimulant (6×10^4 cells/well) or with 1 μ g/mL of stimulating anti-CD40 antibody (Invitrogen) and IL-4 (10,000U/mL) for seven days. Supernatants were then harvested and analyzed for IgG1 and IgE production by ELISA. Cells were also stimulated with 50 μ g/mL of LPS (sigma). Four days later, supernatants were harvested and analyzed for IgM and IgG3 production.

Proliferation

To determine proliferation, cells were isolated and stimulated as described (see above). After 96 hours in culture, cells were pulsed with 1 μ Ci/well of tridiated ($[^3\text{H}]$)-thymidine (Perkin Elmer) for 24 hrs. Plates were then harvested onto GFC plates using a filtermate cell harvester. Plates were dried for at least 2 hours, 25 μ L of scintillation fluid was added, and counts were determined using the Topcount Plate Counter (Perkin Elmer, Waltham, MA). Proliferation was reported as counts per minute (CPM).

2.8 Migration Assay

For B cell migration studies, spleens were harvested and made into a single cell suspension, as previously described in section 2.4. Cells were washed in migration media (RPMI containing 0.5% fatty acid free BSA and 10mM HEPES)

and then incubated in this medium for 30 minutes at 37°C prior to the assay. Migration assay was performed using 5µm transwell (Corning Costar Corp.). Briefly, 600µl of media containing 1µg/mL of CXCL13 (Peprotech), 300ng/mL of CXCL12 (Peprotech), or just media was added to the lower chamber. After insertion of the filter, 1×10^6 cells were added to the top chamber in 100µL. After 3 hours at 37°C, cells in the lower chamber were analyzed by flow cytometry. Cells were stained and resuspended in 60µL of FACS buffer. Events were collected at a high flow rate for 60 seconds. For the calculations of specific migration, a known number of cells were added to the bottom chamber. Chemotaxis is expressed as percent of input cells.

2.9 Statistical analysis.

When dealing with two groups, p-values were calculated using unpaired two-tailed Student's t-tests in Graphpad Prism, unless samples did not form a Gaussian distribution, indicated by a failed D'Agostino and Pearson omnibus normality test. 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 with a Tukey post-test. Error bars represent the standard error of the mean (SEM) between samples. $p < 0.05$ is considered significant.

CHAPTER 3: ADAM10 AND ITS ROLE IN EARLY B CELL DEVELOPMENT, GERMINAL CENTER FORMATION AND PLASMA CELL FUNCTION

3.1 Introduction to ADAM10

ADAM10 is a member of a family of disintegrin and metalloproteinases (ADAMs). ADAMs belong to the metzincin superfamily, along with matrix metalloproteinases (MMPs) and ADAM-thromospondins (ADAM-TS). While all functionally active metzincin proteases contain a zinc binding motif, ADAMs are unique in that they also contain a transmembrane domain and are active while membrane bound. ADAMs are therefore responsible for the processing of receptors and ligands expressed on the cell surface. ADAMs can mediate ectodomain shedding and regulated intramembrane proteolysis (RIP) of transmembrane proteins. Shedding of extracellular domains releases soluble fragments into the extracellular space. This cleavage event can lead to downregulation of events that depend on transmembrane receptor-ligand expression or can activate paracrine and/or autocrine signaling by the production of a soluble mediator, such as necrosis factor- α (TNF- α). While ectodomain shedding is thought to occur constitutively, RIP requires receptor-ligand interaction¹⁵³. The prototypical ADAM is composed of a prodomain, metalloprotease domain, a disintegrin domain that confers substrate specificity, a cysteine-rich region, a transmembrane domain, and a cytoplasmic tail^{87,154}. A prototypical ADAM is depicted in Figure 2.

Ever since ADAM10 and its *Drosophila* homolog, *Kuzbanian* (kuz), were

discovered, their key role in developmental pathways has been noted. *Kuz* was initially identified in 1996. Studies of *kuz*-deficient embryos revealed that *kuz* was required for the development of peripheral and central nervous system. Cloning analysis demonstrated that the *kuz* gene encodes for a metalloprotease-disintegrin, highly homologous to bovine metalloprotease, later named ADAM10^{80,155,156}. Subsequent studies demonstrated that nervous system defects were due to defective Notch receptor signaling in the absence of *kuz* mediated RIP¹⁵⁷. The importance of ADAM10 for mammalian development has also been demonstrated, likely due to the key role of ADAM10 in the initiation of Notch signaling. Murine studies revealed that ADAM10-deficient embryo die very early in development, embryonic day 9.5, with multiple defects of the developing center nervous system and cardiovascular system^{84,158}

ADAM10 has emerged as a key mediator of ectodomain shedding and RIP of a large number of substrates, such as Notch receptors, CD23, and TNF- α , (more ADAM10 substrates are discussed in Table 4)^{87,153}. While it is well accepted that ADAM10 mediates cleavage of membrane-bound substrates, recent evidence demonstrates that ADAM10 activity is not restricted to the plasma membrane. Indeed, several reports have demonstrated that ADAM10 and a few of its substrates are present in exosomes, which are small secreted vesicles^{159,160}. Moreover, it has been reported that ADAM10 is highly expressed in the endoplasmic reticulum (ER)¹⁶¹. Whether the ER represents a reservoir for ADAM10 or if ADAM10 is functional within this organelle has yet to be determined. Furthermore, it has been recently demonstrated that the intracellular

domain of ADAM10 can be processed by ADAM9, ADAM15 and γ -secretase, allowing for the ADAM10 intracellular domain (ICD) to translocate to the nucleus and modulate gene expression¹⁶². Recently, it has been demonstrated that ADAM10-ICD is involved in human prostate cancer progression and in human immunodeficiency virus Type-1 replication^{163,164}.

Despite ADAM10's key role in development, relatively little is known regarding its regulation. ADAM10 is expressed as a zymogen and is subsequently activated by cleavage of the pro-domain by a furin protease^{100,165}. Following activation, ADAM10 activity can be regulated in a variety of ways. Tissue inhibitors of metalloproteases (TIMP), including TIMP1 and TIMP3, have been demonstrated to inhibit ADAM10 activity *in vitro*^{166,167}. Several studies have demonstrated that ADAM10 proteolytic activity can be enhanced by calcium influx, retinoic acid receptor signaling, PKC signaling, cholesterol depletion, and N-glycosylation¹⁶⁸⁻¹⁷².

Some of the most well characterized ADAM10 substrates are Notch receptors. As mentioned earlier, Notch receptors regulate cellular differentiation. Following Notch receptor-ligand interaction, including Delta-like 1-4 and Jagged 1-2, Notch signaling is initiated by ADAM-mediated proteolysis of the extracellular domain of the Notch receptor. The extracellular domain is released and endocytosed by adjacent ligand-expressing cells. This cleavage event, termed S2 cleavage, produces a substrate that can then be cleaved by the γ -secretase complex (S3 cleavage). Subsequently, the Notch intracellular domain (NICD) is released from the cell membrane. NICD then translocates to the

nucleus where it complexes with the transcription factor RBP-Jk and induces the transcription of Notch target genes, such as CD21, Hes 1, Hes 5 and *deltex-1*¹⁷³. Notch1 and Notch2 are the main Notch receptors involved in lymphocyte biology. It has been demonstrated that ADAM10 can mediate their cleavage^{24,174}. Notch1 and Notch2 have been shown to mediate different functions. For example, while Notch1 is required for T cell development, Notch2 signaling is necessary for the development of marginal zone B cells^{25,174}. These cleavage events are depicted in Figure 2B. Recent studies have demonstrated that ADAM10 can also mediate *trans*-cleavage, however, these activity has only been demonstrated for one substrate, ephrin, and it is not thought to be ADAM10's main mechanism of action¹⁷⁵.

Studies have demonstrated that Notch1 signaling is essential for commitment to T cell lineage, and given its role in Notch1 signaling, ADAM10 also plays a key role in T cell development^{174,176}. Moreover, ADAM10 is essential for MZ B cell development due to its role in the initiation of Notch2 signaling^{24,25}. Notch signaling has been recently implicated in mature B cell activation and function. Thomas *et al.* demonstrated that Notch signaling synergizes with B-cell receptor (BCR) and CD40 signaling to enhance B cell activation¹⁷⁷. Further studies by Santos *et al.* showed that Notch signaling contributes to B cell differentiation into antibody secreting cells (ASC) *in vitro* and also, under some conditions, promotes class switching¹⁷⁸. In addition to B-cell activation and differentiation into ASCs, Notch signaling has also been implicated in GC formation. Yoon *et al.* recently demonstrated that Notch signaling protects GC B

cells from apoptosis. These reports demonstrate the importance of Notch signaling in regulating B cell survival, activation, and differentiation into ASCs¹⁷⁹. These studies failed to determine whether the results observed were due to Notch1 or Notch2 activation. Moreover, these results were obtained through *in vitro* studies that might not represent *in vivo* biology. Interestingly, B-cell specific RBP-Jk deficient mice showed no defect in T-dependent or T-independent antibody responses¹⁸⁰, thus suggesting that Notch signaling might affect B cell terminal differentiation in a RBP-Jk independent-manner, RBP-Jk deficiency can be compensated *in vivo* or Notch signaling might not be important *in vivo*.

Here we aimed to address the role of ADAM10 in early B cell development, in GC formation, and PC development and function. We conditionally deleted ADAM10 in B cells at three distinct developmental stages and assessed the impact in B cell development and function in each of the mouse lines. Our results demonstrate that early deletion of ADAM10, starting at the pro/pre-B cell stage (ADAM10 $\Delta\Delta$ mb1-cre^{+/-} mice), does not alter immature B cell development. However, consistent with previous work, MZ B cell development was impaired²⁴. ADAM10 deletion in mature B cells (ADAM10 $\Delta\Delta$ CD19-cre^{+/-} mice) resulted in a dramatic defect in GC formation and antibody responses, accompanied with changes in splenic and LN architecture¹⁸¹. On the other hand, deletion of ADAM10 within germinal centers (ADAM10 $\Delta\Delta$ IgG1-cre^{+/-} mice) did not impair GC B cells numbers nor lymphoid architecture, but still led to dramatic defects in humoral responses due to altered PC gene expression.

Table 4. List of Relevant ADAM10 substrates

Substrate	Function	Reference
CD23	Low affinity IgE receptor; Important for regulating IgE production	182
IL-6 Receptor	Ubiquitously expressed; Involved in pro-inflammatory responses	183
Notch Receptors	Important for cell differentiation	24,174
CXCL16	Chemokine produced by dendritic cells in T cell area of secondary lymphoid tissues	184
TNF- α	Pro-inflammatory cytokine, also involved in lymphoid tissue development	185
Fas Ligand (CD95L)	Cytokine involved in cell death; interacts with Fas	186
CD40 Ligand	Protein important for B cell activation. T cell expressed.	187
RANK Ligand	Important for dendritic cell maturation, LN development and osteogenesis	188
CX3CL1	Important for leukocyte migration	189

Figure 2. ADAM structure.

ADAMs are composed of multiple domains, as depicted in this figure.

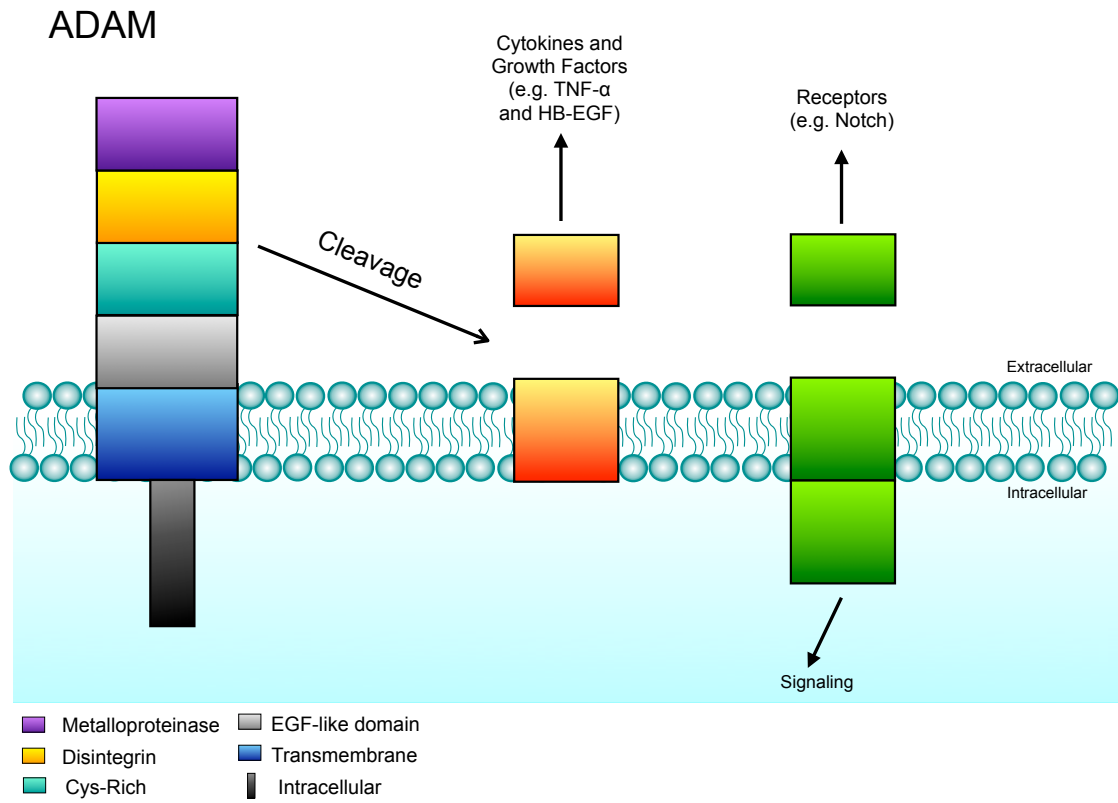
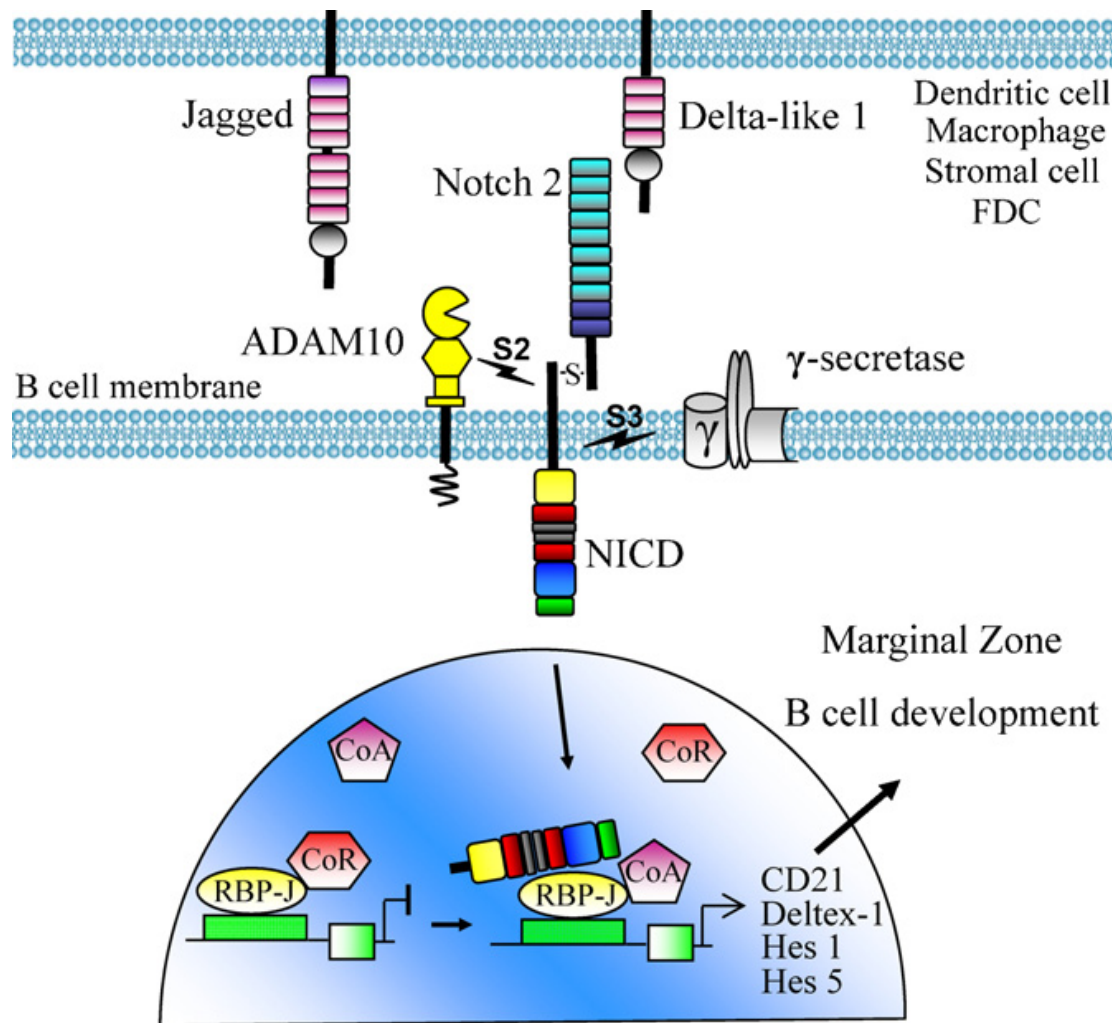


Figure 3. Notch signaling

ADAM10-mediated RIP of Notch2 is required for MZ B cell development²⁵. The Notch2 heterodimer on B cells binds ligands, Jagged 1–2 and Delta-like 1, present on stromal and antigen presenting cells. Binding initiates sequential cleavage events by ADAM10 (S2 cleavage) and γ -secretase complex (S3 cleavage). Cleavage releases the Notch2 intracellular domain (N2ICD). Transport of the N2ICD to the nucleus followed by binding to the transcription factor RBP-Jk allows the release of co-repressors (CoR) and attraction of co-activators (CoA) to the transcriptional complex. The activated complex transcribes Notch target genes, including CD21/35, Deltex-1, Hes 1, and Hes 5, that promote development of MZ B cells. Deletion of ADAM10, Notch2, and RBP-Jk from B cells, or Delta-like 1 from stromal cells prevents MZ B cell development.



3.2 ADAM10 and early B cell development

ADAM10 expression in early B cell subsets

ADAM10 plays a key role in Notch signaling. While it is well established that Notch1 signaling is essential for the commitment to T-cell lineage¹⁷⁶, whether or not ADAM10 plays a role in early B cell development post-lineage commitment has not been formally investigated. It has been previously reported that ADAM10 is expressed in B cell populations within the spleen, including MZBs and FOB cells²⁴. In order to further characterize ADAM10 expression in the B cell lineage, bone marrow cells were isolated and analyzed by flow cytometry for ADAM10 surface expression. This study revealed that ADAM10 is expressed in early B cell progenitors, including pro-B cells, pre-B cells, and immature B cells (Figure 4). This data suggested that ADAM10 might be involved in early B cell development.

Generation of early B cell specific ADAM10 deficient mice

Our lab has recently generated B cell-specific ADAM10 knockout mice (ADAM10^{Δ/Δ}CD19-cre⁺) by crossing mice with homozygous floxed ADAM10 (ADAM10-flox) with CD19-cre animals (CD19-cre⁺)¹⁹⁰. Flow cytometry and immunohistochemical analysis revealed that ADAM10^{Δ/Δ}CD19-cre⁺ lacked marginal zone B cells (MZBs), had dramatically reduced numbers of pre-MZBs, and a significant increase in follicular B cells (FOB)²⁴.

While CD19 expression can be detected starting at the pro-B cell stage⁸, CD19-cre mediates efficient recombination of loxP-flanked genes in mature cells and, to a lesser degree, in immature B and pre-B cells (see

introduction, section 1.1), leading to recombination in a limited number of bone marrow B cells¹⁴⁷.

Given that cre recombinase is poorly expressed in early B cells when under the control of the CD19-promoter, we investigated another B cell specific cre mouse line. The mb1-cre mouse line was obtained from the Reth laboratory¹⁴⁷. The *mb1* gene encodes the Igα signaling subunit of the B cell receptor¹⁹¹ and it is expressed very early in B cell lineage, beginning at the pro-B cell stage⁴⁹. Couple to Igβ, it facilitates antigen binding¹⁹². In order to compare the level of recombination that occurs in the BM when Cre recombinase is expressed under the control of CD19 promoter or mb1 promoter, we took advantage of YFP reporter mice. The YFP reporter mouse line expresses YFP after cre-mediated recombination. BM cells were isolated from mb1-cre⁺YFP⁺ and CD19-cre⁺YFP⁺ mice. The percentage of CD19⁺YFP⁺ B cells was then compared between the two lines. Consistent with published results, mb1-cre mice showed much more efficient recombination (Figure 5). While nearly all CD19⁺ B cells (98.9%) were YFP⁺ in BM of mb1-cre⁺ mice, only 10% of CD19⁺ B cells had undergone cre-mediated recombination in CD19-cre⁺ mice. These results support the concept that CD19-cre mouse models are not well suited for the generation early B cell-specific knockout mice.

ADAM10 is dispensable for early B cell development

Spleen and BM cells were isolated from 8- to 12- week old ADAM10^{Δ/Δ}mb1-cre⁺ animals and control animals (ADAM10^{Δ/Δ}mb1-cre⁻ mice). B

cell populations were analyzed by flow cytometry. In the BM, the percentage of immature B cells, defined as B220⁺IgM⁺ and pro/pre-B cells, defined as B220⁺IgM⁻, was comparable between ADAM10^{Δ/Δ}mb1-cre^{+/-} and WT controls (Figure 6). Consistent with this finding, recently published work demonstrated that B cells developed normally in the presence of ADAM10-inhibitors in an *in vitro* model of hematopoiesis¹⁹³. These results demonstrate that ADAM10 is dispensable for early B cell development. Consistent with previously published results, ADAM10^{Δ/Δ}mb1-cre^{+/-} mice lacked MZ B cells in the spleen (Figure 7) (MZ B cells are described in section 1.2)²⁴.

Figure 4. ADAM10 expression in bone marrow B cells.

Bone marrow derived cells isolated from C57BL/6 mice were stained for B220, IgM, CD43, and ADAM10. B cell subsets were defined as follows: Pro-B cells: B220⁺CD43⁺IgM⁻; Pre-B: B220⁺CD43⁻IgM⁺; and immature B cell: B220⁺IgM⁺. Blue represents sample while gray represent isotype control. MFI; mean fluorescent intensity. Experiment was carried out 3 times with similar results. Two mice of each genotype per experiment.

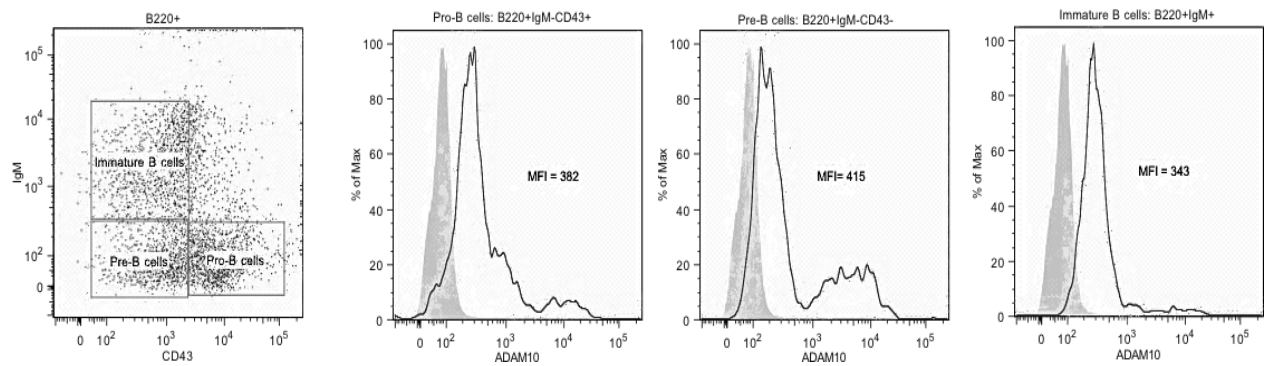


Figure 5. Comparison of recombination efficiency between mb1-cre and CD19-cre mouse models.

Bone marrow cells from CD19-cre⁺YFP⁺ or mb-1-cre⁺YFP⁺ mice were stained for CD19 and analyzed by flow cytometry. The percentage of CD19⁺YFP⁺ is displayed in the upper right quadrant of each FACS plot.

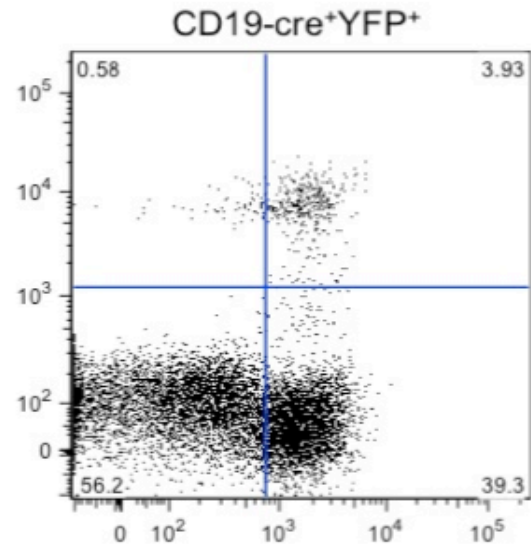
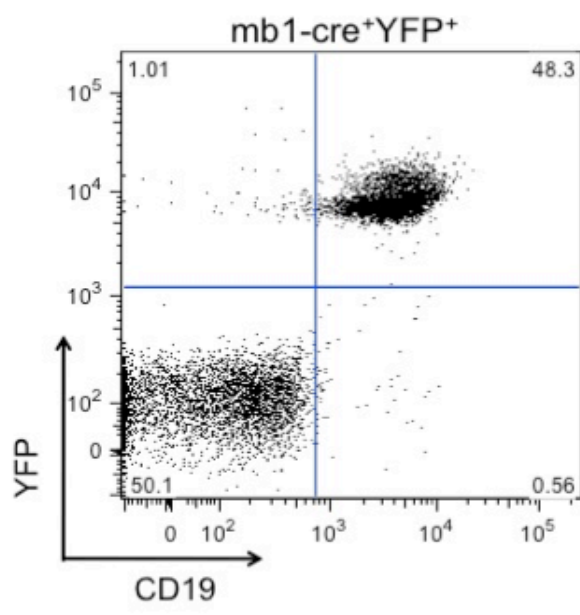


Figure 6. ADAM10 is dispensable for early B cell development.

Bone marrow cells were isolated from ADAM10 Δ/Δ mb1-cre $^{+/-}$ and WT mice and analyzed via flow cytometry for immature (B220 $^{+}$ IgM $^{+}$) and pro/pre-B cells (B220 $^{+}$ IgM $^{-}$). Representative contour plots are shown. Experiment was carried out 3 times with similar results. Two mice of each genotype per experiment.

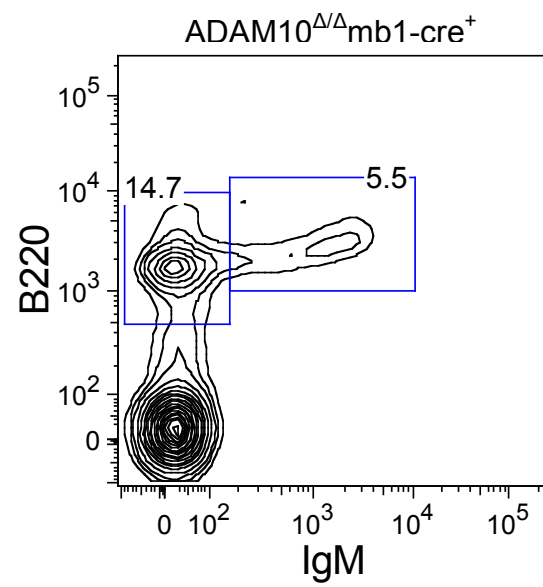
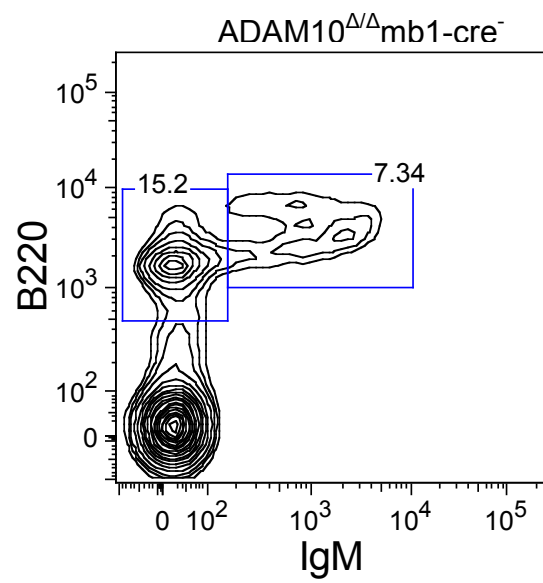
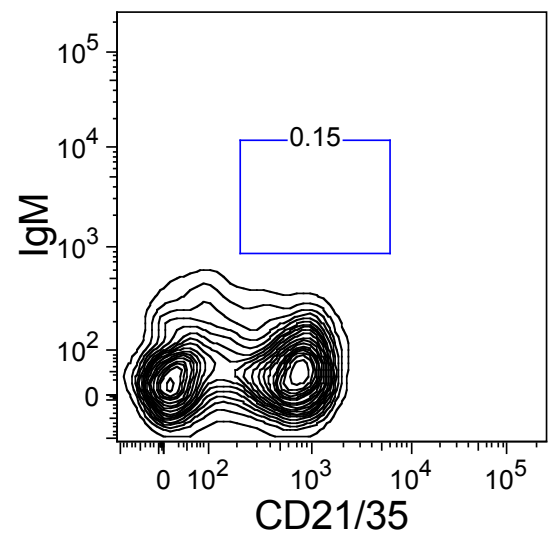
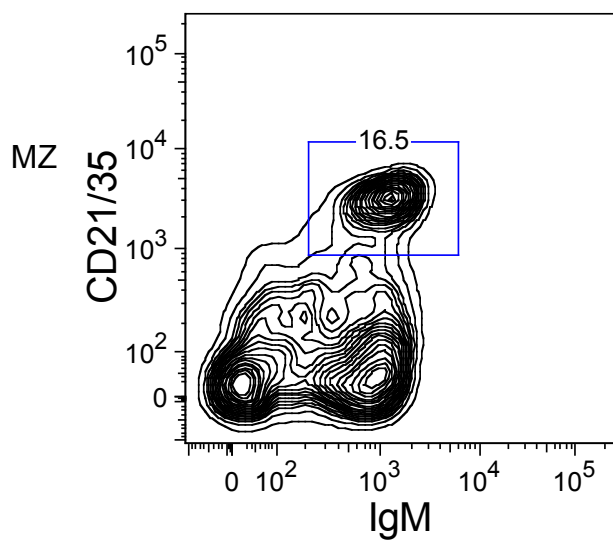
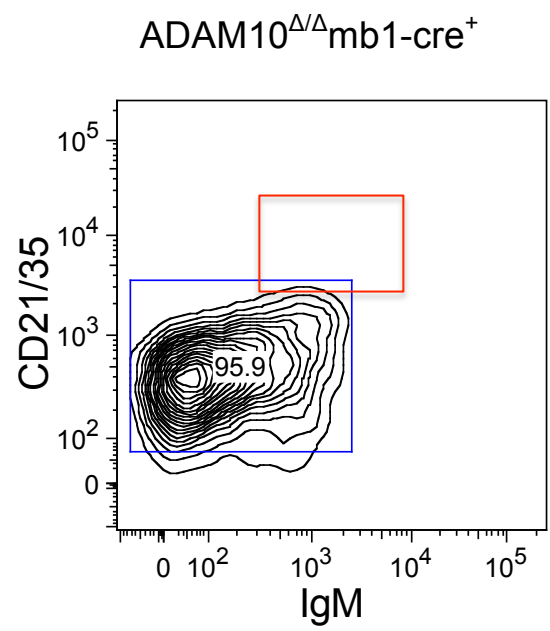
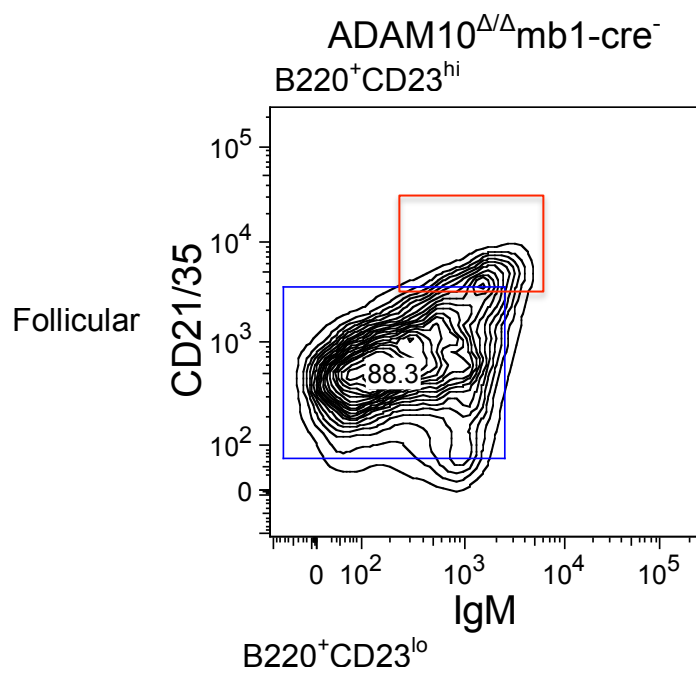


Figure 7. ADAM10 is required for marginal zone B cell development.

Spleen cells were isolated from ADAM10^{Δ/Δ}mb1-cre^{+/-} and WT mice and analyzed via flow cytometry for follicular (B220⁺CD23^{hi}CD21/35⁺IgM⁺) (blue) and pre-MZB (B220⁺CD23^{hi}CD21/35^{hi}IgM^{hi}) (red) [top] and marginal zone B cells (B220⁺CD23^{lo}CD21/35^{hi}IgM⁺) [bottom]. Representative contour plots are shown.



3.3 ADAM10 is essential for Germinal Center formation and maintenance of lymphoid architecture

ADAM10 is highly expressed in GC B cells

ADAM10 cleaves a variety of substrates that have been associated with humoral responses, such as TNF- α , Notch1, Notch2, and CD23^{174,182,185}. We therefore sought to determine the role of ADAM10 in GC formation and antibody production. Delineation of ADAM10 expression patterns in B cells is an important step toward understanding the potential role of the proteinase in humoral immunity. Flow cytometry analysis demonstrated that within Peyer's patches, while less than 2% of naïve B cells expressed ADAM10, over 80% of GC B cells were ADAM10⁺ (Figure 8A,B). The flow cytometry results were confirmed by immunohistochemistry of Peyer's patches stained for ADAM10, PNA, and IgD. Consistent with our flow cytometry results, ADAM10 was highly expressed by cells within GCs (Figure 8C). Peyer's patches were chosen due to the expected high level of GC activation.

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

Given the significant expression of ADAM10 in GC B cells, we investigated its role in humoral responses by using B cell specific ADAM10-deficient mice (ADAM10^{B-/-}). Basal levels of serum IgM, IgG1, IgG2a, and IgG2b were significantly reduced in ADAM10^{B-/-} mice when compared to WT, suggesting a defect in antibody production (Figure 9A). To directly assess antigen-specific

antibody production, ADAM10^{B/-} mice were immunized i.p. with NP-KLH, and serum levels of NP-specific antibodies were examined. ADAM10^{B/-} mice showed reduced levels of NP-specific IgM 7 days post-immunization. However, levels reached WT values by day 21 and through the remainder of the experiment (Figure 9B). In contrast, NP-specific IgG levels, both total and high affinity, were reduced for 4 weeks post-immunization (Figure 9B). This reduction in IgG was not specific to a particular isotype, as antigen-specific IgG1, IgG2a, and IgG2b were dramatically reduced in ADAM10^{B/-} mice (Figure 10A). Furthermore, even when immunized with a high antigen dose of 1mg of NP-KLH, ADAM10^{B/-} mice failed to mount a normal antibody response. This result demonstrates that the defect in antibody production cannot be overcome by simply providing more antigen (Figure 10B).

To test if memory responses were also impaired in ADAM10^{B/-} mice, WT controls and ADAM10^{B/-} mice were immunized and boosted 42 days later. Consistent with data shown in Figure 8B, ADAM10^{B/-} mice had decreased levels of antigen-specific antibodies after primary immunization. Moreover, while WT controls exhibited a 12-fold increase in anti-NP IgG antibodies 5 days after secondary immunization (from 38.34 +/- 25 to 461.5 +/- 72.45), ADAM10^{B/-} mice only had a 4-fold increase (from 3.133 +/- 2.888 to 12.36 +/- 6.4460). Furthermore, 5 days after boost, WT mice produced 16 times more high affinity antigen-specific IgG and 37 times more total NP-specific IgG than ADAM10^{B/-} mice (Figure 9C). These results demonstrate that in ADAM10^{B/-} mice both primary and recall antibody responses are impaired.

Fewer NP-specific Antibody Secreting Cells are observed in ADAM10^{B-/-} mice

Given the severe defect in antibody production observed in ADAM10^{B-/-} mice we hypothesized that the generation of antigen-specific PCs was impaired. Examination of antibody secreting cells (ASCs) by ELISPOT assays showed that immunized ADAM10^{B-/-} mice had drastically reduced numbers of NP-specific ASCs in the spleens and BM 21 days post-immunization (Figure 11A,B). Similar results were observed in spleens 14 and 28 days after immunization (Figure 11D,E). Together, these results demonstrate that the decrease in splenic ASCs is not due to altered migration or preferential localization to the BM, but likely due to impairments in PC differentiation and/or survival.

A critical component of the humoral response is affinity maturation. Thus, we next examined affinity maturation in immunized ADAM10^{B-/-} mice. While 21 days after immunization most ASCs were secreting high affinity antibodies in WT controls, the few ASCs that were present in ADAM10^{B-/-} mice were secreting low affinity Ab. WT controls had 12-fold higher number of NP-specific ASCs and 20-fold higher number of NP-specific ASCs producing high affinity antibodies when compared to ADAM10^{B-/-} mice. Moreover, when the ratio of high affinity and total ASCs was calculated, it was evident that the generation of high affinity ASCs in ADAM10^{B-/-} mice was especially affected (Figure 11C). These results suggest a defect in affinity maturation in ADAM10^{B-/-} mice.

GC formation is impaired in ADAM10^{B-/-} mice

As demonstrated in Figure 10, that antigen-specific ASCs were reduced in immunized ADAM10^{B-/-} mice. Being that long-lived PCs and memory cells are generated within GCs³¹, GC formation in ADAM10^{B-/-} mice was examined. GC B cells, defined as IgM^{lo}IgD^{lo}B220⁺IgG1⁺CD38^{lo} were enumerated by flow cytometry¹⁹⁴ (Figure 12A). Remarkably, the number and percentage of GC B cells within the spleen was dramatically decreased in ADAM10^{B-/-} mice (Figure 12B,C). Similar results were obtained when GC B cells were defined as B220⁺GL7⁺Fas^{hi} (Figure 12D). Activation induced cytosine deaminase (AID) is essential for SHM and CSR, two events that occur within GCs^{80,195}. Therefore, consistent with a decrease in the number of GC B cells, AID expression was also decreased in draining LN of ADAM10^{B-/-} mice compared to that of WT (Figure 13).

ADAM10^{B-/-} mice have altered splenic and lymph node architecture

In order to better understand the relationship between decreased GC B cells and the defects in antibody production observed, GC formation in immunized mice was assessed by immunohistochemistry. As depicted in Figure 14A, while several GCs could be detected in WT controls, as determined by GL-7⁺ positive cells, draining LN of ADAM10^{B-/-} mice contained a paucity of GL7⁺ clusters. Interestingly, GL7⁺ cells were scattered throughout the LN of ADAM10^{B-/-} mice (Figure 14A). Given the unusual pattern of GL-7 expression, we analyzed B- and T cell localization within the LN. Surprisingly, the distribution of B cells

and T cells was aberrant, suggesting that B cell specific ADAM10 deletion leads to changes in LN structure (Figure 14B). In contrast, LN structure in non-draining nodes or unimmunized mice was normal with regards to B- and T cell segregation. However, B cell follicles appeared thin, reminiscent of TNF- α and LT β - deficient mice (Figure 14D)^{14,196}.

Given that FDCs play a crucial role in GC formation and lymphoid tissue structure^{18,197,198}, we stained draining LN sections for FDC with CD21/35 (CR2). FDCs are known to upregulate CD21/35 subsequent to activation¹⁹⁹. Consistent with an absence in GCs and disorganized lymphoid architecture, FDC networks were not detected in LN and spleen from ADAM10^{B-/-} mice (Figure 14C, Figure 15D). Unlike the draining LN, GL7 staining was not detected in spleen of ADAM10^{B-/-} mice (Figure 15A). However, although normal in unimmunized mice, splenic architecture was also clearly altered post-immunization (Figure 15B,C).

ADAM10^{B-/-} mice have dysregulated chemokine expression following antigen challenge

The organization of secondary lymphoid tissues is highly regulated by chemokine gradients. To determine if the changes in structure observed were accompanied by altered chemokine expression, chemokine expression in draining and non-draining LN was assessed by qPCR. As expected, non-draining LN from WT and ADAM10^{B-/-} mice had similar CXCL13, CCL19 and CCL21 expression (Figure 16A). In sharp contrast, draining LN isolated from ADAM10^{B-/-} mice exhibited increased CCL21 expression, while CXCL13 levels were

comparable. CCL19 levels trended higher, but the increase was not statistically significant (Figure 16B). Analysis of CCL21 expression by immunohistochemistry revealed an expanded area of CCL21 staining in the ADAM10^{B/-} LN (Figure 16C), consistent with the localization of T cells within draining lymph nodes (Figure 14B). These results demonstrate that B cell expressed ADAM10 is important for maintenance of lymphoid architecture and regulation of chemokine expression during active immune responses.

Despite having normal levels CXCL13 expression, ADAM10^{B/-} showed an abnormal pattern of B-cell localization secondary to antigen challenge. We therefore decided to investigate whether ADAM10-deficient B cells showed altered migration to the B cell chemoattractants, CXCL13 and CXCL12. Interestingly, ADAM10-deficient B cells showed enhanced migration to CXCL13, but not CXCL12 (Figure 17), despite having normal receptor expression (Figure 18).

B cell specific ADAM10 deletion leads to structural changes in naïve LN

Given the abnormal appearance of B cell follicles in LN isolated from ADAM10^{B/-} mice, we decided to look at other features of lymph node architecture, such as collagen deposition and stromal cell populations. While collagen is heavily deposited within the T-cell zone of lymph nodes, B cell follicles seem to have very few collagen fibers²⁰⁰. As depicted in Figure 19, LN isolated from ADAM10^{B/-} showed excessive collagen deposition when compared to WT LN. Where in WT LN, B cell follicles showed sparse collagen deposition,

collagen was detected throughout the LN of ADAM10^{B-/-} mice. ADAM10 has been demonstrated to have collagenase capacity²⁰¹, so it is possible that B cell expressed ADAM10 is required for collagen breakdown within B cell follicles; therefore, in the absence of ADAM10, collagen deposition is increased. These results are consistent with abnormal LN development.

There are several subsets of stromal cells present in LN. They are CD45⁻, as they are not of hematopoietic origin, and can be further divided into four categories based on their expression of CD31 and gp38, also known as podoplanin or pdp. Fibroblastic reticular cells (FRCs) are defined as gp38⁺CD31⁻; lymph endothelial cells (LECs) are defined as gp38⁺CD31⁺ and blood endothelial cells (BECs) are defined as gp38⁻CD31⁺. gp38⁻CD31⁻ cells are a heterogeneous population of stromal cells—FDCs are thought to be represented in this population¹⁹. The FRC subset is mostly composed of T cell zone reticular cells, also referred to TRCs. These are the cells responsible for producing CCL21 and CCL19, and thus, for the regulation of T cell homing¹⁹. Given the increase in CCL21 and CCL19 in draining LN from ADAM10^{B-/-} and the thinning of the B cell follicles, we hypothesized that LNs from ADAM10^{B-/-} would have increased FRC numbers. Indeed, there was a relative increase in FRCs in LN from aged ADAM10^{B-/-} (16-20 weeks) (Figure 20). Interestingly, when the experiment was repeated with young mice 4 weeks of age, there was a reduction in the proportion of FRCs and an increase in BECs (Figure 21). These results demonstrate that ADAM10 deletion from B cells leads to loss of FDCs as well as alterations in the FRC compartment.

ADAM10-deficient B cells secrete less TNF- α following in vitro stimulation

As previously mentioned, the LN appearance of ADAM10^{B-/-} mice was similar to that of TNF- α deficient mice. Being that ADAM10 has been shown to cleave TNF- α and that B-cell TNF- α is important for lymphoid tissue development^{185,202}, we sought to determine whether ADAM10-deficient B cells produce less TNF- α following activation. Indeed, studies showed that subsequent to LPS stimulation a significantly decreased amount of TNF- α could be detected in supernatant of ADAM10-deficient B cells, despite normal TNF- α mRNA expression (Figure 22). Further studies are needed to determine whether decrease TNF- α release is the mechanism underlying the changes in architecture and defects in humoral responses observed in ADAM10^{B-/-} mice.

Defect in humoral responses in ADAM10^{B-/-} mice is Notch2 and CD23-independent.

Recent studies have suggested that Notch signaling was involved in antibody production and ASC differentiation^{177,178}. ADAM10 is critical for the initiation of Notch signaling. To determine if the antibody production defects were due to impaired Notch signaling, we generated mice that lack ADAM10 and have constitutively active Notch2 signaling in a B cell specific manner (ADAM10^{B-/-} N2ICD-Tg^{B+}). As expected, expression of N2ICD recovered MZ B cell development in these mice, demonstrating that the transgene was indeed active (Figure 23A). WT, ADAM10^{B-/-} and ADAM10^{B-/-} N2ICD-Tg^{B+} mice were

immunized. Consistent with previous experiments, ADAM10^{B-/-} mice had significantly reduced levels of NP-specific antibodies. Importantly, introduction of N21CD transgene failed to rescue the defect in antibody production and GC formation. Moreover, it did not prevent changes in LN architecture (Figure 23B,C). These results demonstrate that increased Notch signaling cannot rescue the defects in humoral responses.

We have recently reported that ADAM10 is responsible for the cleavage of the low affinity IgE receptor, CD23¹⁸², and consequently, ADAM10-deficient B cells have increased CD23 expression^{24,123}. Studies have revealed that CD23 overexpression leads to decreased IgG1 and IgE production^{55,145}. However, when CD23Tg mice were immunized with NP-KLH, analysis of NP-specific IgG levels indicated that CD23Tg mice had a normal antibody response to NP-KLH (Figure 24). Furthermore, it has been previously reported that CD23Tg mice form GCs following immunization with T-dependent antigens. It should be noted that the GCs observed in CD23Tg mice had a more globular appearance than WT²⁰³. These results suggest that the humoral defect observed in ADAM10^{B-/-} mice is not secondary to CD23 overexpression, however, this should be more carefully addressed. Another relevant ADAM10 substrate is CX3CL1 (see below).

CX3CR1 deficient mice have enhanced humoral responses

Another ADAM10 substrate is CX3CL1, also known as fractalkine in humans and neurotactin in mice^{204,205}. CX3CL1 mediates its function through binding to its receptor CX3CR1^{68,70,148}. CX3CL1 is a chemokine that exists in two

forms; the membrane bound form mediates leukocyte adhesion, while the soluble form acts as a conventional chemokine, attracting monocytes, NK cells, T cells and B cells^{125,206,207}. Recent studies have suggested a role of CX3CR1/CX3CL1 signaling axis in GC formation and antibody responses. Studies have demonstrated that GC B cells migrate to CX3CL1^{34,208}. Moreover, it has been suggested that T_{FH} and FDCs secrete CX3CL1 and that through this mechanism, they promote GC B cell survival^{35,207}. Interestingly, CX3CR1-deficient mice showed decreased basal levels of IgM but increased IgG1 levels (Figure 25). These results suggest enhanced class switch recombination.

Given that CX3CR1-deficient mice had altered basal antibody levels, we decided to assess antigen specific antibody responses. To this end, WT and CX3CR1-deficient mice were immunized with NP-KLH and GC formation and antibody production were determined. Despite having a slight, but statistically significant decrease in the percentage of GC B cells, CX3CR1-deficient mice had an enhanced antibody response to NP-KLH immunization (Figure 26). Further studies will be required in order to elucidate the mechanism underlying this phenotype.

Antibody production in ADAM10^{B-/-} mice can be partially rescued by repeated immunization

Very often, murine immunization protocols involve an initial immunization and a challenge two weeks later. When this immunization schedule was carried out with ADAM10^{B-/-} mice, we were surprised to see that total NP-specific IgG

levels were similar between WT and ADAM10^{B/-} mice. On the other hand, high affinity NP-specific IgG levels were still significantly lower following the boost (Figure 27A). Similar results were obtained when NP-specific PCs were quantified by ELISPOT (Figure 27B). Immunohistochemistry analysis should be carried out in this system in order to determine whether repeated immunization rescues GC formation and changes in architecture.

A key difference between a primary immunization and secondary immunization is the presence of pre-existing antibodies and the formation of immune complexes. Given that antibody production was partially rescued by repeated immunization, we hypothesized that the antibody defects observed in ADAM10^{B/-} mice could be rescued by immunization with immune complexes. WT and ADAM10^{B/-} mice were immunized with NP-OVA or NP-OVA and anti-OVA antibodies (IC). Mice were bled five days later and the presence of NP-specific IgM and IgG1 was assessed by ELISA. Surprisingly, WT mice, whether they received antigen alone or immune complexes produced significantly more NP-specific IgM than ADAM10^{B/-} mice (Figure 28A). Regarding NP-specific IgG1, levels were only detectable in WT mice that received immune complexes. In ADAM10^{B/-} mice, NP-specific IgG1 was not detected in either group (Figure 28B). These results demonstrate that immunization with immune complexes does not rescue antibody production defect. However, it is possible that if mice were analyzed at a later time point, antibody production might be restored to WT levels, thus suggesting a kinetics defect.

Defective antibody production seen in ADAM10^{B-/-} mice might be explained by decreased B cell help

We have demonstrated that ADAM10^{B-/-} mice have impaired antibody responses to T-dependent antigens as well as changes in splenic and LN architecture. In order to assess whether the decreased antibody levels result from a B cell intrinsic defect, splenocytes were stimulated with CD40L and IL-4 *in vitro*. Interestingly, WT and ADAM10-null B cells made comparable amounts of IgG1 (Figure 29A) demonstrating that when adequately stimulated, ADAM10-null B cells are able to class switch normally.

T_{FH} cells provide B cell help via CD40L, IL-21 and IL-4 and play important roles in GC B cell survival, affinity maturation and terminal differentiation to PCs^{51,52,209,210}. T_{FH} cells are characterized by high expression of CXCR5 and PD-1, and IL-21 production²¹⁰. Given our *in vitro* findings and the changes in architecture observed in ADAM10^{B-/-} mice, we hypothesized that the defects in humoral responses observed resulted from inadequate B cell stimulation and decreased T cell help. Results revealed that T_{FH} development and/or maintenance was impaired in ADAM10^{B-/-} mice, as fewer T_{FH} cells were present in draining lymph nodes of ADAM10^{B-/-} mice (Figure 29B). Consistent with decreased number of T_{FH} cells, IL-21 message levels were also reduced *in vivo* (Figure 29C). These results demonstrate that while ADAM10 deficient B cells can produce normal levels of antibody *in vitro* when stimulated with anti-CD40 and IL-

4, they are unable to class switch efficiently *in vivo* likely due to decreased T cell help.

Surprisingly, when ADAM10-deficient B cells were stimulated *in vitro* with LPS, they failed to produce normal antibody levels despite normal proliferations (Figure 30). These results demonstrate that ADAM10-deficient B cells respond differently to different types of stimuli. Moreover, *in vitro* stimulation is very different from *in vivo* B cell stimulation and it has been reported to yield different results regarding PC differentiation^{72,135}. Therefore, the role of ADAM10 in PC differentiation and function warranted a more careful evaluation. ADAM10 regulation of PC function will be discussed in section 3.4.

Figure 8. ADAM10 expression on GC B cells.

(A-B) Expression of ADAM10 on gated germinal center $\text{PNA}^{\text{hi}} \text{IgD}^{\text{lo}}$ (blue) and naive $\text{PNA}^{\text{lo}} \text{IgD}^{\text{hi}}$ (red) B (CD19^+) cells isolated from Peyer's patches. Flow cytometry gates used to define the germinal center and naïve B cell populations are indicated in left. To demonstrate specificity of staining, an isotype-matched monoclonal antibody was used (isotype). (A) Gating protocol is shown. (B) Percentage of ADAM10^+ cells in naive and GC B cells. (C) Frozen serial sections of Peyer's patches were stained to detect B cell follicles (B220^+ , blue), germinal centers (PNA^+ , green) and ADAM10 (red) (right panel). To demonstrate specificity of staining, isotype-matched mAb were used instead of mAb against ADAM10 (left panel). Original magnification: x20. The mucosal epithelium (E) also reacts with PNA and anti-ADAM10. Figure courtesy of Joanna Cichy, from Jagiellonian University, Krakow, Poland.

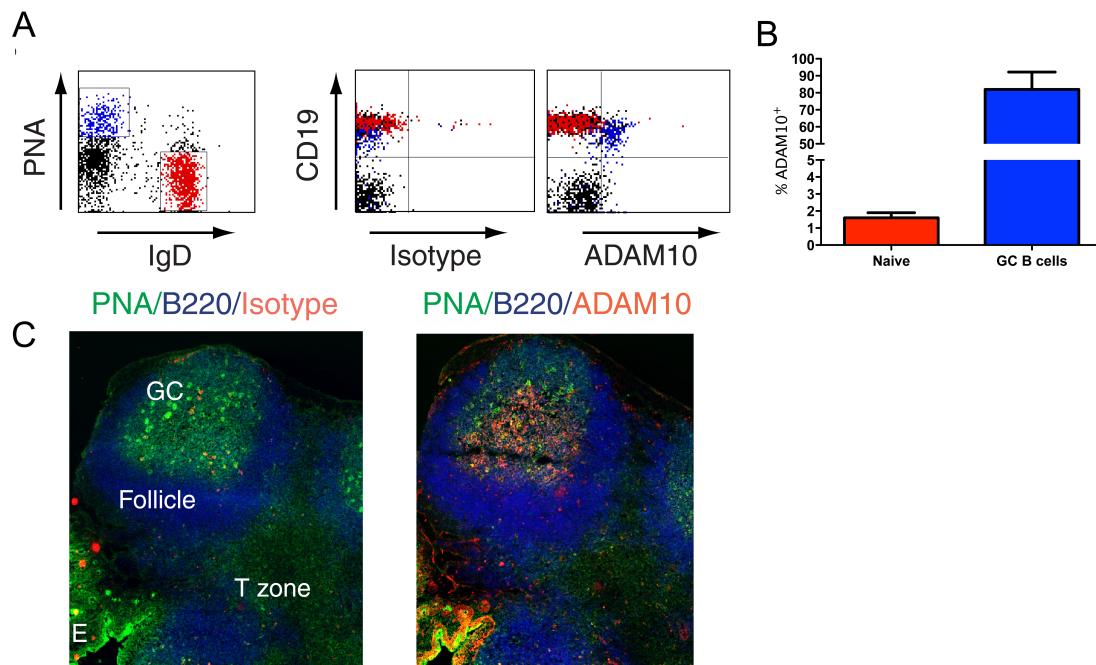


Figure 9. ADAM10^{B/-} mice have impaired humoral responses.

(A) Serum IgM, IgG1, IgG2a and IgG2b were measured by ELISA from non-manipulated 8-12 week old mice. (B) ADAM10^{B/-} mice and WT controls were immunized with 10µg NP-KLH emulsified in alum. At the indicated times, serum samples were collected and total NP-specific IgM, total IgG and high affinity IgG antibody titers were determined by ELISA with NP₁₄BSA as capture antigen for total and NP₄BSA for high affinity ELISA. (C) Mice were immunized with NP-KLH emulsified in alum, rested for 42 days, and boosted with 10µg NP-KLH for 5 days. Mice were bled weekly throughout the course of the experiment. Total and high affinity IgG levels were measured by ELISA at each time point. The relative unit (RU) values for alum-injected mice were less than 0.001. Bars represent the mean ± SE of 5-9 mice per group (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). Data represent results obtained in at least two independent experiments.

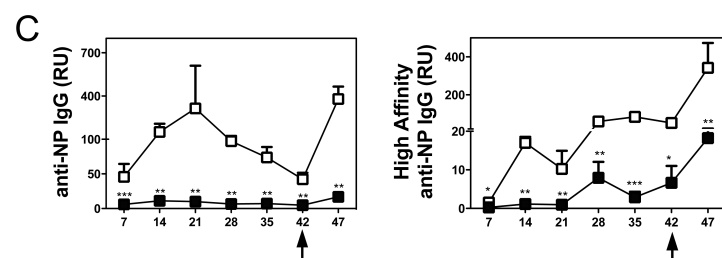
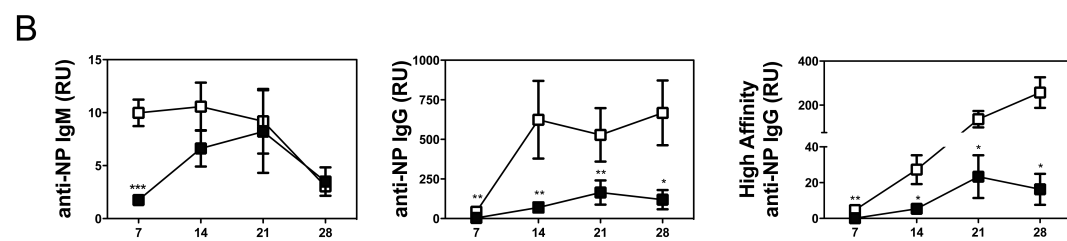
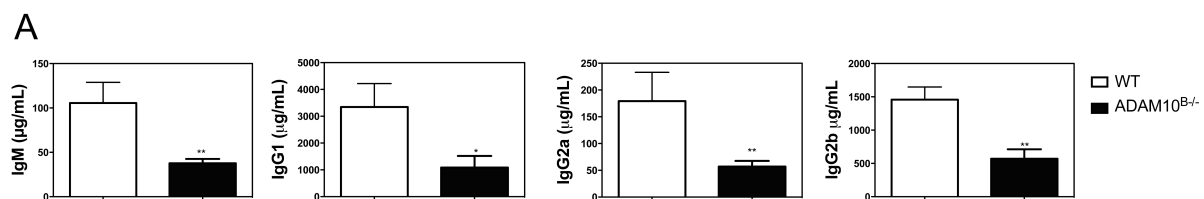


Figure 10. Decreased NP-specific IgG secretion is not IgG subset and cannot be overcome by high antigen dose

(A) ADAM10^{B-/-} mice and WT controls were immunized with 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 NP₁₄BSA 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 less than 0.001. Bars represent the mean \pm SEM of 5 mice per group. Data represent results obtained in two independent experiments. (*p<0.05, **p<0.01, ***p<0.001)

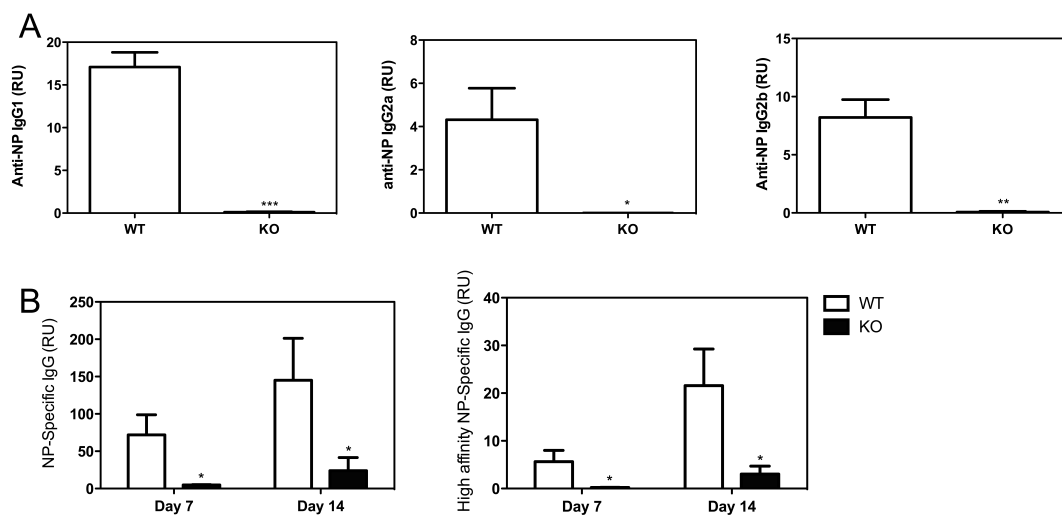


Figure 11. ADAM10^{B-/-} mice have decreased number of antigen specific ASCs.

ELISPOT analysis of cells producing (A) total (both low and high affinity), (B) high-affinity NP-specific IgG and (C) high versus total ratio present in spleen (top) and bone marrow (bottom) isolated from WT and ADAM10^{B-/-} mice 21 days post-immunization with NP-KLH emulsified in alum. (D-E) ELISPOT analysis of cells in spleen producing (D) total and (E) high affinity NP-specific IgG 14 and 28 days post-immunization. (*p <0.05, **p<0.01, ***p <0.001). Bars represent the mean \pm SE of five mice per group. Data represent results obtained in two independent experiments.

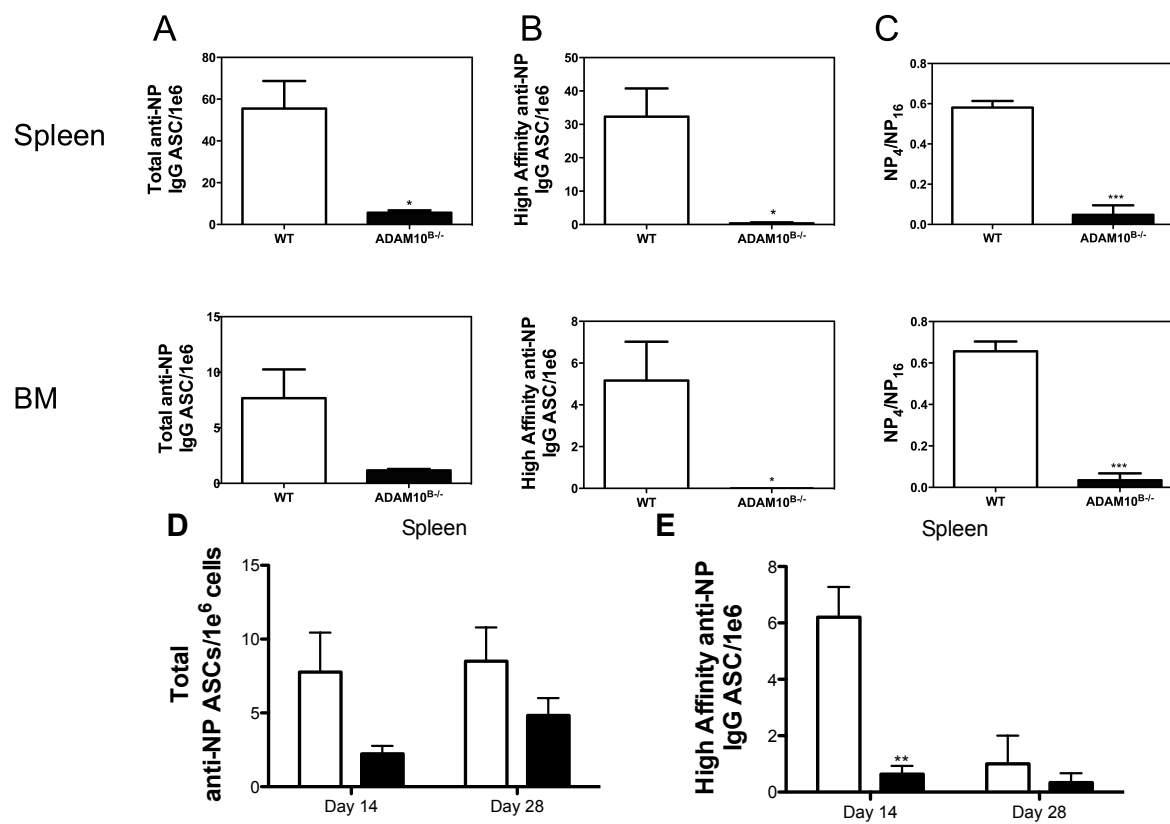


Figure 12. GC formation development following immunization

ADAM10^{B/-} and WT mice were immunized with 10 µg NP-KLH emulsified in alum. Fourteen and twenty one days post immunization flow cytometry was carried out and the presence of GC B cells (IgM^{lo}IgD^{lo}B220⁺IgG1⁺CD38⁻) in spleen was assessed. Staining protocol is depicted (A). Both percentage (B) and total number (C) of GCs were enumerated. (D) Mice were immunized as in A-C. GC B cells were quantified by flow cytometry as B220⁺GL7⁺Fas^{hi} fourteen days post-immunization. (*p<0.05, **p<0.01). Bars represent the mean ± SE of 3-8 mice per group. Data are representative of 3 independent experiments.

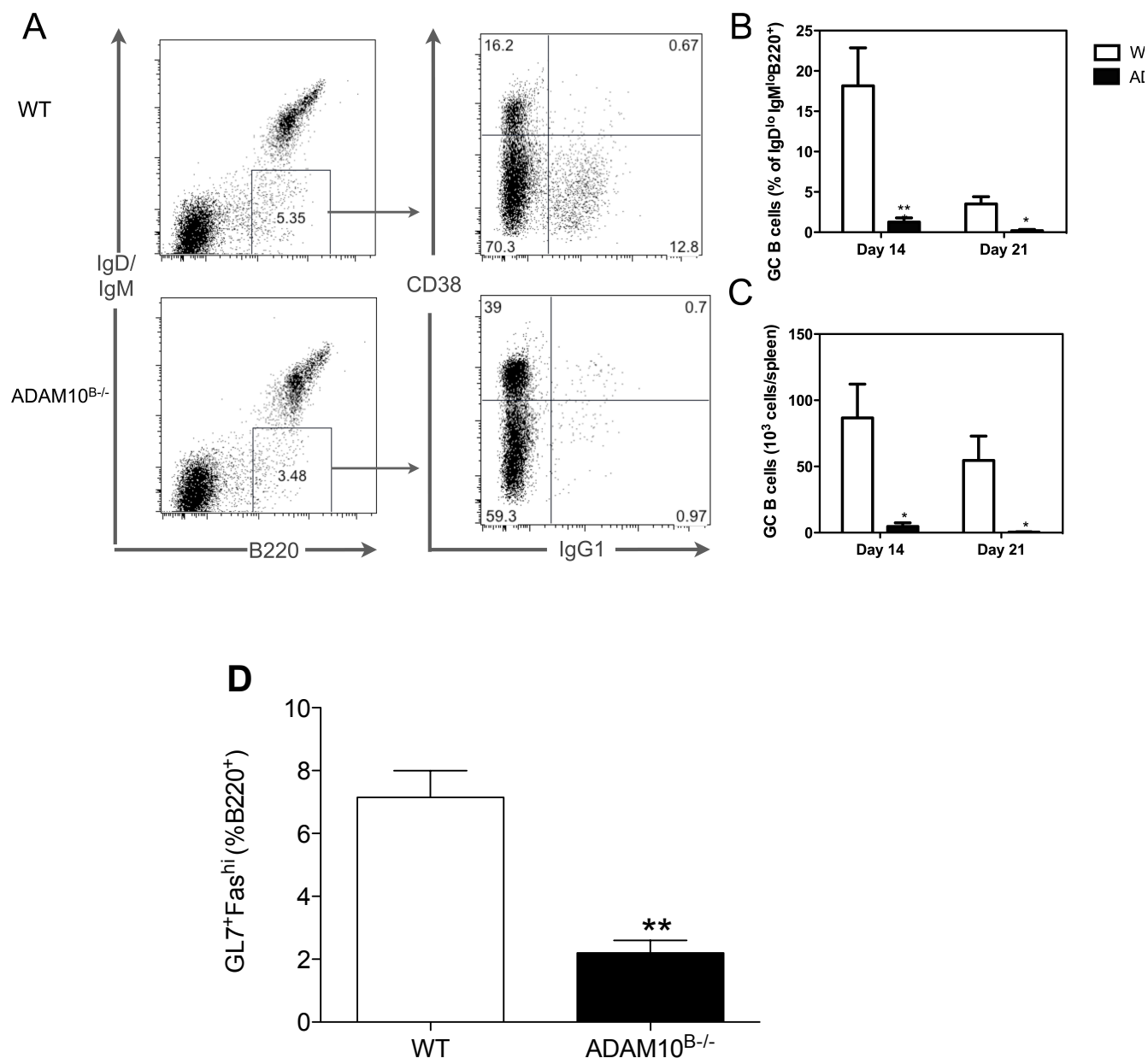


Figure 13. *Aidca* expression in draining lymph nodes.

Mice were immunized in the footpad with 10 μ g of NP-KLH. Fourteen days after immunization, draining LN were isolated and *Aidca* expression was analyzed by qPCR. Bars represent the mean \pm SE of 8 mice per group. Data are representative of 2 independent experiments.

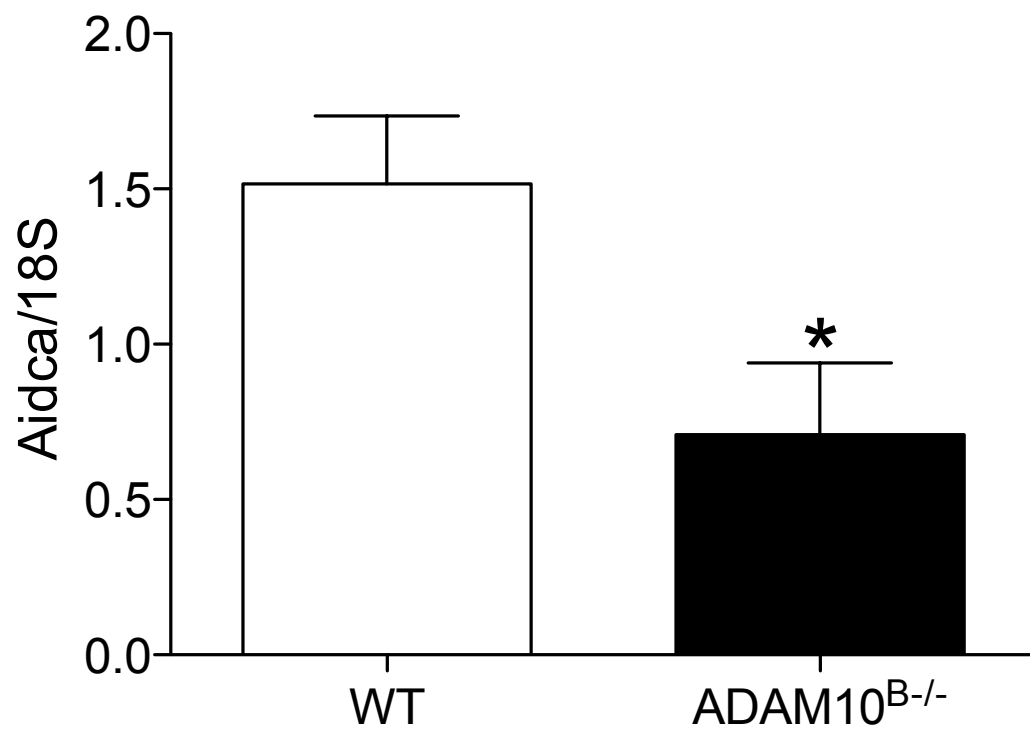


Figure 14. Immunohistochemistry analysis of GC formation and lymph node architecture following T-dependent immunization.

ADAM10^{B-/-} and WT mice were immunized with 10µg NP-KLH emulsified in alum. Fourteen days after immunization, draining LN were sectioned and stained for (A) GL7 (green) and IgD (red); (B) Thy1.2 (green) and B220 (red); (C) CD21/35 (green) and B220 (red). (D) non-draining LN were also stained with CD3 (green) and B220 (red). Pictures are representative of 3 independent experiments. Magnification displayed is 20x.

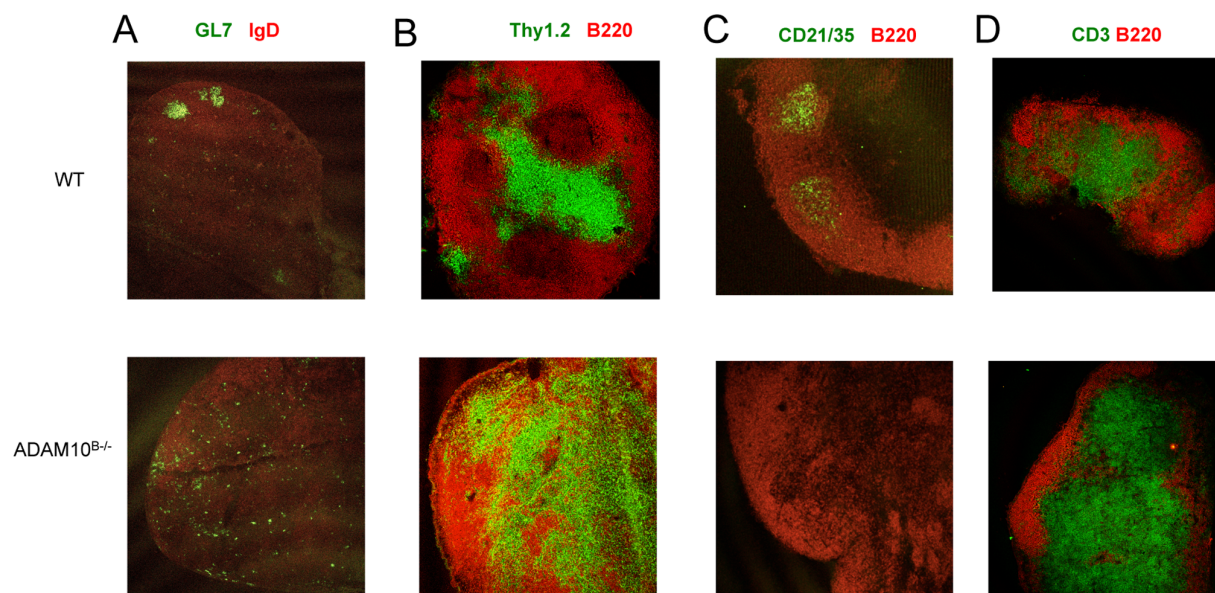


Figure 15. Immunohistochemistry analysis of GC formation and splenic architecture following T-dependent immunization.

ADAM10^{B-/-} and WT mice were immunized with 10µg NP-KLH emulsified in alum. Fourteen days after NP-KLH immunization, spleens were isolated and sectioned. Sections stained for (A) GL7 (green) and IgD (red) or (C) Thy 1.2 (green) and B220 (red) or (D) B220 (red) and CD21/35 (green). (B) Spleens isolated from naïve animals, sectioned and stained for Thy 1.2 (green) and B220 (red). Pictures are representative of 3 independent experiments. Magnification displayed is 20x for A and B, and 10x for C and D.

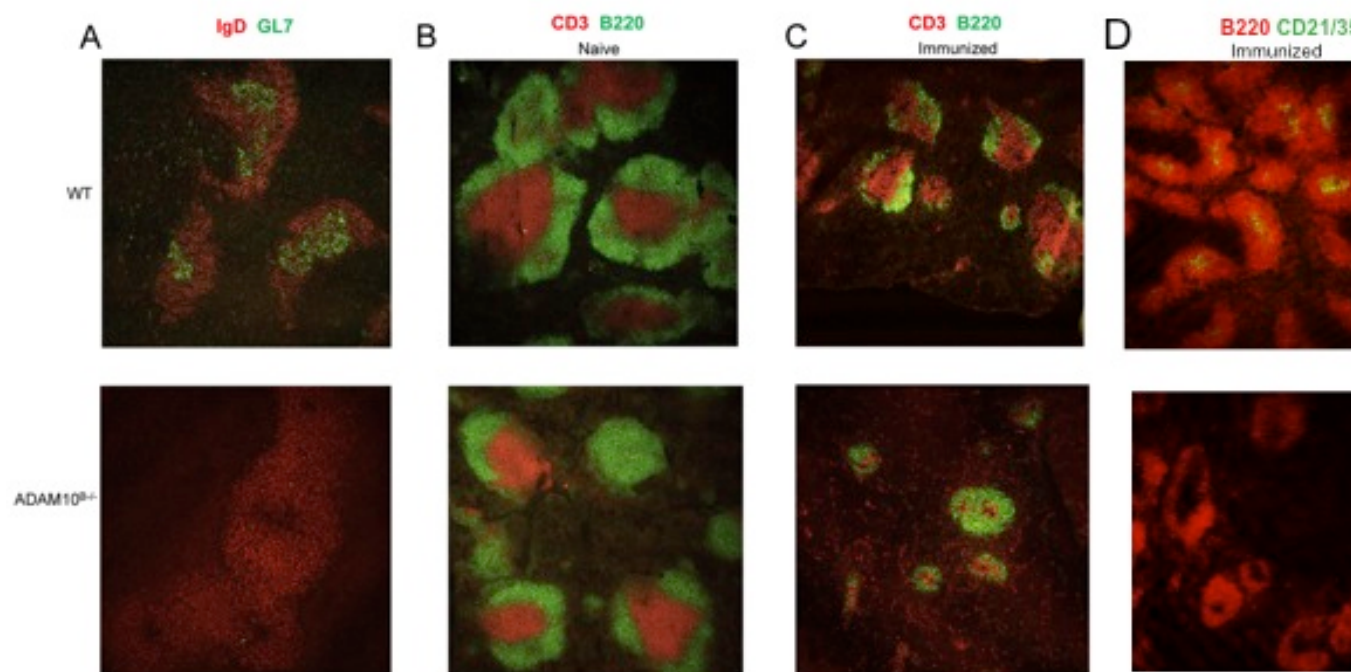


Figure 16. Chemokine expression in draining lymph nodes.

Mice were immunized in the footpad with 10µg of NP-KLH. Fourteen days after immunization, draining and non-draining LN were isolated and chemokine expression was analyzed by qPCR for CCL19, CCL21 and CXCL13. Non-draining (A) and draining (B) LN are shown. (*p<0.05). Bars represent the mean ± SE of 8 mice per group. Data are representative of 2 independent experiments. (C) Draining LN were stained for CCL21. White line depicts lymph node capsule. Pictures are representative of 3 mice per group.

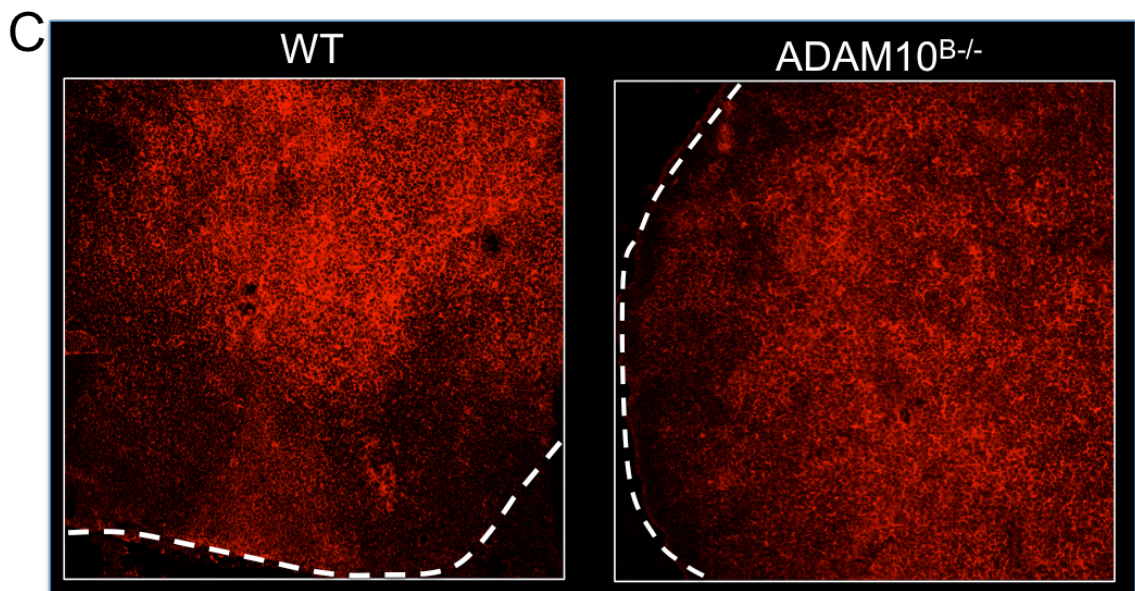
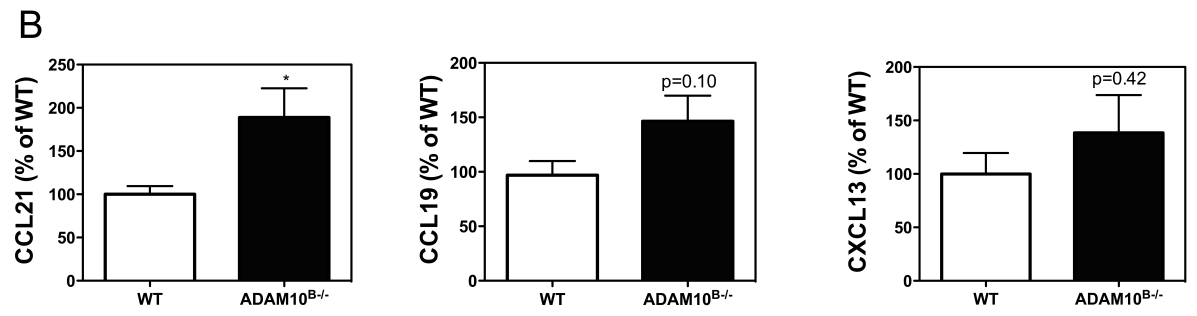
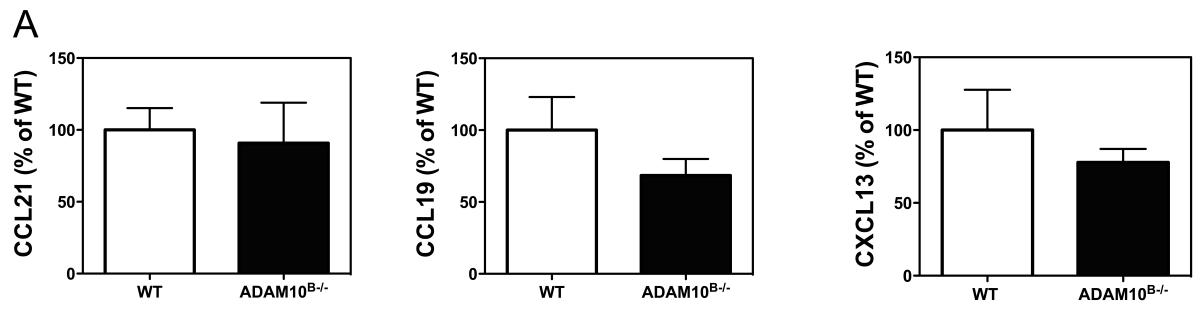


Figure 17. ADAM10-deficient B cells show enhanced migration to CXCL13 but not CXCL12.

Splenocytes from WT and ADAM10 were isolated and migration was assayed as described in methods section. (Top) 1 μ g/mL of CXCL13; (bottom) 300ng/mL of CXCL12. (**p<0.001, 1-way ANOVA with Tukey post-test). Bars represent the mean \pm SE of 4-6 mice per group. Data are representative of 3 independent experiments.

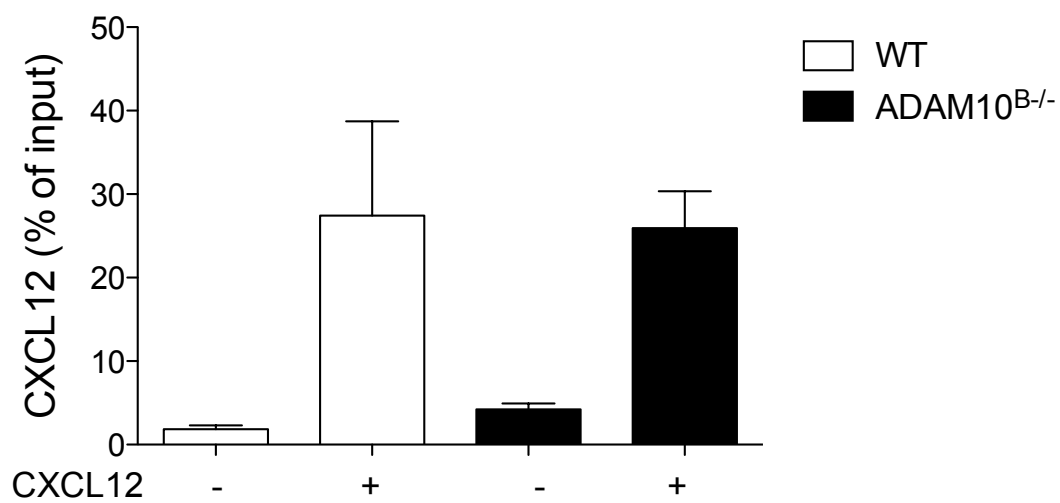
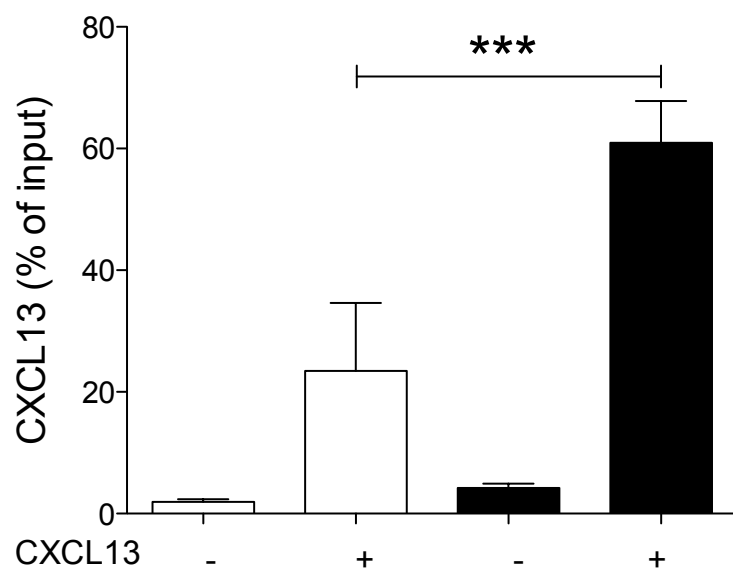
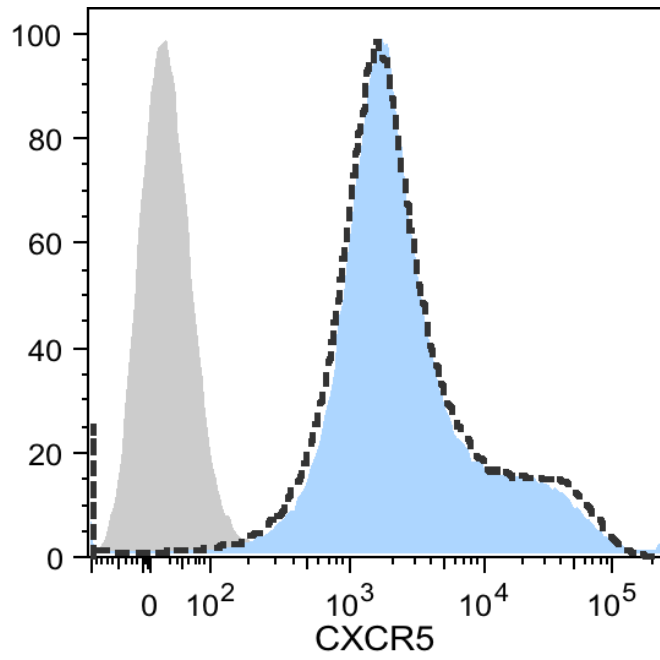


Figure 18. ADAM10-deficient B cells have normal CXCR5 expression.

Splenocytes were isolated and stained for B220 and CXCR5. (A) Histogram demonstrating CXCR5 B cells expression in WT (---) and ADAM10-deficient B cells (shaded light blue). Isotype control is also depicted (shaded light gray). (B) Quantification of mean fluorescence intensity (MFI). Bars represent the mean \pm SE of 4-6 mice per group. Data are representative of 3 independent experiments.

A



B

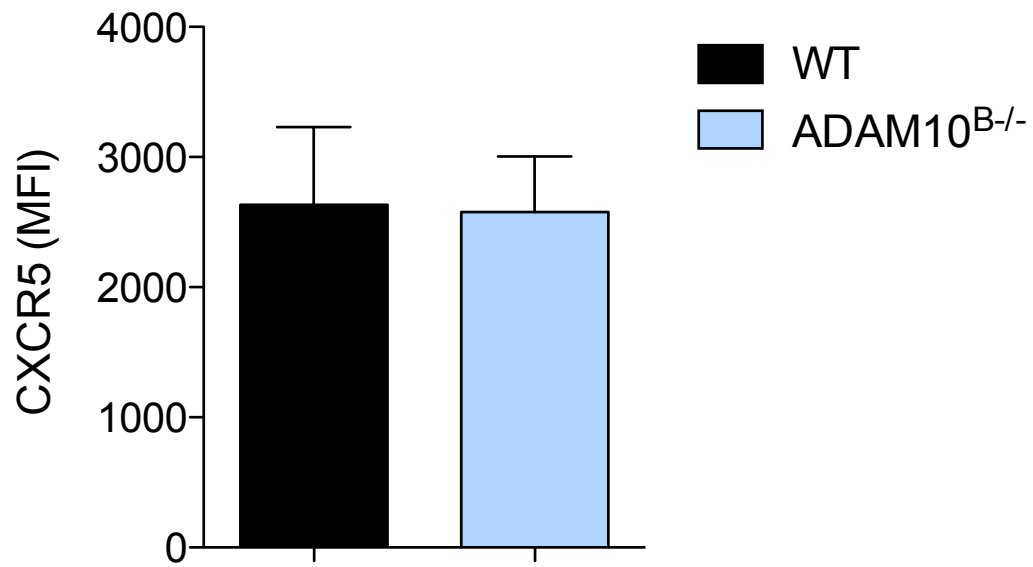
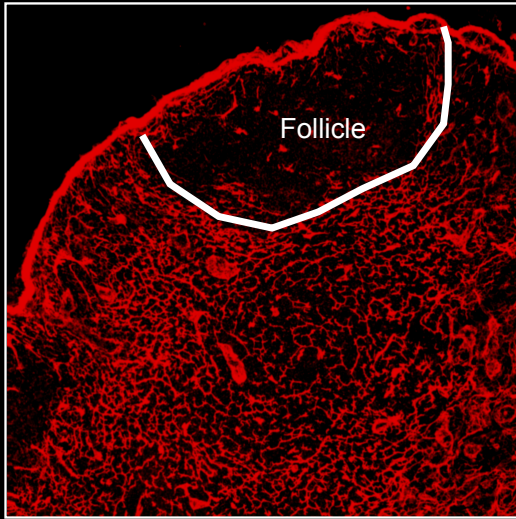


Figure 19. Lymph nodes isolated from ADAM10^{B-/-} mice show abnormal collagen deposition.

Lymph nodes were isolated from naïve WT and ADAM10^{B-/-} mice, sectioned and stained for collagen type II. Representative pictures are displayed. Immunohistochemistry was performed and analyzed by Mohey Eldin El Shikh.

WT



ADAM10^{B-/-}

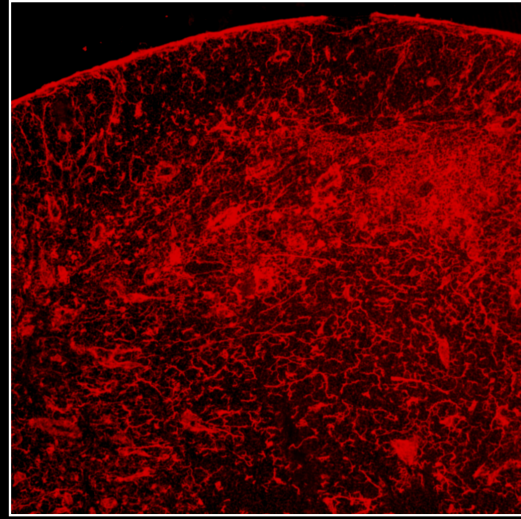


Figure 20. B cell-specific ADAM10 deletion alters stromal cell populations in aged mice.

Naïve LN were isolated from 16-20 week old mice and single cell suspension was generated by enzymatic digestion, as described in methods section. Cells were stained and stromal cell populations were analyzed. FRCs are gp38⁺CD31⁻; LECs are gp38⁺CD31⁺, BECs are gp38⁻CD31⁺. Double negative (DN) cells are gp38⁻CD31⁻ (A) Representative contour plot. (B) quantification. Bars represent the mean \pm SE of 4-6 mice per group. Red boxes represent populations that are different between WT and ADAM10^{B-/-} mice. Data are representative of 3 independent experiments. (*p<0.05, ***p<0.001).

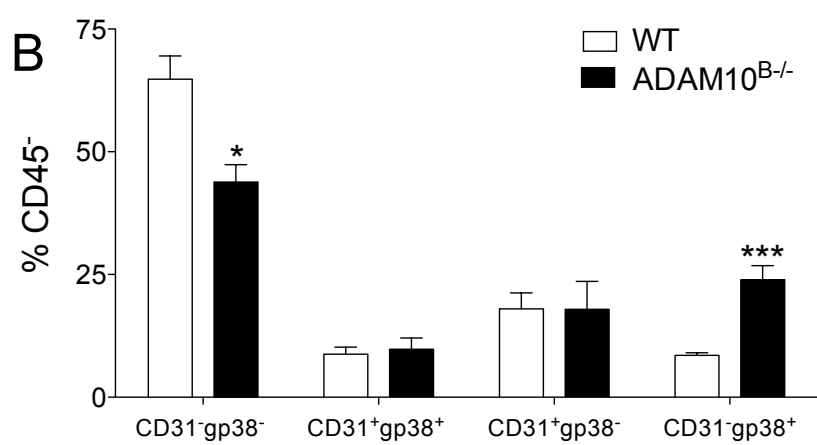
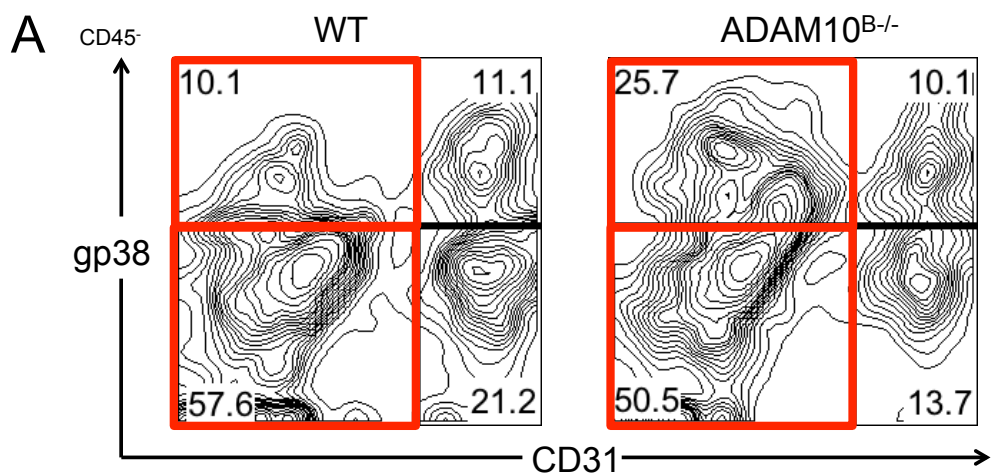


Figure 21. B cell-specific ADAM10 deletion alters stromal cell populations in young mice

Naïve LN were isolated from 4 week old mice and single cell suspension was generated by enzymatic digestion, as described in methods section. Cells were stained and stromal cell populations were analyzed. FRCs are gp38⁺CD31⁻; LECs are gp38⁺CD31⁺, BECs are gp38⁻CD31⁺. Double negative (DN) cells are gp38⁻CD31⁻ (A) Representative contour plot. (B) quantification. Bars represent the mean \pm SE of 4-6 mice per group. Red boxes represent populations that are different between WT and ADAM10^{B-/-} mice. Data are representative of 3 independent experiments. (*p<0.05, ***p<0.001).

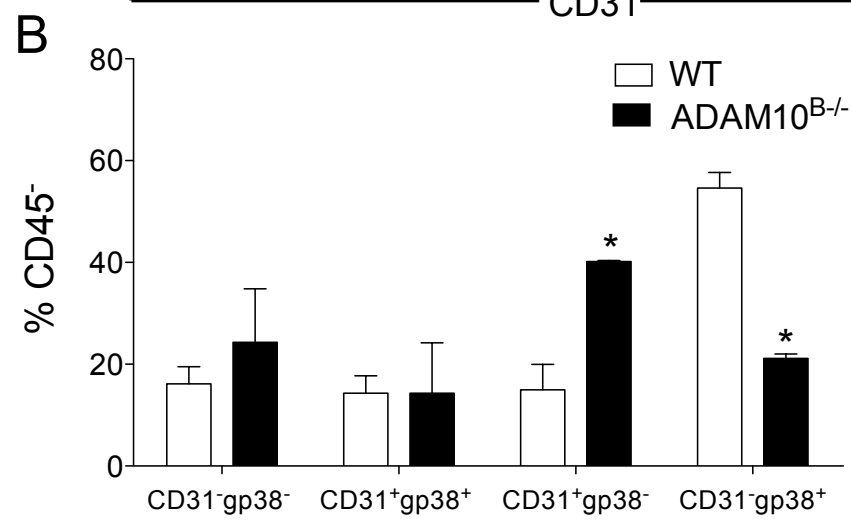
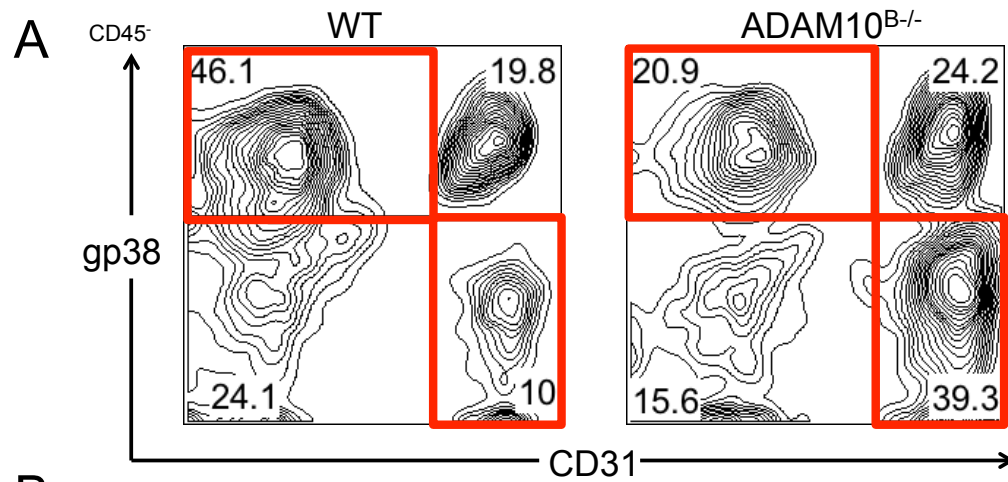


Figure 22. ADAM10 deficient B cells secrete reduced amounts of TNF- α following LPS stimulation.

WT and ADAM10-deficient naïve B cells were isolated via magnetic bead separation. Cells were stimulated with 10 μ g/mL of LPS. Three days after stimulation (A) supernatant was harvest and TNF- α was measured by ELISA and (B) TNF- α mRNA expression was determined by qPCR. Bars represent the mean \pm SE of 6 mice per group. Data represent results obtained in three independent experiments. (**p<0.001).

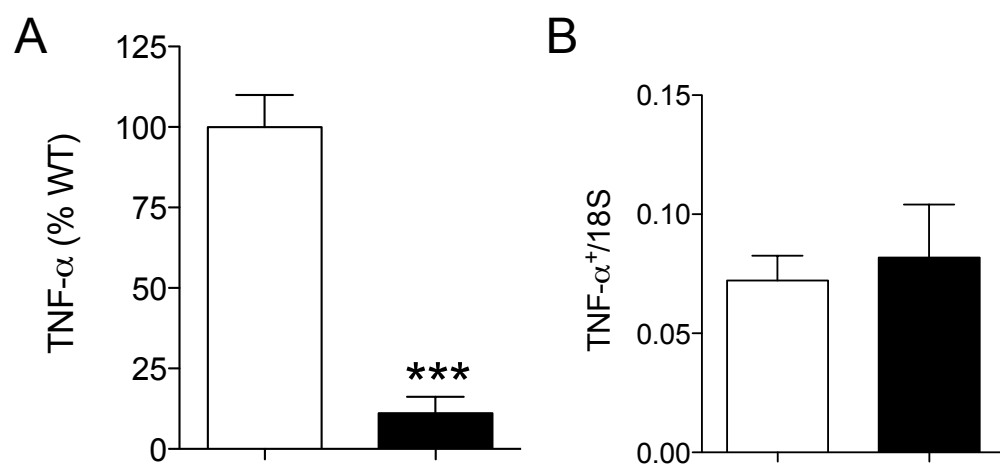


Figure 23. Defect in humoral responses in ADAM10^{B-/-} mice seems to be Notch2-independent.

(A) Splenocytes isolated from ADAM10^{B-/-}, ADAM10^{B-/-N2ICD-TgB+} and WT mice were stained with B220, CD23, CD21/35 and IgM. Marginal zone B cells were defined as B220⁺CD23^{-/lo}CD21/35^{hi}IgM⁺. (B) Mice were immunized in the footpad with NP-KLH, 14 days later draining LN were isolated and stained for GL7 and IgD (top) and Thy1.2 and B220 (bottom). (C) ADAM10^{B-/-}, ADAM10^{B-/-N2ICD-TgB+} and WT mice were immunized i.p. with NP-KLH emulsified in alum. At the indicated times, serum samples were collected and total NP-specific IgG was determined by ELISA. Bars represent the mean \pm SE of 5 mice per group. Data represent results obtained in two independent experiments. (*p<0.05, **p<0.01, ***p<0.001, One-way ANOVA with Turkey post-test). Significance refers to ADAM10^{B-/-} compared to WT and ADAM10^{B-/-N2ICD-TgB+} compared to WT. ADAM10^{B-/-} and ADAM10^{B-/-N2ICD-TgB+} were not different from each other at any time point.

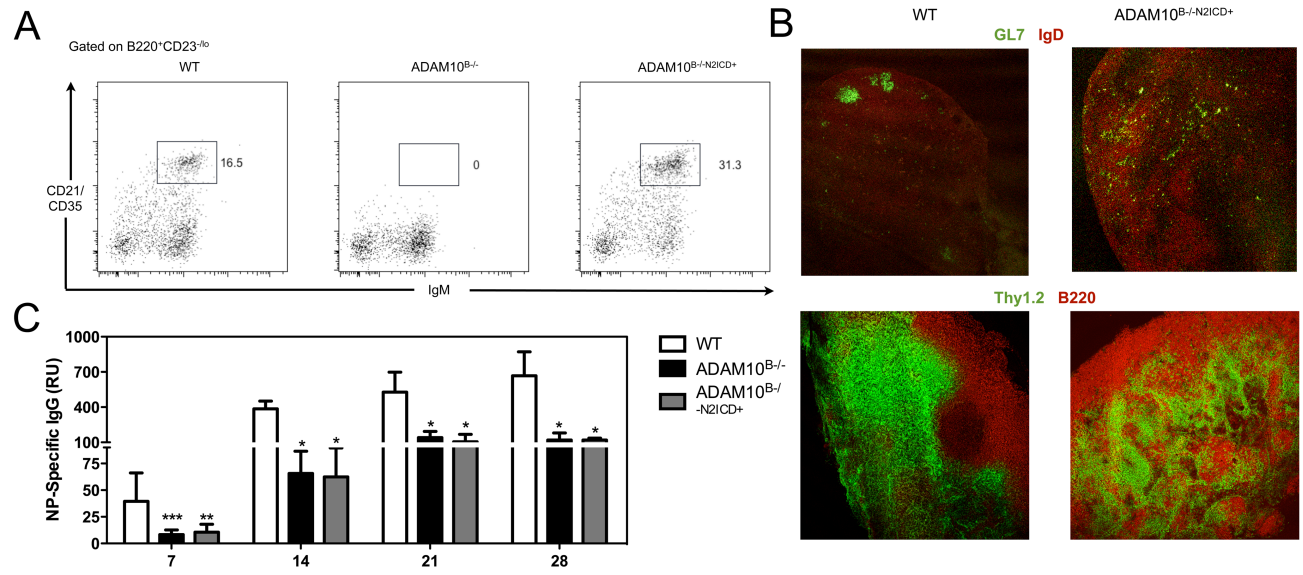


Figure 24. CD23Tg mice have a normal IgG response to NP-KLH

CD23Tg and WT mice were immunized with NP-KLH emulsified in alum. At the indicated times, serum samples were collected and total NP-specific IgG was determined by ELISA. Bars represent the mean \pm SE of 8 mice per group. Data represent results obtained in two independent experiments.

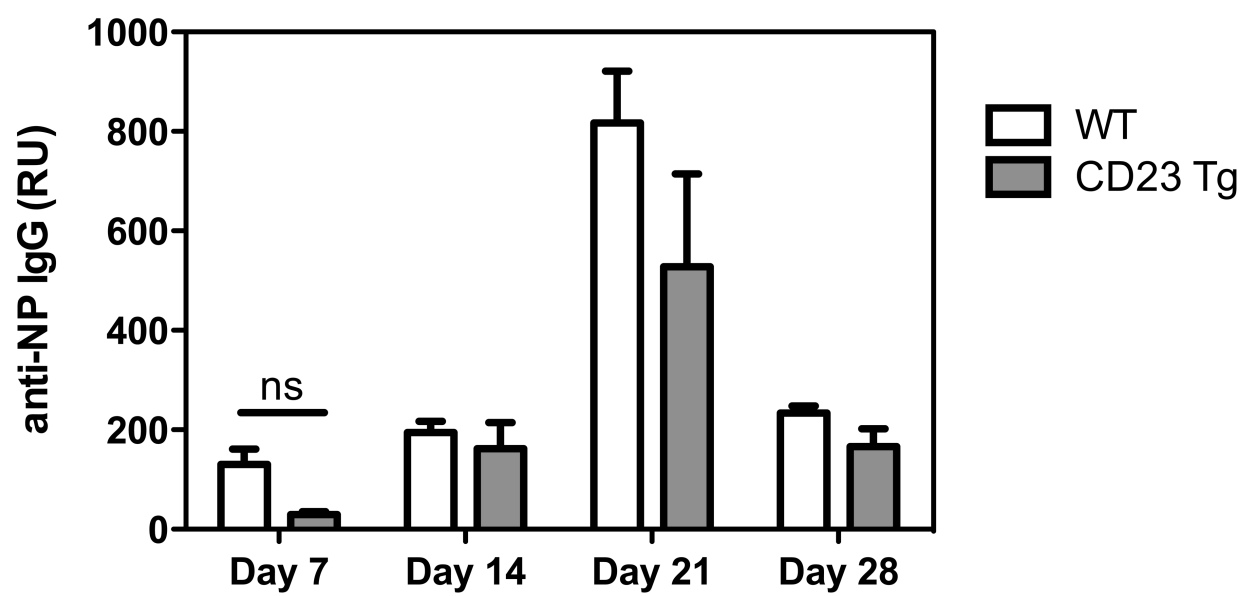


Figure 25. Basal antibody levels in CX3CR1-deficient mice.

Serum (A) IgM, and (B) IgG1 were measured by capture ELISA from non-manipulated 8-12 week old mice. Bars represent the mean \pm SE of 9-15 mice per group ($*p < 0.05$). Data represent results obtained in at least two independent experiments.

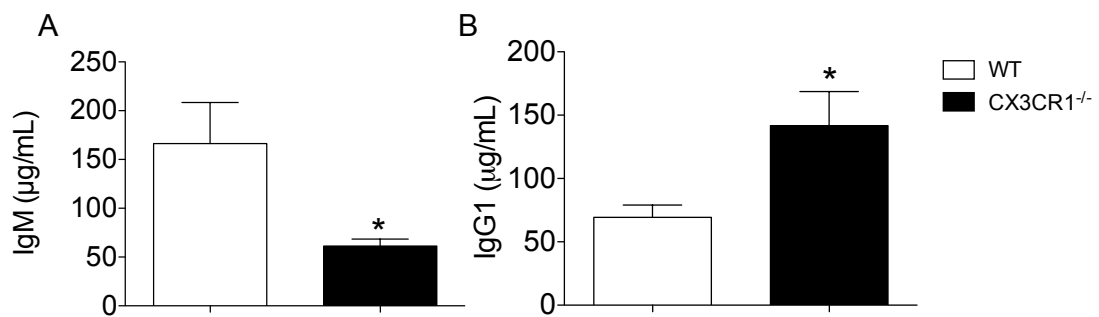


Figure 26. CX3CR1-deficient mice have enhanced antibody responses.

CX3CR1-deficient mice and WT controls were immunized with 10 μ g NP-KLH emulsified in alum. At the indicated times, serum samples were collected and total NP-specific (A) total IgG and (B) high affinity IgG antibody titers were determined by ELISA with NP₁₄BSA as capture antigen for total and NP₄BSA for high affinity ELISA. (C) Mice were immunized with NP-KLH emulsified in alum, fourteen days post-immunization spleens were harvested and analyzed for GC B cells by flow cytometry. Quantified results are showed. The relative unit (RU) values for alum-injected mice were less than 0.001. Bars represent the mean \pm SE of 3-10 mice per group (* p < 0.05, ** p <0.01, *** p <0.001).

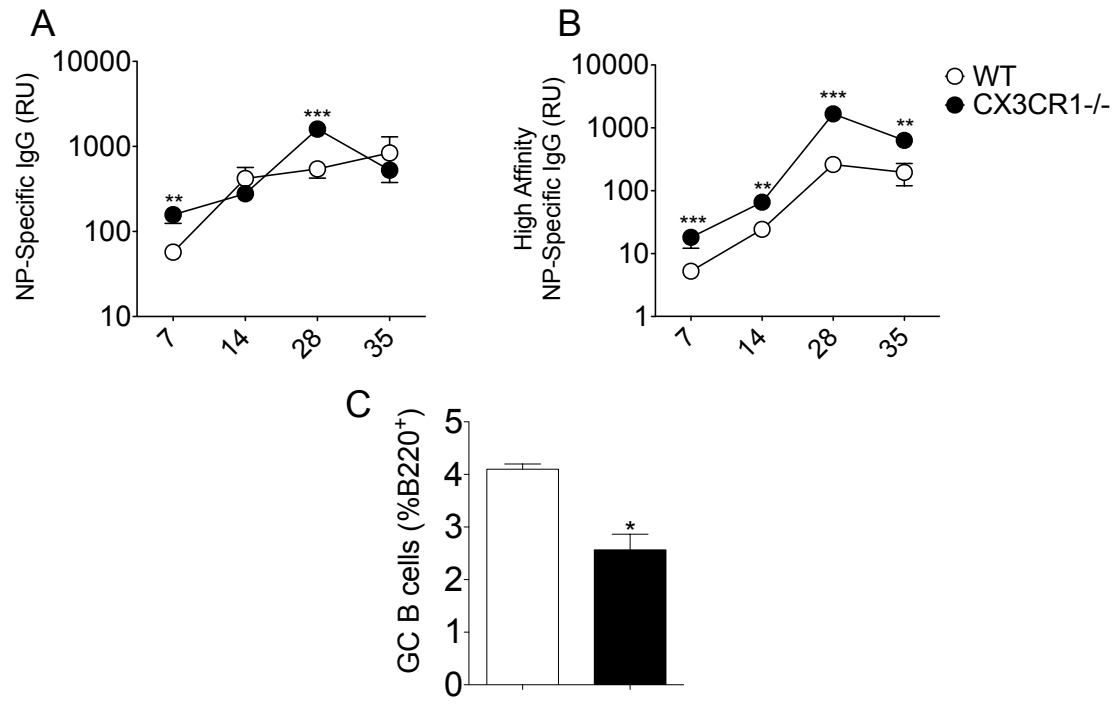
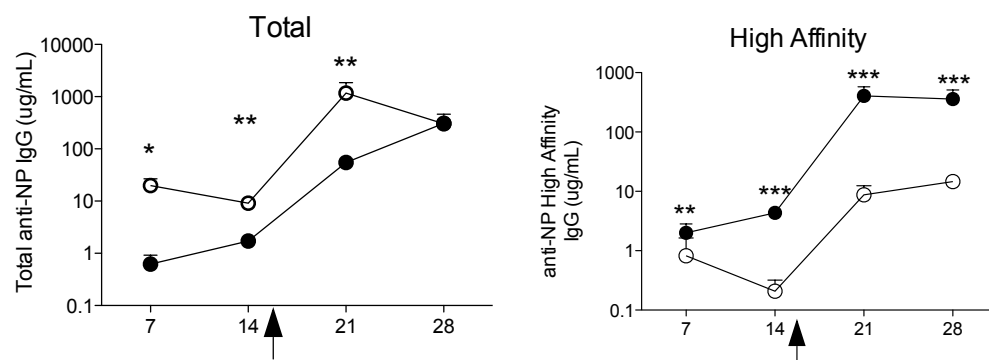


Figure 27. Repeated immunization can partially rescue antibody production defect in ADAM10^{B/-} mice.

ADAM10^{B/-} and WT mice were immunized with 10µg NP-KLH emulsified in alum. Fourteen days after primary immunization, mice were boosted with 10µg NP-KLH emulsified in alum. (A) Mice were bled weekly and total and high-affinity NP-specific IgG levels were measured by ELISA. (B) Twenty-eight days following immunization mice were sacrificed and antibody-secreting cells in spleen and bone marrow were quantified by ELISPOT. The relative unit (RU) values for alum-injected mice were less than 0.001. Bars represent the mean ± SE of 8-10 mice per group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

A



B

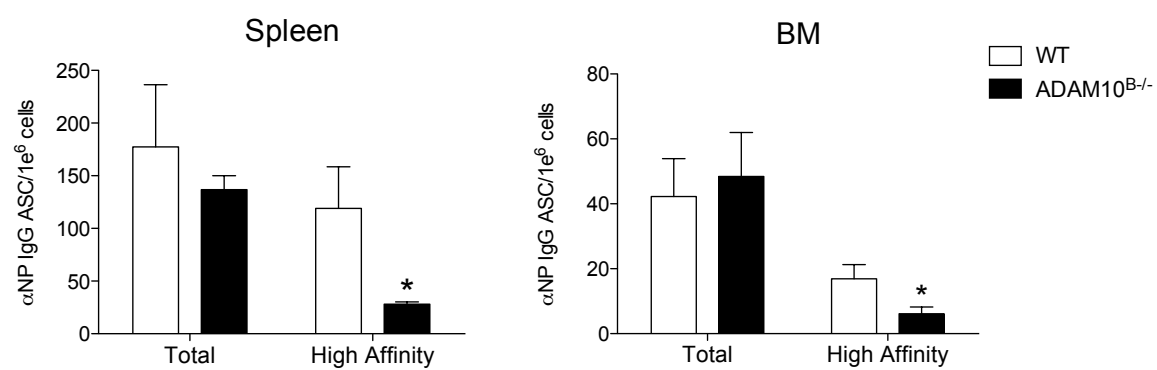


Figure 28. Immunization with immune complexes does not rescue antibody production defects seen in ADAM10^{B-/-} mice.

WT and ADAM10^{B-/-} were passively immunized subcutaneously with 6mg of rabbit polyclonal anti-OVA antibodies or PBS. Twelve hours later, mice were injected with 10 μ of NP-OVA in alum. Six days later, mice were sacrificed and cardiac puncture. Antigen-specific (A) IgM and (B) IgG1 were measured by ELISA. Bars represent the mean \pm SE of 4 mice per group (* p < 0.05, ** p <0.01).

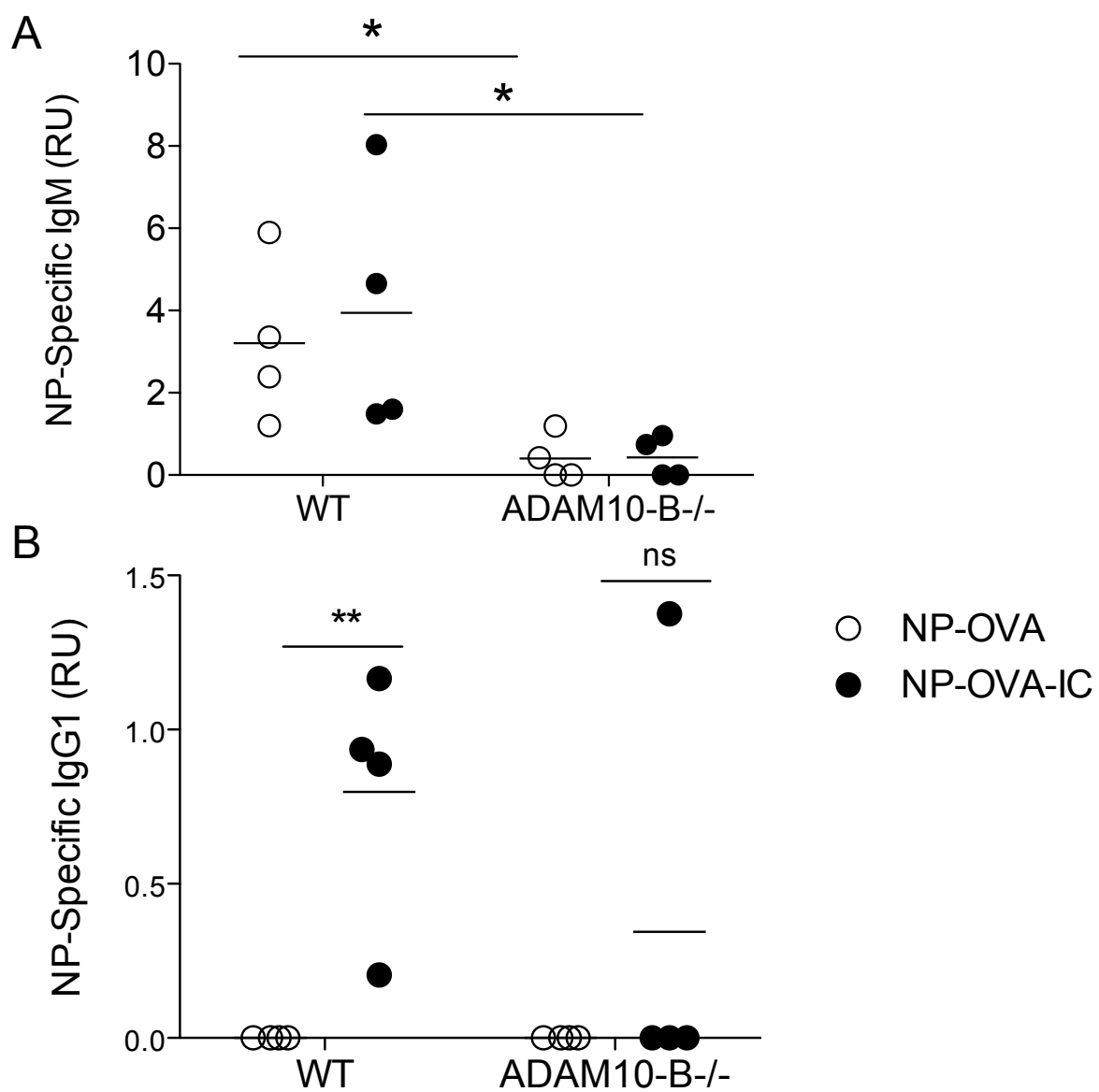


Figure 29. Normal responses of ADAM10 deficient B cells under T dependent stimulation *in vitro*, but decreased T_{FH} cell numbers *in vivo*.

(A) Splenocytes were cultured (12.5×10^6 cells/well) with CD40L-transfected Chinese hamster ovary (CHO) cells as a B cell stimulant (6×10^4 cells/well) for seven days. Supernatants were then harvested and analyzed for IgG1 expression by ELISA. (B-D) Mice were immunized with NP-KLH. Fourteen days post immunization flow cytometry was carried out and the presence of T_{FH} (B220⁻ CD4⁺ CXCR5^{hi} PD-1^{hi}) in the spleen of WT and ADAM10^{B-/-} was assessed. (B). Staining protocol is depicted. (C). Percentage of T_{FH} out of CD4⁺ was enumerated (D) Draining lymph nodes were analyzed for IL-21 mRNA by quantitative PCR (*p<0.05, **p<0.01). Bars represent the mean \pm SE of 6 mice per group. Data are representative of 2 independent experiments.

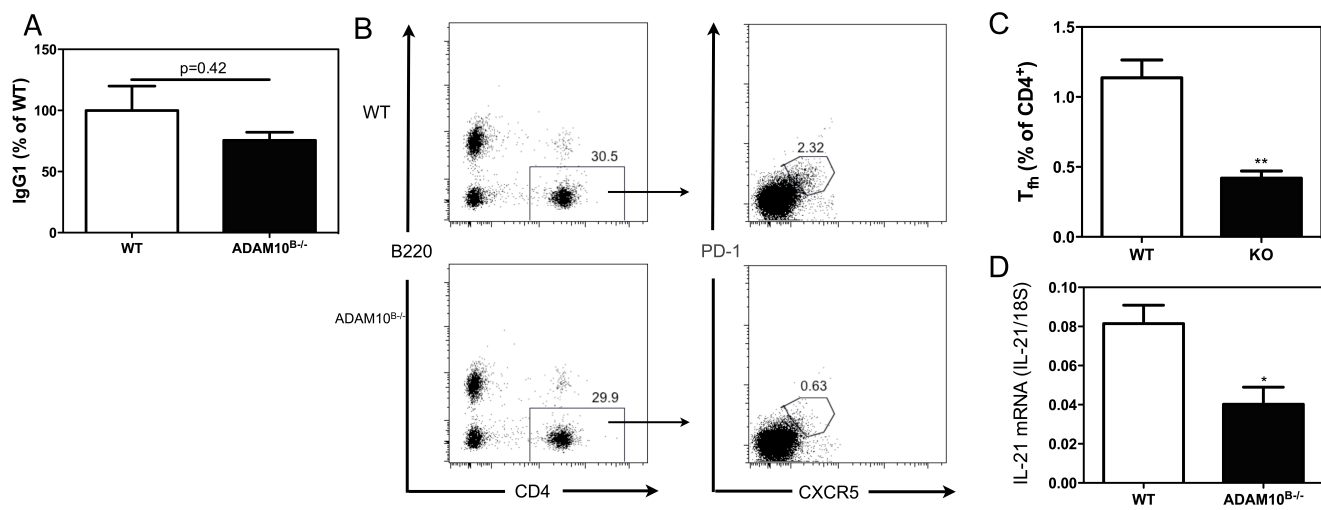
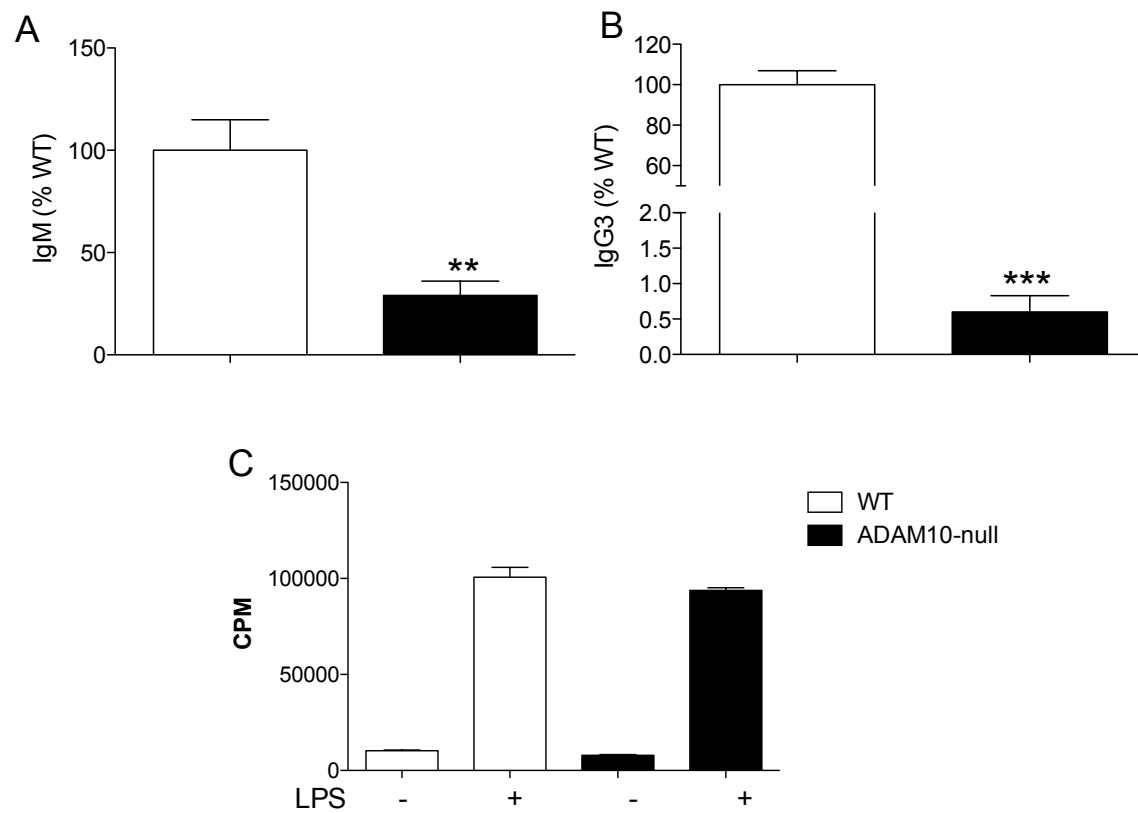


Figure 30. ADAM10-deficient B cells produce reduced antibody amounts following LPS stimulation.

Naïve B cells were isolated via magnetic bead separation and stimulated with 10µg/mL of LPS. Four days following stimulation, supernatant were harvest and analyzed for (A) IgM and (B) IgG1 by ELISA. (C) Two days following challenge, tritiated thymidine was added to cells. Cells were incubated for 24 hours and then harvested. Thymidine incorporation was then measured. (**p<0.01, ***P<0.005). Bars represent the mean ± SE of 6 mice per group. Data are representative of at least 2 independent experiments.



3.4 ADAM10 regulates plasma cell function

Generation of ADAM10^{ΔΔ}IgG1^{+/-}

Members of the ADAM family regulate a variety of functions, including, but not limited to, cell migration, proliferation and adhesion²¹¹. We have previously generated mice that lacked ADAM10 in all peripheral B cells (ADAM10^{B-/-} mice) by crossing a knockin mouse strain containing *loxP* sites surrounding exon 9 of *adam10* (ADAM10^{ΔΔ}) allele with mice expressing Cre recombinase under the control of the CD19 promoter²⁴. As discussed in section 3.3, the study of these mice demonstrated a severe defect in GC formation and changes in lymphoid architecture¹⁸¹. In order to determine whether ADAM10 plays a role in PC development or the defects in antibody production observed in ADAM10^{B-/-} were secondary to changes in architecture, we crossed ADAM10^{ΔΔ} with IgG1-cre transgenic mice and generated ADAM10^{ΔΔ}IgG1-cre^{+/-} mice¹²⁸. Previous studies have demonstrated that IgG1-cre transgene shows specificity for GC B cells with approximately 75% of GC B cells expressing Cre recombinase. Introduction of the IgG1-cre transgene has been shown to affect IgG1 production; therefore, we also generated controls that have cre expression but have WT ADAM10 alleles (ADAM10^{+/+}IgG1-cre^{+/-} mice). In order to track cells that had undergone cre-mediated recombination, we crossed ADAM10^{ΔΔ}IgG1-cre^{+/-} and ADAM10^{+/+}IgG1-cre^{+/-} mice with R26R-EYFP⁺ mice, thus, generating ADAM10^{ΔΔ}IgG1-cre^{+/-}R26R-EYFP⁺ (ADAM10^{ΔΔ}IgG1-cre^{+/-}-YFP⁺) and ADAM10^{+/+}IgG1-cre^{+/-}-YFP⁺ (control) mice that express EYFP transgene following cre-mediated recombination^{24,74-76}.

Antibody production and GC formation in ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ mice

Our previous study demonstrated that ADAM10-deletion in all peripheral B cells (ADAM10^{B-/-} mice) led to severe impairments in antibody production, as evidenced from decreased antibody responses to T-dependent antigens. Moreover, GC formation was severely affected in those mice. Furthermore, T_{FH} cell numbers were also diminished in ADAM10^{B-/-} mice¹⁸¹. In order to determine if deletion of ADAM10 within GCs affected basal antibody levels, mice were bled and IgM, IgG1 and IgE levels were measured by ELISA. As depicted in Figure 31A-C, there were no differences between ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ and controls mice with regards to basal antibody levels.

We then examined whether ADAM10 deletion within GCs affected GC maintenance and antigen-specific antibody production. ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ mice and controls were immunized with 10μg of NP-KLH emulsified in alum. The number of GC B cells, defined as B220⁺GL7⁺Fas^{hi}, 14 days post-immunization was quantified by flow cytometry. The percentage of GC B cells was comparable between ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ and controls (Figure 31D-E). Immunohistochemistry analysis also showed normal GCs, defined as clusters of GL7⁺ within B cell follicle and normal splenic follicle architecture (Figure 31F). Furthermore, T_{FH} numbers were also normal (Figure 31G).

Interestingly, despite normal GC B cell numbers, antigen specific IgG1 was severely diminished (Figure 32B), while antigen-specific IgM levels were comparable to control mice (Figure 32A). The normal IgM levels were not surprising, as it has been reported that less than 2% of IgM⁺ cells undergo cre-

mediated recombination¹²⁸. These results demonstrate a defect in class-switched antibody production.

Mice were also immunized with 100µg of NP-LPS, a T-independent antigen. Surprisingly, antibody responses to these antigens were also severely impaired (Figure 33). These results are rather unexpected because cre-expression has been shown to occur in only 7% of IgG3-expressing cells¹²⁸. Further experiments will be needed in order to determine ADAM10-deletion in IgG3-expressing cells in our system. Our results seem to suggest that the majority of IgG3 secreting cells are ADAM10-deficient, as IgG3 production in ADAM10 Δ/Δ IgG1-cre^{+/-}YFP⁺ mice is so dramatically impaired.

Plasma cell development in ADAM10 Δ/Δ IgG1-cre^{+/-}YFP⁺ mice

Given that ADAM10 Δ/Δ IgG1-cre^{+/-}YFP⁺ mice had impaired antibody responses but normal GCs, we hypothesized that the defect resulted from aberrant plasmablast and PC differentiation. CD138 is a marker for antibody-secreting plasmablast and PCs (B220^{int/low}CD138⁺ cells). Accordingly, we immunized ADAM10 Δ/Δ IgG1-cre^{+/-}YFP⁺ and ADAM10^{+/+}IgG1-cre^{+/-}YFP⁺ mice with 10µg of NP-KLH and determined the percentage of cre-expressed plasmablast and PCs (B220^{int/low}CD138⁺YFP⁺ cells) in the spleen (Figure 34B), peripheral blood (Figure 34C) and bone marrow (Figure 34D)¹³². The gating protocol is depicted in Figure 34A. Surprisingly, even though ADAM10 Δ/Δ IgG1-cre^{+/-}YFP⁺ mice had markedly impaired antigen-specific IgG1 responses, they had plasmablast and PC percentages comparable to that of controls. Interestingly,

the number of IgG1 expressing cells in the spleen of ADAM10 Δ/Δ IgG1-cre $^{+/-}$ YFP $^{+}$ was significantly reduced (Figure 35). These results suggest that either ADAM10-deletion impairs class switching to IgG1 or more likely, ADAM10-deletion leads to impaired antibody production. Previous studies revealed a similar phenotype when humoral responses were studied in B cell specific XBP-1-deficient mice¹³⁵. XBP-1 is a protein involved in ER-stressed and is required for antibody-secretion¹³⁵. These data suggested that ADAM10 might regulate antibody production by modulating XBP-1 expression in PCs.

Abnormal gene expression in plasma cells isolated from ADAM10 Δ/Δ IgG1-cre $^{+/-}$ YFP $^{+}$

Given that the phenotype observed in ADAM10 Δ/Δ IgG1-cre $^{+/-}$ YFP $^{+}$ mice resembled that of B cell specific XBP-1-deficient mice, we sought to determine if XBP-1 levels were altered in PCs isolated from ADAM10 Δ/Δ IgG1-cre $^{+/-}$ YFP $^{+}$ mice. Interestingly, *Xbp-1* message levels were significantly reduced when compared to controls (Figure 36C). Studies have demonstrated that *Xbp-1* expression is preceded by the downregulation of Bcl6 and the expression of Blimp-1 and IRF4¹³⁵. Thus, we next looked at the expression of these genes in the ADAM10 Δ/Δ IgG1-cre $^{+/-}$ YFP $^{+}$ mice compared to controls. PC isolated from ADAM10 Δ/Δ IgG1-cre $^{+/-}$ YFP $^{+}$ mice also showed a reduction in message levels for *Prdm1* (Figure 36A) (the gene encoding for Blmp-1) and *Irf4* (Figure 36B). Moreover, *Bcl6* levels were significantly higher than in control PCs (Figure 36D). Even more striking, while PC isolated from controls had ~60 fold more *Prdm1*

message than *Bcl-6*, PCs isolated from ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ mice showed only ~3 fold more *Prdm1* than *Bcl6* (Figure 36E). These results demonstrate that ADAM10 is required for the proper downregulation of *Bcl-6* and upregulation of *Prdm1*, *Irf4* and *Xbp1*, and thus for proper PC function and optimal humoral responses.

Consistent with the gene expression results, flow cytometry analysis revealed the presence of a B220^{lo/-}CD138⁺Bcl6⁺ population in the spleens of ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice following immunization (Figure 37). These results demonstrate that in the absence of ADAM10, Bcl6 is overexpressed at both the message and protein level.

The results are summarized in Figure 38. In the absence of ADAM10, there is a dysregulation in gene expression, leading to abnormal PC function and impaired antibody production. Further studies are needed in order to determine the underlying mechanisms connecting ADAM10 to Bcl6, Prdm1, Xbp1 and Irf4.

Figure 31. ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice have normal germinal center formation.

(A-C) Naïve mice were bled and basal antibody levels were measured by ELISA. (A) IgM, (B) IgG1 and (C) IgE. (D-G) Mice were immunized with NP-KLH emulsified in alum and 14 days post-immunization GC formation and T_{FH} cell numbers were assessed by flow cytometry and immunohistochemistry. (D) Representative dot plot (gated on B220⁺ cells). (E) Quantification of GC B cells, representative of 5 mice from at least two independent studies. (F) Fourteen days after NP-KLH immunization, spleens were isolated and sectioned. Sections stained for GL7 (green) and IgD (red). (G) Quantification of T_{FH} cells, representative of 5 mice from at least two independent studies

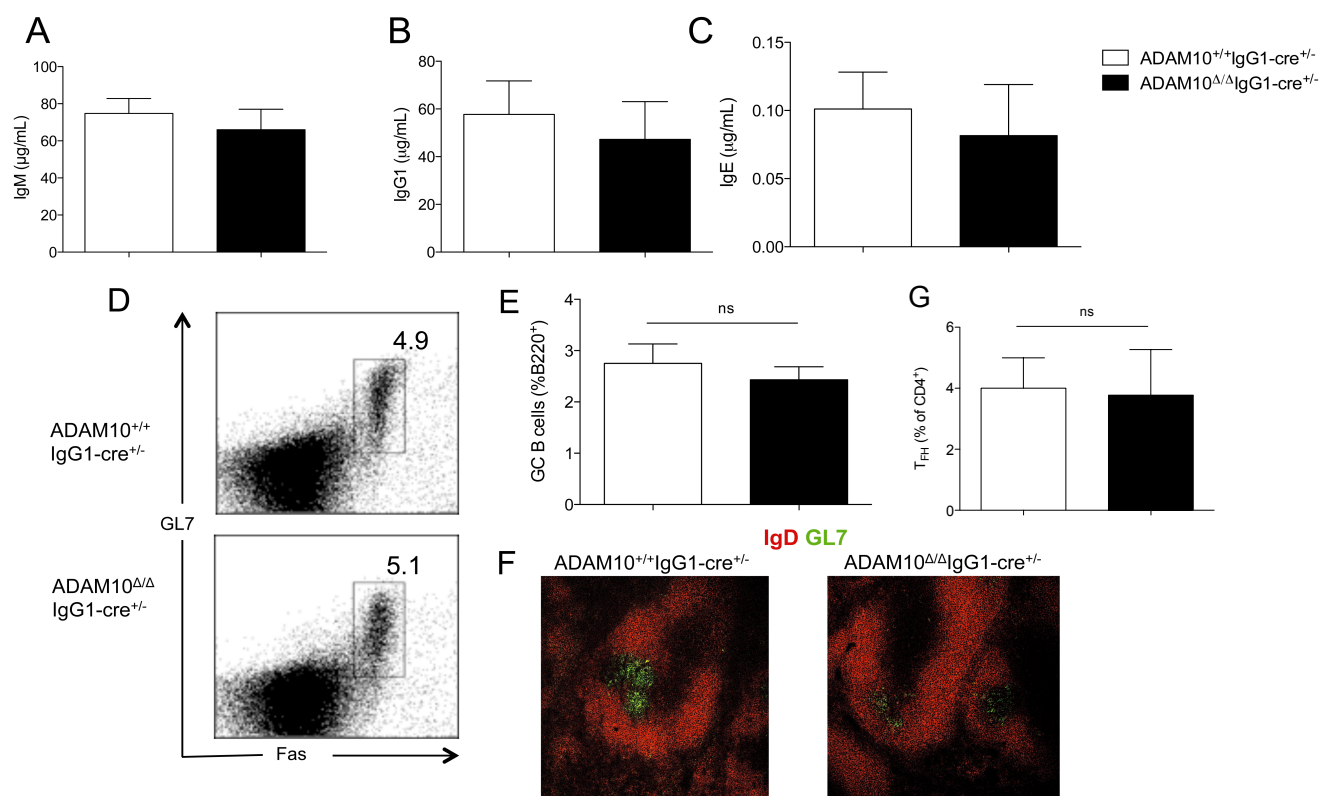
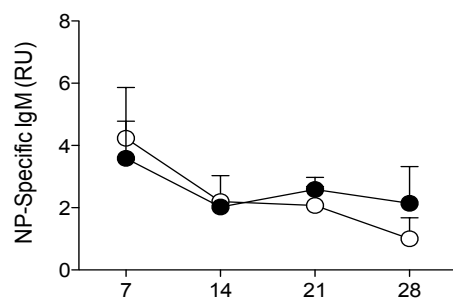


Figure 32. ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice show impaired antibody responses to T-dependent antigens.

ADAM10^{Δ/Δ}IgG1-cre^{+/-} (●) and controls (○) were immunized with NP-KLH emulsified in alum. At the indicated times, serum samples were collected and NP-specific (A) IgM and (B) IgG1 were measured by capture ELISA. Bars represent the mean ± SE of 5-9 mice per group (*p<0.05). Data represent results obtained in at least two independent experiments.

A



B

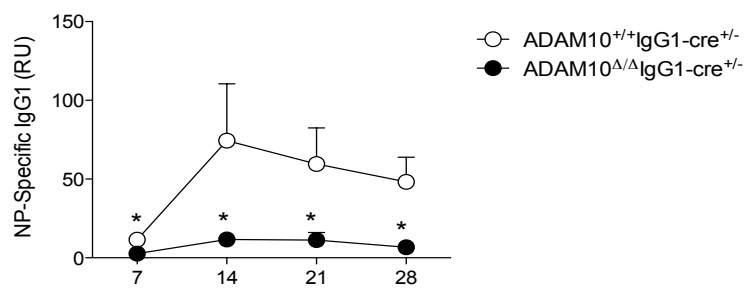


Figure 33. ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice show impaired antibody responses to T-independent antigens.

ADAM10^{Δ/Δ}IgG1-cre^{+/-} (●) and controls (○) were immunized with NP-LPS. At the indicated times, serum samples were collected and NP-specific (A) IgG3 and (B) IgG1 were measured by capture ELISA. Bars represent the mean ± SE of 5-9 mice per group (*p<0.05, **p<0.01). Data represent results obtained in at least two independent experiments.

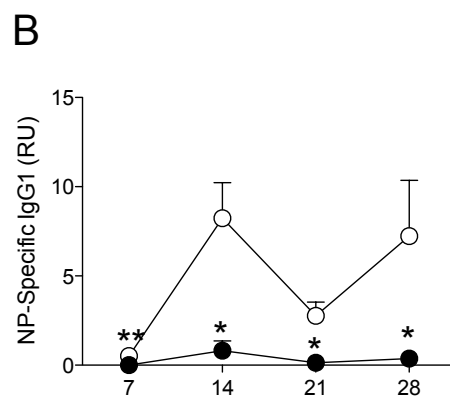
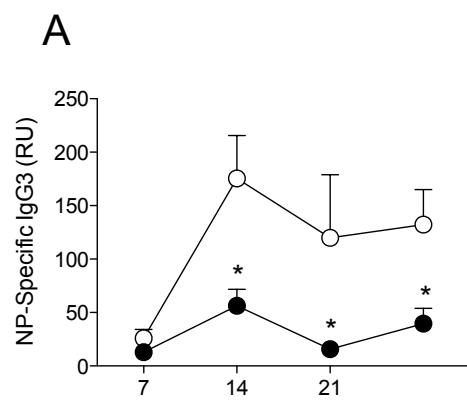


Figure 34. ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice have normal plasma cell percentages.

ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ and controls were immunized with NP-KLH emulsified in alum. Twenty-one days following immunization, tissues were harvested and PC numbers were analyzed. (A) Representative FACS staining of ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ (bottom) and controls (top).. Quantified results from (B) spleen, (C) peripheral blood and (D) bone marrow. Bars represent the mean \pm SE of 4-5 mice per group. Data represent results obtained in at least two independent experiments.

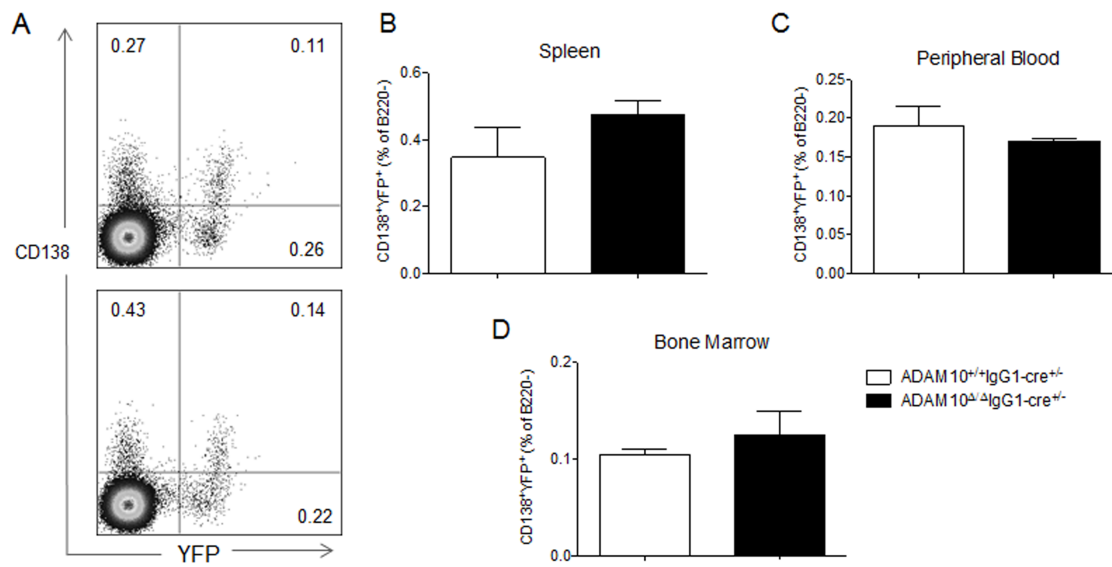


Figure 35. ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice have decreased numbers of IgG1⁺ cells.

ADAM10^{Δ/Δ}IgG1-cre^{+/-} and controls were immunized with NP-KLH emulsified in alum. Fourteen days following immunization, spleens were harvested and the percentage of B220⁺IgD^{lo}IgG1⁺ was quantified by flow cytometry. Quantified results are depicted. Bars represent the mean \pm SE of 4-5 mice per group. Data represent results obtained in at least two independent experiments. (*p<0.05)

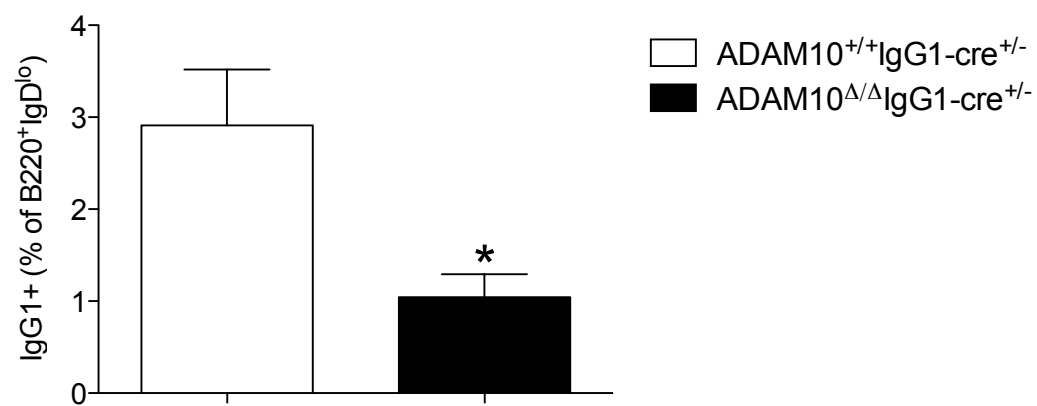


Figure 36. Plasma Cells isolated from ADAM10^{Δ/Δ}IgG1-cre^{+/-} have altered gene expression.

ADAM10^{Δ/Δ}IgG1-cre^{+/-} and controls were immunized with NP-KLH emulsified in alum. Twenty-one days following immunization, splenic PCs were isolated via magnetic bead isolation. mRNA was isolated and (A) *Prdm1*, (B) *Irf4*, (C) *Xbp1* and (D) *Bcl6* message levels were determined by qPCR. (E) The ratio of *Prdm1* to *Bcl6* was calculated. Bars represent the mean ± SE of 3 independent studies; cells from 3 mice from each genotype were pooled in each study. (*p<0.05, **p<0.01, ***p<0.001).

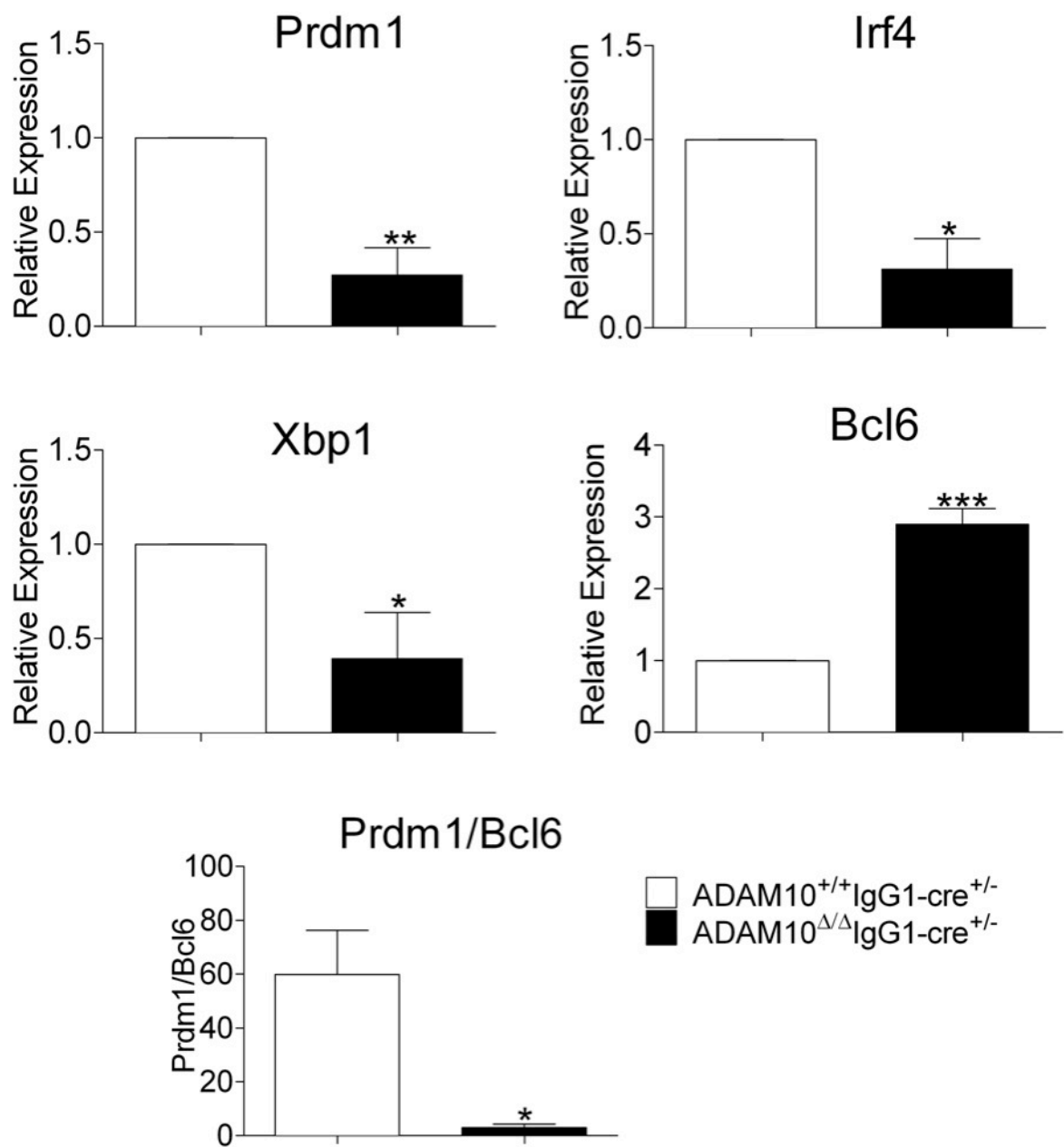


Figure 37. Plasma cells isolated from ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice express Bcl6 protein

ADAM10^{Δ/Δ}IgG1-cre^{+/-} and controls were immunized with NP-KLH emulsified in alum. Twenty-one days following immunization, splenic plasma cells were analyzed by Bcl6 protein expression via flow cytometry. (A) CD138⁺B220^{lo/-} B cells were gated and analyzed for either (B) non-specific staining (isotype) or (C) Bcl6 staining. (D) Quantified results are depicted. Bars represent the mean ± SE of 4-5 mice. (**p<0.01).

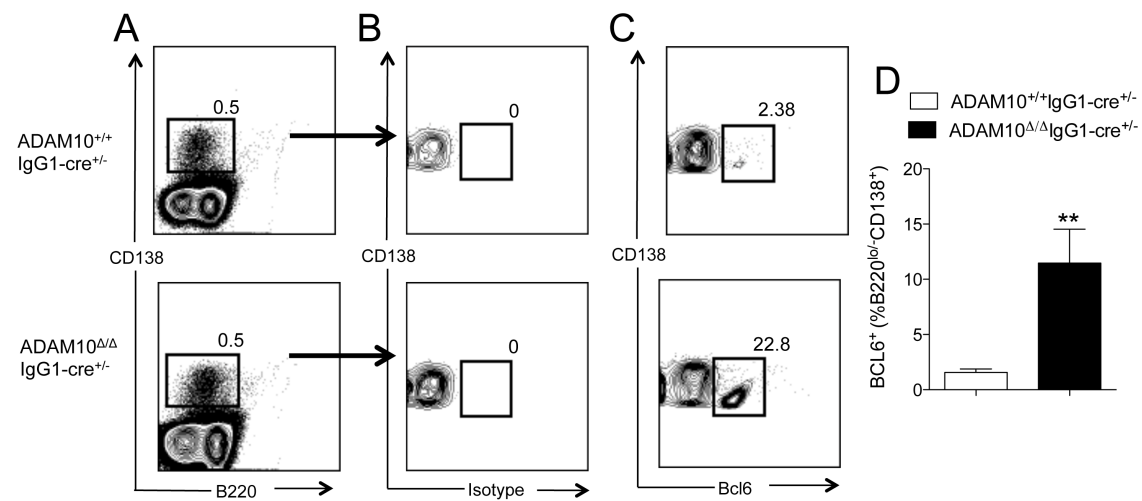
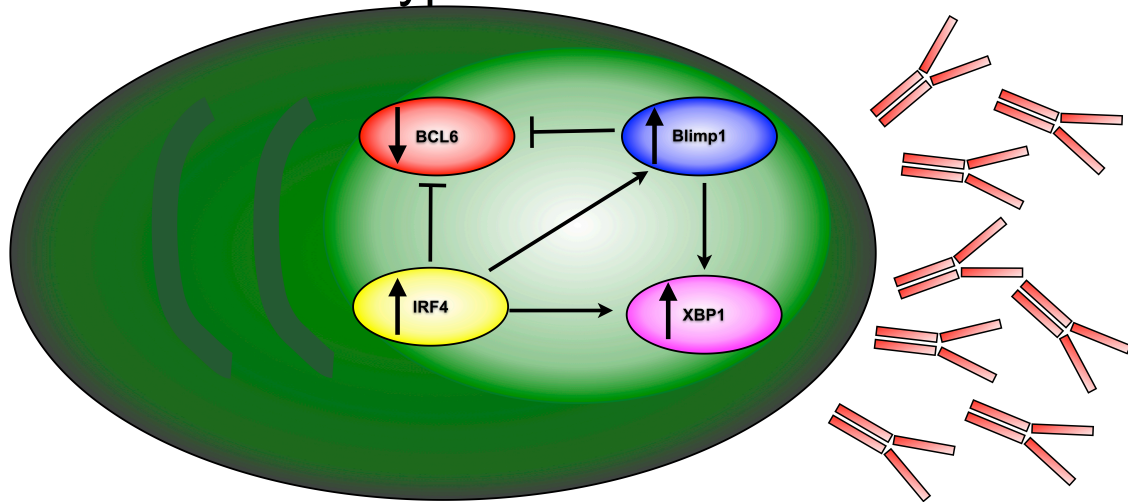


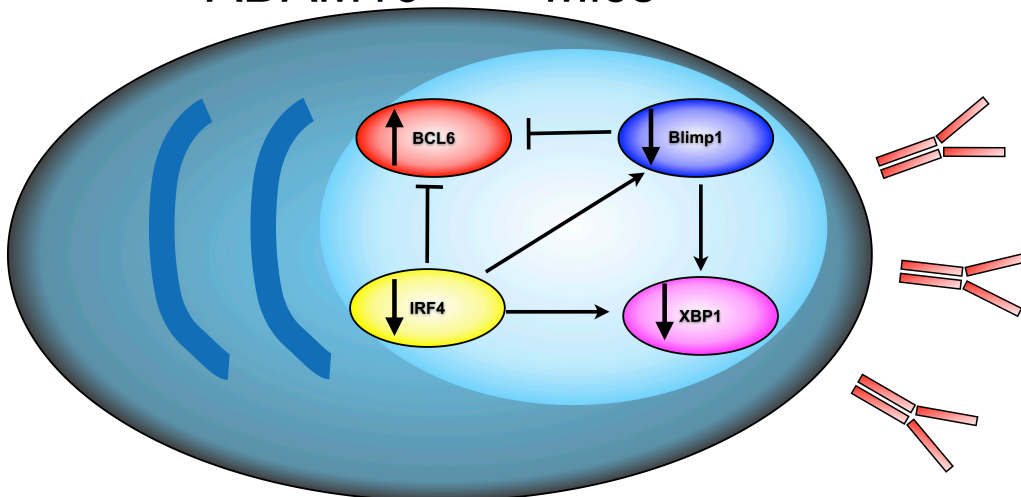
Figure 38. Proposed Model: Adam10 Regulations Of Plasma Cell Function

Wild type plasma cells express much higher levels of Blimp-1, IRF4 and XBP-1 that at previous developmental stages, while Bcl6 is repressed. This allows for antibody secretion. In the case of ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice, Bcl6 levels are much higher than seen in wild type. Moreover, Blimp-1, IRF4 and XBP-1 expression are decreased, leading to impaired antibody secretion

Wild Type Mice



ADAM10^{IgG1}^{-/-} Mice



3.5 Discussion

Members of the ADAM family regulate a wide range of functions, including cell migration, proliferation and adhesion²¹². ADAM10, in particular, has been recently shown to be critical for lymphocyte development through initiation of the canonical Notch signaling pathway (see Figure 3)^{24,174}. Here we report that ADAM10 is dispensable for early B cell development within the bone marrow. However, deletion of ADAM10 from all peripheral B cells or in post-switch B cells leads to severe impairments in antibody responses. When ADAM10 was deleted from all peripheral B cells a decrease in antigen specific IgG production was seen both with respect to serum levels and IgG ASCs, indicating that PC differentiation is influenced. Cells producing high affinity antibodies were particularly affected, consistent with defects in GC reactions and markedly repressed FDC-reticula. Moreover, changes in lymphoid architecture were also observed. On the other hand, when ADAM10 was deleted in post-switch B cells, GC formation and lymphoid architecture were not impaired. Despite normal architecture, however, antibody production was still affected, likely due to abnormal gene expression in ADAM10-deficient PCs. Consistent with this hypothesis, PCs isolated from ADAM10^{Δ/Δ}IgG1-cre^{+/-} showed decreased expression of genes that facilitate plasma cell differentiation and function and increased expression of Bcl6, an inhibitor of PC differentiation.

ADAM10 is dispensable for early B cell development

ADAM10 is critical for the development of T cells due to its role in Notch1 signaling. Studies revealed that ADAM10-deficient common lymphoid progenitor cells (CLPs) failed to develop into T cells, while B cell development was not impaired¹⁷⁴. We decided to carry out similar experiments in order to assess whether ADAM10 is needed for early B cell development by knocking out ADAM10 in a B cell specific manner at the pro-/pre-B cell stage. Our results demonstrate that ADAM10 is dispensable for immature B cell development. Consistent with this finding, it has been recently reported that the use of an ADAM10 inhibitor in an *in vitro* system of hematopoiesis did not affect B cell development¹⁹³.

Impaired Notch signaling and increased CD23 expression are not likely to contribute to the phenotype observed in ADAM10^{B-/-} mice

As previously mentioned, here we demonstrate that ADAM10 is essential for mature B cell function. ADAM10^{B-/-} mice showed severely impaired humoral responses and alterations in lymphoid tissue architecture. In order to determine the mechanism underlying the observed phenotype, we investigated the role of two well described B cell expressed ADAM10 substrates: Notch and CD23^{24,174,182}. The role of Notch signaling in mature B cell function is controversial. While some studies have clearly shown a role for Notch in antibody production, others failed to identify such a role^{177,180}. In order to assess the role of Notch signaling in lymphoid tissue organization and antibody production, we

expressed N2ICD transgene in a B cell specific manner, by conditionally expressing N2ICD in a cre-dependent manner, under the control of CD19-cre. We have demonstrated that restoration of Notch2 signaling in ADAM10^{B-/-} mice is not sufficient to rescue antibody production or GC formation. Studies have shown that N2ICD and N1ICD have redundant functions. Our results, therefore, suggest that the phenotype observed in ADAM10^{B-/-} mice is not dependent Notch1 nor Notch2 signaling²¹³, however, whether impaired Notch1 signaling contributes to the phenotype observed in ADAM10^{B-/-} mice should be formally studied.

Another feature of ADAM10-deficient B cells is decreased expression of CD21²⁴. Since Notch signaling enhances CD21 expression, the finding that the introduction of the N2ICD transgene failed to rescue antibody production and GC formation in ADAM10^{B-/-} mice also suggests that CD21 is not involved in the ADAM10^{B-/-} phenotype, N2ICD transgene expression led to increase CD21 expression. In addition, unlike CD21/CD35^{-/-} mice, ADAM10^{B-/-} mice had impaired affinity maturation and failed to mount antibody responses comparable to controls following immunization even at large antigen dosages (i.e. 1mg)²¹⁴.

CD23, also known as the low affinity IgE receptor, is another B cell expressed ADAM10 substrate¹⁸². We have recently demonstrated that CD23 surface expression is significantly enhanced after deletion of ADAM10 in B cells²⁴. Defects observed in ADAM10^{B-/-} could result from CD23 overexpression. Previous studies with CD23Tg mice, however, demonstrated that elevated CD23 levels resulted in inhibition of IgE and IgG1 production, while other classes were not significantly affected. On the other hand, in ADAM10^{B-/-} mice, the production

of all classes of IgG was impaired. Moreover, when immunized with NP-KLH, CD23Tg mice had Ig levels comparable to that of WT. In contrast, ADAM10^{B-/-} mice showed diminished levels of both low and high affinity anti-NP antibodies. These results suggest that elevated CD23 levels do not explain the phenotype of ADAM10^{B-/-} mice. However, in order to demonstrate that the phenotype observed in ADAM10^{B-/-} mice is CD23-independent, we are generating mice that lack both CD23 and B cell expressed ADAM10 by mating CD23^{-/-} mice and ADAM10^{B-/-} mice²¹⁵. It is possible that another B cell expressed ADAM10 substrate is responsible for the phenotype observed in ADAM10^{B-/-} mice.

ADAM10 is important for B cell follicle formation and maintenance of lymphoid architecture

In lymphoid organs, the correct positioning of B cells and T cells as well as the migration during an immune response are critical for adequate interaction between them²⁸. As previously mentioned, interactions between cognate B cells and T cells play an essential role for adequate responses to T-dependent antigens. Our results demonstrate that while B cell expressed ADAM10 is dispensable for B cell homing during LN and spleen development, ADAM10 is critical for the maintenance of B cell and T cell positioning subsequent to immune stimulation. However, ADAM10 also is needed for normal B cell follicle formation, as a thinning of B cell follicular areas was observed in LN of ADAM10^{B-/-} mice. The aberrant B cell follicle structure is accompanied with excessive collagen deposition and a relative increase in FRCs.

TNF- α is another ADAM10 substrate and it has been implicated in the formation of primary B cell follicles and GCs¹⁸⁵. Unlike ADAM10^{B-/-} mice, TNF- α deficient mice have a more dramatic disruption of splenic architecture prior to immunization¹⁴. However, TNF- α deficient or TNF- α receptor-deficient mice also had normal B cell homing but defective B cell follicle formation^{216,217}. Moreover, studies have demonstrated that secreted TNF- α is important for lymph node architecture, while membrane bound TNF- α is sufficient to support normal splenic architecture development²¹⁷. Furthermore, B cells were found to be an essential source of secreted TNF- α during LN development²⁰². Interestingly, despite normal TNF- α expression, ADAM10-deficient B cells secreted significantly less TNF- α than WT B cells following *in vitro* stimulation. These results suggest that the abnormal appearance of B cell follicles in LN of ADAM10^{B-/-} is likely due to a defect in B cell TNF- α shedding.

Lymphotoxin-alpha (LT) is another TNF- α family member that is involved in lymphoid architecture. The phenotype of ADAM10^{B-/-} mice is also different to that seen with LT-deficient mice, however, as LT-deficient mice lack peripheral LN and have severely disrupted splenic architecture¹². Interestingly, LT has been shown to be important for the maintenance of splenic architecture in adult mice²¹⁸. These results suggest that during an active immunization, lymphotoxin production by ADAM10-deficient B cells might be impaired.

CCL21, CCL19 and CXCL13 have been shown to play a key role in inducing and maintaining normal cellular compartments¹⁸. Draining LNs from ADAM10^{B-/-} mice showed increased CCL21 mRNA expression. This increase in

CCL21 was also confirmed at the protein level by immunohistochemistry. Interestingly, ADAM10^{B-/-} mice had a larger area of CCL21 staining, consistent with the aberrant T cell localization seen in draining LNs. The cells responsible for CCL21 production in LN are fibroblastic reticular cells (FRCs)^{219,220}. Despite having normal CCL21 mRNA levels, compared to WT LN, FRCs made up a larger percentage of the stromal cells present in LN of ADAM10^{B-/-} mice. Recent studies have demonstrated that following immunization, FRCs expand in a B cell dependent manner. Therefore, it is likely that due to the basal increase in FRCs observed in ADAM10^{B-/-} LN, an increase in CCL21 is also apparent. FRC numbers following immunization should be studied in ADAM10^{B-/-} mice.

FDCs are important for GC development, CSR, SHM and affinity maturation as well as induction of recall responses^{105,221-225}. FDCs also secrete CXCL13 and are thus important for recruitment of CXCR5-expressing B cells and T cells into the follicle²²⁶. Moreover, they are required for the maintenance of B cell and T cell zones²⁸. The loss of B cell and T cell segregation and the lack of GCs in the draining secondary lymphoid tissues of ADAM10^{B-/-} mice are thus consistent with a decreased number of FDC networks. The fact that CXCL13 mRNA levels were not altered, despite the lack of FDCs was surprising. However, recent published reports have also noted that CXCL13 expression is not affected by FDC depletion²²⁷. Together, these results challenge that idea that FDCs are the main producer of CXCL13.

Studies have demonstrated that FDCs are required for GC B selection within GCs. If GC B cells fail to interact with immune complexes displayed in the

surface of FDCs, the cells will undergo apoptosis⁹⁶. FDC-mediated GC B cell selection is consistent with the finding that in the absence of FDCs GC B cell numbers are diminished, as observed in ADAM10^{B-/-} mice.

The changes in architecture correlate with the decrease in T_{FH} cells. T_{FH} cells provide B cell help and are thus essential for PC differentiation, memory cell generation and affinity maturation²²⁸. A decrease in the number of T_{FH} cells leads to decreased B cells help, thus leading to diminished isotype switching.

ADAM10 might regulate B cell activation

Decreased B cell activation is consistent with the phenotype we observe in the ADAM10^{B-/-} mice and it is likely to contribute to the defect in humoral responses. When B cells were stimulated with LPS, ADAM10-deficient B cells showed impaired antibody productions. However, when ADAM10-deficient B cells were stimulated with anti-CD40 and IL-4, antibody production was not affected. These results suggest that ADAM10 might be involved in B cell activation, but its involvement might be dependent on the specific stimulation. B cell activation *in vitro* occurs very differently from antigen-specific *in vivo* responses, thus making it difficult to correlate *in vitro* and *in vivo* findings. Discrepancies between *in vivo* and *in vitro* results might relate to differences in short lived and long-lived plasma cells. Moreover, *in vitro* differentiation assays often lead to a heterogeneous population of cells, displaying different degrees of activation thus making the study of B cell responses difficult. Furthermore, *in vitro* activation might not precisely reflect *in vivo* stimulation²²⁹.

ADAM10 is required for plasma cell function

The changes in architecture observed in ADAM10^{B-/-} mice did not allow us to discern whether ADAM10 is important for antibody production or if the defects in antibody responses were secondary to architectural changes. Here we demonstrate that mice with ADAM10-deletion in class-switched B cells (ADAM10^{Δ/Δ}IgG1-cre^{+/-} mice) have normal GC formation and show no changes in lymphoid architecture. These mice, however, showed defective antibody responses to T-dependent and T-independent antigens. Furthermore, we show that despite having normal PC percentages, defined as CD138-expressing cells, expression levels of transcription factors important for PC development, *Prdm1*, *xbp1* and *Irf4* were altered, while housekeeping gene levels remained unchanged. In addition, the GC transcription factor *Bcl6* was not properly downregulated in ADAM10^{Δ/Δ}IgG1cre^{+/-} mice. These results demonstrate that ADAM10 is required for proper downregulation of *Bcl6*, upregulation of *Prdm1*, *Irf4* and *Xbp1*. Subsequently, antibody secretion is abnormal in ADAM10^{fl/fl}IgG1cre^{+/-} mice. Thus ADAM10 is important for proper PC function *in vivo*. Interestingly, when gene expression was assessed in *in vitro* activated B cells, gene expression varied from sample to sample, thus making it difficult to discern differences between WT and ADAM10-deficient B cells. These results are consistent with the concept that *in vitro* activation leads to heterogeneous populations of cells²²⁹.

The transcription factor *Bcl6* is necessary for GC formation and B cell proliferation. While it is well established that *Bcl6* must be downregulated for PC

differentiation to occur²³⁰, the factors mediating its downregulation are not fully understood. It has been previously demonstrated that STAT3, downstream of IL-21, can trigger Blimp-1 expression by competing with Bcl6 for DNA binding sites¹⁰⁹. Moreover, it has been proposed that B-cell receptor (BCR) and CD40 signaling lead to Bcl-6 degradation¹¹⁰. Recent studies demonstrated that ectopic STAT3 signaling could induce Blimp-1 expression even in the presence of high Bcl-6 levels; however, PC differentiation did not proceed until Bcl6 levels were reduced¹⁰⁹. Here we demonstrate that ADAM10 is essential for Bcl6 downregulation at least *in vitro* and that ADAM10 deficiency leads to impaired antibody responses.

Studies have demonstrated that IL-6 signaling can induce Bcl6 expression^{99,103}. In the context of a GC, IL-6 is produced by activated FDCs and promotes SHM and affinity maturation¹⁰⁵. Given that ADAM10 is responsible for the cleavage of the IL-6 receptor (IL-6R)¹⁸³, one could speculate that increased IL-6R expression could lead to increased IL-6 signaling, thus leading to Bcl6 overexpression.

Along with defective Bcl-6 downregulation observed in PCs isolated from ADAM10^{B-/-} mice, ADAM10 deletion also resulted in decreased levels of *Prdm1*, the gene encoding for Blimp-1, *Irf4* and *Xbp-1*. Blimp-1 and IRF4 lead to cell cycle arrest and promote PC development¹³³, while XBP-1 is required for endoplasmic reticulum (ER) expansion and immunoglobulin production and secretion¹³⁴. Moreover, Blimp-1 and IRF4 permit XPB1 expression^{132,134}. Furthermore, it has been very well documented that Bcl-6 can inhibit Blimp-1⁷¹. It

is therefore possible that ADAM10 is involved in a pathway that controls both Blimp-1 and IRF4 expression, and decreased Blimp-1 and IRF4 then leads to decreased XBP-1 expression.

As demonstrated in this study, humoral responses were severely impaired in ADAM $\Delta\Delta$ IgG1-cre $^{+/-}$ mice. Surprisingly, the number of PCs was comparable between ADAM $\Delta\Delta$ IgG1-cre $^{+/-}$ mice and controls. Previous studies revealed a similar phenotype when humoral responses were assessed in B cell specific XBP-1-deficient mice. As previously mentioned, XBP-1 is a transcription factor involved in ER-stress and is required for antibody-secretion. PCs isolated from ADAM $\Delta\Delta$ IgG1-cre $^{+/-}$ mice had decreased *XBP-1* message levels, suggesting that the phenotype observed in ADAM $\Delta\Delta$ IgG1-cre $^{+/-}$ mice is partly due to decreased XBP-1 expression and impaired antibody secretion.

As previously mentioned, ADAM10 is critical for Notch1 and Notch2 cleavage and the initiation of the canonical Notch signaling pathway (see Figure 2)^{24,174}. The role of Notch signaling in GC formation and PC differentiation, however, remains controversial. *In vivo* studies of B cell specific RBP-Jk-deficient mice, a key mediator of Notch signaling, failed to reveal a defect in antibody production¹⁸⁰. Consistent with this finding, a recently published report demonstrated that B cell-specific Notch2-deficient mice have normal GC formation²³¹. Moreover, mice with constitutively active Notch2 intracellular domain (Notch2-ICD) showed defective antibody responses to T-dependent and T-independent antigens and impaired GC formation²³². On the other hand, *in vitro* studies have demonstrated that Notch signaling enhances B cell activation

and supports B cell survival¹⁷⁸. Moreover, recent studies have shown that Notch signaling synergizes with B-cell receptor (BCR) and CD40 signaling to enhance murine B cell activation¹⁷⁷. Further studies will be needed to elucidate the role of Notch1 and Notch2 in PC differentiation and function and to determine whether the phenotype observed in ADAM^{Δ/Δ}IgG1-cre^{+/-} mice results from defective Notch signaling.

ADAM10: Not just a sheddase

Cleavage of membrane-bound proteins, such as Notch receptors, is not the only way that ADAM10 is capable of modulating gene expression. Recent experiments have demonstrated that ADAM10 not only mediates receptor intramembrane proteolysis (RIP), but it is also subject to RIP. ADAM9 and ADAM15 have been identified as the proteases responsible for releasing the ADAM10 ectodomain, while gamma-secretase mediates the release of the ADAM10 intracellular domain (ICD). ADAM10-ICD then translocates to the nucleus and modulates gene expression¹⁶². Studies have demonstrated that nuclear ADAM10 can interact with androgen receptors and act like a transcription factor²³³. It is thus possible that ADAM10 regulates Bcl-6 expression and PC function, not by shedding of membrane proteins but through its involvement in gene regulation. These recent findings really expand the potential roles for ADAM10 in regulating cellular functions. ADAM10-ICD regulation of gene expression will require further study.

Several reports have demonstrated that ADAM10 and a few of its substrates are present in exosomes, which are small secreted vesicles^{159,160}. It is possible that ADAM10-expressing B cell secreted exosomes play a role in regulating GC formation and PC differentiation. However, further experiments are necessary to determine the mechanism by which B cell expressed ADAM10 controls humoral responses, lymph node development and the maintenance of lymphoid architecture.

Conclusions and future directions

In conclusion, while ADAM10 is dispensable for early B cell development, our studies have demonstrated that ADAM10 is critical for the maintenance of splenic and lymph node architecture during active immune responses. ADAM10-deletion from all peripheral B cells (ADAM10^{B-/-} mice) led to decreased numbers of T_{FH} cells, leading to diminished T cell help and impaired GC formation, affinity maturation and CSR. On the other hand, deletion of ADAM10 in GC B cells does not impair GC formation. However, despite normal PC numbers, ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ mice showed impaired antibody responses to T-dependent and T-independent antigens. Gene expression analysis demonstrated that PCs isolated from ADAM10^{Δ/Δ}IgG1-cre^{+/-}YFP⁺ mice expressed lower levels of *Prdm-1*, *Irf4* and *Xbp-1* and expressed 3-fold more *Bcl6* than WT counterparts. These results thus demonstrate that ADAM10 is important for PC function, independently of the changes in architecture observed in ADAM10^{B-/-} mice.

While the studies described here have really expanded our knowledge of the role of B-cell expressed ADAM10 in B cell biology and the development and maintenance of lymphoid tissue architecture, there are many questions that have not been addressed. While we know that ADAM10-mediate B cell follicle formation, likely through TNF- α shedding, this has not been proven. Mixed bone marrow chimera with WT and ADAM10^{B-/-} BM and ADAM10^{B-/-} and TNF- α ^{-/-} BM will be used to determine whether impaired TNF- α release in ADAM10^{B-/-} mice results in impaired B cell follicle formation. While LN from WT/ADAM10^{B-/-} mice should be normal because WT B cells can provide enough TNF- α ^{-/-} to allow for normal B cell follicle formation, we would expect that LN from ADAM10^{B-/-}/TNF- α ^{-/-} would have impaired follicle formation, as ADAM10-deficient B cells nor TNF- α -deficient B cells could release TNF- α and support B cell follicle formation.

The work described here focuses on the role of ADAM10 in conventional or B2 B cells. Another B cell subset are B1 B cells. B1 B cells are located mostly within the peritoneal cavity and produce natural antibodies. Natural antibodies are antibodies that recognize very common bacterial antigens, such as phosphorylcholine²³⁴. It would be interesting to determine whether ADAM10 also plays a role in B1 B cell function.

Secondary lymphoid organs are positioned such that they facilitate immune responses to foreign antigens. In states of chronic inflammation, lymphoid cell aggregates that have a similar organization to secondary lymphoid organs can develop; these structures are known as tertiary lymphoid structures. These structures have been described in patients with atherosclerosis and

rheumatoid arthritis²⁸. Tertiary lymphoid organs have organized B cell compartments, which often include GCs, and T cell compartments, in which antigen-presenting cells can be detected. When tertiary organs develop at sites where autoantigens are continuously being presented, they may lead to unnecessary tissue destruction by facilitating the activation of autoreactive lymphocytes²⁸. Our results demonstrate that ADAM10 is critical for the structural maintenance of secondary lymphoid organs during active immune responses and thus, suggest a role for ADAM10 in the maintenance of tertiary lymphoid organs. Further experiments are needed to determine whether ADAM10 plays a role in tertiary lymphoid tissue formation and whether ADAM10 is a potential therapeutic target for the treatment of diseases involving tertiary lymphoid tissues. As complete loss of ADAM10 is lethal *in utero*¹⁵⁸, systemic ADAM10 blockade could have severe side effects. We have recently shown that intranasal administration of ADAM10 inhibitors reduced symptomology in the mouse asthma model without causing any obvious deleterious effects²³⁵. Thus, it may be possible to use local ADAM10 inhibition in some B-cell mediated autoimmune scenarios to decrease autoimmune symptoms. ADAM10 inhibition is not only likely to affect tertiary lymphoid structure, but also to affect autoantibody production within these tissues.

CHAPTER 4: FYN IS REQUIRED FOR OPTIMAL GERMINAL CENTER FORMATION AND ANTIBODY PRODUCTION

4.1 Introduction to Fyn

The gene *SH2D1A* encodes for SLAM-associated protein (SAP). Mutations in this gene resulting in SAP-deficiency or SAP protein destabilization cause X-linked lymphoproliferative disease (XLP). Patients with XLP often have difficulties clearing infections, such as EBV, measles virus and *Neisseria meningitides*. They also show progressive hypogammaglobulinemia and increased incidence of B cell lymphomas²³⁶.

SAP interacts with SLAM (CD150) through binding to the cytoplasmic tail. Subsequently, SAP recruits the Src protein kinase Fyn. Studies have demonstrated that SAP directly binds Fyn via an unconventional SH3 domain-binding surface²³⁷⁻²³⁹. This binding requires arginine 78 residue (R78) on the SAP protein^{237,239}. Recruitment of Fyn leads to the phosphorylation of tyrosine residues in the cytoplasmic domain of SLAM. This phosphorylation event generates docking sites for several proteins and initiates signaling cascades²⁴⁰.

Fyn is a member of the Src protein tyrosine kinase. Fyn is widely expressed in many cell types, including lymphocytes²⁴¹. Fyn has been shown to interact with both the B cell and T cell receptor (BCR and TCR, respectively)^{242,243}. While Fyn-deletion did not impair the development of immature T cells and B cells, TCR signaling was altered in mature T cells^{143,244}.

Recent studies have suggested that SAP regulates NKT cell development in a Fyn-dependent and independent manner. Experiments demonstrated that

SAP-deficient mice have a reduction in the number of NKT cells. Mice expressing SAP R78A, a mutant SAP that is unable to bind Fyn (SAP^{R78A} mice), still had diminished NKT cell numbers, but the defect in NKT cell development was not as pronounced as that observed in SAP-deficient mice²⁴⁵.

Another feature of SAP-deficient mice is a severe impairment in GC development accompanied with impaired generation of memory B cells and long-lived plasma cells²⁴⁶. Through the use of conditional SAP-knockouts and adoptive transfer approaches, two groups demonstrated that T cell SAP expression was important for humoral responses^{67,246}. B cell specific SAP deficiency did not impair antigen-specific immunoglobulin production or GC formation. Indeed, GC B cell numbers were enhanced in SAP^{Δ/Δ}CD19-cre⁺ mice⁶⁷. Further studies demonstrated that SAP was dispensable for the interaction between T cells and DCs. However, SAP-deficient T cells were shown to be unable to make stable interactions with cognate B cells⁶⁶. It was later determined that these defects in stable B-T interactions resulted in impaired T_{FH} development and diminished T cell help^{247,248}.

Interestingly, it was demonstrated that SAP regulates T_{FH} development in a Fyn-independent manner, as evidenced by the fact that SAP^{R78A} mice had normal T_{FH} and GC B cell numbers^{247,249}. Experiments with Fyn-deficient mice (Fyn-KO) demonstrated that they are able to form GCs. The authors of these studies concluded that Fyn is dispensable for GC formation, however, how GC B cell numbers compare to that of wild type cannot be deduce from these studies being that Fyn-KO mice are in a B6.129 background and no B6.129 wild type

mice were studied in those experiments²⁴⁹.

The SAP-Fyn axis has also been demonstrated to mediate many aspects of T cell biology. Studies have demonstrated that both Fyn and SAP are important for Th2 differentiation. Studies have demonstrated that Fyn-KO and SAP-deficient T cells produced little to no IL-4 following anti-CD3 and anti-CD28 stimulation²⁵⁰. Moreover, it has been reported that T_{FH} cells present within GCs (GC T_{FH} cells) depend on the SAP-Fyn axis for IL-4 production²⁴⁸.

The data regarding the role of Fyn in cytokine production and Th2 cell differentiation is conflicting. While some have reported that Fyn-KO mice are impaired in IL-4 production²⁵⁰, other have reported that Fyn-KO T cells preferentially differentiate into Th2 cells²⁵¹. Furthermore, it was reported that Fyn-KO T cells produced increased levels of IL-4 but reduced IFN- γ levels^{251,252}. Consistent with these findings, Fyn-KO T cells showed exaggerated responses in a mouse model of allergic airway inflammation²⁵³. Interestingly, while naïve Fyn-deficient mice had reduced IgE levels, immunized mice had comparable IgE levels to that of WT²⁵³. Others have demonstrated that Fyn-KO mice have elevated IgE following immunization²⁵⁴. It is possible that Fyn is involved in the secretion of both IL-4 and IFN- γ and which cytokine is more impaired might depend on the situation.

While much of the research regarding Fyn in lymphocyte biology has focused on T cells, Fyn is also expressed in B cells²⁵⁵. As previously mentioned, Fyn interacts with the BCR. Moreover, Fyn interacts with the BCR co-receptor CD19 (See Chapter 1)²⁵⁶. Interestingly, Fyn-KO B cells showed only a mild

impairment in BCR signaling²⁵⁷. Moreover, humoral responses to T-dependent antigens 7 days following immunization were lowered in Fyn-KO mice, although the difference was not statistically significant, likely due to the fact that only few mice per group were used in these experiments²⁵⁷. On the other hand, Fyn-KO mice showed a marked decrease in Ig subsequent to T-independent antigens^{244,258}.

Fyn has also been associated with IL-5 receptor^{142,257}. IL-5 is a pleotropic cytokine. With regard to B cells, IL-5 signaling has been shown to contribute to induce IgA production by antigen-primed B cells and LPS stimulated B cells^{259,260}. Moreover, in the presence of IL-4, IL-5 can induce IgG1 and IgE secretion. It has been demonstrated that in combination with IL-4, IL-5 leads to the accumulation of $\gamma 1$ and ϵ chain transcripts²⁶¹. Fyn-KO B cells have been shown to have impaired IL-5 signaling¹⁴².

Fyn has also been shown to associate with CD23, the low affinity IgE receptor, also known as Fc ϵ RII. Studies of CD23-deficient and CD23-transgenic mice demonstrated that CD23 is the natural regulator of IgE production²⁶². The role of Fyn in CD23 signaling and IgE production should be carefully assessed.

While a role for Fyn in cytokine production and antibody responses has been reported, the role of Fyn in humoral responses remains controversial. We thus decided to examine humoral responses in Fyn-KO mice. Our results demonstrate that Fyn-KO mice have significantly low basal IgG1 and IgG2a. Additionally, these mice displayed delayed kinetics in the production of NP-specific IgG1 and IgG2b, and significantly low NP-specific IgG2a after a T-

dependent immunization protocol. Defects in antibody production correlated with significantly reduced numbers of GC B cells, T_{FH} cells and spleen plasma cells. Moreover, Fyn-KO B cells showed decreased antibody production following *in vitro* activation. Our results thus demonstrate that Fyn-mediated signaling is necessary for optimal humoral responses.

4.2 Results

Fyn deficient animals have low basal IgG1 and IgG2a

In order to better understand Fyn's role in humoral immunity, we analyzed antibody levels in the serum of naïve Fyn-KO and WT B6.129 mice. Compared to WT, Fyn-KO mice had normal IgM levels and IgG2b, but significantly decreased basal IgG1 levels and IgG2a (Figure 39). IgE levels were below the levels of detection in both WT and Fyn-KO mice. These findings implicated Fyn in antibody production.

Fyn kinase deficiency affects antigen-specific antibody production

To directly assess antigen-specific antibody production, Fyn-KO and WT mice were immunized i.p. with NP-KLH in alum and the generation of NP-specific antibodies was monitored by ELISA. Surprisingly, while Fyn-KO mice showed normal antigen-specific IgM responses (Figure 40A), they had a severely impaired NP-specific IgG2a response (Figure 40C). With regards to NP-specific IgG1 and IgG2b, Fyn-KO mice demonstrated a delayed in Ig production, but had

normal levels of NP-specific Ig four weeks following immunization (Figure 40B,D), suggesting abnormal antibody production kinetics.

Splenic PC numbers are reduced in Fyn deficient mice

Given the diminished antibody response observed in Fyn-KO mice, we hypothesized that CSR or PC development were impaired in these mice. In order to determine whether PC generation was abnormal in Fyn-KO mice, we immunized mice with NP-KLH and determined the number of PC by flow cytometry 14 days post-immunization. Consistent with the defects in antibody production, Fyn-KO mice showed decreased in the proportion of splenic PCs (CD138⁺B220^{lo/-} cells) (Figure 41).

GC formation is impaired in Fyn deficient mice

A large portion of PCs in response to T-dependent antigens are derived from GCs, we therefore assessed GC formation in Fyn-KO mice. GC B cells, defined as B220⁺GL7⁺Fas^{hi} were enumerated by flow cytometry 14 days following NP-KLH immunization. Our results demonstrate that 14 days post-immunization, Fyn-KO mice showed significantly decreased levels of GC B cells compared to WT controls (Figure 42).

T_{FH} formation is impaired in Fyn deficient mice

T_{FH} cells provide B cell help via CD40L, IL-21 and IL-4. They play a key

role in GC B cells survival, affinity maturation, and plasma cell differentiation. T_{FH} cells are characterized by high expression of CXCR5 and programmed death-1 (PD-1). We therefore examined whether the decreased number on GC B cells was accompanied with decrease T_{FH} number. T_{FH} cells were enumerated by flow cytometry 14 days following NP-KLH immunization. Interestingly, T_{FH} numbers were significantly reduced in Fyn-KO mice (Figure 43). Our data reveal a novel role of Fyn kinase. Our experiments demonstrate that Fyn is required for optimal GC B cell and T_{FH} cell development or maintenance.

Fyn kinase is required for B cell antibody production in vitro

In order to determine whether Fyn-deletion affected antibody production in a B cell intrinsic manner, WT and Fyn-KO B cells were stimulated with anti-CD40 and IL-4. Proliferation and antibody production were then measured. Compared to WT B cells, Fyn-KO B cells produced significantly lower amounts of IgG1 and IgE (Figure 44A,B). These results demonstrate that, at least *in vitro*, Fyn-KOB cells have impaired activation. Interestingly, Fyn-KO B cells showed no alteration in proliferation (Figure 44C). It is likely that the phenotype observed *in vivo* results from both a B cell-intrinsic defect and impaired cytokine production.

Figure 39. Fyn deficient mice have impaired humoral responses.

Serum (A) IgM, (B) IgG1, (C) IgG2a and (D) IgG2b were measured by ELISA from non-manipulated 8-12 week old mice. Bars represent the mean \pm SE of 10 mice per group (** $p < 0.001$). Data represent results obtained in at least two independent experiments.

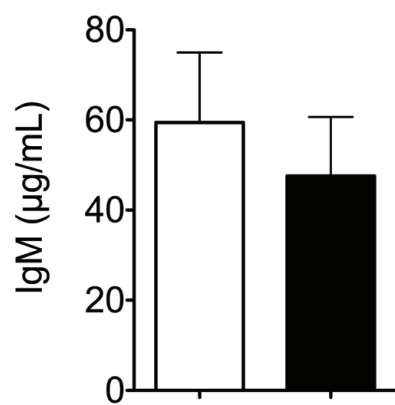
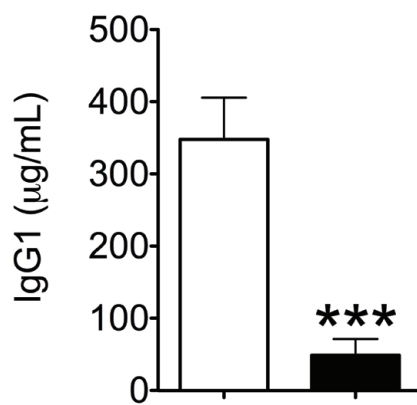
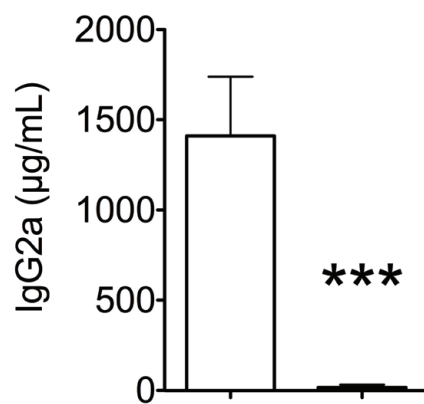
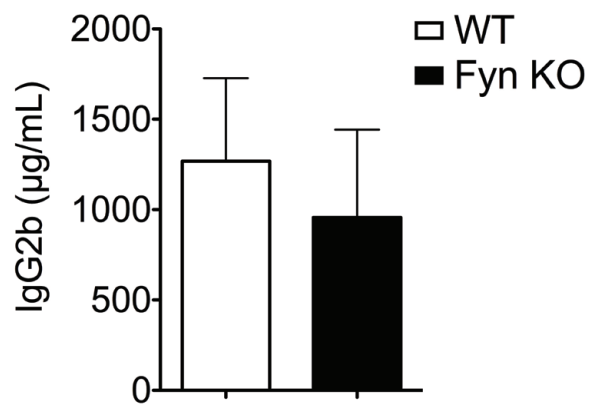
A**B****C****D**

Figure 40. Fyn-KO mice have impaired humoral responses to T-dependent antigens

Fyn-KO mice and WT controls were immunized with 10µg NP-KLH emulsified in alum. At the indicated times, serum samples were collected and NP-specific (A) IgM, (B) IgG1, (C) IgG2a and (D) IgG2b antibody titers were determined by ELISA. Bars represent the mean \pm SE of 5-9 mice per group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data represent results obtained in at least two independent experiments.

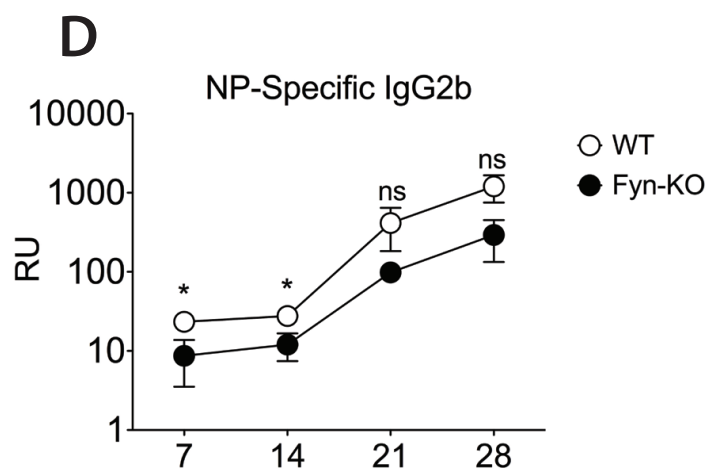
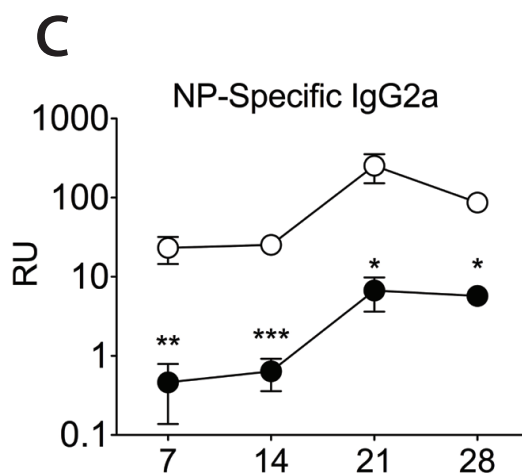
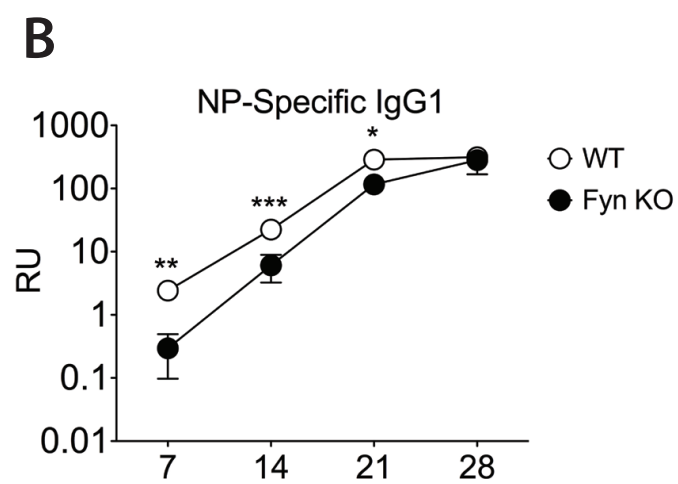
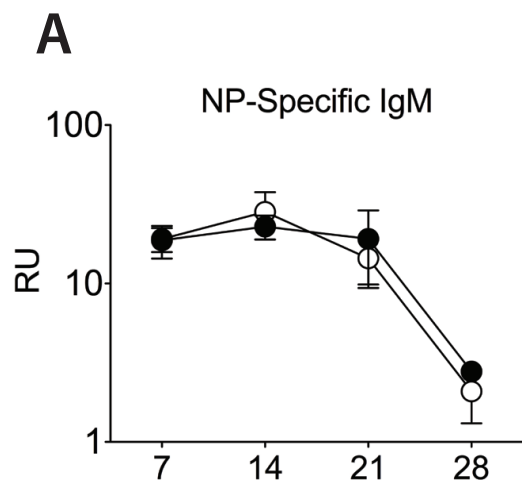


Figure 41. Fyn-KO mice have reduced plasma cell percentages.

Fyn-KO and WT mice were immunized with NP-KLH emulsified in alum. Twenty-one days following immunization, spleens were harvested and PC numbers were analyzed. (A) Representative FACS staining. (B) Quantified results from spleen. Bars represent the mean \pm SE of 6 mice per group. Data represent results obtained in at least two independent experiments. ($p < 0.01$)

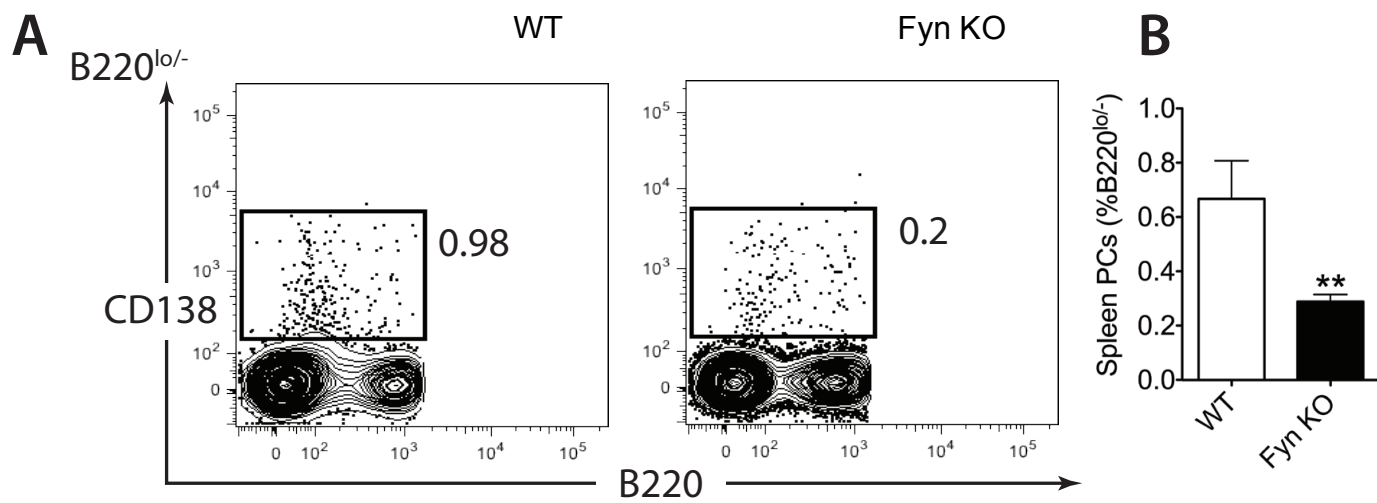


Figure 42. Fyn deficient mice have impaired germinal center formation.

Mice were immunized with NP-KLH emulsified in alum and 14 days post-immunization GC formation was assessed by flow cytometry. (A) Representative dot plot (gated on B220⁺ cells). (B) Quantification of GC B cells. Bars represent the mean \pm SE of 6 mice per group. Data represent results obtained in at least two independent experiments. ($p < 0.001$)

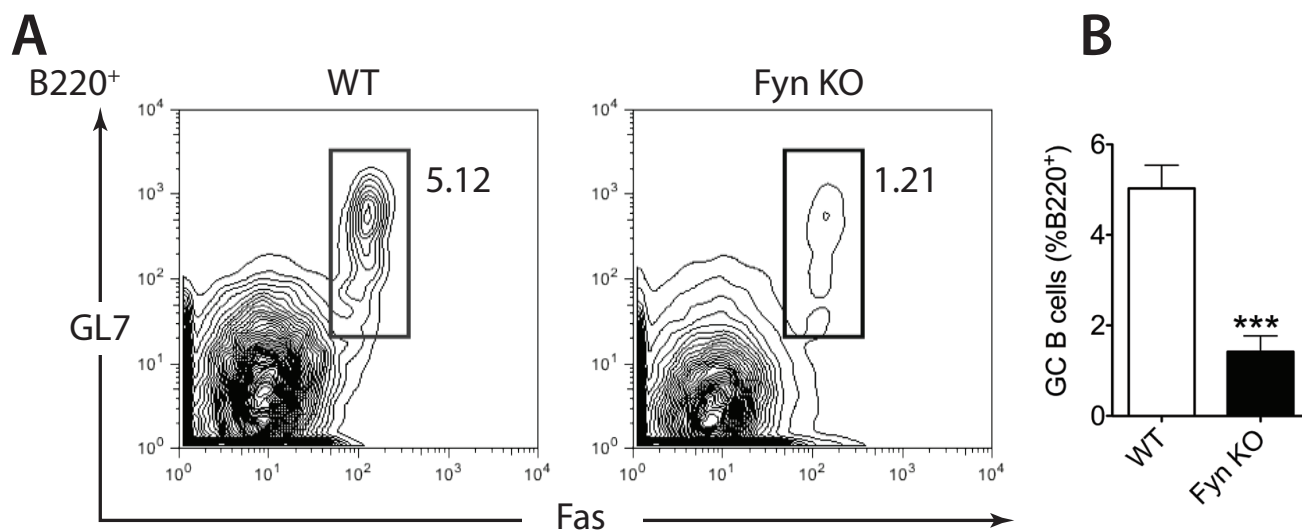


Figure 43. Fyn deficient mice have reduced T_{FH} numbers

Mice were immunized with NP-KLH emulsified in alum and 14 days post-immunization T_{FH} cell numbers were assessed by flow cytometry. (A) Representative dot plot (gated on CD4⁺ cells) (B) Quantification of T_{FH} cells. Bars represent the mean \pm SE of 6 mice per group. Data represent results obtained in at least two independent experiments. ($p < 0.001$).

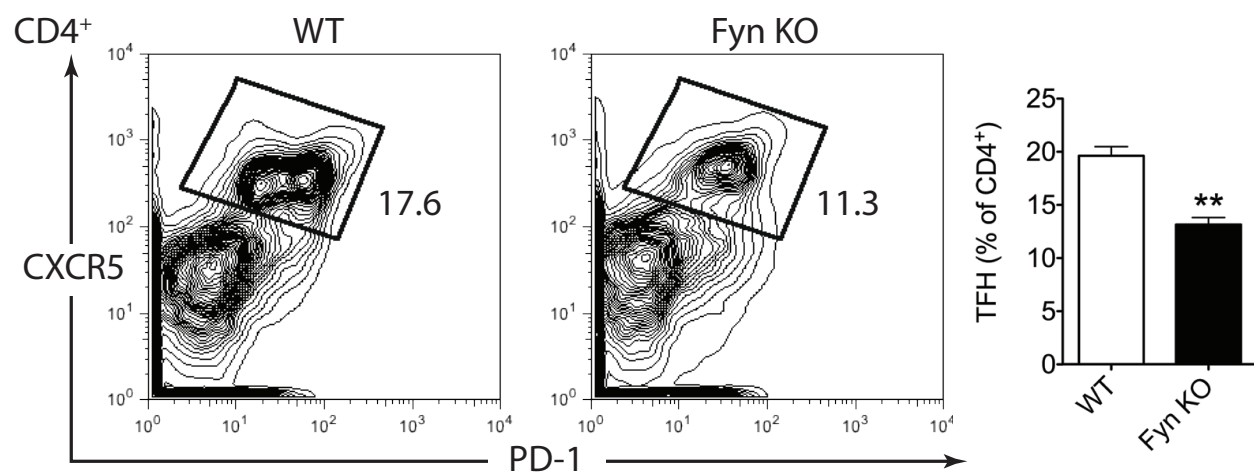
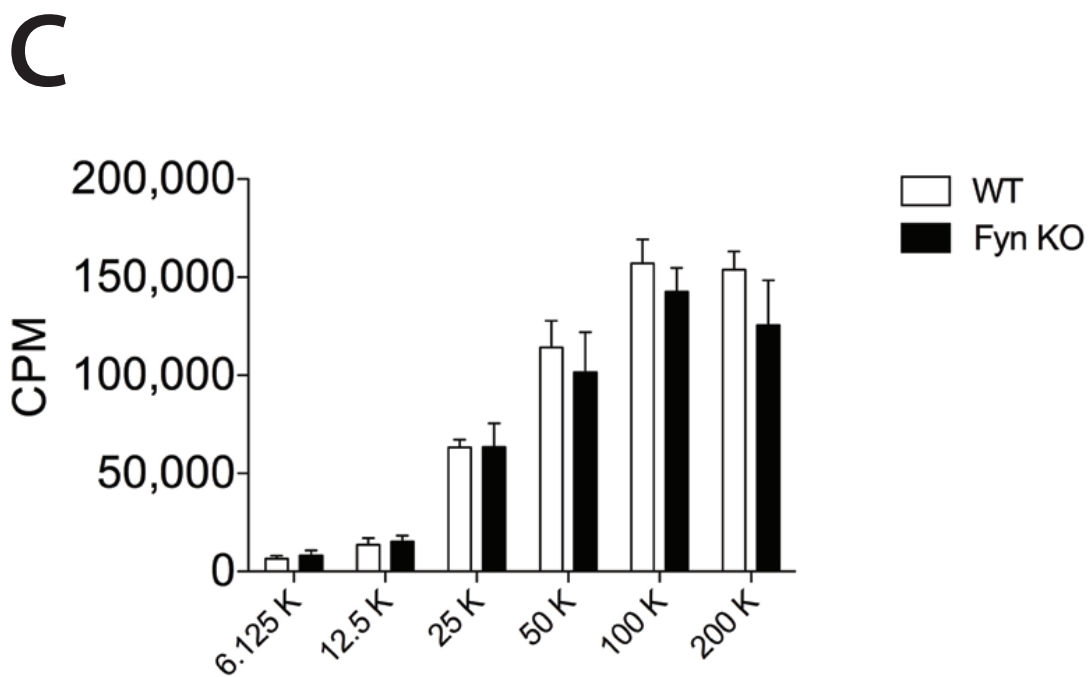
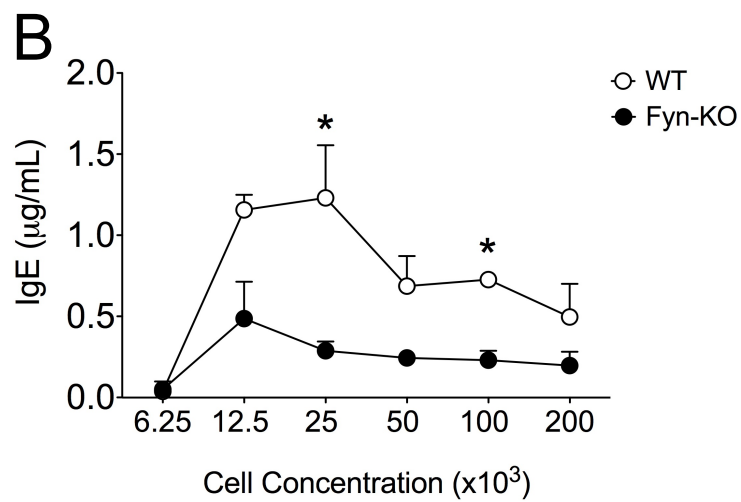
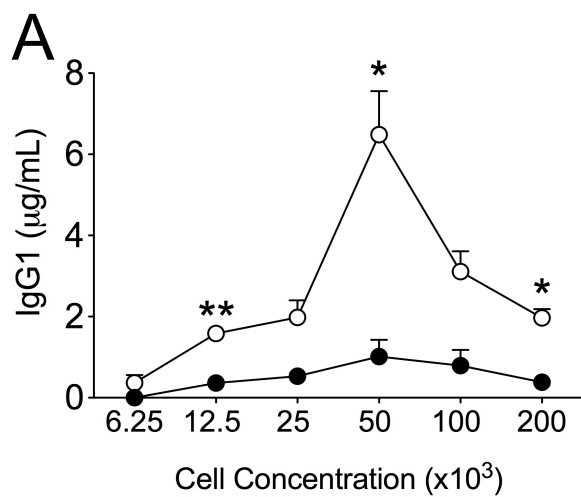


Figure 44. Fyn deficient B cells produce reduced antibody levels despite normal proliferation following *in vitro* stimulation.

B cells were isolated and cultured with IL-4 and anti-CD40L. Eight days following stimulation, supernatant were harvest and analyzed for (A) IgG1 and (B) IgE by ELISA. (C) Three days following challenge, tritiated thymidine was added to cells. Cells were incubated for 24 hours and then harvested. Thymidine incorporation was then measured. Experiment has been performed three times with similar results. (* $p < 0.05$, ** $p < 0.01$).



4.3 Discussion

Fyn is a member of the Src protein tyrosine kinase family. Previous work has demonstrated that Fyn-deficient T cells have impaired cytokine production that is accompanied by impaired antibody production. However, several conflicting reports have been published. Here we demonstrate that Fyn-KO mice showed decreased basal IgG1 and IgG2a levels. Additionally, following immunization, these mice had altered kinetics regarding NP-specific IgG1 and IgG2b productions. Moreover, Fyn-deficient mice displayed a dramatic defect in NP-specific IgG2a production. These defects correlated with significantly reduced numbers of GC B cells, T_{FH} cells and spleen PCs. Moreover, Fyn-KO B cells showed decreased antibody production following *in vitro* activation. Our results thus demonstrate that Fyn-mediated signaling is necessary for optimal humoral responses.

The role of Fyn in GC formation has been previously assessed. Cannons, et al. immunized C57B6 WT, 129 WT mice and Fyn-KO mice in a mixed C57B6.129 background. In these experiments, C57B6 WT mice had fewer GC B cells than 129 WT mice, while Fyn-KO mice had GC B cell numbers comparable to C57B6 WT mice. Based on these results, the authors concluded that Fyn was dispensable for GC formation²⁴⁹. However, it is difficult to draw conclusions from these experiments given that the mice were not background matched. Our results demonstrate that Fyn is important for GC B cell differentiation.

It has been previously reported that Fyn is involved in IL-4 secretion. While some report that Fyn-deficient T cells are unable to secrete IL-4, others

demonstrate that Fyn-deficient T cells are skewed toward a Th2 phenotype, results in increased IL-4 production and decreased IFN γ ^{250,251,254,263}. These cytokines promote the production of different Ig isotypes. While IL-4 promotes IgG1 and IgE class switching, IFN γ induces IgG2a production^{40-42,80}. Here we demonstrate that Fyn-KO mice have dramatically reduced basal and antigen-specific IgG2a levels. These results are consistent with the notion that IFN γ production is impaired in Fyn-KO mice.

We also noted that Fyn-KO mice have a delayed kinetics in the production of antigen-specific IgG1 and IgG2b. Moreover, these mice had fewer numbers of GC B cells following immunization. IL-4 has been implicated in GC formation. Indeed, IL-4-deficient mice have smaller GCs than WT mice²⁶⁴. Given the fact that Fyn is important for IL-4 production, it is possible that reduced IL-4 leads to fewer GC B cells resulting in impaired Ig kinetics.

Along with defective GC formation, we noticed a decrease in splenic PCs and T_{FH} cells. It is likely that they are both secondary to the decrease in GC B cells. Previous work demonstrated that SAP-Fyn interactions were dispensable for T_{FH} development, as T_{FH} cells developed normally in SAP^{R78A} mice²⁴⁹. Therefore, our results suggest that Fyn regulated T_{FH} differentiation in a SAP-independent manner.

Given that Fyn is involved cytokine production, it is hard to delineate whether the defects in immunoglobulin production observed result from B cell intrinsic defects or if they are due to altered cytokine production. However, the fact that Fyn-deficient B cells had impaired antibody production *in vitro* indicates

that Fyn plays a role in B cell activation. Interestingly, Fyn-deficient B cells proliferated normally, suggesting a role for Fyn in antibody class switching.

Future studies will be needed to identify the mechanism underlying the defects in humoral responses in Fyn-deficient mice. While our *in vitro* studies demonstrate a B cell intrinsic defect, we have not demonstrated a B cell defect *in vivo*. In order to identify whether the phenotype observed results from Fyn-deficiency in B cells or T cells, mice that lack lymphocytes (RAG2^{-/-} mice) will be reconstituted with a combination of WT B cells and Fyn-deficient T cells or vice versa. These mice will then be immunized and antibody responses will be assessed. It is likely that both B cell and T cell-expressed Fyn play a role in humoral responses.

Fyn-deficient mice have been studied extensively. It has been demonstrated that Fyn kinase is required for NKT cell development²⁴⁵. Moreover, it has been demonstrated that Fyn is also involved in T cell activation and cytokine production^{143,242,254}. Moreover, some studies have demonstrated that Fyn is important for antibody production *in vivo*, although others failed to identify such role^{252,254,265}. Our results further extend our knowledge of Fyn's role in lymphocyte biology. Here we demonstrate that Fyn is important for optimal humoral responses by regulating GC formation and possibly CSR. Moreover, our data show that Fyn mediates antibody production via B cell intrinsic and extrinsic manners.

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