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Germinal Center B Cell Expression Of Aire Regulates Antibody Diversification And Autoimmunity

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GERMINAL CENTER B CELL EXPRESSION OF AIRE REGULATES ANTIBODY DIVERSIFICATION AND AUTOIMMUNITY

by

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DEDICATION

To whom do I dedicate this charming new booklet, polished over the past several years with the coarse grain of hard work, perseverance, and long hours? To those who have supported me throughout my studies. To those who have challenged me, constantly, to improve myself both academically and personally. For these are the individuals who were accustomed to believe that my trifles were something. Thus, have for yourselves, whatever this is, and may it remain scientifically sound throughout the ages.

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It should go without saying that those who have given me the gift of their time must be acknowledged, but, nonetheless, I find that it is crucial I thank them for their service to my education and my development as a scientist. To the faculty who have devoted their careers to the training of students and have committed themselves to developing the future generation of scientists both within the institution and without, thank you for your continued support and dedication. To the lab personnel who have contributed to this work, thank you for your hard work. Finally, to the core facilities, and the people who run them, which have allowed me unfettered access to excellently maintained equipment, thank you for trusting me with such expensive toys. None of what has been written here would be possible without the support an expertise of the groups mentioned above as they are the foundation of the scientific edifice that we as early stage researchers are attempting to erect. Of course, omission of any individual in this brief paragraph is not due to a lack of gratitude, but, rather, due to my own forgetfulness.

PREFACE

Contained within these pages lies the dissertation, on which I have dedicated the majority of my effort during my graduate training, written for the purpose of fulfilling the graduation requirements of the Molecular Genetics and Genomics program at Wayne State University. I took over this project in the laboratory of Dr. Kang Chen after the departure of a previous research associate, Bo Pei. Together with Dr. Bihui Huang, a postdoctoral researcher at the time who assisted with many of the biochemical assays, we worked this project to completion, dedicating enormous effort to its success. Though the work was difficult, I felt that the opportunities I had to learn through, not just the hands on labor of performing experiments, but the highly cerebral activities of designing experiments, developing ideas, and discussing our work with other researchers at Wayne State and around the world, have allowed me to grow and mature into, I believe, a slightly more competent scientist than before.

To those who have decided to tolerate my prose and are still reading, I commend your ambition and wish you the best of luck in the coming chapters. The goal of my writing is to, hopefully, teach you a little bit about B cell biology and the mechanisms of antibody diversification. This will not be an easy task and has taken me several years to learn and, though my use of jargon and my bizarre syntax may be a bit overwhelming, I must insist that you embrace the suffering as I have these past years while constructing this opus and give me the pleasure of educating others as others have educated me.

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LIST OF ABBREVIATIONS

1. ADCC	Antibody Dependent Cellular Cytotoxicity
2. AID	Activation Induced Cytidine Deaminase
3. Aire	Autoimmune Regulator
4. APC	Antigen Presenting Cell
5. APE	AP Endonuclease
6. APECED	Autoimmune Polyendocrinopathy Candadiasis Ectodermal Dystrophy
7. APS-1	Autoimmune Polyglandular Syndrome Type 1
8. BCR	B Cell Receptor
9. BER	Base Excision Repair
10. BET	Bromodomain and Extraterminal Domain
11. Blimp-1	B-Lymphocyte-Induced Maturation Protein 1
12. CARD	Caspase Recruitment Domain
13. CBP	CREB Binding Protein
14. CCL	C-C motif ligands
15. CCR	C-C chemokine receptors
16. CD	Cluster of Differentiation
17. CDK9	Cyclin Dependent Kinase 9
18. CFA	Complete Freund's Adjuvant
19. CLP	Common Lymphoid Progenitor
20. CMP	Common Myeloid Progenitor
21. CRM1	Chromosomal Maintenance 1
22. CSR	Class Switch Recombination

23. cTEC	Cortical Thymic Epithelial Cell
24. DAMP	Danger Associated Molecular Pattern
25. DC	Dendritic Cells
26. DEAF-1	Deformed Epidermal Autoregulatory Factor-1
27. DN	Double Negative
28. DNA	Deoxyribose Nucleic Acid
29. DP	Double Positive
30. DSB	Double Stranded Break
31. DSIF	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole Sensitivity Inducing Factor
32. DZ	Dark Zone
33. Ebi2	Epstein-Barr virus-induced G-protein coupled receptor 2
34. eTAC	Extra Thymic Aire Expressing Cell
35. FACS	Fluorescence Activated Cell Sorting
36. FBS	Fetal Bovine Serum
37. FDC	Follicular Dendritic Cell
38. Flt3	fms-like tyrosine kinase receptor-3
39. Foxp3	Forkhead Box Protein p3
40. GC	Germinal Center
41. GZ	Gray Zone
42. HSC	Hematopoietic Stem Cell
43. ICOS	Inducible T-cell Costimulator
44. IEC	Intestinal Epithelial Cell
45. IFA	Incomplete Freund’s Adjuvant

46. IFN-β	Interferon-β
47. Ig	Immunoglobulin
48. IL	Interleukin
49. ILC3	Type 3 Innate Lymphoid Cell
50. IP	Immunoprecipitation
51. Irf	Interferon Regulatory Factor
52. JMJD6	Jumonji Domain Containing 6
53. KO	Knockout
54. LMPP	Lymphoid Primed Multipotent Progenitor
55. LZ	Light Zone
56. MAPK	Mitogen Activated Protein Kinase
57. MHC	Major Histocompatability
58. mLN	Mesenteric Lymph Node
59. MPP	Multipotent Progenitor
60. mRNA	Messenger Ribonucleic Acid
61. mTEC	Medullary Thymic Epithelial Cell
62. mTORC1	Mechanistic Target of Rapamycin Complex 1
63. MZ	Marginal Zone
64. NELF	Negative Elongation Factor
65. NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
66. NHEJ	Non-Homologous End Joining
67. NLR	Nod Like Receptor
68. NLS	Nuclear Localization Signal

69. OT-I	$CD8^+$ OVA specific T cells
70. OVA	Chicken Ovalbumin
71. PCNA	Proliferating Cell Nuclear Antigen
72. PD-1	Programmed Cell Death Protein 1
73. PHD	Plant Homeodomain
74. PI3K	Phosphatidylinositol-3' Kinase
75. PIP2	Phosphatidylinositol 4,5-Bisphosphate
76. PIP3	Phosphatidylinositol 3,4,5-Triphosphate
77. Pol-II	RNA Polymerase II
78. PP	Peyer Patch
79. PRR	Pattern Recognition Receptor
80. P-TEFb	Positive Transcriptional Elongation Factor-b
81. Rag	Recombination Activating Gene
82. RBC	Red Blood Cell
83. RSS	Recombination Signal Sequence
84. SAND	Sp100, Aire, NucP41/75, and Deformed epidermal autoregulatory factor-1 [DEAF-1]
85. SHM	Somatic Hypermutation
86. SLE	Systemic Lupus Erythematosus
87. snRNA	Small Nuclear RNA
88. SP	Single Positive
89. SRBC	Sheep Red Blood Cells
90. Tbx21	T-box Transcription Factor
91. TCF7	Transcription Factor 7

92. TCR	T Cell Receptor
93. Tfh	T Follicular Helper Cell
94. Tfr	T Follicular Regulatory Cell
95. Th	T Helper Cell
96. TOP	Topoisomerase
97. Treg	Regulatory T cell
98. TSA	Tissue Specific Antigen
99. UNG	Uracil N-glycosylase
100. WB	Western Blot
101. WT	Wild Type

CHAPTER 1: INTRODUCTION

1.1 Autoimmune Regulator

1.1.1 APECED/APS-1

In the 1960s APECED was first described as a form of autoimmune Addison's disease, a pathology defined by adrenal insufficiency, by Blizzard and Kyle who found that these patients present with circulating autoreactive antibodies against adrenal antigens (Blizzard and Kyle, 1963). Over time, autoimmune Addison's disease expanded and became associated with a number of different symptoms such as chronic mucocutaneous candidiasis, diabetes, hypoparathyroidism, and hypogonadism, and the category of polyglandular autoimmune syndrome was, eventually split into three separate disorders: type 1, which we now know as autoimmune polyendocrinopathy candidiasis ecotodermal dystrophy (APECED), type 2, or Schmidt's syndrome, and type 3, including patients with autoimmune thyroid disease, but do not present with Addison's disease (Neufeld et al., 1981).

APECED or autoimmune polyglandular syndrome type 1 (APS-1) is an autosomal recessive autoimmune disorder occurring in approximately 1 out of 90,000-200,000 individuals in most populations, but 10 times more frequently in Jewish, Sardinian, and Finnish populations according to the NIH. Typically, APECED is diagnosed based on the presence of two out of three major symptoms: chronic mucocutaneous candidiasis (chronic infections of the fungal pathogen, *Candida albicans*), hypoparathyroidism, or adrenal insufficiency, of which chronic candidiasis is the most prevalent (Husebye et al., 2009). Additionally, if a patient has a sibling who was diagnosed with APECED, then only one of the symptoms is needed for diagnosis and, more recently, due to the identification of the causal genetic lesions, testing for mutations in the gene,

autoimmune regulator (*AIRE*) (Finnish-German, 1997; Husebye et al., 2009; Nagamine et al., 1997).

In most cases, the onset of APECED symptoms will occur at an early age with candidiasis occurring just a few years after birth and, in most cases, primarily affects the mucosal tissues. This is thought to be due to the production of high affinity, neutralizing, autoreactive antibodies against cytokines such as IL-17 and IL-22, which are essential for controlling fungal pathogens (Kisand et al., 2010; Meyer et al., 2016; Puel et al., 2010) and dysfunction in the Th17 compartment (Ng et al., 2010). Other features of the disease result from direct immune activation against the endocrine system leading to hormonal dysregulation and endocrine dysfunction. Overall, this disease gives us valuable insight to the inner workings of immune tolerance and the mechanisms involved in establishing immune tolerance and, as a result, a significant body of work describing these mechanisms has been erected. Our goal in this dissertation is to add to that body and further improve our understanding of immune regulation and tolerance.

1.1.2 T cell development and tolerance

T cells sit at the center of the adaptive immune system, orchestrating a myriad of interactions and pathways involved in a proper, controlled immune response. These cells express a collection of surface receptors, generated through DNA recombination, which allow them to recognize, virtually, any protein antigen presented by the appropriate antigen presentation machinery. Due to their importance, it is critical that these cells are, not only capable of being activated by antigen presentation, but are able to discriminate between self and non-self. As a result, the survival of the developing T cell is dependent upon the surface receptors it has created.

T cells arise from a self-renewing population of c-Kit and Sca-1 expressing, CD34 variable hematopoietic stem cells (HSC) (Ikuta and Weissman, 1992; Osawa et al., 1996; Spangrude et al.,

1988; Uchida and Weissman, 1992) that lose their ability to self-renew as they differentiate into multipotent progenitors (MPP) (Ikuta and Weissman, 1992; Morrison and Weissman, 1994) and begin to upregulate fms-like tyrosine kinase receptor-3 (Flt3) (Adolfsson et al., 2001) as they develop into lymphoid-primed multipotent progenitors (LMPP). These cells retain the ability to differentiate into most immune cell types, but are not able to significantly contribute erythroid and megakaryocytic progeny compared to their Flt3 deficient counterparts (Adolfsson et al., 2005). These cells will, eventually, further differentiate into common myeloid progenitors (CMP) or common lymphoid progenitors (CLP), depending on the expression of IL-7 receptor (Akashi et al., 2000; Kondo et al., 1997), the latter of which will lead to the development of T cells. In the late 2000s, CLPs, which were originally thought to have been a homogenous population, were subject to novel (at the time) computational methods to determine developmentally regulated genes and it was discovered that the CLP compartment was not homogeneous at all and that CLPs could be divided into two groups based on Ly6d expression: one positive and one negative (Inlay et al., 2009). Based on data generated from the reconstitution of sublethally irradiated mice, only the $Ly6d^{+}$ CLPs contributed significantly to the T cell pool whereas the $Ly6d^{-}$ fraction only contributed to the development of B cells (Inlay et al., 2009). CLPs were then further analyzed using single cell sequencing and divided into Rag-1^{low} $\lambda5^{-}$, Rag-1^{high} $\lambda5^{-}$, and Rag-1^{high} $\lambda5^{+}$ populations where Rag-1 experienced cells almost exclusively differentiated into B cells whereas the Rag-1low $\lambda5^{-}$ population retained T cell and NK cell potential (Mansson et al., 2010). Of note, the Rag-1 high cells showed higher surface expression of Ly6d, consistent with previous findings (Mansson et al., 2010). To further differentiate into T cells, the CLPs need to migrate into the thymus. To this end, the cells upregulate CCR9, a chemokine receptor for CCL25 (Uehara et al., 2002; Zlotoff et al., 2010) expressed by thymic $CD11c^{+}$ dendritic cells (DC) (Vicari et al., 1997)

and thymic endothelial cells (Gossens et al., 2009), as well as CD43 (P-Selectin glycoprotein ligand-1) which recognizes CD62P (P-selectin) (Rossi et al., 2005; Scimone et al., 2006), also expressed by thymic epithelial cells (Gossens et al., 2009). Additionally, it has been observed that, in the case of CCR9 deficiency, some cells still migrate to the thymus, leading to the discovery of CCR7 as an additional signal to achieve migration (Misslitz et al., 2004; Zlotoff et al., 2010).

Once in the thymus, progenitor cells develop into double negative 1 cells (DN1) that express neither CD4 nor CD8. These cells can be subdivided into two groups based on their expression of c-Kit and further divided by their expression of CD24 (Porritt et al., 2004). Additionally, as the cells transition into the thymus, there is a dependence Notch signaling to maintain the T cell fate decision as deficiency can lead to differentiation into the B cell lineage (Sambandam et al., 2005; Schmitt et al., 2004; Wilson et al., 2001). As the cells continue to differentiate into DN2 and DN3, the myeloid potential is lost and, by the DN3 stage, the cell will completely commit to T cell differentiation. During this time, the DN cells will begin to rearrange the T cell receptor (TCR) through VDJ recombination starting with TCRβ for the conventional αβT cells. The process of VDJ recombination depends on Rag-1 and Rag-2 that will begin by joining a Dβ to a Jβ and subsequently joining the resulting construct to a Vβ and a pre-Tα with the signaling CD3 components that may rescue the cells from cell death (von Boehmer and Fehling, 1997) or maturational arrest as a result of defective signaling from the pre-TCR complex (Clements et al., 1998; Negishi et al., 1995; van Oers et al., 1996). Once developing T cells recombine the TCRβ and begin to receive reduced amount of IL-7 signaling, they progress into the double positive (DP) stage of development, expressing both CD4 and CD8, and upregulate Bcl11b, which has been shown to be essential for commitment into the T cell lineage as well as for positive selection (Albu et al., 2007; Ikawa et al., 2010). Upon differentiation into the DP stage,

the T cells will undergo a second round of recombination to generate the α chain of the TCR (Wilson et al., 1994) by joining a Vα and Jα segment. After rearrangement of the TCRα chain, the T cell will have a fully constructed TCR that needs to be tested for functionality by means of positive selection – selecting against T cells with non-functional TCRs where T cells must induce survival mechanisms by reaching a TCR signaling threshold.

As it is known that self-peptides are required for positive selection, there seems to be a paradox in which, while positive selection promotes survival of self-specific T cells, negative selection would remove those same T cells. To untangle this paradox, several hypotheses regarding the generation of antigens for positive selection. At first, it was proposed that the antigen pool expressed by cortical thymic epithelial cells (cTECs) that are critical for antigen presentation during positive selection, compared to other antigen presenting cells (APCs) in the thymic medulla or anywhere else in the body (Marrack and Kappler, 1987). This model, dubbed the "altered peptide" model, largely faded away as other APCs such as splenic DCs were shown to present the same peptides as cTECs and that these peptides were capable of promoting positive selection in developing T cells (Ebert et al., 2009; Hogquist et al., 1997; Marrack et al., 1993). In addition to the altered peptide model, two additional models came to light: the avidity and affinity models. The avidity model suggested that the quantity of peptide-MHC complexes presented by the cTECs determined the selective capacity of the peptides themselves and this was supported by the finding that peptides that efficiently activate mature T cells can support positive or negative selection depending on the concentration (Ashton-Rickardt et al., 1994; Sebzda et al., 1994); however, it was later observed that T cells selected this way had altered reactivity (Sebzda et al., 1996) and the avidity model fell out of favor. Since then, there has been data supporting the affinity model, the idea that the quality of the interaction between the peptide-MHC complex and TCR is

responsible for the selection of the T cells (Alam et al., 1996; Lo et al., 2009; Moran et al., 2011), though, in the mid-2000s, some data was presented to bring back the altered peptide model (Murata et al., 2007). As of now, it seems that there is a combination of these models in that cTECS express unique molecular machinery that allow for differential processing of peptides to generate a completely unique pool of peptide-MHC molecules and that the affinity of the TCRs to these unique molecules is what allows for positive selection (Klein et al., 2009).

After the developing T cell has established a working TCR, the cells must undergo negative selection to remove the self-reactive TCRs from the mature repertoire. This has, classically, been thought to occur exclusively in the thymic medulla, but there have been reports of negative selection of DP thymocytes in the thymic cortex as well (Stritesky et al., 2013). In the medulla, medullary thymic epithelial cells (mTECs) and thymic B cells (Yamano et al., 2015) generate tissue specific antigens (TSAs), which are then presented either by the mTECs or thymic B cells and DCs. Of note, mTECs do not efficiently present conventional MHC-II substrates and have developed mechanisms for MHC-II loading to focus on self-antigens such as macroautophagy (Aichinger et al., 2013; Atibalentja et al., 2009; Klein et al., 2001). Single positive (SP) thymocytes that have migrated to the medulla and committed to either a CD4 or CD8 lineage that recognize the self-peptide-MHC complexes presented during this stage are then negatively selected via cell death or regulatory T cell conversion (Malchow et al., 2016). This process of negatively selecting T cells that recognize self-peptides is essential for a healthy immune system as it establishes central T cell tolerance, dysregulation of which leads to systemic autoimmune disorders such as APS-1.Following negative selection in the medulla, the tolerized T cells can be released into the periphery.

1.1.3 Autoimmune regulator

AIRE, a gene located on chromosome 21q22.3, was first discovered as the gene responsible for the autoimmune disorder, APECED (Finnish-German, 1997; Nagamine et al., 1997). The gene encodes for a 58kDa protein that includes a caspase recruitment domain (CARD) on the N-terminus, a nuclear localization signal (NLS), a SAND (Sp100, Aire, NucP41/75, and deformed epidermal autoregulatory factor-1 [DEAF-1]) domain, and two plant homeodomain (PHD) zinc finger domains along with four LXXLL motifs. Based on the structure of the protein and the domains contained within it, there seems to be a necessity for oligomerization and DNA interaction for the function of the protein to remain intact as many of these domains are associated with DNA binding and transcriptional regulation (Ferguson et al., 2008; Halonen et al., 2004; Heery et al., 1997).

As it is clear that Aire is involved in the etiology of APECED, it is not unreasonable to infer that Aire plays a critical role in the regulation and establishment of immune tolerance (Finnish-German, 1997; Nagamine et al., 1997) and, based on its high level of expression in mTECs, it is likely to function as a mechanism for T cell negative selection (Hubert et al., 2008; Kishimoto and Sprent, 1997). Additionally, there had also been previous reports that a collection of peripheral tissue antigens are ectopically expressed by mTECs (Kyewski and Klein, 2006). This led to the hypothesis that Aire regulates the expression of PTAs by mTECs, thereby enforcing T cell negative selection against self-antigens and, with the development of an Aire deficient mouse model, this hypothesis could be tested (Ramsey et al., 2002). From my own experience, it seems that the phenotype in Aire KO mice is somewhat mild as there does not seem to be any obvious macroscopic defect; however, on a molecular level, multiorgan autoimmunity can be observed (Anderson et al., 2002). In addition to multiorgan autoimmunity, it was also shown that Aire

promotes the expression of PTAs in the thymus as Aire deficient mTECs showed a reduction in the transcription of PTAs (Anderson et al., 2002). Similarly, Aire was found to be expressed in thymic B cells that upregulate the expression of TSAs to assist in T cell negative selection (Yamano et al., 2015). These B cells also seem to lose Aire expression and, as a result, TSA expression with age (Cepeda et al., 2018), consistent with previous literature, which showed that expression of Aire during the perinatal period is both necessary and sufficient to establish immune tolerance and that the mechanisms controlled by Aire are largely dispensable in adults (Guerau-de-Arellano et al., 2009).

Further interrogation of Aire function seems to indicate that the expression of PTAs was not the only function for Aire in the thymus. Reports had been published showing that, even in Aire sufficient systems, autoreactive T cells were still observed in the thymus as well as the periphery (Taniguchi et al., 2012). In APECED patients, Treg suppressive capacity is also diminished due to a reduction in the expression of forkhead box protein p3 (Foxp3) (Laakso et al., 2010) and, in mice, Aire deficiency is associated with a slight reduction in thymic Tregs as well as alterations in the development and repertoire of thymic Tregs (Anderson et al., 2005; Lei et al., 2011; Malchow et al., 2013; Perry et al., 2014). In contrast, some groups have observed that, in an Aire deficient model, Treg populations and functionality are retained (Anderson et al., 2005; Kuroda et al., 2005; Liston et al., 2003). To determine the role of Aire in the development of Tregs, one group performed follow up experiments and confirmed that Aire was essential for the conversion of autoreactive T cells into Tregs, describing another path of T cell negative selection and development of central T cell tolerance (Malchow et al., 2016).

In short, the classical model of Aire in mTECs states that the protein induces the expression of TSAs to generate a self-antigen pool. Developing T cells use this antigen pool for negative

selection and the cells that activate when presented a self-peptide-MHC molecule will undergo either clonal deletion or Treg conversion (Figure 1.1). To add to this model, studies have reported Aire acting to promoting immune tolerance in other manners such as inhibiting the generation of IL-17 producing γδT cells through modulation of IL-7 (Fujikado et al., 2016) production and regulation of thymocyte migration through its control of CCR4 and CCR7 (Laan et al., 2009). Although there does seem to be quite a diversity in the downstream effects of Aire, it should be noted that many of these findings were first observed in global knockout models or human patients, therefore, more precise tools such as the recently generated $Aire^{fl/fl}$ model (Dobes et al., 2018) must be utilized as a confirmation method to determine the relative contribution of Aire expression in individual cell types.

1.1.4 Role of Aire in the periphery

The majority of previous literature primarily focused on Aire expression in the thymus, particularly in mTECs as it seemed to be the most consequential Aire expressing cell type in the context of central T cell tolerance, however, several studies have shown the importance of Aire in the peripheral stromal (Gardner et al., 2008; Lee et al., 2007) and hematopoietic (Gardner et al., 2013; Heino et al., 2000; Kogawa et al., 2002; Poliani et al., 2010) populations.

The function of Aire in stromal cells was first explored to follow up on the observation that, when $CD8^+$ T cells specific for OVA (chicken ovalbumin) (OT-I) are transferred into iFABP-tOVA mice, a mouse strain which expresses OVA as a self-antigen in intestinal epithelial cells (IEC), the OT-I cells, expectedly, responded poorly in the spleen after mice were fed OVA, but, surprisingly, responded strongly in the mesenteric lymph nodes (mLN), Peyer patches (PP), and the extraintestinal lymph nodes (Lee et al., 2007). It was then found that, by blocking T cell exit from the lymph nodes, there was no change in extraintestinal T cell proliferation in the lymph

nodes, indicating that T cells were receiving OVA stimulation within the lymph nodes outside of the intestines. This led to the discovery that, although $CD8^+$ DCs present OVA in the mLN, lymph node UEA-1^+ stromal cells presented OVA, and other intestinal tissue associated antigens in the extraintestinal lymph nodes, similar to mTECs (Lee et al., 2007). Follow up experiments showed that lymph node stromal cells express Aire and that they promote deletion of OT-I T cells in iFABP-tOVA mice, thus confirming that T cell negative selection occurs in the periphery and can be mediated by non-hematopoietic cell types expressing Aire(Lee et al., 2007). These findings were confirmed using an Aire driven *Igrp-Gfp* ($Aire^{Adig}$) mice in which a transgene with exon 2 of Aire is replaced with GFP, including a 90kb upstream region and 85kb downstream region is inserted into the genome (Gardner et al., 2008).

In the hematopoietic compartment, Aire was first discovered to be expressed by $CD14^+$ monocytes as well as DCs (Kogawa et al., 2002). In monocytes, expression of Aire was associated with upregulation of genes such as CCL22, CD25, ICAM-1, and RelB, which occur during DC maturation; however, the major autoantigens associated with APECED were not found to be significantly upregulated in these cell types as a result of Aire expression indicating that the role of Aire in peripheral hematopoietic cells is different compared to thymic cells (Fergusson et al., 2018; Sillanpaa et al., 2004). Following experiments showed some expression of TSAs in Aire expressing DCs as well as expression of IL-10 suggesting that these DCs may play a role in tolerance by inducing Tregs in the periphery (Poliani et al., 2010). An alternative method for the induction of tolerance by extra thymic Aire expressing cells (eTAC) that show low expression of DC specific markers such as CD11c and Zbtb46, is the expression of peptide-MHC complexes that engage T cells and promote anergy through the absence of co-stimulation (Gardner et al., 2013), though it is still uncertain the mechanism by which Aire regulates this pathway.

In the mid-2010s, it was discovered that, in the intestines, type 3 innate lymphoid cells (ILC3) express MHC-II and present bacterial antigens to promote negative selection of $CD4^+$ T cells that are responsive to the microbiome (Hepworth et al., 2015; Hepworth et al., 2013). These studies did not, however, determine whether the intestinal MHC-II^+ ILC3 responsible for T cell selection express Aire. A more recent found that ILC3-like cells in secondary lymphoid organs that express high levels of MHC-II and co-stimulatory molecules also express Aire, although, in the same study, it was observed that these ILC3-like cells are the only extrathymic cell that expresses Aire protein (Yamano et al., 2019). Overall, these findings seem to challenge the body of literature describing the expression and function of Aire in peripheral hematopoietic cells such as DCs and additional experiments will need to be performed to confirm these results.

In non-lymphoid tissues such as the uterus (Soumya et al., 2016), ovaries (Jasti et al., 2012), and testes (Radhakrishnan et al., 2016), Aire expression has been observed. It seems that in the ovaries and testes, Aire expression has some control over fertility and T cell infiltration into the reproductive organs and, in the uterus, it is responsible for maintaining homeostasis during pregnancy; however, its function in these tissues is largely unknown as the majority of literature is focused on its regulation of immune response in lymphoid organs. Additionally, its expression was noted in the brain, but, similar to reproductive organs, its function has not been significantly interrogated (Eldershaw et al., 2011).

1.1.5 Mechanisms of Aire function

In the nucleus, Aire localizes into punctate structures directly adjacent to nuclear speckles (Su et al., 2008), subnuclear structures containing RNA splicing factors and other proteins involved in pre-mRNA processing. These nuclear bodies do not contain chromatin or RNA polymerase II (Pol-II) (Pitkanen et al., 2005) which means that they are not sites of active

transcription, but it is possible that these nuclear bodies are acting to modify chromatin organization or recruiting transcriptional complexes to specific regions of the genome(Tao et al., 2006) and based on the chromosomal clustering of downstream genes (Johnnidis et al., 2005), the protein likely has a more general function in the regulation of transcription.

In the context of transcriptional regulation, the first protein that was found to interact directly with Aire was CREB-binding protein (CBP) (Pitkanen et al., 2000). Together, these proteins regulate transcription of interferon-β (IFN-β) (Pitkanen et al., 2005) and may play a role in the development of mTECs (Ferguson et al., 2008). After the discovery of CBP, more Aire interacting partners were uncovered. Among these partners were protein inhibitor of activated STAT1 (PIAS1) (Ilmarinen et al., 2008) and the DNA-dependent protein kinase (DNA-PK) complex (Liiv et al., 2008) and interaction with these proteins suggests that Aire plays a role in matrix-mediated transcriptional regulation (Peterson et al., 2008).

In an attempt to discover mechanisms by which Aire regulates such a large number of genes, it was found that Aire is associated with a number of transcriptional programs. One of these programs is the regulation of transcriptional elongation by Pol-II via positive transcriptional elongation factor-b (P-TEFb).

Early studies associating human immunodeficiency virus (HIV) with c-*myc* and expression of heat shock genes indicated that several mechanisms for regulating gene expression actually regulated transcriptional elongation rather than initiation (Bentley and Groudine, 1986) and that Pol-II pauses proximally to promoter regions as a method to rapidly modify transcriptional activity (Rougvie and Lis, 1988, 1990). This transcriptional pausing is mediated by the pausing factors, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole sensitivity inducing factor (DSIF), made up

of a heterodimeric complex of suppressor of Ty5 homolog (Spt5) and Spt4, and negative elongation factor (NELF) that bind to Pol-II enriched for phosphorylation of serine 5 in the C-terminal domain heptapeptide repeats (Yamaguchi et al., 2013). Importantly, it was found that Spt5 can function as an adaptor molecule to assist in targeting of transcriptional factors and enzymes to the Pol-II complex (Lindstrom et al., 2003; Mayer et al., 2012; Pavri et al., 2010; Pei and Shuman, 2002). To release Pol-II and promote transcriptional elongation, P-TEFb, a complex of Cyclin T and Cyclin dependent kinase 9 (CDK9) (Peng et al., 1998), is released from 7SK small nuclear RNA (snRNA) (Nguyen et al., 2001; Yang et al., 2001) and phosphorylates serine 2 in the heptapeptide C-terminal repeats on Pol-II (Marshall et al., 1996) as well as NELF and DSIF which then leads to dissociation of the inhibitory complex and converts DSIF into an elongation promoting factor (Fujinaga et al., 2004; Liu et al., 2015; Wada et al., 1998; Yamada et al., 2006). The association between Aire and this mechanism was first noticed when mTECs treated with I-BET151, a small molecule bromodomain inhibitor that displaces bromodomain and extraterminal domain (BET) proteins such as Brd4 from the chromatin, showed significant influence on their transcriptional profiles (Yoshida et al., 2015). Based on these results, additional experiments were performed to determine the connection between Aire and BET proteins. These experiments found that Aire, through its CARD domain, interacts with Brd4 and that mutations disrupting this interaction prevent the interaction between Aire and P-TEFb to promote the release of Pol-II at Aire induced genes (Yoshida et al., 2015). These data tell us that Aire promotes transcription by trafficking Brd4 to P-TEFb to release stalled Pol-II at promoter proximal regions on TSAs.

More recently, advanced genomic and biochemical techniques have been applied to investigate the mechanism of Aire function. These experiments showed that there was strong association between Aire and H3K27ac as well as H3K27me1, modifications which are indicative

of super-enhancers, stretches of DNA binding a high density of transcription factors (Hnisz et al., 2013; Whyte et al., 2013), and active transcription (Ferrari et al., 2014; Hnisz et al., 2013), but very little association with H3K27me3, a modification associated with downregulation of nearby genes (Bansal et al., 2017; Cui et al., 2009; Org et al., 2009). At these super-enhancers, Aire and its critical interacting partners, topoisomerase 1 (TOP1) and topoisomerase 2 (TOP2), promote chromatin availability and induce mTEC gene transcription. The importance of this mechanism is highlighted by the fact that treatment of mice with the etoposide, topotecan, to interrupt the interaction between Aire and TOP1/2 leads to dysfunctional negative selection of autoreactive T cells; however did not disrupt Aire independent negative selection (Bansal et al., 2017). These data seem to provide a second function of Aire independent of its regulation of transcriptional elongation via P-TEFb and, instead, acts as an epigenetic regulator to increase chromatin availability.

Previous studies have shown that, in hypothetical functional networks, Aire acts in cooperation with the switch/sucrose non-fermentable (SWI/SNF) complex, which is known to assist in chromatin remodeling (Giraud et al., 2014). Based on this information, experiments were performed to test the relationship between Aire and Brg1, a known transcriptional activator and catalytic subunit of SWI/SNF. This revealed that the accessibility of TSAs is promoted by Brg1 whereas Aire acts as a repressor to restrict chromatin availability, in contrast to the previous findings that Aire acts as a promoter of chromatin availability (Hnisz et al., 2013; Koh et al., 2018). It was also observed that Brg1 deficiency, similar to Aire deficiency, leads to multiorgan autoimmunity due to the activation of autoreactive T cells, suggesting that Brg1 is essential for T cell negative selection (Koh et al., 2018). It was observed that Aire restricts transcriptional amplitude to, potentially, prevent the resulting proteins from perturbing physiological processes.

Overall, it seems that Aire acts as a repressor and promoter of chromatin availability as well as an activator of transcriptional elongation and that such an important regulatory protein must, itself, be tightly regulated.

1.1.6 Regulation of Aire

The regulation of a gene/protein can occur transcriptionally by modifying the initiation or continuation of transcription, translationally, and post-translationally by adding or removing functional groups to the protein altering the protein function. To regulate transcriptional initiation of Aire in the thymus, few mechanisms have been characterized; however, that the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is essential (Chin et al., 2003; Haljasorg et al., 2015; Heino et al., 2000) and likely binds to conserved noncoding sequence 1 (CNS1) upstream of Aire (Haljasorg et al., 2015), though this mechanism is not sufficient to activate Aire expression on its own. In the thymus, this signaling can be initiated by either receptor activator of NF-κB ligand (RANKL) signaling through RANK in mTECs (Rossi et al., 2007) or through CD40 engagement of CD40L expressed by developing thymocytes in thymic B cells (Yamano et al., 2015). In addition to NF-kB, several other transcription factors such as the interferon regulatory factor (Irf) 4, Irf8, T-box transcription factor 21 (Tbx21), and transcription factor 7 (Tcf7) have been implicated in the regulation of Aire expression (Herzig et al., 2017). Interestingly, it has also been found that CCCTC-binding factor (CTCF), a protein which regulates chromatin looping, is also responsible for regulating Aire and that removal of CTCF from the Aire locus is required for transcription (Herzig et al., 2017). According to the UCSC genome browser, there is also a consensus binding site for transcription factor PU.1 (Spi1) and early B cell factor 1 (Ebf1) upstream of the Aire gene; however it is unclear whether these proteins are involved in regulating the gene (Figure 1.2).

There are very few studies describing the regulation of translation for Aire mRNA; however, a recent study showed that ineffective splicing of intron 2 by Jumonji Domain Containing 6 (JMJD6), a member of the JmjC-domain containing proteins, which have been implicated in a demethylation of histones by means of an oxidative mechanism involving iron and alpha-ketogluterate (Klose et al., 2006), improves Aire translation by promoting splicing of intron 2 resulting in a premature stop codon (Yanagihara et al., 2015). Although, currently, the substrate for JMJD6 in the context of Aire intron retention is currently unknown, one candidate could be U2AF65, a protein associated with intron splicing(Webby et al., 2009). This may not be the only regulation of intron splicing for Aire since it is a protein activated by NF-kB, its translation may be temporally regulated, though this regulation for Aire still needs to be tested (Hao and Baltimore, 2013; Werner et al., 2008).

The NLS of Aire has been suggested to actually be two separate NLSs, one from lysine 110 to lysine 130 and another between lysines 157 to 159 (Saltis et al., 2008). Lysine residues in the NLS are particularly important as, in some proteins, acetylation of the residues improves nuclear localization whereas, in others, nuclear localization is reduced, potentially as a result of changing interaction with nuclear import proteins. For Aire, it seems as though acetylation of the NLS by CBP leads to nuclear retention and regulatory function and that Aire can be regulated by histone deacetylase (HDAC) 1 and HDAC2 (Incani et al., 2014). In addition to acetylation phosphorylation by DNA-PK at threonine 69 and serine 156 (Liiv et al., 2008), the former being a residue in the CARD domain, which is important for interaction with CBP (Yoshida et al., 2015). These findings suggest that phosphorylation by DNA-PK in the CARD domain leads to interaction with CBP and subsequent acetylation of lysine residues in the NLS, thus improving nuclear retention and transcriptional regulation.

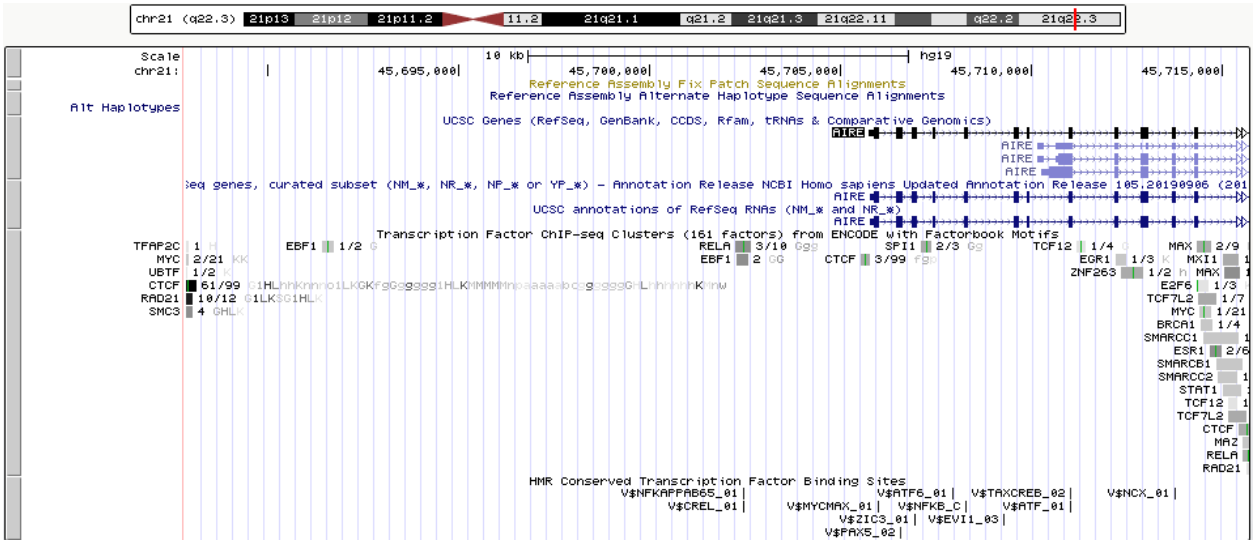

Figure 1.1: UCSC genome browser tracks for transcription factor binding to AIRE promoter. Both RelA and CTCF have consensus sequences in the Aire promoter. Additionally, Spi1 (PU.1) has a consensus sequence and Ebf1 has a binding site; however, the functional relevance is yet to be determined.

1.2 B Cells and Antibodies

1.2.1 B cells

Early B cell research first began through the discovery of antibodies by Tiselius and Kabat who ran electrophoretic analysis on serum (Tiselius and Kabat, 1938). Eventually, plasma cells, the cells which produce the antibodies, were discovered (Fagraeus, 1948) and two competing hypotheses of antibody formation developed. The first hypothesis was Niels Jerne's natural selection theory, which stipulated that antibodies were merely carriers of antigen to a cell that was then able to replicate that antibody (Jerne, 1955). The second hypothesis was Sir Macfarlane Burnet's clonal selection theory, in which each antibody was made from a different cell specific for a unique antigen (Burnet, 1976). The latter theory eventually gained prominence as data came out to show that, when single cells are simultaneously stimulated with two different antigens, they could only form antibody to one or the other, not both, meaning each individual cell only had the capacity to generate antibody to a single antigen (Nossal, 1959). Eventually, B cells were defined when studying the bursal lymphoid system in chickens (Cooper et al., 1965) and, as they were first discovered in the bursa, were subsequently named "B cells", though, at this time, it was still uncertain what the function of these cells was.

Not long after the discovery of B cells, there became a link between them and antibody production when it was discovered that surface immunoglobulin (Ig) could be used as a marker for both normal and leukemic B cells (Coombs et al., 1969; Froland et al., 1971). Eventually, monoclonal antibodies were developed, and we were able to characterize the cells with significantly more detail and, to standardize the nomenclature, each monoclonal antibody was given a designation as a cluster of differentiation (CD).

Once the nomenclature was standardized and laboratories had the capability of characterizing cells with greater precision, it was found that B cells were not a homogenous cell type and that they came in many different forms with different functions. In the 1980s, B-1 cells, a unique subset of B cells was first described as a $CD5^+$ population (Hayakawa et al., 1983). These B cells were then further subdivided into a $CD5^+$(B-1a) and $CD5^-$(B-1b) populations and it was discovered that these cells develop separately from B-2 B cells; however, their origin has been a point of contention (Dorshkind and Montecino-Rodriguez, 2007), though we, currently, believe that the majority of these cells arise from the fetal liver with very few coming from the adult bone marrow (Montecino-Rodriguez et al., 2006). The B-1a and B-1b cell types were then functionally defined and it was found that B-1a cells make natural antibodies, antibodies which are predominantly IgM and do not require immunization for production, whereas B-1b cells mediate adaptive antibody responses to polysaccharides and other T cell independent antigens (Haas et al., 2005). Although B cells have been known to produce antibodies, these cells have functions that help maintain tissue homeostasis through the production of cytokines such as progesterone induced blocking factor during pregnancy in the case of B-1 B cells (Huang et al., 2017) and IL-10 in the case of regulatory B cells (B10 or Breg) (Tedder, 2015).

In the spleen, a region surrounding the splenic follicles, separating the white pulp and red pulp, known as the marginal zone (MZ) houses a group of MZ B cells, macrophages, and reticular cells. In mice, MZ B cells are a lineage, separate from follicular B cells or B-1 B cells, which can be characterized as $IgM^{hi}IgD^{lo}CD21^{hi}CD23^-CD1d^{hi}$ and express polyreactive, non-mutated B cell receptors (BCR) (Cerutti et al., 2013). Additionally, mouse MZ B cells express high levels of toll like receptors (TLR) (Rubtsov et al., 2008; Treml et al., 2007) that, when coupled with BCR signaling, induces production of low affinity antibodies (Pone et al., 2012). In humans, MZ B cells

are $IgM^{hi}IgD^{lo}CD1c^{+}CD21^{hi}CD23^{-}CD27^{+}$and occupy subcapsular regions of lymph nodes and other lymphoid tissues (Dono et al., 2000; Puga et al., 2011; Tierens et al., 1999). In contrast to mouse MZ B cells, they express both CD27 and mutated BCRs indicating that they have experienced germinal center (GC) reactions prior to undergoing class switch recombination (CSR) and that they may be equivalent to memory B cells (Dunn-Walters et al., 1995; Klein et al., 1998; Seifert and Kuppers, 2009; Tierens et al., 1999), though they have a distinct repertoire and are not as dependent on T cell help compared to canonical memory B cells (Berkowska et al., 2011; Weller et al., 2001; Weller et al., 2008). Functionally, since MZ B cells are located in between the B cell follicle rich white pulp and the blood-filled red pulp making them ideal for responding to blood antigens. Additionally, due to their high levels of CD21, they are capable of efficiently capturing complement (Guinamard et al., 2000) and, since they have elevated levels of Blimp-1 as well as a pre-activated state, they are capable of rapidly dividing into plasmablasts (Martin et al., 2001; Sintes et al., 2017).

The B cells that typically respond to T dependent antibody responses and generate much of the high affinity antibodies in the body are follicular B cells. These cells have the most diverse BCR repertoire and can undergo further diversification once activated. Once they mature, they can either be found circulating in the blood and lymph or they can be found in established B cell follicles in secondary lymphoid organs. Those that have encountered antigen in these follicles can then be activated by nearby T cells. Alternatively, though not as common, follicular B cells can home to the bone marrow to form perivascular sinusoids that assist in response to blood pathogens (Cariappa et al., 2007; Cariappa et al., 2005).

Over the past several decades, since the discovery of antibodies, the study of B cells has been one of the most productive avenues of research resulting in significant growth of our

understanding of the immune system as well as improving therapies and clinical outcomes. We have learned the origin of antibodies and how they help the body clear pathogens. Additionally, we have discovered numerous different cell types and characterized their functions as well as consequences of dysfunction. Continued research will help us understand, not just B cell biology, but allow us to create better treatments for disease and create more effective methods for vaccination.

1.2.2 B cell development and tolerance

In the bone marrow, the differentiation of B cells begins similarly to that of T cells. Hematopoietic stem cells differentiate and, eventually, become c-Kit and IL-7R CLPs. As the CLPs differentiate, they become restricted to the B cell lineage with the upregulation of B220 in the early pre-pro-B cell stage without CD19, the molecule common to all later B cell stages, or BCR rearrangement(Allman et al., 1999; Li et al., 1996; Ogawa et al., 2000). As these cells continue to differentiate into pro-B cells, that begin to upregulate CD19 and CD40, IL-7, signaling through Janus kinases, JAK1 and JAK3, and signal transducers and activators of transcription (STAT), initiates numerous processes critical for B cell commitment such as proliferation (Kittipatarin et al., 2006; Morrow et al., 1992), survival (Li et al., 2004; Lu et al., 1999), and IgH recombination (Chowdhury and Sen, 2003; Corcoran et al., 1998); however, although IL-7 signaling is critical for the development of B cells in mice, generation of B cells in humans is not dependent of IL-7 or IL-7R as humans with mutations in IL-7Rα and downstream signaling develop a lack of T cells, but have normal numbers of NK and B cells (Macchi et al., 1995; Puel et al., 1998; Russell et al., 1995).

Recombination of the BCR is one of the most important developmental checkpoints for B cell maturation. The VDJ recombination mechanism can be divided into two separate stages:

cleavage and repair – the genomic DNA sequences are broken and recombined through repair mechanisms. To begin cleavage, the recombination activating (Rag) 1 and Rag2 recombinases search for a recombination signal sequence (RSS) following the 12/23 rule, in which a heptamer and nonamer are separated by either 12 or 23 nucleotides (Schatz and Swanson, 2011). The recombination event begins with a nick formed on the 5' end of the RSS to form a 3' OH group that then attacks a phosphate group on the opposing strand to form a double stranded break containing a hairpin coding region and a blunt end signaling region (McBlane et al., 1995; van Gent et al., 1996). The hairpin coding ends are then opened by Artemis and DNA-PK prior to being processed via non-homologous end joining (NHEJ) before being joined (Lieber, 2010).

Once the heavy chain is recombined during the pro-B cell phase, a surrogate light chain, λ5 and VpreB, combines with the heavy chain to form a pre-BCR that, necessarily, recognizes self-antigen, and the cell differentiates into a large pre-B cell and is subjected to its first round of positive selection, in which cells without a functioning pre-BCR either continue recombination on the other chromosome or halt their development, as patients who cannot make functional surrogate light chain develop agammaglobulinemia due to a complete block of differentiation in the B cell compartment (Minegishi et al., 1998). In contrast, cells with a pre-BCR that is capable of transmitting signal into the cell will rapidly proliferate (Winkler and Martensson, 2018), specifically, the cells will undergo approximately five to six cell divisions (Decker et al., 1991). It is possible that this proliferative phase assists in allelic exclusion – prevention of recombination in the second chromosome – as it has been observed that Rag2 protein is destabilized during S phase of proliferative cells (Grawunder et al., 1995; Lin and Desiderio, 1994). Ultimately, signaling from the pre-BCR will lead to activation of SLP-65 that then downregulates PI3K

activity, exit from the proliferative phase, and allows the cell to differentiate into a small-pre-B cell (Jumaa et al., 1999; Koretzky et al., 2006; Pappu et al., 1999).

In addition to bringing the developing B cells out of the proliferative phase, SLP65 regulation of PI3K is a critical differentiation stage. PI3K is a powerful inhibitor of the FoxO transcription factors, which are key activators of Rag1 and Rag2 (Amin and Schlissel, 2008; Herzog et al., 2008). As Rag1 and Rag2 are reactivated and proliferation is halted, the light chain is recombined to generate the complete, conventional BCR, which replaces the pre-BCR, signaling is required for the maintenance of mature B cells since deletion of the BCR results in loss of mature B cells (Lam et al., 1997). Once the B cell expresses a mature BCR on the cell surface, they become classified as immature B cells.

Due to the variable nature of VDJ recombination, a mature B cell pool with the capacity to recognize a staggering, almost unlimited number of antigens can be generated and, therefore, must undergo a second round of selection to, not only be certain that the BCR is operational, but also that self-reactive B cells are not released into the periphery as previous reports show that the majority of early immature B cells express BCRs with self-reactivity (Wardemann et al., 2003). One mechanism for ensuring self-tolerance is by halting development at the immature B cell stage. It has been shown that when self-reactive B cells are treated with self-antigens, continued B cell development is completely halted (Nemazee and Burki, 1989), though, should the recombination take too much time, the cells may undergo cell death. A second mechanism for establishing B cell tolerance is through receptor editing. B cells with receptors that strongly interact with multivalent self-antigen will reactivate Rag1 and Rag2 to continue rearranging the VDJ region for the opportunity to generate a new antigen binding region that is not self-reactive (Gay et al., 1993; Koralov et al., 2006). Finally, strong activation of immature B cells to self-antigens may render

the B cells inert, a state known as anergy (Goodnow et al., 1988). Generally, these B cells remain inert; however, recently, it has been shown that stimulation with high-density foreign antigen can lead to hypermutation and affinity maturation away from self-reactivity, a phenomenon termed "antibody redemption" (Burnett et al., 2018). Though, as mentioned, there are several mechanisms for the tolerization of B cells and removal of autoreactive cells from the repertoire, a significant population of B cells in the thymus expressing self-reactive BCRs indicating that there are mechanisms for B cells to escape tolerance(Rother et al., 2016). These autoreactive B cells are thought to assist in the negative selection of T cells through the presentation of self-antigens to thymocytes. Additionally, B cell precursors, whose development is influenced by microbial colonization, have been discovered in the intestines, though it is unclear how the antigen pool on which these cells are selected is generated (Wesemann et al., 2013). Once the cells are tolerized, they leave the bone marrow as transitional B cells and home to their respective compartments.

Although most B cells develop in the way mentioned above, B-1 B cells, generally, follow a separate path compared to conventional B cells. These cells are the predominant B cell formed in the fetus and arise from the fetal liver rather than adult bone marrow(Hardy and Hayakawa, 1991). Importantly, during VDJ recombination in B-1 B cell precursors, there is an increase in expression of terminal deoxynucleotidyl transferase (TdT), not seen in adult bone marrow precursors, which allows for additional diversification in the BCR repertoire (Desiderio et al., 1984). Although it is clear that these cells arise from the fetal liver, there is data to indicate that they can potentially be replenished by the bone marrow in adults (Gu et al., 1990; Tornberg and Holmberg, 1995); however, transgenic BCR mice have shown that B-1 and B-2 B cells have distinct receptors where the receptor for one type cannot generate cells of the other type (Lam and

Rajewsky, 1999). When fully functional, these cells will reside in the peritoneum and, when activated, migrate to the spleen to differentiate into antibody secreting cells (Baumgarth, 2011).

1.2.3 B cell activation

Activation of B cells can occur in either a T dependent or T independent manner, though in most cases, BCR signaling is critical. The BCR is a membrane bound antibody generated by the B cell to recognize cognate antigen. Once these receptors recognize their respective antigens, the receptors are crosslinked leading to activation of Src family kinases like Lyn as well as activation of the kinase, Syk(Takata et al., 1994); however, there is an argument to be made that BCR does not crosslink when activated, but is crosslinked when inactive and this interaction is dissociated during activation (Klasener et al., 2014; Volkmann et al., 2016). Following phosphorylation of the key kinases downstream of the BCR, additional signaling molecules such as phosphatidylinositol-3' kinase (PI3K), which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP3), NF-kB, and mitogen activated protein kinase (MAPK). These signals lead to B cell proliferation and effector function whereas inhibitory signals such as FcγRIIB and SH2 domain-containing inositol 5′-phosphatase repress overstimulation through the BCR, which can prove fatal for the cell.

In the case of T dependent activation, B cells that have captured antigen with their BCRs internalize the antigen, processes them, and presents them as peptide-MHC-II. During this time, Epstein-Barr virus-induced G-protein coupled receptor 2 (Ebi2) and CCR7, a chemokine receptor for CCL19 and CCL21, home the activated B cells to the T zone in the lymphoid tissues (Gatto et al., 2009; Gatto et al., 2011; Kelly et al., 2011; Pereira et al., 2009) where the peptide-MHC-II expressed by the B cell will be presented to the T cell and engage the TCR. Should the B cell have a relatively low affinity receptor, intercellular adhesion molecule (ICAM) 1 and ICAM2 can

interact with Lymphocyte function-associated antigen 1 (LFA1) to improve the interaction between the B and T cells (Zaretsky et al., 2017). The T cells provide B cell help by expressing CD40L, a member of the TNF family ligands, which engages the CD40 expressed by B cells leading to additional NF-kB signaling and continued activation of the B cells to allow additional antibody diversification mechanisms to activate such as somatic hypermutation (SHM) and class switch recombination (CSR) or undergo differentiation into plasma cells or memory cells.

For the most part, the mechanisms of T independent activation are employed by B-1 cells and MZ B cells that respond to two distinct classes of signals: one being cytokine activation or danger associated molecular pattern (DAMP) activation, which signal through cytokine receptors or pattern recognition receptors respectively, and the second being stimulation through the BCR or complement. B-1 B cells act as members of the innate immune system and help with cellular signaling events as well as antibody production. When peritoneal B cells are activated, they migrate into the mLN (Fagarasan et al., 2000; Okamoto et al., 1992) where they will undergo proliferation and differentiation into plasma cells. The activation of these cells is critical for generation of IgA against bacteria at mucosal surfaces (Macpherson et al., 2000) making them crucial for the interactions between the host and microbiome. MZ B cells play a critical role in T-independent responses to type two antigens (Guinamard et al., 2000). C3 deficiency or deficiency in complement receptors on MZ B cells diminishes their ability to bind antigen and their pre-activated state makes them more sensitive to stimulation (Sintes et al., 2017). Additionally, a population of B helper neutrophils were discovered in the spleen and express B-cell activating factor (BAFF), A proliferation-inducing ligand (APRIL), and IL-21, which assist MZ B cells in differentiating into IgM, IgG, or IgA producing plasma cells independently of CD40 engagement (Puga et al., 2011).

Based on studies of T dependent B cell activation, it would seem that B cells are subservient to T cells and that they depend on T cells for their function. This, however, is not the case as there are numerous mechanisms by which B cells can be activated independently of T cells. Evolutionarily, it is likely that these T independent mechanisms preceded the T dependent mechanisms as they are methods to generate quick antibody and cytokine responses to environmental stimuli. Further research in this area may give us a better understanding of the evolution of B cells and the mechanisms regulating their function.

1.2.4 Antibody functions

As previously mentioned, antibodies were first discovered in the late 1930s through electrophoresis of serum (Tiselius and Kabat, 1938); however, the functions of antibodies and their physiological effects have been known. In the early 1700s, Lady Mary Wortley Montagu, Emanuel Timoni and James Pylarini first attempted to inoculate small amounts of infectious smallpox material into healthy subjects to prevent disease. Later, in 1798, Edward Jenner used cowpox as a means of achieving a similar result, only, his treatment was significantly safer compared to that of previous attempts at vaccination due to his use of cowpox (Weiss and Esparza, 2015). Following the success of Jenner, in the late 1800s, the first reference to antibodies was made when Emil von Behring when he showed that transfer of serum from animals immunized against diphtheria could cure animals with the disease, a technique we now call, “passive immunization” (Kaufmann, 2017). Since then, our understanding of antibody function has advanced considerably and, with it, our ability to take advantage of these functions to produce more favorable clinical outcomes.

At the time of writing this dissertation, a novel coronavirus, SARS-CoV2, has ravaged the world. To alleviate the situation, groups have been searching for potential therapies to prevent the spread of disease, one of which is the discovery, purification, and transfer of neutralizing

antibodies to patients at risk for contracting the virus (Ju et al., 2020; Liu et al., 2020; Rogers et al., 2020). Neutralizing antibodies are typically thought to interfere with ligand/receptor interactions, thus preventing pathogen attachment to the host; however, in reality, there are multiple mechanisms by which antibodies can provide neutralizing function. These mechanisms can be categorized as either pre-attachment neutralization in which the antibody interferes with the functioning of the pathogen by agglutination/aggregation (Brioen et al., 1983; Phalipon et al., 2002; Thomas et al., 1986), immobilization (separately of agglutination)(Bishop et al., 2010; Campodonico et al., 2010), or by impacting cellular functions (LaRocca et al., 2009; McClelland et al., 2010; Pachl et al., 2006; Watanabe and Blobel, 1989). The second classification for neutralization mechanisms is post-attachment refers to the classical definition of neutralization, interference of pathogen binding and invasion. Finally, neutralizing antibodies can act via post-attachment mechanisms that inhibit pathogens after they have attached to their target cells, including inhibition of intracellular processes as well as viral release. In light of the alternative mechanisms for neutralization, a more accurate definition of neutralizing antibodies would be those that are capable of modifying the ability of a pathogen to infect the host (Corthesy et al., 2006; Hughey et al., 1995; Maciejewski et al., 1995; Mallery et al., 2010; Thouvenin et al., 2001; Varghese et al., 2004).

In addition to neutralization, antibodies, particularly IgG and IgM, have the ability to activate the complement cascade through the classical complement pathway. In this pathway, antibody-antigen complexes bind to C1q via the antibody Fc region, thus activating C1r and C1s serine proteases that cleave C2 and C4 (Dunkelberger and Song, 2010). The products of this cleavage form a complex, C4bC2a, also known as the C3 convertase, which is capable of cleaving C3 into C3a and C3b to assist in the construction of the membrane attack complex (Dunkelberger

and Song, 2010). This mechanism is particularly important as it can assist in numerous innate immune functions such as promoting inflammation, opsonization, cytotoxicity, and antigen presentation. It is important to note that functions of antibodies are not mutually exclusive and that antibodies, which are capable of fixing complement, are also capable of performing the other functions mentioned here as well.

The third major function of antibodies is the activation of Fc receptors. These receptors recognize the Fc regions of antibodies and are, typically, isotype specific and, although they tend to have many different functions depending on environmental context, two major pathways stand out as particularly important: antibody dependent cellular cytotoxicity (ADCC) and phagocytosis. ADCC was first discovered in the context of HSV1 infection (Shore et al., 1974) and occurs when an antibody bound to an infected cell interacts with Fc receptors on an effector cells, such as NK cells and cytotoxic T cells, leading to the activation of cytotoxic effector pathways and death of the target cell. In recent years, researchers have taken advantage of this mechanism to generate monoclonal antibodies such as Herceptin and Rituximab, which target and kill cancer cells (Clynes et al., 2000; Petricevic et al., 2013; Weiner, 2010). In contrast to ADCC, phagocytosis acts, not on infected cell, but on, antibody coated material (Flannagan et al., 2012). Phagocytes such as macrophage, DCs, and neutrophils bind the antibodies, distributed around the entire surface of the molecule, via Fc receptors and engulf the material (Griffin et al., 1975; Griffin et al., 1976), forming a phagosome. The phagosome then fuses with a lysosome to form a phagolysosome that digests whatever material was phagocytosed. Molecules such as peptides resulting from the enzymatic degradation within the phagolysosome can be used as activating signals for T cells when presented on MHC-II or cross presented on MHC-I.

Since the discovery of the physiological consequences of antibodies in the early 1700s, and since the first discovery of the protein complexes, themselves, in the 1930s, significant progress has been made in characterizing the function of antibodies. However, although we have a sense of their functions in general, we have yet to fully characterize the specific functions of all the different isotypes. Future research will continue to elucidate these functions and mechanisms as well as further utilizing them to treat disease.

1.2.5 Antibodies and Autoimmunity

In the 1950s, autoreactive antibodies were first discovered when an observation was made that a mysterious serum factor with the characteristics of IgG in patients with systemic lupus erythematosus (SLE) reacted specifically with nuclei and was able to fix complement (Miescher and Fauconnet, 1954; Robbins et al., 1957). Today, it is increasingly clear that autoreactive antibodies play a critical role in the pathogenesis of numerous autoimmune diseases by mediating both tissue injury and inflammation (Mackay, 2010), thus a significant body of literature pertaining to the generation of autoreactive antibodies has been generated over the past several decades.

As previously mentioned, during B cell development, precautions are in place to prevent autoreactivity such as receptor editing, anergy, cell death, and developmental arrest; however, autoreactive B cells are not uncommon in healthy individuals indicating that these mechanisms that establish central tolerance are not sufficient to completely tolerize the B cell repertoire. Although it has been well-established that many autoreactive B cells belong to the B-1 and MZ subclasses (Batten et al., 2000; Pao et al., 2007; Thien et al., 2004), it has been observed that autoreactive antibodies in systemic autoimmune disease are class switched and harbor large numbers of somatic mutations, indicating that they have likely undergone T dependent antibody diversification (Diamond and Scharff, 1984; Mietzner et al., 2008; Schroder et al., 1996;

Wellmann et al., 2005). Additionally, previous findings have shown defects in early B cell tolerance mechanisms in patients with autoimmune disease (Samuels et al., 2005; Yurasov et al., 2005); however, more recent data suggests that autoreactive memory antibodies arise from non-autoreactive, but polyreactive precursors (Mietzner et al., 2008). These data show that patients with autoimmune disorders suffer from defects in tolerance mechanisms, but this deficiency does not necessarily lead to the generation of pathogenic, autoreactive antibodies and that mechanisms of regulating antibody diversification play a critical role. Accordingly, should autoreactive T cells escape the thymus, B cell diversification would be directed toward autoreactivity in T dependent responses.

Interestingly, many antigens targeted by autoreactive antibodies, such as DNA, RNA, and oxidized phospholipids, are also ligands for pattern recognition receptors (PRRs) including TLRs and Nod like receptors (NLRs) suggesting a role for these receptors in the generation of these antibodies and the breakdown of tolerance (Horkko et al., 1997; Imai et al., 2008; Zhang et al., 2009; Zhou et al., 2011). In support of this theory, studies have shown that deficiency in TLR7 and TLR9 as well as MyD88, the adaptor molecule for most TLR-associated signals is protective, at least in part, against the development of anti-nuclear antibodies in SLE models (Christensen et al., 2006; Ehlers et al., 2006; Groom et al., 2007; Hua et al., 2014). These receptors, when activated by oxidized mitochondrial DNA in DCs can also induce pathogenic T dependent antibody production due to the activation of CD4 T cells that help B cells through IL10 and succinate (Caielli et al., 2019). Additionally, in T independent mechanisms, BCR signaling is synergistic with TLR recognition of nuclear antigens, therefore, self-antigens capable of activating these pathways can induce B cell activation (Chaturvedi et al., 2008; Lau et al., 2005; Viglianti et al.,

2003) and, in developing B cells, dysregulate the establishment of central tolerance (Aranburu et al., 2010; Azulay-Debby et al., 2007; Giltiay et al., 2013).

Taken together, these findings show that autoreactive antibodies, which carry with them the potential to initiate immune responses in the same way as antibodies against foreign bodies, can be generated through both T dependent and T independent mechanisms. Generally, it has been thought that these antibodies are a result of dysfunctional central and peripheral B cell tolerance; however, recent work has shown that autoreactivity can also arise from non-autoreactive B cells, putting into perspective the importance of regulating peripheral antibody diversification mechanisms. Further study into the creation of these antibodies can allow us to provide improved treatments for patients with autoimmune disease such as SLE or rheumatoid arthritis.

1.3 Germinal Centers

1.3.1 Germinal centers

The immune system is capable of recognizing an infinite array of foreign substances through the production of antibodies that bind with remarkably high affinity and specificity. For many years, the mechanism by which a genome, limited by its size, is capable of producing such a diverse pool of immunoglobulin specificities. Further research on the topic uncovered VDJ recombination as the method to generate the original B cell repertoire (Hozumi and Tonegawa, 1976); however, studies observing antibodies of animals showed that antibody affinity increased significantly over time, a process now known as affinity maturation (Eisen and Siskind, 1964; Jerne, 1951). Following investigations found that the antibodies that emerge later in the immune response were not a result of continued VDJ recombination, but, rather, highly mutated versions of relatively lower affinity VDJ sequences (Berek and Milstein, 1987; McKean et al., 1984;

Weigert et al., 1970) resulting from a combination of somatic hypermutation (SHM) followed by selective pressure.

For the most part, the affinity maturation process occurs in microanatomical structures known as germinal centers (GC), first described in 1884 by Walter Flemming. This was discovered when single cells from GCs were dissected and shown to have high levels of somatic antibody mutations (Berek et al., 1991; Jacob et al., 1991). GCs can be characterized by a mantle zone, a ring of naïve B cells that are pushed aside as the GC expands, a light zone (LZ) proximal to the capsule or marginal zone, containing FDCs and T cells, a gray zone (GZ), and a dark zone (DZ) of actively proliferating cells (Figure 1.3).

FDCs are a radioresistant cell type that form a reticular network in both primary and GCs where they are primarily localized to the LZ (Mandel et al., 1980). These cells are known to retain antigens in a complement receptor 1 and 2 dependent manner (Barrington et al., 2002) and display in intact forms on the cell surface in the form of immune complexes for long periods of time (Mandel et al., 1981), therefore, it is believed that they act as a reservoir for antigens in the GC from which B cells with BCRs specific for the antigens displayed are selected. Although FDCs seem to play a critical role in the GC reaction, the necessity of FDCs for GC development has been questioned as affinity maturation can still occur in mice with defective FDCs (Haberman and Shlomchik, 2003). Currently, the data suggests that FDCs are essential for promoting affinity maturation under suboptimal conditions in which antigen is scarce; however, are less important when antigen is more abundant (Victora and Nussenzweig, 2012). In addition to antigen retention, FDCs also express the Fc receptor, FcγRIIB, which impacts GC B cell development by acting as an IgG sink to limit the number of Fc regions available to interact with the inhibitory receptors on GC B cells (Ravetch and Lanier, 2000). Finally, FDCs assist in GC B cell migration into the light

zone through the production of CXCL13 (Cyster et al., 2000), a ligand for CXCR5, which is upregulated in GC B cells after DZ expansion, as well as other molecules such as IL-6 and BAFF, which may play critical roles in B cell survival and development within the GCs (Allen and Cyster, 2008; Kopf et al., 1998; Wu et al., 2009).

T cells make up a small fraction of cells in the GC, but they are essential for GC formation, maintenance, and affinity maturation as athymic mice cannot form GCs without adoptive transfer of T cells prior to immunization (Jacobson et al., 1974). Specifically, these cells are critical due to their expression of CD40L, which provides survival and activation signals to B cells. The importance of this pathway is highlighted by experiments showing that injection of CD40-CD40L blocking antibodies into mice dissolves ongoing GC reactions (Han et al., 1995) and, in humans, loss of function mutations in either CD40 or CD40L lead to hyper IgM type 3 or type 1 respectively, in which B cells are unable to undergo GC reactions and antibody diversification processes do not occur. In recent years, a subset of GC T cells, T follicular helper cells (Tfh), the cells responsible for providing T cell help within the GC , have been under intense investigation (Vinuesa et al., 2005). Originally, these cells were found to express CXCR5 (Ansel et al., 1999), a receptor that is required for GC entry, and CD28 family co-stimulatory molecules, inducible T-cell costimulatory (ICOS) and programmed cell death protein 1 (PD-1) (Haynes et al., 2007; Hutloff et al., 1999), which are essential for their GC localization and maintenance (Shi et al., 2018). Other T cell types in the GCs that are less studied include T follicular cytotoxic cells, which express CD8, and are thought to help control viral infections (Yu and Ye, 2018) and T follicular regulatory cells, which help to limit Tfh function and GC reactions to prevent production of pathogenic antibodies (Clement et al., 2019). As the importance of these other T cell types becomes more apparent, more

research is focused on the mechanisms by which they assist in GC reactions and antibody production.

Although GCs are made up of several different cell types, the vast majority of cells are activated B cells. In contrast to their naïve counterparts, GC B cells divide rapidly with a division time of between 6 and 12 hours (Allen et al., 2007; Hauser et al., 2007). They can be characterized by their expression of Fas and n-glycolylneuraminic acid, which binds the antibody GL-7, as well as binding of peanut agglutinin, loss of IgD expression, and changes in expression of CD38, which is increased in human GC B cells, but reduced in mice (Cervenak et al., 2001; Naito et al., 2007; Oliver et al., 1997; Rose et al., 1980; Yoshino et al., 1994). Additionally, Ebi2, an oxysterol receptor is highly expressed by naïve B cells, but not GC B cells (Gatto et al., 2009; Pereira et al., 2009) whereas S1P2, a sphingosine 1 phosphate receptor, that shows the opposite trend (Green et al., 2011). Though there are many phenotypic differences between GC B cells and other B cell types, one of the most critical transcriptional regulators expressed by GC B cells, but not naïve B cells is Bcl-6 (Basso and Dalla-Favera, 2010). This protein is generally thought to be a transcriptional repressor and mice lacking Bcl-6 are incapable of producing high affinity antibodies (Dent et al., 1997; Ye et al., 1997) as this molecule regulates several major GC B cell functions including cell survival (Ci et al., 2009; Saito et al., 2009), repression of p53 and DNA damage molecules to allow the B cell to tolerate the mutational burdens of antibody diversification (Phan and Dalla-Favera, 2004; Ranuncolo et al., 2007), silencing of B-lymphocyte-induced maturation protein 1 (Blimp-1) to block differentiation to plasma cells (Shaffer et al., 2000), and downregulation of BCR and CD40 signaling molecules (Basso et al., 2010).

1.3.2 Germinal center dynamics

Germinal center reactions can be summarized into five major phases: entry, proliferation and diversification, migration, selection, and, finally, differentiation. GC B cells that can continuously cycle through the different phases to promote the generation of higher and higher affinity antibodies through affinity maturation. Although GC B cells do not secrete much antibody, they contribute to long lived responses through differentiation into long lived plasma cells. Due to the importance of GC reactions to humoral immunity, many researchers have focused primarily on describing the mechanisms by which GC B cells undergo affinity maturation and differentiation as well as the mechanisms regulating these critical functions.

B cell entry and participation in the GC first requires that the cell recognizes cognate antigen via the BCR, which can come directly from the environment or presented on the surface of other cell types such as FDCs, DCs, and macrophage (Allen and Cyster, 2008; Carrasco and Batista, 2007; Phan et al., 2007). Once the BCR has bound to cognate antigen, the B cell internalizes the antigens, processes them, and presents peptide fragments on MHC-II. Concomitantly, the B cells upregulate CCR7, a receptor for the chemokines CCL19 and CCL21 expressed in the T zone and begin migration (Okada et al., 2005). In the T zone, the B cells form mobile conjugates with T cells (Okada et al., 2005) that express CD40L, ICOS, and PD-1. Engagement of these receptors and ligands with B cells presenting peptide-MHC leads to activation of mechanisms to undergo CSR prior to expansion and GC formation (Roco et al., 2019). In T cells, engagement of ICOS by ICOSL expressed on B cells promotes the expression of CXCR5 to localize the cells into the LZ of the GC.

After entry into the GC, B cells undergo cycles of interzonal migration. Using radiolabeled nucleotides, cells were observed *in vivo* to first undergo expansion in the DZ and are then detected

in the LZ several hours later (Hanna, 1964). These findings were later confirmed by Victora et al. who used photoactivated GFP to also show less than 10% of LZ cells recirculate back into the DZ whereas over 50% of the cells from the DZ migrate into the LZ within 4 hours, though the speed of interzonal migration seems to be equal in both directions at approximately 7μm/min (Victora et al., 2010). Furthermore, this study showed that cells having migrated to the DZ from the LZ underwent clonal expansion, providing solid evidence for the cyclic re-entry theory, which suggested that a fraction of positively selected B cells in the LZ return to the DZ for further rounds of mutation and proliferation (Kepler and Perelson, 1993; Victora et al., 2010).

The evolutionary process of antibody affinity maturation and B cell selection in GCs is a competition between B cells in which lower affinity cells are eliminated by means of cell death and higher affinity cells survive resulting in an increased average affinity of BCRs in the GC (Rajewsky, 1996). Once the B cells are in the GC, there is no minimum required affinity for survival, the affinity must be higher relative to other B cells specific for the same antigen; however, low affinity B cells are unable to form GCs when high affinity competitors are present (Dal Porto et al., 2002; Schwickert et al., 2011) suggesting a competitiveness to GC formation as well as affinity maturation. In the case of GCs, this competition can occur in the form of limitations on antigen availability and presentation by FDCs that would cause higher affinity B cells to deplete antigen pools or blocking lower affinity cells from accessing the antigens (Liu et al., 1989; Tarlinton and Smith, 2000). This mechanism by which high affinity B cells act as a sink for antigen and limit antigen availability is critical for the positive selection process since apoptosis is the default fate for LZ GC B cells (Liu et al., 1989), therefore, low affinity B cells are not only incapable of continuing through the GC reaction, but will undergo cell death leaving only the higher affinity cells alive (Liu et al., 1989; Mayer et al., 2017). An alternative theory to this direct

competition model suggests that Tfh are the limiting factor in GC B cell selection. In this model, higher affinity B cells are capable of binding a greater amount of antigen, internalizing the antigen, and presenting a greater concentration of peptide-MHC-II to Tfh compared to lower affinity B cells (Batista et al., 2001). This suggests that all GC B cells can survive, even with limited BCR signaling, but only those with sufficient levels of surface peptide-MHC-II would be able to engage the limited number of Tfh for T cell help, thus the T cells would selectively give help to higher affinity B cells (Meyer-Hermann et al., 2006). Previously, it was uncertain which model was more favorable as determining the relative importance of T cell help compared to BCR signaling since B cells that lose BCR expression as a result of hypermutation undergo rapid cell death (Mayer et al., 2017). Consistently, both models require BCR signaling as a mechanism of cell survival; however, experiments performed using α-DEC205-OVA, which created a situation in which all GC B cells displayed the same amount of peptide-MHC-II, thus blinding T cells to BCR affinity, showed a loss of selective pressure against low affinity BCRs thereby indicating that strong BCR signaling, alone, is not sufficient for GC B cell selection (Victora et al., 2010).

After the cells receive T cell help, the cell will either choose to differentiate into a plasma or memory cell, or continued GC cycling. In the case of continued GC cycling, the B cell will upregulate mechanistic target of rapamycin complex 1 (mTORC1) and Myc to activate a cell growth program necessary for continued clonal expansion with the number of proliferative cycles being proportional to the amount of Myc expressed and the size of the cell after the growth cycle (Ersching et al., 2017; Finkin et al., 2019). The cells then upregulate CXCR4, a receptor for CXCL12, and migrates into the DZ to undergo both SHM and proliferation. The proliferation continues through the DZ and into the GZ (Kennedy et al., 2020) until the new BCR is expressed and signals through the serine/threonine kinase, Akt, to phosphorylate FoxO1 to expel it from the

nucleus and allow migration back into the LZ (Luo et al., 2018) using CXCR5, where it will undergo the same selection process as before. Over time, through continuing cycles of selection and expansion, GCs will begin to lose diversity as a single clone dominates the reaction due to having the highest affinity receptor for the immunizing antigen (Tas et al., 2016).

It has been nearly three decades since the emergence of a GC model that accounts for affinity maturation. Based on what we currently understand, B cells undergo an activation stage in which they encounter antigen and migrate into T cell zones to receive CD40-CD40L engagement. This activation induces CSR prior and subsequent GC formation in which B cells undergo an evolutionary process of SHM, proliferation, and selective pressure known as affinity maturation (Figure 1.3). These mechanisms of antibody diversification allow our bodies to generate high affinity antibodies to a virtually infinite number of antigens. Future research will continue to study these mechanisms to describe, in more detail, the functions of lesser studied GC cell types such as Tfr and the processes controlling the emergence of pathogenic autoreactive antibodies.

1.3.3 Activation induced cytidine deaminase

Activation induced cytidine deaminase (AID) was first discovered by the Honjo group in 1999 as a novel member of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family of mRNA editing enzymes, sharing homology with APOBEC-1 and inducing cytosine to uracil mutations, expressed specifically in splenic B cells that had been activated through immunization (Muramatsu et al., 1999). Following studies showed the importance of the enzyme in inducing CSR and SHM as patients presenting with hyper IgM, later categorized as hyper IgM type 2, were found to have loss of function mutations in this enzyme (Muramatsu et al., 2000; Revy et al., 2000). At this time, due to its homology with APOBEC proteins, it was still thought to regulate these diversification processes through deamination of RNA and it was not

until the Neuberger group, in 2002, published their findings showing that expression of AID in *Escherichia coli* leads to nucleotide transitions in the DNA rather than RNA that the true target of AID was discovered. Due to the discovery of its mutagenic potential, much of the research regarding AID has been aimed toward describing the regulatory networks that control the targeting and expression of the enzyme as dysregulation can lead to severe pathologies such as autoimmunity and cancers such as Burkitt's lymphoma in which the Myc oncogene is recombined into the IgH locus leading to constitutive expression (Adams et al., 1983; Klein et al., 2011; Ramiro et al., 2004).

Transcriptionally, AID is under the control of transcriptional regulators such as NF-kB (Park et al., 2013), HoxC4 (Park et al., 2013; Park et al., 2009), and Irf8 (Qi et al., 2009), of these, NF-kB being one of the more important signals as it is downstream of nearly all activation signals including CD40, BCRs, TLRs, Transmembrane activator and CAML interactor (TACI), APRIL, and BAFF. Notably, all of these signaling cascades, excluding BCR signaling, are capable of inducing AID expression (He et al., 2004; Kim et al., 2011; Park et al., 2013; Pone et al., 2012). Additionally, signals such as CD40 induce both canonical p65 NF-kB activation as well as non-canonical p52 both of which play a critical role in AID expression as non-canonical NF-kB can be recruited to the AID promoter while the canonical p65 can be recruited to enhancer regions upstream of the gene (Park et al., 2009; Tran et al., 2010). Interestingly, AID expression peaks approximately 2-3 days after stimulation, which mirrors the activation kinetics of the non-canonical NF-kB pathway (Pone et al., 2012), which may act to provide sustained expression levels whereas the canonical pathway, which is more rapidly induced, may provide an immediate, short term burst in expression (Zan and Casali, 2013). To prevent aberrant expression, two major repressor proteins, Myb and E2F, are thought to be capable of silencing the gene as they both have

binding sites in *cis*-regulatory regions adjacent to the gene (Tran et al., 2010). As cells become activated, Pax5 and E2A act as promoters for transcription; however, these factors can be antagonized by Blimp-1 and Id2 (Xu et al., 2007).

After the protein is transcribed and translated, subcellular localization of the enzyme heavily favors the cytoplasm rather than the nucleus, even though, at 24 kDa, the protein is small enough to freely diffuse through the nuclear pores. The protein contains a nuclear export signal that allows transport out of the nucleus in an chromosomal Maintenance 1 (CRM1) dependent manner (McBride et al., 2004)and, in the cytosol, forms a complex with eEF1A and HSP90 (Hasler et al., 2011; Ito et al., 2004; McBride et al., 2004; Orthwein et al., 2010). Additionally, in contrast to cytosolic AID, the nuclear protein is rapidly targeted by proteasomes in both ubiquitin and non-ubiquitin mediated degradation pathways (Aoufouchi et al., 2008; Uchimura et al., 2011). Although some factors have been found to assist in AID localization, few cytosolic mechanisms maintaining compartmentalization of AID have been discovered and the details of its cytosolic retention are still a mystery, though it is clear that his regulation is essential for avoiding off target genome mutagenesis and genomic instability as nuclear restricted forms of the protein are associated with increased mutagenesis both at the Ig locus and at non-Ig loci (Aoufouchi et al., 2008).

One of the most common mechanisms for regulating protein function is through post-translational modifications – the addition or subtraction of functional groups from a protein. Previous studies on AID targeting have shown that DNA can be deaminated by phosphorylated AID, but not by non-phosphorylated forms of the protein (Chaudhuri et al., 2004). This suggests that phosphorylation of AID is important for its targeting to its DNA substrate. Additionally, due to the reduction in phosphorylation of AID purified from non-B cells (Basu et al., 2005; McBride

et al., 2006), there seems to be B cell specific mechanisms by which the protein is phosphorylated. In B cells, AID can be phosphorylated on Ser38, a requirement for normal CSR and SHM (Basu et al., 2005; Cheng et al., 2009; McBride et al., 2006), Thr27, also important for AID activity(Basu et al., 2005), Thr 140, which is also important for both CSR and SHM, but preferentially favors SHM(McBride et al., 2006), and Ser3, which seems to have a negative impact on the protein function (Gazumyan et al., 2011). Consistent with previous findings, mutation of either Ser38 or Thr140 do not impact catalytic activity, but likely interferes with targeting (McBride et al., 2006). In addition to these residues, AID can also be phosphorylated on Tyr184 (Basu et al., 2005; McBride et al., 2006); however the functional relevance of this phenomenon is still unclear as the mutation of this residue to alanine does not seem to significantly affect CSR in activated B cells (Basu et al., 2005).

Since AID is a deaminase that generates mutations in the genome, targeting of the enzyme is crucial for preventing off target effects that can lead to severe pathologies such as cancer resulting from deleterious genomic translocations. Early studies investigating AID targeting looked for target sequences that may be unique to the Ig locus. These studies showed that switch regions contained high frequencies of AGCT repeats, which are targeted by 14-3-3 adaptor proteins that recruit AID to the DNA (Xu et al., 2010). Following these findings, the Nussenzweig group showed that AID preferentially localizes to regions of stalled transcriptional elongation and that it interacts with the DSIF component, Spt5 (Pavri et al., 2010) indicating that, not only does AID recognize a specific target sequence, but it also requires that the target region initiates transcription, but does not efficiently elongate the RNA product, which may provide an abundance of ssDNA substrate as well as a greater amount of time for AID to interact with the DNA.

More recently, it was discovered by the Chaudhuri group that AID targeting can be mediated through interaction with non-coding RNAs generated following lariat branching (Zheng et al., 2015). It was found that switch regions generate a transcript that contains G-quadruplexes, secondary structures formed by sequences rich in guanines, and that these structures interact with AID to assist in targeting (Zheng et al., 2015). Following this study, additional data showed that AID can also bind G-quadruplexes in DNA and that AID oligomerizes at these genomic regions to generate clustered mutations that may facilitate the formation of double stranded breaks (DSB) (Qiao et al., 2017). Additionally, targeting of AID to these genomic structures was more robust than targeting to the AGCT hotspot sequence or TTCT cold spot sequence as linear ssDNA with or without the target sequences did not have any effect on the deamination of G-quadruplex structured substrates (Qiao et al., 2017). Interestingly, it was observed that c-Myc, the transcription factor that is commonly recombined to the IgH locus in B cell lymphoma, contains genomic G-quadruplexes in the first exon and intron that may be responsible for this off target AID effect (Duquette et al., 2004; Duquette et al., 2005). In general, the targeting of AID seems to be similar to that of clustered regularly interspaced short palindromic repeats (CRISPR) in which a guide RNA is used to target a DNA modifying enzyme to a specific target sequence; however, AID targeting includes the ability to bind DNA as well as RNA.

1.3.4 Somatic hypermutation

To make high affinity antibodies, B cells undergo a Darwinian-like process of selection within germinal centers in which higher affinity B cells outcompete lower affinity B cells for survival. Between rounds of selection in the GC LZ, B cells undergo SHM in the DZ, which allows them to mutate the antigen binding region of the BCR and generate new BCRs. This process begins with a deamination event caused by AID leading to a mismatched G:U in the DNA. The cell could

simply replicate the mismatch, which would restrict the potential mutations to transitions – C to T or G to A. Other types of mutations would depend on recognition of the lesion and activation of DNA repair mechanisms such as base excision repair (BER) or mismatch repair and subsequent recruitment of low fidelity DNA polymerases.

When the cell recognizes a G:U DNA lesion, the most common mechanism for its removal is through BER. To initiate this pathway, the cell must first excise the uracil from the genome using an enzyme such as uracil-DNA glycosylase (UNG), which leaves an abasic site that is typically repaired through faithful BER mechanisms, thus generating no mutations (Krokan and Bjoras, 2013). This is highlighted by the finding that deficiency in UNG leads to increased mutational burdon at the Ig and non-Ig loci (Di Noia and Neuberger, 2002; Liu et al., 2008; Rada et al., 2004). Alternatively, the abasic site can be cleaved by AP endonuclease (APE) 2 (Stavnezer et al., 2014) and the following DNA synthesis is performed using a specialized DNA polymerase such as Rev1, which can only introduce cytosines and is responsible for most C:G transversions (Jansen et al., 2006), or Polη, which plays a major role in the generation of mutations at A:T pairs (Delbos et al., 2007; Delbos et al., 2005).

During SHM, the majority of A:T mutations are dependent on MutSα and the exonuclease, EXO1, components of the MMR pathway, and do not seem to be affected by UNG deficiency (Rada et al., 2002). In this pathway, MSH2 and MSH6 recognize AID mediated U:G mismatches and EXO1 recognizes a nick in the DNA breaks down a portion of the DNA in the 5’-3’ direction; however, this step may not be necessary as mice with deficiency in EXO1 are still capable of performing SHM (Schaetzlein et al., 2013). The DNA gaps formed after exonuclease activity are then filled in by low fidelity polymerases such as Polη, Polζ, Polκ, and Polι (Faili et al., 2009;

Maul et al., 2016; Zanotti and Gearhart, 2016). Of these polymerases, Polη makes the largest contribution and deficiency in the rest does not significantly affect SHM (Figure 1.4).

The recruitment of low fidelity polymerases is key to the process of SHM, though the mechanism by which they are recruited is not well understood. One major molecular event that determines the recruitment of Polη and Rev1 is the monoubiquitination of proliferating cell nuclear antigen (PCNA) at Lys164 (Guo et al., 2006; Kannouche et al., 2004; Moldovan et al., 2007). Consistently, mutation of Lys164 on PCNA leads to deficiencies in SHM due to a reduction in the recruitment of Polη, which results in decreased levels of mutations at A:T pairs, compensated by an increase in mutations at C:G pairs (Langerak et al., 2007; Roa et al., 2008). Though it is clear that PCNA plays a major role in SHM and that the ubiquitination at Lys164 is critical for the recruitment of low fidelity polymerases, the mechanism by which generation or excision of uracil lesions leads to the ubiquitination of PCNA is still a mystery.

1.3.5 Class switch recombination

Determined by the structure of the heavy chain constant region (C_H), Antibodies have several major isotypes, IgM, IgD, IgG, IgE, and IgA, each of which has its own unique effector functions. Excluding IgM and IgD, which are expressed on mature naïve B cells by default, B cells must undergo CSR to make the different antibody isotypes. This process involves the deletional recombination of C_H genes in the IgH locus, therefore, cells that have switched to a downstream isotype cannot return to a previous isotype. Previously, it was believed that CSR occurred in the LZ of the GC while SHM occurred in the DZ; however, recently, it was found that CSR occurs prior to GC formation, around 2.5 days after antigen exposure, whereas the GC forms around day 3.5 and contains very few cells actively undergoing CSR (Roco et al., 2019).

The initiation of CSR is similar to that of SHM – AID generates C to U mutations in the switch regions upstream of each C_H gene that become transcriptionally active depending on the cytokine signaling experienced by the B cell. These mutations are then recognized by DNA damage machinery including UNG and, in leu of APE2 as was seen in SHM, APE1. This step of uracil removal by UNG, although it was optional in SHM, is critical for generating the necessary DSBs to initiate recombination as CSR is almost entirely absent in UNG deficient mice and patients (Imai et al., 2003; Petersen-Mahrt et al., 2002; Rada et al., 2002; Schrader et al., 2005). Once the DSBs are generated, donor and acceptor S regions are joined by either NHEJ, which includes the essential proteins Ku70 and Ku80 as well as the ligase complex containing XRCC4 and DNA ligase IV (Casellas et al., 1998; Manis et al., 1998; Pan-Hammarstrom et al., 2005; Reina-San-Martin et al., 2003; Soulas-Sprauel et al., 2007) or alternative end joining (A-EJ), though NHEJ is the main pathway (Bothmer et al., 2010; Ehrenstein et al., 2001; Yan et al., 2007). In the case of NHEJ, the Ku70-Ku80 complex binds the DSB and helps recruit recombination enzymes including XRCC4 and DNA ligase IV that join the two DNA ends (Figure 1.4).

After the discovery of these mechanisms, there was much speculation as to the mechanism by which donor and acceptor regions would be properly aligned during the recombination process and whether they were pre associated prior to DSB formation. Recently, the Alt group showed that this process involved cis IgH organizational features (Dong et al., 2015), the same group later characterized this as chromatin loop extrusion in which coshesin aligns the acceptor and donor regions in preparation for AID mediated DSB formation (Zhang et al., 2019). This finding not only answers the question of how acceptor and donor sites are aligned for proper recombination, but also how the DSBs generated during CSR are recombined in a deletional manner rather than inversional.

1.3.6 Plasma cells

Antibodies are made by a subset of terminally differentiated B cells known as plasma cells that can be characterized by their expression of CD138 and the transcription factor Blimp-1. Plasma cells can be put into two major categories: short-lived plasma cells and long-lived plasma cells. Short lived plasma cells are generally products of extrafollicular responses, therefore, they undergo very little SHM and can be generated by B-1 cells, MZ B cells, and follicular B cells (MacLennan et al., 2003). On the other hand, long-lived plasma cells are generated through GC responses in a T dependent manner (Victora and Nussenzweig, 2012). Since these cells arise late during the GC response (Weisel et al., 2016), they experience significant SHM and affinity maturation and are, usually, very high affinity compared to short-lived plasma cells. After differentiation in the secondary lymphoid organs, long-lived plasma cells migrate to the bone marrow where they can survive for many years, providing long term immunity.

In the GCs, plasma cell precursors have been found in the LZ and were characterized as cells that have low expression of Bcl-6 and high expression of CD69, Irf4, and Myc (Ise et al., 2018). These cells make up approximately 1-3% of LZ B cells and were enriched for high affinity BCRs compared to Bcl-6^{hi}CD69^{hi} cells that had relatively low BCR affinity (Ise et al., 2018). Interestingly, Bcl-6^{lo}Irf4^{+}CD69^{hi} cells in the LZ do not express the classical plasma cell markers, CD138 and Blimp-1, indicating that they may be early plasma cell precursors and not bona fide plasma cells (Ise et al., 2018). Additionally, a population of Bcl-6^{lo}CD69^{hi} cells with higher expression of Irf4 and Blimp-1 were identified in the DZ (Ise et al., 2018; Krautler et al., 2017), which may suggest that plasma cell precursors migrate from the LZ to the DZ to continue the maturation process prior to exiting the GC from the DZ, consistent with prior models (Fooksman et al., 2010; Meyer-Hermann et al., 2012).

Since plasma cells generated from GC B cells tend to produce very high affinity antibodies, the B cells that develop into plasma cells must have high affinity BCRs and should receive greater or more consistent BCR signaling compared to lower affinity B cells. In addition to increased BCR signaling, these cells would outcompete lower affinity cells for antigen and should present the greatest amount of peptide-MHC-II on their cell surfaces, providing the greatest potential for T cell help through CD40 engagement. Indeed, transcriptomic analysis of plasma cell precursors in the LZ showed an enrichment of BCR and CD40 signaling signatures (Ise et al., 2018). In contrast, a recent study using an MHC-II$^{+/+}$ and MHC-II$^{+/-}$ chimeric mouse model, it was shown that a 50% reduction in surface expression of peptide-MHC-II did not significantly affect GC B cell selection, proliferation, or plasma cell differentiation (Yeh et al., 2018). This seems to contradict the previous understanding; however, it is entirely possible that a two-fold difference in antigen presentation is not significant to GC B cells and GC selection and differentiation is not sensitive to small differences in peptide-MHC-II density.

Unlike T cell help, the role of BCR signaling in the selection and differentiation of plasma cells is still unclear. Based on recent data, GC B cells seem to have attenuated BCR signaling compared to naïve B cells; however, BCR signaling is still capable of selectively activating the AKT pathway, which leads to phosphorylation and nuclear exclusion of FoxO1 (Luo et al., 2018), therefore, it is possible that the duration and amplitude of BCR signaling may have finer control over FoxO1 activity. Additionally, CD40 signaling induces nuclear localization of NF-kB, a driver for Irf4 expression (Luo et al., 2018). Presumably, strong BCR signaling works synergistically to direct GC B cells to commit to the plasma cell fate by inducing Myc and Irf4 while inhibiting FoxO1. In support of this hypothesis, strong BCR and CD40 signaling led to degradation of Cbl ubiquitin ligases that mediate ubiquitin dependent protein degradation of Irf4 (Li et al., 2018).

1.3.7 Memory B cells

One of the defining features of the adaptive immune response is the ability to generate memory to previous antigens. Humoral immune memory is maintained, in part, by long-lived plasma cells in the bone marrow secreting antibody into circulation as well as by the formation of memory B cells that can quickly respond to antigen. Previously, it was thought that these memory B cells would recognize antigen and reenter GCs to boost affinity and differentiate into plasma cells as they were assumed to be higher affinity compared to naïve B cells due to prior GC experience; however, memory B cells generally arise from early GC reactions, or prior to GC formation (Weisel et al., 2016), which allows for a high percentage of low affinity clones to persist in the compartment compared to plasma cells that exit later during GC response and are, therefore, higher affinity due to a greater amount of affinity maturation (Smith et al., 1997; Takahashi et al., 2001). Additionally, recent findings show that, although the secondary antibody response is dominated by few high affinity memory B cell clones, these cells rarely participate in recall GCs (Mesin et al., 2020).

In humans, memory B cells have been characterized as cells expressing CD27 on the cell surface as $CD27^+$ cells show many characteristics of memory B cell identity such as isotype switch and the presence of V region mutations (Klein et al., 1997; Tangye et al., 1998). Importantly, though, it does not seem that memory B cells are exclusively $CD27^+$ since minor populations have been found that do not express the molecule (Ehrhardt et al., 2005; Fecteau et al., 2006). In mice, memory B cells can be defined by their expression of CD80 and PD-L2, which is a product of the strength of CD40 signaling provided during differentiation (Koike et al., 2019): double positive cells are more prone to differentiate into plasma cells whereas double negative cells favor reentry

into GCs while $CD80^{-}PD\text{-}L2^{+}$ cells are more similar to double negative cells, but may be an intermediate between the two (Zuccarino-Catania et al., 2014).

Memory B cells must provide protection against pathogens for a significant amount of time. This led to questions regarding the mechanism by which these cells are maintained. A major advance in understanding of the longevity of memory B cells was the finding that they do not require BAFFR signals in neither humans nor mice (Benson et al., 2008; Scholz et al., 2008), but Syk is necessary for their survival, potentially due to the need for tonic BCR signaling (Ackermann et al., 2015; Kraus et al., 2004). In contrast to this, it does not seem as though antigen recognition is important for memory B cell survival, as has been suggested (Gray and Skarvall, 1988), since switching mouse BCRs to ones that do not recognized antigen complexes trapped on FDCs does not affect the half-life of these cells (Maruyama et al., 2000). Additionally, T cells are not required for their maintenance as T cell depletion does not affect recall responses (Vieira and Rajewsky, 1990). To date, it is still uncertain what external signals provide support for the maintenance of memory B cells. Several factors have been suggested to be important for their survival such as Puma (Clybouw et al., 2011) and Mcl-1 (Vikstrom et al., 2010); however, it is unclear whether these molecules are important for the longevity of memory B cells or their formation (Weisel and Shlomchik, 2017).

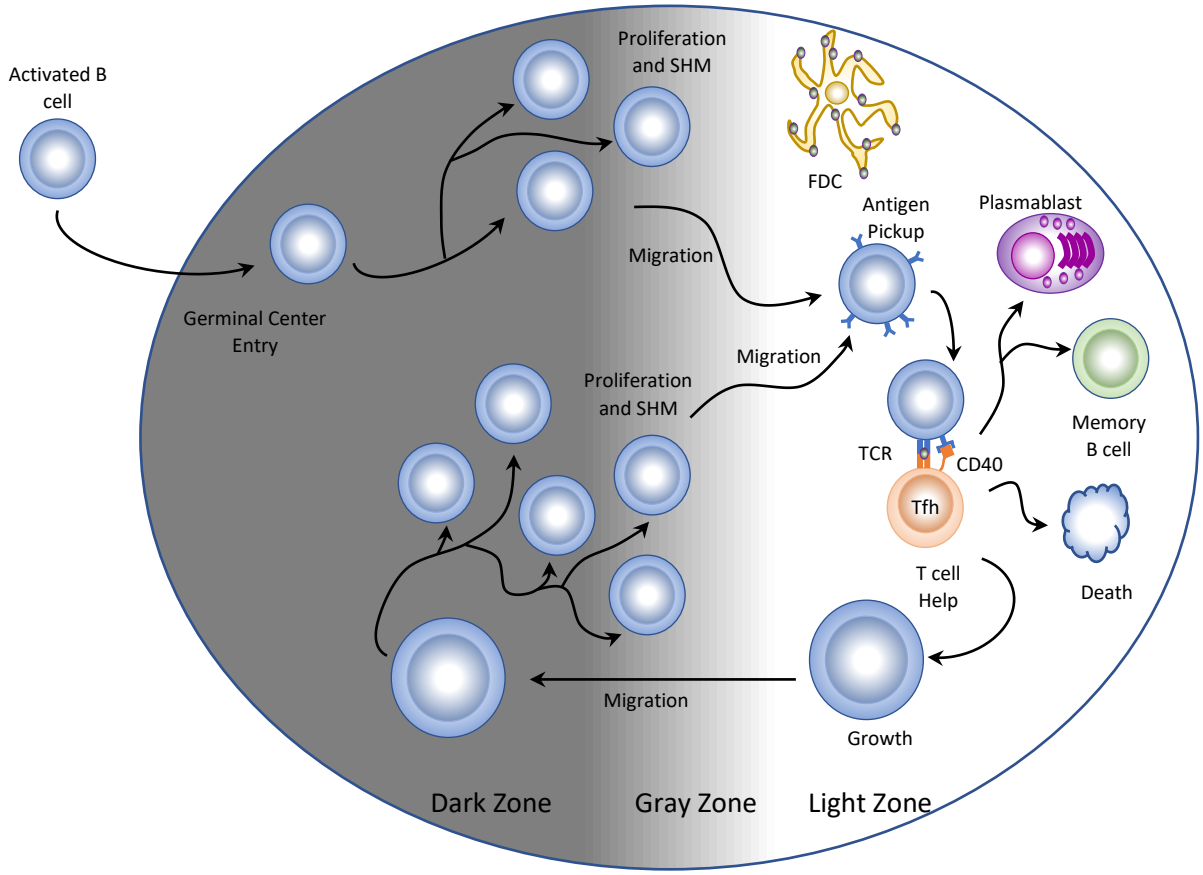

Figure 1.2: Germinal centers. GC B cells undergo proliferation and SHM in the DZ, then migrate into the light zone where they will pick up antigen from FDCs and look for T cell help. After receiving T cell help, they can either undergo differentiation or continued SHM. If the cell does not receive sufficient T cell help, then it will undergo cell death. This image was adapted from (De Silva and Klein, 2015).

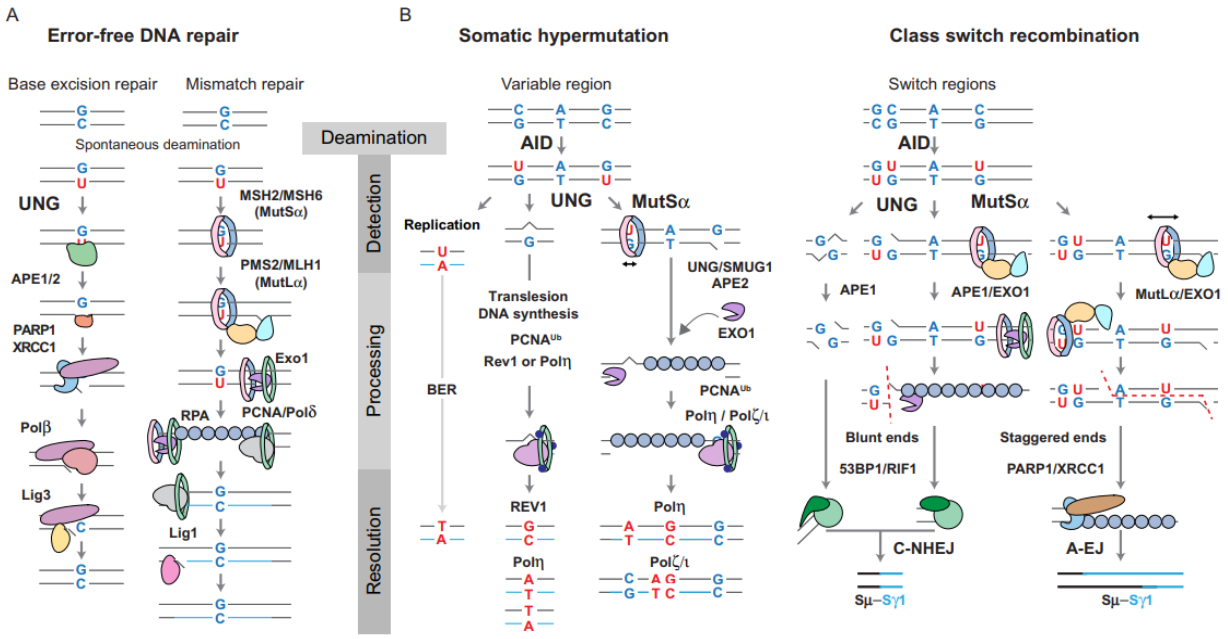

Figure 1.3: Mechanism of SHM and CSR. A) Error free mismatch repair. B) Error inducing DNA polymerases repair DNA lesion to introduce mutations in the VDJ region to promote SHM. CSR occurs when upstream switch regions are recombined with downstream regions via NHEJ. This image was taken from (Methot and Di Noia, 2017) with permission from Elsevier.

1.4 Hypothesis and Specific Aims

To test our hypothesis that Aire expression in germinal center B cells acts as a checkpoint to regulate the processes of antibody diversification, my thesis work was split into three aims in chapters 2-4 respectively:

Specific Aim 1: To determine the function of Aire in germinal center B cells. Our working hypothesis was that since germinal centers are the sites in which B cells undergo SHM and CSR, Aire expression will likely have regulatory effects on the mechanisms of antibody diversification.

Specific Aim 2: To determine the mechanism by which Aire regulates antibody diversification. Our working hypothesis was that, if Aire is capable of regulating the processes of antibody diversification, then it will have regulatory functions on AID, the enzyme that initiates both processes.

Specific Aim 3: To determine the role of Aire in the regulation of autoreactive antibodies. Our working hypothesis is that if Aire negatively regulates the process of SHM, then the absence of Aire will likely lead to the generation of autoreactive B cell receptors. These B cell receptors may then escape immune tolerance mechanisms and, eventually, lead to the generation of autoreactive antibodies similar to what is seen in APS-1 patients.

CHAPTER 2: AIRE EXPRESSION IN GC B CELLS

2.1 Introduction

Autoimmune Regulator (Aire) is a protein that has been shown to be expressed in a number of different cell types both in the thymus as well as in the periphery. In the thymus, expression in medullary thymic epithelial cells (mTECs) and B cells drives expression of peripheral tissue antigens to generate an antigen pool on which developing thymocytes are negatively selected (Anderson et al., 2002; Yamano et al., 2015). In the periphery, Aire has been observed in a subset of extra thymic Aire expressing cells (eTACs) that play a role in regulating T cell activation through the expression of peptide-MHC without co-stimulation (Gardner et al., 2013). As its name suggests, in most contexts, Aire functions as a regulatory of immune response to prevent autoreactivity.

Aire expression in the thymus is dependent on the transcription factor, NF-kB (Chin et al., 2003; Haljasorg et al., 2015; Heino et al., 2000). In mTECs, this signaling can come from the receptor, receptor activator of NF-kB ligand (RANKL) (Rossi et al., 2007) whereas, in thymic B cells, it can be activated through engagement of CD40 by CD40L expressed on developing thymocytes (Yamano et al., 2015). Due to the ability of CD40 signaling to activate Aire expression in thymic B cells, it stands to reason that peripheral B cells activated by CD40-CD40L engagement would also express Aire.

Germinal centers are structures composed primarily of activated B cells. These structures can be divided into several major regions: a mantle zone composed of naïve B cells that get pushed aside as activated GC B cells expand, a dark zone (DZ) in which GC B cells undergo proliferation and somatic hypermutation (SHM), a gray zone (GZ) in which B cells continue proliferation, and a light zone in which B cells receive survival signals and T cell help (Kennedy et al., 2020; Victora

and Nussenzweig, 2012). GC B cells can be characterized by their expression of the transcription factor, Bcl-6, and absence of surface IgD as the majority of these cells have already undergone class switch recombination (CSR). Due to the dependence of B cells on T cell help in the form of CD40-CD40L engagement with T follicular helper cells (Tfh) and due to the reduced BCR signaling in GC B cells, we hypothesize that GC B cells would express Aire.

2.2 Materials and Methods

2.2.1 Patient Samples

Human tonsil samples were taken after pediatric tonsillectomy from the Children's Hospital of Michigan in collaboration with Dr. Janet Poulik. Peripheral blood was purchased from the Red Cross in the form of blood filters.

2.2.2 Mice

Aire^{Adig} mice were previously described (Gardner et al., 2008) and provided by Dr. Mark Anderson from University of California San Francisco. Mice were housed in the Division of Laboratory Animal Resources (DLAR) facility at Wayne State University under the institutional animal care and use committee (IACUC) protocol number, 18-07-0732. All mice were genotyped by PCR to ensure inheritance of the transgene.

2.2.3 Cell lines

CH12 cells were cultured in RPMI-1640 medium further supplemented with 5% (v/v) NCTC and 50 μM β-mercaptoethanol. To stimulate Aire expression and CSR, cells were activated with 5μg/ml anti-CD40 + 100ng/ml IL-4 + 1ng/ml TGF-β1 (R&D 7666-MB/CF) for up to 3 days.

2.2.4 Immunofluorescence

Tissue blocks were frozen in OCT prior to cutting 6µm sections. Sections were thawed in cold PBS prior to fixation with 4% paraformaldehyde (PFA) for 15 minutes and permeabilization with 0.2% Triton X-100 for 15 minutes. Slides were then blocked with either 5% BSA in PBS + 0.1% Tween or 10% serum of the secondary antibody source in the same buffer with 0.1mg/ml human IgG for 1 hour at room temperature prior to antibody staining. Primary antibodies for IgD (Southern 2032-02), Aire (Fisher 13-9534-82 or Miltenyi 130-105-401), Pax5 (BioLegend 649711), Bcl-6 (Ventana 227M), and CD23 (Beckman IM0529) were stained overnight at 4°C. Secondary antibodies were stained the following day for 1 hour at room temperature. Slides were sealed and imaged on a Leica TCS SP5 in the Wayne State MICR core facility.

2.2.5 Flow Cytometry

For human peripheral blood samples, cells were purified purified by gradient centrifugation using Histopaque-1077 (Sigma 10771) prior to red blood cell (RBC) lysis. Tonsil samples were minced and pushed through a 40µm cell strainer to generate single cell suspensions. Samples were blocked using Fc blocking reagent (Miltenyi 130-059-901) prior to staining with fluorescently labeled antibody and either GV510 (Tonbo 13-0870-T500) or 7AAD (Tonbo 13-6993-T500) for 1 hour at room temperature. To show human tonsil expression of Aire, cells were stained with CD19 (BioLegend 302204), CD38 (BioLegend 303516), Aire (Miltenyi 130-105-401), IgD, CD27 (BioLegend 302812), and CD24 (eBioscience 47-0247). Events were captured on a Fortessa LSR II and analyzed using Treestar FlowJo 7 or 10 software.

For mouse cells, spleens were harvested after intraperitoneal immunization with either 100µg NP_{32}-KLH (Biosearch N-5060) mixed with Complete Freund's Adjuvant (CFA) for the first

immunization and Incomplete Freund's Adjuvant (IFA) (Fisher 77145) for 3 subsequent immunizations or $4x10^8$ SRBC mixed with or without Complete Freund's Adjuvant (CFA) (Fisher 77140). Spleens were then minced and pushed through a 40µm cell strainer prior to RBC lysis. Staining was performed for 1 hour at room temperature with either GV510 or 7AAD after Fc block (Tonbo 70-0161) for 10 minutes. To show Aire expression, cells were stained with a combination of CD19 (BioLegend 115523), GL-7 (BD 561529), Fas (BD 554258), B220 (BioLegend 103224), CXCR4 (eBioscience 13-9991), and CD83 (BioLegend 121515). Events were captured on a Fortessa LSR II and analyzed using Treestar FlowJo 7 or 10 software. For sorting, cells were sorted using a SONY Biotechnology SH800 cell sorter at the Wayne State MICR core facility.

2.2.6 RT-qPCR

RNA was purified using either a RNeasy purification kit (Qiagen 74004) or Trizol (Fisher 15596026) purification method. Reverse transcription was performed using an iScript reverse transcription kit (BioRad 1708840) according to the manufacturer's instructions. qPCR was performed using either SYBR Green (BioRad 4367660) or with an iTaq universal SYBR Green one-step kit (BioRad 172-5150) according to the manufacturer's instructions. PCR was performed using a StepOnePlus instrument (Applied Biosystems) and analyzed using the StepOne software.

2.2.7 Activation of cells in the presence of CAPE

Cells from peripheral blood were purified by gradient centrifugation using Histopaque-1077 (Sigma 10771) prior to RBC lysis. B cells were purified by incubating peripheral blood mononuclear cells with anti-IgD-Biotin (Southern 2032-08) for 10 minutes prior to conjugation with anti-biotin magnetic beads (Miltenyi 130-090-485) and passage through a magnetic column.

Cells were stimulated with 500ng/ml CD40L with or without 100ng/ml IL-4 and 25μM Caffeic Acid (CAPE) (Cayman 70750).

Naïve B cells were purified from splenocytes using a B cell isolation kit (Miltenyi 130-090-862) prior to stimulation with 500ng/ml CD40L (Peprotech 315-15) with or without 100ng/ml IL-4 (Peprotech 214-14) or 25μM CAPE (Cayman 70750).

2.3 Results

2.3.1 Human GC Aire expression

To test Aire expression in GC B cells, human tonsil sections were stained with IgD to show the mantle zone, Pax5, which indicates B cells, and Aire (**Figure 2.1**). To confirm these cells are, in fact GC B cells, co-staining with Bcl-6 was performed (**Figure 2.2**). Staining in GC B cells shows Aire staining in GC B cells with a speckled appearance, consistent with what is seen in thymic epithelial cells due to the interaction of Aire with nuclear bodies (Ilmarinen et al., 2008; Pitkanen et al., 2005); however no staining in mantle zone B cells was observed. These data were confirmed with flow cytometry analysis (**Figure 2.3**) which shows that, in healthy human tonsils, the majority of Aire positive cells in the $CD19^+$ compartment are CD38 positive, indicating that they are GC B cells (Oliver et al., 1997). Further analysis of the Aire positive cells show that they are IgD negative indicating that they have already undergone CSR, consistent with what is expected of GC B Cells (Roco et al., 2019). With the expression of Aire in GC B cells, we sought to determine whether other B cell types also expressed Aire. To this end, peripheral blood naïve (IgD^+CD27^-), MZ (IgD^+CD27^+), switched memory (IgD^-CD27^+), switched non-memory (IgD^-CD27^-) B cells as well as transitional ($CD24^{hi}CD38^{hi}$), mature ($CD24^{int}CD38^{int}$), memory ($CD24^{hi}CD38^-$) B cells, and plasma cells ($CD24^-CD38^{hi}$) were analyzed for Aire expression by

flow cytometry (**Figure 2.4**). None of these peripheral blood B cell types showed detectable expression of Aire, therefore, we conclude that Aire expression is unique to GC B cells in the periphery.

2.3.2 Mouse GC Aire expression

To test whether Aire expression in GC B cells shows the same characteristics in mice as it does in humans, we utilized a transgenic reporter mouse, *Aire*^{Adig}, henceforth referred to as "Adig", in which a transgene containing an Aire driven Igrp GFP uses the *Aire* locus to drive expression of GFP (Gardner et al., 2008). Mice were immunized with NP_{32}-KLH with CFA and boosted with NP_{32}-KLH with IFA three times prior to tissue harvest. Splenocytes analyzed by flow cytometry showed no expression of the GFP reporter in either non-GC B cells or plasma cells but showed clear upregulation of GFP in GC B cells (**Figure 2.5**). These data were surprising as previous studies have shown no GFP expression using this model (Yamano et al., 2015). To determine the reason for this inconsistency, we attempted to perform the experiment using the published immunization protocol that did not include adjuvant compared to immunization with adjuvant; however, the data from repeating the published experiment were consistent with our previous data showing upregulation of GFP in GC B cells, but no in blood, peritoneal, or non-GC B cells from either the spleen or peripheral lymph nodes (**Figure 2.6**). To confirm the correlation between GFP expression and Aire expression, GFP positive and negative cells were sorted from immunized Adig mice and RNA was purified for qPCR. Aire transcript is upregulated in GFP positive GC B cells compared to GFP negative non-GC B cells (**Figure 2.7**) confirming Aire expression in GC B cells and that the GFP reporter accurately represents Aire expression in the Adig model. Therefore, the reason for the inconsistency between our data and that which was previously published is still unclear.

2.3.3 LZ/DZ expression

As previously mentioned, GCs are split up into several regions: a LZ, DZ, GZ, and mantle zone (Kennedy et al., 2020; Victora and Nussenzweig, 2012). To determine the distribution of Aire expression in GC B cells, human tonsils were analyzed by immunofluorescence, staining for CD23 to indicate follicular dendritic cells (FDCs), whichexist predominantly in the LZ and IgD expressed by the naïve B cells in the mantle zone. Aire expression in both the LZ and DZ with what appears to be an even distribution between both regions; however, Aire expression was not observed in the mantle zone, confirming our previous results (**Figure2.8**).

To quantify this distribution, Adig mice were immunized with NP_{32}-KLH with CFA and boosted with NP_{32}-KLH with IFA three times. Spleens were taken from the immunized mice and flow cytometry analysis was performed to determine the distribution of GFP between the LZ and DZ. Although the trend seemed to slightly favor $CD83^+$ LZ localization, there was no significant difference between the proportion of LZ to $CXCR4^+$ DZ cells compared to total ratios (**Figure 2.9**) indicating no difference in the percentage of Aire expressing cells between the LZ and DZ.

2.3.4 Regulation of Aire expression

In the thymus, B cells express Aire as a result of CD40-CD40L signaling through NF-kB with thymic T cells (Yamano et al., 2015). Due to the dependence of GC reactions on this same signaling pathway, it is reasonable that GC B cells may also utilize this pathway for inducing Aire expression. To test this hypothesis, human IgD^+ B cells were purified and stimulated with CD40L + IL-4 with and without an NF-kB specific inhibitor, Caffeic acid (CAPE). Upregulation of Aire transcripts was observed after CD40L stimulation, which was ablated when CAPE was added (**Figure 2.10**). Protein expression showing upregulation of Aire after CD40 stimulation and

subsequent ablation was performed via western blot analysis (**Figure 2.11**). This effect was, largely, the same in mouse B cells as purified Adig B cells showed upregulation of GFP signal after CD40L treatment, which was absent when CAPE was added (**Figure 2.12**). Additionally, CH12 mouse B cell lines treated with CD40L+IL-4+TGF-β showed increase in Aire RNA and protein (**Figures 2.13** and **2.14**). These data are consistent with previous findings and indicate that (Yamano et al., 2015), in B cells, CD40 signaling through NF-kB induces Aire expression.

2.4 Discussion

Our immunofluorescence, flow cytometry, qPCR, and western blot data suggest that the transcriptional regulator, Aire, is expressed in both human and mouse GC B cells and that this expression is not seen in peripheral blood B cell types. The expression of Aire seems to be evenly distributed between the LZ and DZ and can be induced via CD40 signaling through NF-kB. This finding is particularly surprising since previous reports using the same Adig mice showed no Aire expression in GC B cells (Yamano et al., 2015). To determine the cause of this inconsistency, we attempted to replicate previous experiments; however, these attempts showed that, using the published protocols, Aire expression can still be observed in GC B cells. The conclusions drawn from the previous paper suggest that Aire expression is repressed by high levels of BCR signaling and that this repression is what leads to the lack of Aire in GC B cells (Yamano et al., 2015). This is an interesting idea, but does not take into account the findings that GC B cell BCR signaling is significantly reduced compared to naïve B cells (Khalil et al., 2012; Luo et al., 2018). Additionally, although, treatment of B cells with a high concentration of anti-IgM does, in fact, reduce CD40 mediated Aire expression, it is not completely inhibitory with approximately 20% of cells still showing positive GFP signal (Yamano et al., 2015). Therefore, we believe that it is entirely possible for Aire to be expressed in GC B cells; despite BCR signaling.

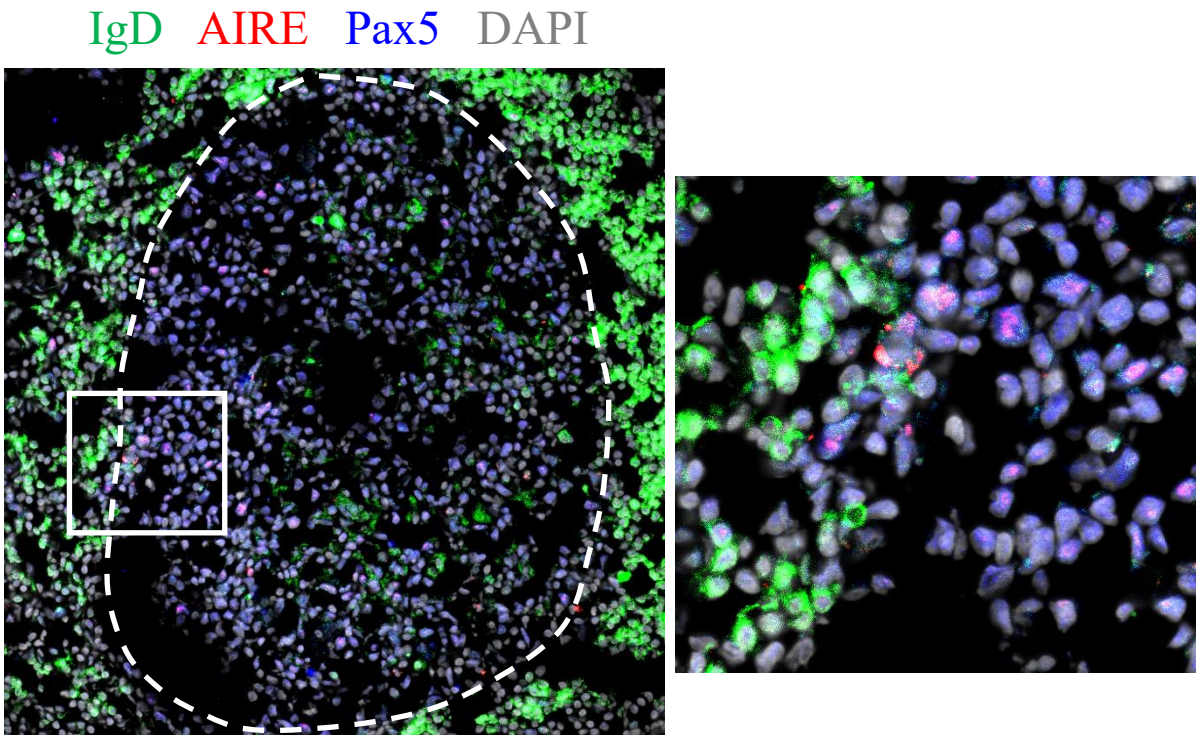

Figure 2.1: Expression of Aire in human GC B cells. Immunofluorescence staining of a germinal center in human tonsil.

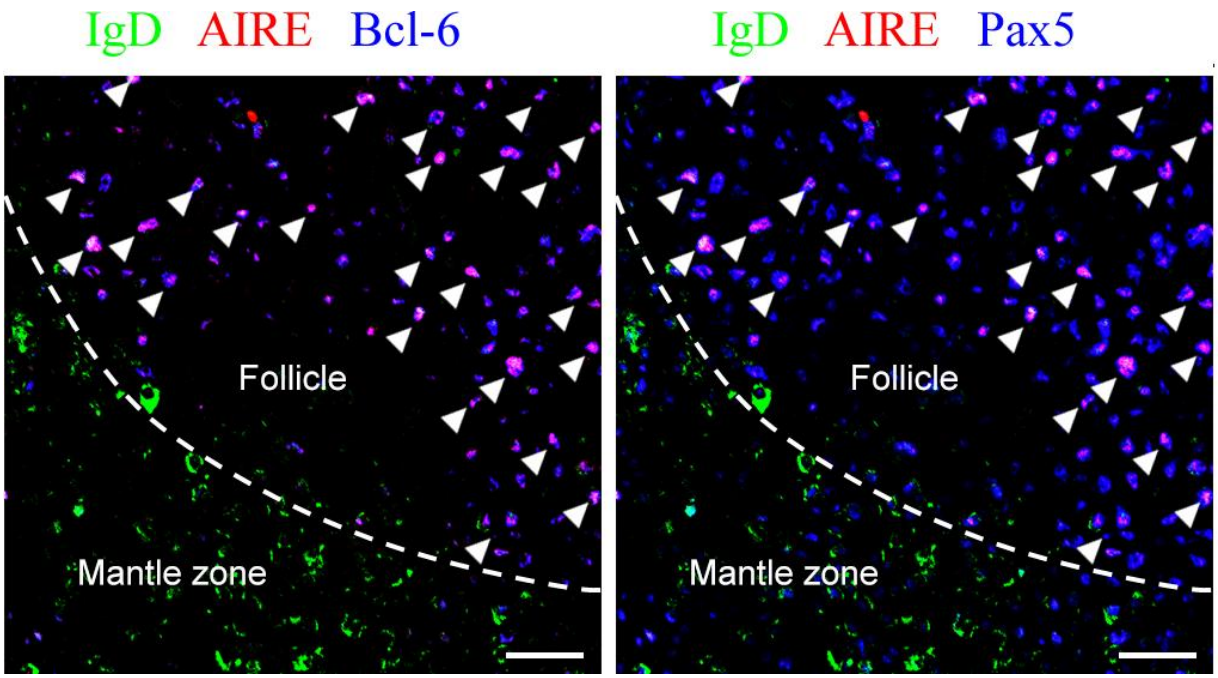

Figure 2.2: Aire expression in human tonsil Bcl-6 positive cells. Immunofluorescence staining of a germinal center in human tonsil to confirm Bcl-6 expression in Aire positive GC B cells

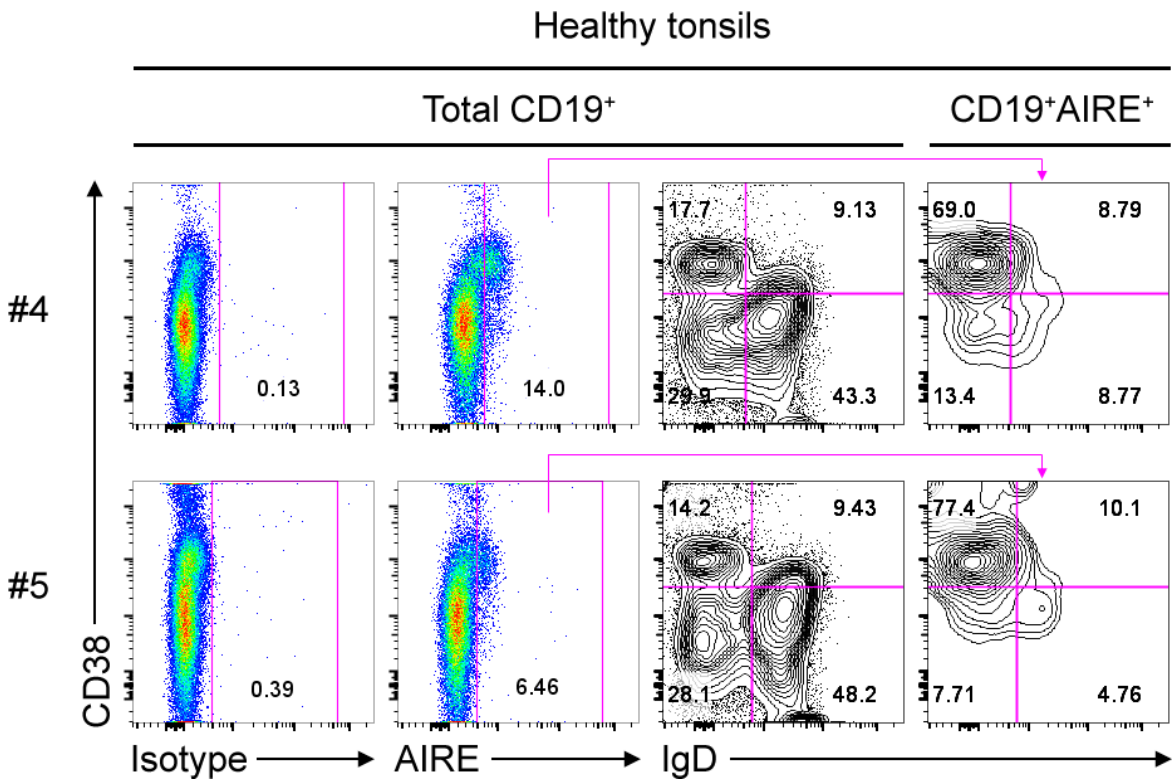

Figure 2.3: Aire expression in human GC B cells by flow cytometry. Flow cytometry analysis of Aire positive cells in human tonsils. Aire positive cells are positive for CD38 and negative for IgD indicating they have undergone CSR.

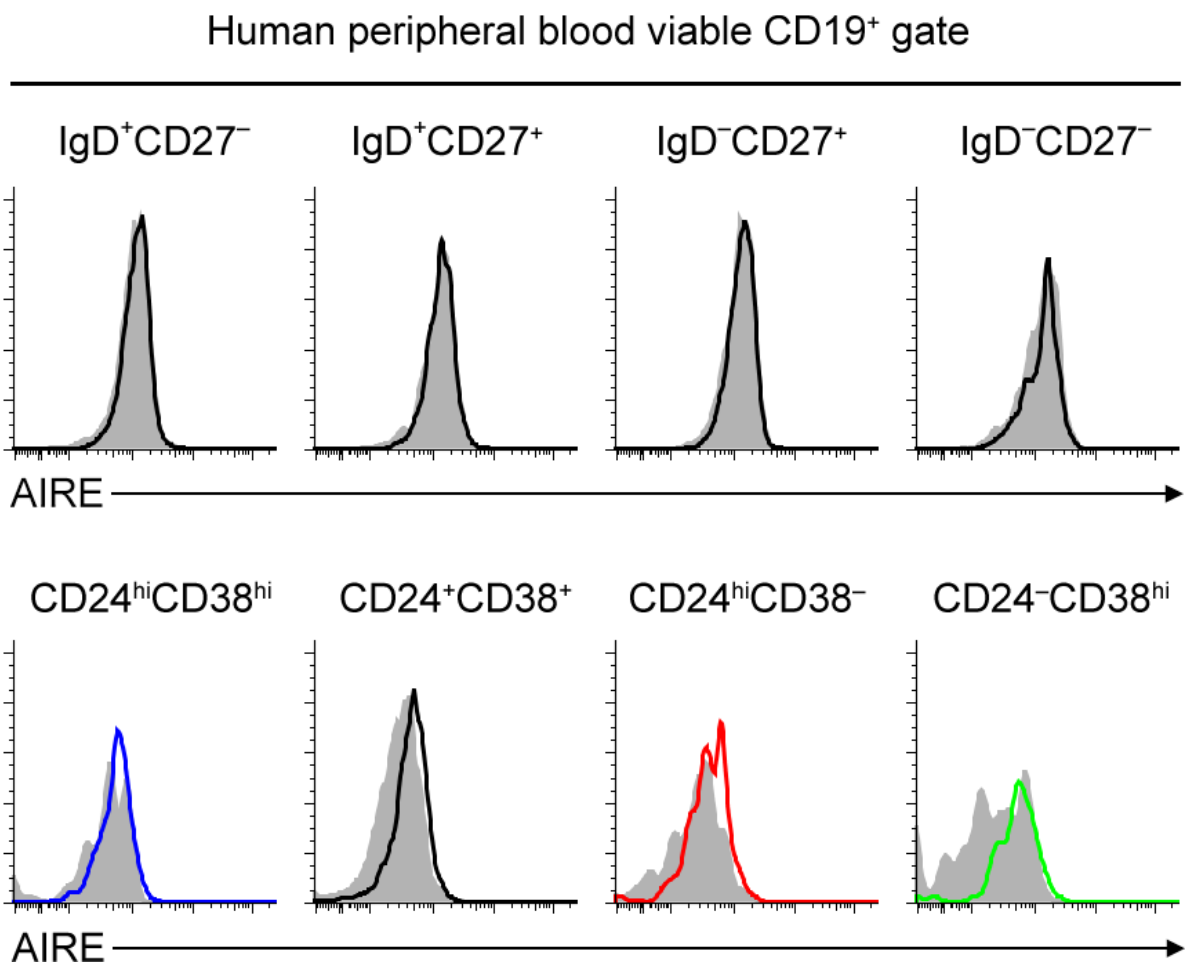

Figure 2.4: Aire is not expressed in human peripheral blood B cells. Flow cytometry analysis of peripheral blood memory, plasma, and naïve B cells show no Aire expression in these subpopulations.

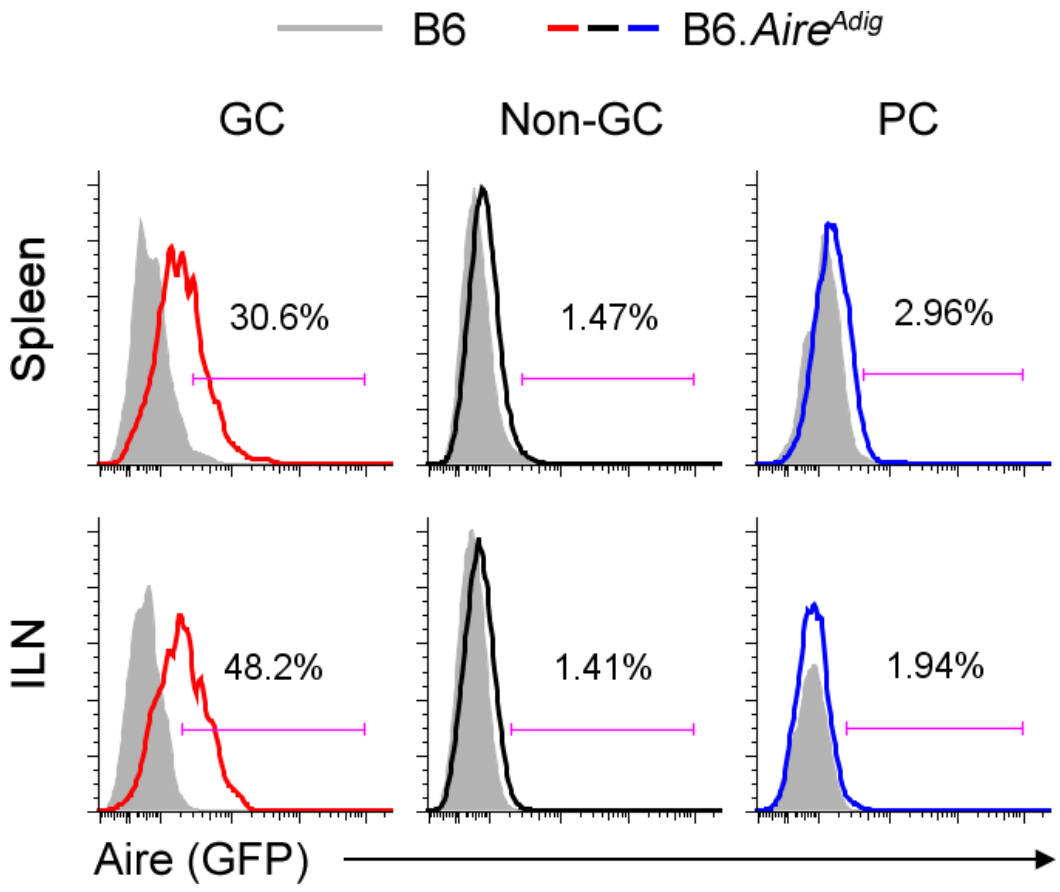

Figure 2.5: Aire expression in mouse B cells by flow cytometry. Immunized Adig mice show GFP in GC B cells from spleen and ILN, but not in non-GC B cells or PCs.

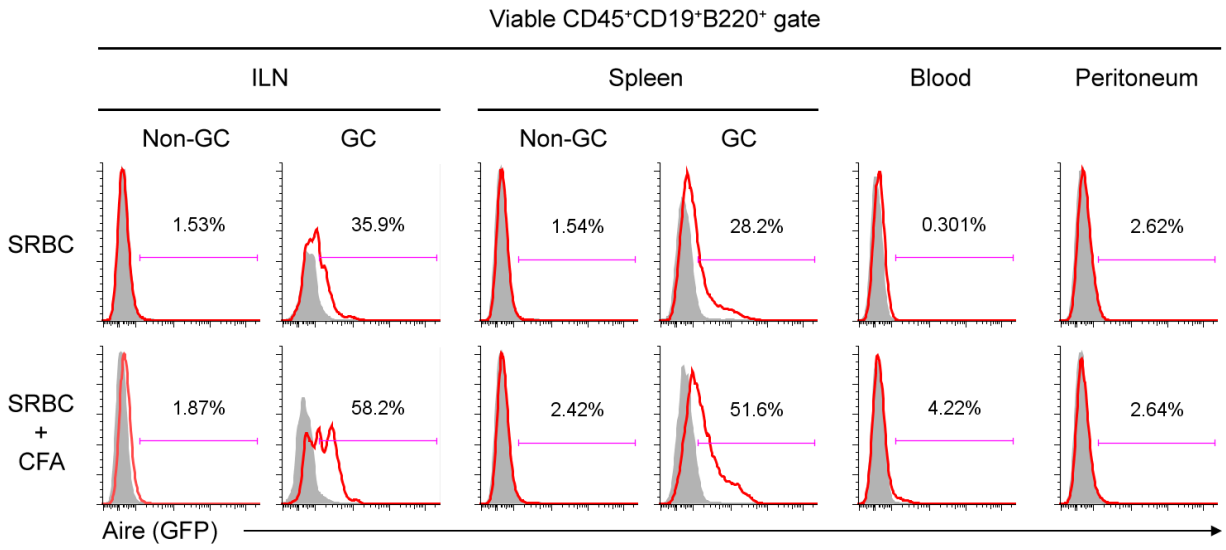

Figure 2.6: Comparison of immunization protocols. Adig mice immunized with or without CFA to determine whether immunization protocol explains the discrepancy between our data and those of other groups.

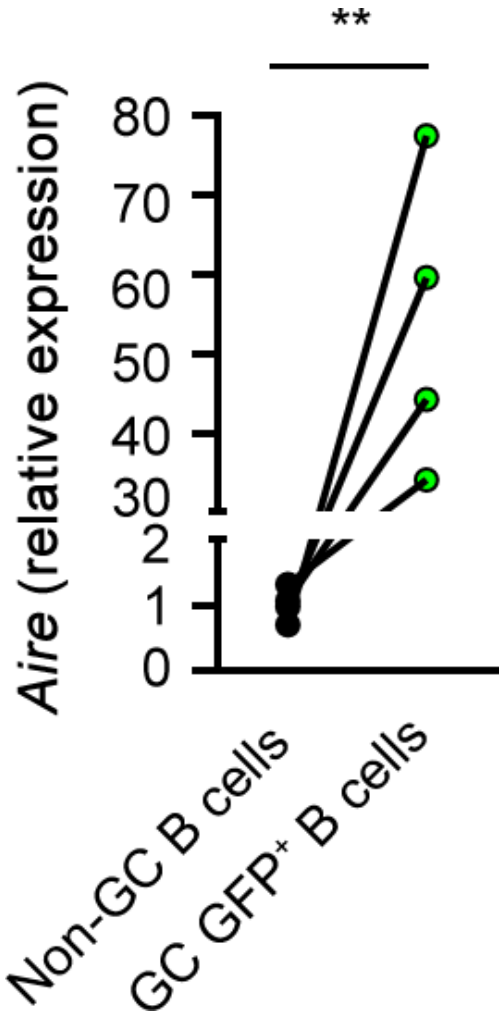

Figure 2.7: Aire expression in mouse B cells by qPCR. GFP^+ GC B cells and non-GC B cells were sorted from Adig mice to confirm the association between GFP expression and Aire mRNA.

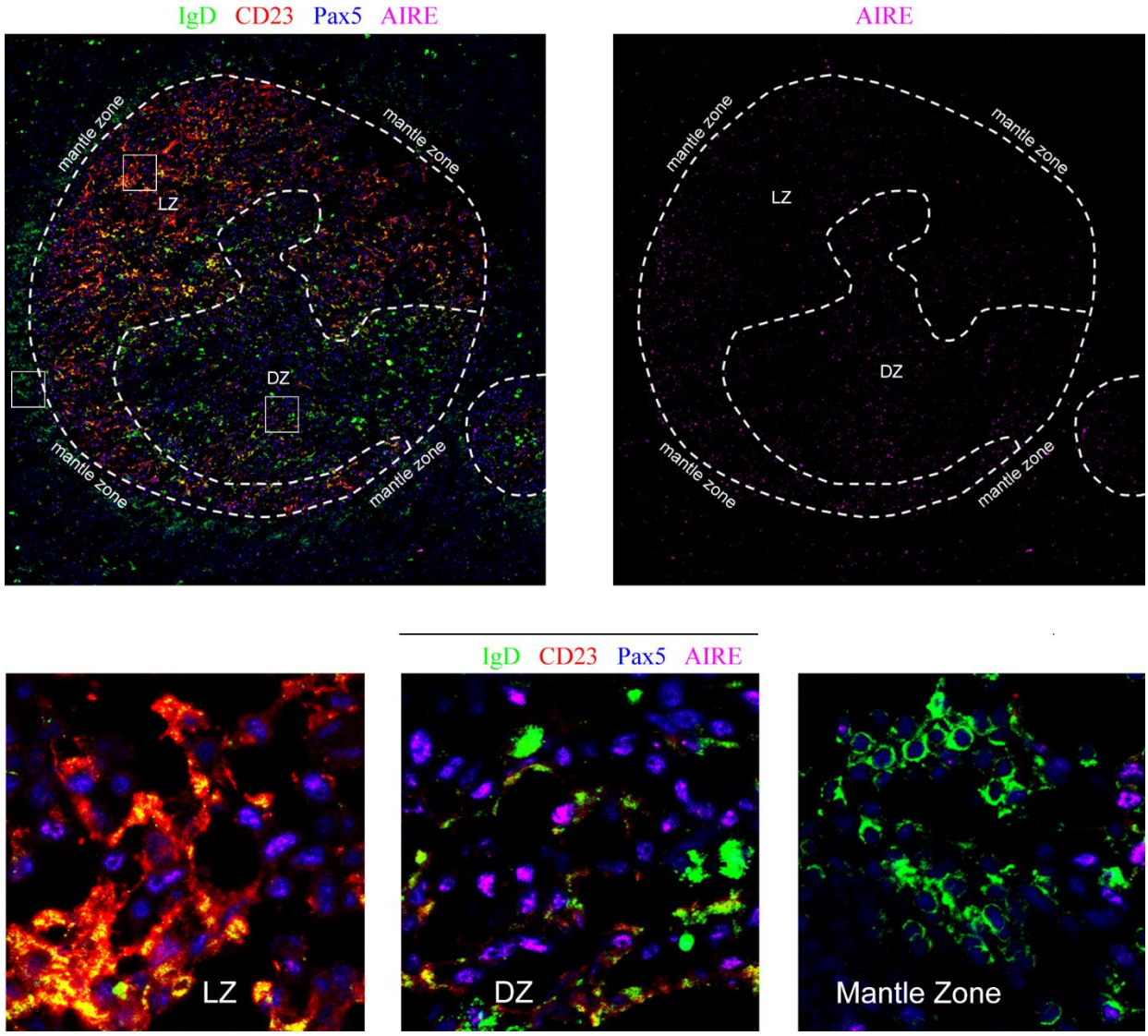

Figure 2.8: Aire expression in both LZ and DZ in human GCs. Immunofluorescence staining to show distribution of Aire in both DZ and LZ of human GC. Aire seems to be evenly distributed between the LZ and DZ.

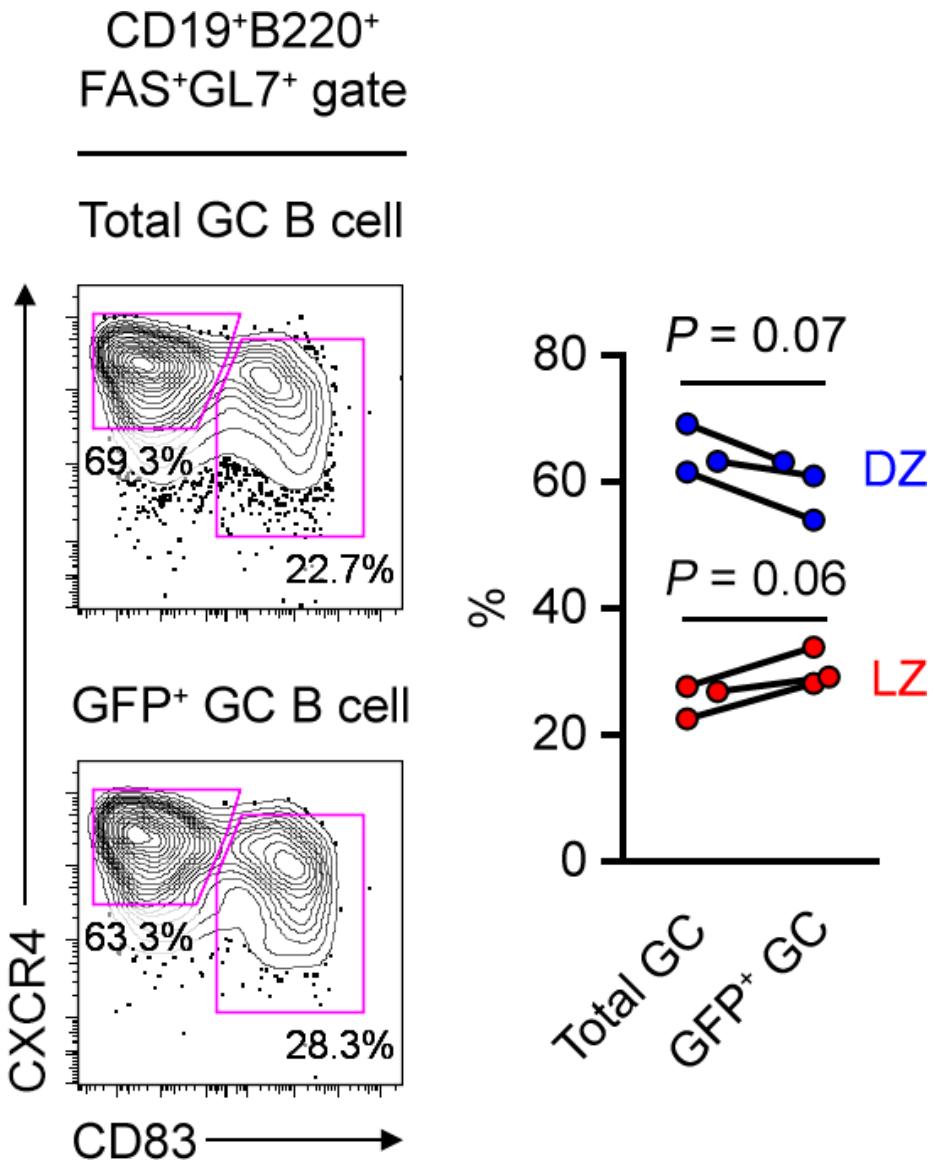

Figure 2.9: Aire expression in LZ vs. DZ B cells in mouse GCs. Comparison of LZ and DZ expression of Aire in immunized Adig mice. Aire expression seems evenly distributed between the LZ and DZ.

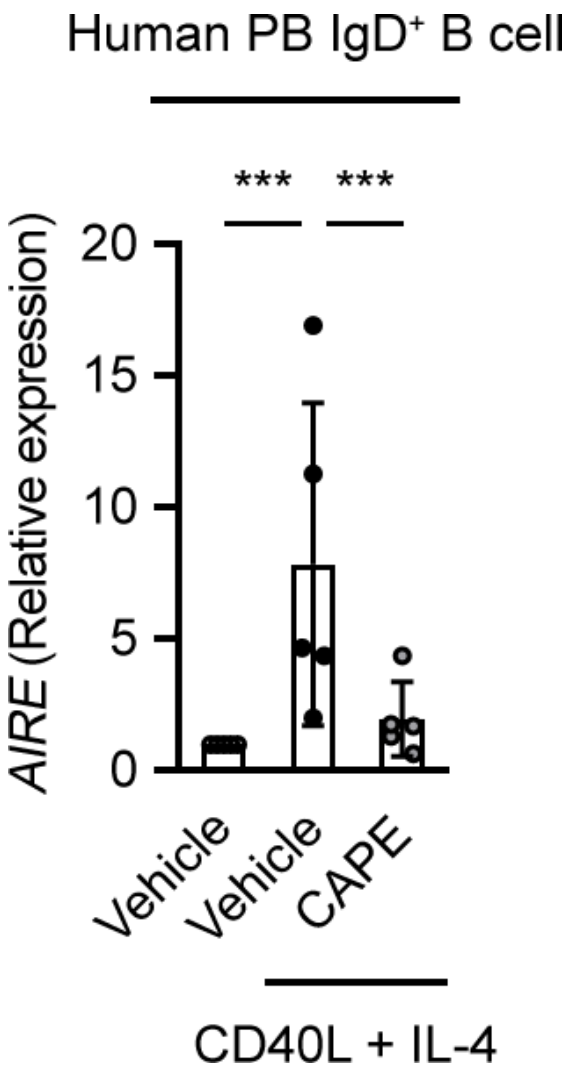

Figure 2.10: Human B cell Aire expression is induced by CD40 signaling through NF-kB.

Aire mRNA is upregulated in human B cells after stimulation with CD40L+IL-4. This upregulation is inhibited with the addition of an NF-kB specific inhibitor.

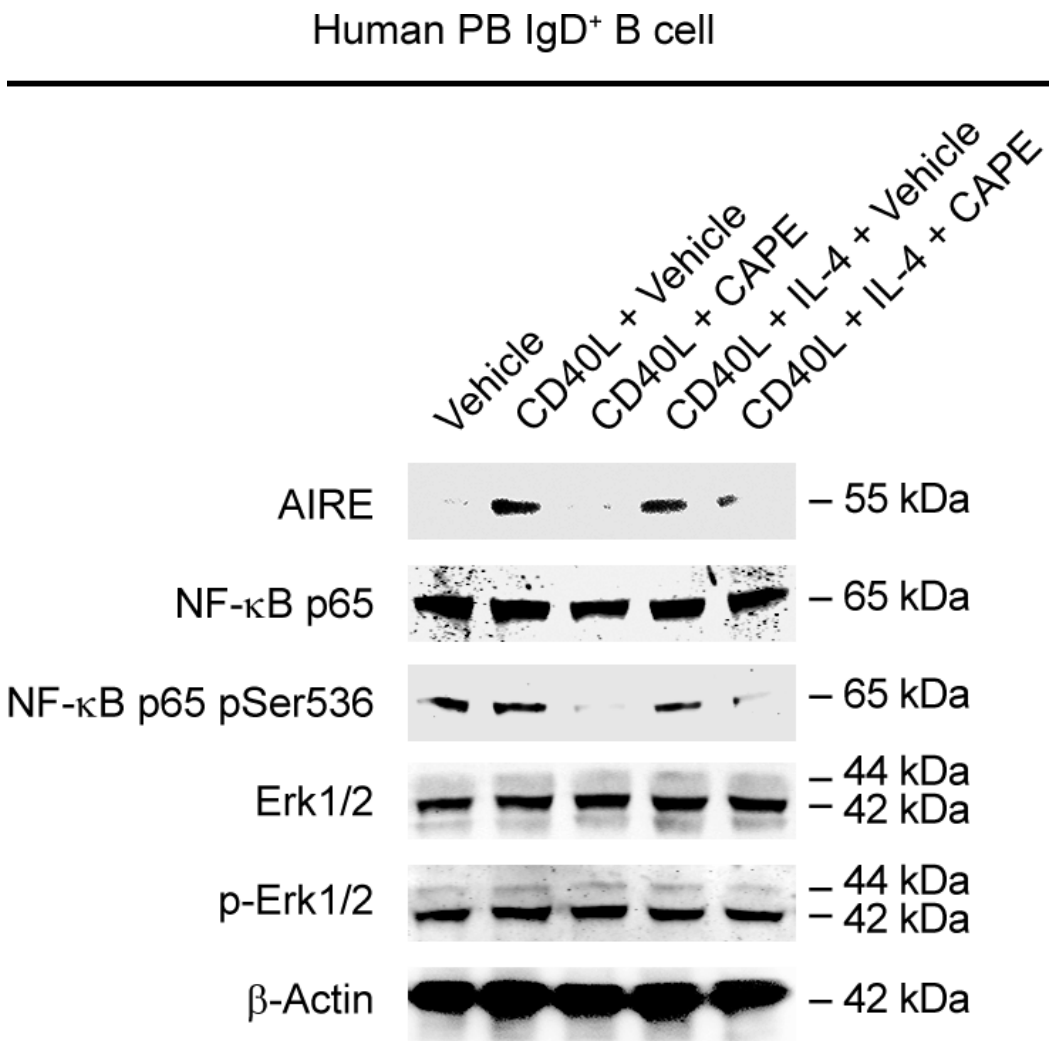

Figure 2.11: Human B cell Aire protein is upregulated by CD40 signaling through NF-kB.

Aire protein is upregulated in human B cells after stimulation with CD40L alone as well as with CD40L+IL-4. This upregulation is inhibited with the addition of an NF-kB specific inhibitor.

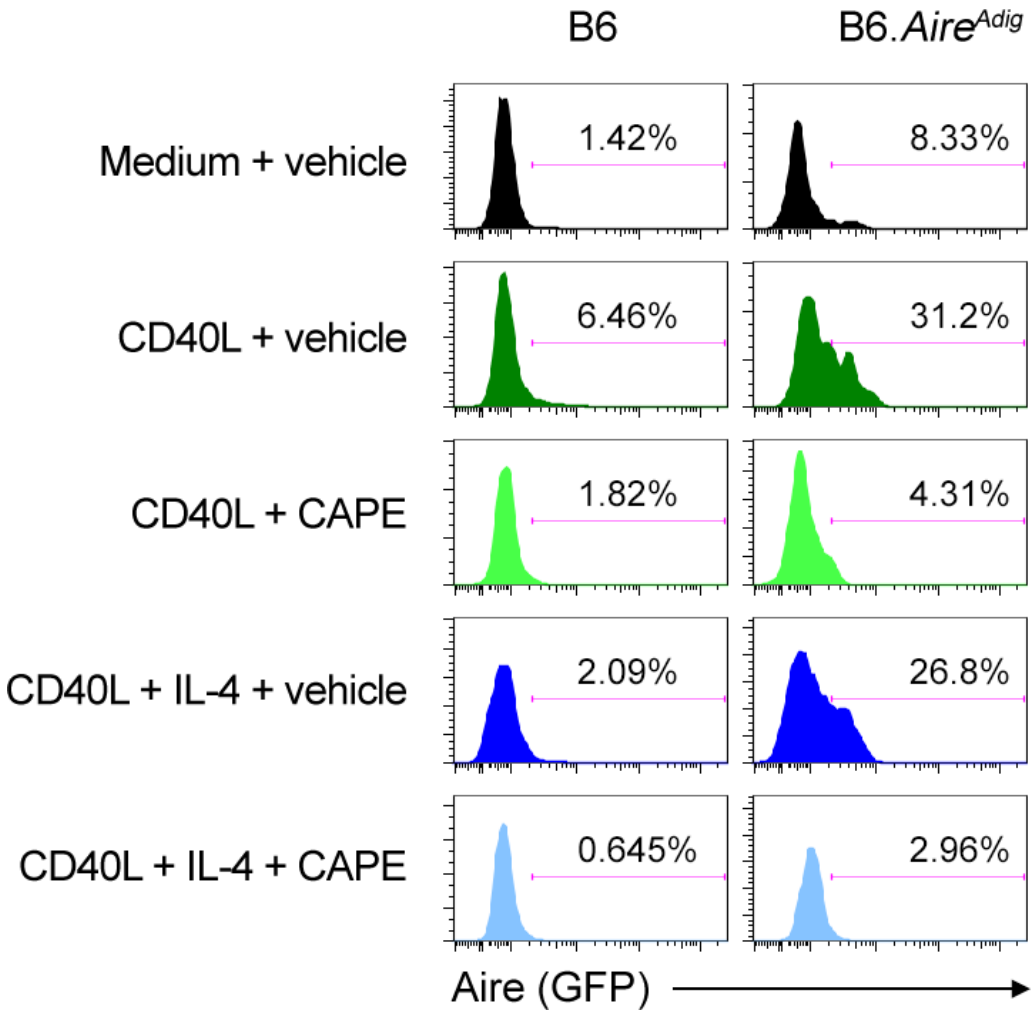

Figure 2.12: Reporter mouse upregulates GFP whith CD40 signaling through NF-kB. B cells purified from Adig mice show upregulation of GFP after stimulation with CD40L alone as well as with CD40L+IL-4. This upregulation is inhibited with the addition of an NF-kB specific inhibitor.

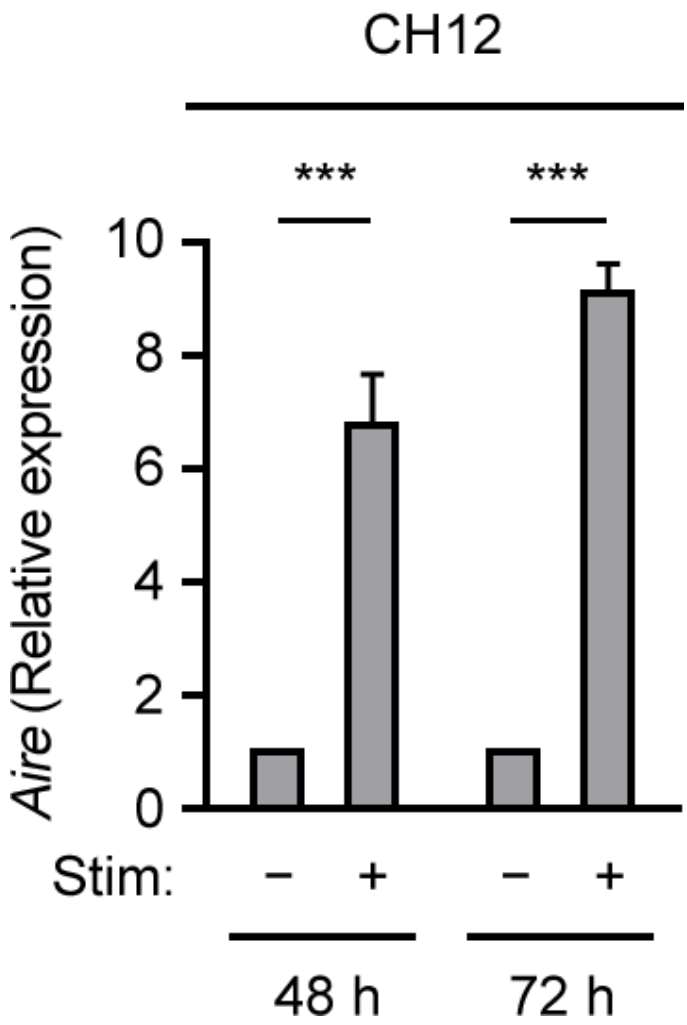

Figure 2.13: CH12 cells upregulate Aire RNA when activated through CD40. CH12 cells show upregulation of Aire mRNA after stimulation with αCD40+IL-4+TGF-β for 48 and 72 hours.

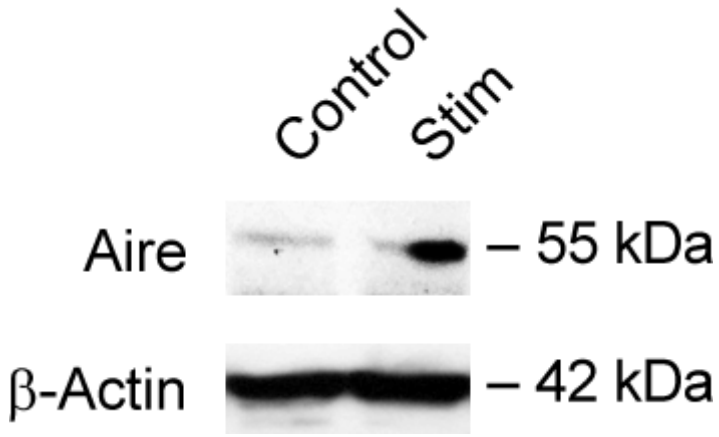

Figure 2.14: CH12 cells upregulate Aire protein when activated through CD40. CH12 cells show upregulation of Aire protein after stimulation with αCD40+IL-4+TGF-β.

CHAPTER 3: FUNCTION OF AIRE IN GC B CELLS

3.1 Introduction

Generally, Aire functions as a transcriptional regulator that helps prevent autoreactivity in T cells and B cells. In the thymus, Aire expressed by mTECs induces the expression of tissue restricted antigens on which developing thymocytes are negatively selected. This prevents the release of autoreactive T cells into the periphery as well as generates a population of thymic regulatory T cells that prevent self-activation in the periphery (Malchow et al., 2016).

Germinal centers are structures in which B cells undergo antibody diversification. GC B cells, activated by T cell engagement, activate mechanisms for CSR and SHM that allow them to generate high affinity antibodies of various antibody isotypes. This allows the B cells to continuously alter antibody affinity and, with help from Tfh, significantly increase the affinity of their BCRs over time (Victora and Nussenzweig, 2012). Based on the knowledge that, in nearly all contexts, Aire acts as a promoter of immune tolerance and regulator of immune activation, we hypothesize that Aire in GC B cells acts as a regulator of B cell function and antibody diversification.

3.2 Materials and Methods

3.2.1 Patient samples

Autoimmune polyglandular syndrome 1 (APS-1) patients harboring loss of function mutations in *AIRE* were enrolled with an approved protocol from the Ethics Committee of Medicine from the Hospital District of Helsinki and Uusimaa, Finland. Samples were processed and naïve B cells were sorted before being sent to Detroit from Finland. Healthy human blood was purchased as previously mentioned.

3.2.2 Mice

C57BL/6J, Aire$^{-/-}$, CD45.2, and μMT mice were purchased from the Jackson Laboratory. Mice were housed as mentioned above. Age and sex matched mice were randomly assigned to control and experimental groups for experimentation.

3.2.3 Cell Lines

CH12 cells were cultured in RPMI-1640 medium supplemented with 5% (v/v) NCTC and 50 μM β-mercaptoethanol. To stimulate Aire expression and CSR, cells were activated with 5μg/ml anti-CD40 + 100ng/ml IL-4 + 1ng/ml TGF-β1 for up to 3 days. Aire knockout CH12 cells were generated by Guang Wen Sun at School of Applied Science in Singapore.

3.2.4 Flow Cytometry

Mouse spleens were harvested after intraperitoneal immunization with 100μg NP_{32}-KLH mixed with Complete Freund's Adjuvant (CFA) for the first immunization and Incomplete Freund's Adjuvant (IFA) for 3 subsequent immunizations. Spleens were then minced and pushed through a 40μm cell strainer prior to RBC lysis. Staining was performed for 1 hour at room temperature with either GV510 or 7AAD after Fc block for 10 minutes. To show Aire expression, cells were stained with a combination of CD19, GL-7, Fas, B220, CD3 (Tonbo 25-0032), CD4 (BioLegend 100406 or 100434), CXCR5 (BioLegend 145504), PD-1 (eBioscience 11-9985 or 25-9985), CD45.1 (Tonbo 60-0453), CD45.2 (Tonbo 65-0454), IgD (BioLegend 405727 or eBioscience 25-5993), IgM (BioLegend 406509), NP8 (BioSearch N-5050F), and NP36 (BioSearch N-5070-1) for 1 hour at 4°C. For NP staining, cells were first stained with NP8 for 10 minutes on ice prior to staining with NP36. Events were captured on a Fortessa LSR II and

analyzed using Treestar FlowJo 7 or 10 software. For sorting, cells were sorted using a Sony Biotechnology SH800 cell sorter at the Wayne State MICR core facility.

3.2.5 RT-qPCR

APS-1 patient cells were pelleted and RNAlater was removed. Cells were washed with PBS prior to RNA isolation using the Cells-to-CT 1-step SYBR Green kit (Fisher A25601) as per manufacturer's instructions. RNA was then amplified using iTaq Universal SYBR Green One-Step kit as previously mentioned.

For CH12 samples, RNA was purified using a Trizol purification method. Reverse transcription was performed using an iScript reverse transcription kit according to the manufacturer's instructions. qPCR was performed using either SYBR Green. PCR was performed using a StepOnePlus instrument and analyzed using the StepOne software.

3.2.6 Adoptive Transfers

For transfer of *Aire*$^{-/-}$ or *Aire*$^{+/+}$ cells to µMT hosts, naïve resting B cells were purified from spleens using a B cell isolation kit as previously mentioned. 2.5x10^{7} B cells were then injected into the tail vein of each recipient µMT host. Mice were then immunized intraperitoneally with 100µg NP_{32}-KLH mixed with Complete Freund's Adjuvant (CFA) for the first immunization and Incomplete Freund's Adjuvant (IFA) for 3 subsequent immunizations.

For bone marrow chimeras, bone marrow cells were isolated from femurs and tibias of CD45.1 *Aire*$^{+/+}$ and CD45.2 *Aire*$^{-/-}$ mice. After RBC lysis, B220+ bone marrow cells were depleted by magnetic separation using a biotinylated anti-B220 antibody and anti-biotin magnetic beads. WT and KO bone marrow cells were then mixed in a 1:1 ratio prior to transfer into µMT mice that had received 10Gy total body irradiation the previous day. Each recipient received

$1.5x10^7$ cells bone marrow cells and were allowed to reconstitute for 28 days prior to tissue harvest and purification of naïve resting B cells from splenocytes for secondary transfer. Mice were then immunized using the above procedure.

3.2.7 Mutation Sequencing

Live naïve (IgM^+IgD^+) or switched (IgM^-IgD^-) NP specific B cells from spleens of μMT mice, which have received either Aire WT or KO B cells and were immunized as mentioned above, were sorted using a SONY Biotechnology SH800 cell sorter at the Wayne State MICR core facility. High throughput IgHV profiling by RNA-seq was performed by iRepertoire, Inc. (Huntsville, AL, USA). Sequences were analyzed using the IMonitor 1.1.0 pipeline as previously published (Zhang et al., 2015). Mutation rate for each position was calculated for sequences with read depth $\geqslant 10$ and frequency of nucleotide substitutions was computed for each isotype.

3.2.8 ELISA

After adoptive transfer and immunization, μMT blood was collected and serum was extracted by centrifugation. Samples were titrated on NP_{29}-BSA (BioSearch N-5050H-10)and NP_4-BSA (BioSearch N-5050L-10) coated microtiter plates to determine the ratio of NP_{29} to NP_4 binding. Bound antibodies were probed using HRP conjugated anti mouse IgG1 (Jackson 115-035-205), IgG2b (Jackson 115-035-207), IgG3 (Jackson 115-035-209), or IgM (Bethyl A90-101P) prior to color development and quantitation on a microplate reader (BioTek Epoch) at 450nm.

3.3 Results

3.3.1 Effect of Aire on GC populations

To determine whether Aire expression in B cells had any effect on GC cell populations, μMT mice were adoptively transferred with either Aire WT or KO B cells prior to immunization. No differences in GC entry (**Figure 3.1**), Tfh (**Figure 3.2**), or Tfr (**Figure 3.3**) populations were detected indicating that Aire does not affect GC biology by disrupting the overall number of cells.

3.3.2 Bone marrow chimera

As previously mentioned, GCs are regions in which B cells undergo antibody diversification. One of the most important processes that occur in these structures is SHM and affinity maturation. To determine whether GC B cell Aire affects this process, bone marrow chimeras using CD45.1 Aire WT and CD45.2 Aire KO bone marrow, depleted of all $B220^+$ cells (**Figure 3.5**) were transferred to μMT hosts that had been irradiated the previous day (**Figure 3.4**). Mice were allowed to reconstitute for 4 weeks prior to tissue harvest. Naïve resting B cells from spleens were isolated and tested for purity (**Figure 3.6**) prior to secondary transfer into a new μMT host in a 1:1 ratio of WT to KO cells (**Figure 3.7**). Additionally, these cells did not have any differences in expression of stimulatory molecules (**Figure 3.8**), nor did they show any difference in affinity to NP (**Figure 3.9**). Mice were then immunized via intraperitoneal injection of NP_{32}-KLH three times. After immunization, analysis of GC B cells showed a greater proportion of Aire KO GC B cells (**Figure 3.10**), suggestive of improved GC survivability and selection. Analysis of BCR affinity using fluorescently labeled NP_8 and NP_{36} by flow cytometry (**Figure 3.11**) showed increased BCR affinity of Aire KO B cells compared to Aire WT B cells (**Figure 3.12** and **3.13**)

as shown by increased NP_8 binding ratios. These data suggest that Aire negatively regulates the process of Affinity maturation.

For CH12, cells were harvested and blocked using Fc blocking reagent for 10 minutes on ice prior to staining with either GV510 or 7AAD and primary antibodies for CD19, B220, IgM (BioLegend 406509), and IgA (BD 559354).

3.3.2 Effect of Aire on serum antibody affinity and mouse CSR

The second antibody diversification process associated with GC B cells is CSR. Although CSR does not frequently occur within the GC, it is activated by the same signals that activate Aire expression and can potentially fall under the same regulatory mechanism. To determine whether Aire also regulates the process of CSR, adoptive transfer of either Aire WT or KO B cells into µMT mice was performed. The mice were then immunized against NP prior to harvest of spleen and blood (**Figure 3.14**). After immunization, mice that receive Aire KO B cells showed a greater percentage of $IgD^{-}IgM^{-}$ cells in both the total B cell compartment as well as in the NP specific B cell compartment indicating increased CSR (**Figure 3.15**). Consistent with previous experiments, serum antibodies from these mice showed increased affinity for NP in the switched IgG isotypes, but not in IgM (**Figure 3.16**).

3.3.2 Effect of Aire on SHM

As previous experiments showed the regulation of affinity maturation by Aire expression, we attempted to determine whether this phenomenon was due to a change in SHM. To answer this question, µMT mice received either Aire WT or Aire KO B cells prior to immunization against NP (**Figure 3.14**). After immunization, naïve $IgD^{+I}gM^{+}$ and activated $IgD^{-}IgM^{-}$ NP specific B cells were sorted and sent for repertoire sequencing (**Figure 3.17**). Sequencing results show that

Aire KO B cells acquired more mutations in switched isotypes such as IgG and IgE compared to Aire WT B cells (**Figure 3.18**). These data suggest that Aire negatively affects affinity maturation by regulating SHM.

3.3.2 APS-1 patient CSR

Our adoptive transfer experiments show that, in mice, Aire negatively regulates CSR. To show that this is also the case in humans, APS-1 and health control patient B cells were stimulated with either CD40L+IL-4 or CD40L+IFN-γ, inducing the cells to undergo CSR to either IgG1 or IgG3 respectively. In these experiments, APS-1 patient B cells showed greater upregulation of Iμ-Cγ1 and Cγ3 transcripts (**Figure 3.19** and **3.20**) indicating increased CSR, consistent with our mouse data.

3.3.2 Effect of Aire on CH12 cell CSR

Since these findings were consistent in both mice and humans, we attempted to show that they could be replicated in cell culture as well. To this end, CH12 cells, a mouse B cell line, were used to generate Aire KO clones 43, 53, and 69. Aire deficiency was confirmed by western blot (**Figure 3.21**). WT CH12 as well as the KO clones were stimulated to undergo CSR to IgA. After stimulation, the Aire KO clones showed an increased percentage of cells that have experienced CSR compared to the WT clone (**Figure 3.22**). This was confirmed by qPCR to detect switch circles, which shows increased expression after stimulation of Aire KO cells (**Figure 3.23**). to show a causal relationship between this increase in CSR and Aire, a WT Aire-GFP construct was transfected into an Aire KO clone, 69. After cells were stimulated to undergo CSR, cells that received the Aire-GFP construct showed a reduction in CSR compared to cells that did not receive

the construct, thus rescuing the phenotype. These data indicate that Aire causes a reduction in CSR in CH12 cells.

3.4 Discussion

In this chapter, we show that Aire negatively regulates the processes of antibody diversification associated with GC B Cells in mice, humans, and cell culture. B cell deficient mice that receive Aire KO B cells show increased SHM and affinity maturation as well as an increased rate of CSR, which is also observed in APS-1 patients and Aire KO CH12 cells. We did not, however, test SHM in APS-1 patient B cells since there is no mechanism to show affinity maturation *in vitro* and sorting GC B Cells from APS-1 patients would give wildly variable mutation rates due to complicated infection history. Therefore, we do not believe that SHM data using APS-1 patient GC B cells would provide meaningful results as it is known that these patients suffer from an increased rate of infections compared to healthy patients. Additionally, we do not observe increased SHM in mouse IgA after intraperitoneal immunization of NP (**Figure 3.18**), in fact, the mutation rates may be slightly lower in Aire KO B cells compared to Aire WT B cells. This inconsistency may be due to the nature of the immunogen and the route of immunization. IgA is largely made at mucosal surfaces whereas we were testing B cells purified from the spleen. Therefore, the mutation data for IgA may not accurately represent the total IgA repertoire and only a very small percentage of the IgA positive cells that may skew the results (Bemark et al., 2016).

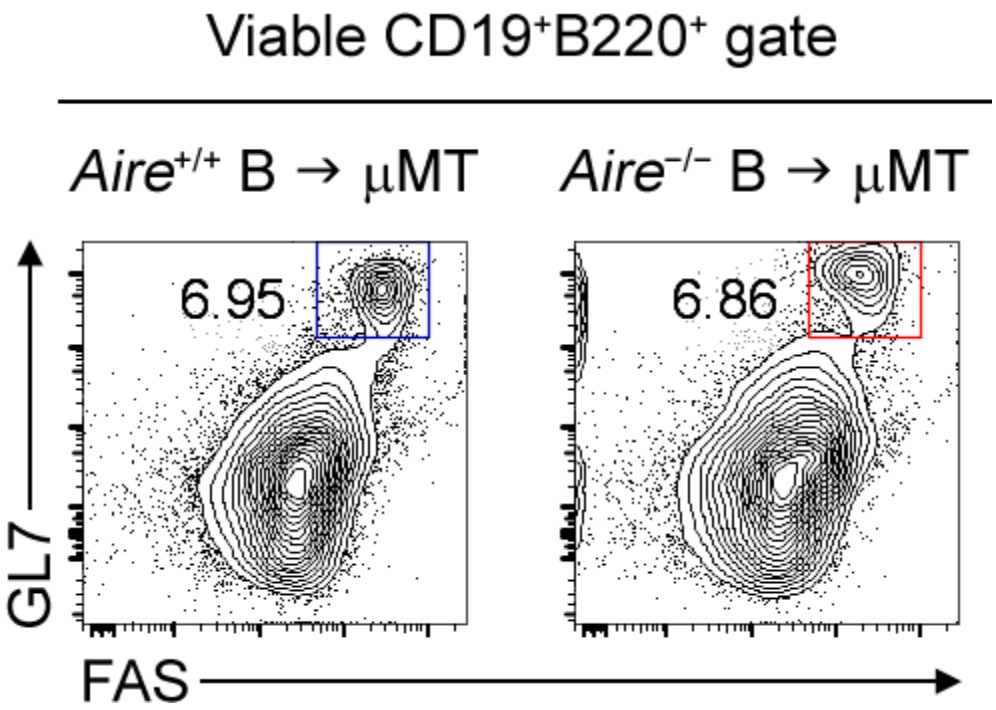

Figure 3.1: Aire expression in GC B cells does not affect B cell entry into the GC. $GL7^{+}Fas^{+}$ B cells after immunization does not change between μMT mice that receive Aire WT and Aire KO B cells

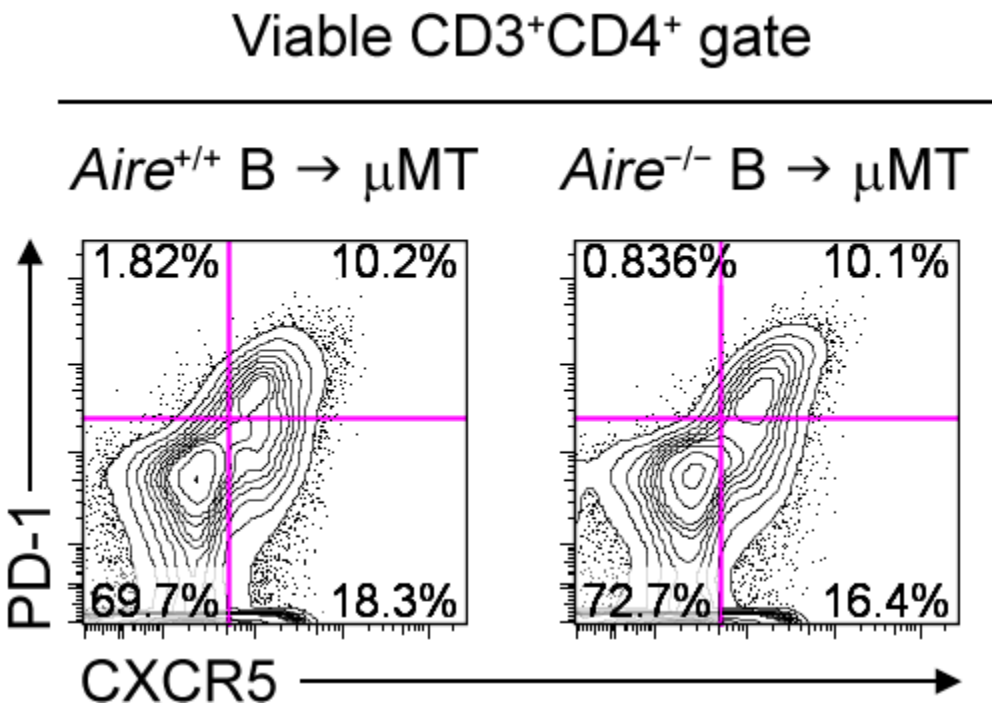

Figure 3.2: Aire expression in GC B cells does not affect Tfh proportions. PD-1+CXCR5+ T cells in secondary lymphoid organs does not change after immunization of µMT mice that receive Aire WT and Aire KO B cells

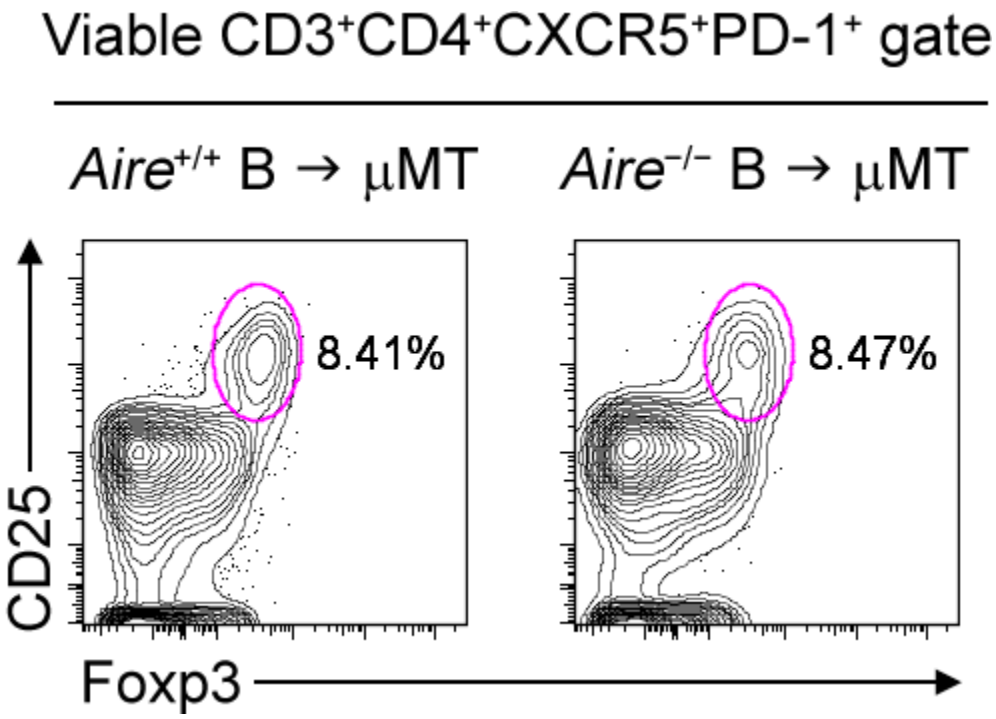

Figure 3.3: Aire expression in GC B cells does not affect Tfr proportions. PD-1+CXCR5+ regulatory T cells in secondary lymphoid organs does not change after immunization of µMT mice that receive Aire WT and Aire KO B cells

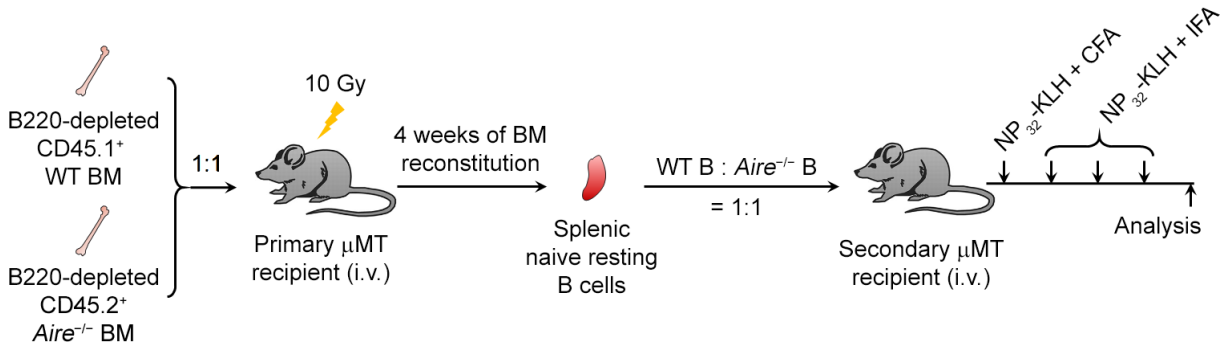

Figure 3.4: Chimera experimental setup. Bone marrow from CD45.1 Aire WT and CD45.2 Aire KO mice were depleted of B220 cells prior to transfer into a µMT irradiated host. Mice were reconstituted for 4 weeks and naïve resting B cells were purified. B cells were then transferred into a second µMT host that was immunized against NP.

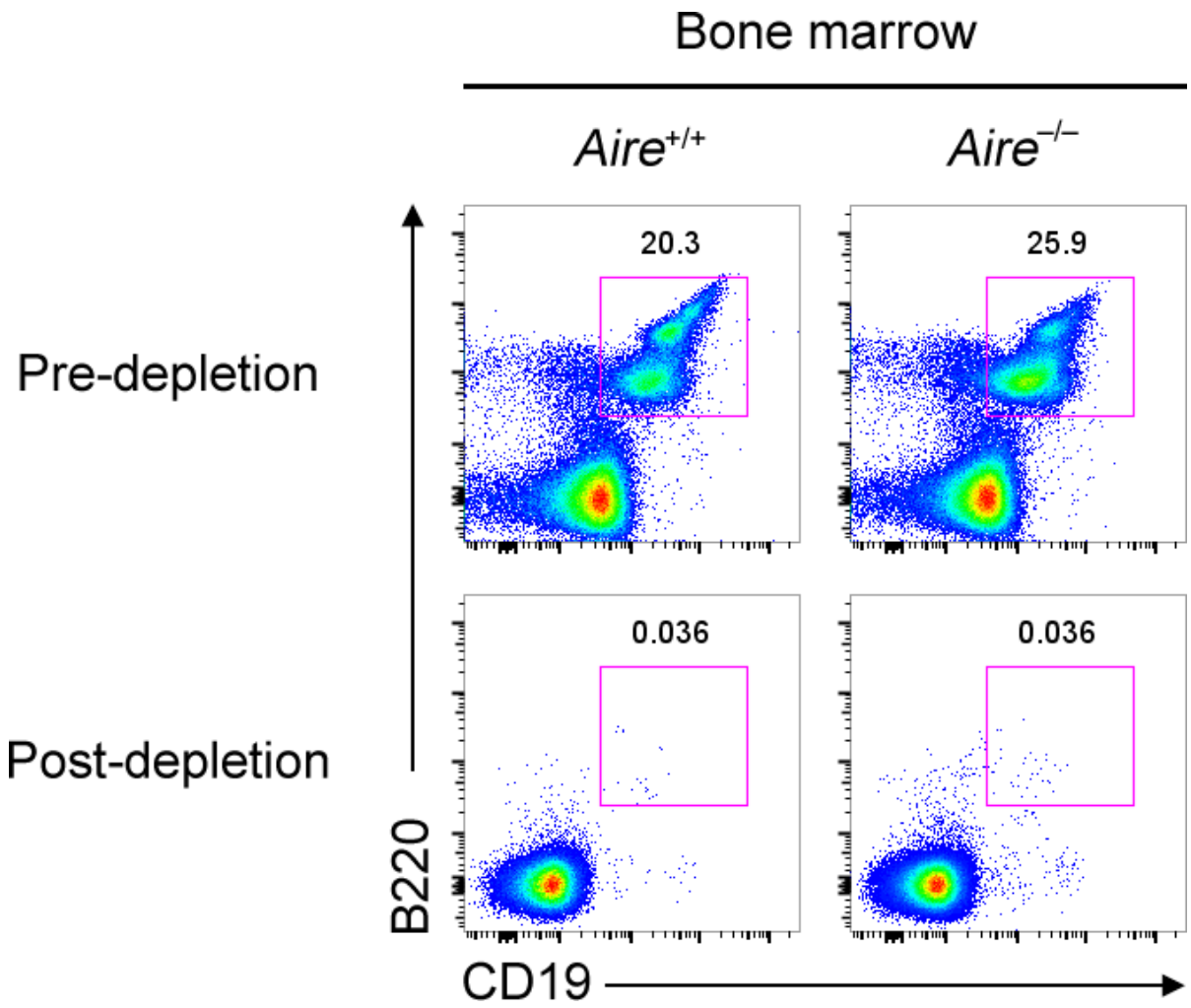

Figure 3.5: B cell depletion in bone marrow. Bone marrow B cells after depletion using αB220 MACS separation.

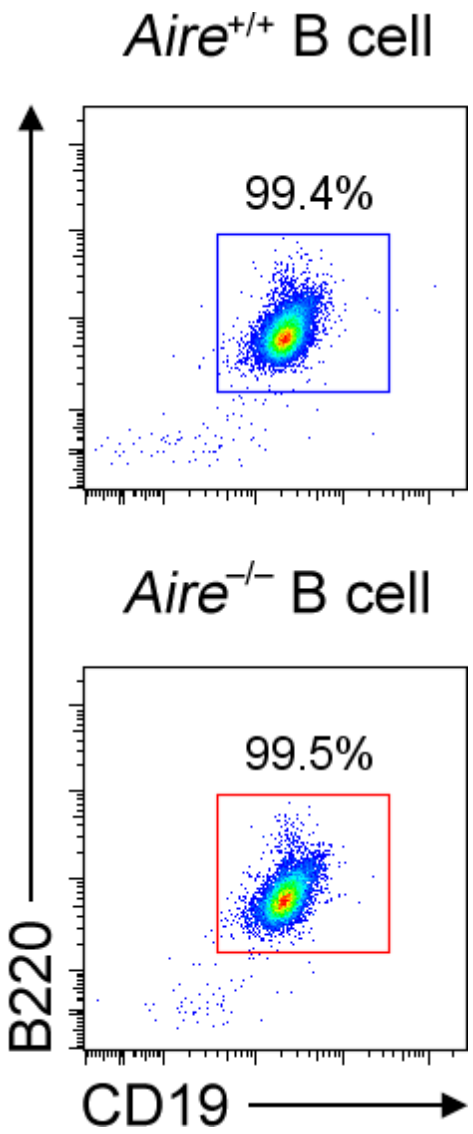

Figure 3.6: Purity of transferred B cells. After MACS separation of B cells from host spleens, purity was over 99%.

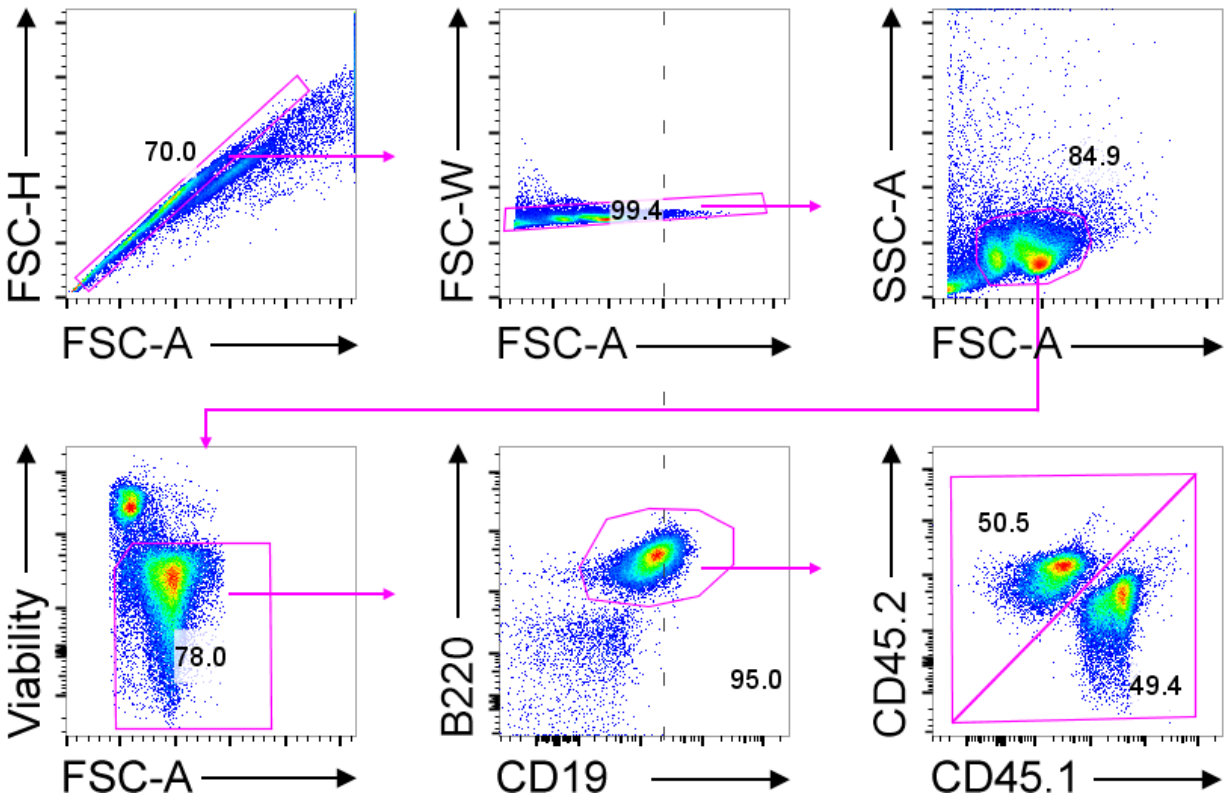

Figure 3.7: CD45.1/.2 ratios prior to secondary transfer. After reconstitution, B cells were transferred at a 1:1 ratio to secondary µMT hosts.

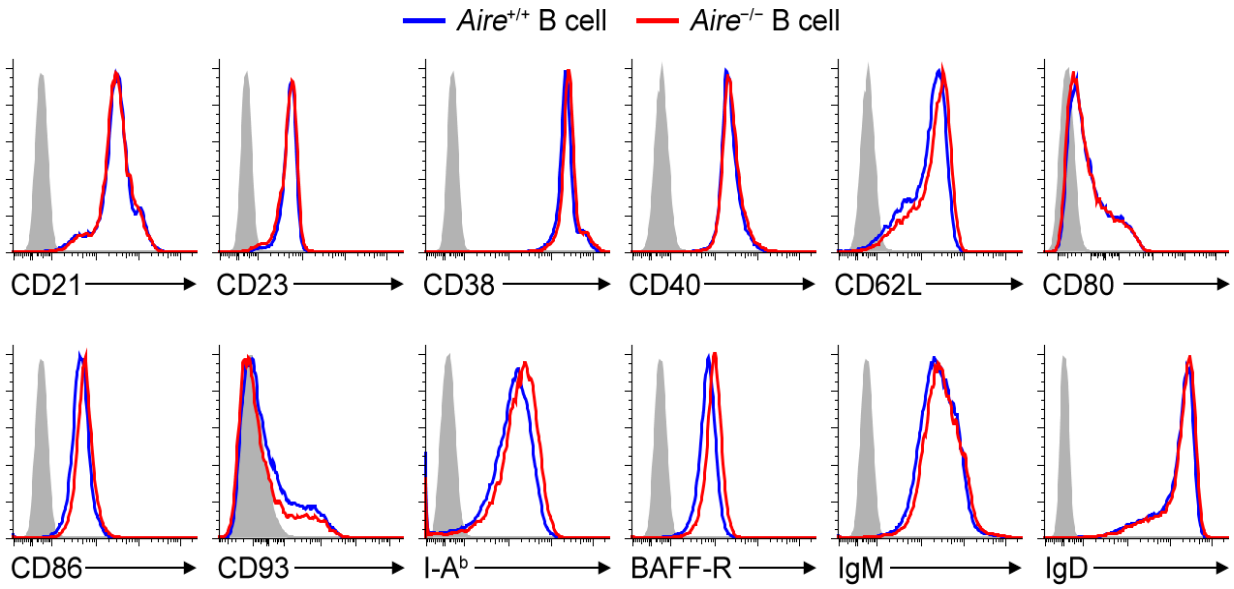

Figure 3.8: Phenotype of transferred B cells prior to transfer. Aire WT and KO cells show similar expression of surface markers.

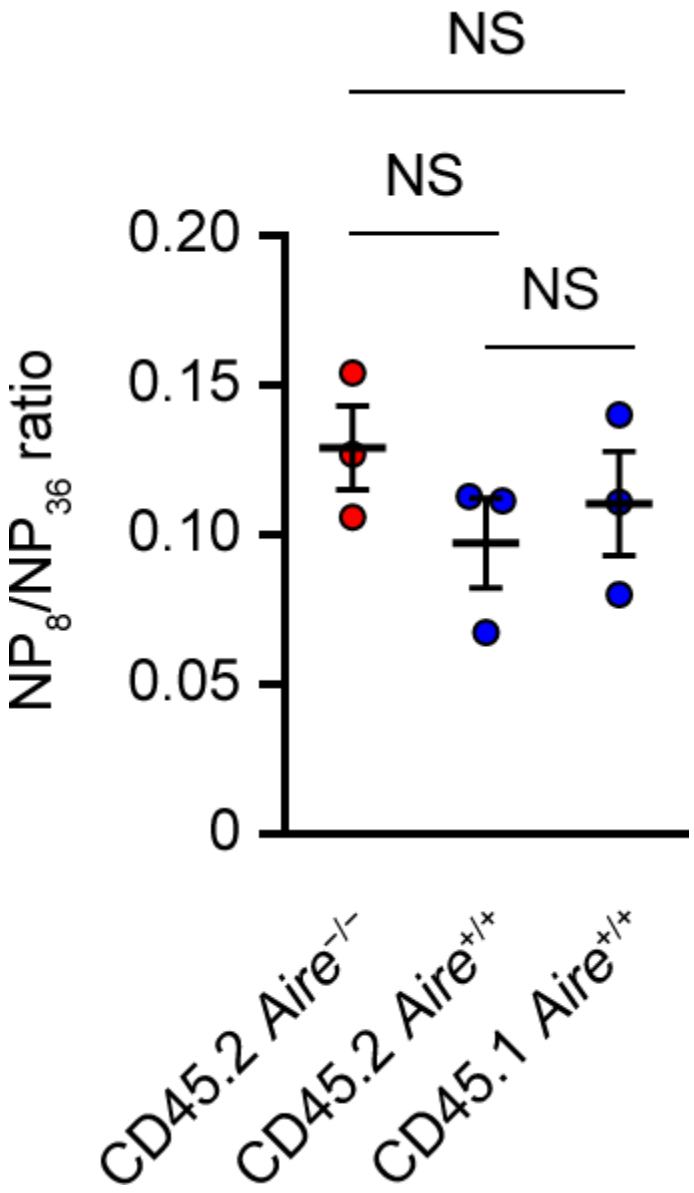

Figure 3.9: NP binding characteristics prior to transfer. Aire WT and KO cells show similar NP binding characteristics prior to adoptive transfer.

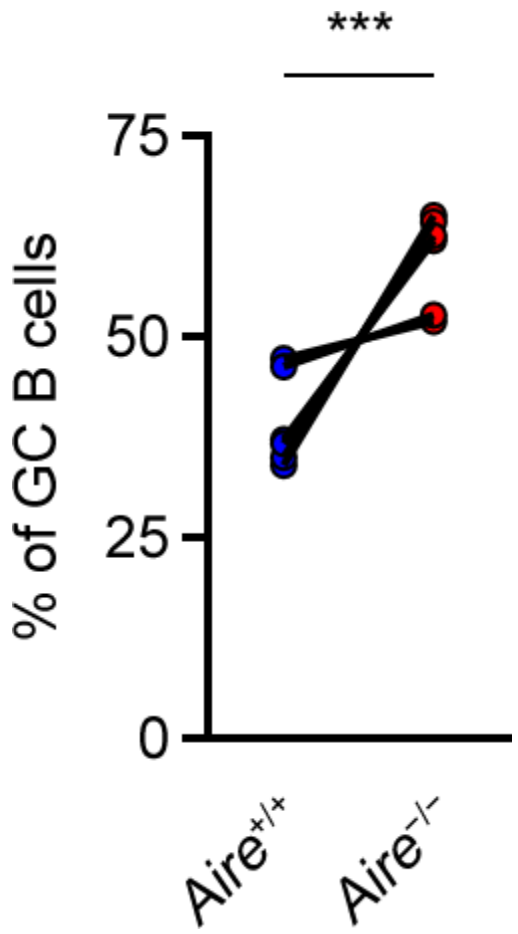

Figure 3.10: Percent GC B cells after immunization. Aire KO B cells represented a greater proportion of cells within GCs after immunization.

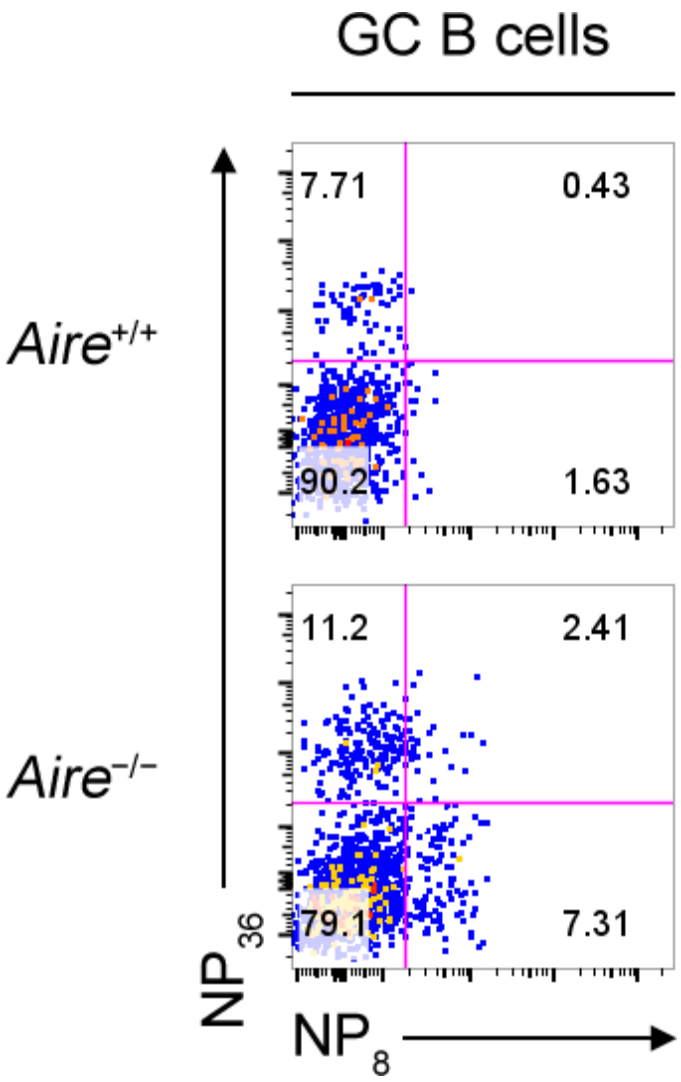

Figure 3.11: Method for analyzing NP ratios. GL7^{+}Fas^{+} GC B cells were stained using fluorescent NP8 and NP36 molecules to determine binding affinity of BCRs.

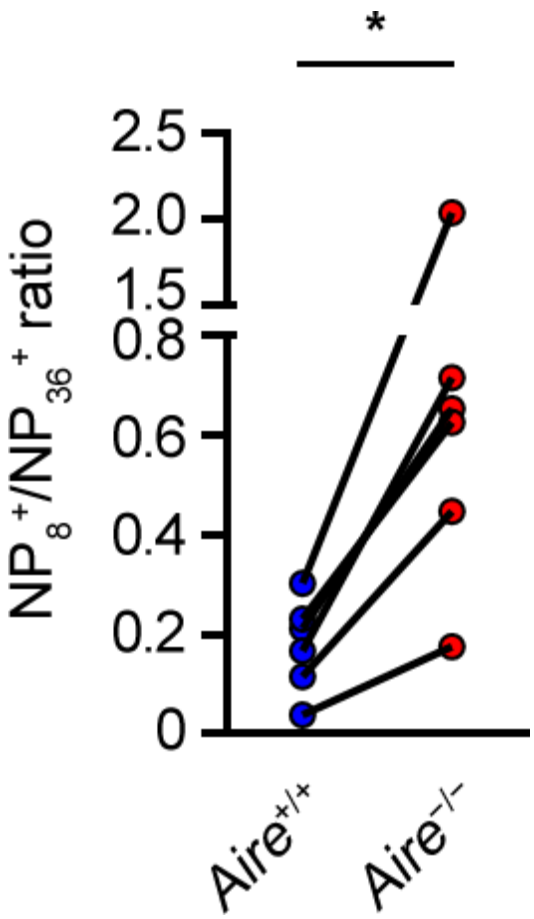

Figure 3.12: NP8/NP36 binding ratios. Aire KO GC B cells had greater NP8/NP36 ratios compared to Aire WT B cells in the same host.

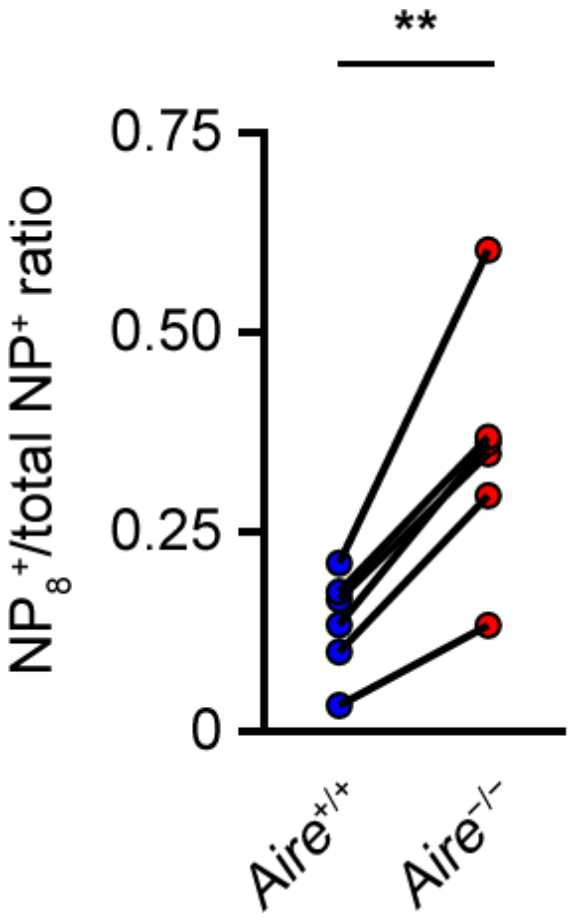

Figure 3.13: NP8/total binding ratios. Aire KO B cells had greater NP8 binding as a ratio of total GC B cells.

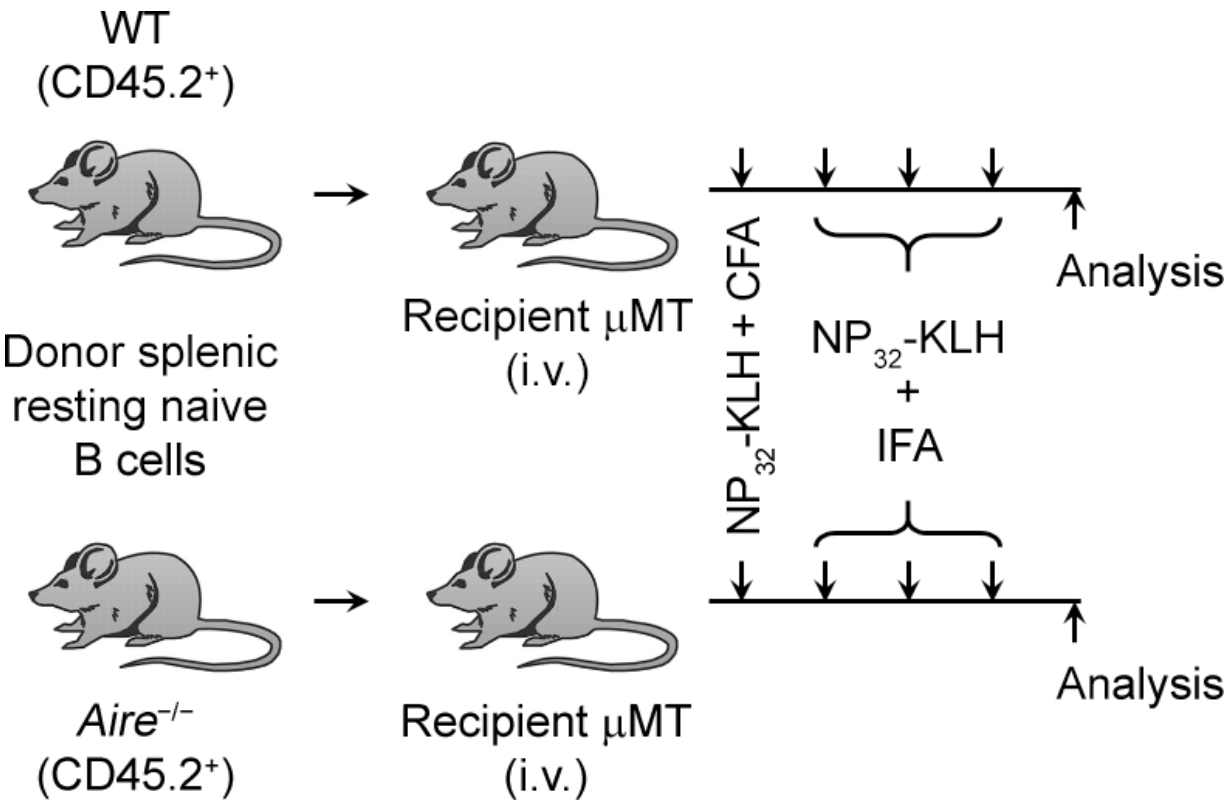

Figure 3.14: Experimental setup for second transfer experiment. Aire WT and KO cells are separately transferred into μMT hosts prior to immunization to NP.

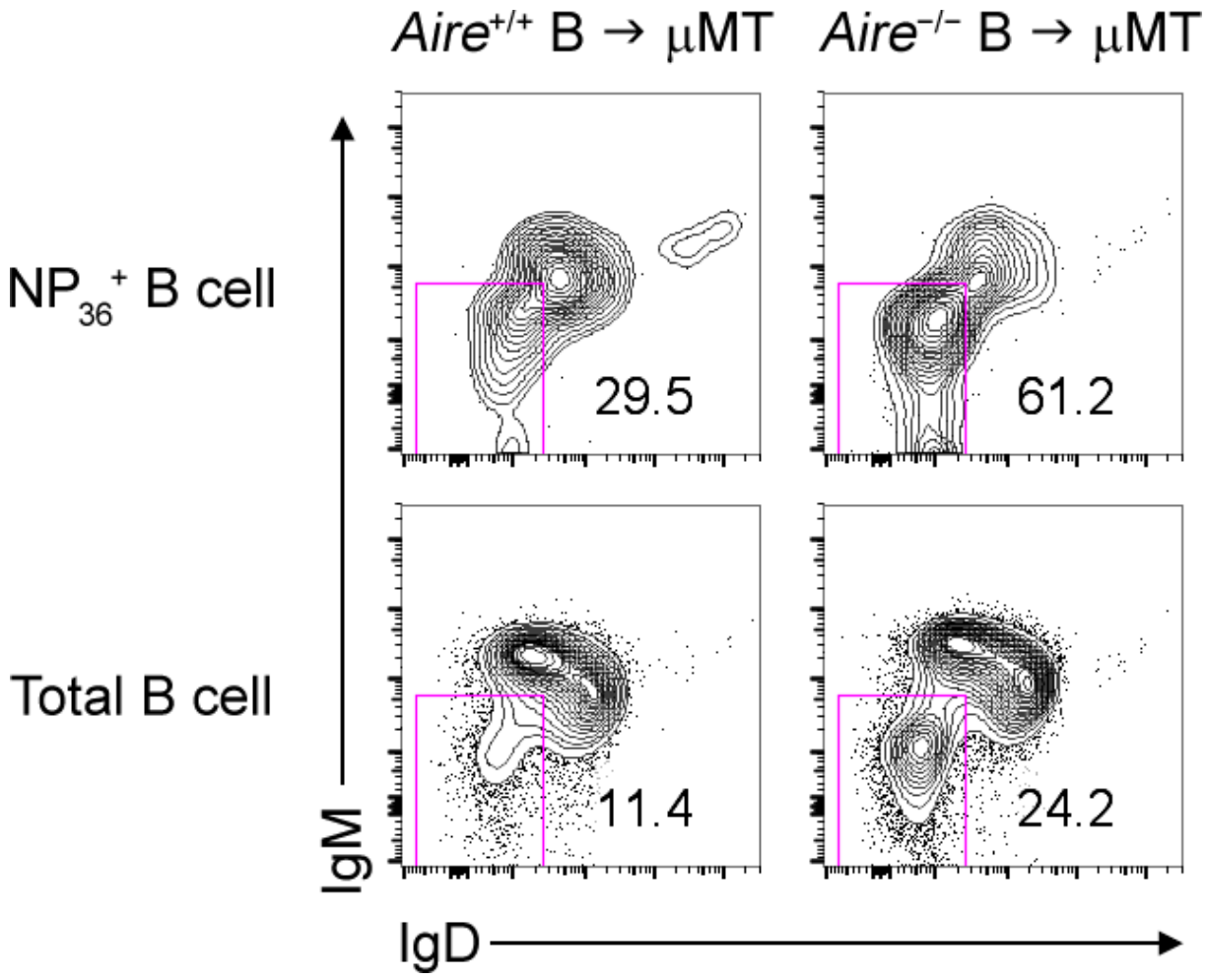

Figure 3.15: Effect of B cell Aire on CSR. Mice that receive Aire KO B cells show increased populations of $IgM^{-}IgD^{-}$ cells indicating increased CSR.

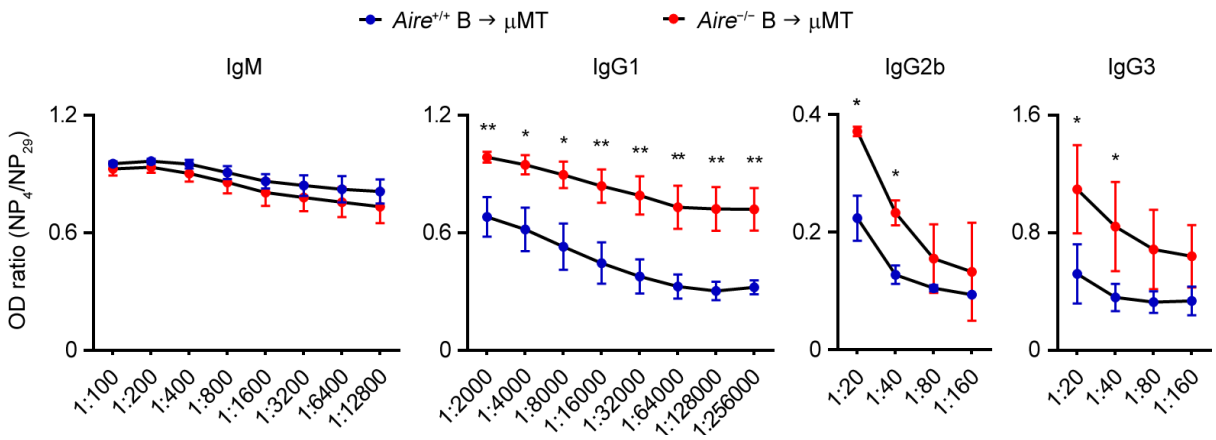

Figure 3.16: Effect of B cell Aire on Affinity maturation. Class switched isotypes from mice that receive Aire KO B cells are higher affinity for NP, indicated by a greater NP8/NP29 ratio, compared to those from mice that receive Aire WT B cells

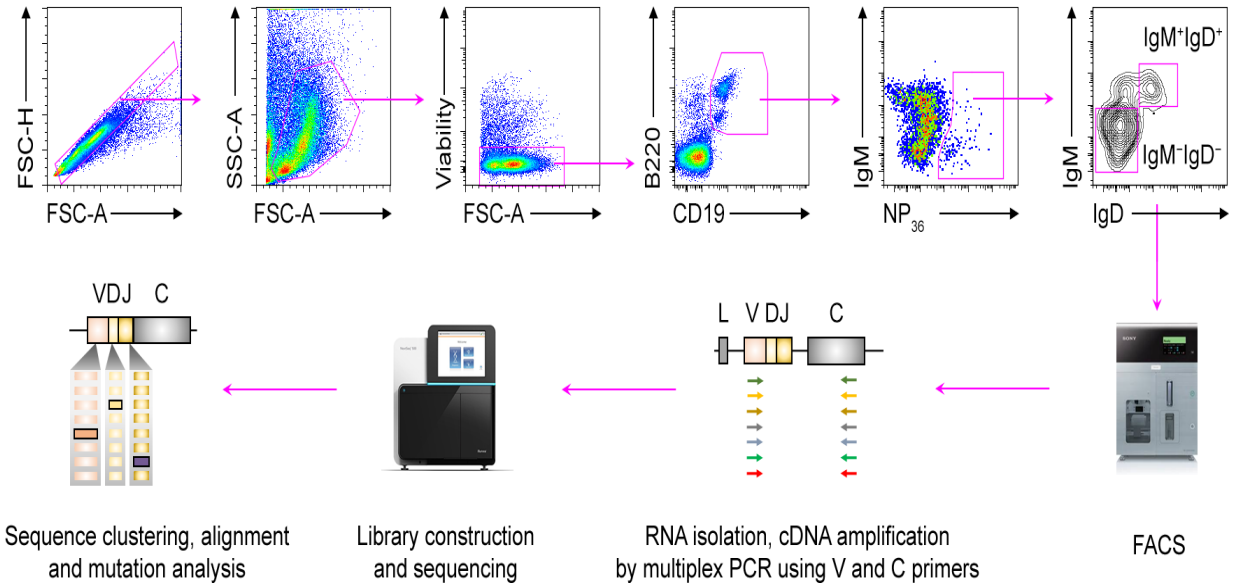

Figure 3.17: Experimental setup for sequencing experiment. $IgM^{+}IgD^{+}$ and $IgM^{-}IgD^{-}$ cells were sorted from µMT mice immunized against NP after adoptive transfer of Aire WT or Aire KO B cells. RNA from cells was isolated and amplified by PCR prior to library construction and sequencing of VDJ regions.

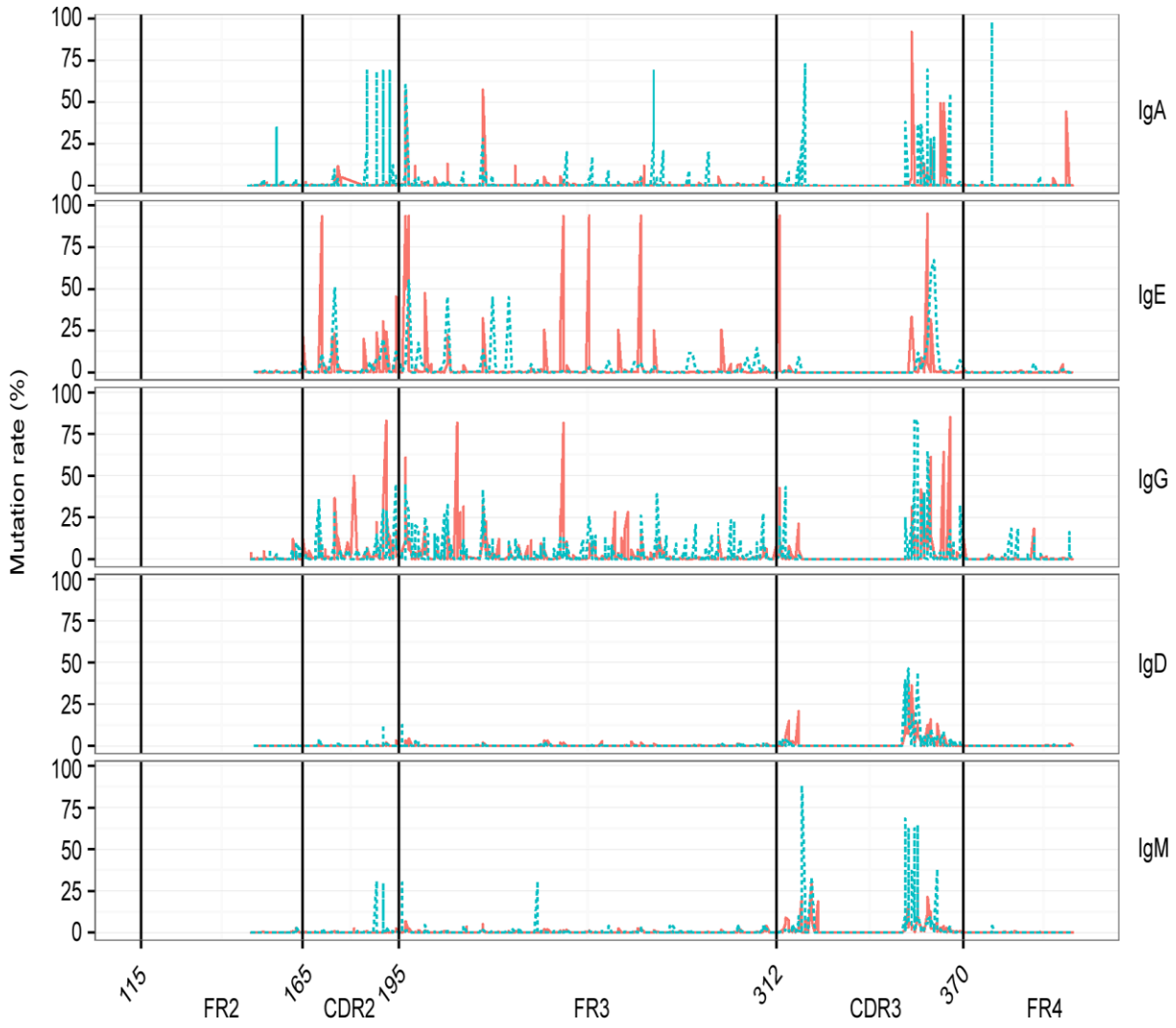

Figure 3.18: Effect of B cell Aire on SHM. Sequencing analysis shows increased mutation frequency in switched isotypes IgG and IgE, but not in unswitched isotypes IgD and IgM.

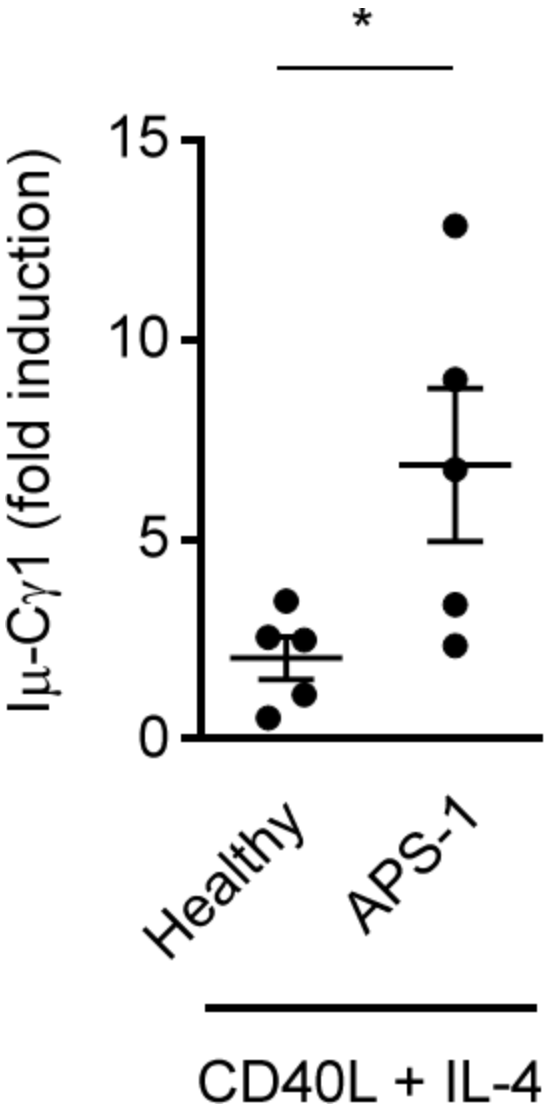

Figure 3.19: CSR to IgG1 in APS-1 patients. APS-1 patient B cells were treated with CD40L+IL-4 to stimulate switching to IgG1. APS-1 patient B cells show increased switch transcripts after stimulation compared to healthy patient B cells.

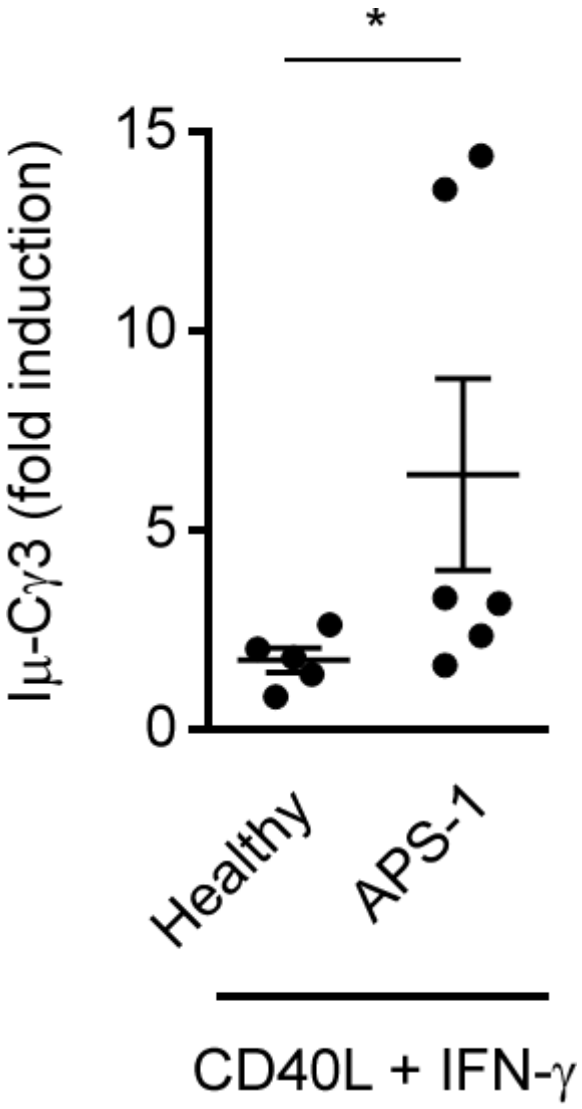

Figure 3.20: CSR to IgG3 in APS-1 patients. APS-1 patient B cells were treated with CD40L+IFN-γ to stimulate switching to IgG3. APS-1 patient B cells show increased switch transcripts after stimulation compared to healthy patient B cells.

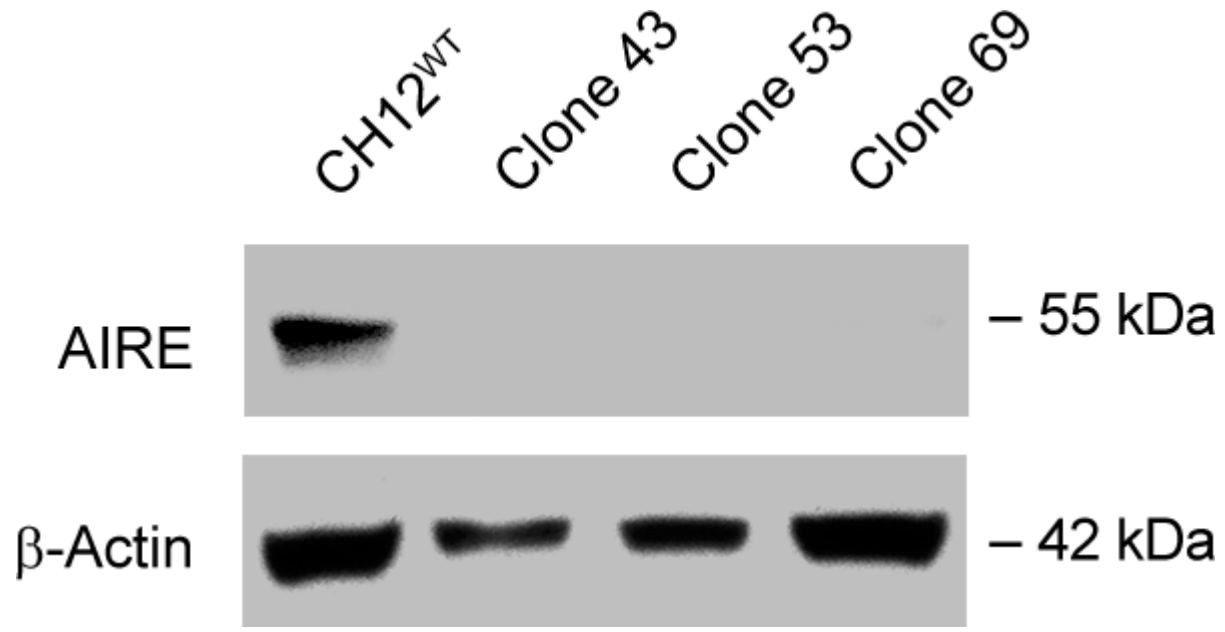

Figure 3.21: Confirming Aire KO in CH12 cells. CH12 Aire KO cells show no upregulation of Aire protein after stimulation.

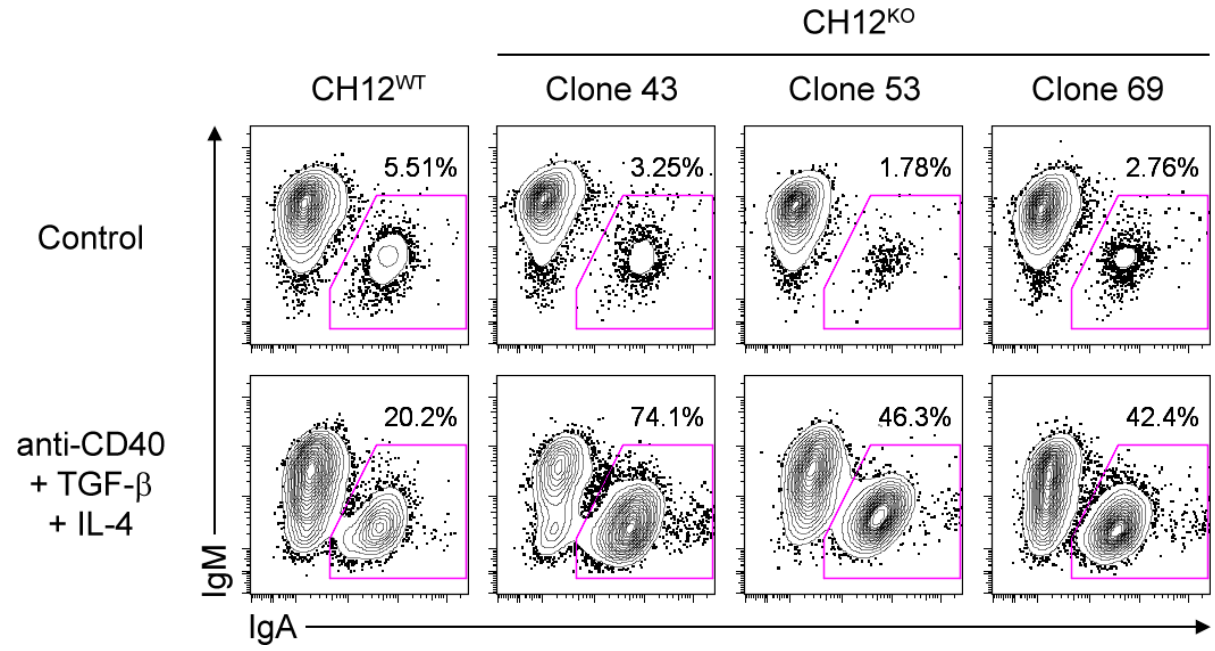

Figure 3.22: Effect of Aire deficiency on CH12 CSR. Aire deficient CH12 cells show increased CSR compared to WT.

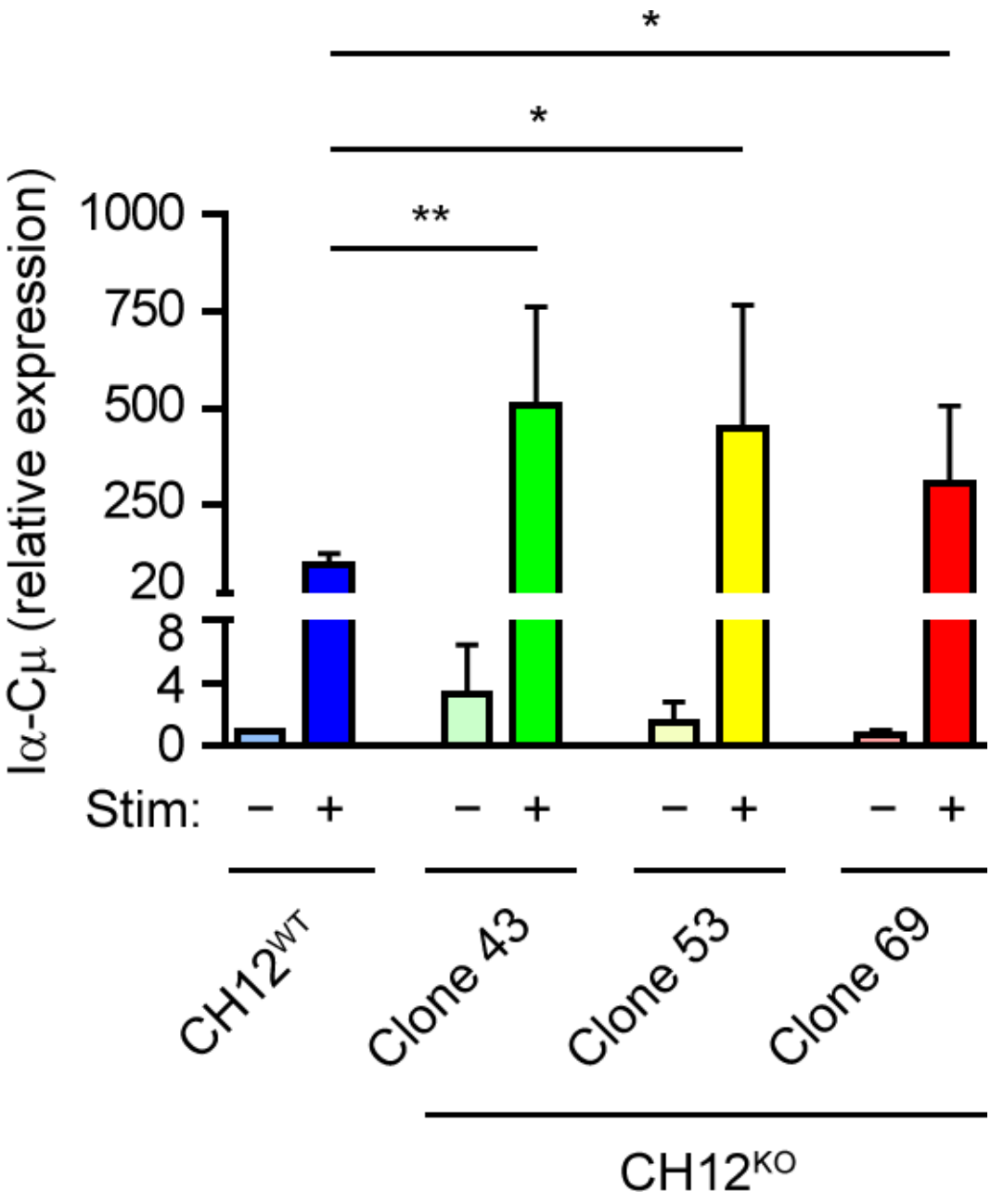

Figure 3.23: Expression of IgA switch circle in Aire deficient CH12 cells. Aire deficient CH12 cells show increased expression of switch circles compared to WT.

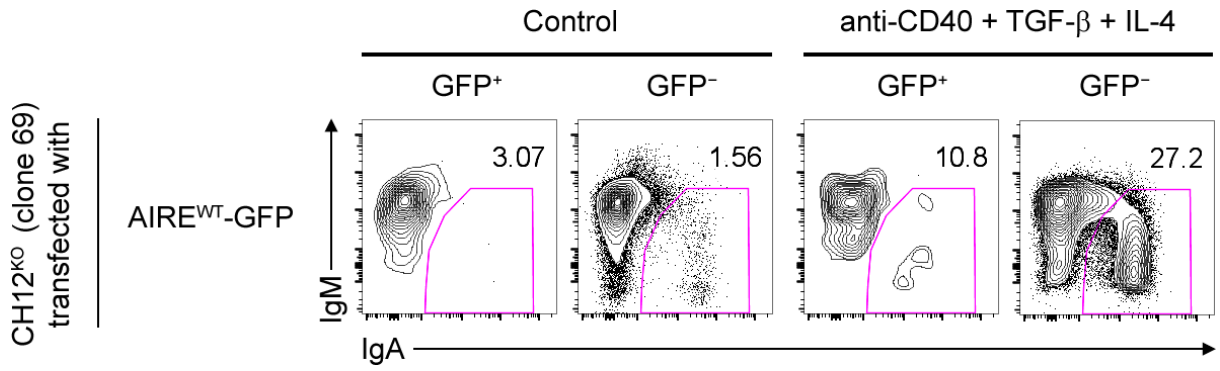

Figure 3.24: Rescue assay using WT Aire-GFP construct. Aire KO cells were transfected with a WT Aire-GF construct prior to stimulation. GFP+ cells show reduced CSR compared to GFP– cells.

CHAPTER 4: MECHANISM OF AIRE IN GC B CELLS

4.1 Introduction

AID is known to initiate the processes of SHM and CSR by generating C to U mutations in either the VDJ region or the switch regions (Petersen-Mahrt et al., 2002). These DNA lesions are then excised and repaired in a manner that generates mutations in the case of SHM or recombination in the case of CSR. There are several mechanisms by which AID is targeted to the DNA to allow for the deamination event to take place. One of the most important mechanisms is the initiation of transcription of AID target regions. The importance of the transcriptional machinery at AID targets has three major functions: first, it opens the DNA to create single strands so that AID can have access to the substrate, second, it creates a transcript that is utilized by AID as a guide to target the enzyme to the proper location (Zheng et al., 2015), and third, it recruits AID through interaction with stalled Pol II (Pavri et al., 2010). Due to the observation that Aire negatively regulates both SHM and CSR, it is likely that it affects the function of AID either through one of these mechanisms or through direct inhibition of catalytic activity.

4.2 Materials and Methods

4.2.1 Patient samples

Human tonsil samples were taken after pediatric tonsillectomy from the Children's Hospital of Michigan in collaboration with Dr. Janet Poulik.

4.2.2 Mice

C57BL/6J and Aire$^{+/-}$ mice were purchased from the Jackson Laboratory. Mice were housed as mentioned above. Age and sex matched mice were randomly assigned to control and experimental groups for experimentation.

4.2.3 Cell lines

CH12 cells were cultured in RPMI-1640 medium supplemented with 5% (v/v) NCTC and 50 μM β-mercaptoethanol. To stimulate Aire expression and CSR, cells were activated with 5μg/ml anti-CD40 + 100ng/ml IL-4 + 1ng/ml TGF-β1 for up to 3 days. Aire knockout CH12 cells were generated by Guang Wen Sun at School of Applied Science in Singapore.

HKB-11 cells, a human embryonic kidney cell/Burkitt's lymphoma fusion, was cultured in DMEM/F12 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 10% FBS.

4.2.4 Primary cell purification

Human tonsils were minced and pushed through a 40μm cell strainer. B cells were purified by magnetic separation using anti-IgD-Biotin and anti-Biotin magnetic beads. To purify mouse splenocytes, mouse spleens were minced and passed through a 40μm strainer prior to RBC lysis.

4.2.5 Flow cytometry

Tonsil samples were blocked using Fc blocking reagent for 10 minutes on ice prior to staining with fluorescently labeled antibody and either GV510, 7AAD, or 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) for 1 hour at room temperature. To show human B cell subcellular localization of Aire and AID, cells were stained with CD19, CD38, Aire, IgD, and AID (BD 565785). For imaging flow, cells were imaged on an ImageStream X Mark II imaging flow cytometer (Amnis) and analyzed using IDEAS 6.1 (Amnis).

For CH12, cells were harvested and blocked using Fc blocking reagent for 10 minutes on ice prior to staining with either GV510 or 7AAD and primary antibodies for CD19, B220, IgM, and IgA.

4.2.6 Immunoprecipitation

Cells were harvested and washed prior to lysis with CelLytic M buffer (Sigma C2978) containing protease inhibitor cocktail and phosphatase inhibitor for 1 hour on ice. Lysates were centrifuged at 18,000 x g for 15 minutes at 4°C to pellet debris. Protein concentration was measured using a BCA protein assay kit (Fisher 23225) as per the manufacturer's instructions. Lysates were precleared with protein G magnetic beads (Cell Signaling 8740 or Fisher 88847). IP antibodies against AID, Aire (Santa Cruz sc-17986), or Myc (BioLegend 626802) were incubated with magnetic beads prior to mixing with precleared lysates. Beads were incubated with lysates overnight at 4°C while shaking. To precipitate flag, anti-flag magnetic beads were used instead (Sigma F3165). Western blot analysis was performed using primary antibodies against Aire (Santa Cruz sc-373703), AID (eBioscience 14-5959), Flag (Cell Signaling 2368), or His (BioLegend 652502).

4.2.7 RT-qPCR

For CH12 samples, RNA was purified using a Trizol purification method. Reverse transcription was performed using an iScript reverse transcription kit according to the manufacturer's instructions. qPCR was performed using either SYBR Green. PCR was performed using a StepOnePlus instrument and analyzed using the StepOne software.

4.2.8 Transfection

HKB-11 cells were cultured to 70-90% confluence prior to transfection with 4µg plasmid. Transfection was performed using Lipofectamine 30000 (Fisher L3000015) in Opti-MEM (Fisher 31985070) according to manufacturer's instruction. CH12 cells were transfected using an Amaxa cell line nucleofector kit V (Lonza VCA-1003) according to manufacturer's instructions.

4.2.9 Uracil dot blot

Genomic uracil content was analyzed as described previously (Wei et al., 2017; Wei et al., 2015).

4.2.10 ChIP-qPCR

CH12 cells were stimulated to undergo CSR. Chromatin immunoprecipitation (ChIP) was performed with a ChIP assay kit (EMD Millipore 17-295) according to manufacturer's instructions. DNA was eluted from agarose after precipitation using 1% SDS, 0.1 M NaHCO3, pH 8.0 and reverse-crosslinked from protein by incubation in 200mNaCl at 65°C for 4 hours. Samples were cleaned with RNaseA (Sigma R6513) for 30 minutes at 37°C followed by treatment with 40µg/ml proteinase K (Qiagen 9133) for 1 hour at 45°C. DNA was then purified using phenol/chloroform extraction and ethanol precipitation prior to qPCR analysis as described above.

4.3 Results

4.3.1 Expression of germline transcripts

Germline transcription is critical for AID targeting and function. AID can be recruited into transcriptional bubbles generated by Pol II and be assisted by the transcripts generated (Pavri et al., 2010; Zheng et al., 2015). Additionally, it is well known that Aire is a transcriptional regulator (Anderson et al., 2002; Bansal et al., 2017; Yoshida et al., 2015), therefore, to determine whether Aire influences the transcription of AID or switch regions, qPCR CH12 cells were stimulated to

undergo CSR to IgA. qPCR analysis showed no difference in transcription of either AID or switch region genes, suggesting that Aire regulation of CSR is not a result of transcriptional repression at AID or switch regions (**Figure 4.1 – 4.3**).

4.3.2 Aire interaction with AID

Due to the known functions of AID and Aire, both proteins must exist in the nucleus. Indeed, this has been observed by many groups, though it is unclear whether both proteins are in the nucleus together. To test whether both proteins co-localize in the nucleus, imaging flow was performed on healthy human tonsils. Co-localization was observed in the nucleus of $CD19^{+}IgD^{-}CD38^{+}$ GC B cells (**Figure 4.4**). Since both proteins seem to exist in the nucleus, we sought to determine whether these proteins interact with each other. To this end, human total ($CD19^{+}$), naïve (IgD^{+}), and switched ($CD19^{+}IgD^{-}$) B cells were purified by magnetic separation from tonsil samples. Cells were lysed and immunoprecipitation was performed on the purified protein. AID was shown to co-immunoprecipitate with Aire in both the $CD19^{+}$ and the $CD19^{+}IgD^{-}$ fractions, but little to no AID was pulled down from the IgD+ fraction, therefore, little to no Aire was detected (**Figure 4.5**). To confirm these findings in mice, Aire KO and WT mice were immunized to SRBC via four intraperitoneal injections with adjuvant and splenocytes were harvested. Immunoprecipitation on purified protein was performed and western blot analysis shows interaction of Aire with AID in the WT mice; however, due the deficiency of Aire in the KO mice, no Aire was pulled down, showing the specificity of the antibody (**Figure 4.6**).

Since it is known that both of these proteins bind DNA, it is possible that they interact through a DNA complex and not a protein-protein interaction. To determine whether these proteins can interact directly, purified Aire-Flag and AID-His were incubated with an irrelevant protein, IL-33. Immunoprecipitation of Flag and His were performed to show that the two purified proteins

interact directly *in vitro* (**Figure 4.7**). Using HKB-11 cells transfected with various Aire and AID constructs (**Figures 4.8** and **4.10**), it was determined that the interaction between Aire and AID requires the CARD and NLS domains of Aire (**Figure 4.9**) as well as the catalytic and APOBEC-like domains of AID (**Figure 4.11**). To determine whether this interaction was responsible for the functional phenotypes described above, a WT Aire-GFP construct as well as an Aire-GFP with a deletion in the CARD domain (ΔCARD) was transfected into the Aire deficient CH12 clone, 69, prior to stimulation. The cells that received the Aire WT construct rescued the WT phenotype in which CSR is reduced in the GFP+ cells; however, cells that received the ΔCARD construct did not show any change relative to the GFP^- cells (**Figure 4.12**). These data indicate that Aire interaction with AID is important for its regulatory function.

4.3.3 Regulation of AID function by Aire

The function of AID as a DNA deaminase has been well established over the past couple of decades (Petersen-Mahrt et al., 2002). Our data suggests that interaction between Aire and AID negatively regulates CSR, therefore, it is likely that AID function is affected by this interaction. We utilized a uracil dot blot assay (**Figure 4.13**) that measures genomic uracil content by fluorescence signal (**Figure 4.14**). Using this assay, we show that there is an accumulation of genomic uracil in Aire KO CH12 cells compared to the WT controls (**Figure 4.15**) Additionally, this accumulation of genomic uracil is lost in AID deficient CH12 cells indicating that this assay specifically recognizes AID directed mutagenesis. These data suggest that AID mediated C to U mutations is reduced in the presence of Aire.

4.3.4 Regulation of AID targeting by Aire

To determine whether the regulation of AID by Aire is due to inhibition of catalytic activity or targeting of AID to the DNA substrate, immunoprecipitation of AID was performed in CH12 cells to show interaction with the transcriptional machinery. In Aire KO CH12 cells, increased association of AID with Spt5 and pSer5 Pol II was detected indicating increased targeting of AID to stalled Pol II in activated cells compared to the WT (**Figure 4.16**). Additionally, ChIP-qPCR analysis shows enrichment of Sμ interaction with AID in stimulated Aire KO CH12 cells compared to the WT, consistent with the finding that Aire reduces its targeting to stalled Pol II (**Figure 4.17**). Taken together, these data suggest that Aire interaction with AID negatively regulates its targeting to the DNA.

4.4 Discussion

Here, we describe a mechanism by which Aire negatively regulates AID activity. We did not find any significant differences in transcription of AID or switch region transcripts between Aire KO and Aire WT CH12 cells after stimulation to undergo CSR; however, we did observe a direct protein-protein interaction between Aire and AID, in which the CARD and NLS of Aire interacted with the catalytic and APOBEC-like domains of AID, that was necessary for protein function. Genomic uracil quantification showed significantly increased uracil content in Aire KO CH12 cells, confirming that Aire negatively regulates AID activity and immunoprecipitation showed increased association of AID with the transcriptional machinery indicating that Aire prevents AID targeting to the DNA. These findings were then confirmed with ChIP-qPCR to show increased AID binding to DNA in Aire deficient CH12 cells.

At this time, it is still unclear exactly how this interaction between Aire and AID prevent AID targeting. Several possibilities exist, though the most attractive is the sequestering of nuclear AID, thus immobilizing the protein. Although the stoichiometry of this interaction is still unclear, it should not require very high concentrations of Aire to inhibit AID due to the cytoplasmic retention of the protein compared to the nuclear localization of Aire. Importantly, Aire expression does not completely inhibit, but reduces AID function, thereby still allowing CSR and SHM to take place. These data seem to suggest that Aire acts as an additional regulatory mechanism by which AID is controlled to prevent excessive AID activity that could lead to autoimmunity and cancer.

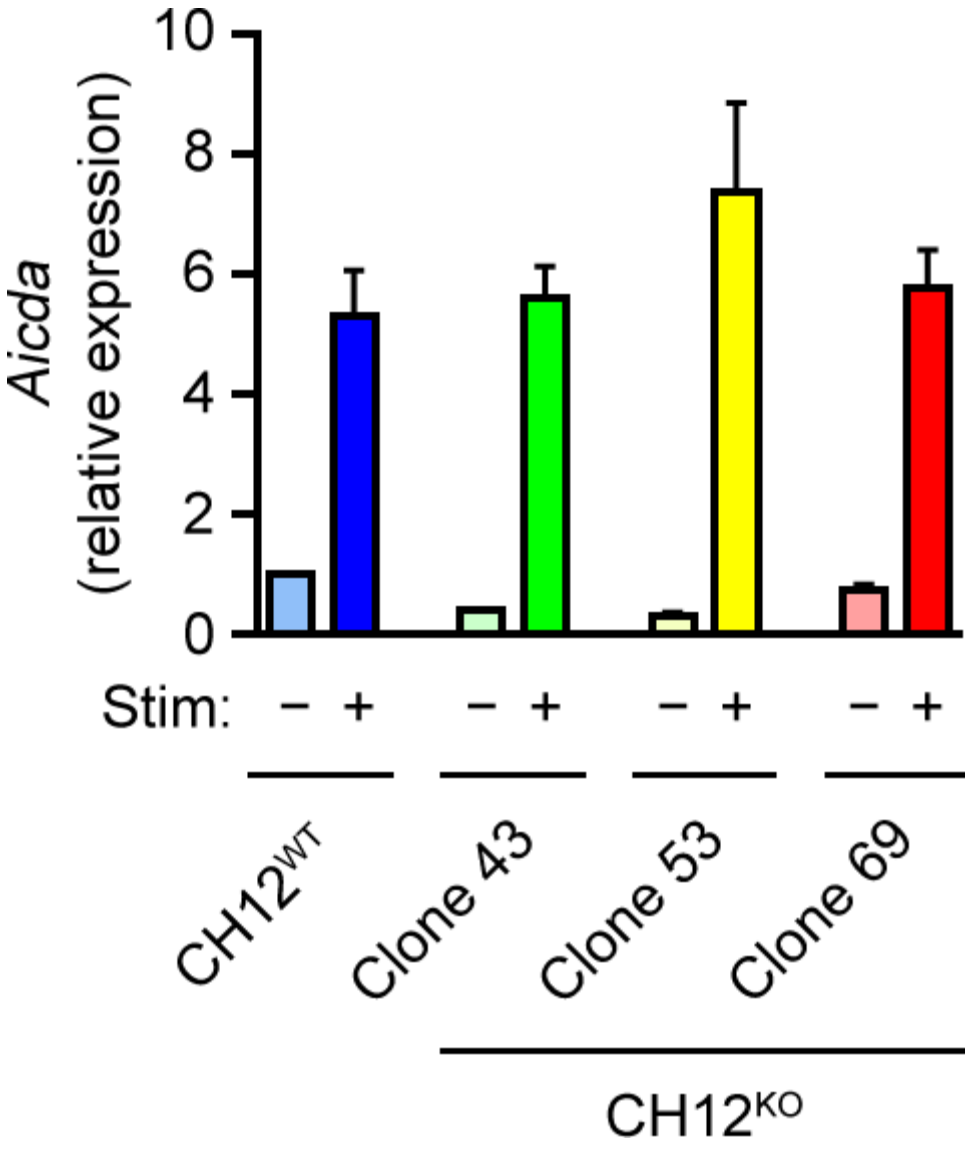

Figure 4.1: Expression of AID in Aire deficient CH12 cells. AID expression is the same between WT and KO CH12 cells.

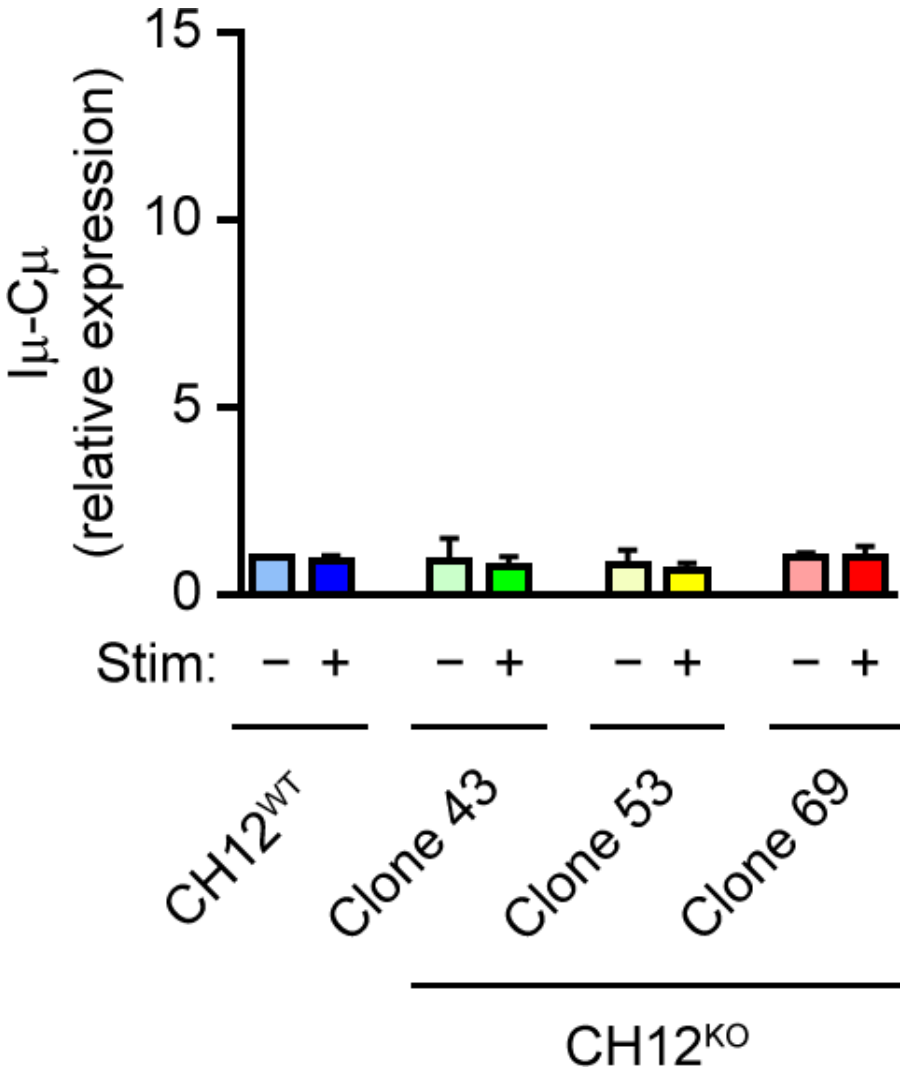

Figure 4.2: Expression of IgM germline transcripts in Aire deficient CH12 cells. IgM germline transcript expression is the same between WT and KO CH12 cells.

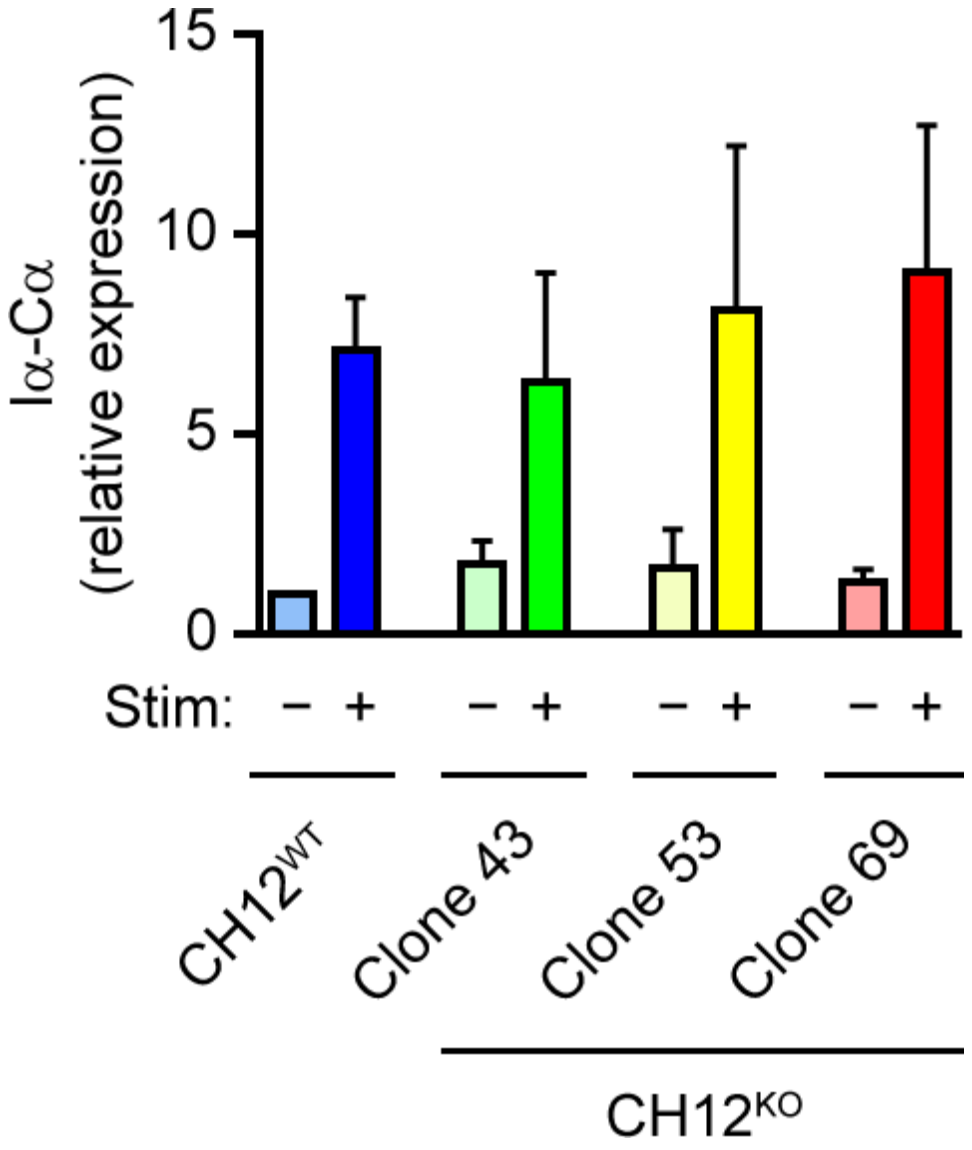

Figure 4.3: Expression of IgA germline transcripts in Aire deficient CH12 cells. IgA germline transcript expression is the same between WT and KO CH12 cells.

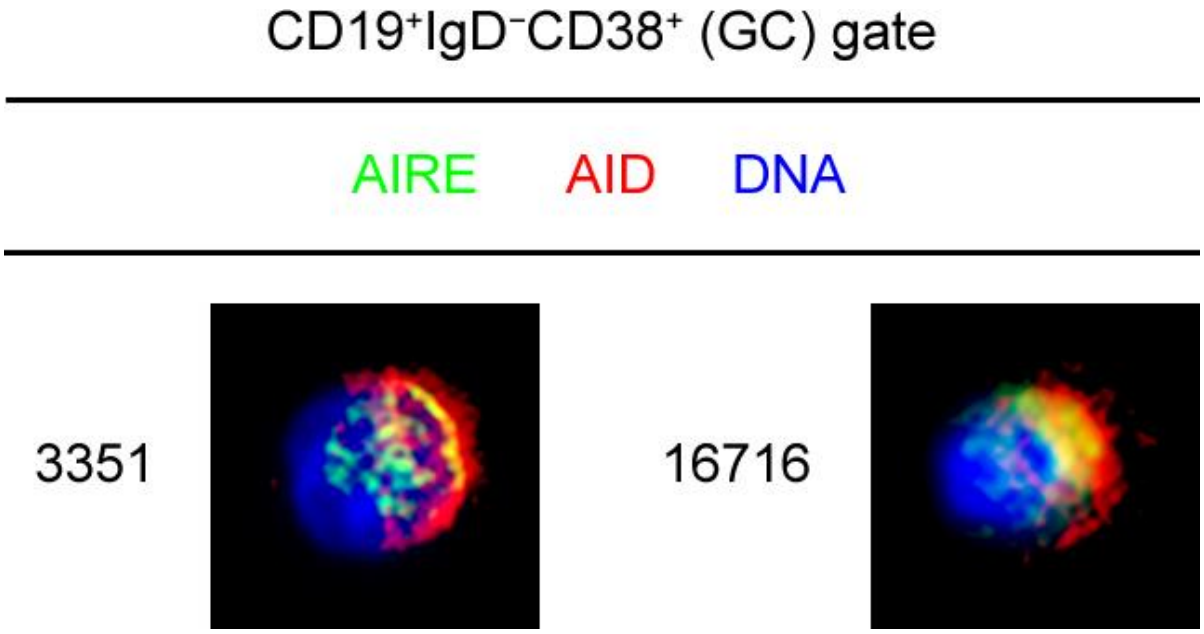

Figure 4.4: Aire and AID co-localization. Human GC B cells analyzed by imaging flow show co-localization of Aire and AID in the nucleus.

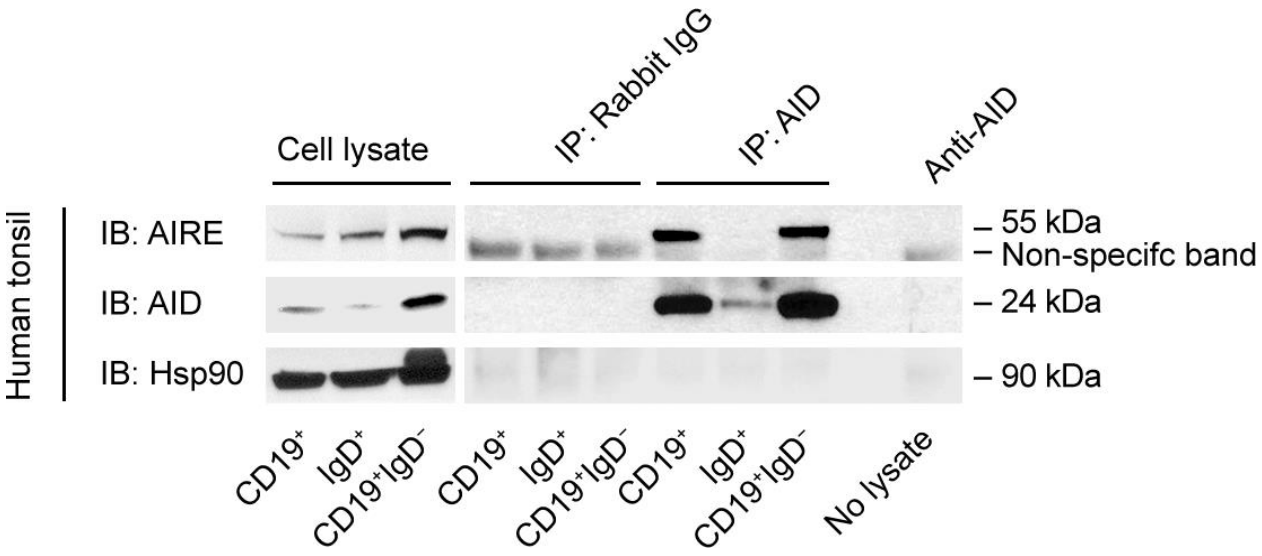

Figure 4.5: Aire and AID co-immunoprecipitation in human cells. $CD19^+$ total B cells, IgD^+ naïve B cells, and $CD19^+IgD^-$ B cells were purified from patient tonsils. From the cell lysates, AID was immunoprecipitated and western blot analysis was performed showing Aire interaction with AID.

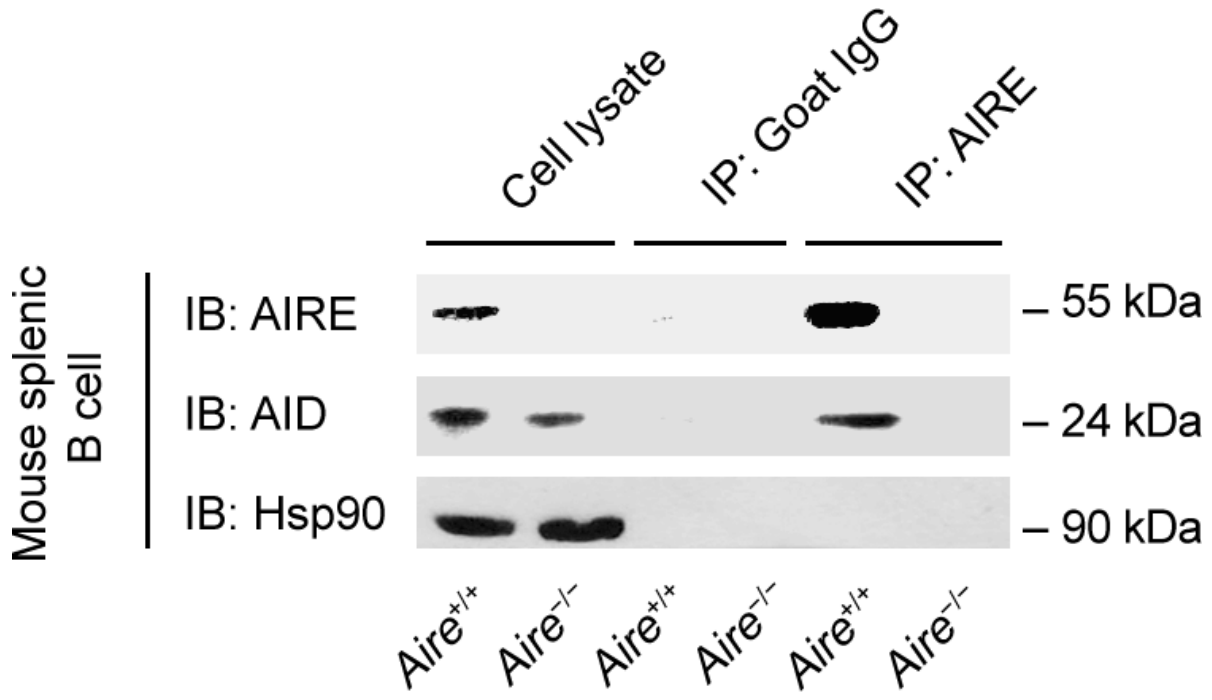

Figure 4.6: Aire and AID co-immunoprecipitation in mouse cells. Aire WT and KO mice were immunized with SRBC and splenic B cells were purified. Aire was immunoprecipitated from the lysates and western blot analysis was performed to show interaction with AID.

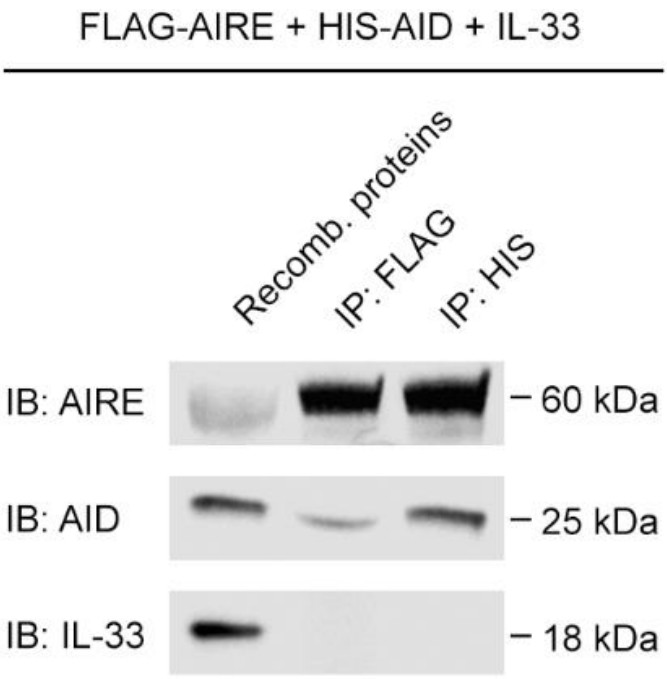

Figure 4.7: Aire interacts directly with AID. Flag tagged Aire and His tagged AID proteins were incubated with an irrelevant protein, IL-33. Immunoprecipitation was performed for Flag and His prior to western blot to show Aire interaction with AID.

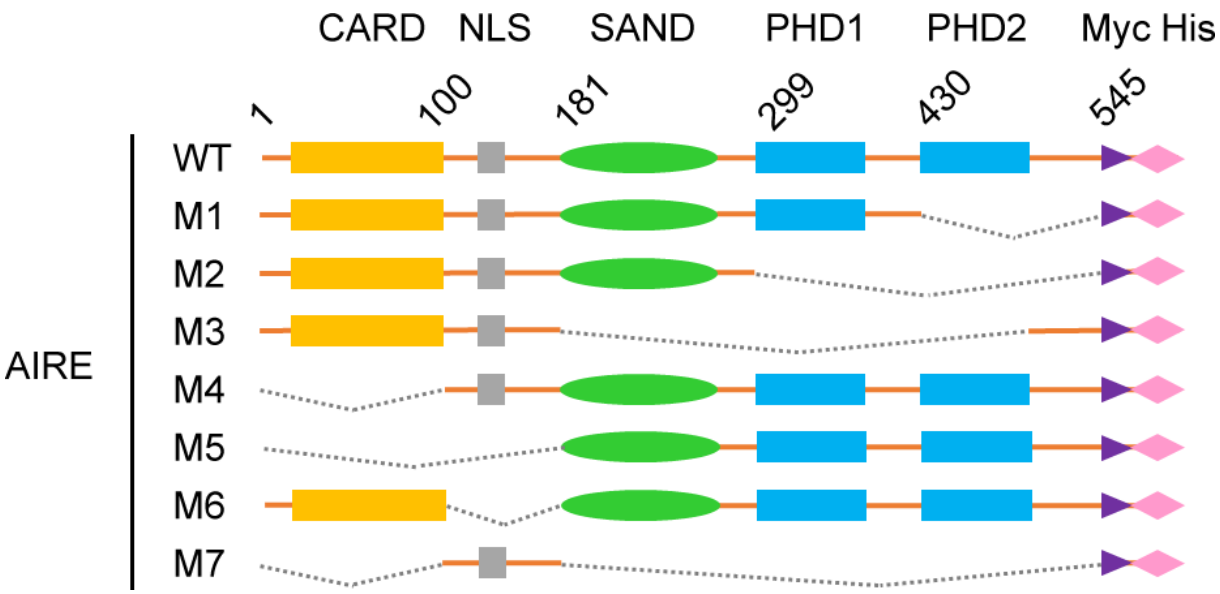

Figure 4.8: Aire domain constructs. Myc His tagged Aire constructs with deletions in specific domains used for interaction assay with AID.

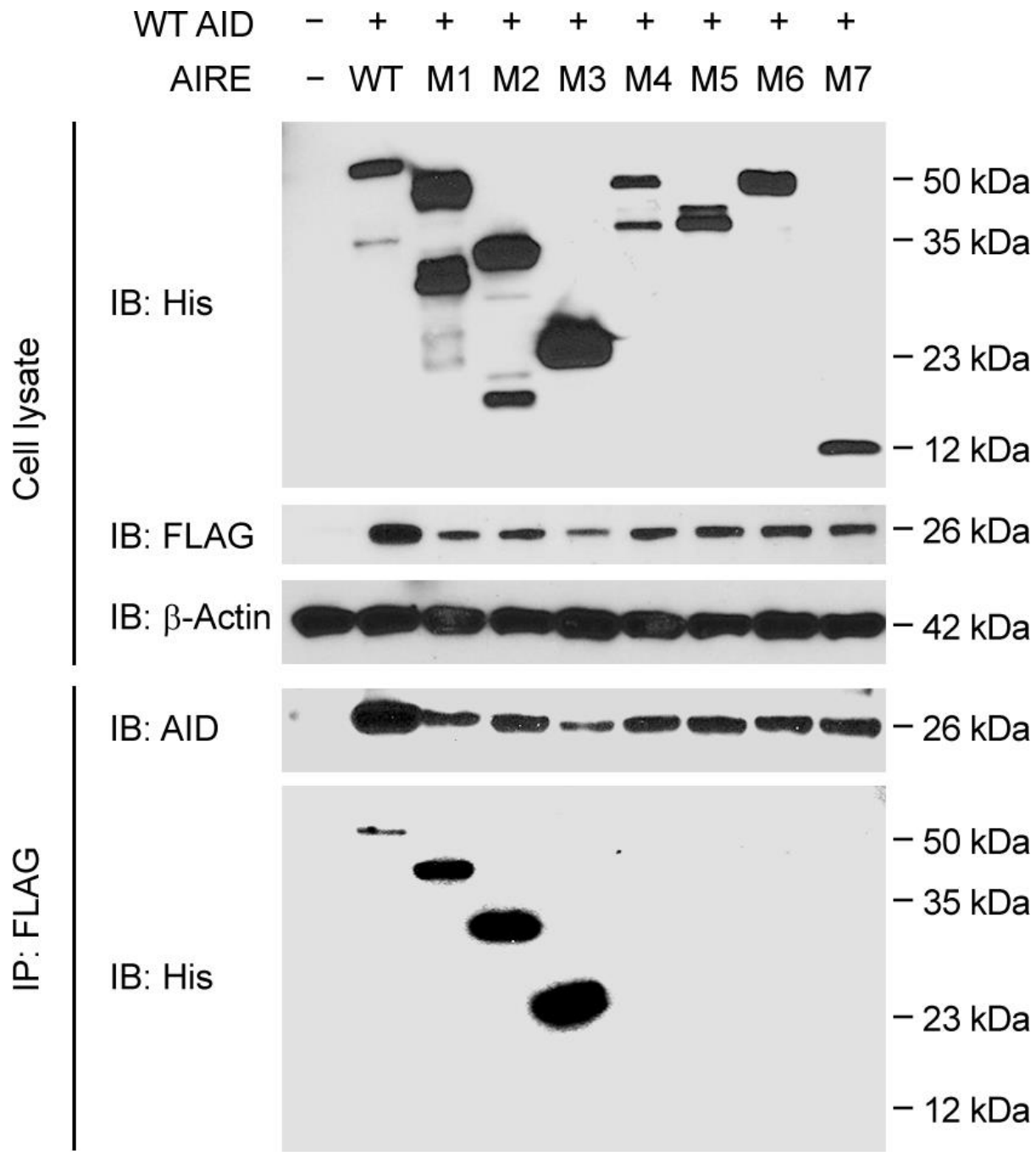

Figure 4.9: Aire domains interacting with AID. Flag tagged AID was immunoprecipitated with Myc His tagged Aire. M4-M7 show no interaction between AID and the Aire construct indicating the necessity of the NLS and CARD domains of Aire.

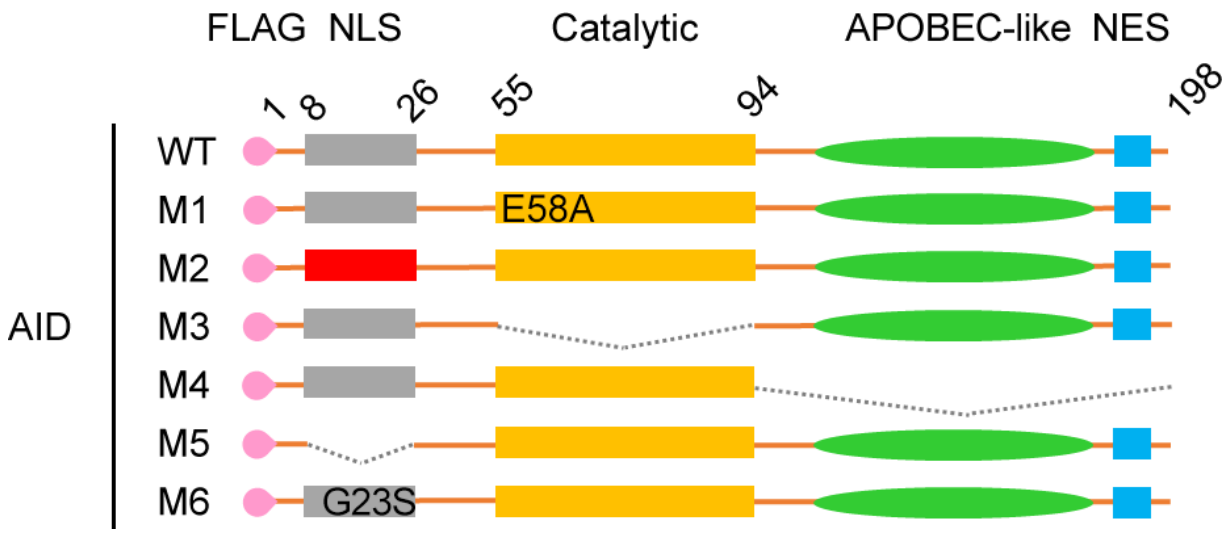

Figure 4.10: AID domain constructs. Flag tagged Aire constructs with deletions in specific domains used for interaction assay with AID. M1 indicates a loss of function point mutation in the catalytic domain. M2 indicates NLS is replaced with the NLS of nucleoplasmin. M6 indicates a loss of function mutation in the NLS whereby the protein does not localize to the nucleus.

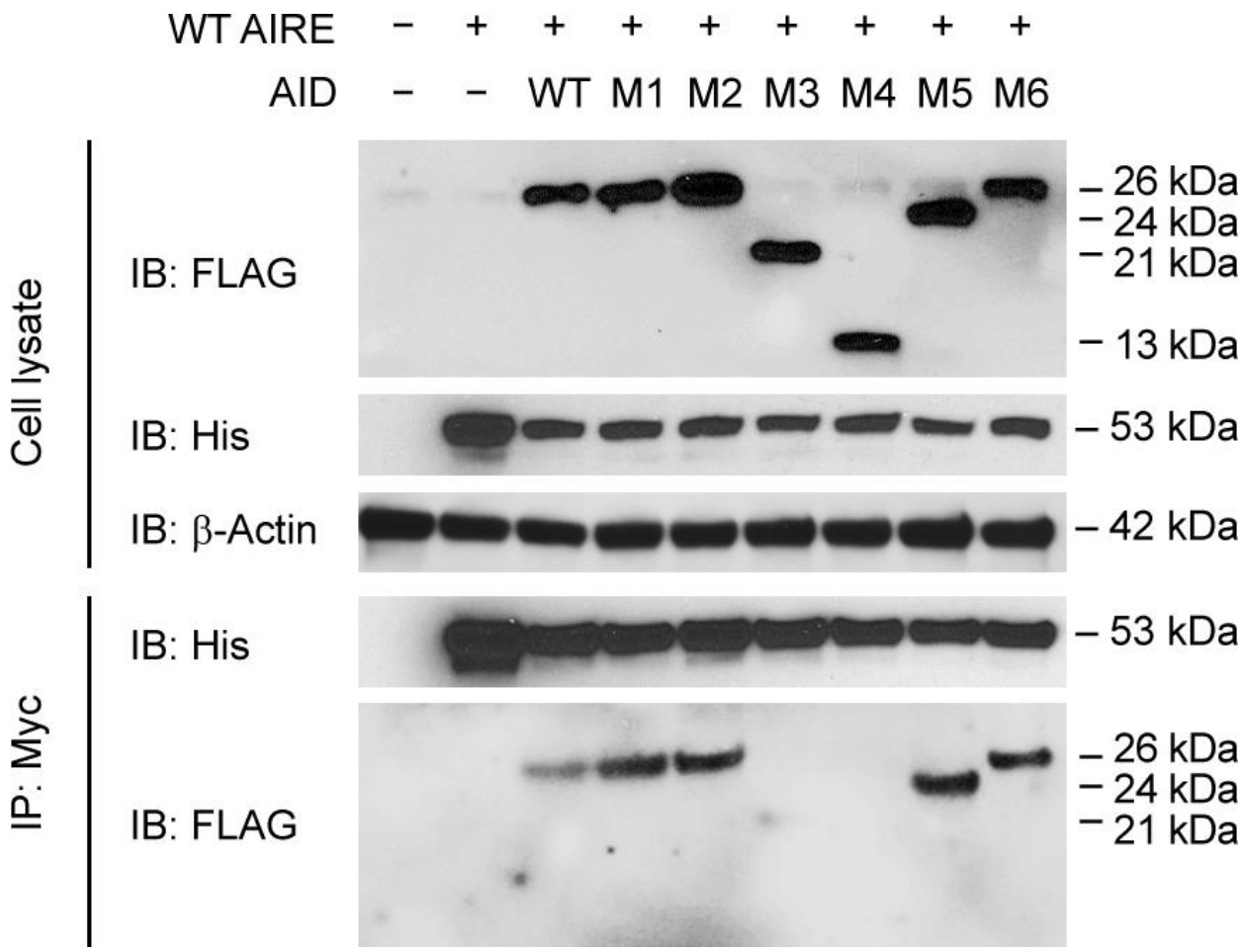

Figure 4.11: AID domains interacting with Aire. Flag tagged AID was immunoprecipitated with Myc His tagged Aire. M3 and M4 show no interaction between Aire and AID indicating a necessity for both the catalytic domain and APOBEC like domain.

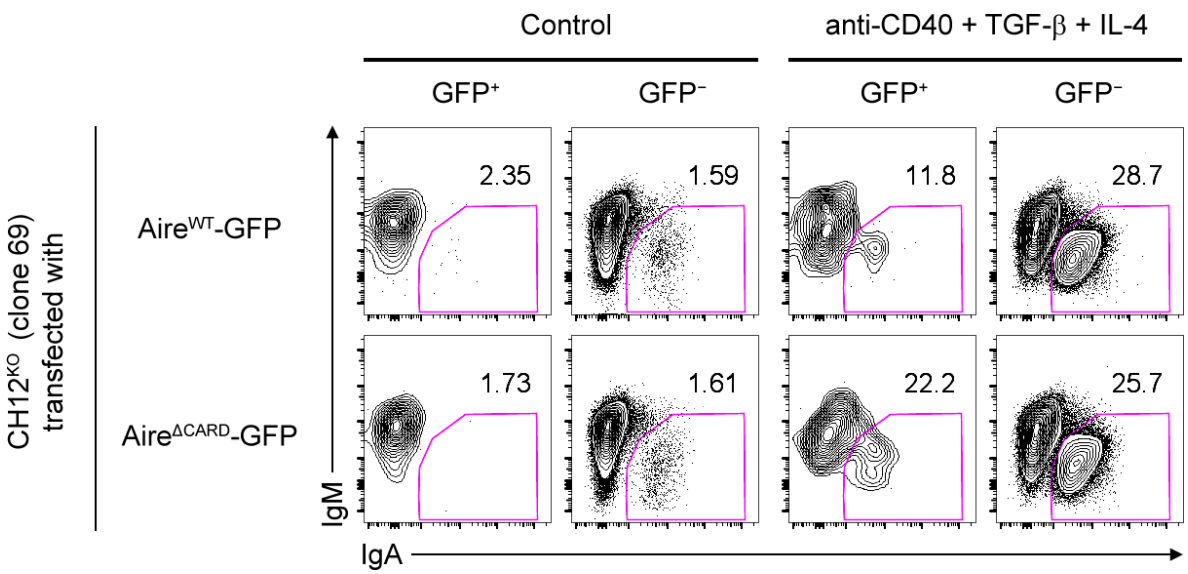

Figure 4.12: Domain specific rescue assay. Aire KO cells were transfected with either a WT Aire-GF or AireΔCARD-GFP construct prior to stimulation. GFP^+ cells show reduced CSR in the WT, but not AireΔCARD-GFP when compared to GFP^- cells.

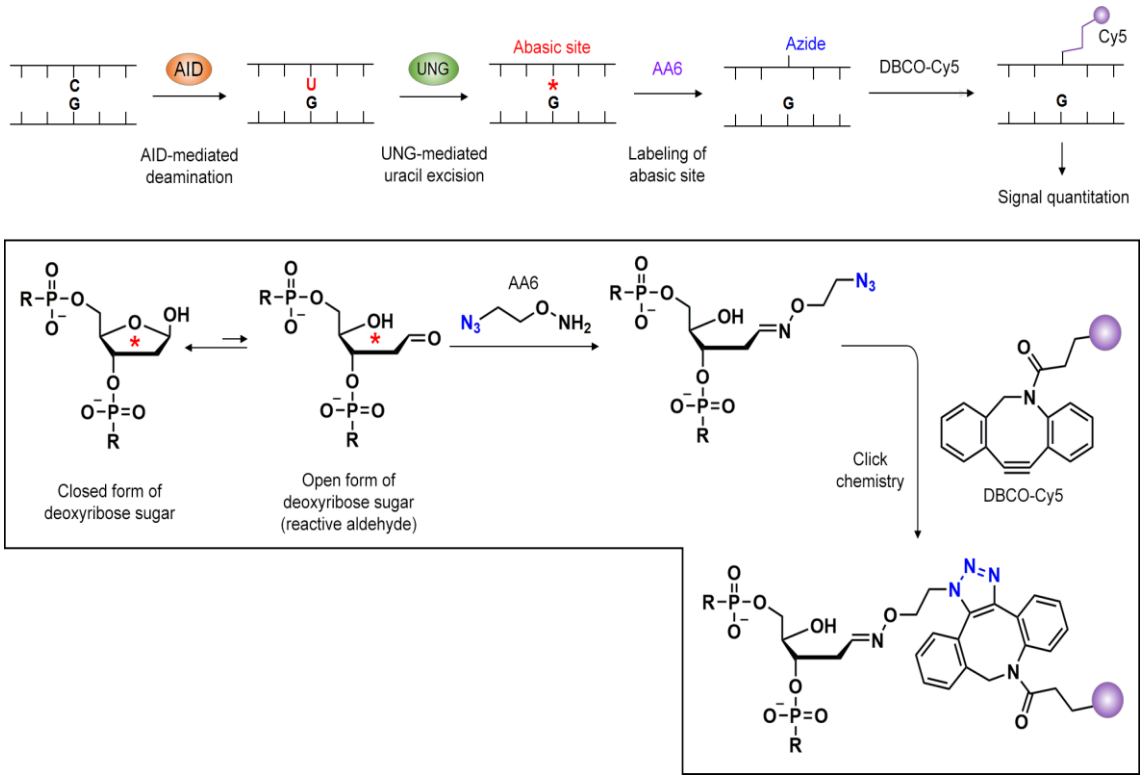

Figure 4.13: Uracil assay chemistry. Uracil assay designed by the lab of Dr. Bhagwat to quantify genomic uracil content.

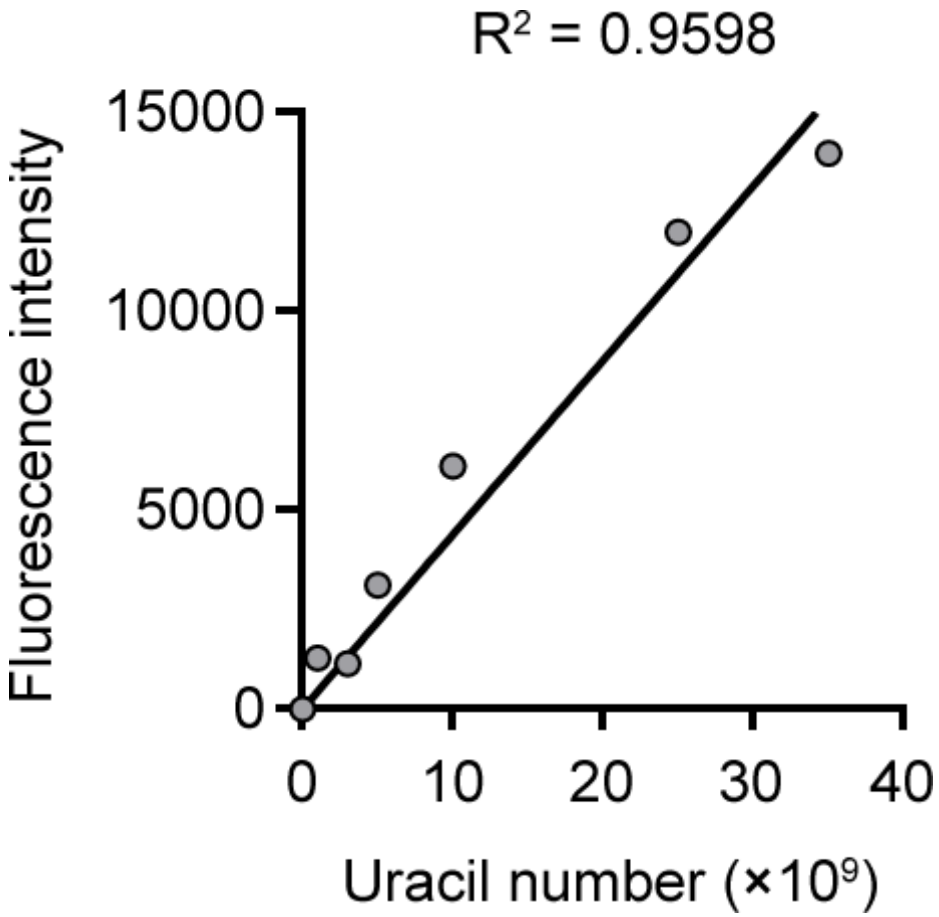

Figure 4.14: Correlation between fluorescence intensity and uracil content. Fluorescence intensity of uracil dot blot strongly correlates with genomic uracil content.

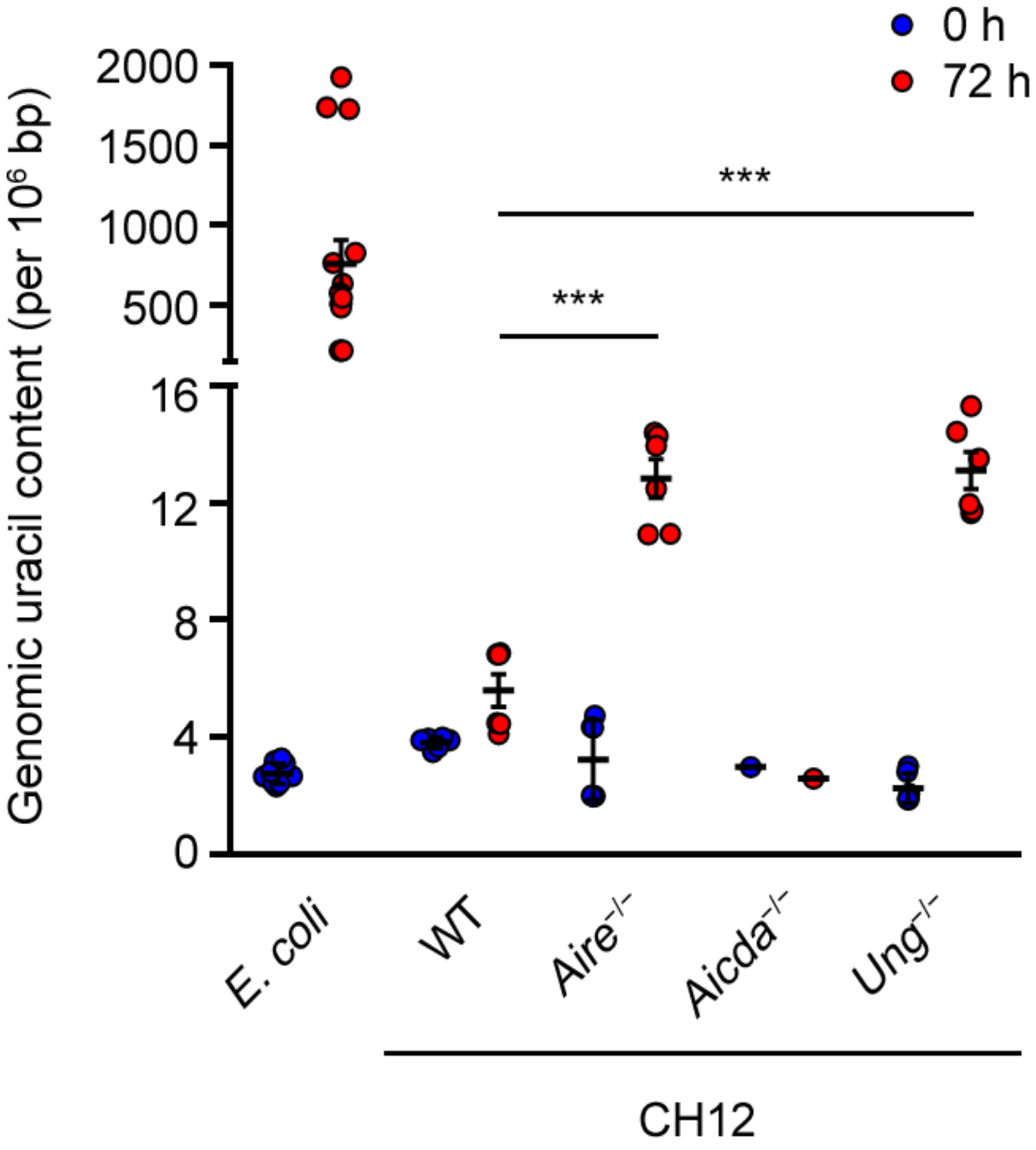

Figure 4.15: Effect of Aire on genomic AID mediate genomic uracil generation. Aire KO CH12 cells harbor significantly more genomic uracils compared to WT CH12 cells.

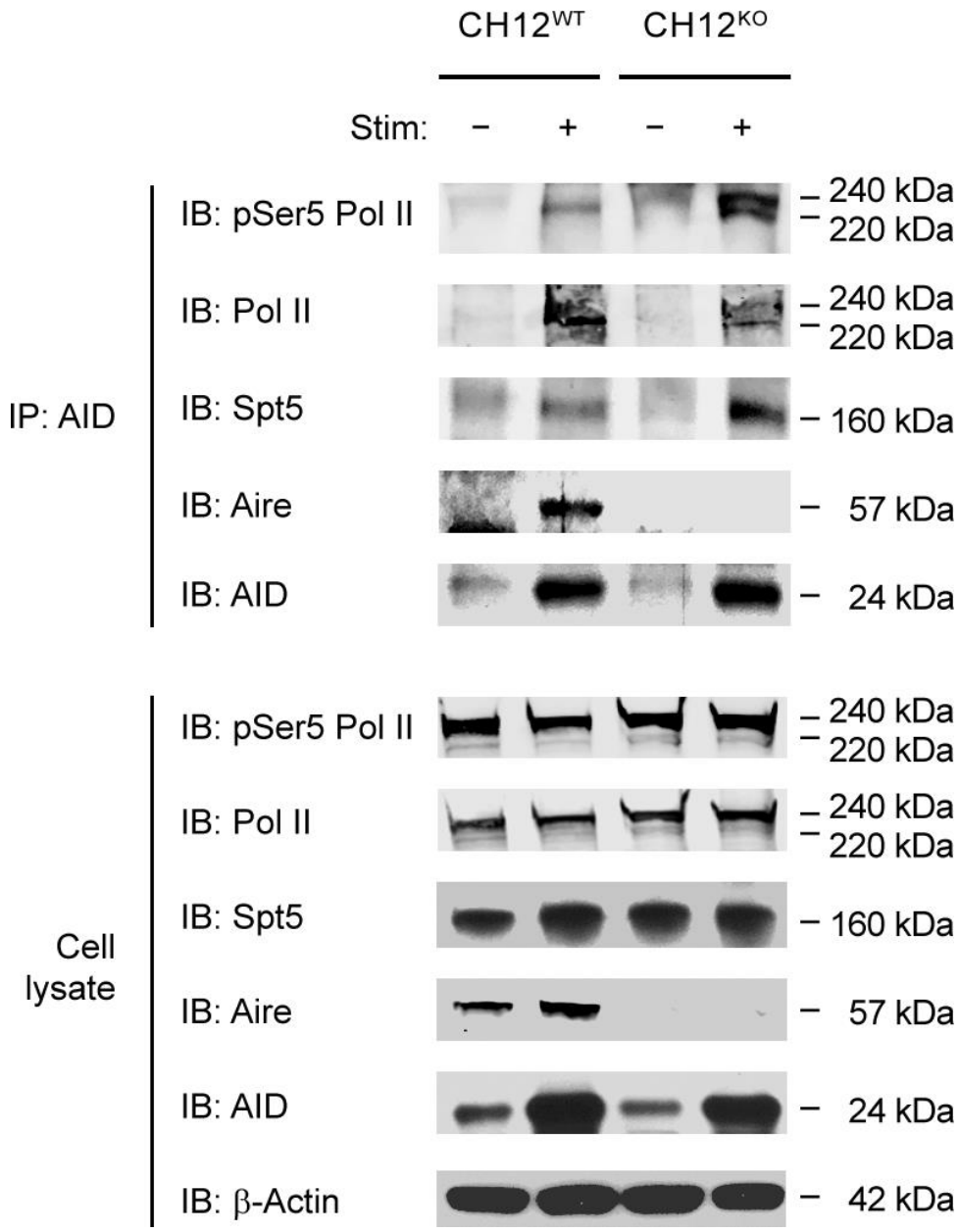

Figure 4.16: AID Co-immunoprecipitation with stalled RNA-Pol II. AID shows increased association with stalled Pol-II in Aire KO CH12 cells compared to Aire WT.

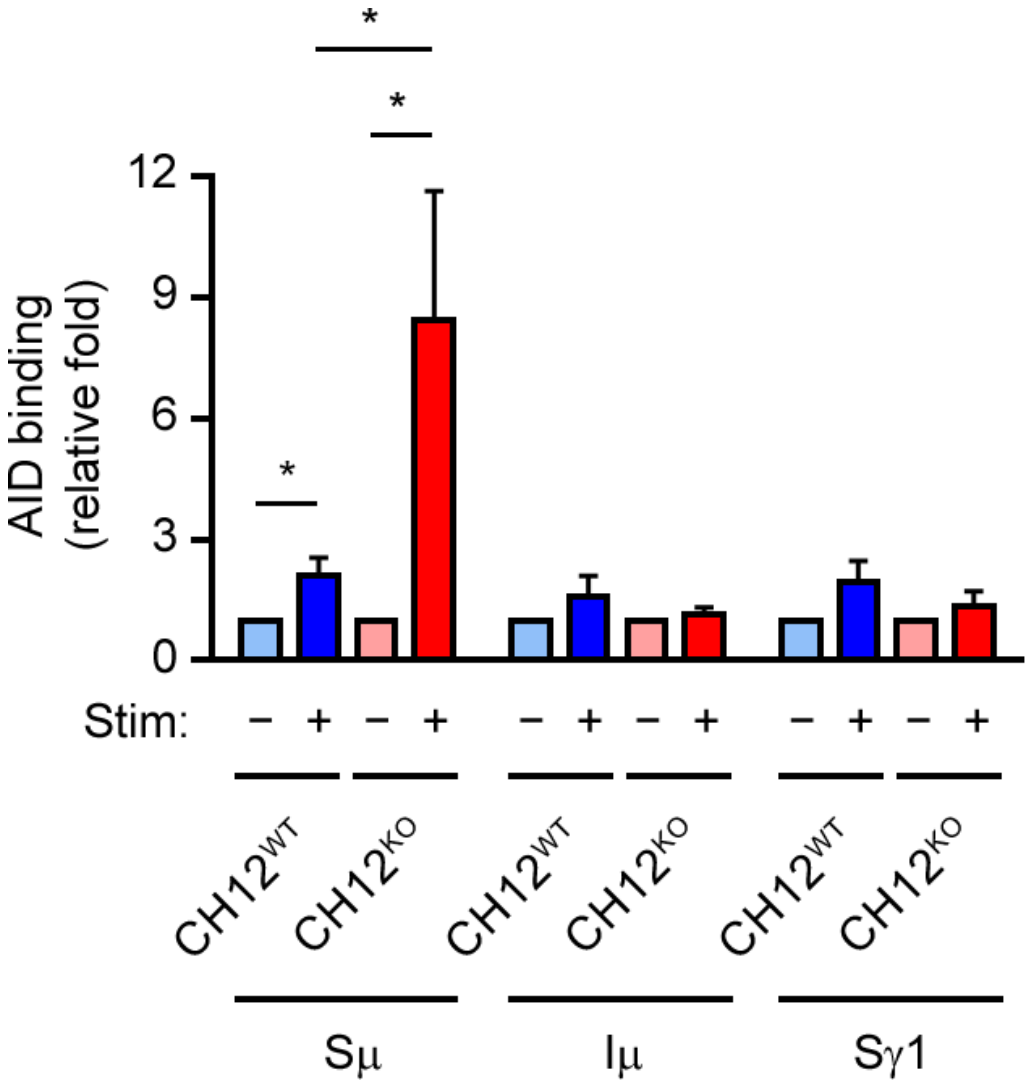

Figure 4.17: Effect of Aire on AID interaction with DNA. ChIP-qPCR analysis with WT and Aire KO CH12 cells. Increased AID interaction with switch regions in Aire KO cells compared to Aire WT after stimulation.

CHAPTER 5: INSERT TITLE HERE

5.1 Introduction

One of the hallmarks of autoimmune polyglandular syndrome type 1 (APS-1) is the appearance of chronic mucocutaneous candidiasis, chronic infection of the mucosal surfaces with *Candida albicans*. Interestingly, it has been observed that this immunodeficiency phenotype is, in fact, a result of an autoimmune reaction in which autoreactive B cells generate high affinity, neutralizing antibodies against Th17 cytokines such as IL-17 and IL-22 which inhibit anti-fungal immune response (Kisand et al., 2010; Meyer et al., 2016; Puel et al., 2010). Additionally, Th17 development may be hindered in APS-1 patients as, although APS-1 patient peripheral blood cells have normal differentiation capacity to Th17, incubation of cells with APS-1 patient serum impairs IL-17 production and Th17 proliferation (Ng et al., 2010). Generally, these phenotypes are thought to be a result of T cell autoimmunity directing B cell diversification, but, with the novel finding that B cells also have the capacity to express Aire, it will be important to test whether B cell specific deficiency in Aire plays a role.

5.2 Materials and Methods

5.2.1 Mice

Aire$^{+/-}$, and μMT mice were purchased from the Jackson Laboratory. To generate Aire$^{+/+}$ and Aire$^{-/-}$ mice, Aire$^{+/-}$ mice were bred and the offspring were genotyped. Age and sex matched mice were randomly assigned to control and experimental groups for experimentation.

5.2.2 Adoptive transfer

For transfer of *Aire*$^{-/-}$ or *Aire*$^{+/+}$ cells to µMT hosts, naïve resting B cells were purified from spleens using a B cell isolation kit as previously mentioned. 5x10^{7} B cells were then injected into the tail vein of each recipient µMT host.

5.2.3 *Candida albicans* culture

C. albicans was cultured in YPD broth at 30°C overnight with shaking at 220 rpm. Culture was then diluted 1:10 with fresh YPD broth containing 10% fetal bovine serum and incubated at 37°C for 3 hours with shaking. Once approximately 95% of cells transitioned into the pseudohyphal form, the culture was centrifuged at 4000 rpm for 10 minutes, washed, and resuspended at a concentration of 5×10^{6} CFU per 50 µl. These cells were either used for intradermal infection or heat killed at 95°C for 2 hours prior to sonication on ice at 30% power for 5 seconds.

5.2.4 Fungal load analysis

µMT mice adoptively transferred with either Aire WT or KO B cells were immunized with 5 doses of 10^{6} CFU heat-killed *C. albicans* pseudohyphae intraperitoneally every 4 days. Four days after the final boost, mice were infected with 5×106 CFU live pseudohyphae in 50µl PBS per spot adjacent to the spine. Four days after infection, fungal lesions were excised and either fixed in formalin overnight for staining or minced and crushed using a pestle and mortar to free the fungi. Live fungi were then used for serial dilution and plated on YPD agar to determine total CFU for every milligram of tissue taken.

5.2.5 ELISA

Blood was taken from μMT mice after immunization and serum was separated. Microtiter plates were coated with 1μg/ml recombinant mouse IL-17A (Peprotech 210-17A), IL-17F (Peprotech 210-17F) or IL-22 (Peprotech 210-22). Plates were blocked with 10% BSA, washed, and incubated with the serum samples prior to probing with an anti-mouse IgG antibody (Vector AP-2000) conjugated to alkaline phosphatase. Reaction was developed using BluePhos (KPL 50-88-02) and quantitated as at 620nm.

For neutralization assays, mouse serum was incubated with overnight with recombinant HRP conjugated (Abcam Ab102890) IL-17A, IL-17F, and IL-22. Microtiter plates were coated with either IL-17RA (R&D 4481-MR-100) or IL-22Rα1 (R&D 4294-MR-050) prior to blocking with 10% BSA. Serum-cytokine mixture was then added to the wells and incubated for 1 hour at room temperature. Wells were washed prior to detection of HRP signal on a microplate reader at 450nm.

5.2.6 Flow cytometry

Excised fungal lesions were washed and digested using collagenase II (Worthington LS004177) in HBSS at 37°C with shaking for 1 hour. Supernatant was collected and solid tissue was pushed through a 100μm cell strainer. Cells were then restimulated with 500ng/ml PMA (Sigma P1585-1MG), 500ng/ml ionomycin (Fisher I24222), and 1ug/ml Golgi plug (BD 55029) at 37°C for 5 hours. Cells were collected, blocked with Fc blocking reagent, and stained with GV510, CD45 (Tonbo 60-0459), CD3, and TCR-β (BioLegend 109227) for 1 hour on ice. Cells were then fixed and permeabilized before staining for IL-17A (BioLegend 506929), and IL-22

(BioLegend 516406) for 1 hour on ice. Events were captured on a Fortessa LSR II and data were analyzed on FlowJo 10.

5.3 Results

5.3.1 Effect of B cell Aire on fungal clearance

APS-1 patients often suffer from chronic mucocutaneous candidiasis, chronic infections of the fungal pathogen, *C. albicans*. To determine the role of B cell Aire in controlling *C. albicans* infection, we developed a model in which μMT mice were adoptively transferred with either Aire WT or Aire KO B cells. Mice were then immunized with heat killed *C. albicans* prior to intradermal infection of live, pathogenic fungi. Four days after infection, injection sites and blood were taken for analysis (**Figure 5.1**). After intradermal challenge, it was observed that mice that received Aire KO B cells were unable to clear the fungal pathogen as effectively as mice that received Aire WT B cells (**Figures 5.2** and **5.3**) indicating that Aire deficiency in B cells leads to a similar immunodeficiency phenotype as seen in APS-1 patients.

5.3.2 Generation of autoreactive antibodies

As previously mentioned, it is thought that the chronic mucocutaneous candidiasis phenotype observed in APS-1 patients is a result of the production of autoreactive, neutralizing antibodies against Th17 cytokines, which are critical for clearing fungal pathogens (Meyer et al., 2016). To determine whether susceptibility to *C. albicans* infection in our model is a result of autoreactive antibodies, serum samples were tested for antibodies against IL-17A, IL-17F, and IL-22. Indeed, we observed an increase in the titers of autoreactive antibodies against these cytokines in mice that received Aire KO B cells compared to mice that received Aire WT B cells (**Figures 5.4**-**5.6**). Additionally, these antibodies seem to have some slight increase in neutralization

capacity in the KO model (**Figures 5.7-5.9**). These data suggest that Aire deficiency, specifically in the B cell compartment, can lead to an increased production of autoreactive, neutralizing antibodies against Th17 cytokines.

5.3.3 Th17 dysfunction

Due to the importance of Th17 cells in the clearance of fungal pathogens, we sought to determine whether Aire deficiency in B cells had any effect on the Th17 compartment. Lesions resulting from fungal infection were excised from mice and digested to generate a single cell suspension. After re-stimulation with PMA/Ionomycin, IL-17A and IL-22 producing T cells were analyzed by flow cytometry. These data show a significant decrease in the $CD45^{+}TCR\text{-}\beta^{+}$ IL-$17A^{+}IL22^{+}$, IL-$17A^{+}IL22^{-}$, and IL-$17A^{-}$IL-22+ fractions in mice that received Aire KO B cells compared to Aire WT B cells suggesting Th17 deficiency in these mice.

5.4 Discussion

To show that the chronic mucocutaneous candidiasis phenotype seen in APS-1 patients can, in part, be attributed to B cell deficiency in Aire, we devised this adoptive transfer infection model to show the production of neutralizing autoreactive antibodies as well as dysfunctional fungal clearance. Our findings suggest that B cell Aire contributes to the overall protection against production of anti-IL-17 and IL-22 antibodies responsible for the mucosal immunodeficiency phenotype in APS-1; however, due to the seemingly small effect sizes, we believe that this mechanism only contributes to the phenotype and is not the major pathway by which it arises and that T cell autoimmunity must have even greater significance. Additionally, in accordance with previous findings (Ng et al., 2010), we also see a reduction in Th17 cells, though this is likely not a result of antibodies against IL-17A, IL-17F, or IL-22, but rather to a different cytokine, possibly

type 1 interferon (Mourik et al., 2017) as an abundance of high affinity, neutralizing antibodies against type 1 interferons have been described in APS-1 patients (Meyer et al., 2016). However, due to the limited amount of sample we were able to extract from each mouse, we did not test for anti-interferon antibodies.

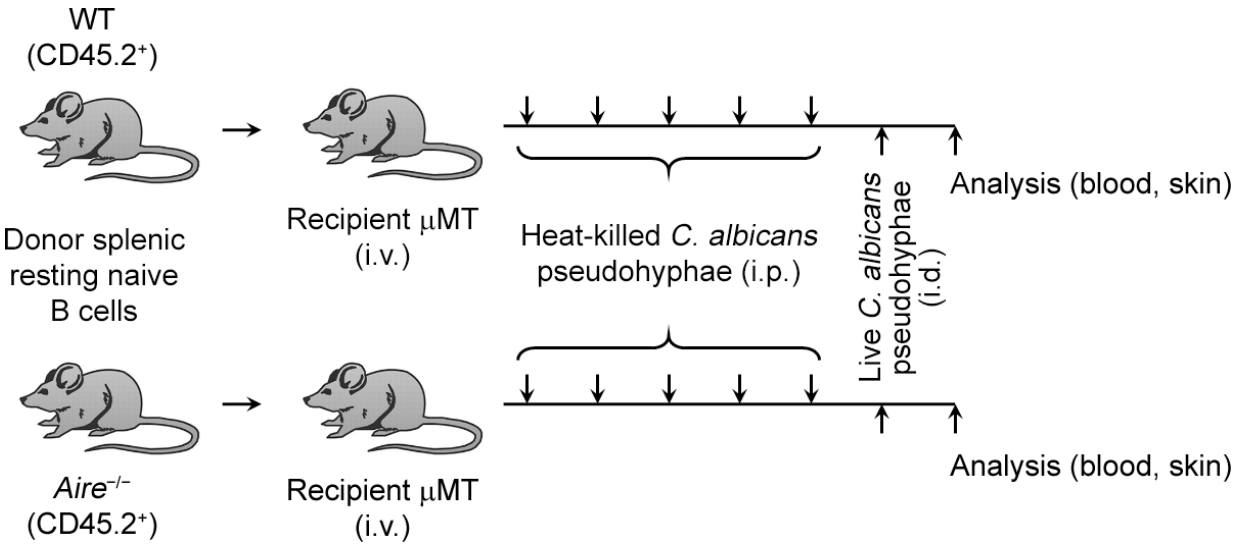

Figure 5.1: C. albicans infection model. μMT mice were adoptively transferred with either Aire WT or Aire KO B cells prior to intraperitoneal immunization with heat-killed *C. albicans*. After immunization, mice were challenged intradermally with live, pathogenic fungi.

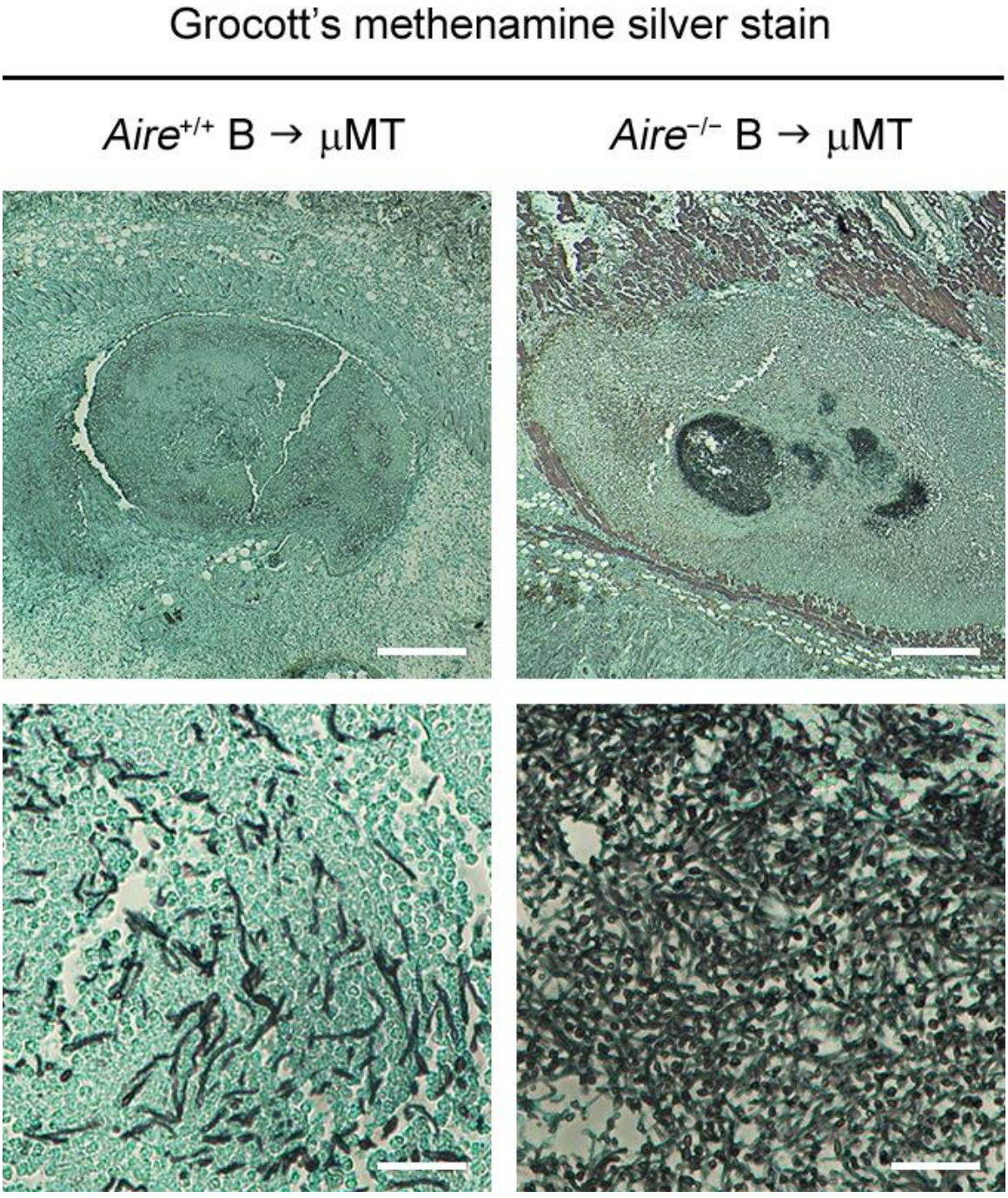

Figure 5.2: Silver staining of fungal lesion. Silver staining of fungal lesions shows increased fungal load in mice that receive Aire deficient B cells compared to mice that receive Aire WT B cells

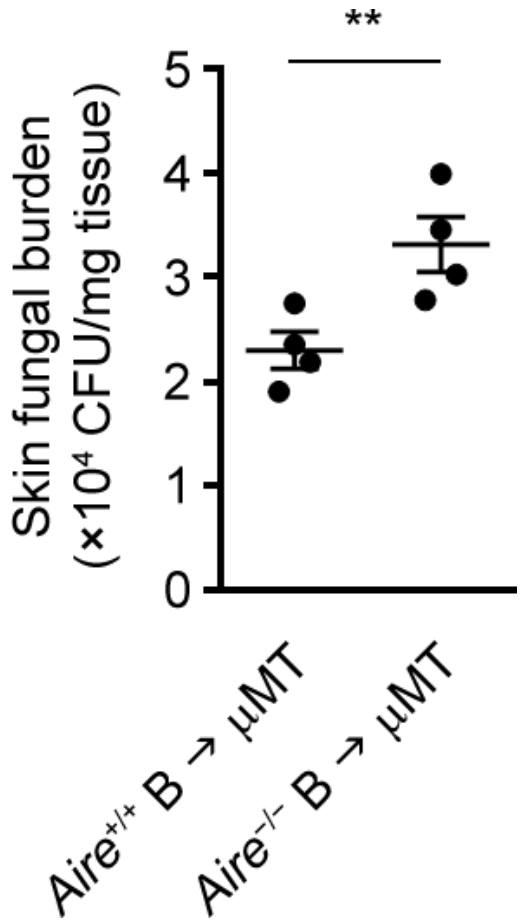

Figure 5.3: Effect of B cell Aire on fungal clearance. Skin lesions were excised and crushed using a pestle and mortar. Serial dilutions were made to determine the number of CFU/mg tissue in each lesion. Mice that receive Aire KO B cells showed an increased CFU/mg tissue compared to mice that receive WT B cells.

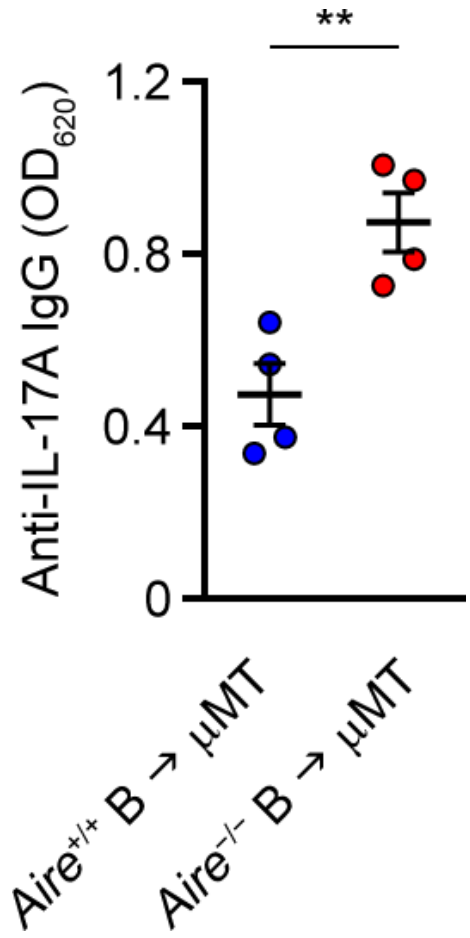

Figure 5.4: Effect of B cell Aire on autoreactive IL-17A antibodies. Mice that receive Aire KO B cells show increased serum antibodies against IL-17A.

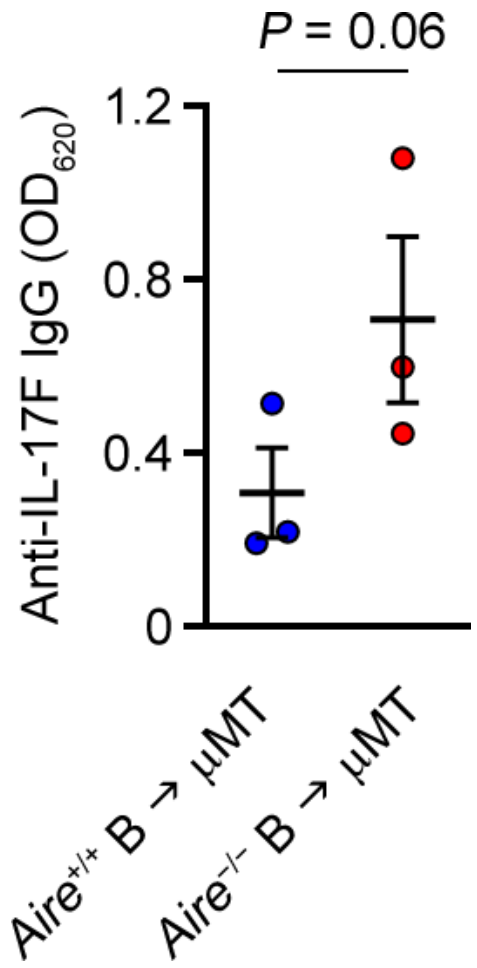

Figure 5.5: Effect of B cell Aire on autoreactive IL-17F antibodies. Mice that receive Aire KO B cells show slightly increased, but not significant serum antibodies against IL-17F.

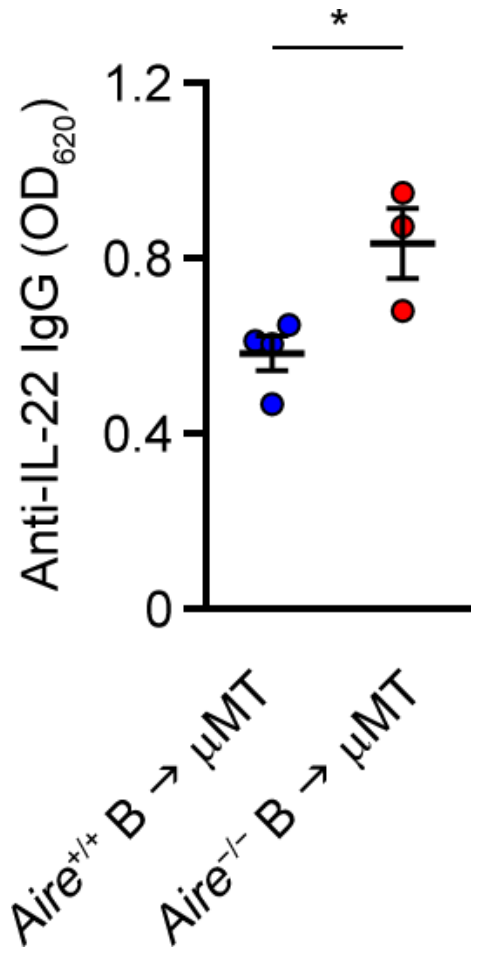

Figure 5.6: Effect of B cell Aire on autoreactive IL-22 antibodies. Mice that receive Aire KO B cells show increased serum antibodies against IL-22

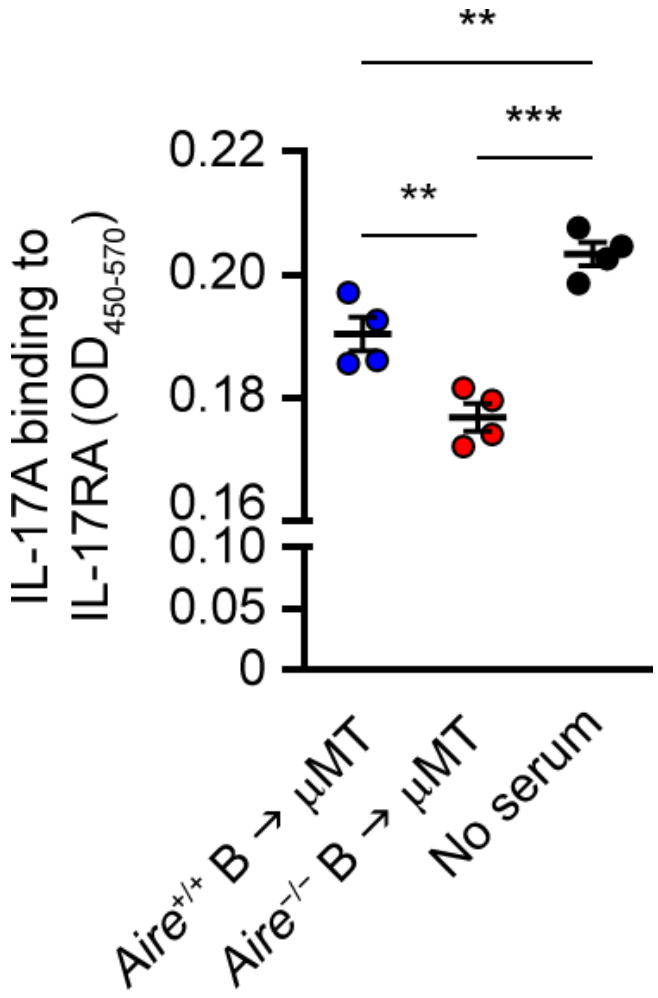

Figure 5.7: Neutralization capacity of anti-IL-17A antibodies. Serum from mice that received Aire KO B cells show increased neutralization capacity to IL-17A compared to mice that receive Aire WT B cells

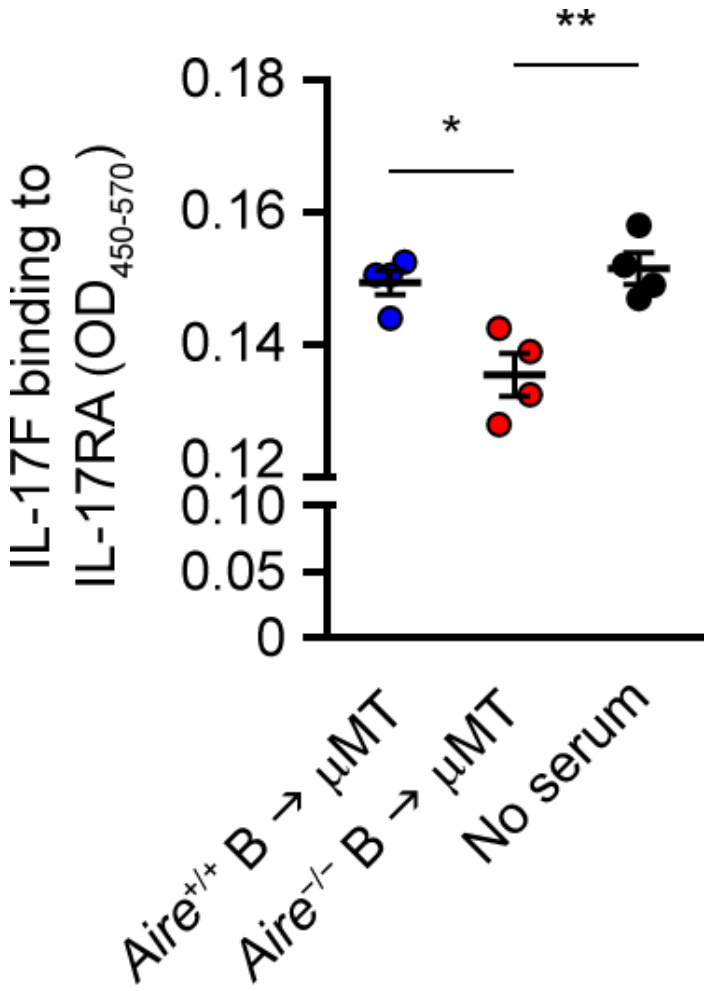

Figure 5.8: Neutralization capacity of anti-IL-17F antibodies. Serum from mice that received Aire KO B cells show increased neutralization capacity to IL-17F compared to mice that receive Aire WT B cells

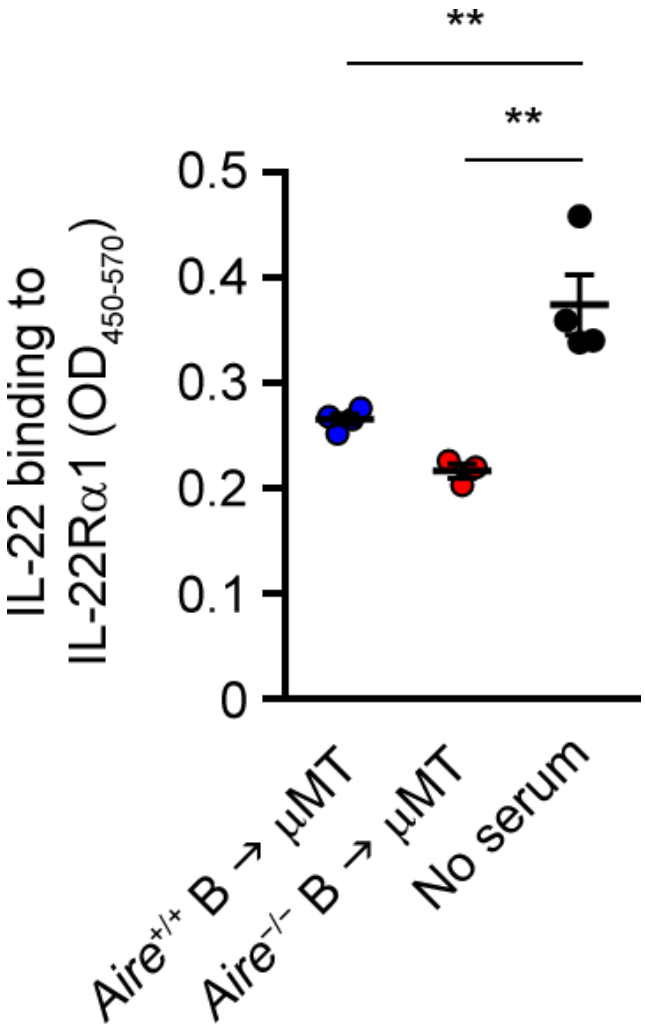

Figure 5.9: Neutralization capacity of anti-IL-22A antibodies. Serum from mice that received Aire KO B cells show increased neutralization capacity to IL-22 compared to mice that receive Aire WT B cells

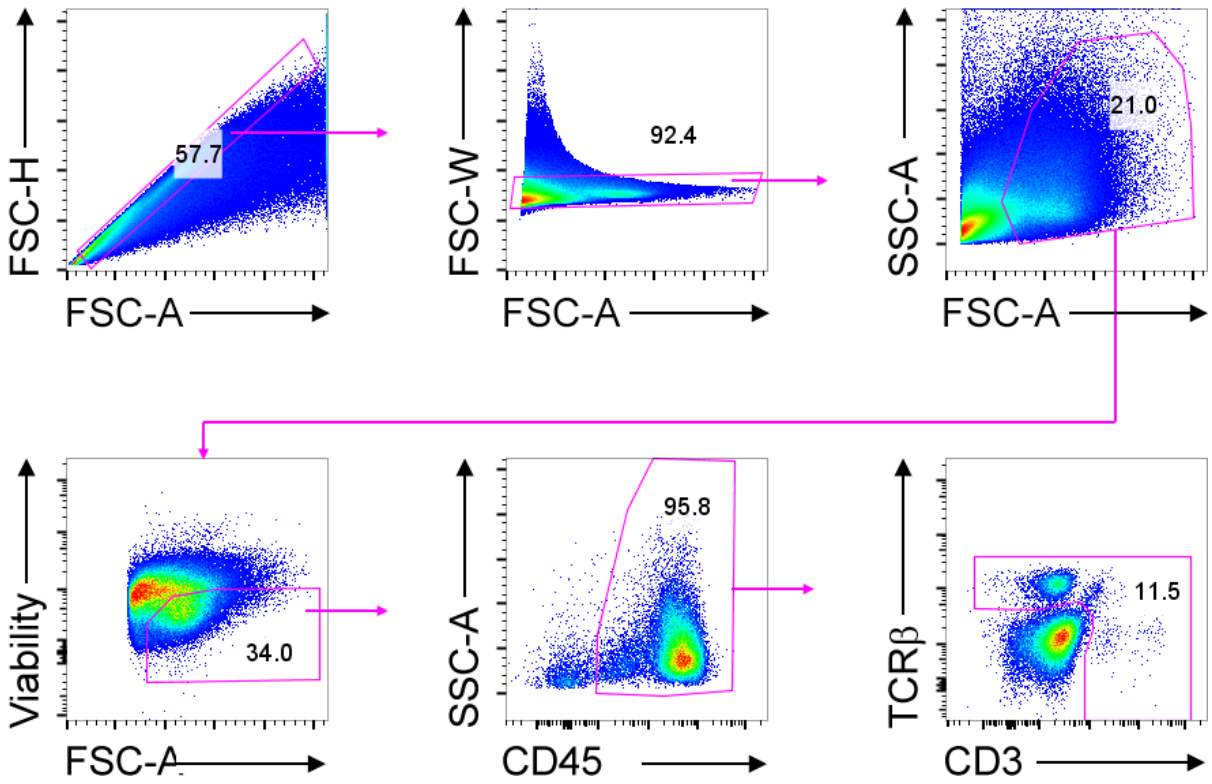

Figure 5.10: Gating strategy for Th17 cells. Single cells taken from the fungal lesion were gated as $CD45^{+}CD3^{+}TCR\beta^{+}$ prior to analysis of IL-22 and IL-17A expression.

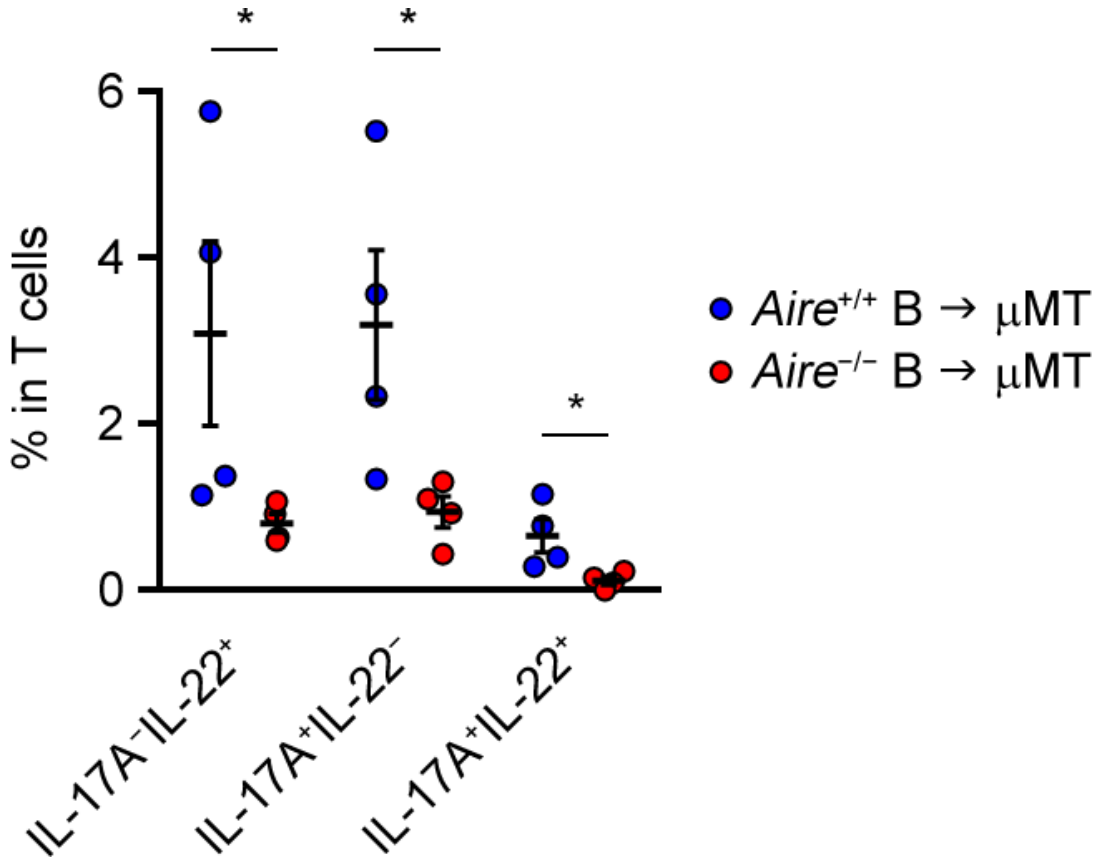

Figure 5.11: Effect of B cell Aire on Th17 populations. Analysis of IL-22 and IL-17A expression by T cells. Mice that received Aire WT B cells have significantly increased IL-17 and IL-22 producing T cells compared to mice that received Aire KO B cells

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions and significance

Here, we describe a novel mechanism for the regulation of antibody diversification in GC B Cells. We find that Aire, a protein associated with establishing and maintaining immune tolerance, is expressed in GC B cells in a CD40 dependent manner. Aire deficiency in B cells leads to increased affinity maturation and SHM as well as increased CSR through a mechanism in which a protein-protein interaction with AID, inhibits AID targeting to the substrate and subsequent deamination events initiating the diversification process. Additionally, Aire deficiency in B cells is associated with an increased serum titer of autoreactive, neutralizing antibodies against Th17 cytokines such as IL-17A, IL-17F, and IL-22 which are critical for clearing fungal pathogens, highlighted by the reduction in Th17 cells and inability of mice with B cell Aire deficiency to clear *C. albicans* as effectively as WT controls. Importantly, this mechanism does not seem to be specific for self-antigens as immunization with the foreign antigen, NP, shows increased antibody affinity, thus this mechanism can be utilized to generate high affinity antibodies for both therapeutic and commercial purposes.

6.2 Future directions

To further characterize the importance and mechanism by which Aire regulates the mechanisms of antibody diversification, it will be important for additional studies to focus on the production of high affinity antibodies to a more diverse array of antigens. Currently, it is unclear whether GC B cell Aire drives the expression of tissue restricted antigens and whether it has any effect on the T helper cell compartment. Though our data seem to suggest that there is no effect on the total number of T cells in the GC, it is possible that the repertoire is shifted. Additionally, it will be interesting to see whether Aire deficient models can be utilized for the production of

antibody vaccines for passive immunization, since the importance of efficient methods for this is in extremely high demand due to the current pandemic. Finally, future research on this mechanism may provide invaluable insight into the molecular characteristics of detrimental pathologies associated with dysregulation of the GC B cell compartment such as cancer and autoimmunity.

REFERENCES

Ackermann, J.A., Nys, J., Schweighoffer, E., McCleary, S., Smithers, N., and Tybulewicz, V.L. (2015). Syk tyrosine kinase is critical for B cell antibody responses and memory B cell survival. J Immunol *194*, 4650-4656.

Adams, J.M., Gerondakis, S., Webb, E., Corcoran, L.M., and Cory, S. (1983). Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proceedings of the National Academy of Sciences of the United States of America *80*, 1982-1986.

Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S.E. (2001). Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity *15*, 659-669.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., *et al.* (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295-306.

Aichinger, M., Wu, C., Nedjic, J., and Klein, L. (2013). Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance. The Journal of experimental medicine *210*, 287-300.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., and Gascoigne, N.R. (1996). T-cell-receptor affinity and thymocyte positive selection. Nature *381*, 616-620.

Albu, D.I., Feng, D., Bhattacharya, D., Jenkins, N.A., Copeland, N.G., Liu, P., and Avram, D. (2007). BCL11B is required for positive selection and survival of double-positive thymocytes. The Journal of experimental medicine *204*, 3003-3015.

Allen, C.D., and Cyster, J.G. (2008). Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. Semin Immunol *20*, 14-25.

Allen, C.D., Okada, T., Tang, H.L., and Cyster, J.G. (2007). Imaging of germinal center selection events during affinity maturation. Science *315*, 528-531.

Allman, D., Li, J., and Hardy, R.R. (1999). Commitment to the B lymphoid lineage occurs before DH-JH recombination. The Journal of experimental medicine *189*, 735-740.

Amin, R.H., and Schlissel, M.S. (2008). Foxo1 directly regulates the transcription of recombination-activating genes during B cell development. Nat Immunol *9*, 613-622.

Anderson, M.S., Venanzi, E.S., Chen, Z., Berzins, S.P., Benoist, C., and Mathis, D. (2005). The cellular mechanism of Aire control of T cell tolerance. Immunity *23*, 227-239.

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., *et al.* (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395-1401.

Ansel, K.M., McHeyzer-Williams, L.J., Ngo, V.N., McHeyzer-Williams, M.G., and Cyster, J.G. (1999). In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. The Journal of experimental medicine *190*, 1123-1134.

Aoufouchi, S., Faili, A., Zober, C., D'Orlando, O., Weller, S., Weill, J.C., and Reynaud, C.A. (2008). Proteasomal degradation restricts the nuclear lifespan of AID. The Journal of experimental medicine *205*, 1357-1368.

Aranburu, A., Ceccarelli, S., Giorda, E., Lasorella, R., Ballatore, G., and Carsetti, R. (2010). TLR ligation triggers somatic hypermutation in transitional B cells inducing the generation of IgM memory B cells. J Immunol *185*, 7293-7301.

Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Van Kaer, L., Pircher, H.P., Zinkernagel, R.M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T cell selection in the thymus. Cell *76*, 651-663.

Atibalentja, D.F., Byersdorfer, C.A., and Unanue, E.R. (2009). Thymus-blood protein interactions are highly effective in negative selection and regulatory T cell induction. J Immunol *183*, 7909-7918.

Azulay-Debby, H., Edry, E., and Melamed, D. (2007). CpG DNA stimulates autoreactive immature B cells in the bone marrow. Eur J Immunol *37*, 1463-1475.

Bansal, K., Yoshida, H., Benoist, C., and Mathis, D. (2017). The transcriptional regulator Aire binds to and activates super-enhancers. Nat Immunol *18*, 263-273.

Barrington, R.A., Pozdnyakova, O., Zafari, M.R., Benjamin, C.D., and Carroll, M.C. (2002). B lymphocyte memory: role of stromal cell complement and FcgammaRIIB receptors. The Journal of experimental medicine *196*, 1189-1199.

Basso, K., and Dalla-Favera, R. (2010). BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. Adv Immunol *105*, 193-210.

Basso, K., Saito, M., Sumazin, P., Margolin, A.A., Wang, K., Lim, W.K., Kitagawa, Y., Schneider, C., Alvarez, M.J., Califano, A., *et al.* (2010). Integrated biochemical and computational approach identifies BCL6 direct target genes controlling multiple pathways in normal germinal center B cells. Blood *115*, 975-984.

Basu, U., Chaudhuri, J., Alpert, C., Dutt, S., Ranganath, S., Li, G., Schrum, J.P., Manis, J.P., and Alt, F.W. (2005). The AID antibody diversification enzyme is regulated by protein kinase A phosphorylation. Nature *438*, 508-511.

Batista, F.D., Iber, D., and Neuberger, M.S. (2001). B cells acquire antigen from target cells after synapse formation. Nature *411*, 489-494.

Batten, M., Groom, J., Cachero, T.G., Qian, F., Schneider, P., Tschopp, J., Browning, J.L., and Mackay, F. (2000). BAFF mediates survival of peripheral immature B lymphocytes. The Journal of experimental medicine *192*, 1453-1466.

Baumgarth, N. (2011). The double life of a B-1 cell: self-reactivity selects for protective effector functions. Nat Rev Immunol *11*, 34-46.

Bemark, M., Hazanov, H., Stromberg, A., Komban, R., Holmqvist, J., Koster, S., Mattsson, J., Sikora, P., Mehr, R., and Lycke, N.Y. (2016). Limited clonal relatedness between gut IgA plasma cells and memory B cells after oral immunization. Nat Commun *7*, 12698.

Benson, M.J., Dillon, S.R., Castigli, E., Geha, R.S., Xu, S., Lam, K.P., and Noelle, R.J. (2008). Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. J Immunol *180*, 3655-3659.

Bentley, D.L., and Groudine, M. (1986). A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature *321*, 702-706.

Berek, C., Berger, A., and Apel, M. (1991). Maturation of the immune response in germinal centers. Cell *67*, 1121-1129.

Berek, C., and Milstein, C. (1987). Mutation drift and repertoire shift in the maturation of the immune response. Immunol Rev *96*, 23-41.

Berkowska, M.A., Driessen, G.J., Bikos, V., Grosserichter-Wagener, C., Stamatopoulos, K., Cerutti, A., He, B., Biermann, K., Lange, J.F., van der Burg, M., *et al.* (2011). Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood *118*, 2150-2158.

Bishop, A.L., Schild, S., Patimalla, B., Klein, B., and Camilli, A. (2010). Mucosal immunization with Vibrio cholerae outer membrane vesicles provides maternal protection mediated by antilipopolysaccharide antibodies that inhibit bacterial motility. Infect Immun *78*, 4402-4420.

Blizzard, R.M., and Kyle, M. (1963). Studies of the Adrenal Antigens and Antibodies in Addison's Disease. J Clin Invest *42*, 1653-1660.

Bothmer, A., Robbiani, D.F., Feldhahn, N., Gazumyan, A., Nussenzweig, A., and Nussenzweig, M.C. (2010). 53BP1 regulates DNA resection and the choice between classical and alternative end joining during class switch recombination. The Journal of experimental medicine *207*, 855-865.

Brioen, P., Dekegel, D., and Boeye, A. (1983). Neutralization of poliovirus by antibody-mediated polymerization. Virology *127*, 463-468.

Burnet, F.M. (1976). A modification of Jerne's theory of antibody production using the concept of clonal selection. CA Cancer J Clin *26*, 119-121.

Burnett, D.L., Langley, D.B., Schofield, P., Hermes, J.R., Chan, T.D., Jackson, J., Bourne, K., Reed, J.H., Patterson, K., Porebski, B.T., *et al.* (2018). Germinal center antibody mutation trajectories are determined by rapid self/foreign discrimination. Science *360*, 223-226.

Caielli, S., Veiga, D.T., Balasubramanian, P., Athale, S., Domic, B., Murat, E., Banchereau, R., Xu, Z., Chandra, M., Chung, C.H., *et al.* (2019). A CD4(+) T cell population expanded in lupus blood provides B cell help through interleukin-10 and succinate. Nat Med *25*, 75-81.

Campodonico, V.L., Llosa, N.J., Grout, M., Doring, G., Maira-Litran, T., and Pier, G.B. (2010). Evaluation of flagella and flagellin of Pseudomonas aeruginosa as vaccines. Infect Immun *78*, 746-755.

Cariappa, A., Chase, C., Liu, H., Russell, P., and Pillai, S. (2007). Naive recirculating B cells mature simultaneously in the spleen and bone marrow. Blood *109*, 2339-2345.

Cariappa, A., Mazo, I.B., Chase, C., Shi, H.N., Liu, H., Li, Q., Rose, H., Leung, H., Cherayil, B.J., Russell, P., *et al.* (2005). Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes. Immunity *23*, 397-407.

Carrasco, Y.R., and Batista, F.D. (2007). B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. Immunity *27*, 160-171.

Casellas, R., Nussenzweig, A., Wuerffel, R., Pelanda, R., Reichlin, A., Suh, H., Qin, X.F., Besmer, E., Kenter, A., Rajewsky, K., *et al.* (1998). Ku80 is required for immunoglobulin isotype switching. EMBO J *17*, 2404-2411.

Cepeda, S., Cantu, C., Orozco, S., Xiao, Y., Brown, Z., Semwal, M.K., Venables, T., Anderson, M.S., and Griffith, A.V. (2018). Age-Associated Decline in Thymic B Cell Expression of Aire and Aire-Dependent Self-Antigens. Cell Rep *22*, 1276-1287.

Cerutti, A., Cols, M., and Puga, I. (2013). Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. Nat Rev Immunol *13*, 118-132.

Cervenak, L., Magyar, A., Boja, R., and Laszlo, G. (2001). Differential expression of GL7 activation antigen on bone marrow B cell subpopulations and peripheral B cells. Immunol Lett *78*, 89-96.

Chaturvedi, A., Dorward, D., and Pierce, S.K. (2008). The B cell receptor governs the subcellular location of Toll-like receptor 9 leading to hyperresponses to DNA-containing antigens. Immunity *28*, 799-809.

Chaudhuri, J., Khuong, C., and Alt, F.W. (2004). Replication protein A interacts with AID to promote deamination of somatic hypermutation targets. Nature *430*, 992-998.

Cheng, H.L., Vuong, B.Q., Basu, U., Franklin, A., Schwer, B., Astarita, J., Phan, R.T., Datta, A., Manis, J., Alt, F.W., *et al.* (2009). Integrity of the AID serine-38 phosphorylation site is critical for class switch recombination and somatic hypermutation in mice. Proceedings of the National Academy of Sciences of the United States of America *106*, 2717-2722.

Chin, R.K., Lo, J.C., Kim, O., Blink, S.E., Christiansen, P.A., Peterson, P., Wang, Y., Ware, C., and Fu, Y.X. (2003). Lymphotoxin pathway directs thymic Aire expression. Nat Immunol *4*, 1121-1127.

Chowdhury, D., and Sen, R. (2003). Transient IL-7/IL-7R signaling provides a mechanism for feedback inhibition of immunoglobulin heavy chain gene rearrangements. Immunity *18*, 229-241.

Christensen, S.R., Shupe, J., Nickerson, K., Kashgarian, M., Flavell, R.A., and Shlomchik, M.J. (2006). Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity *25*, 417-428.

Ci, W., Polo, J.M., Cerchietti, L., Shaknovich, R., Wang, L., Yang, S.N., Ye, K., Farinha, P., Horsman, D.E., Gascoyne, R.D., *et al.* (2009). The BCL6 transcriptional program features repression of multiple oncogenes in primary B cells and is deregulated in DLBCL. Blood *113*, 5536-5548.

Clement, R.L., Daccache, J., Mohammed, M.T., Diallo, A., Blazar, B.R., Kuchroo, V.K., Lovitch, S.B., Sharpe, A.H., and Sage, P.T. (2019). Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses. Nat Immunol *20*, 1360-1371.

Clements, J.L., Yang, B., Ross-Barta, S.E., Eliason, S.L., Hrstka, R.F., Williamson, R.A., and Koretzky, G.A. (1998). Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. Science *281*, 416-419.

Clybouw, C., Fischer, S., Auffredou, M.T., Hugues, P., Alexia, C., Bouillet, P., Raphael, M., Leca, G., Strasser, A., Tarlinton, D.M., *et al.* (2011). Regulation of memory B-cell survival by the BH3-only protein Puma. Blood *118*, 4120-4128.

Clynes, R.A., Towers, T.L., Presta, L.G., and Ravetch, J.V. (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. Nat Med *6*, 443-446.

Coombs, R.R., Feinstein, A., and Wilson, A.B. (1969). Immunoglobulin determinants on the surface of human lymphocytes. Lancet *2*, 1157-1160.

Cooper, M.D., Peterson, R.D., and Good, R.A. (1965). Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken. Nature *205*, 143-146.

Corcoran, A.E., Riddell, A., Krooshoop, D., and Venkitaraman, A.R. (1998). Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. Nature *391*, 904-907.

Corthesy, B., Benureau, Y., Perrier, C., Fourgeux, C., Parez, N., Greenberg, H., and Schwartz-Cornil, I. (2006). Rotavirus anti-VP6 secretory immunoglobulin A contributes to protection via intracellular neutralization but not via immune exclusion. J Virol *80*, 10692-10699.

Cui, K., Zang, C., Roh, T.Y., Schones, D.E., Childs, R.W., Peng, W., and Zhao, K. (2009). Chromatin signatures in multipotent human hematopoietic stem cells indicate the fate of bivalent genes during differentiation. Cell Stem Cell *4*, 80-93.

Cyster, J.G., Ansel, K.M., Reif, K., Ekland, E.H., Hyman, P.L., Tang, H.L., Luther, S.A., and Ngo, V.N. (2000). Follicular stromal cells and lymphocyte homing to follicles. Immunol Rev *176*, 181-193.

Dal Porto, J.M., Haberman, A.M., Kelsoe, G., and Shlomchik, M.J. (2002). Very low affinity B cells form germinal centers, become memory B cells, and participate in secondary immune responses when higher affinity competition is reduced. The Journal of experimental medicine *195*, 1215-1221.

De Silva, N.S., and Klein, U. (2015). Dynamics of B cells in germinal centres. Nat Rev Immunol *15*, 137-148.

Decker, D.J., Boyle, N.E., Koziol, J.A., and Klinman, N.R. (1991). The expression of the Ig H chain repertoire in developing bone marrow B lineage cells. J Immunol *146*, 350-361.

Delbos, F., Aoufouchi, S., Faili, A., Weill, J.C., and Reynaud, C.A. (2007). DNA polymerase eta is the sole contributor of A/T modifications during immunoglobulin gene hypermutation in the mouse. The Journal of experimental medicine *204*, 17-23.

Delbos, F., De Smet, A., Faili, A., Aoufouchi, S., Weill, J.C., and Reynaud, C.A. (2005). Contribution of DNA polymerase eta to immunoglobulin gene hypermutation in the mouse. The Journal of experimental medicine *201*, 1191-1196.

Dent, A.L., Shaffer, A.L., Yu, X., Allman, D., and Staudt, L.M. (1997). Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science *276*, 589-592.

Desiderio, S.V., Yancopoulos, G.D., Paskind, M., Thomas, E., Boss, M.A., Landau, N., Alt, F.W., and Baltimore, D. (1984). Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature *311*, 752-755.

Di Noia, J., and Neuberger, M.S. (2002). Altering the pathway of immunoglobulin hypermutation by inhibiting uracil-DNA glycosylase. Nature *419*, 43-48.

Diamond, B., and Scharff, M.D. (1984). Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity. Proceedings of the National Academy of Sciences of the United States of America *81*, 5841-5844.

Dobes, J., Edenhofer, F., Voboril, M., Brabec, T., Dobesova, M., Cepkova, A., Klein, L., Rajewsky, K., and Filipp, D. (2018). A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire. Eur J Immunol *48*, 546-548.

Dong, J., Panchakshari, R.A., Zhang, T., Zhang, Y., Hu, J., Volpi, S.A., Meyers, R.M., Ho, Y.J., Du, Z., Robbiani, D.F., *et al.* (2015). Orientation-specific joining of AID-initiated DNA breaks promotes antibody class switching. Nature *525*, 134-139.

Dono, M., Zupo, S., Leanza, N., Melioli, G., Fogli, M., Melagrana, A., Chiorazzi, N., and Ferrarini, M. (2000). Heterogeneity of tonsillar subepithelial B lymphocytes, the splenic marginal zone equivalents. J Immunol *164*, 5596-5604.

Dorshkind, K., and Montecino-Rodriguez, E. (2007). Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential. Nat Rev Immunol *7*, 213-219.

Dunkelberger, J.R., and Song, W.C. (2010). Complement and its role in innate and adaptive immune responses. Cell Res *20*, 34-50.

Dunn-Walters, D.K., Isaacson, P.G., and Spencer, J. (1995). Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells. The Journal of experimental medicine *182*, 559-566.

Duquette, M.L., Handa, P., Vincent, J.A., Taylor, A.F., and Maizels, N. (2004). Intracellular transcription of G-rich DNAs induces formation of G-loops, novel structures containing G4 DNA. Genes Dev *18*, 1618-1629.

Duquette, M.L., Pham, P., Goodman, M.F., and Maizels, N. (2005). AID binds to transcription-induced structures in c-MYC that map to regions associated with translocation and hypermutation. Oncogene *24*, 5791-5798.

Ebert, P.J., Jiang, S., Xie, J., Li, Q.J., and Davis, M.M. (2009). An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. Nat Immunol *10*, 1162-1169.

Ehlers, M., Fukuyama, H., McGaha, T.L., Aderem, A., and Ravetch, J.V. (2006). TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. The Journal of experimental medicine *203*, 553-561.

Ehrenstein, M.R., Rada, C., Jones, A.M., Milstein, C., and Neuberger, M.S. (2001). Switch junction sequences in PMS2-deficient mice reveal a microhomology-mediated mechanism of Ig class switch recombination. Proceedings of the National Academy of Sciences of the United States of America *98*, 14553-14558.

Ehrhardt, G.R., Hsu, J.T., Gartland, L., Leu, C.M., Zhang, S., Davis, R.S., and Cooper, M.D. (2005). Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. The Journal of experimental medicine *202*, 783-791.

Eisen, H.N., and Siskind, G.W. (1964). Variations in Affinities of Antibodies during the Immune Response. Biochemistry *3*, 996-1008.

Eldershaw, S.A., Sansom, D.M., and Narendran, P. (2011). Expression and function of the autoimmune regulator (Aire) gene in non-thymic tissue. Clin Exp Immunol *163*, 296-308.

Ersching, J., Efeyan, A., Mesin, L., Jacobsen, J.T., Pasqual, G., Grabiner, B.C., Dominguez-Sola, D., Sabatini, D.M., and Victora, G.D. (2017). Germinal Center Selection and Affinity Maturation Require Dynamic Regulation of mTORC1 Kinase. Immunity *46*, 1045-1058 e1046.

Fagarasan, S., Shinkura, R., Kamata, T., Nogaki, F., Ikuta, K., Tashiro, K., and Honjo, T. (2000). Alymphoplasia (aly)-type nuclear factor kappaB-inducing kinase (NIK) causes defects in secondary lymphoid tissue chemokine receptor signaling and homing of peritoneal cells to the gut-associated lymphatic tissue system. The Journal of experimental medicine *191*, 1477-1486.

Fagraeus, A. (1948). The plasma cellular reaction and its relation to the formation of antibodies in vitro. J Immunol *58*, 1-13.

Faili, A., Stary, A., Delbos, F., Weller, S., Aoufouchi, S., Sarasin, A., Weill, J.C., and Reynaud, C.A. (2009). A backup role of DNA polymerase kappa in Ig gene hypermutation only takes place in the complete absence of DNA polymerase eta. J Immunol *182*, 6353-6359.

Fecteau, J.F., Cote, G., and Neron, S. (2006). A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. J Immunol *177*, 3728-3736.

Ferguson, B.J., Alexander, C., Rossi, S.W., Liiv, I., Rebane, A., Worth, C.L., Wong, J., Laan, M., Peterson, P., Jenkinson, E.J., *et al.* (2008). AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. J Biol Chem *283*, 1723-1731.

Fergusson, J.R., Morgan, M.D., Bruchard, M., Huitema, L., Heesters, B.A., van Unen, V., van Hamburg, J.P., van der Wel, N.N., Picavet, D., Koning, F., *et al.* (2018). Maturing Human CD127+ CCR7+ PDL1+ Dendritic Cells Express AIRE in the Absence of Tissue Restricted Antigens. Front Immunol *9*, 2902.

Ferrari, K.J., Scelfo, A., Jammula, S., Cuomo, A., Barozzi, I., Stutzer, A., Fischle, W., Bonaldi, T., and Pasini, D. (2014). Polycomb-dependent H3K27me1 and H3K27me2 regulate active transcription and enhancer fidelity. Mol Cell *53*, 49-62.

Finkin, S., Hartweger, H., Oliveira, T.Y., Kara, E.E., and Nussenzweig, M.C. (2019). Protein Amounts of the MYC Transcription Factor Determine Germinal Center B Cell Division Capacity. Immunity *51*, 324-336 e325.

Finnish-German, A.C. (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet *17*, 399-403.

Flannagan, R.S., Jaumouille, V., and Grinstein, S. (2012). The cell biology of phagocytosis. Annu Rev Pathol *7*, 61-98.

Fooksman, D.R., Schwickert, T.A., Victora, G.D., Dustin, M.L., Nussenzweig, M.C., and Skokos, D. (2010). Development and migration of plasma cells in the mouse lymph node. Immunity *33*, 118-127.

Froland, S., Natvig, J.B., and Berdal, P. (1971). Surface-bound immunoglobulin as a marker of B lymphocytes in man. Nat New Biol *234*, 251-252.

Fujikado, N., Mann, A.O., Bansal, K., Romito, K.R., Ferre, E.M.N., Rosenzweig, S.D., Lionakis, M.S., Benoist, C., and Mathis, D. (2016). Aire Inhibits the Generation of a Perinatal Population of Interleukin-17A-Producing gammadelta T Cells to Promote Immunologic Tolerance. Immunity *45*, 999-1012.

Fujinaga, K., Irwin, D., Huang, Y., Taube, R., Kurosu, T., and Peterlin, B.M. (2004). Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element. Mol Cell Biol *24*, 787-795.

Gardner, J.M., Devoss, J.J., Friedman, R.S., Wong, D.J., Tan, Y.X., Zhou, X., Johannes, K.P., Su, M.A., Chang, H.Y., Krummel, M.F., *et al.* (2008). Deletional tolerance mediated by extrathymic Aire-expressing cells. Science *321*, 843-847.

Gardner, J.M., Metzger, T.C., McMahon, E.J., Au-Yeung, B.B., Krawisz, A.K., Lu, W., Price, J.D., Johannes, K.P., Satpathy, A.T., Murphy, K.M., *et al.* (2013). Extrathymic Aire-expressing cells are a distinct bone marrow-derived population that induce functional inactivation of CD4(+) T cells. Immunity *39*, 560-572.

Gatto, D., Paus, D., Basten, A., Mackay, C.R., and Brink, R. (2009). Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses. Immunity *31*, 259-269.

Gatto, D., Wood, K., and Brink, R. (2011). EBI2 operates independently of but in cooperation with CXCR5 and CCR7 to direct B cell migration and organization in follicles and the germinal center. J Immunol *187*, 4621-4628.

Gay, D., Saunders, T., Camper, S., and Weigert, M. (1993). Receptor editing: an approach by autoreactive B cells to escape tolerance. The Journal of experimental medicine *177*, 999-1008.

Gazumyan, A., Timachova, K., Yuen, G., Siden, E., Di Virgilio, M., Woo, E.M., Chait, B.T., Reina San-Martin, B., Nussenzweig, M.C., and McBride, K.M. (2011). Amino-terminal phosphorylation of activation-induced cytidine deaminase suppresses c-myc/IgH translocation. Mol Cell Biol *31*, 442-449.

Giltiay, N.V., Chappell, C.P., Sun, X., Kolhatkar, N., Teal, T.H., Wiedeman, A.E., Kim, J., Tanaka, L., Buechler, M.B., Hamerman, J.A., *et al.* (2013). Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells. The Journal of experimental medicine *210*, 2773-2789.

Giraud, M., Jmari, N., Du, L., Carallis, F., Nieland, T.J., Perez-Campo, F.M., Bensaude, O., Root, D.E., Hacohen, N., Mathis, D., *et al.* (2014). An RNAi screen for Aire cofactors reveals a role for Hnrnpl in polymerase release and Aire-activated ectopic transcription. Proceedings of the National Academy of Sciences of the United States of America *111*, 1491-1496.

Goodnow, C.C., Crosbie, J., Adelstein, S., Lavoie, T.B., Smith-Gill, S.J., Brink, R.A., Pritchard-Briscoe, H., Wotherspoon, J.S., Loblay, R.H., Raphael, K., *et al.* (1988). Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature *334*, 676-682.

Gossens, K., Naus, S., Corbel, S.Y., Lin, S., Rossi, F.M., Kast, J., and Ziltener, H.J. (2009). Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. The Journal of experimental medicine *206*, 761-778.

Grawunder, U., Leu, T.M., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F., and Winkler, T.H. (1995). Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. Immunity *3*, 601-608.

Gray, D., and Skarvall, H. (1988). B-cell memory is short-lived in the absence of antigen. Nature *336*, 70-73.

Green, J.A., Suzuki, K., Cho, B., Willison, L.D., Palmer, D., Allen, C.D., Schmidt, T.H., Xu, Y., Proia, R.L., Coughlin, S.R., *et al.* (2011). The sphingosine 1-phosphate receptor S1P(2) maintains the homeostasis of germinal center B cells and promotes niche confinement. Nat Immunol *12*, 672-680.

Griffin, F.M., Jr., Griffin, J.A., Leider, J.E., and Silverstein, S.C. (1975). Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to

specific receptors on the macrophage plasma membrane. The Journal of experimental medicine *142*, 1263-1282.

Griffin, F.M., Jr., Griffin, J.A., and Silverstein, S.C. (1976). Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-coated bone marrow-derived lymphocytes. The Journal of experimental medicine *144*, 788-809.

Groom, J.R., Fletcher, C.A., Walters, S.N., Grey, S.T., Watt, S.V., Sweet, M.J., Smyth, M.J., Mackay, C.R., and Mackay, F. (2007). BAFF and MyD88 signals promote a lupuslike disease independent of T cells. The Journal of experimental medicine *204*, 1959-1971.

Gu, H., Forster, I., and Rajewsky, K. (1990). Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. EMBO J *9*, 2133-2140.

Guerau-de-Arellano, M., Martinic, M., Benoist, C., and Mathis, D. (2009). Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. The Journal of experimental medicine *206*, 1245-1252.

Guinamard, R., Okigaki, M., Schlessinger, J., and Ravetch, J.V. (2000). Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. Nat Immunol *1*, 31-36.

Guo, C., Sonoda, E., Tang, T.S., Parker, J.L., Bielen, A.B., Takeda, S., Ulrich, H.D., and Friedberg, E.C. (2006). REV1 protein interacts with PCNA: significance of the REV1 BRCT domain in vitro and in vivo. Mol Cell *23*, 265-271.

Haas, K.M., Poe, J.C., Steeber, D.A., and Tedder, T.F. (2005). B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. Immunity *23*, 7-18.

Haberman, A.M., and Shlomchik, M.J. (2003). Reassessing the function of immune-complex retention by follicular dendritic cells. Nat Rev Immunol *3*, 757-764.

Haljasorg, U., Bichele, R., Saare, M., Guha, M., Maslovskaja, J., Kond, K., Remm, A., Pihlap, M., Tomson, L., Kisand, K., *et al.* (2015). A highly conserved NF-kappaB-responsive enhancer is critical for thymic expression of Aire in mice. Eur J Immunol *45*, 3246-3256.

Halonen, M., Kangas, H., Ruppell, T., Ilmarinen, T., Ollila, J., Kolmer, M., Vihinen, M., Palvimo, J., Saarela, J., Ulmanen, I., *et al.* (2004). APECED-causing mutations in AIRE reveal the functional domains of the protein. Hum Mutat *23*, 245-257.

Han, S., Hathcock, K., Zheng, B., Kepler, T.B., Hodes, R., and Kelsoe, G. (1995). Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. J Immunol *155*, 556-567.

Hanna, M.G., Jr. (1964). An Autoradiographic Study of the Germinal Center in Spleen White Pulp during Early Intervals of the Immune Response. Lab Invest *13*, 95-104.

Hao, S., and Baltimore, D. (2013). RNA splicing regulates the temporal order of TNF-induced gene expression. Proceedings of the National Academy of Sciences of the United States of America *110*, 11934-11939.

Hardy, R.R., and Hayakawa, K. (1991). A developmental switch in B lymphopoiesis. Proceedings of the National Academy of Sciences of the United States of America *88*, 11550-11554.

Hasler, J., Rada, C., and Neuberger, M.S. (2011). Cytoplasmic activation-induced cytidine deaminase (AID) exists in stoichiometric complex with translation elongation factor 1alpha (eEF1A). Proceedings of the National Academy of Sciences of the United States of America *108*, 18366-18371.

Hauser, A.E., Junt, T., Mempel, T.R., Sneddon, M.W., Kleinstein, S.H., Henrickson, S.E., von Andrian, U.H., Shlomchik, M.J., and Haberman, A.M. (2007). Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. Immunity *26*, 655-667.

Hayakawa, K., Hardy, R.R., Parks, D.R., and Herzenberg, L.A. (1983). The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. The Journal of experimental medicine *157*, 202-218.

Haynes, N.M., Allen, C.D., Lesley, R., Ansel, K.M., Killeen, N., and Cyster, J.G. (2007). Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. J Immunol *179*, 5099-5108.

He, B., Qiao, X., and Cerutti, A. (2004). CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. J Immunol *173*, 4479-4491.

Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733-736.

Heino, M., Peterson, P., Sillanpaa, N., Guerin, S., Wu, L., Anderson, G., Scott, H.S., Antonarakis, S.E., Kudoh, J., Shimizu, N., *et al.* (2000). RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD mouse. Eur J Immunol *30*, 1884-1893.

Hepworth, M.R., Fung, T.C., Masur, S.H., Kelsen, J.R., McConnell, F.M., Dubrot, J., Withers, D.R., Hugues, S., Farrar, M.A., Reith, W., *et al.* (2015). Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells. Science *348*, 1031-1035.

Hepworth, M.R., Monticelli, L.A., Fung, T.C., Ziegler, C.G., Grunberg, S., Sinha, R., Mantegazza, A.R., Ma, H.L., Crawford, A., Angelosanto, J.M., *et al.* (2013). Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature *498*, 113-117.

Herzig, Y., Nevo, S., Bornstein, C., Brezis, M.R., Ben-Hur, S., Shkedy, A., Eisenberg-Bord, M., Levi, B., Delacher, M., Goldfarb, Y., *et al.* (2017). Transcriptional programs that control expression of the autoimmune regulator gene Aire. Nat Immunol *18*, 161-172.

Herzog, S., Hug, E., Meixlsperger, S., Paik, J.H., DePinho, R.A., Reth, M., and Jumaa, H. (2008). SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway. Nat Immunol *9*, 623-631.

Hnisz, D., Abraham, B.J., Lee, T.I., Lau, A., Saint-Andre, V., Sigova, A.A., Hoke, H.A., and Young, R.A. (2013). Super-enhancers in the control of cell identity and disease. Cell *155*, 934-947.

Hogquist, K.A., Tomlinson, A.J., Kieper, W.C., McGargill, M.A., Hart, M.C., Naylor, S., and Jameson, S.C. (1997). Identification of a naturally occurring ligand for thymic positive selection. Immunity *6*, 389-399.

Horkko, S., Miller, E., Branch, D.W., Palinski, W., and Witztum, J.L. (1997). The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and beta2 glycoprotein 1 (and other proteins). Proceedings of the National Academy of Sciences of the United States of America *94*, 10356-10361.

Hozumi, N., and Tonegawa, S. (1976). Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proceedings of the National Academy of Sciences of the United States of America *73*, 3628-3632.

Hua, Z., Gross, A.J., Lamagna, C., Ramos-Hernandez, N., Scapini, P., Ji, M., Shao, H., Lowell, C.A., Hou, B., and DeFranco, A.L. (2014). Requirement for MyD88 signaling in B cells and dendritic cells for germinal center anti-nuclear antibody production in Lyn-deficient mice. J Immunol *192*, 875-885.

Huang, B., Faucette, A.N., Pawlitz, M.D., Pei, B., Goyert, J.W., Zhou, J.Z., El-Hage, N.G., Deng, J., Lin, J., Yao, F., *et al.* (2017). Interleukin-33-induced expression of PIBF1 by decidual B cells protects against preterm labor. Nat Med *23*, 128-135.

Hubert, F.X., Kinkel, S.A., Webster, K.E., Cannon, P., Crewther, P.E., Proeitto, A.I., Wu, L., Heath, W.R., and Scott, H.S. (2008). A specific anti-Aire antibody reveals aire expression is restricted to medullary thymic epithelial cells and not expressed in periphery. J Immunol *180*, 3824-3832.

Hughey, P.G., Roberts, P.C., Holsinger, L.J., Zebedee, S.L., Lamb, R.A., and Compans, R.W. (1995). Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. Virology *212*, 411-421.

Husebye, E.S., Perheentupa, J., Rautemaa, R., and Kampe, O. (2009). Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. J Intern Med *265*, 514-529.

Hutloff, A., Dittrich, A.M., Beier, K.C., Eljaschewitsch, B., Kraft, R., Anagnostopoulos, I., and Kroczek, R.A. (1999). ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature *397*, 263-266.

Ikawa, T., Hirose, S., Masuda, K., Kakugawa, K., Satoh, R., Shibano-Satoh, A., Kominami, R., Katsura, Y., and Kawamoto, H. (2010). An essential developmental checkpoint for production of the T cell lineage. Science *329*, 93-96.

Ikuta, K., and Weissman, I.L. (1992). Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proceedings of the National Academy of Sciences of the United States of America *89*, 1502-1506.

Ilmarinen, T., Kangas, H., Kytomaa, T., Eskelin, P., Saharinen, J., Seeler, J.S., Tanhuanpaa, K., Chan, F.Y., Slattery, R.M., Alakurtti, K., *et al.* (2008). Functional interaction of AIRE with PIAS1 in transcriptional regulation. Mol Immunol *45*, 1847-1862.

Imai, K., Slupphaug, G., Lee, W.I., Revy, P., Nonoyama, S., Catalan, N., Yel, L., Forveille, M., Kavli, B., Krokan, H.E., *et al.* (2003). Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. Nat Immunol *4*, 1023-1028.

Imai, Y., Kuba, K., Neely, G.G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., Ermolaeva, M., Veldhuizen, R., Leung, Y.H., Wang, H., *et al.* (2008). Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. Cell *133*, 235-249.

Incani, F., Serra, M., Meloni, A., Cossu, C., Saba, L., Cabras, T., Messana, I., and Rosatelli, M.C. (2014). AIRE acetylation and deacetylation: effect on protein stability and transactivation activity. J Biomed Sci *21*, 85.

Inlay, M.A., Bhattacharya, D., Sahoo, D., Serwold, T., Seita, J., Karsunky, H., Plevritis, S.K., Dill, D.L., and Weissman, I.L. (2009). Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. Genes Dev *23*, 2376-2381.

Ise, W., Fujii, K., Shiroguchi, K., Ito, A., Kometani, K., Takeda, K., Kawakami, E., Yamashita, K., Suzuki, K., Okada, T., *et al.* (2018). T Follicular Helper Cell-Germinal Center B Cell Interaction Strength Regulates Entry into Plasma Cell or Recycling Germinal Center Cell Fate. Immunity *48*, 702-715 e704.

Ito, S., Nagaoka, H., Shinkura, R., Begum, N., Muramatsu, M., Nakata, M., and Honjo, T. (2004). Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. Proceedings of the National Academy of Sciences of the United States of America *101*, 1975-1980.

Jacob, J., Kelsoe, G., Rajewsky, K., and Weiss, U. (1991). Intraclonal generation of antibody mutants in germinal centres. Nature *354*, 389-392.

Jacobson, E.B., Caporale, L.H., and Thorbecke, G.J. (1974). Effect of thymus cell injections on germinal center formation in lymphoid tissues of nude (thymusless) mice. Cell Immunol *13*, 416-430.

Jansen, J.G., Langerak, P., Tsaalbi-Shtylik, A., van den Berk, P., Jacobs, H., and de Wind, N. (2006). Strand-biased defect in C/G transversions in hypermutating immunoglobulin genes in Rev1-deficient mice. The Journal of experimental medicine *203*, 319-323.

Jasti, S., Warren, B.D., McGinnis, L.K., Kinsey, W.H., Petroff, B.K., and Petroff, M.G. (2012). The autoimmune regulator prevents premature reproductive senescence in female mice. Biol Reprod *86*, 110.

Jerne, N.K. (1951). A study of avidity based on rabbit skin responses to diphtheria toxin-antitoxin mixtures. Acta Pathol Microbiol Scand Suppl *87*, 1-183.

Jerne, N.K. (1955). The Natural-Selection Theory of Antibody Formation. Proceedings of the National Academy of Sciences of the United States of America *41*, 849-857.

Johnnidis, J.B., Venanzi, E.S., Taxman, D.J., Ting, J.P., Benoist, C.O., and Mathis, D.J. (2005). Chromosomal clustering of genes controlled by the aire transcription factor. Proceedings of the National Academy of Sciences of the United States of America *102*, 7233-7238.

Ju, B., Zhang, Q., Ge, J., Wang, R., Sun, J., Ge, X., Yu, J., Shan, S., Zhou, B., Song, S., *et al.* (2020). Human neutralizing antibodies elicited by SARS-CoV-2 infection. Nature *584*, 115-119.

Jumaa, H., Wollscheid, B., Mitterer, M., Wienands, J., Reth, M., and Nielsen, P.J. (1999). Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. Immunity *11*, 547-554.

Kannouche, P.L., Wing, J., and Lehmann, A.R. (2004). Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. Mol Cell *14*, 491-500.

Kaufmann, S.H. (2017). Remembering Emil von Behring: from Tetanus Treatment to Antibody Cooperation with Phagocytes. mBio *8*.

Kelly, L.M., Pereira, J.P., Yi, T., Xu, Y., and Cyster, J.G. (2011). EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and nonlymphoid tissues. J Immunol *187*, 3026-3032.

Kennedy, D.E., Okoreeh, M.K., Maienschein-Cline, M., Ai, J., Veselits, M., McLean, K.C., Dhungana, Y., Wang, H., Peng, J., Chi, H., *et al.* (2020). Novel specialized cell state and spatial compartments within the germinal center. Nat Immunol *21*, 660-670.

Kepler, T.B., and Perelson, A.S. (1993). Cyclic re-entry of germinal center B cells and the efficiency of affinity maturation. Immunol Today *14*, 412-415.

Khalil, A.M., Cambier, J.C., and Shlomchik, M.J. (2012). B cell receptor signal transduction in the GC is short-circuited by high phosphatase activity. Science *336*, 1178-1181.

Kim, H.A., Seo, G.Y., and Kim, P.H. (2011). Macrophage-derived BAFF induces AID expression through the p38MAPK/CREB and JNK/AP-1 pathways. J Leukoc Biol *89*, 393-398.

Kisand, K., Boe Wolff, A.S., Podkrajsek, K.T., Tserel, L., Link, M., Kisand, K.V., Ersvaer, E., Perheentupa, J., Erichsen, M.M., Bratanic, N., *et al.* (2010). Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. The Journal of experimental medicine *207*, 299-308.

Kishimoto, H., and Sprent, J. (1997). Negative selection in the thymus includes semimature T cells. The Journal of experimental medicine *185*, 263-271.

Kittipatarin, C., Li, W.Q., Bulavin, D.V., Durum, S.K., and Khaled, A.R. (2006). Cell cycling through Cdc25A: transducer of cytokine proliferative signals. Cell Cycle *5*, 907-912.

Klasener, K., Maity, P.C., Hobeika, E., Yang, J., and Reth, M. (2014). B cell activation involves nanoscale receptor reorganizations and inside-out signaling by Syk. Elife *3*, e02069.

Klein, I.A., Resch, W., Jankovic, M., Oliveira, T., Yamane, A., Nakahashi, H., Di Virgilio, M., Bothmer, A., Nussenzweig, A., Robbiani, D.F., *et al.* (2011). Translocation-capture sequencing reveals the extent and nature of chromosomal rearrangements in B lymphocytes. Cell *147*, 95-106.

Klein, L., Hinterberger, M., Wirnsberger, G., and Kyewski, B. (2009). Antigen presentation in the thymus for positive selection and central tolerance induction. Nat Rev Immunol *9*, 833-844.

Klein, L., Roettinger, B., and Kyewski, B. (2001). Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. Eur J Immunol *31*, 2476-2486.

Klein, U., Kuppers, R., and Rajewsky, K. (1997). Evidence for a large compartment of IgM-expressing memory B cells in humans. Blood *89*, 1288-1298.

Klein, U., Rajewsky, K., and Kuppers, R. (1998). Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region

genes: CD27 as a general marker for somatically mutated (memory) B cells. The Journal of experimental medicine *188*, 1679-1689.

Klose, R.J., Kallin, E.M., and Zhang, Y. (2006). JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet *7*, 715-727.

Kogawa, K., Nagafuchi, S., Katsuta, H., Kudoh, J., Tamiya, S., Sakai, Y., Shimizu, N., and Harada, M. (2002). Expression of AIRE gene in peripheral monocyte/dendritic cell lineage. Immunol Lett *80*, 195-198.

Koh, A.S., Miller, E.L., Buenrostro, J.D., Moskowitz, D.M., Wang, J., Greenleaf, W.J., Chang, H.Y., and Crabtree, G.R. (2018). Rapid chromatin repression by Aire provides precise control of immune tolerance. Nat Immunol *19*, 162-172.

Koike, T., Harada, K., Horiuchi, S., and Kitamura, D. (2019). The quantity of CD40 signaling determines the differentiation of B cells into functionally distinct memory cell subsets. Elife *8*.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Kopf, M., Herren, S., Wiles, M.V., Pepys, M.B., and Kosco-Vilbois, M.H. (1998). Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. The Journal of experimental medicine *188*, 1895-1906.

Koralov, S.B., Novobrantseva, T.I., Konigsmann, J., Ehlich, A., and Rajewsky, K. (2006). Antibody repertoires generated by VH replacement and direct VH to JH joining. Immunity *25*, 43-53.

Koretzky, G.A., Abtahian, F., and Silverman, M.A. (2006). SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. Nat Rev Immunol *6*, 67-78.

Kraus, M., Alimzhanov, M.B., Rajewsky, N., and Rajewsky, K. (2004). Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer. Cell *117*, 787-800.

Krautler, N.J., Suan, D., Butt, D., Bourne, K., Hermes, J.R., Chan, T.D., Sundling, C., Kaplan, W., Schofield, P., Jackson, J., *et al.* (2017). Differentiation of germinal center B cells into plasma cells is initiated by high-affinity antigen and completed by Tfh cells. The Journal of experimental medicine *214*, 1259-1267.

Krokan, H.E., and Bjoras, M. (2013). Base excision repair. Cold Spring Harb Perspect Biol *5*, a012583.

Kuroda, N., Mitani, T., Takeda, N., Ishimaru, N., Arakaki, R., Hayashi, Y., Bando, Y., Izumi, K., Takahashi, T., Nomura, T., *et al.* (2005). Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. J Immunol *174*, 1862-1870.

Kyewski, B., and Klein, L. (2006). A central role for central tolerance. Annu Rev Immunol *24*, 571-606.

Laakso, S.M., Laurinolli, T.T., Rossi, L.H., Lehtoviita, A., Sairanen, H., Perheentupa, J., Kekalainen, E., and Arstila, T.P. (2010). Regulatory T cell defect in APECED patients is associated with loss of naive FOXP3(+) precursors and impaired activated population. J Autoimmun *35*, 351-357.

Laan, M., Kisand, K., Kont, V., Moll, K., Tserel, L., Scott, H.S., and Peterson, P. (2009). Autoimmune regulator deficiency results in decreased expression of CCR4 and CCR7 ligands and in delayed migration of CD4+ thymocytes. J Immunol *183*, 7682-7691.

Lam, K.P., Kuhn, R., and Rajewsky, K. (1997). In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell *90*, 1073-1083.

Lam, K.P., and Rajewsky, K. (1999). B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. The Journal of experimental medicine *190*, 471-477.

Langerak, P., Nygren, A.O., Krijger, P.H., van den Berk, P.C., and Jacobs, H. (2007). A/T mutagenesis in hypermutated immunoglobulin genes strongly depends on PCNAK164 modification. The Journal of experimental medicine *204*, 1989-1998.

LaRocca, T.J., Holthausen, D.J., Hsieh, C., Renken, C., Mannella, C.A., and Benach, J.L. (2009). The bactericidal effect of a complement-independent antibody is osmolytic and specific to Borrelia. Proceedings of the National Academy of Sciences of the United States of America *106*, 10752-10757.

Lau, C.M., Broughton, C., Tabor, A.S., Akira, S., Flavell, R.A., Mamula, M.J., Christensen, S.R., Shlomchik, M.J., Viglianti, G.A., Rifkin, I.R., *et al.* (2005). RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. The Journal of experimental medicine *202*, 1171-1177.

Lee, J.W., Epardaud, M., Sun, J., Becker, J.E., Cheng, A.C., Yonekura, A.R., Heath, J.K., and Turley, S.J. (2007). Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. Nat Immunol *8*, 181-190.

Lei, Y., Ripen, A.M., Ishimaru, N., Ohigashi, I., Nagasawa, T., Jeker, L.T., Bosl, M.R., Hollander, G.A., Hayashi, Y., Malefyt Rde, W., *et al.* (2011). Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. The Journal of experimental medicine *208*, 383-394.

Li, W.Q., Jiang, Q., Khaled, A.R., Keller, J.R., and Durum, S.K. (2004). Interleukin-7 inactivates the pro-apoptotic protein Bad promoting T cell survival. J Biol Chem *279*, 29160-29166.

Li, X., Gadzinsky, A., Gong, L., Tong, H., Calderon, V., Li, Y., Kitamura, D., Klein, U., Langdon, W.Y., Hou, F., *et al.* (2018). Cbl Ubiquitin Ligases Control B Cell Exit from the Germinal-Center Reaction. Immunity *48*, 530-541 e536.

Li, Y.S., Wasserman, R., Hayakawa, K., and Hardy, R.R. (1996). Identification of the earliest B lineage stage in mouse bone marrow. Immunity *5*, 527-535.

Lieber, M.R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem *79*, 181-211.

Liiv, I., Rebane, A., Org, T., Saare, M., Maslovskaja, J., Kisand, K., Juronen, E., Valmu, L., Bottomley, M.J., Kalkkinen, N., *et al.* (2008). DNA-PK contributes to the phosphorylation of AIRE: importance in transcriptional activity. Biochim Biophys Acta *1783*, 74-83.

Lin, W.C., and Desiderio, S. (1994). Cell cycle regulation of V(D)J recombination-activating protein RAG-2. Proceedings of the National Academy of Sciences of the United States of America *91*, 2733-2737.

Lindstrom, D.L., Squazzo, S.L., Muster, N., Burckin, T.A., Wachter, K.C., Emigh, C.A., McCleery, J.A., Yates, J.R., 3rd, and Hartzog, G.A. (2003). Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol Cell Biol *23*, 1368-1378.

Liston, A., Lesage, S., Wilson, J., Peltonen, L., and Goodnow, C.C. (2003). Aire regulates negative selection of organ-specific T cells. Nat Immunol *4*, 350-354.

Liu, L., Wang, P., Nair, M.S., Yu, J., Rapp, M., Wang, Q., Luo, Y., Chan, J.F., Sahi, V., Figueroa, A., *et al.* (2020). Potent Neutralizing Monoclonal Antibodies Directed to Multiple Epitopes on the SARS-CoV-2 Spike. bioRxiv.

Liu, M., Duke, J.L., Richter, D.J., Vinuesa, C.G., Goodnow, C.C., Kleinstein, S.H., and Schatz, D.G. (2008). Two levels of protection for the B cell genome during somatic hypermutation. Nature *451*, 841-845.

Liu, X., Kraus, W.L., and Bai, X. (2015). Ready, pause, go: regulation of RNA polymerase II pausing and release by cellular signaling pathways. Trends Biochem Sci *40*, 516-525.

Liu, Y.J., Joshua, D.E., Williams, G.T., Smith, C.A., Gordon, J., and MacLennan, I.C. (1989). Mechanism of antigen-driven selection in germinal centres. Nature *342*, 929-931.

Lo, W.L., Felix, N.J., Walters, J.J., Rohrs, H., Gross, M.L., and Allen, P.M. (2009). An endogenous peptide positively selects and augments the activation and survival of peripheral CD4+ T cells. Nat Immunol *10*, 1155-1161.

Lu, L., Chaudhury, P., and Osmond, D.G. (1999). Regulation of cell survival during B lymphopoiesis: apoptosis and Bcl-2/Bax content of precursor B cells in bone marrow of mice with altered expression of IL-7 and recombinase-activating gene-2. J Immunol *162*, 1931-1940.

Luo, W., Weisel, F., and Shlomchik, M.J. (2018). B Cell Receptor and CD40 Signaling Are Rewired for Synergistic Induction of the c-Myc Transcription Factor in Germinal Center B Cells. Immunity *48*, 313-326 e315.

Macchi, P., Villa, A., Giliani, S., Sacco, M.G., Frattini, A., Porta, F., Ugazio, A.G., Johnston, J.A., Candotti, F., O'Shea, J.J., *et al.* (1995). Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). Nature *377*, 65-68.

Maciejewski, J.P., Weichold, F.F., Young, N.S., Cara, A., Zella, D., Reitz, M.S., Jr., and Gallo, R.C. (1995). Intracellular expression of antibody fragments directed against HIV reverse transcriptase prevents HIV infection in vitro. Nat Med *1*, 667-673.

Mackay, I.R. (2010). Travels and travails of autoimmunity: a historical journey from discovery to rediscovery. Autoimmun Rev *9*, A251-258.

MacLennan, I.C., Toellner, K.M., Cunningham, A.F., Serre, K., Sze, D.M., Zuniga, E., Cook, M.C., and Vinuesa, C.G. (2003). Extrafollicular antibody responses. Immunol Rev *194*, 8-18.

Macpherson, A.J., Gatto, D., Sainsbury, E., Harriman, G.R., Hengartner, H., and Zinkernagel, R.M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science *288*, 2222-2226.

Malchow, S., Leventhal, D.S., Lee, V., Nishi, S., Socci, N.D., and Savage, P.A. (2016). Aire Enforces Immune Tolerance by Directing Autoreactive T Cells into the Regulatory T Cell Lineage. Immunity *44*, 1102-1113.

Malchow, S., Leventhal, D.S., Nishi, S., Fischer, B.I., Shen, L., Paner, G.P., Amit, A.S., Kang, C., Geddes, J.E., Allison, J.P., *et al.* (2013). Aire-dependent thymic development of tumor-associated regulatory T cells. Science *339*, 1219-1224.

Mallery, D.L., McEwan, W.A., Bidgood, S.R., Towers, G.J., Johnson, C.M., and James, L.C. (2010). Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). Proceedings of the National Academy of Sciences of the United States of America *107*, 19985-19990.

Mandel, T.E., Phipps, R.P., Abbot, A., and Tew, J.G. (1980). The follicular dendritic cell: long term antigen retention during immunity. Immunol Rev *53*, 29-59.

Mandel, T.E., Phipps, R.P., Abbot, A.P., and Tew, J.G. (1981). Long-term antigen retention by dendritic cells in the popliteal lymph node of immunized mice. Immunology *43*, 353-362.

Manis, J.P., Gu, Y., Lansford, R., Sonoda, E., Ferrini, R., Davidson, L., Rajewsky, K., and Alt, F.W. (1998). Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. The Journal of experimental medicine *187*, 2081-2089.

Mansson, R., Zandi, S., Welinder, E., Tsapogas, P., Sakaguchi, N., Bryder, D., and Sigvardsson, M. (2010). Single-cell analysis of the common lymphoid progenitor compartment reveals functional and molecular heterogeneity. Blood *115*, 2601-2609.

Marrack, P., Ignatowicz, L., Kappler, J.W., Boymel, J., and Freed, J.H. (1993). Comparison of peptides bound to spleen and thymus class II. The Journal of experimental medicine *178*, 2173-2183.

Marrack, P., and Kappler, J. (1987). The T cell receptor. Science *238*, 1073-1079.

Marshall, N.F., Peng, J., Xie, Z., and Price, D.H. (1996). Control of RNA polymerase II elongation potential by a novel carboxyl-terminal domain kinase. J Biol Chem *271*, 27176-27183.

Martin, F., Oliver, A.M., and Kearney, J.F. (2001). Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity *14*, 617-629.

Maruyama, M., Lam, K.P., and Rajewsky, K. (2000). Memory B-cell persistence is independent of persisting immunizing antigen. Nature *407*, 636-642.

Maul, R.W., MacCarthy, T., Frank, E.G., Donigan, K.A., McLenigan, M.P., Yang, W., Saribasak, H., Huston, D.E., Lange, S.S., Woodgate, R., *et al.* (2016). DNA polymerase iota functions in the generation of tandem mutations during somatic hypermutation of antibody genes. The Journal of experimental medicine *213*, 1675-1683.

Mayer, A., Schreieck, A., Lidschreiber, M., Leike, K., Martin, D.E., and Cramer, P. (2012). The spt5 C-terminal region recruits yeast 3' RNA cleavage factor I. Mol Cell Biol *32*, 1321-1331.

Mayer, C.T., Gazumyan, A., Kara, E.E., Gitlin, A.D., Golijanin, J., Viant, C., Pai, J., Oliveira, T.Y., Wang, Q., Escolano, A., *et al.* (2017). The microanatomic segregation of selection by apoptosis in the germinal center. Science *358*.

McBlane, J.F., van Gent, D.C., Ramsden, D.A., Romeo, C., Cuomo, C.A., Gellert, M., and Oettinger, M.A. (1995). Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell *83*, 387-395.

McBride, K.M., Barreto, V., Ramiro, A.R., Stavropoulos, P., and Nussenzweig, M.C. (2004). Somatic hypermutation is limited by CRM1-dependent nuclear export of activation-induced deaminase. The Journal of experimental medicine *199*, 1235-1244.

McBride, K.M., Gazumyan, A., Woo, E.M., Barreto, V.M., Robbiani, D.F., Chait, B.T., and Nussenzweig, M.C. (2006). Regulation of hypermutation by activation-induced cytidine deaminase phosphorylation. Proceedings of the National Academy of Sciences of the United States of America *103*, 8798-8803.

McClelland, E.E., Nicola, A.M., Prados-Rosales, R., and Casadevall, A. (2010). Ab binding alters gene expression in Cryptococcus neoformans and directly modulates fungal metabolism. J Clin Invest *120*, 1355-1361.

McKean, D., Huppi, K., Bell, M., Staudt, L., Gerhard, W., and Weigert, M. (1984). Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. Proceedings of the National Academy of Sciences of the United States of America *81*, 3180-3184.

Mesin, L., Schiepers, A., Ersching, J., Barbulescu, A., Cavazzoni, C.B., Angelini, A., Okada, T., Kurosaki, T., and Victora, G.D. (2020). Restricted Clonality and Limited Germinal Center Reentry Characterize Memory B Cell Reactivation by Boosting. Cell *180*, 92-106 e111.

Methot, S.P., and Di Noia, J.M. (2017). Molecular Mechanisms of Somatic Hypermutation and Class Switch Recombination. Adv Immunol *133*, 37-87.

Meyer-Hermann, M., Mohr, E., Pelletier, N., Zhang, Y., Victora, G.D., and Toellner, K.M. (2012). A theory of germinal center B cell selection, division, and exit. Cell Rep *2*, 162-174.

Meyer-Hermann, M.E., Maini, P.K., and Iber, D. (2006). An analysis of B cell selection mechanisms in germinal centers. Math Med Biol *23*, 255-277.

Meyer, S., Woodward, M., Hertel, C., Vlaicu, P., Haque, Y., Karner, J., Macagno, A., Onuoha, S.C., Fishman, D., Peterson, H., *et al.* (2016). AIRE-Deficient Patients Harbor Unique High-Affinity Disease-Ameliorating Autoantibodies. Cell *166*, 582-595.

Miescher, P., and Fauconnet, M. (1954). [Absorption of L. E. factor by isolated cell nuclei]. Experientia *10*, 252-253.

Mietzner, B., Tsuiji, M., Scheid, J., Velinzon, K., Tiller, T., Abraham, K., Gonzalez, J.B., Pascual, V., Stichweh, D., Wardemann, H., *et al.* (2008). Autoreactive IgG memory antibodies in patients with systemic lupus erythematosus arise from nonreactive and polyreactive precursors. Proceedings of the National Academy of Sciences of the United States of America *105*, 9727-9732.

Minegishi, Y., Coustan-Smith, E., Wang, Y.H., Cooper, M.D., Campana, D., and Conley, M.E. (1998). Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. The Journal of experimental medicine *187*, 71-77.

Misslitz, A., Pabst, O., Hintzen, G., Ohl, L., Kremmer, E., Petrie, H.T., and Forster, R. (2004). Thymic T cell development and progenitor localization depend on CCR7. The Journal of experimental medicine *200*, 481-491.

Moldovan, G.L., Pfander, B., and Jentsch, S. (2007). PCNA, the maestro of the replication fork. Cell *129*, 665-679.

Montecino-Rodriguez, E., Leathers, H., and Dorshkind, K. (2006). Identification of a B-1 B cell-specified progenitor. Nat Immunol *7*, 293-301.

Moran, A.E., Holzapfel, K.L., Xing, Y., Cunningham, N.R., Maltzman, J.S., Punt, J., and Hogquist, K.A. (2011). T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. The Journal of experimental medicine *208*, 1279-1289.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661-673.

Morrow, M.A., Lee, G., Gillis, S., Yancopoulos, G.D., and Alt, F.W. (1992). Interleukin-7 induces N-myc and c-myc expression in normal precursor B lymphocytes. Genes Dev *6*, 61-70.

Mourik, B.C., Lubberts, E., de Steenwinkel, J.E.M., Ottenhoff, T.H.M., and Leenen, P.J.M. (2017). Interactions between Type 1 Interferons and the Th17 Response in Tuberculosis: Lessons Learned from Autoimmune Diseases. Front Immunol *8*, 294.

Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y., and Honjo, T. (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell *102*, 553-563.

Muramatsu, M., Sankaranand, V.S., Anant, S., Sugai, M., Kinoshita, K., Davidson, N.O., and Honjo, T. (1999). Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J Biol Chem *274*, 18470-18476.

Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y., and Tanaka, K. (2007). Regulation of CD8+ T cell development by thymus-specific proteasomes. Science *316*, 1349-1353.

Nagamine, K., Peterson, P., Scott, H.S., Kudoh, J., Minoshima, S., Heino, M., Krohn, K.J., Lalioti, M.D., Mullis, P.E., Antonarakis, S.E., *et al.* (1997). Positional cloning of the APECED gene. Nat Genet *17*, 393-398.

Naito, Y., Takematsu, H., Koyama, S., Miyake, S., Yamamoto, H., Fujinawa, R., Sugai, M., Okuno, Y., Tsujimoto, G., Yamaji, T., *et al.* (2007). Germinal center marker GL7 probes activation-dependent repression of N-glycolylneuraminic acid, a sialic acid species involved in the negative modulation of B-cell activation. Mol Cell Biol *27*, 3008-3022.

Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A.C., and Loh, D.Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature *376*, 435-438.

Nemazee, D.A., and Burki, K. (1989). Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature *337*, 562-566.

Neufeld, M., Maclaren, N.K., and Blizzard, R.M. (1981). Two types of autoimmune Addison's disease associated with different polyglandular autoimmune (PGA) syndromes. Medicine (Baltimore) *60*, 355-362.

Ng, W.F., von Delwig, A., Carmichael, A.J., Arkwright, P.D., Abinun, M., Cant, A.J., Jolles, S., and Lilic, D. (2010). Impaired T(H)17 responses in patients with chronic mucocutaneous candidiasis with and without autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Allergy Clin Immunol *126*, 1006-1015, 1015 e1001-1004.

Nguyen, V.T., Kiss, T., Michels, A.A., and Bensaude, O. (2001). 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. Nature *414*, 322-325.

Nossal, G.J. (1959). Antibody production by single cells. III. The histology of antibody production. Br J Exp Pathol *40*, 301-311.

Ogawa, M., ten Boekel, E., and Melchers, F. (2000). Identification of CD19(-)B220(+)c-Kit(+)Flt3/Flk-2(+)cells as early B lymphoid precursors before pre-B-I cells in juvenile mouse bone marrow. Int Immunol *12*, 313-324.

Okada, T., Miller, M.J., Parker, I., Krummel, M.F., Neighbors, M., Hartley, S.B., O'Garra, A., Cahalan, M.D., and Cyster, J.G. (2005). Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. PLoS Biol *3*, e150.

Okamoto, M., Murakami, M., Shimizu, A., Ozaki, S., Tsubata, T., Kumagai, S., and Honjo, T. (1992). A transgenic model of autoimmune hemolytic anemia. The Journal of experimental medicine *175*, 71-79.

Oliver, A.M., Martin, F., and Kearney, J.F. (1997). Mouse CD38 is down-regulated on germinal center B cells and mature plasma cells. J Immunol *158*, 1108-1115.

Org, T., Rebane, A., Kisand, K., Laan, M., Haljasorg, U., Andreson, R., and Peterson, P. (2009). AIRE activated tissue specific genes have histone modifications associated with inactive chromatin. Hum Mol Genet *18*, 4699-4710.

Orthwein, A., Patenaude, A.M., Affar el, B., Lamarre, A., Young, J.C., and Di Noia, J.M. (2010). Regulation of activation-induced deaminase stability and antibody gene diversification by Hsp90. The Journal of experimental medicine *207*, 2751-2765.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Pachl, J., Svoboda, P., Jacobs, F., Vandewoude, K., van der Hoven, B., Spronk, P., Masterson, G., Malbrain, M., Aoun, M., Garbino, J., *et al.* (2006). A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. Clin Infect Dis *42*, 1404-1413.

Pan-Hammarstrom, Q., Jones, A.M., Lahdesmaki, A., Zhou, W., Gatti, R.A., Hammarstrom, L., Gennery, A.R., and Ehrenstein, M.R. (2005). Impact of DNA ligase IV on nonhomologous end joining pathways during class switch recombination in human cells. The Journal of experimental medicine *201*, 189-194.

Pao, L.I., Lam, K.P., Henderson, J.M., Kutok, J.L., Alimzhanov, M., Nitschke, L., Thomas, M.L., Neel, B.G., and Rajewsky, K. (2007). B cell-specific deletion of protein-tyrosine phosphatase Shp1 promotes B-1a cell development and causes systemic autoimmunity. Immunity *27*, 35-48.

Pappu, R., Cheng, A.M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B.P., and Chan, A.C. (1999). Requirement for B cell linker protein (BLNK) in B cell development. Science *286*, 1949-1954.

Park, S.R., Kim, P.H., Lee, K.S., Lee, S.H., Seo, G.Y., Yoo, Y.C., Lee, J., and Casali, P. (2013). APRIL stimulates NF-kappaB-mediated HoxC4 induction for AID expression in mouse B cells. Cytokine *61*, 608-613.

Park, S.R., Zan, H., Pal, Z., Zhang, J., Al-Qahtani, A., Pone, E.J., Xu, Z., Mai, T., and Casali, P. (2009). HoxC4 binds to the promoter of the cytidine deaminase AID gene to induce AID expression, class-switch DNA recombination and somatic hypermutation. Nat Immunol *10*, 540-550.

Pavri, R., Gazumyan, A., Jankovic, M., Di Virgilio, M., Klein, I., Ansarah-Sobrinho, C., Resch, W., Yamane, A., Reina San-Martin, B., Barreto, V., *et al.* (2010). Activation-induced cytidine deaminase targets DNA at sites of RNA polymerase II stalling by interaction with Spt5. Cell *143*, 122-133.

Pei, Y., and Shuman, S. (2002). Interactions between fission yeast mRNA capping enzymes and elongation factor Spt5. J Biol Chem *277*, 19639-19648.

Peng, J., Zhu, Y., Milton, J.T., and Price, D.H. (1998). Identification of multiple cyclin subunits of human P-TEFb. Genes Dev *12,* 755-762.

Pereira, J.P., Kelly, L.M., Xu, Y., and Cyster, J.G. (2009). EBI2 mediates B cell segregation between the outer and centre follicle. Nature *460,* 1122-1126.

Perry, J.S.A., Lio, C.J., Kau, A.L., Nutsch, K., Yang, Z., Gordon, J.I., Murphy, K.M., and Hsieh, C.S. (2014). Distinct contributions of Aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus. Immunity *41,* 414-426.

Petersen-Mahrt, S.K., Harris, R.S., and Neuberger, M.S. (2002). AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification. Nature *418,* 99-103.

Peterson, P., Org, T., and Rebane, A. (2008). Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. Nat Rev Immunol *8,* 948-957.

Petricevic, B., Laengle, J., Singer, J., Sachet, M., Fazekas, J., Steger, G., Bartsch, R., Jensen-Jarolim, E., and Bergmann, M. (2013). Trastuzumab mediates antibody-dependent cell-mediated cytotoxicity and phagocytosis to the same extent in both adjuvant and metastatic HER2/neu breast cancer patients. J Transl Med *11,* 307.

Phalipon, A., Cardona, A., Kraehenbuhl, J.P., Edelman, L., Sansonetti, P.J., and Corthesy, B. (2002). Secretory component: a new role in secretory IgA-mediated immune exclusion in vivo. Immunity *17,* 107-115.

Phan, R.T., and Dalla-Favera, R. (2004). The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature *432,* 635-639.

Phan, T.G., Grigorova, I., Okada, T., and Cyster, J.G. (2007). Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. Nat Immunol *8,* 992-1000.

Pitkanen, J., Doucas, V., Sternsdorf, T., Nakajima, T., Aratani, S., Jensen, K., Will, H., Vahamurto, P., Ollila, J., Vihinen, M., *et al.* (2000). The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein. J Biol Chem *275*, 16802-16809.

Pitkanen, J., Rebane, A., Rowell, J., Murumagi, A., Strobel, P., Moll, K., Saare, M., Heikkila, J., Doucas, V., Marx, A., *et al.* (2005). Cooperative activation of transcription by autoimmune regulator AIRE and CBP. Biochem Biophys Res Commun *333*, 944-953.

Poliani, P.L., Kisand, K., Marrella, V., Ravanini, M., Notarangelo, L.D., Villa, A., Peterson, P., and Facchetti, F. (2010). Human peripheral lymphoid tissues contain autoimmune regulator-expressing dendritic cells. Am J Pathol *176*, 1104-1112.

Pone, E.J., Zhang, J., Mai, T., White, C.A., Li, G., Sakakura, J.K., Patel, P.J., Al-Qahtani, A., Zan, H., Xu, Z., *et al.* (2012). BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF-kappaB pathway. Nat Commun *3*, 767.

Porritt, H.E., Rumfelt, L.L., Tabrizifard, S., Schmitt, T.M., Zuniga-Pflucker, J.C., and Petrie, H.T. (2004). Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. Immunity *20*, 735-745.

Puel, A., Doffinger, R., Natividad, A., Chrabieh, M., Barcenas-Morales, G., Picard, C., Cobat, A., Ouachee-Chardin, M., Toulon, A., Bustamante, J., *et al.* (2010). Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. The Journal of experimental medicine *207*, 291-297.

Puel, A., Ziegler, S.F., Buckley, R.H., and Leonard, W.J. (1998). Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. Nat Genet *20*, 394-397.

Puga, I., Cols, M., Barra, C.M., He, B., Cassis, L., Gentile, M., Comerma, L., Chorny, A., Shan, M., Xu, W., *et al.* (2011). B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nat Immunol *13*, 170-180.

Qi, C.F., Li, Z., Raffeld, M., Wang, H., Kovalchuk, A.L., and Morse, H.C., 3rd (2009). Differential expression of IRF8 in subsets of macrophages and dendritic cells and effects of IRF8 deficiency on splenic B cell and macrophage compartments. Immunol Res *45*, 62-74.

Qiao, Q., Wang, L., Meng, F.L., Hwang, J.K., Alt, F.W., and Wu, H. (2017). AID Recognizes Structured DNA for Class Switch Recombination. Mol Cell *67*, 361-373 e364.

Rada, C., Di Noia, J.M., and Neuberger, M.S. (2004). Mismatch recognition and uracil excision provide complementary paths to both Ig switching and the A/T-focused phase of somatic mutation. Mol Cell *16*, 163-171.

Rada, C., Williams, G.T., Nilsen, H., Barnes, D.E., Lindahl, T., and Neuberger, M.S. (2002). Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in UNG-deficient mice. Curr Biol *12*, 1748-1755.

Radhakrishnan, K., Bhagya, K.P., Kumar, A.T., Devi, A.N., Sengottaiyan, J., and Kumar, P.G. (2016). Autoimmune Regulator (AIRE) Is Expressed in Spermatogenic Cells, and It Altered the Expression of Several Nucleic-Acid-Binding and Cytoskeletal Proteins in Germ Cell 1 Spermatogonial (GC1-spg) Cells. Mol Cell Proteomics *15*, 2686-2698.

Rajewsky, K. (1996). Clonal selection and learning in the antibody system. Nature *381*, 751-758.

Ramiro, A.R., Jankovic, M., Eisenreich, T., Difilippantonio, S., Chen-Kiang, S., Muramatsu, M., Honjo, T., Nussenzweig, A., and Nussenzweig, M.C. (2004). AID is required for c-myc/IgH chromosome translocations in vivo. Cell *118*, 431-438.

Ramsey, C., Winqvist, O., Puhakka, L., Halonen, M., Moro, A., Kampe, O., Eskelin, P., Pelto-Huikko, M., and Peltonen, L. (2002). Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. Hum Mol Genet *11*, 397-409.

Ranuncolo, S.M., Polo, J.M., Dierov, J., Singer, M., Kuo, T., Greally, J., Green, R., Carroll, M., and Melnick, A. (2007). Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. Nat Immunol *8*, 705-714.

Ravetch, J.V., and Lanier, L.L. (2000). Immune inhibitory receptors. Science *290*, 84-89.

Reina-San-Martin, B., Difilippantonio, S., Hanitsch, L., Masilamani, R.F., Nussenzweig, A., and Nussenzweig, M.C. (2003). H2AX is required for recombination between immunoglobulin switch regions but not for intra-switch region recombination or somatic hypermutation. The Journal of experimental medicine *197*, 1767-1778.

Revy, P., Muto, T., Levy, Y., Geissmann, F., Plebani, A., Sanal, O., Catalan, N., Forveille, M., Dufourcq-Labelouse, R., Gennery, A., *et al.* (2000). Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). Cell *102*, 565-575.

Roa, S., Avdievich, E., Peled, J.U., Maccarthy, T., Werling, U., Kuang, F.L., Kan, R., Zhao, C., Bergman, A., Cohen, P.E., *et al.* (2008). Ubiquitylated PCNA plays a role in somatic hypermutation and class-switch recombination and is required for meiotic progression. Proceedings of the National Academy of Sciences of the United States of America *105*, 16248-16253.

Robbins, W.C., Holman, H.R., Deicher, H., and Kunkel, H.G. (1957). Complement fixation with cell nuclei and DNA in lupus erythematosus. Proc Soc Exp Biol Med *96*, 575-579.

Roco, J.A., Mesin, L., Binder, S.C., Nefzger, C., Gonzalez-Figueroa, P., Canete, P.F., Ellyard, J., Shen, Q., Robert, P.A., Cappello, J., *et al.* (2019). Class-Switch Recombination Occurs Infrequently in Germinal Centers. Immunity *51*, 337-350 e337.

Rogers, T.F., Zhao, F., Huang, D., Beutler, N., Burns, A., He, W.T., Limbo, O., Smith, C., Song, G., Woehl, J., *et al.* (2020). Isolation of potent SARS-CoV-2 neutralizing antibodies and protection from disease in a small animal model. Science *369*, 956-963.

Rose, M.L., Birbeck, M.S., Wallis, V.J., Forrester, J.A., and Davies, A.J. (1980). Peanut lectin binding properties of germinal centres of mouse lymphoid tissue. Nature *284*, 364-366.

Rossi, F.M., Corbel, S.Y., Merzaban, J.S., Carlow, D.A., Gossens, K., Duenas, J., So, L., Yi, L., and Ziltener, H.J. (2005). Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. Nat Immunol *6*, 626-634.

Rossi, S.W., Kim, M.Y., Leibbrandt, A., Parnell, S.M., Jenkinson, W.E., Glanville, S.H., McConnell, F.M., Scott, H.S., Penninger, J.M., Jenkinson, E.J., *et al.* (2007). RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. The Journal of experimental medicine *204*, 1267-1272.

Rother, M.B., Schreurs, M.W., Kroek, R., Bartol, S.J., van Dongen, J.J., and van Zelm, M.C. (2016). The Human Thymus Is Enriched for Autoreactive B Cells. J Immunol *197*, 441-448.

Rougvie, A.E., and Lis, J.T. (1988). The RNA polymerase II molecule at the 5' end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged. Cell *54*, 795-804.

Rougvie, A.E., and Lis, J.T. (1990). Postinitiation transcriptional control in Drosophila melanogaster. Mol Cell Biol *10*, 6041-6045.

Rubtsov, A.V., Swanson, C.L., Troy, S., Strauch, P., Pelanda, R., and Torres, R.M. (2008). TLR agonists promote marginal zone B cell activation and facilitate T-dependent IgM responses. J Immunol *180*, 3882-3888.

Russell, S.M., Tayebi, N., Nakajima, H., Riedy, M.C., Roberts, J.L., Aman, M.J., Migone, T.S., Noguchi, M., Markert, M.L., Buckley, R.H., *et al.* (1995). Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. Science *270*, 797-800.

Saito, M., Novak, U., Piovan, E., Basso, K., Sumazin, P., Schneider, C., Crespo, M., Shen, Q., Bhagat, G., Califano, A., *et al.* (2009). BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. Proceedings of the National Academy of Sciences of the United States of America *106*, 11294-11299.

Saltis, M., Criscitiello, M.F., Ohta, Y., Keefe, M., Trede, N.S., Goitsuka, R., and Flajnik, M.F. (2008). Evolutionarily conserved and divergent regions of the autoimmune regulator (Aire) gene: a comparative analysis. Immunogenetics *60*, 105-114.

Sambandam, A., Maillard, I., Zediak, V.P., Xu, L., Gerstein, R.M., Aster, J.C., Pear, W.S., and Bhandoola, A. (2005). Notch signaling controls the generation and differentiation of early T lineage progenitors. Nat Immunol *6*, 663-670.

Samuels, J., Ng, Y.S., Coupillaud, C., Paget, D., and Meffre, E. (2005). Impaired early B cell tolerance in patients with rheumatoid arthritis. The Journal of experimental medicine *201*, 1659-1667.

Schaetzlein, S., Chahwan, R., Avdievich, E., Roa, S., Wei, K., Eoff, R.L., Sellers, R.S., Clark, A.B., Kunkel, T.A., Scharff, M.D., *et al.* (2013). Mammalian Exo1 encodes both structural and catalytic functions that play distinct roles in essential biological processes. Proceedings of the National Academy of Sciences of the United States of America *110*, E2470-2479.

Schatz, D.G., and Swanson, P.C. (2011). V(D)J recombination: mechanisms of initiation. Annu Rev Genet *45*, 167-202.

Schmitt, T.M., Ciofani, M., Petrie, H.T., and Zuniga-Pflucker, J.C. (2004). Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. The Journal of experimental medicine *200*, 469-479.

Scholz, J.L., Crowley, J.E., Tomayko, M.M., Steinel, N., O'Neill, P.J., Quinn, W.J., 3rd, Goenka, R., Miller, J.P., Cho, Y.H., Long, V., *et al.* (2008). BLyS inhibition eliminates primary B cells but leaves natural and acquired humoral immunity intact. Proceedings of the National Academy of Sciences of the United States of America *105*, 15517-15522.

Schrader, C.E., Linehan, E.K., Mochegova, S.N., Woodland, R.T., and Stavnezer, J. (2005). Inducible DNA breaks in Ig S regions are dependent on AID and UNG. The Journal of experimental medicine *202*, 561-568.

Schroder, A.E., Greiner, A., Seyfert, C., and Berek, C. (1996). Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. Proceedings of the National Academy of Sciences of the United States of America *93*, 221-225.

Schwickert, T.A., Victora, G.D., Fooksman, D.R., Kamphorst, A.O., Mugnier, M.R., Gitlin, A.D., Dustin, M.L., and Nussenzweig, M.C. (2011). A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. The Journal of experimental medicine *208*, 1243-1252.

Scimone, M.L., Aifantis, I., Apostolou, I., von Boehmer, H., and von Andrian, U.H. (2006). A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus. Proceedings of the National Academy of Sciences of the United States of America *103*, 7006-7011.

Sebzda, E., Kundig, T.M., Thomson, C.T., Aoki, K., Mak, S.Y., Mayer, J.P., Zamborelli, T., Nathenson, S.G., and Ohashi, P.S. (1996). Mature T cell reactivity altered by peptide agonist that induces positive selection. The Journal of experimental medicine *183*, 1093-1104.

Sebzda, E., Wallace, V.A., Mayer, J., Yeung, R.S., Mak, T.W., and Ohashi, P.S. (1994). Positive and negative thymocyte selection induced by different concentrations of a single peptide. Science *263*, 1615-1618.

Seifert, M., and Kuppers, R. (2009). Molecular footprints of a germinal center derivation of human IgM+(IgD+)CD27+ B cells and the dynamics of memory B cell generation. The Journal of experimental medicine *206*, 2659-2669.

Shaffer, A.L., Yu, X., He, Y., Boldrick, J., Chan, E.P., and Staudt, L.M. (2000). BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity *13*, 199-212.

Shi, J., Hou, S., Fang, Q., Liu, X., Liu, X., and Qi, H. (2018). PD-1 Controls Follicular T Helper Cell Positioning and Function. Immunity *49*, 264-274 e264.

Shore, S.L., Nahmias, A.J., Starr, S.E., Wood, P.A., and McFarlin, D.E. (1974). Detection of cell-dependent cytotoxic antibody to cells infected with herpes simplex virus. Nature *251*, 350-352.

Sillanpaa, N., Magureanu, C.G., Murumagi, A., Reinikainen, A., West, A., Manninen, A., Lahti, M., Ranki, A., Saksela, K., Krohn, K., *et al.* (2004). Autoimmune regulator induced changes in the gene expression profile of human monocyte-dendritic cell-lineage. Mol Immunol *41*, 1185-1198.

Sintes, J., Gentile, M., Zhang, S., Garcia-Carmona, Y., Magri, G., Cassis, L., Segura-Garzon, D., Ciociola, A., Grasset, E.K., Bascones, S., *et al.* (2017). mTOR intersects antibody-inducing signals from TACI in marginal zone B cells. Nat Commun *8*, 1462.

Smith, K.G., Light, A., Nossal, G.J., and Tarlinton, D.M. (1997). The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. EMBO J *16*, 2996-3006.

Soulas-Sprauel, P., Le Guyader, G., Rivera-Munoz, P., Abramowski, V., Olivier-Martin, C., Goujet-Zalc, C., Charneau, P., and de Villartay, J.P. (2007). Role for DNA repair factor XRCC4 in immunoglobulin class switch recombination. The Journal of experimental medicine *204*, 1717-1727.

Soumya, V., Padmanabhan, R.A., Titus, S., and Laloraya, M. (2016). Murine uterine decidualization is a novel function of autoimmune regulator-beyond immune tolerance. Am J Reprod Immunol *76*, 224-234.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *241*, 58-62.

Stavnezer, J., Linehan, E.K., Thompson, M.R., Habboub, G., Ucher, A.J., Kadungure, T., Tsuchimoto, D., Nakabeppu, Y., and Schrader, C.E. (2014). Differential expression of APE1 and APE2 in germinal centers promotes error-prone repair and A:T mutations during somatic hypermutation. Proceedings of the National Academy of Sciences of the United States of America *111*, 9217-9222.

Stritesky, G.L., Xing, Y., Erickson, J.R., Kalekar, L.A., Wang, X., Mueller, D.L., Jameson, S.C., and Hogquist, K.A. (2013). Murine thymic selection quantified using a unique method to capture deleted T cells. Proceedings of the National Academy of Sciences of the United States of America *110*, 4679-4684.

Su, M.A., Giang, K., Zumer, K., Jiang, H., Oven, I., Rinn, J.L., Devoss, J.J., Johannes, K.P., Lu, W., Gardner, J., *et al.* (2008). Mechanisms of an autoimmunity syndrome in mice caused by a dominant mutation in Aire. J Clin Invest *118*, 1712-1726.

Takahashi, Y., Ohta, H., and Takemori, T. (2001). Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. Immunity *14*, 181-192.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kurosaki, T. (1994). Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. EMBO J *13*, 1341-1349.

Tangye, S.G., Liu, Y.J., Aversa, G., Phillips, J.H., and de Vries, J.E. (1998). Identification of functional human splenic memory B cells by expression of CD148 and CD27. The Journal of experimental medicine *188*, 1691-1703.

Taniguchi, R.T., DeVoss, J.J., Moon, J.J., Sidney, J., Sette, A., Jenkins, M.K., and Anderson, M.S. (2012). Detection of an autoreactive T-cell population within the polyclonal repertoire that undergoes distinct autoimmune regulator (Aire)-mediated selection. Proceedings of the National Academy of Sciences of the United States of America *109*, 7847-7852.

Tao, Y., Kupfer, R., Stewart, B.J., Williams-Skipp, C., Crowell, C.K., Patel, D.D., Sain, S., and Scheinman, R.I. (2006). AIRE recruits multiple transcriptional components to specific genomic regions through tethering to nuclear matrix. Mol Immunol *43*, 335-345.

Tarlinton, D.M., and Smith, K.G. (2000). Dissecting affinity maturation: a model explaining selection of antibody-forming cells and memory B cells in the germinal centre. Immunol Today *21*, 436-441.

Tas, J.M., Mesin, L., Pasqual, G., Targ, S., Jacobsen, J.T., Mano, Y.M., Chen, C.S., Weill, J.C., Reynaud, C.A., Browne, E.P., *et al.* (2016). Visualizing antibody affinity maturation in germinal centers. Science *351*, 1048-1054.

Tedder, T.F. (2015). B10 cells: a functionally defined regulatory B cell subset. J Immunol *194*, 1395-1401.

Thien, M., Phan, T.G., Gardam, S., Amesbury, M., Basten, A., Mackay, F., and Brink, R. (2004). Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. Immunity *20*, 785-798.

Thomas, A.A., Vrijsen, R., and Boeye, A. (1986). Relationship between poliovirus neutralization and aggregation. J Virol *59*, 479-485.

Thouvenin, E., Schoehn, G., Rey, F., Petitpas, I., Mathieu, M., Vaney, M.C., Cohen, J., Kohli, E., Pothier, P., and Hewat, E. (2001). Antibody inhibition of the transcriptase activity of the rotavirus DLP: a structural view. J Mol Biol *307*, 161-172.

Tierens, A., Delabie, J., Michiels, L., Vandenberghe, P., and De Wolf-Peeters, C. (1999). Marginal-zone B cells in the human lymph node and spleen show somatic hypermutations and display clonal expansion. Blood *93*, 226-234.

Tiselius, A., and Kabat, E.A. (1938). Electrophoresis of Immune Serum. Science *87*, 416-417.

Tornberg, U.C., and Holmberg, D. (1995). B-1a, B-1b and B-2 B cells display unique VHDJH repertoires formed at different stages of ontogeny and under different selection pressures. EMBO J *14*, 1680-1689.

Tran, T.H., Nakata, M., Suzuki, K., Begum, N.A., Shinkura, R., Fagarasan, S., Honjo, T., and Nagaoka, H. (2010). B cell-specific and stimulation-responsive enhancers derepress Aicda by overcoming the effects of silencers. Nat Immunol *11*, 148-154.

Treml, L.S., Carlesso, G., Hoek, K.L., Stadanlick, J.E., Kambayashi, T., Bram, R.J., Cancro, M.P., and Khan, W.N. (2007). TLR stimulation modifies BLyS receptor expression in follicular and marginal zone B cells. J Immunol *178*, 7531-7539.

Uchida, N., and Weissman, I.L. (1992). Searching for hematopoietic stem cells: evidence that Thy-1.1lo Lin- Sca-1+ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. The Journal of experimental medicine *175*, 175-184.

Uchimura, Y., Barton, L.F., Rada, C., and Neuberger, M.S. (2011). REG-gamma associates with and modulates the abundance of nuclear activation-induced deaminase. The Journal of experimental medicine *208*, 2385-2391.

Uehara, S., Grinberg, A., Farber, J.M., and Love, P.E. (2002). A role for CCR9 in T lymphocyte development and migration. J Immunol *168*, 2811-2819.

van Gent, D.C., Mizuuchi, K., and Gellert, M. (1996). Similarities between initiation of V(D)J recombination and retroviral integration. Science *271*, 1592-1594.

van Oers, N.S., Lowin-Kropf, B., Finlay, D., Connolly, K., and Weiss, A. (1996). alpha beta T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. Immunity *5*, 429-436.

Varghese, R., Mikyas, Y., Stewart, P.L., and Ralston, R. (2004). Postentry neutralization of adenovirus type 5 by an antihexon antibody. J Virol *78*, 12320-12332.

Vicari, A.P., Figueroa, D.J., Hedrick, J.A., Foster, J.S., Singh, K.P., Menon, S., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Bacon, K.B., *et al.* (1997). TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. Immunity *7*, 291-301.

Victora, G.D., and Nussenzweig, M.C. (2012). Germinal centers. Annu Rev Immunol *30*, 429-457.

Victora, G.D., Schwickert, T.A., Fooksman, D.R., Kamphorst, A.O., Meyer-Hermann, M., Dustin, M.L., and Nussenzweig, M.C. (2010). Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell *143*, 592-605.

Vieira, P., and Rajewsky, K. (1990). Persistence of memory B cells in mice deprived of T cell help. Int Immunol *2*, 487-494.

Viglianti, G.A., Lau, C.M., Hanley, T.M., Miko, B.A., Shlomchik, M.J., and Marshak-Rothstein, A. (2003). Activation of autoreactive B cells by CpG dsDNA. Immunity *19*, 837-847.

Vikstrom, I., Carotta, S., Luthje, K., Peperzak, V., Jost, P.J., Glaser, S., Busslinger, M., Bouillet, P., Strasser, A., Nutt, S.L., *et al.* (2010). Mcl-1 is essential for germinal center formation and B cell memory. Science *330*, 1095-1099.

Vinuesa, C.G., Tangye, S.G., Moser, B., and Mackay, C.R. (2005). Follicular B helper T cells in antibody responses and autoimmunity. Nat Rev Immunol *5*, 853-865.

Volkmann, C., Brings, N., Becker, M., Hobeika, E., Yang, J., and Reth, M. (2016). Molecular requirements of the B-cell antigen receptor for sensing monovalent antigens. EMBO J *35*, 2371-2381.

von Boehmer, H., and Fehling, H.J. (1997). Structure and function of the pre-T cell receptor. Annu Rev Immunol *15*, 433-452.

Wada, T., Takagi, T., Yamaguchi, Y., Ferdous, A., Imai, T., Hirose, S., Sugimoto, S., Yano, K., Hartzog, G.A., Winston, F., *et al.* (1998). DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. Genes Dev *12*, 343-356.

Wardemann, H., Yurasov, S., Schaefer, A., Young, J.W., Meffre, E., and Nussenzweig, M.C. (2003). Predominant autoantibody production by early human B cell precursors. Science *301*, 1374-1377.

Watanabe, M., and Blobel, G. (1989). Site-specific antibodies against the PrlA (secY) protein of Escherichia coli inhibit protein export by interfering with plasma membrane binding of preproteins. Proceedings of the National Academy of Sciences of the United States of America *86*, 1895-1899.

Webby, C.J., Wolf, A., Gromak, N., Dreger, M., Kramer, H., Kessler, B., Nielsen, M.L., Schmitz, C., Butler, D.S., Yates, J.R., 3rd, *et al.* (2009). Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with RNA splicing. Science *325*, 90-93.

Wei, S., Perera, M.L.W., Sakhtemani, R., and Bhagwat, A.S. (2017). A novel class of chemicals that react with abasic sites in DNA and specifically kill B cell cancers. PLoS One *12*, e0185010.

Wei, S., Shalhout, S., Ahn, Y.H., and Bhagwat, A.S. (2015). A versatile new tool to quantify abasic sites in DNA and inhibit base excision repair. DNA Repair (Amst) *27*, 9-18.

Weigert, M.G., Cesari, I.M., Yonkovich, S.J., and Cohn, M. (1970). Variability in the lambda light chain sequences of mouse antibody. Nature *228*, 1045-1047.

Weiner, G.J. (2010). Rituximab: mechanism of action. Semin Hematol *47*, 115-123.

Weisel, F., and Shlomchik, M. (2017). Memory B Cells of Mice and Humans. Annu Rev Immunol *35*, 255-284.

Weisel, F.J., Zuccarino-Catania, G.V., Chikina, M., and Shlomchik, M.J. (2016). A Temporal Switch in the Germinal Center Determines Differential Output of Memory B and Plasma Cells. Immunity *44*, 116-130.

Weiss, R.A., and Esparza, J. (2015). The prevention and eradication of smallpox: a commentary on Sloane (1755) 'An account of inoculation'. Philos Trans R Soc Lond B Biol Sci *370*.

Weller, S., Faili, A., Garcia, C., Braun, M.C., Le Deist, F.F., de Saint Basile, G.G., Hermine, O., Fischer, A., Reynaud, C.A., and Weill, J.C. (2001). CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. Proceedings of the National Academy of Sciences of the United States of America *98*, 1166-1170.

Weller, S., Mamani-Matsuda, M., Picard, C., Cordier, C., Lecoeuche, D., Gauthier, F., Weill, J.C., and Reynaud, C.A. (2008). Somatic diversification in the absence of antigen-driven responses is the hallmark of the IgM+ IgD+ CD27+ B cell repertoire in infants. The Journal of experimental medicine *205*, 1331-1342.

Wellmann, U., Letz, M., Herrmann, M., Angermuller, S., Kalden, J.R., and Winkler, T.H. (2005). The evolution of human anti-double-stranded DNA autoantibodies. Proceedings of the National Academy of Sciences of the United States of America *102*, 9258-9263.

Werner, S.L., Kearns, J.D., Zadorozhnaya, V., Lynch, C., O'Dea, E., Boldin, M.P., Ma, A., Baltimore, D., and Hoffmann, A. (2008). Encoding NF-kappaB temporal control in response to TNF: distinct roles for the negative regulators IkappaBalpha and A20. Genes Dev *22*, 2093-2101.

Wesemann, D.R., Portuguese, A.J., Meyers, R.M., Gallagher, M.P., Cluff-Jones, K., Magee, J.M., Panchakshari, R.A., Rodig, S.J., Kepler, T.B., and Alt, F.W. (2013). Microbial colonization influences early B-lineage development in the gut lamina propria. Nature *501*, 112-115.

Whyte, W.A., Orlando, D.A., Hnisz, D., Abraham, B.J., Lin, C.Y., Kagey, M.H., Rahl, P.B., Lee, T.I., and Young, R.A. (2013). Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell *153*, 307-319.

Wilson, A., Held, W., and MacDonald, H.R. (1994). Two waves of recombinase gene expression in developing thymocytes. The Journal of experimental medicine *179*, 1355-1360.

Wilson, A., MacDonald, H.R., and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. The Journal of experimental medicine *194*, 1003-1012.

Winkler, T.H., and Martensson, I.L. (2018). The Role of the Pre-B Cell Receptor in B Cell Development, Repertoire Selection, and Tolerance. Front Immunol *9*, 2423.

Wu, Y., El Shikh, M.E., El Sayed, R.M., Best, A.M., Szakal, A.K., and Tew, J.G. (2009). IL-6 produced by immune complex-activated follicular dendritic cells promotes germinal center reactions, IgG responses and somatic hypermutation. Int Immunol *21*, 745-756.

Xu, Z., Fulop, Z., Wu, G., Pone, E.J., Zhang, J., Mai, T., Thomas, L.M., Al-Qahtani, A., White, C.A., Park, S.R., *et al.* (2010). 14-3-3 adaptor proteins recruit AID to 5'-AGCT-3'-rich switch regions for class switch recombination. Nat Struct Mol Biol *17*, 1124-1135.

Xu, Z., Pone, E.J., Al-Qahtani, A., Park, S.R., Zan, H., and Casali, P. (2007). Regulation of aicda expression and AID activity: relevance to somatic hypermutation and class switch DNA recombination. Crit Rev Immunol *27*, 367-397.

Yamada, T., Yamaguchi, Y., Inukai, N., Okamoto, S., Mura, T., and Handa, H. (2006). P-TEFb-mediated phosphorylation of hSpt5 C-terminal repeats is critical for processive transcription elongation. Mol Cell *21*, 227-237.

Yamaguchi, Y., Shibata, H., and Handa, H. (2013). Transcription elongation factors DSIF and NELF: promoter-proximal pausing and beyond. Biochim Biophys Acta *1829*, 98-104.

Yamano, T., Dobes, J., Voboril, M., Steinert, M., Brabec, T., Zietara, N., Dobesova, M., Ohnmacht, C., Laan, M., Peterson, P., *et al.* (2019). Aire-expressing ILC3-like cells in the lymph node display potent APC features. The Journal of experimental medicine *216*, 1027-1037.

Yamano, T., Nedjic, J., Hinterberger, M., Steinert, M., Koser, S., Pinto, S., Gerdes, N., Lutgens, E., Ishimaru, N., Busslinger, M., *et al.* (2015). Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction. Immunity *42*, 1048-1061.

Yan, C.T., Boboila, C., Souza, E.K., Franco, S., Hickernell, T.R., Murphy, M., Gumaste, S., Geyer, M., Zarrin, A.A., Manis, J.P., *et al.* (2007). IgH class switching and translocations use a robust non-classical end-joining pathway. Nature *449*, 478-482.

Yanagihara, T., Sanematsu, F., Sato, T., Uruno, T., Duan, X., Tomino, T., Harada, Y., Watanabe, M., Wang, Y., Tanaka, Y., *et al.* (2015). Intronic regulation of Aire expression by Jmjd6 for self-tolerance induction in the thymus. Nat Commun *6*, 8820.

Yang, Z., Zhu, Q., Luo, K., and Zhou, Q. (2001). The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. Nature *414*, 317-322.

Ye, B.H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., de Waard, R., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R.S., *et al.* (1997). The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. Nat Genet *16*, 161-170.

Yeh, C.H., Nojima, T., Kuraoka, M., and Kelsoe, G. (2018). Germinal center entry not selection of B cells is controlled by peptide-MHCII complex density. Nat Commun *9*, 928.

Yoshida, H., Bansal, K., Schaefer, U., Chapman, T., Rioja, I., Proekt, I., Anderson, M.S., Prinjha, R.K., Tarakhovsky, A., Benoist, C., *et al.* (2015). Brd4 bridges the transcriptional regulators, Aire and P-TEFb, to promote elongation of peripheral-tissue antigen transcripts in thymic stromal cells. Proceedings of the National Academy of Sciences of the United States of America *112*, E4448-4457.

Yoshino, T., Kondo, E., Cao, L., Takahashi, K., Hayashi, K., Nomura, S., and Akagi, T. (1994). Inverse expression of bcl-2 protein and Fas antigen in lymphoblasts in peripheral lymph nodes and activated peripheral blood T and B lymphocytes. Blood *83*, 1856-1861.

Yu, D., and Ye, L. (2018). A Portrait of CXCR5(+) Follicular Cytotoxic CD8(+) T cells. Trends Immunol *39*, 965-979.

Yurasov, S., Wardemann, H., Hammersen, J., Tsuiji, M., Meffre, E., Pascual, V., and Nussenzweig, M.C. (2005). Defective B cell tolerance checkpoints in systemic lupus erythematosus. The Journal of experimental medicine *201*, 703-711.

Zan, H., and Casali, P. (2013). Regulation of Aicda expression and AID activity. Autoimmunity *46*, 83-101.

Zanotti, K.J., and Gearhart, P.J. (2016). Antibody diversification caused by disrupted mismatch repair and promiscuous DNA polymerases. DNA Repair (Amst) *38*, 110-116.

Zaretsky, I., Atrakchi, O., Mazor, R.D., Stoler-Barak, L., Biram, A., Feigelson, S.W., Gitlin, A.D., Engelhardt, B., and Shulman, Z. (2017). ICAMs support B cell interactions with T follicular helper cells and promote clonal selection. The Journal of experimental medicine *214*, 3435-3448.

Zhang, P., Cox, C.J., Alvarez, K.M., and Cunningham, M.W. (2009). Cutting edge: cardiac myosin activates innate immune responses through TLRs. J Immunol *183*, 27-31.

Zhang, W., Du, Y., Su, Z., Wang, C., Zeng, X., Zhang, R., Hong, X., Nie, C., Wu, J., Cao, H., *et al.* (2015). IMonitor: A Robust Pipeline for TCR and BCR Repertoire Analysis. Genetics *201*, 459-472.

Zhang, X., Zhang, Y., Ba, Z., Kyritsis, N., Casellas, R., and Alt, F.W. (2019). Fundamental roles of chromatin loop extrusion in antibody class switching. Nature *575*, 385-389.

Zheng, S., Vuong, B.Q., Vaidyanathan, B., Lin, J.Y., Huang, F.T., and Chaudhuri, J. (2015). Non-coding RNA Generated following Lariat Debranching Mediates Targeting of AID to DNA. Cell *161*, 762-773.

Zhou, R., Yazdi, A.S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature *469*, 221-225.

Zlotoff, D.A., Sambandam, A., Logan, T.D., Bell, J.J., Schwarz, B.A., and Bhandoola, A. (2010). CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. Blood *115*, 1897-1905.

Zuccarino-Catania, G.V., Sadanand, S., Weisel, F.J., Tomayko, M.M., Meng, H., Kleinstein, S.H., Good-Jacobson, K.L., and Shlomchik, M.J. (2014). CD80 and PD-L2 define functionally distinct memory B cell subsets that are independent of antibody isotype. Nat Immunol *15*, 631-637.

ABSTRACT

GERMINAL CENTER B CELL EXPRESSION OF AIRE REGULATES ANTIBODY DIVERSIFICATION AND AUTOIMMUNITY

by

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Major: Molecular Genetics and Genomics

Degree: Doctor of Philosophy

B cells are a unique subset of immune cells that, in response to antigen, diversify their antibody repertoire to generate progressively higher affinity antibodies of different isotypes through the processes of affinity maturation/somatic hypermutation (SHM) and class switch recombination (CSR). One of the major sites in which this diversification occurs is in T cell dependent microanatomical structures known as germinal centers (GC). Here, we find that GC B cells express the protein, autoimmune regulator (Aire) in a CD40 dependent manner. In these cells, Aire interacts with activation induced cytidine deaminase (AID), the protein responsible for initiating the processes of diversification through the deamination of the immunoglobulin locus and prevents AID function by inhibiting its targeting to the DNA substrate. Mice that receive Aire deficient B cells are unable to clear the fungal pathogen *Candida albicans* as effectively as mice with wild type B cell Aire and produce increased levels of autoreactive antibodies against Th17 cytokines. These results describe a novel mechanism for the regulation of antibody diversification and potentially provide a new approach to generating high affinity antibodies for both therapeutic and commercial applications.

AUTOBIOGRAPHICAL STATEMENT

Jordan Z. Zhou

During my undergraduate career, I studied both classical traditions and biomedical sciences with an emphasis in microbiology. It was difficult to balance two completely separate majors, but with the outstanding guidance from excellent professors at Grand Valley State University, I was able to manage, though, according to some, just barely. My original goal was to attend medical school and become a physician; however, once I began research training under the watchful eye of Dr. Aaron Baxter, working on describing the mechanisms of pathogenicity in *Salmonella enterica* and biofilm formation in *Escherichia coli*, my course quickly shifted. It was under his tutelage that I developed a passion for inquisition and creative problem solving and, for that, I am deeply grateful as it drove me down the path I am on today.

After beginning my graduate work in the lab of Dr. Kang Chen at Wayne State University in May 2016, my curiosity, and my determination to learn has grown at an exponential rate. Over the past several years, while studying the mechanisms of antibody diversification and immune tolerance, I have learned more than I could have ever imagined as an undergrad, though, I must admit that, at the time, I had never imagined I would be interested in immunology as it had always been my least favorite subject. Today, my drive to learn more about the immune system and to develop new ideas can only be described as an unhealthy obsession: one that I am more than happy to bear.