

The role of extra-thymic AIRE-expressing cells (eTACs) in AIRE-propagated immune tolerance



Lindsey Belle Rosen

Balliol College

University of Oxford

NIH Oxford-Cambridge Scholars Program

A thesis submitted for the degree of

Doctor of Philosophy

in Biomedical Sciences

Table of Contents

Abstract	5
Acknowledgements	7
1 Introduction	8
1.1 Immune system.....	8
1.1.1 Innate immunity	8
1.1.2 Adaptive immunity	9
1.2 Tolerance	11
1.2.1 Central tolerance	11
1.2.2 Peripheral tolerance	15
1.2.3 Autoimmunity.....	17
1.3 AIRE.....	18
1.3.1 Promiscuous gene expression	19
1.3.2 Mouse models of AIRE-deficiency	20
1.3.3 APECED	21
1.4 Anti-cytokine autoantibodies.....	22
1.4.1 Anti-cytokine autoantibodies of known etiology	23
1.4.2 Anti-cytokine autoantibodies of unknown etiology	24
1.5 Hypotheses & Aims.....	26
2 Materials & Methods.....	28
2.1 Mice	28
2.1.1 C57BL/6	28
2.1.2 OT.1	28
2.1.3 <i>Aire</i> ^{-/-}	28
2.1.4 <i>Aire</i> (flox/flox).....	29
2.1.5 <i>Foxn1</i> (Cre)	29
2.1.6 <i>Mb1</i> (Cre).....	30
2.1.7 <i>Rorc</i> (Cre).....	30
2.2 General reagents.....	30
2.3 Genotyping.....	31
2.3.1 DNA isolation and amplification	31
2.3.2 Restriction enzyme digestion	33
2.3.3 Gel electrophoresis.....	33
2.4 Flow Cytometry.....	34
2.4.1 Lymph node digestion and staining.....	34
2.4.2 Splenocyte isolation and staining	34
2.4.3 Thymocyte isolation and staining	35
2.4.4 mTEC isolation and staining.....	36
2.5 Molecular biology	38
2.5.1 RNA extraction.....	38
2.5.2 cDNA synthesis	39
2.5.3 qPCR	39
2.6 Imaging	40
2.6.1 Immunofluorescence on murine lymph nodes.....	40

2.6.2	Intranuclear AIRE immunofluorescence	41
2.6.3	Organ-specific autoantibodies by immunofluorescence	42
2.6.4	Histology	43
2.6.4.1	Organ collection and sectioning	43
2.6.4.2	H&E staining	44
2.6.4.3	Histological scoring	44
2.7	<i>In vitro</i> experiments	44
2.7.1	Lymph node digestion and isolation of APCs	44
2.7.2	CD8 ⁺ T cell isolation	45
2.7.3	Proliferation dye labeling	45
2.7.4	APC priming with OVA peptide	46
2.7.5	Cell culture and proliferation analysis	46
2.8	Anti-cytokine autoantibody analyses	46
2.8.1	Subjects and samples	46
2.8.2	Multiplex particle-based assay for anti-cytokine autoantibody detection	47
2.8.3	Functional evaluation of anti-cytokine autoantibodies	48
2.9	Statistics	49
3	Identification of extra-thymic AIRE-expressing cells (eTACs) in secondary lymphoid tissues	50
3.1	Identification of AIRE expression in murine lymph nodes by immunofluorescence	51
3.2	Identification of two distinct eTAC populations by flow cytometry	53
3.3	Identification of AIRE-expressing cells in sorted eTAC populations by confocal microscopy	55
4	Antigen presentation by extra-thymic AIRE-expressing cells (eTACs)	62
4.1	eTACs express high levels of costimulatory molecules	64
4.2	eTACs are effective antigen presenting cells <i>in vitro</i>	65
4.3	AIRE is not required for antigen presentation by eTACs	69
5	Phenotypic analyses of constitutive and cell lineage-specific loss-of-AIRE mutant mice	72
5.1	Generation of cell lineage-specific loss-of-AIRE mice	74
5.2	Loss of AIRE in the thymus affects thymocyte development	77
5.2.1	Normal early thymocyte development in cell-specific loss-of-AIRE mice	79
5.2.2	Loss of AIRE in the thymus affects thymocyte negative selection	83
5.2.3	Thymic T _{reg} and re-circulating T cells affected by loss of AIRE	87
5.3	Peripheral T cell compartment affected by loss of AIRE	92
5.4	Organ-specific autoantibodies more prevalent in the sera of mice lacking AIRE in mTECs	100
5.5	Thymic loss-of-AIRE affects inflammatory infiltration of organs	103
5.5.1	Increased lymphocytic infiltration in the salivary glands of mice lacking AIRE in the thymus	103
5.5.2	More severe retinal degeneration in mice lacking AIRE in mTECs	105

6	Anti-cytokine autoantibodies.....	108
6.1	Anti-cytokine autoantibodies in healthy individuals.....	108
6.2	APECED patients have a distinct anti-cytokine autoantibody profile	111
6.3	Autoantibodies against type I IFNs in COVID-19 patients	114
7	Discussion.....	134
	References	151

Abstract

The autoimmune regulator (AIRE) is a facilitator of gene transcription found primarily in the thymus where its expression is localized primarily to mature medullary thymic epithelial cells (mTECs). AIRE modulates the promiscuous gene expression of tissue-restricted antigens (TRAs) and thus ensures that maturing T cells in the thymus are exposed to a broad array of peptides derived from self-antigens typically detected in peripheral tissues. Autoreactive thymocytes are deleted from the emerging repertoire upon high affinity recognition of TRAs, a process known as negative selection that consequently establishes central tolerance. The deletion of autoreactive thymocytes by mTECs is, however, incomplete, allowing a significant number of self-reactive T cells to mature fully and exit to the periphery. This unwanted “escape” necessitates the existence of peripheral tolerance mechanisms operative outside of the thymus that collectively prevent autoreactivity and thus the likelihood of overt autoimmune destruction of tissues. In addition to its expression in mTECs, AIRE is also detected in two distinct hematopoietic cell populations, namely activated thymic B cells and extra-thymic AIRE-expressing cells (eTACs). However, the function of AIRE in these cells in general and their role in maintaining peripheral T cell tolerance remain largely unexplored.

The results of my research provide experimental evidence that AIRE is expressed under physiological conditions in the peripheral lymph nodes of wildtype mice. To examine the functional importance of these cells, I assessed their ability to present exogenously added antigen *in vitro* and compared this capacity to that of professional antigen presenting cells. Antigen-loaded eTACs and conventional dendritic cells demonstrated a comparable

capacity for activating antigen-specific T cells *in vitro*, indicating a potential role for eTACs in peripheral tolerance mechanisms. To better understand the contribution of AIRE to central and peripheral tolerance, I generated lineage-specific, loss-of-AIRE transgenic mouse strains. Loss of AIRE expression limited to only mTECs resulted in changes in thymocytes and peripheral T cells that were comparable to constitutively AIRE-deficient mice, including a spectrum of autoimmune manifestations. In aggregate, the experimental data here presents evidence that while eTACs are potentially capable of contributing to peripheral tolerance via the presentation of antigen to T cells, AIRE-expressing mTECs are the primary players in AIRE-regulated immune tolerance.

Finally, due to the observation of anti-cytokine autoantibodies in patients with AIRE-deficiency and the finding of heterozygous *AIRE* mutations in some patients with COVID-19, I assessed the sera/plasmas of healthy control individuals, patients with AIRE-deficiency, and COVID-19 patients for the presence of anti-cytokine autoantibodies. I found that neutralizing autoantibodies against type I interferons are a risk factor for developing life-threatening COVID-19. These findings provide an explanation for some of the clinical variability observed in COVID-19 patients and pave the way for prevention and treatment when autoantibodies are identified early in the course of disease.

Acknowledgements

I would like to sincerely thank my mentors on either side of the Atlantic, Prof. Georg Holländer and Dr. Steve Holland, for their support, wisdom, and kindness.

I would also like to thank my parents for always encouraging and loving me.

Finally, to Kyle, my everything, whose unwavering patience, support, and love got me through.

1 Introduction

1.1 Immune system

The role of the immune system is to protect the body from infection and diseases caused by foreign pathogens. The immune system can be divided into two categories: innate immunity and adaptive immunity.

1.1.1 Innate immunity

The innate immune system allows for an immediate, albeit non-specific, response to foreign pathogens. The first line of defense consists of physical and chemical barriers of the body such as epithelial surfaces and stomach acid, which prevent the invasion of foreign pathogens. If pathogens evade the body's primary defenses, a second line of non-specific defense is invoked via a signaling cascade of cytokines and chemokines that results in inflammation and pathogen elimination by phagocytes (Aderem & Underhill, 1999).

An important part of the innate immune response is the complement cascade, whereby small proteins synthesized by the liver, circulating as inactive precursors, are stimulated initiating a chain reaction of activation that leads to bacterial membrane attack, inflammation, and phagocyte recruitment/activation (Murphy & Weaver, 2017). Phagocytic sensor cells (macrophages, neutrophils, and dendritic cells) are able to detect invasion by foreign pathogens due to pattern recognition receptors (PRRs) on their surfaces. These PRRs are able to recognize pathogen-associated molecular patterns (PAMPs), which are specific to foreign pathogens and have been conserved throughout evolution (Janeway & Medzhitov, 2002; Murphy & Weaver, 2017), making them ideal targets. Once activated,

sensor cell PRRs stimulate processes such as phagocytosis and cytokine/chemokine production, working to clear the host of pathogens and initiate an inflammatory response. Cytokines are secreted proteins that activate cells possessing the cognate receptor for that cytokine, while chemokines are another class of secreted proteins that serve as chemoattractants to bring other immune cells to the site of infection (Akira & Takeda, 2004; Murphy & Weaver, 2017).

Although effective and fast-acting, the innate immune response is limited by its lack of specificity and is thus accompanied by the slower-acting, but highly specific, adaptive immune response.

1.1.2 Adaptive immunity

The adaptive immune system relies upon immunological memory and is thus an acquired and specific response to pathogens. Adaptive immunity is mediated by lymphocytes, a subset of white blood cells that can be further divided into B cells, T cells, and natural killer (NK) cells. The major players in adaptive immunity are B cells and T cells, which possess antigen receptors on their surface. Whether the antigen receptor has been activated by its cognate antigen determines whether the cell is a naïve lymphocyte (has not seen its antigen) or an effector lymphocyte (has seen and been activated by its antigen) (Murphy & Weaver, 2017).

Humoral adaptive immunity is facilitated by B lymphocytes, which each have membrane-bound immunoglobulin that is specific for a unique antigen. Secreted immunoglobulins, or antibodies, help to protect the host by either direct neutralization of the pathogen,

opsonization, or complement activation. Opsonization is the process by which pathogens are marked for phagocytosis, allowing macrophages and neutrophils to eliminate the opsonized molecule via Fc receptor-mediated phagocytosis. Complement proteins can make this process more efficient and allow for lysis and ingestion of pathogens via complement receptors on phagocytes (Bardhan & Kaushik, 2021; Murphy & Weaver, 2017).

Cell-mediated adaptive immunity is facilitated by T lymphocytes, which possess a T cell receptor (TCR) on their surface. The TCR repertoire is stochastic and vast, allowing for the recognition of both non-self and self-antigens. During their development in the thymus T cells must be educated and self-reactive T cells deleted from the repertoire, a process called tolerance which will be discussed in greater detail below (Section 1.2). There are two types of mature T cells, named for the expression of the glycoproteins CD4 or CD8. Unlike B cells, T cells require help to recognize their antigen in the form of major histocompatibility complexes (MHC) which are found on antigen-presenting cells (APCs). MHC molecules bind antigen peptides and then form complexes with T cells by binding to their TCR. There are two classes of MHC molecules: MHC class I (MHC-I) and MHC class II (MHC-II) (Rock *et al.*, 2016). Cells expressing MHC-I molecules present antigen to CD8 T cells while cells expressing MHC-II molecules present antigen to CD4 T cells.

The humoral and cell-mediated adaptive immune responses work together to enhance immunological memory. When a memory B cell recognizes its antigen it can internalize, process, and present antigenic peptide on its surface via MHC-II. Upon interaction with this peptide:MHC-II complex, antigen-specific T cells proliferate and secrete cytokines, activating B cells to secrete more antibodies. It is through long-lived memory B and T cells

and circulating antibodies that immunological memory persists, making long-term protection from pathogens through vaccination possible (Gourley *et al.*, 2004; Murphy & Weaver, 2017).

Additionally, with T cell help, B cells can undergo affinity maturation and class switching, two processes that ensure more robust humoral immunity through antibody diversification. Affinity maturation occurs in germinal centers where B cells that have been triggered by an antigen undergo somatic hypermutation to alter the variable region of their receptor. B cells recognizing their antigen with high affinity are then selected for by T follicular helper cells. Alternatively, the constant region of the B cell receptor (BCR) can be altered while the antigen-specific variable region stays the same, resulting in immunoglobulin production of different isotypes, or class switching (Z. Li *et al.*, 2004; Murphy & Weaver, 2017; Papavasiliou & Schatz, 2002).

1.2 Tolerance

Establishing and maintaining tolerance to self-antigens can be classified into central tolerance, which occurs in the thymus and bone marrow for T and B cells, respectively, and peripheral tolerance, which occurs in peripheral secondary lymphoid tissues. A breakdown in the induction or maintenance of self-tolerance results in autoimmunity, although the exact mechanisms leading to disease remain elusive (Kamradt & Mitchison, 2001).

1.2.1 Central tolerance

Central tolerance occurs in the thymus for T cells and refers to the elimination of self-reactive cells from the repertoire. This process is essential for ensuring that developing T

cells are able to recognize a diverse array of foreign peptides while not reacting to self-antigens (Mouchess & Anderson, 2014). Developing thymocytes are educated with the help of stromal cells called thymic epithelial cells (TECs). TECs are subcategorized into cortical TECs (cTECs) or medullary TECs (mTECs) based on the physical compartments in which they reside. cTECs and mTECs play distinct and critical roles in the development and education of thymocytes.

T cells precursors come from the bone marrow and enter the thymus at the cortico-medullary junction (CMJ) via post-capillary venules where they proliferate in the thymic cortex to generate the early thymic progenitor (ETP) population. ETPs retain their expression of stem cell markers, such as cKit and Sca1, and are not yet committed to the T cell lineage (Wu *et al.*, 1991). Developing thymocytes can be divided into subpopulations based on their expression of the surface markers CD4 and CD8, where ~5% express neither (double negative (DN) thymocytes), ~80% express both CD4 and CD8 (double positive (DP) thymocytes), and the remaining ~15% express either CD4 or CD8 alone (Ceredig *et al.*, 1983).

DN thymocytes can be subdivided into four populations, DN1-DN4, based on their surface expression of CD44 and CD25 (D. I. Godfrey *et al.*, 1993). DN1 thymocytes (CD44⁺ CD25⁻) are a heterogeneous population further divided into five subsets based on their CD90 and CD127 expression (Porritt *et al.*, 2004). DN2 thymocytes (CD44⁺ CD25⁺) proliferate extensively (Kawamoto *et al.*, 2003) and begin to undergo rearrangement of their TCR genes, mediated by the recombinase activating genes *Rag1* and *Rag2* (Rothenberg *et al.*, 2008). It is in the DN2 stage that thymocytes become committed to the T cell lineage

(Hozumi *et al.*, 2008; Koch *et al.*, 2008), mediated by signals through *Notch1* (Pui *et al.*, 1999; Radtke *et al.*, 1999). Thymocytes undergo β -selection upon reaching the DN3 stage ($CD44^{\text{low}} CD25^+$), whereby the rearranged β -chain of the TCR must successfully pair with the invariant α -chain, forming the pre-TCR (Hogquist *et al.*, 2005). The DN3 stage of development can be further divided into DN3a and DN3b based on the expression of the transferrin receptor CD71, which is upregulated following β -selection (Kelly *et al.*, 2007). When TCR β chains pair with pre-TCR α chains, the pre-TCR is then assembled and expressed by DN3b cells, along with CD71. Thymocytes expressing the pre-TCR downregulate CD25 and are called DN4 cells ($CD44^- CD25^-$).

Following the DN4 stage, developing thymocytes express CD8 and are termed CD8 immature single positive (CD8 ISP) cells before then expressing CD4 to become double positive thymocytes (Xiong *et al.*, 2011). Immature DP thymocytes, still in the thymic cortex, are now able to rearrange their TCR α chain and are positively selected based on which MHC molecules they bind. Those that bind MHC-I will become CD8 $^+$ cells while those that bind MHC-II will become CD4 $^+$ cells, a process mediated by cortical thymic epithelial cells (cTECs) (Jameson *et al.*, 1995). Immature thymocytes that do not interact with MHC molecules after multiple rounds of TCR α chain rearrangement undergo apoptosis due to the absence of a survival signal, or “death by neglect”, and are thus removed from the repertoire. It is thought that up to 90% of DP thymocytes die in the thymus this way due to their expression of a useless TCR (Szondy *et al.*, 2012). cTECs can interact with 20 or more thymocytes at a time, forming multicellular complexes called thymic nurse cells (Wekerle *et al.*, 1980).

While cTECs do present self-peptides to developing thymocytes, they are limited to those that are normally found in the thymus. Thus, AIRE-expressing mTECs are required to expose single positive thymocytes to tissue-restricted antigens (TRAs), or antigens found outside the thymus in peripheral tissues. This process is referred to negative selection and takes place in the thymic medulla. Thymocytes that have survived selection in the cortex upregulate expression of chemokine receptor 7 (CCR7), which is critical for their migration into the medulla (Ueno *et al.*, 2004). Through the promiscuous gene expression (PGE) of TRAs, the collective population of mTECs stochastically express a broad range of tissue-specific genes encompassing nearly the entire molecular repertoire of an individual (Derbinski *et al.*, 2001).

Thymocytes binding self-antigens with high affinity are removed from the repertoire primarily through clonal deletion (Palmer, 2003), although tolerance can also be achieved through anergy induction (Hammerling *et al.*, 1991) and receptor editing (McGargill *et al.*, 2000; Wang *et al.*, 1998). Thymocytes with moderate TCR affinities for self-antigens are instructed by mTECs to become T regulatory cells (T_{reg}) (J. Lin *et al.*, 2016). Single positive thymocytes that survive the negative selection process then leave the thymus through the post-capillary venules of the CMJ and enter the periphery as recent thymic emigrants (RTE). It was once thought that T cells unilaterally migrate out of the thymus into the periphery, however studies have shown that mature T cells are able to re-enter the thymus (Michie *et al.*, 1988).

1.2.2 Peripheral tolerance

Although wide-ranging, central tolerance is nonetheless incomplete and requires additional fail-safe mechanisms to achieve effective immunological tolerance to self-antigens expressed in the periphery. Thymic deletion is only 60-70% effective at removing self-reactive T cells from the repertoire, leaving a significant number of autoreactive T cells to enter the peripheral T cell pool (Bouneaud *et al.*, 2000; Gallegos & Bevan, 2006). Several peripheral tolerance mechanisms serve as checkpoints to eliminate these autoreactive cells, thus avoiding the risk of autoimmune responses. These tolerance mechanisms include clonal deletion, anergy, and clonal diversion (or T_{reg} conversion). Alternatively, some autoreactive T cells never encounter their antigen and thus do not pose a risk of inciting autoimmunity, referred to as immunologic ignorance.

When autoreactive naïve T cells are activated by an antigen in the absence of costimulation, the majority undergo apoptosis (clonal deletion) while the remaining minority become unresponsive (anergic) (Kyburz *et al.*, 1993; Liblau *et al.*, 1996; Pape *et al.*, 1998). Studies where antigen was injected into TCR transgenic mice have provided evidence for the peripheral deletion of autoreactive clones via death receptors and the upregulation of pro-apoptotic molecules (Bonfoco *et al.*, 1998; Hildeman *et al.*, 2002; Pinkoski *et al.*, 2002; Sytwu *et al.*, 1996). Clonal deletion is thought to be mediated through the interaction of the Fas receptor with Fas ligand (Suda *et al.*, 1993). Anergy is a state of unresponsiveness that can occur in self-reactive T cells that repeatedly encounter their antigen, have prolonged exposure to their antigen, and/or receive antigenic stimulation in the absence of CD28 costimulatory receptor ligation (Choi & Schwartz, 2007; Harding *et al.*, 1992; Mueller *et al.*, 1989). Anergy can be maintained by the inhibitory receptors cytotoxic

T lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD-1) or through the proteolytic degradation of TCR-associated proteins by ubiquitin ligases (Choi & Schwartz, 2007; Khan & Ghazanfar, 2018; Kumar *et al.*, 2010).

In contrast to clonal deletion, which removes autoreactive T cells from the repertoire, clonal diversion, or T_{reg} conversion, instead shunts self-reactive T cell clones into a suppressive or regulatory role (Xing & Hogquist, 2012). T_{reg} are a subset of CD4⁺ T cells that express Forkhead box P3 (FOXP3) and suppress immune responses through a variety of mechanisms (Shevach, 2009). Mutations leading to FOXP3-deficiency result in immune dysregulation and multisystem autoimmunity in mice and humans (V. L. Godfrey *et al.*, 1991; Torgerson & Ochs, 2007) that is thought to be due to the uncontrolled activity of autoreactive effector CD4⁺ T cells (Suscovich *et al.*, 2012). Studies have shown that T_{reg} conversion is dependent on the recognition of self-reactive TCR ligands and while the majority of T_{reg} are derived from the thymus, T_{reg} conversion can also occur in the periphery (Curotto de Lafaille & Lafaille, 2009).

In the circumstance of immunologic ignorance cells can, under normal conditions, ignore their antigens, either because the amount of antigen is low enough as to not incite the activation of T cells (Ferber *et al.*, 1994), or because the antigen resides in an immunologically privileged site, such as the brain or eye, resulting in physical separation from potentially autoreactive T cells (Barker & Billingham, 1977). Immunologically privileged organs like the eye are susceptible to autoimmune attack by self-reactive cells that escape central tolerance mechanisms because peripheral tolerance mechanisms cannot operate efficiently in the absence of sequestered eye-specific antigens (Caspi,

2008). Indeed, lack of the interphotoreceptor retinoid-binding protein (IRBP) in the thymus due to AIRE-deficiency or IRBP-deficiency results in spontaneous eye-specific autoimmunity in mice (DeVoss *et al.*, 2006).

1.2.3 Autoimmunity

The initial TCR repertoire is stochastically generated by DNA recombination during thymocyte maturation and hence includes specificities that are potentially harmful as they may recognize self-antigens. The mechanisms outlined above to either remove self-reactive T cells from the repertoire or keep them in check are critical in maintaining a state of self-tolerance. A breakdown in any of these mechanisms can result in the attack of host tissues by autoreactive cells leading to severe damage, or autoimmune disease.

Autoimmune disorders are complex and heterogeneous and can be organ-specific or systemic in nature (Khan & Ghazanfar, 2018). Organ-specific autoimmune disorders target antigens with expression limited to one tissue or organ, such as pancreatic β -cell destruction in type 1 diabetes mellitus (DiMeglio *et al.*, 2018) or the attack of melanocytes in the depigmenting disorder vitiligo (Ezzedine *et al.*, 2015). Systemic autoimmune disorders target antigens that are ubiquitously found throughout the body and thus involve many organs.

Mutations in genes known to play a role in tolerance checkpoints result in autoimmune manifestations. For instance, autoimmune lymphoproliferative syndrome (ALPS) is an autoimmune disorder characterized by the increased proliferation of lymphocytes due to defective Fas-mediated apoptosis (Fisher *et al.*, 1995; Kuehn *et al.*, 2011; Rieux-Laucat *et*

al., 1995), while patients with CTLA4 deficiency suffer from immune dysregulation and lymphoproliferation (Kuehn *et al.*, 2014). The existence of monogenic autoimmune diseases, such as those caused by mutations in *AIRE* or *FOXP3* have provided insights into the underlying mechanisms of tolerance and autoimmunity (Theofilopoulos *et al.*, 2017).

1.3 AIRE

The autoimmune regulator (AIRE) is a transcription facilitator primarily expressed in the thymus in mature medullary thymic epithelial cells (mTECs). AIRE modulates, in collaboration with several other nuclear factors, the promiscuous expression of self-antigens that are typically detected in peripheral tissues. The ectopic expression, processing, and MHC-mediated presentation of TRAs ensures that maturing T cells are exposed in the thymus to a wide variety of peptides derived from self-antigens which are employed to delete thymocytes with a strongly self-reactive TCR specificity, thus successfully establishing central tolerance (Sansom *et al.*, 2014). It has been shown that the specific set of TRAs induced by AIRE can vary depending on the cellular environment (Guerau-de-Arellano *et al.*, 2008), which may explain the varied autoimmune phenotypes observed in humans (Ferre *et al.*, 2016; Peterson *et al.*, 1998) and mice (Jiang *et al.*, 2005) with AIRE-deficiency.

AIRE expression is not confined to the population of mature mTECs but can also be detected in thymic B cells and outside the confines of the thymus in a subpopulation of dendritic-like hematopoietic cells within peripheral lymphoid tissues. However, both precise cell phenotypes and micro-anatomical locations of these extra thymic AIRE-expressing cells (eTACs) remain, at present, insufficiently defined (Eldershaw *et al.*, 2011).

1.3.1 Promiscuous gene expression

To establish tolerance to self, it is critical that T cells are exposed to antigens that are expressed outside the thymus in peripheral tissues. This is accomplished through the promiscuous expression of TRAs, primarily by mTECs in the thymus. Failure of mTECs to express even a single TRA appropriately results in organ-specific autoimmunity. For example, loss of thymic interphotoreceptor retinoid-binding protein (IRBP) expression triggers spontaneous eye-specific autoimmunity in mice (DeVoss *et al.*, 2006), while loss of thymic insulin expression predisposes mice to autoimmune diabetes (Chentoufi & Polychronakos, 2002; Fan *et al.*, 2009). Many tissue-specific antigens have been detected in the thymus; from the neuroendocrine antigens oxytocin and neurophysin (Geenen *et al.*, 1986), to the endocrine regulator somatostatin (Fuller & Verity, 1989).

In addition to AIRE, another transcription regulator, FEZ family Zinc Finger 2 (FEZF2), is licensed to express TRAs in mTECs. FEZF2-deficient mice develop severe autoimmunity mediated by cell infiltration of peripheral tissues and autoantibody production (Takaba *et al.*, 2015). AIRE expression is thought to be regulated through the RANK and CD40 pathways (Desanti *et al.*, 2012) while FEZF2 expression is regulated through the LT β R pathway, and while some TRA genes are both AIRE-dependent and FEZF2-dependent, the majority are controlled by one or the other (Takaba *et al.*, 2015). In contrast to AIRE, which lacks an obvious DNA binding domain (Abramson *et al.*, 2010), FEZF2 is thought to play a direct role in transcription regulation (Takaba *et al.*, 2015). Additionally, AIRE is required for mTEC differentiation (Yano *et al.*, 2008), while FEZF2 is dispensable in this process (Takaba *et al.*, 2015).

1.3.2 Mouse models of AIRE-deficiency

Several mouse models have been generated in an attempt to recapitulate the clinical manifestations seen in patients with *AIRE* mutations. The genetic background of mice has been shown to influence the severity of autoimmunity, with the most severe manifestations observed in AIRE-deficient (*Aire*^{-/-}) mice on the non-obese diabetic (NOD) background and most mild phenotype seen in *Aire*^{-/-} mice on the C57BL/6 (B6) background (Jiang *et al.*, 2005). In addition to differential severities, genetically diverse mice also exhibit variation in target organs. *Aire*^{-/-} NOD mice develop organ-specific autoantibodies against multiple organs, including the pancreas, eye, salivary gland, and stomach, while *Aire*^{-/-} BALB/c and *Aire*^{-/-} B6 mice are prone to autoantibodies against the stomach and eye, respectively. Phenotypic variations are observed not only between *Aire*^{-/-} mice on different genetic backgrounds, but also in *Aire*^{-/-} mice backcrossed to the same genetic background. For instance, mice derived from 129 embryonic stem (ES) cells, even when backcrossed to C57BL/6, display a different phenotype from those derived from C57BL/6 ES cells (Hubert *et al.*, 2009). This has been attributed to the retention of the flanking ES cell-derived chromosome (Schalkwyk *et al.*, 2007) which is enough to maintain the phenotype of the ES cell strain.

AIRE-deficient mice on a C57BL/6 background have increased mature-to-immature mTEC ratios despite the total number of mTECs being unchanged from AIRE-sufficient controls (Anderson *et al.*, 2002; Hubert *et al.*, 2009; Ramsey *et al.*, 2002). AIRE expression likely contributes to mature mTEC turnover, as AIRE-deficient mature mTECs have an increased capacity for survival than do wildtype mature mTECs (Gray *et al.*, 2007), perhaps to

increase the array of TRAs expressed throughout an organism's lifespan. Defects in the thymic negative selection of single positive CD4 T cells have been observed (Anderson *et al.*, 2005; Anderson *et al.*, 2002), as have increased activation/memory markers on peripheral CD4 T cells (Anderson *et al.*, 2002; Hubert *et al.*, 2009). TCR repertoire analyses of splenic-T cells have revealed minimal abnormalities (Ramsey *et al.*, 2002), however more recent studies specifically focused on pathogenic T cells infiltrating target organs have demonstrated that infiltrating T cells clonotypes in *Aire*^{-/-} mice match those preferentially expressed by T_{reg} in wildtype mice (Malchow *et al.*, 2016). This suggests a role for AIRE in the diversion of autoreactive T cells into T_{reg}.

Autoimmune manifestations in AIRE-deficient mice on a C57BL/6 background vary, with the highest prevalence of cell infiltration by histology noted in salivary glands, eyes, prostate, ovaries, lung, and liver (Anderson *et al.*, 2002; Hassler *et al.*, 2006; Hubert *et al.*, 2009; Jiang *et al.*, 2005; Kuroda *et al.*, 2005; Ramsey *et al.*, 2002). Gonadal failure leading to infertility has been observed in 85% of homozygous AIRE-deficient mice (Kuroda *et al.*, 2005; Ramsey *et al.*, 2002), necessitating the use of heterozygous mice for breeding purposes.

1.3.3 APECED

Human AIRE is a 13 kDa protein encoded by a gene located on chromosome 21 in the q22.3 region. In 1997 it was identified that mutations in the gene encoding AIRE result in a rare autoimmune disease known in humans as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, also known as autoimmune polyendocrinopathy syndrome 1, APS-1) (Nagamine *et al.*, 1997). The clinical presentation of APECED is characteristically marked by the triad of chronic mucocutaneous candidiasis, hypoparathyroidism, and

adrenal insufficiency, although the criteria required for the diagnosis of APECED have recently been expanded (Ferre *et al.*, 2016). As a consequence of a lack of AIRE-dependent expression of TRAs, APECED patients develop biologically active autoantibodies to many tissue-specific proteins and cytokines (Meyer *et al.*, 2016), implicating antibody-mediated organ-specific damage to cause (at least in part) organ destruction and loss of function.

There are parallels in both clinical presentation and laboratory findings between APECED and diseases that result in a loss of regular thymic stromal function. For example, thymic epithelial cell tumors do not adequately express AIRE, and consequently lack a normal complementation of TRAs, including acetylcholine receptor, titin, and other muscle antigens, resulting in a higher frequency of myasthenia gravis in patients with thymoma (Wolff *et al.*, 2014). Similarly, ineffective thymopoiesis leading to defects in T cell maturation due to a partial loss of RAG1 or RAG2 function can lead to diminished AIRE expression, inadequate expression of TRAs, and thus, autoimmunity (Milner *et al.*, 2008).

1.4 Anti-cytokine autoantibodies

Anti-cytokine autoantibodies have been described in many contexts and, when neutralizing, often present as phenocopies of genetic mutations within the cognate cytokine pathway. For instance, both mutations in the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor and neutralizing anti-GM-CSF autoantibodies cause the lung disorder pulmonary alveolar proteinosis (Kitamura *et al.*, 1999; Martinez-Moczygemba *et al.*, 2008; S. H. Rosen *et al.*, 1958; T. Suzuki *et al.*, 2008). Similarly, interferon-gamma (IFN γ) receptor mutation and anti-IFN γ autoantibody patients are susceptible to the same infectious pathogens due to the blockade of IFN γ signaling

(Browne, Burbelo, *et al.*, 2012; Doffinger *et al.*, 2004; Dorman & Holland, 2000; Hoflich *et al.*, 2004). Anti-cytokine autoantibodies have been reported in both health and disease, with varying degrees of pathogenicity, likely dependent on autoantibody titer and avidity which may wax and wane over time (Browne & Holland, 2010).

1.4.1 Anti-cytokine autoantibodies of known etiology

Anti-cytokine autoantibodies occur in diseases in which there is known T cell developmental impairment, such as APECED, thymic malignancies, and RAG deficiency. Notably, the autoantibody profiles of these patients are both overlapping and distinctly different. For instance, APECED patients have autoantibodies to type I IFNs and the IL-17 family of cytokines (IL-17A, IL-17F, and IL-22) (Meager *et al.*, 2006; Meyer *et al.*, 2016; Puel *et al.*, 2010), while thymoma patients share these autoreactivities but also have autoantibodies against IL-12 (Bagnato *et al.*, 2004; Burbelo *et al.*, 2010; Meager *et al.*, 1997; Meager *et al.*, 2003; Shiono *et al.*, 2003; Yoshikawa *et al.*, 2006; W. Zhang *et al.*, 2003). Anti-cytokine autoantibodies are less prevalent in RAG deficiency but have been detected in patients with delayed-onset combined immune deficiency and granulomatous/autoimmune manifestations against type I IFNs, and occasionally IL-12 and IL-17 (Walter *et al.*, 2015).

Patients with thymic malignancies have been shown to have impaired AIRE expression (Cheng *et al.*, 2010; Strobel *et al.*, 2007; E. Suzuki *et al.*, 2008), while patients with RAG deficiency have abnormalities in thymic architecture, mTEC differentiation, and AIRE expression (Cavadini *et al.*, 2005; Poliani *et al.*, 2009). It should be noted, however, that anti-cytokine autoantibodies are not found in all patients with RAG deficiency. In fact,

there is an association between the presence of neutralizing anti-IFN α , -IFN ω , and -IL-12 autoantibodies and a history of severe viral infections (Walter *et al.*, 2015).

It was thought that neutralizing IL-17A/IL-17F/IL-22 autoantibodies conferred increased susceptibility to chronic mucocutaneous candidiasis (CMC) (Kisand *et al.*, 2010; Puel *et al.*, 2010), a common feature of APECED and thymoma patients. However, it was later observed that IL-17 autoantibodies did not track with CMC in APECED patients and it has been recently demonstrated that increased IFN γ production, rather than impaired Th17 responses, at the oral mucosa led to mucosal fungal infection susceptibility (Break *et al.*, 2021).

1.4.2 Anti-cytokine autoantibodies of unknown etiology

Other anti-cytokine autoantibodies are of unknown etiology, yet their contributions to disease are well established. Neutralizing autoantibodies against GM-CSF have been recognized to cause an autoimmune form of pulmonary alveolar proteinosis (PAP), a rare lung disorder characterized by the accumulation of proteinaceous material in the alveolar spaces (Kitamura *et al.*, 1999; S. H. Rosen *et al.*, 1958). This proteinaceous accumulation is due to defective surfactant clearance by pulmonary macrophages, a GM-CSF-dependent process (Trapnell *et al.*, 2009; Yoshida *et al.*, 2001). Mutations in the GM-CSF receptor cause a hereditary form of PAP (Martinez-Moczygemba *et al.*, 2008; T. Suzuki *et al.*, 2008) while genetically GM-CSF-deficient mice develop a lung phenotype similar to that of human PAP (Dranoff *et al.*, 1994; Stanley *et al.*, 1994). Additionally, injection of pathogenic anti-GM-CSF autoantibodies into non-human primates recapitulated the lung pathology of PAP patients (Sakagami *et al.*, 2009), confirming the causative nature of anti-GM-CSF

autoantibodies in autoimmune PAP. Later, anti-GM-CSF autoantibodies were found to be a risk factor for cryptococcal meningitis and disseminated nocardiosis (L. B. Rosen *et al.*, 2013; L. B. Rosen *et al.*, 2015).

IFN γ is a cytokine produced by T cells and recognized by macrophages which express the IFN γ receptor (Dorman & Holland, 2000). IFN γ signaling plays a critical role in host defense against intracellular pathogens and neutralizing anti-IFN γ autoantibodies are associated with severe opportunistic infections in otherwise immunocompetent individuals (Browne, Burbelo, *et al.*, 2012; Doffinger *et al.*, 2004; Hoflich *et al.*, 2004; Kampmann *et al.*, 2005). There is a known human leukocyte antigen (HLA) association between HLA-DRB1*16:02 and HLA-DQB1*05:02 and neutralizing anti-IFN γ autoantibodies in patients from Southeast Asia (Chi *et al.*, 2013; Ku *et al.*, 2016). However, this observation does not explain the occurrence of these autoantibodies in all patients. It has also been reported that exposure to *Aspergillus* can cause anti-IFN γ autoantibodies, due to a highly homologous epitope shared between the *Aspergillus* Noc2 protein and a region of the C terminus of IFN γ , which is recognized by anti-IFN γ autoantibodies (C. H. Lin *et al.*, 2016).

Beyond molecular mimicry, it is theoretically possible that infection causes upregulation in IFN γ generation, inducing production of neutralizing antibodies to IFN γ in the environment, leading to increased infection susceptibility. Similarly, in the case of anti-GM-CSF autoantibodies, infection by *Nocardia* led to autocrine release of GM-CSF (L. B. Rosen *et al.*, 2015), which could trigger the production of neutralizing anti-GM-CSF autoantibodies, leading to impairment of GM-CSF-dependent surfactant clearance by alveolar macrophages, ultimately leading to lung damage and PAP. Against this hypothesis, APECED

patients, who have been shown to have increased levels of IFN γ associated with organ damage (Break *et al.*, 2021), do not develop autoantibodies to IFN γ . This implies the presence of a specific inciting mechanism(s) that first causes the production of autoantibodies against a certain cytokine target, which could then be exacerbated by autocrine production of said cytokine, perpetuating epitope spreading, affinity maturation, and increased autoantibody titers.

Coronavirus disease 2019 (COVID-19) is caused by infection with the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The clinical severity of COVID-19 varies immensely between individuals, ranging from rapid death to completely asymptomatic infection. Although it is observed that older age, male sex, and presence of underlying medical conditions put individuals at greater risk of morbidity and mortality from SARS-CoV-2 infection, the specific mechanisms that lead to severe COVID-19 are still being elucidated. Anti-cytokine autoantibodies have been associated with severe viral infections (Ansari *et al.*, 2020; Walter *et al.*, 2015), however, their role in COVID-19 pathogenesis remains to be examined.

1.5 Hypotheses & Aims

AIRE expression is detected in mature mTECs, activated B cells, and eTACs. However, little is known about the functional importance of AIRE expression outside the thymus. This research project aims to identify and characterize eTACs and elucidate their functional role using cell lineage-specific AIRE-deficient transgenic mice to better understand the mechanisms of AIRE-regulated tolerance. Based on a growing body of literature and preliminary data, I hypothesize that expression of AIRE in the periphery plays a non-

redundant role with thymic AIRE expression in establishing and maintaining immune tolerance.

During the course of this project, the COVID-19 pandemic temporarily restricted all non-COVID-19-related laboratory work. Thus, my NIH laboratory engaged in a research project relevant to the pandemic, aimed at examining the innate and adaptive immune responses during acute COVID-19. The Holland lab had previously shown the role of neutralizing anti-cytokine autoantibodies in the susceptibility to various infectious diseases. Therefore, we sought to assess blood samples from COVID-19 patients for the presence of anti-cytokine autoantibodies. Because of my past experience in anti-cytokine autoantibody screening and functional testing, and in light of the extraordinary circumstances of the global pandemic, I was asked to spearhead this project. This effort resulted in a publication in *Science*, on which I am co-first author, and the findings have been included as the final chapter of my thesis with the consent of both of my mentors.

My research objectives are:

1. To identify extra-thymic AIRE-expressing cells (eTACs) in peripheral lymphoid tissues and investigate their functional role in wildtype mice.
2. To generate and assess cell lineage-specific loss-of-AIRE mutant mice for thymocyte development, T cell maturation, and autoimmunity.
3. To characterize the anti-cytokine autoantibody profiles of healthy individuals, APECED patients, and individuals with SARS-CoV-2 infection.

2 Materials & Methods

2.1 Mice

Mouse colonies were maintained at the University of Oxford and at the National Institutes of Health in accordance with UK and US regulations.

2.1.1 C57BL/6

For experiments performed in the UK, C57BL/6 mice were obtained from the in-house colony maintained by the University of Oxford. For experiments performed in the US, C57BL/6 mice were purchased from Jackson Laboratories and maintained as a colony in the NIH Animal Facility 14BS.

2.1.2 OT.1

C57BL/6NAi-[Tg]TCR OT-1-[KO]RAG1 (OT.1) mice were initially purchased from Taconic and maintained as a colony by the NIAID Intramural Research Repository. These mice are homozygous for a transgene encoding a TCR specific for ovalbumin 257-264 (SIINFEKL) on a recombination activating gene 1 (RAG1)-deficient background. RAG1-deficiency ensures these mice do not develop mature T or B cells with endogenous receptor expression.

2.1.3 *Aire*^{-/-}

Aire^{-/-} (*Aire*^{tm1.1Doi/J}) mice were obtained from Jackson Laboratories (stock #004743). These mice were generated by Christophe Benoist and Diane Mathis by the insertion of a *loxP*-flanked P_{gk}-neo cassette, which drives expression of a neomycin-resistant gene under control of phosphoglycerate kinase I (P_{gk}), an enzyme necessary for glycolysis. Embryonic

stem (ES) cells were transfected by electroporation with the targeting construct and correctly targeted embryonic stem cells were injected into C57BL/6 blastocytes. Chimeric mice were then crossed to mice expressing Cre under control of a human cytomegalovirus (CMV) (CMV(Cre) mice) which allowed for deletion of the *loxP*-flanked region in all tissues, resulting in the excision of exon 2 of *Aire* (Anderson *et al.*, 2002).

2.1.4 *Aire*(flox/flox)

Aire(fl/fl) mice were a generous gift from Dominik Filipp who generated them by inserting a *loxP*-flanked Pgk-neo/kanR cassette, which drives expression of the neomycin/kanamycin-resistance gene under control of Pgk, before exon 6 and a *loxP* site after exon 7 of *Aire*. ES cells were transfected by electroporation with the targeting vector and chimeras were bred to C57BL/6NCrl mice. The Pgk-neo/kanR cassette was permanently removed and correctly targeted mice retaining *loxP*-flanked exons 6-7 were selected (Dobes *et al.*, 2018). During the course of this project, *Aire*(fl/fl) mice were made available through Jackson Laboratories (stock #031409).

2.1.5 *Foxn1*(Cre)

Foxn1(Cre) mice were generated by Saulius Zuklys via the insertion of the iCre coding sequence into a *Foxn1* P1 artificial chromosome clone (RPC121-436p24) which was then injected into pronuclei of fertilized B6D2F1 oocytes (Zuklys *et al.*, 2009). Heterozygous mutants of this B6;D2-Tg(*Foxn1*-Cre)8Ghr transgenic line, designated *Foxn1*(Cre), were crossed to *Aire*(fl/fl) mice to generate mice deficient for AIRE in FOXN1-expressing cells (*Aire*(fl/fl):*Foxn1*(Cre+)).

2.1.6 *Mb1*(Cre)

Mb1(Cre) mice were purchased from Jackson Laboratories (stock #020505). They were originally generated by Elias Hobeika via the insertion of a flip-recombinase target (FRT) site flanked neomycin phosphotransferase-thymidine kinase (neo-tk) cassette, followed by a SV40 poly(A) signal which replaced exons 2-3 and resulted in the truncation of exon 1. ES cells were transfected by electroporation and correctly targeted ES cells were injected into C57BL/6J blastocytes. The FRT flanked neo cassette was then excised by crossing mice to FLPe delete mice (Branda & Dymecki, 2004) to yield heterozygous mutants that express Cre-recombinase in MB1-expressing cells (Hobeika *et al.*, 2006). Heterozygous *Mb1*(Cre+) mice were crossed to *Aire*(fl/fl) mice to generate mice deficient for AIRE in MB1-expressing cells (*Aire*(fl/fl):*Mb1*(Cre+)).

2.1.7 *Rorc*(Cre)

Rorc(Cre) mice were purchased from Jackson Laboratories (stock #022791). They were originally generated by Dan Littman by the modification of the bacterial artificial chromosome (BAC) clone I15 to express Cre-recombinase under control of the mouse *Rorc* gene. The vector was injected into FBV embryos and backcrossed onto the C57BL/6 background (Eberl & Littman, 2004). Heterozygous *Rorc*(Cre+) mice were crossed to *Aire*(fl/fl) mice to generate (*Aire*(fl/fl):*Rorc*(Cre+)).

2.2 General reagents

Various standard buffers and media were made in-house and used throughout different experiments. Table 2.1 lists standard buffer and media recipes.

Table 2.1. Standard buffers and media

Buffer or media	Ingredients	Company	Final concentration
Fluorescence-activated cell sorting (FACS) buffer	Phosphate-buffered saline (PBS)	Lonza	1X
	Fetal calf serum (FCS)	Corning	2%
Liberase TL digestion buffer	PBS	Lonza	1X
	Liberase TL	Roche	75µg/mL
	DNase I	Roche	300µg/mL
Erythrocyte lysis buffer	Ammonium chloride (NH ₄ Cl)	Sigma	1.66%
Magnetic-activated cell sorting (MACS) buffer, degassed	PBS	Lonza	1X
	Bovine serum albumin (BSA)	Sigma	0.5%
	Ethylenediaminetetraacetic acid (EDTA)	Sigma	2 mM
Cell culture media	RPMI	Gibco	1X
	FCS	Corning	10%
	Penicillin-streptomycin	Sigma	1X
	L-glutamine	Sigma	1X
	HEPES	Corning	10 mM
	Sodium pyruvate	Gibco	1 mM
	Non-essential amino acids	Gibco	1X
	2-mercaptoethanol	Sigma	50 µM

2.3 Genotyping

2.3.1 DNA isolation and amplification

Genomic DNA was extracted from ear punches using BioLink's FastLink PCR Genotyping Kit.

Tissue samples were incubated in a final volume of 100µL of Fastlink lysis buffer for 5 minutes at 75°C. The proteinase was then inactivated via a 10-minute incubation at 95°C.

Prior to centrifugation to remove debris, 900µL of water was added to each sample. The supernatant was then collected, and genomic DNA amplified by polymerase chain reaction (PCR) under the standard or touchdown conditions listed below (Table 2.3 and Table 2.4, respectively), where the annealing temperature (T_a) is 5°C below the lowest melting temperature (T_m) of the primer pair. Biolink 2X Genotyping Mastermix, 0.5µL of 10 mM forward primer, 0.5µL of 10 mM reverse primer, and 1.5µL of genomic DNA was combined

and brought up to a final volume of 25µL with nuclease-free water. Primers used for genotyping are listed in Table 2.2.

Table 2.2. Genotyping primers

Strain	Primer type	Sequence 5' to 3'	Approximate product size
<i>Aire</i> ^{-/-}	Common fwd	AGACTAGGTGTTCCCTCCCAACCTCAG	Mutant= 140 bp Het= 140 bp, 195 bp Wildtype= 195 bp
	Wildtype rev	GGAGACTTGCCTATTCCTGTC	
	mutant rev	CCGGCGGATTTGTCCTAC	
<i>Aire</i> (fl/fl)	<i>loxP1</i> fwd	ATCTGCCATTGGGCTCAACA	Mutant= 900 bp Het= 900 bp, 600 bp, 300 bp Wildtype= 600 bp, 300 bp
	<i>loxP1</i> rev	GTCCCTTCCCACACCTTGAC	
	<i>loxP2</i> fwd	CTAGGGCTAACGGGAAACCC	Mutant= 555 bp Het= 555 bp, 336 bp, 219 bp Wildtype= 219 bp
	<i>loxP2</i> rev	CTTAGGAATGCCCTCAGAGA	
<i>Foxn1</i> (Cre)	Transgene fwd	TCTGATGAAGTCAGGAAGAACC	Transgene= 500 bp Int ctl= 220 bp
	Transgene rev	GAGATGTCCTTCACTCTGATTC	
	Int ctl fwd	CAAATGTTGCTTGTCTGGTG	
	Int ctl rev	GTCAGTCGAGTGCACAGTTT	
<i>Mb1</i> (Cre)	Mutant fwd	CATTTTCGAGGGAGCTTCA	Mutant= 230 bp Het= 197 bp, 230 bp Wildtype= 197 bp
	Wildtype fwd	CTCTTTACCTTCCAAGCACTGA	
	Common rev	ACTGAGGCAGGAGGATTGG	
<i>Rorc</i> (Cre)	Transgene fwd	TTCCGGTTATTCAACTTGCAC	Transgene= 250 bp Int ctl= 324 bp
	Transgene rev	TGTCCTGGGCTACCCTACTG	
	Int ctl fwd	CTAGGCCACAGAATTGAAAGATCT	
	Int ctl rev	GTAGGTGGAAATTCTAGCATCATCC	

Abbreviations: fwd= forward; rev= reverse; Int ctl= Internal positive control; Het= Heterozygote; bp= base pairs

Table 2.3. Thermocycler conditions for standard genotyping PCR

Step	# of cycles	Temperature	Duration	Comments
1	1	94°C	5 min	
2	40	94°C	30 sec	$T_a = T_m - 5^\circ\text{C}$
		T_a	30 sec	
		72°C	1 min 15 sec	
3	1	72°C	5 min	
4	1	4°C	hold	

Table 2.4. Thermocycler conditions for touchdown genotyping PCR

Step	# of cycles	Temperature	Duration	Comments
1	1	94°C	2 min	
2	10	94°C	20 sec	
		65°C	15 sec	-0.5°C per cycle decrease
		68°C	10 sec	
3	28	94°C	15 sec	
		60°C	15 sec	
		72°C	10 sec	
4	1	72°C	2 min	
5	1	4°C	hold	

2.3.2 Restriction enzyme digestion

For the *Aire*(fl/fl) mice, the insertion of the *loxP1* and *loxP2* sites into the *Aire* genomic locus disrupted the restriction sites *MfeI* and *Acil*, respectively. Thus, a restriction enzyme digestion step was required for genotyping purposes. After PCR amplification, 4µL of PCR product, 2µL of CutSmart buffer (New England Biolabs, or NEB), 0.5µL of restriction enzyme (NEB), and 13.5µL of water were combined and incubated at 37°C for 1 hour.

2.3.3 Gel electrophoresis

Agarose gels were prepared at 1-2% (depending on expected product size) using UltraPure Agarose (Invitrogen) in 1X Tris-borate-EDTA (TBE) buffer (Corning) with 1X GelRed dye (ThermoScientific). The agarose solution was poured into a gel box fitted with a comb to form wells and allowed to set at room temperature until solidified. The comb was removed and 10µL of amplified PCR product (following restriction enzyme digestion for *Aire*(fl/fl) genotyping) was loaded into wells. The φX174 DNA-Hea III digest DNA ladder (NEB) was used to determine the size of the PCR products by comparison. Gels were visualized on a ChemiDoc MP system using Image Lab software (Bio-Rad).

2.4 Flow Cytometry

2.4.1 Lymph node digestion and staining

Peripheral lymph nodes (inguinal, axillary, sub-axillary, superficial parotid, cervical, and mandibular) from 6- to 12-week-old wildtype C57BL/6 mice were isolated, pooled, and digested at 37°C in Liberase TL digestion buffer (300µg/mL DNase, 75µg/mL Liberase TL in 1x PBS) with gentle agitation. Suspensions were subjected to intermittent up-and-down pipetting with pipette tips of decreasing bore size (first with a p1000 pipette tip that had been cut 4mm from the bottom, then a standard p1000 pipette tip, and finally a p200 pipette tip). Supernatants were collected at 10-minute intervals into FACS buffer on ice and digestion buffer replaced, for a total of 3 rounds or until tissue clumps were no longer visible. Cells were filtered, counted, and stained for FACS with the following fluorochrome-conjugated antibodies (CD45, EpCAM, MHC-II, CD40, CD4, CD8a, CD11b, CD11c) and the following biotin-conjugated antibodies (CD31, CD19, CD14, NK1.1, CD49b, Gr-1, Ter119, TCRγδ, TCRβ), the latter were revealed by fluorochrome-conjugated Streptavidin. 4',6-diamidino-2-phenylindole (DAPI) was added immediately prior to sorting as a marker of cell viability.

2.4.2 Splenocyte isolation and staining

Spleens from at least three 20-week-old wildtype or *Aire*(fl/fl):(Cre-) mice and at least three 20-week-old *Aire*^{-/-} or *Aire*(fl/fl):(Cre+) C57BL/6 mice and were isolated and mechanically digested to yield a single cell suspension. Erythrocytes were lysed in 1.66% NH₄Cl lysis buffer and cells were filtered, counted, and incubated with fluorophore-conjugated surface markers (B220, CD73, CD24, Qa-2, CD44, TCRβ, CD25, FR4, CD62L, CD4, CD8a) for 25 minutes at 4°C. Cells were fixed and permeabilized with the Foxp3/Transcription Factor

Staining Buffer Set (ThermoFisher) according to the manufacturer's instructions and Foxp3 antibody was incubated with cells for 1 hour at 4°C. Viable cells were identified using Live/Dead Aqua fixable viability dye. Cells were acquired on a BD LSR Fortessa cytometer and data analyzed using FlowJo.

2.4.3 Thymocyte isolation and staining

Thymi from at least three 20-week-old wildtype or *Aire*(fl/fl):(Cre-) mice and at least three 20-week-old *Aire*^{-/-} or *Aire*(fl/fl):(Cre+) C57BL/6 mice and were isolated and mechanically digested to yield a single cell suspension. Cells were filtered, counted, and incubated for 30 minutes with CCR7 antibody at 37°C. Without washing, the remaining fluorophore-conjugated surface markers (early thymocyte maturation panel: CD69, CD44, CD24, Sca1, cKit, CD25, TCRβ, CD4, CD8a, CD71; negative selection panel: CD69, PD-1, CD44, CD24, TCRβ, CD25, CD4, CD5, CD8a) and biotinylated lineage markers (early thymocyte maturation panel: CD19, Gr-1, CD49b, NK1.1, TCRγδ, Ter119, MHC-II, CD11b, CD11c, F4/80) were added and cells were incubated for 25 minutes at 4°C. For the early thymocyte maturation panel, biotinylated lineage markers were visualized with streptavidin conjugated to BV711, which was incubated with cells for 15 minutes at 4°C. For the negative selection panel, cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher) according to the manufacturer's instructions and intranuclear antibodies (Helios and Foxp3) were incubated with cells for 1 hour at 4°C. Viable cells were identified using Live/Dead Aqua fixable viability dye. Cells were acquired on a BD LSR Fortessa cytometer and data analyzed using FlowJo.

2.4.4 mTEC isolation and staining

Thymi were collected from wildtype mice and digested with Liberase TL Digestion Buffer (300µg/mL DNase, 75µg/mL Liberase TL in 1x PBS). Cells were filtered, counted and enriched for stromal cells on the AutoMACS using Miltenyi CD45 beads. The CD45-negative fraction was stained for FACS with the following fluorophore-conjugated antibodies (CD45, EpCAM, Ly51, CD80, MHC II, CD4, CD8a), and UEA-1 lectin. DAPI was used to identify viable cells. mTEC^{hi}s were gated as follows: CD45⁻ CD4⁻ CD8a⁻ EpCAM⁺ Ly51⁻ UEA1⁺ MHC-II^{hi} CD80^{hi}.

Table 2.5. FACS antibodies

Antibody Target	Clone	Conjugate(s)	Dilution	Isotype	Source
AIRE	5H12	AF488	1 to 1K	Rat IgG2c, κ	ThermoScientific (eBio)
B220	RA3-6B2	AF700, BV785	1 to 500	Rat IgG2a, κ	BioLegend
CCR7	4B12	PE-Cy7	1 to 200	Rat IgG2a, κ	BioLegend
CD11b	M1/70	biotin, PE-Cy5	1 to 500	Rat IgG2b, κ	BioLegend
CD11c	N418	APC-Cy7, biotin	1 to 1K	Armenian hamster IgG	ThermoScientific (eBio)
CD14	Sa14-2	biotin	1 to 1K	Rat IgG2a, κ	BioLegend
CD19	1D3	APC, biotin	1 to 1K	Rat IgG2a, κ	ThermoScientific (eBio)
CD24	M1/69	APC/Fire-750	1 to 500	Rat IgG2b, κ	BioLegend
CD25	PC61	BV605, PE-Cy5	1 to 1K	Rat IgG1, λ	BioLegend
CD31	390	biotin	1 to 1K	Rat IgG2a, κ	BioLegend
CD4	RM4-5	APC-Cy7, BV510, BV488, PE-Cy5	1 to 1K	Rat IgG2a, κ	BioLegend
CD40	HM40-3	AF647, PE	1 to 500	Armenian hamster IgM	BioLegend
CD44	IM7	BV421, PE-eF610	1 to 500	Rat IgG2b, κ	BioLegend, ThermoScientific (eBio)
CD45	30-F11	AF700	1 to 1K	Rat IgG2b, κ	BioLegend
CD49b	DX5	biotin	1 to 1K	Rat IgM, κ	BioLegend
CD5	53-7.3	PerCP-Cy5.5	1 to 1K	Rat IgG2a, κ	BioLegend
CD62L	MEL-14	PE-Cy7	1 to 1K	Rat IgG2a, κ	BioLegend
CD69	H1.2F3	BV785	1 to 500	Armenian hamster IgG	BioLegend

CD71	RI7217	PE	1 to 200	Rat IgG2a, κ	BioLegend
CD73	TY/11.8	PE	1 to 500	Rat IgG1, κ	BioLegend
CD80	16-10A1	AF647	1 to 500	Armenian hamster IgG	BioLegend
CD86	GL-1	PE-Cy7	1 to 1K	Rat IgG2a, κ	BioLegend
CD8a	53-6.7	AF700, PE, PE/Dazzle-594	1 to 2K	Rat IgG2a, κ	BioLegend
cKit (CD117)	2B8	AF647, PE-Cy5	1 to 500	Rat IgG2b, κ	BioLegend
EpCAM	G8.8	PerCP-Cy5.5	1 to 1K	Rat IgG2a, κ	BioLegend
F4/80	BM8	biotin	1 to 1K	Rat IgG2a, κ	BioLegend
FoxP3	FJK-16s	FITC, PE	1 to 1K	Rat IgG2a, κ	BioLegend
FR4	12A5	PerCP-Cy5.5	1 to 1K	Rat IgG2b, κ	BioLegend
Gr-1	RB6-8C5	biotin	1 to 1K	Rat IgG2b, κ	BioLegend
Helios	22F6	AF647	1 to 160	Armenian hamster IgG	BioLegend
isotype for AIRE	RTK4174	AF488	1 to 1K	Rat IgG2c, κ	BioLegend
Ly51	6C3	PE	1 to 5K	Rat IgG2a, κ	BioLegend
MHC-II (I-A/I-E)	M5/114.15.2	biotin, BV421, BV785, FITC	1 to 1K	Rat IgG2b, κ	BioLegend
MHC-II (I-A/I-E)	M5/114.15.2	APC-eF780	1 to 1K	Rat IgG2b, κ	ThermoScientific (eBio)
NK1.1	PK136	biotin	1 to 1K	Mouse IgG2a, κ	BioLegend
PD-1	29F.1A12	BV605	1 to 500	Rat IgG2a, κ	BioLegend
Qa-2	695H1-9-9	AF647	1 to 500	Mouse IgG2a, κ	BioLegend
Sca-1 (Ly-6A/E)	D7	AF700	1 to 500	Rat IgG2a, κ	BioLegend
TCRβ	H57-597	biotin, BV711, PE-Cy5	1 to 500	Armenian hamster IgG	BioLegend
TCRγδ	GL3	biotin	1 to 1K	Armenian hamster IgG	BioLegend
Ter119	TER-119	biotin	1 to 1K	Rat IgG2b, κ	BioLegend

Abbreviations: AF= AlexaFluor; BV= BrilliantViolet eF = eFluor; eBio= eBiosciences

Table 2.6. Other FACS reagents

Description	Conjugate(s)	Dilution	Source
DAPI	n/a	1 to 100K	ThermoScientific
CellTrace Violet	n/a	1 to 1K	ThermoScientific
Live/Dead Aqua	n/a	1 to 1K	ThermoScientific
Live/Dead NearIR	n/a	1 to 1K	ThermoScientific
Streptavidin	BV605, BV711	1 to 500	BioLegend
UEA-1 lectin	biotin	1 to 5K	Vector Laboratories

Viability staining was performed using Live/Dead Aqua or Live/Dead NearIR for 30 minutes at room temperature in the dark. Where appropriate, 4',6-diamidino-2-phenylindole (DAPI) was added to live cells directly before sorting as a marker of cell viability. Cells were stained at a concentration of 50 million cells per 1 mL. Cells were stained for extracellular targets for 25 minutes at 4°C in the dark. Intracellular targets were stained for 1 hour at 4°C in the dark following fixation and permeabilization. Streptavidin conjugates were incubated with cells for 15 min at 4°C. Antibody concentrations used were based on volume titrations performed in the Holländer lab and correspond to concentrations between 2.5-15µg/mL of cells (up to 50 million cells).

2.5 Molecular biology

2.5.1 RNA extraction

Cells were collected into RLT lysis buffer containing 1% β-mercaptoethanol, vortexed for 1 minute, then stored at -80°C until use. RNA was isolated using Qiagen's RNeasy Plus Micro Kit, which is optimized for low yield RNA extractions, according to the manufacturer's instructions. Briefly, genomic DNA was removed by running lysates through a gDNA Eliminator spin column (Qiagen) and flow-through was diluted in an equal volume of 70% ethanol to facilitate precipitation of RNA out of the sample. Samples were loaded onto the RNeasy MiniElute spin columns (Qiagen) and washed with RW1 buffer (Qiagen), RPE buffer (Qiagen), and finally 80% ethanol. RNA was eluted from the column in 14µL of RNase-free water and used immediately for cDNA synthesis.

2.5.2 cDNA synthesis

cDNA was synthesized using Bioline's SENSIfast cDNA Synthesis Kit according to the manufacturer's instructions. Briefly, 14μL of purified RNA, 4μL of 5X TransAmp buffer, 1μL of reverse transcriptase, and 1μL of nuclease-free water were combined and incubated in a thermocycler for 10 min at 25°C (to allow primers to anneal), 15 min at 42°C (to allow for reverse transcription), and 5 min at 85°C (to inactivate the reverse transcriptase). cDNA was either used immediately for qPCR or stored at -20°C until use.

2.5.3 qPCR

qPCR was carried out using TaqMan Gene Expression Assays for *Aire* (mRNA) and *Gapdh* (mRNA). cDNA from mTEC^{hi} and double positive (DP) thymocytes were used as positive and negative controls for *Aire* (mRNA), respectively. Briefly, 1μL of template cDNA, 12.5μL of 2X TaqMan Universal PCR master mix, 1.25μL of the appropriate 20X TaqMan Gene expression assay (*Aire* Mm00477461_m1; *Gapdh* Mm99999915_g1), and 10.25μL of nuclease-free water was combined and run in an ABI Prism 7500 sequence detector (Applied Biosystems) according to the conditions in Table 2.7. CT values for *Aire* (mRNA) were normalized first to the internal control *Gapdh* (mRNA) to generate the Δ CT, then to the negative control sample (DP thymocytes) to generate the $\Delta\Delta$ CT for each sample. Expression levels were plotted as $2^{-\Delta\Delta CT}$ to demonstrate fold change over the negative control.

Table 2.7. qPCR thermocycler conditions

Step	# of cycles	Temperature	Duration	Comments
1	1	50°C	2 min	Uracil-DNA glycosylate (UNG) incubation
2	1	95°C	10 min	DNA polymerase activation
3	40	95°C	15 sec	Denature
		60°C	1 min	Anneal/extend
4	1	4°C	hold	

2.6 Imaging

Imaging was performed in the NIAID Biological Imaging Core Facility at NIH with kind instrumentation assistance from Dr. Margery Smelkinson.

2.6.1 Immunofluorescence on murine lymph nodes

Multi-color immunofluorescence experiments were carried out with the kind help of Dr. Antonio Pires da Silva Baptista of Dr. Ron Germain's laboratory, NIAID/NIH. Following dissection, inguinal lymph nodes from an 8-week-old wildtype C57BL/6 mouse were fixed and permeabilized overnight at 4°C in Cytofix/Cytoperm buffer (BD Biosciences), equilibrated in 30% sucrose for an additional 24 hours at 4°C, then frozen in OCT Compound (Tissue-Tek). Tissue sections were cut at 16µm using a cryostat, placed onto microscope slides, and stored at -20°C until use. After demarcating tissues with a Pap pen, sections were rehydrated for 10 min at room temperature (RT) then incubated with PBS/1% FCS/1% BSA/0.2% Triton-X-100 for 30 min at RT. Tissue sections were then stained with the appropriate fluorophore-conjugated antibodies (AIRE, B220, CD11c, CD127, CD40, EpCAM, MHC-II) overnight at 4°C in PBS/1% FCS/1% BSA/0.2% Triton-X-100. The next day, slides were washed 3x with PBS and coverslips were mounted with ProLong Diamond mounting media. Slides were left to cure for several hours before multiparameter confocal images were acquired on a Leica SP8 microscope equipped with a 40x 1.3 NA Oil objective and corrected for fluorophore spillover using the built-in Leica Channel Dye Separation module (Leica Microsystems). Images were analyzed in Imaris (Bitplane) using consistent settings across all images.

2.6.2 Intranuclear AIRE immunofluorescence

Glass microscope slides were incubated in 0.01% poly-L-lysine solution (Sigma) for 15 min at RT, washed 3x with PBS, then left to air dry overnight. A Pap pen was used to demarcate the area in which cells were to be sorted on each slide. Cells were sorted into a droplet of FACS buffer to prevent drying out. Slides were then placed on ice for 20 min to allow the cells to adhere to the slides then fixed directly by adding 20 μ L of 4% paraformaldehyde directly to the droplet (15 min at RT). The droplet was carefully removed with a pipette and slides were placed in PBS to be transported between buildings. Cells were permeabilized with 0.2% Triton-X-100 added as a droplet for 10 min at RT, washed 3x with PBS, then blocked in PBS/1% FCS/1% BSA for 30 min at RT. Without washing, the appropriate AF488-conjugated antibody (AIRE, CD40, or isotype (rat IgG2c, κ) control) was added at a 1:100 dilution in PBS/1% FCS/1% BSA, overnight at 4°C in a moisture chamber. The following day, slides were washed 3x with PBS and incubated with 1 μ g/mL DAPI for 10 min at RT. Slides were washed 3x with PBS before coverslips were mounted using ProLong Diamond mounting media. Slides were left to cure for several hours before being imaged. The images below were acquired using a Leica SP8 (690/730) confocal microscope. Images were analyzed as z-stacks in Imaris (Bitplane) using consistent settings across all images.

In order to quantify the number of AIRE-positive cells, images were also acquired using a Leica DMI6000 widefield microscope. A large area of the coverslip was imaged by taking many fields of view and then merging them together using the Navigator feature in the LAS X (Leica) software. Due to differences in cell concentration, a different number of fields were taken per sample. For EpCAM⁺ eTACs, 312 fields of view were imaged encompassing 51 cells; for EpCAM⁻ eTACs, 798 fields of view were taken encompassing 236 cells; for cDCs,

294 fields of view were imaged encompassing 1470 cells. Post-processing analysis was performed using Imaris software where the “Spots” feature was used to automatically quantify the total number of cells from the DAPI signal. The number of cells that had AIRE signal was quantified manually by two individuals and the totals averaged.

2.6.3 Organ-specific autoantibodies by immunofluorescence

Organs were collected and immediately snap-frozen in OCT compound. Tissue sections were cut at 16µm using a cryostat and placed onto microscope slides. Slides were stored at -20°C until use. One slide was stained with hematoxylin and eosin (H&E) to provide an overview of the tissue structure (see 2.6.4.2 for H&E staining).

Slides were allowed to dry in a humidity chamber for 1 hour at RT. A Pap pen was used to demarcate the area around the tissue sections. Tissues were rehydrated in PBS for 10 minutes at RT. Mouse sera was diluted 1:100 in PBS/1% BSA/0.3% Triton-X-100 and incubated with tissues overnight at 4°C in a humidity chamber. The following day tissues were washed 3x with PBS (5 minutes each) and incubated with AF488-conjugated goat anti-mouse IgG (JacksonLabs; diluted 1:500 in PBS/1% BSA/0.3% Triton-X-100) for 2 hours at RT in a humidity chamber. Tissues were washed 3x then incubated with DAPI (1mg/mL in PBS) for 10 minutes at RT. Tissues were again washed 3x before coverslips were mounted with Prolong Diamond mounting media. Slides were imaged using consistent settings for each tissue using a Leica widefield microscope equipped with a PCO camera. Images were analyzed in Imaris using consistent settings.

Post-processing analysis was performed using Imaris software where fluorescence intensity mean was automatically quantified for the total image volume for DAPI and for AF488 using consistent settings across all images. The fluorescence intensity mean for the AF488 signal was normalized to the fluorescence intensity mean for the DAPI signal for that same sample. To compare this result to the results of wildtype mice and decrease the impact of intra-assay variability, fluorescence intensity means were then normalized to the average fluorescence intensity mean for control samples within the same experiment.

Table 2.8. Immunofluorescence antibodies

Antibody Target	Clone	Conjugate(s)	Dilution	Isotype	Source
AIRE	5H12	AF488	1 to 100	Rat IgG2c, κ	ThermoScientific (eBio)
B220	RA3-6B2	eF570	1 to 50	Rat IgG2a, κ	ThermoScientific (eBio)
CD11c	N418	AF594	1 to 25	Armenian hamster IgG	BioLegend
CD127	A7R34	AF647	1 to 100	Rat IgG2a, κ	BioLegend
CD40	3/23	PacBlue	1 to 100	Rat IgG2a, κ	BioLegend
CD40	HM40-3	AF488	1 to 100	Armenian hamster IgM	BioLegend
EpCAM	G8.8	AF700	1 to 100	Rat IgG2a, κ	BioLegend
isotype for AIRE	RTK4174	AF488	1 to 100	Rat IgG2c, κ	BioLegend
MHC-II (I-A/I-E)	M5/114.15.2	eF506	1 to 200	Rat IgG2b, κ	ThermoScientific (eBio)

Abbreviations: AF= AlexaFluor; eF = eFluor; PacBlue = Pacific Blue; eBio= eBioSciences

2.6.4 Histology

2.6.4.1 Organ collection and sectioning

Eyes and salivary glands were collected from 20-week-old mice into 10% neutralized buffered formalin. After 36 hours, organs were transferred into 70% ethanol. Organs were embedded in paraffin and sectioned at 5 μ m thickness.

2.6.4.2 H&E staining

Tissue sections were deparaffinized with xylene, then hydrated through graded alcohols (100% ethanol, 95% ethanol, 80% ethanol) up to water. Slides were stained with Carazzi's hematoxylin solution (ITW Reagents) for 3 min, washed in tap water, then placed in 95% ethanol. Slides were then stained with eosin-phloxine for 30 sec (VWR), dehydrated through graded alcohols (95% ethanol, 100% ethanol), transferred to xylene, and coverslips mounted using Permount mounting media (Fisher Scientific).

2.6.4.3 Histological scoring

For eyes, H&E-stained tissue sections were assessed for retinal degeneration based on the appearance of the seven retinal layers using the following severity scores: normal (7 intact layers), mild (limited disorganization of retinal layers), moderate (evident disorganization and loss of 1 to 3 layers), severe (loss of >3 layers). For salivary glands, H&E-stained tissue sections were assessed for the presence of mononuclear cell infiltration using the following severity scores: normal (no disease), mild (sporadic infiltration; 1 to 2 foci), moderate (clear infiltrate with 3 to 6 foci), or severe (many extensive areas of infiltration; ≥ 7 foci).

2.7 *In vitro* experiments

2.7.1 Lymph node digestion and isolation of APCs

Peripheral lymph nodes from 6- to 12-week-old wildtype or *Aire*^{-/-} mice were isolated, pooled, and digested with Liberase TL Digestion buffer before being stained with the following fluorochrome-conjugated antibodies (CD45, EpCAM, MHC-II, CD40, CD4, CD8a, CD11b, CD11c) and the following biotinylated lineage antibodies (CD31, CD19, CD14, NK1.1, CD49b, Gr-1, Ter119, TCR $\gamma\delta$, TCR β). The latter were revealed by fluorochrome-

conjugated Streptavidin. DAPI was added immediately prior to sorting as a marker of cell viability. The following cell populations were sorted into complete RPMI media using a BD FACS Aria III: (cDCs: CD45⁺ CD11b⁺ EpCAM⁻ CD8a⁻ CD11c⁺ lineage⁻; EpCAM⁺ eTACs: CD45⁺ CD11b⁻ CD4⁻ CD8a⁻ EpCAM⁺ lineage⁻; EpCAM⁻ eTACs: CD45⁺ CD11b⁻ CD4⁻ CD8a⁻ EpCAM⁻ CD40⁺ MHC-II^{hi} lineage⁻).

2.7.2 CD8⁺ T cell isolation

CD8⁺ T cells were isolated from the spleens of 8- to 12-week-old OT-1 mice using the EasySep Mouse CD8⁺ T cell isolation kit (STEMCELL Technologies) according to the manufacturer's instructions. Briefly, 50μL of rat serum was added to 1mL cells (up to 100 million) in FACS buffer followed by 50μL of isolation antibody cocktail. After a 10 min incubation at RT, 125μL beads were added to cells and incubated for 5 min at RT. Cells were topped up to 2.5mL with FACS buffer and placed on a magnet for 2.5 min at RT. The supernatant containing isolated cells was carefully removed and cells counted for subsequent CellTrace labeling.

2.7.3 Proliferation dye labeling

Isolated CD8⁺ OT-I T cells were resuspended in 1mL of additive-free RPMI and gently vortexed while 1mL of 10μM CellTrace Violet solution was added (final CellTrace Violet concentration of 5μM). Cells were incubated at 37°C for 20 minutes in the dark before the reaction was quenched by adding 1mL of ice-cold FCS followed by a 5-minute incubation on ice. Cells were washed and resuspended in complete RPMI medium.

2.7.4 APC priming with OVA peptide

Sorted cDCs, EpCAM⁺ eTACs, and EpCAM⁻ eTACs were resuspended in PBS containing the indicated concentrations of OVA₂₅₇₋₂₆₄ (SIINFEKL) peptide and incubated at 37°C for 30 minutes. Cells were pelleted and resuspended in complete RPMI medium.

2.7.5 Cell culture and proliferation analysis

Cells were plated in a 96-well, flat-bottom plate in triplicate where possible at a 1 to 10 APC to T cell ratio at a final concentration of 1×10^6 cells/mL. T cells alone were incubated in the presence of plate-bound anti-CD3 ϵ (clone 37.51, Syrian hamster IgG, BioLegend) and soluble anti-CD28 (clone 145-2C11, Armenian hamster IgG, BioLegend) as a positive control (2 μ g/mL each). After 3 days in culture, cells were stained for viability and CD8a before being acquired by FACS for CellTrace Violet dilution.

2.8 Anti-cytokine autoantibody analyses

2.8.1 Subjects and samples

Patients and healthy controls were enrolled on Institutional Review Board (IRB)-approved protocols in Italy, France, and the United States. All protocols followed local ethics recommendations and informed consent was obtained when required. Serum and plasma samples from patients and controls were frozen at $\leq -20^\circ\text{C}$ immediately after collection.

Disease severity was assessed for each COVID-19 patient in accordance with the Diagnostics and Treatment for Novel Coronavirus Pneumonia (trial version 7), released by the National Health Commission & State Administration of Traditional Chinese Medicine on March 3, 2020. “Life-threatening COVID-19 pneumonia” was defined as pneumonia

developing in patients with critical disease, whether pulmonary, with mechanical ventilation (continuous positive airway pressure (CPAP), bilevel positive airway pressure (BIPAP), intubation, high-flow oxygen), septic-shock, or damage to any other organ requiring admission to the intensive care unit (ICU). Severe disease was defined as an oxygen saturation at rest 93% or lower OR respiratory rate >30/min OR partial pressure of oxygen/fraction of inspired oxygen (PaO₂/FiO₂) <300 (includes use of low-flow oxygen). Moderate disease was defined as fever and respiratory symptoms PLUS radiological evidence of pneumonia (includes use of low-flow oxygen if O₂ saturation is >93% at rest). Patients with mild disease had modest symptoms and no pneumonia, while asymptomatic controls tested positive by PCR but did not develop symptoms.

2.8.2 Multiplex particle-based assay for anti-cytokine autoantibody detection

Serum/plasma samples were screened for autoantibodies against 18 targets in a multiplex particle-based assay, in which magnetic beads (BioRad) with differential fluorescence were covalently coupled to carrier protein-free recombinant human proteins (2.5 µg/reaction; R&D or Peprotech). Beads were combined and incubated with 1:100 diluted serum/plasma samples for 30 minutes. They were then washed and incubated with PE-labeled goat anti-human IgG (1 µg/mL; ThermoScientific) for an additional 30 minutes. Beads were washed again and run on a BioPlex X200 instrument in a multiplex assay. Patients with a fluorescence intensity (FI) of >1000 for IFN α , IFN β , or IFN ω were tested for blocking activity as were anti-type I IFN autoantibody-positive patients who were also positive for direct binding to another cytokine target (>5000 FI).

2.8.3 Functional evaluation of anti-cytokine autoantibodies

The blocking activity of anti-IFN α , -IFN β , and -IFN ω autoantibodies was determined by assessing signal transducer and activator of transcription 1 (STAT1) phosphorylation in healthy control cells following stimulation with the appropriate cytokines in the presence of 10% healthy control or patient serum/plasma. Surface-stained healthy control PBMCs (350,000/reaction) were cultured in serum-free RPMI medium with 10% healthy control or patient serum/plasma and were either left unstimulated or stimulated with IFN α , IFN β , or IFN ω (10 ng/mL) for 15 minutes at 37°C. Cells were fixed with 4% PFA, permeabilized with 100% ice cold methanol for 10 min on ice, washed with FACS buffer, and stained in Fix & Perm Medium B (ThermoScientific/Invitrogen) for intranuclear phospho-STAT1 (Y701) (pSTAT1). Cells were acquired on a BD LSR Fortessa cytometer with gating on CD14⁺ monocytes and analyzed with FlowJo software.

For IgG-depletion experiments, IgG was captured on an Ab Spin Trap protein G column (GE Healthcare) and the flow-through fraction (IgG-depleted) was then collected and compared to total plasma in the pSTAT1 assay.

The blocking activity of anti-IFN γ , -GM-CSF, -IFN λ 1, -IFN λ 2, -IFN λ 3, -IL-6, -IL-10, -IL-12p70, -IL-22, -IL-17A, -IL-17F, -TNF α , and -TNF β antibodies was assessed with the assays outlined in Table 2.9.

Table 2.9. Functional assays used to test blocking activities of various cytokines

Cytokine (concentration)	Incubation time	Signaling target	Cell type	Method
IFN γ (400 U/mL)	15 min	pSTAT1 (Y701)	CD14 ⁺ monocytes	FACS, surface and IC staining
GM-CSF (10 ng/mL)	30 min	pSTAT5 (Y694)	CD14 ⁺ monocytes	FACS, surface and IC staining
IL-6 (10 ng/mL)	20 min	pSTAT3 (Y705)	CD14 ⁺ monocytes	FACS, surface and IC staining
IL-10 (10 ng/mL)	20 min	pSTAT3 (Y705)	CD14 ⁺ monocytes	FACS, surface and IC staining
IL-12p70 (10 ng/mL)	15 min	pSTAT4 (Y693)	Activated CD4 ⁺ lymphocytes	FACS, surface and IC staining
IL-17A, IL-17F (100 ng/mL)	18 hr (IL-17A) 36 hr (IL-17F)	IL-6	HFF-1 cells	Cytokine detection assay
IL-22 (100 ng/mL)	30 min	pSTAT3 (Y705)	A549 cells	FACS, IC staining
TNF α , TNF β (10 ng/mL)	Overnight	Rat Thy1	Jurkat 3T8 cells	FACS, surface staining
IFN λ 1, IFN λ 2, IFN λ 3 (50 ng/mL)	45 min	pSTAT1 (Y701)	A549 cells	FACS, IC staining

2.9 Statistics

Statistical analyses were performed using Prism (Graphpad) software. Bar graphs represent the mean of individual values with error bars to represent the standard deviation.

Statistical validity was determined by unpaired, two-tailed student's t test (normal distribution) unless otherwise stated where *p<0.05, **p<0.01, ***p<0.001,

****p<0.0001. Where appropriate, the nonparametric one-way ANOVA Kruskal-Wallis or Friedman tests using Dunn's multiple comparisons test were performed.

3 Identification of extra-thymic AIRE-expressing cells (eTACs) in secondary lymphoid tissues

AIRE is expressed primarily in the thymus in mature medullary thymic epithelial cells (mTECs). In addition to mTECs, *Aire* (mRNA) is also found in secondary lymphoid organs, in embryonic liver, and in reproductive tissues (Eldershaw *et al.*, 2011). Additionally, activated thymic B cells were shown to express AIRE following CD40 signals acquired upon interactions with CD4⁺ thymocytes (Yamano *et al.*, 2015). Gardner *et al* have described a novel AIRE-expressing cell population in murine peripheral lymph nodes called extra-thymic AIRE-expressing cells (eTACs) (Gardner *et al.*, 2008; Gardner *et al.*, 2013). eTACs are bone marrow-derived cells with dendritic-like morphology, although phenotypically they did not fit into previously identified dendritic cell (DC) subsets (Gardner *et al.*, 2013).

Methods used to identify eTACs in mice have been primarily limited to transgenic reporter mouse models, with little work done to identify eTACs in wildtype animals. The use of a reporter mouse, whereby a fluorescent protein is expressed under the control of *Aire*, limits the ability to identify and assess eTACs in other mutant strains without first crossbreeding, resulting in increased time and cage space requirements. Identification of eTACs in wildtype animals based on the expression of surface markers would allow for eTAC quantification and functional assessment *in vitro*. Thus, in this chapter I sought to identify eTACs by their surface markers so that they could be sorted for *in vitro* experiments. eTACs were first identified in fixed inguinal lymph nodes from wildtype C57BL/6 mice by immunofluorescence. Using the surface markers found to be expressed by

eTACs from those experiments and previous studies performed in the Holländer lab (MHC-II, CD40, EpCAM), a FACS panel and gating strategy was then designed to identify and isolate eTACs based only on their surface markers. Due to the relatively low expression of AIRE by eTACs compared to mTECs (Yamano *et al.*, 2019) (for which commercially available fluorochrome-conjugated AIRE antibodies were designed), I encountered sensitivity and specificity issues when staining for AIRE by flow cytometry in that all cells appeared to be positive for AIRE when compared to isotype control-stained cells. Thus, to confirm the presence of AIRE-expressing cells in my sorted eTAC populations, I sorted cells directly onto poly-L-lysine-coated microscopy slides and assessed intranuclear AIRE expression with a high-power confocal microscope.

3.1 Identification of AIRE expression in murine lymph nodes by immunofluorescence

AIRE is an intranuclear protein and thus its detection requires fixation and permeabilization, which precludes the option for *in vitro* studies on live cells. Reporter mice that express green fluorescent protein (GFP) under the control of *Aire* have been shown only to express AIRE protein in 10-20% of AIRE-reporter-positive cells (Yamano *et al.*, 2019), making this model suboptimal for functional studies on sorted eTACs. Additionally, reliance on a transgenic AIRE-reporter mouse precludes the ability to isolate and quantify eTACs in other transgenic strains without the need for additional breeding and crosses.

For these reasons I saw a need to identify AIRE-expressing cells based on their surface markers in wildtype murine lymph nodes. In doing so, I retained the possibility of performing *in vitro* functional studies on transgenic mice without first crossing to an AIRE-reporter strain. Using fluorochrome-conjugated antibodies against AIRE, B220, CD40,

CD11c, CD127, MHC-II, and EpCAM, I sought to identify AIRE-expressing cells in wildtype murine lymph nodes by immunofluorescence. Figure 3.1 depicts a representative image of an inguinal lymph node from a wildtype C57BL/6 mouse.

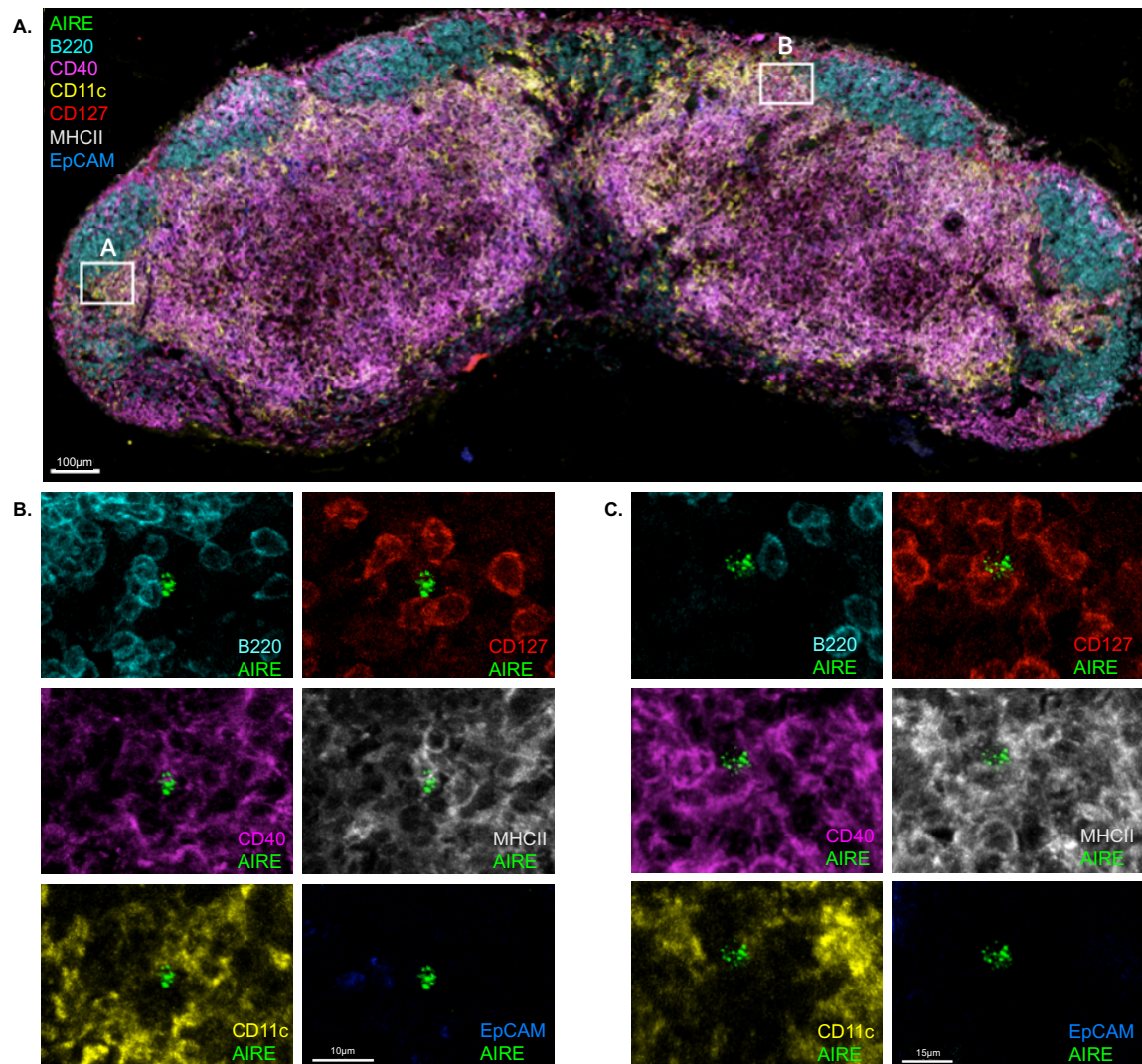


Figure 3.1. Detection of eTACs in a murine lymph node. A) Fluorescent microscopy imaging of an inguinal lymph node taken from an 8-week-old wildtype C57BL/6 mouse. Regions A and B are shown at higher resolution for each surface marker and AIRE in B) and C), respectively.

The nuclear speckling seen is consistent with AIRE staining of mTEC^{hi}s (Halonen *et al.*, 2001; Heino *et al.*, 2000; Rinderle *et al.*, 1999). The AIRE-positive cells were found in the interfollicular area and were B220-negative, confirming eTACs are distinct from AIRE-expressing B cells. Consistent with transcriptomic data from the Holländer lab, the AIRE-

positive cells expressed high levels of MHC class II (MHC-II) and CD40 and seemed to be positive for CD127. In contrast to previous reports that described eTACs as CD11c⁻ and EpCAM⁺ (Gardner *et al.*, 2008; Gardner *et al.*, 2013), I found that cells positive for AIRE by immunofluorescence appeared to be positive for CD11c and negative for EpCAM. EpCAM (epithelial cellular adhesion molecule) is a protein found on epithelial cells that promotes cell adhesion and is involved in cell proliferation, differentiation, and migration (Schnell *et al.*, 2013). Despite previous reports that describe eTACs as EpCAM⁺ cells (Gardner *et al.*, 2008; Gardner *et al.*, 2013), I did not observe AIRE expression in EpCAM⁺ cells by this method. Later, Yamano *et al* reported that AIRE protein was found exclusively in CD11c⁻ EpCAM⁻ AIRE-reporter⁺ cells (10-20% were AIRE⁺ by intracellular staining), utilizing transgenic mice in which the *Aire* locus is modified to drive the expression of green fluorescent protein (Yamano *et al.*, 2019). Given these conflicting findings, it was important to remain agnostic in developing a strategy for sorting eTACs by FACS.

3.2 Identification of two distinct eTAC populations by flow cytometry

I next sought to identify eTACs based on their surface markers to allow for isolation by FACS for subsequent functional analyses. Based on the immunofluorescence data presented in section 3.1 and previous reports (Gardner *et al.*, 2008; Gardner *et al.*, 2013), a gating strategy was employed to identify two potential eTAC populations based on their expression of CD45, EpCAM, MHC-II, CD40, and lineage markers to exclude endothelial cells, B cells, macrophages, NK cells, neutrophils, erythrocytes, and T cells (Figure 3.2).

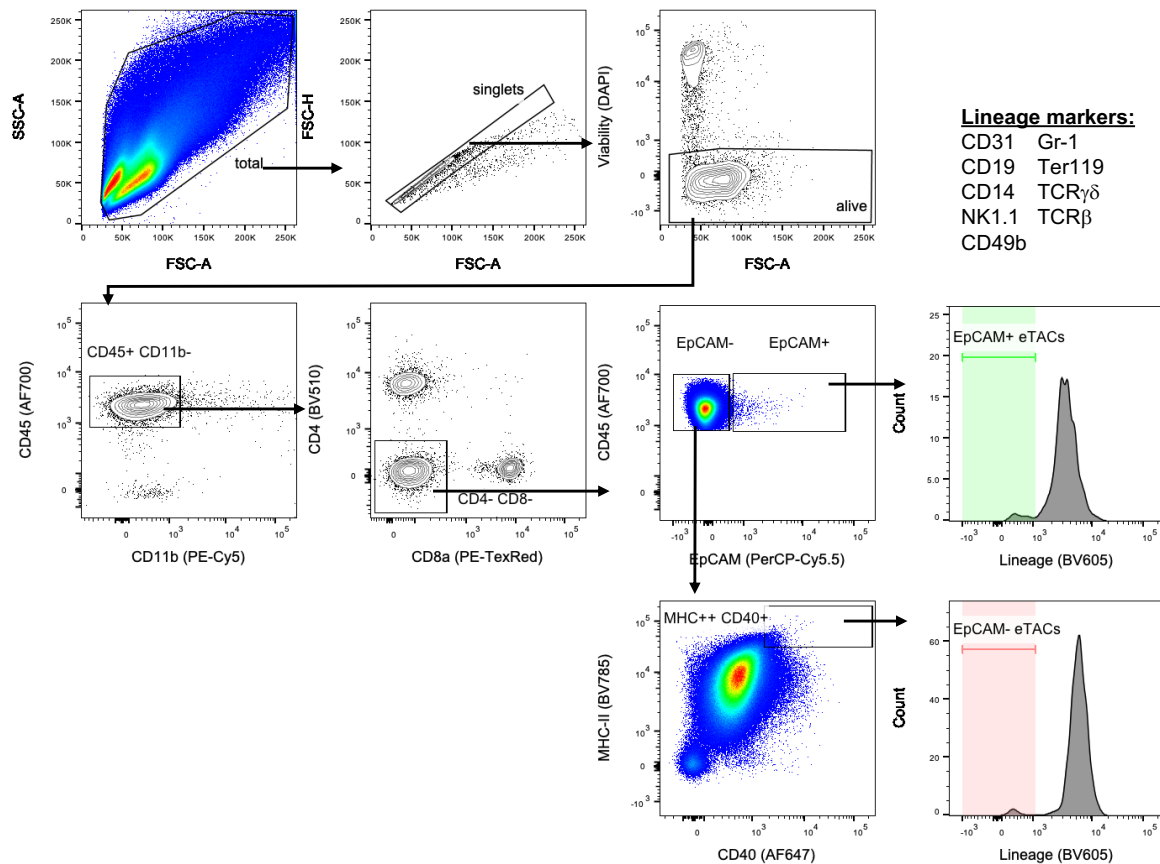


Figure 3.2. Gating strategy to identify eTACs in murine lymph nodes by FACS. Representative FACS plots depicting the gating strategy to identify two distinct phenotypes of AIRE-expressing cells in 6- to 12-week-old wildtype C57BL/6 mice based on their surface markers.

The frequency of viable EpCAM⁺ and EpCAM⁻ eTACs was found to be $0.0062 \pm 0.004\%$ and $0.023 \pm 0.03\%$, respectively, calculated from 8 individual mice across 4 independent experiments. Due to non-specific binding of the commercially available AIRE antibody (clone 5H12), the low AIRE-expressing populations of eTACs could not be stained for AIRE by FACS as all cells appeared positive when comparing to isotype control-stained cells. Instead of staining for AIRE by FACS, the populations were sorted and assessed by qPCR for *Aire* (mRNA) transcript levels (Figure 3.3). Sorted mTEC^{hi}s and double positive (DP) thymocytes were used as positive and negative controls for *Aire* (mRNA) transcript, respectively. Levels of *Aire* (mRNA) expression were calculated using the $\Delta\Delta CT$ method. *Gapdh* (mRNA) was used as an internal control to account for the total amount of mRNA

assessed. Fold change ($2^{-\Delta\Delta CT}$) in *Aire* (mRNA) expression was calculated by normalizing *Aire* (mRNA) transcript levels in all samples to *Aire* (mRNA) transcript levels in DP thymocytes.

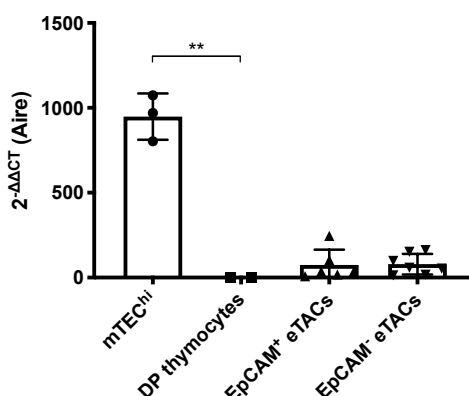


Figure 3.3. *Aire* (mRNA) transcript levels in eTACs. Sorted eTACs were assessed for *Aire* (mRNA) transcript levels compared to mTEC^{hi}s and DP thymocytes. Statistical validity was determined by the nonparametric one-way ANOVA Kruskal-Wallis test using Dunn's multiple comparisons test where **p<0.01.

Aire transcripts were detected in both EpCAM⁺ eTACs and EpCAM⁻ eTACs, which was later independently shown by Yamano *et al* (Yamano *et al.*, 2019). *Aire* (mRNA) levels in eTACs were low compared to mTEC^{hi}s, a result comparable to what has previously been noted (Gardner *et al.*, 2008; Yamano *et al.*, 2019).

3.3 Identification of AIRE-expressing cells in sorted eTAC populations by confocal microscopy

I next sought to identify AIRE protein in sorted eTAC populations by immunofluorescence. There have been conflicting reports on the precise phenotype of eTACs (Gardner *et al.*, 2008; Gardner *et al.*, 2013) and which eTAC populations express AIRE protein (Yamano *et al.*, 2019). AIRE protein was first detected using Cytospin technology, whereby sorted cell populations were concentrated into a monolayer onto microscopy slides using a specialized centrifuge. High cell input requirements, extended processing times, and the cell damage that can occur with the Cytospin - even under ideal experimental conditions - precluded

this technology as a suitable option. To avoid additional damage due to centrifugal force, I instead sorted cells by flow cytometry directly onto poly-L-lysine-coated microscopy slides, utilizing a method from Ema *et al* (Ema *et al.*, 2006), which I adapted in order to sort 3 cell populations simultaneously. Poly-L-lysine coating creates a positively charged surface which allows for negatively charged ions to adhere via enhanced electrostatic interactions. A schematic of how cells were sorted into 3 populations directly onto poly-L-lysine-coated slides is shown in Figure 3.4. Three poly-L-lysine-coated slides were secured to the Automatic Cell Disposition Unit (ACDU) of a FACSARIA III cytometer and the instrument was set up to sort 3 populations with the integral magnet in place (Figure 3.4A). Sorted cells were allowed to adhere before being fixed with paraformaldehyde (PFA), permeabilized with a Triton-X-100 solution, and stained with an AlexaFluor488 (AF488)-conjugated anti-AIRE antibody (clone 5H12) and DAPI (Figure 3.4B-F).

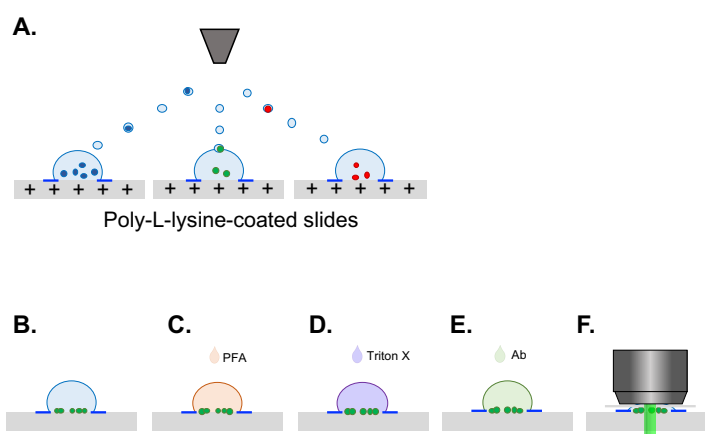


Figure 3.4. Schematic for sorting 3 populations of cells simultaneously into slides for immunofluorescent imaging. A) Poly-L-lysine-coated slides were secured to the Automatic Cell Disposition Unit (ACDU) of a FACSARIA III cytometer, and the instrument set up to sort 3 populations positioned by eye with the integral magnet in place. B) Sorted cells were allowed to settle in the buffer droplet and adhere to slides. C) Adhered cells were fixed in a droplet of paraformaldehyde, D) permeabilized with Triton-X-100 solution, E) stained with fluorescent antibodies, and F) imaged using a confocal microscope.

In a pilot experiment, mTEC^{hi}s were sorted onto slides according to the gating strategy in Figure 3.5A. Cells were either stained with AF488-conjugated antibodies for intranuclear AIRE expression, CD40 expression, or an isotype control of the same species and Ig subtype as the anti-AIRE antibody (rat IgG2c, κ). DAPI was used to identify cell nuclei (Figure 3.5B).

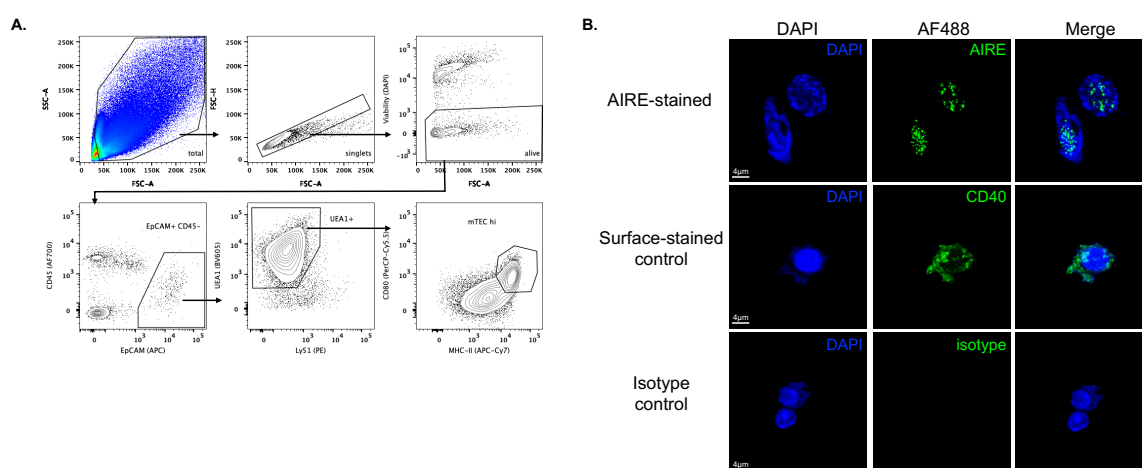


Figure 3.5. Sorted mTEC^{hi}s by immunofluorescent microscopy. A) The gating strategy is shown to sort mTEC^{hi}s from a single cell suspension generated from thymus tissue and depleted of CD45⁺ cells. B) Sorted cells were either stained using AF488-conjugated antibodies for the expression of intranuclear AIRE (top panel), CD40 (middle panel), or a rat IgG2c, κ isotype control (bottom panel). Nuclei were counterstained using DAPI. Thymi were from 6-week-old wildtype C57BL/6 mice.

Staining for AIRE in mTEC^{hi}s resulted in a speckled pattern as previously noted (Halonen *et al.*, 2001; Heino *et al.*, 2000; Rinderle *et al.*, 1999). CD40 was used as a surface-stained control due to its high levels of expression on mTEC^{hi}s, however detection of intracellular CD40 in migration to the cell surface cannot be ruled out using this method.

Having successfully detected AIRE protein in mTEC^{hi}s sorted onto poly-L-lysine-coated slides by immunofluorescence, I next sought to identify AIRE in sorted eTACs using the same method. Due to the low frequency of eTACs (<0.01% of viable lymph node cells), peripheral lymph nodes from four 8-week-old wildtype C57BL/6 mice were pooled,

digested, stained, and the following phenotypes sorted (conventional dendritic cells (cDCs)
cDCs: CD45⁺ CD11b⁺ EpCAM⁻ CD8a⁻ CD11c⁺ lineage⁻; EpCAM⁺ eTACs: CD45⁺ CD11b⁻ CD4⁻
CD8a⁻ EpCAM⁺ lineage⁻; EpCAM⁻ eTACs: CD45⁺ CD11b⁻ CD4⁻ CD8a⁻ EpCAM⁻ CD40⁺ MHC-II^{hi}
lineage⁻). Even when sorting three populations simultaneously, sort times exceeded four
hours, which negatively impacted cell viability and thus sort yields. Importantly, this
method was successful at detecting AIRE in as few as 170 sorted cells. Figure 3.6 shows the
gating strategy used to simultaneously sort three different populations of cells (cDCs,
EpCAM⁺ eTACs, and EpCAM⁻ eTACs) onto three poly-L-lysine-coated slides.

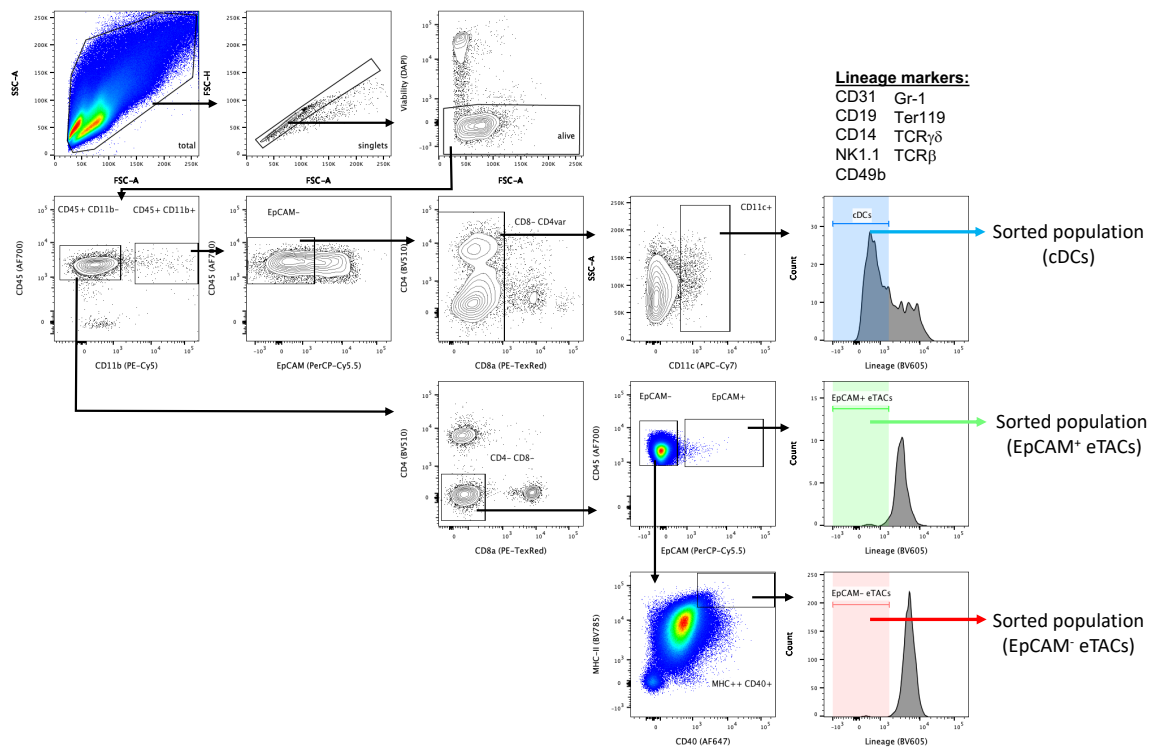


Figure 3.6. Gating strategy to identify cDCs and eTACs. FACS plots depicting the gating strategy to identify cDCs and two distinct phenotypes of AIRE-expressing cells from a single cell suspension generated from lymph node tissue from 8-week-old wildtype C57BL/6 mice based on their surface markers.

cDCs, EpCAM⁺ eTACs, and EpCAM⁻ eTACs were simultaneously sorted onto poly-L-lysine-coated slides and stained with an AIRE or isotype control antibody conjugated to AF488.

Nuclear speckles were observed in the AIRE-stained (Figure 3.7), but not the isotype-stained slides.

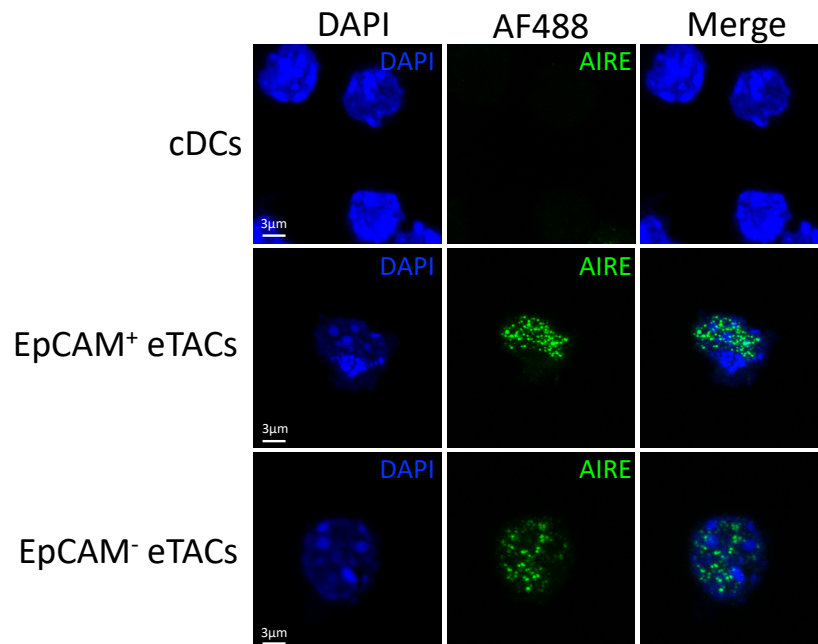


Figure 3.7. AIRE expression in sorted cDCs and eTACs by immunofluorescent microscopy. Sorted cells were stained for intranuclear AIRE using an AF488-conjugated AIRE antibody and DAPI to identify cell nuclei.

Using a widefield microscope, between 300 and 1500 fields were imaged and merged together using the Navigator feature of LAS X (Leica) software. Cells that were double positive for AIRE (AF488) and DAPI were identified and tabulated. The number of AIRE-positive cells was calculated by two individuals in a blinded manner and averaged together. In only smaller fractions of EpCAM⁺ eTACs (15.7%) and EpCAM⁻ eTACs (9.3%), AIRE protein could be detected. AIRE was also detected in 1.4% of the sorted cDC population, an unsurprising finding given the similar phenotypes of eTACs and dendritic cells and previous reports of AIRE expression in dendritic cells (Kogawa *et al.*, 2002; Poliani *et al.*, 2010).

These results indicate that although I was able to capture AIRE⁺ cells using the gating strategy outlined in Figure 3.6, not all of the cells within the eTAC gates are actually positive for AIRE at the protein level. Further, AIRE positivity in a small fraction (1.4%) of sorted cDCs implies AIRE expression in a small population of dendritic cells, in addition to the phenotypically distinct eTACs populations. This could imply that AIRE expression in eTACs and cDCs is transient, and/or that eTACs represent populations of dendritic cells of differential maturity. This is corroborated by single cell RNA sequencing studies in human eTACs that identified varying levels of *AIRE* (mRNA) transcript across cells and concluded that eTACs represent a temporal state in DC maturation during which *AIRE* (mRNA) is transiently expressed (Fergusson *et al.*, 2018).

My findings of AIRE protein in EpCAM⁺ eTACs (9.3%) corroborate the recent findings of Yamano *et al.*, who reported AIRE protein exclusively in CD11c⁺ EpCAM⁺ eTACs (Yamano *et al.*, 2019). However, I also detected AIRE⁺ cells within the EpCAM⁺ eTAC population (15.7%), which Yamano *et al.* concluded were negative for AIRE protein. Correspondingly, my findings by immunofluorescence indicated that AIRE⁺ cells in mouse lymph nodes are negative for EpCAM expression (Figure 3.1), although only a handful AIRE⁺ cells were detected per tissue section by that method. Given the FACS gating method used to identify EpCAM⁺ eTACs (Figure 3.6), it is possible the AIRE-expressing cells detected express low levels of EpCAM that might be considered negative by another technique. Comparable to the findings of Yamano *et al.*, both eTAC populations were negative for CD11c by FACS despite appearing to be positive by immunofluorescence (Figure 3.1).

These results present evidence for two distinct populations of eTACs in the lymph nodes of 6- to 12-week-old wildtype C57BL/6 mice. EpCAM⁺ eTACs are phenotypically defined as CD45⁺ CD11b⁻ CD4⁻ CD8a⁻ EpCAM⁺ lineage⁻ while EpCAM⁻ eTACs are CD45⁺ CD11b⁻ CD4⁻ CD8a⁻ EpCAM⁻ CD40⁺ MHC-II^{hi} lineage⁻. These cells can be identified based on their surface markers, allowing them to be sorted for *in vitro* studies. AIRE protein could be detected in 15.7% of EpCAM⁺ eTACs and 9.3% of EpCAM⁻ eTACs, necessitating the use of AIRE-deficient (*Aire*^{-/-}) control mice in downstream *in vitro* studies in order to elucidate the role of AIRE in eTAC functionality.

4 Antigen presentation by extra-thymic AIRE-expressing cells (eTACs)

Despite clear evidence of AIRE expression in peripheral lymphoid tissues, much remains to be known about the functional activity of AIRE in eTACs and their potential role in peripheral tolerance. Thymic negative selection is only 60-70% effective at removing self-reactive T cells from the repertoire, leaving a significant number of autoreactive T cell clones to enter the periphery (Bouneaud *et al.*, 2000; Gallegos & Bevan, 2006). If eTACs are effective antigen presenting cells, they could be relevant in the peripheral deletion of autoreactive T cells, perhaps specifically in the wake of age-related thymic involution. Further, if AIRE expression in eTACs allows for the promiscuous expression of tissue-restricted antigens (TRAs), eTACs could contribute to the deletion of autoreactive T cells specific for antigens found in immunologically privileged organs, thus preventing autoimmune damage in organs such as the eye.

Several studies to date have assessed the functional capacity of eTACs *in vivo* through the use of transgenic mouse models. For example, a mouse transgenic for the *Aire*-controlled expression of the islet-specific glucose-6-phosphatase-related protein (IGRP; also known as the Adig mouse) targeted the expression of IGRP to mTEC^{hi}s, eTACs, and AIRE-expressing B cells. Upon the transfer of IGRP-specific CD8⁺ T cells into Adig mice, eTAC-mediated IGRP presentation resulted in the rapid proliferation and eventual deletion of these adoptively transferred cells while CD8⁺ T cells with other antigen specificities were not affected (Gardner *et al.*, 2008). Other studies have focused on *in vivo* antigen presentation by eTACs

to CD4⁺ T cells. The *Aire*-driven BDC antigen (AdBDC) transgenic mouse, which expresses the pancreatic antigen BDC under the *Aire* promoter, was utilized to demonstrate the interaction of eTACs with BDC-specific CD4⁺ T cells and subsequent T cell proliferation, ultimately driving tolerance and protection from BDC-mediated diabetes. *In vitro* studies demonstrated the functional inactivation of BDC-specific T cells by eTACs in the absence of CD28 costimulation (Gardner *et al.*, 2013). In yet another study, the *Aire*-HCO transgenic mouse, which expresses influenza hemagglutinin (HA) under the control of *Aire*, deleted HA-specific CD4⁺ T cells upon adoptive transfer, and concluded this effect was due to HA expression in bone marrow-derived cells as the observed effect could be recapitulated by hematopoietic stem cell transplantation (Yamano *et al.*, 2019), thus inferring eTACs are bone marrow-derived. While these studies have demonstrated the antigen presentation capacity of eTACs to both CD4⁺ and CD8⁺ T cells, they did not relate this biological effect to the different eTAC phenotypes as described in Chapter 3, nor did these studies compare eTACs to professional antigen presenting cells (APCs).

Transcriptomic analyses performed by the Holländer lab identified differences and similarities in the gene expression patterns of EpCAM⁺ and EpCAM⁻ eTACs and thus inferred functional differences of biological relevance. EpCAM⁺ eTACs demonstrated an upregulation of genes relevant for MHC class I-mediated antigen processing and antigen presentation, suggesting an increased capacity for these cells to process and present antigen. EpCAM⁻ eTACs demonstrated an upregulation in genes associated with B cell receptor signaling and antigen-receptor mediated signaling. Although the pathway of B cell signaling is not overtly intuitive to be operational and upregulated in EpCAM⁻ eTACs, this

could indicate that EpCAM⁺ eTACs are involved in presenting antigen to adaptive immune cells, a major immune function of B cells.

In this chapter, I sought to assess the *in vitro* antigen presentation capacity of EpCAM⁺ eTACs and EpCAM⁺ eTACs compared to conventional dendritic cells (cDCs). I first assessed the expression of costimulatory molecules on eTACs compared to cDCs, then tested the ability of eTACs to present the foreign exogenous peptide antigen OVA₂₅₇₋₂₆₄ (SIINFEKL). Finally, I assessed APCs from AIRE-sufficient and AIRE-deficient mice for their ability to present antigen *in vitro*.

4.1 eTACs express high levels of costimulatory molecules

To determine whether eTACs possess the molecular features to be effective antigen-presenting cells, I first assessed the expression of distinct costimulatory molecules on the surface of eTACs and cDCs. Figure 4.1 depicts the surface expression of CD86, MHC class II, and CD40 on eTACs and cDCs, as determined by FACS.

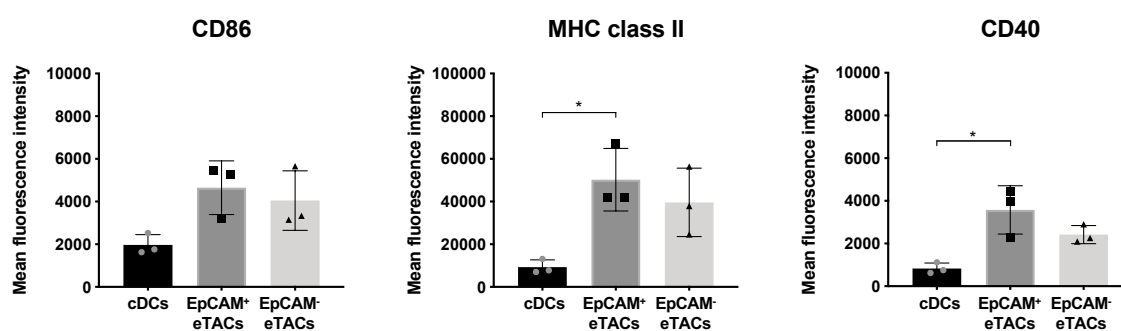


Figure 4.1. Surface expression of costimulatory molecules on eTACs. cDCs, EpCAM⁺ eTACs, and EpCAM⁺ eTACs were assessed for their surface expression of CD86, MHC class II, and CD40 by flow cytometry. Graphs shown represent the mean fluorescence intensity values for each marker across 3 independent experiments on wildtype mice between 6- and 12-weeks of age. Statistical validity was determined by the nonparametric one-way ANOVA Friedman test for matched measures using Dunn's multiple comparisons test where *p<0.05.

Three independent experiments were performed and their analysis employed the nonparametric Friedman test for matched measures, a test that does not assume Gaussian distributions. Dunn's multiple comparisons test was utilized to determine differences between the three cell subsets. A statistically significant increase in the mean fluorescence intensity of MHC class II (*p=0.0429) and CD40 (*p=0.0429) in EpCAM⁺ eTACs compared to cDCs was observed. These findings indicate that eTACs not only possess the necessary costimulatory molecule expression to present antigen, but also that, in the case of EpCAM⁺ eTACs, increased MHC class II and CD40 expression could indicate superior antigen presentation ability compared to cDCs.

4.2 eTACs are effective antigen presenting cells *in vitro*

To test the antigen presentation capability of eTACs I assessed the ability of EpCAM⁺ eTACs, EpCAM⁻ eTACs, or cDCs to activate TCR-transgenic CD8⁺ T cells (OT.1), which are specific to the ovalbumin-derived peptide OVA₂₅₇₋₂₆₄ (SIINFEKL). Antigen presenting cells were sorted according to the gating strategy in Figure 4.2.

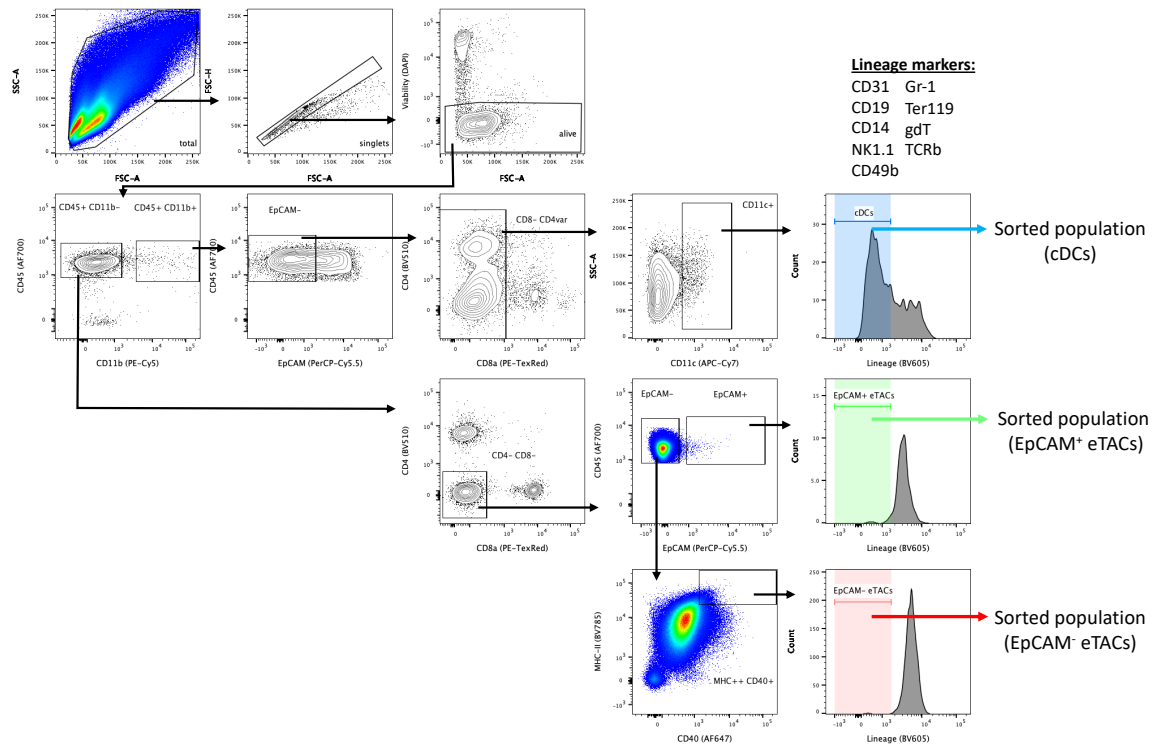


Figure 4.2. Gating strategy used to sort APCs. cDCs, EpCAM⁺ eTACs, and EpCAM⁻ eTACs were sorted from digested lymph nodes from 6- to 12-week-old wildtype C57BL/6 mice based on their expression of various surface markers. cDCs, EpCAM⁺ eTACs, and EpCAM⁻ eTACs constitute $0.13 \pm 0.06\%$, $0.0062 \pm 0.004\%$, and $0.023 \pm 0.03\%$ of viable lymph node cells, respectively.

For experiments performed in Oxford, naïve CD8⁺ T cells were sorted from the spleens of OT.1 mice according to the gating strategy depicted in Figure 4.3. Viable naïve CD8⁺ T cells were isolated based on their combined surface phenotype of TCRβ⁺ CD8a⁺ CD4⁻ CD44^{lo/-} CD62L⁺ viable cells.

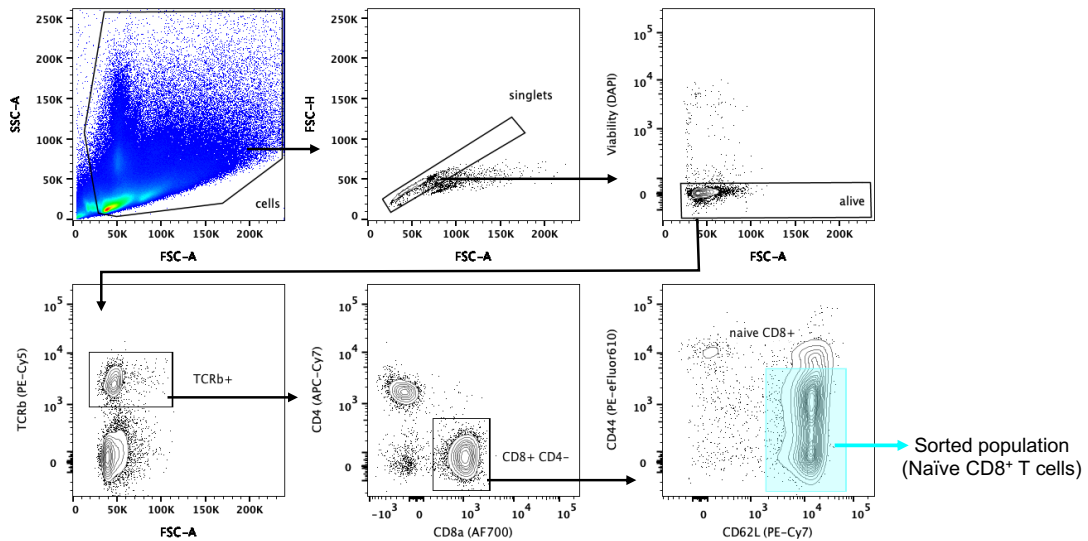


Figure 4.3. Gating strategy used to sort naïve OT.1 CD8⁺ T cells. Naïve CD8⁺ T cells were sorted from spleens of 6- to 12-week-old OT.1 mice. Across three independent experiments, the frequency of naïve CD8⁺ T cells was $17.0 \pm 2.5\%$ of all viable cells.

Experiments performed at NIH required longer sorting times and suffered from increased cell death, thus a gentler CD8⁺ T cell isolation technique was employed (EasySep Mouse CD8⁺ T Cell Isolation Kit, STEMCELL Technologies), which markedly improved day 3 cell viability. Post-isolation purity analyses done for the experiments performed at NIH revealed >95% purity (CD8⁺ out of viable cells). CD8⁺ T cells were labeled with CellTrace Violet (ThermoScientific), a fluorescent dye that covalently binds to cellular amines and is diluted out with each cell division to yield progeny with approximately half the fluorescence of their parental cell. The gating strategy used to assess proliferation in CD8⁺ T cells is shown in Figure 4.5.

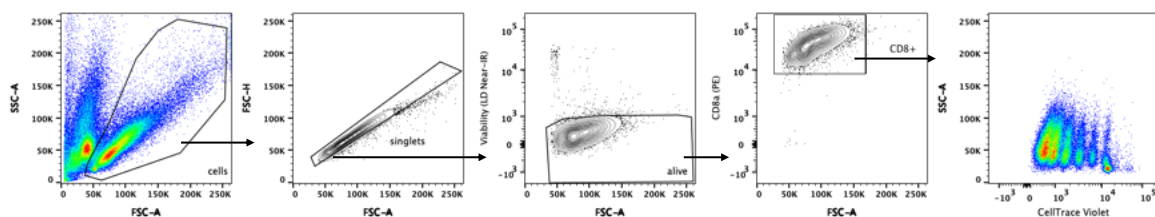


Figure 4.5. CD8⁺ T cell gating strategy and proliferation analysis. On day 3 of culture, OT.1 CD8⁺ T cells incubated in the presence of SIINFEKL-loaded APCs sorted from 6- to 12-week-old wildtype C57BL/6 mice were assessed for proliferation using CellTrace dilution as a readout.

Sorted APCs (cDCs, EpCAM⁺ eTACs, and EpCAM⁻ eTACs) were loaded with SIINFEKL peptide and cultured at a 1:10 ratio with purified CD8⁺ T cells for three days. The proliferation index was calculated for each condition and represents the average number of divisions undergone by responding cells (Roederer, 2011). Figure 4.6 depicts the proliferation index for OT.1 CD8⁺ T cells in the presence of cDCs or eTACs loaded with decreasing amounts of SIINFEKL peptide.

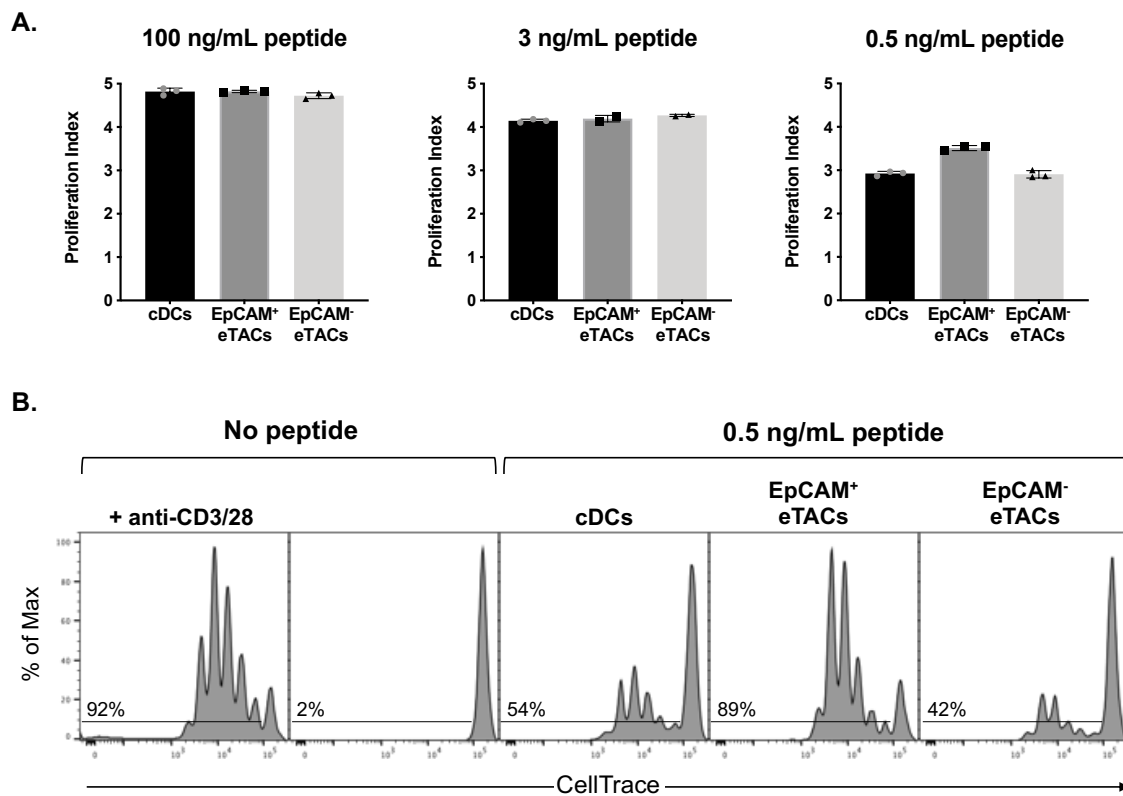


Figure 4.6. Antigen presentation capacity of eTACs *in vitro*. A) The proliferation index was determined for OT.1 CD8⁺ T cells incubated in the presence of cDCs, EpCAM⁺ eTACs, or EpCAM⁻ eTACs loaded with 100 ng/mL, 3 ng/mL, or 0.5 ng/mL SIINFEKL peptide on day 3 of stimulation. Graphs shown represent 2 independent experiments. Statistical validity was determined by the nonparametric one-way ANOVA Kruskal-Wallis test using Dunn's multiple comparisons test. B) Representative examples of CellTrace dilution histograms are shown for each condition. The percentage of divided cells is indicated in the lower left corner of each graph. Graphs shown for each peptide concentration are from a single experiment and are representative of at least two independent experiments.

The proliferation index decreased with decreasing SIINFEKL peptide concentration indicating a dose-dependent response. Each APC population was able to present high SIINFEKL peptide concentrations (100 ng/mL and 3 ng/mL) with comparable efficiency. At 0.5 ng/mL of SIINFEKL, the proliferation index of CD8⁺ T cells cultured in the presence of EpCAM⁺ eTACs is modestly increased compared to that of cDCs and EpCAM⁻ eTACs, but the differences observed were not statistically significant. This difference at low antigen concentration correlated with the heightened expression of the costimulatory molecules MHC-II and CD40 on EpCAM⁺ eTACs when compared to other APCs tested in this experiment (Figure 4.1). However, I would expect an increase in MHC-II expression to influence antigen presentation to CD4⁺ T cells over CD8⁺ T cells, as CD4⁺ T cells recognize antigens presented by MHC-II while CD8⁺ T cells recognize antigens presented by MHC-I (Murphy & Weaver, 2017).

4.3 AIRE is not required for antigen presentation by eTACs

Studies performed in transgenic mice have demonstrated tolerance induction by the AIRE-dependent expression of antigens in peripheral lymphoid tissues (Gardner *et al.*, 2008; Gardner *et al.*, 2013; Lee *et al.*, 2007; Yamano *et al.*, 2019), although a direct contribution by AIRE to the activation of antigen-specific T cells has not been shown. To address the role of AIRE in the presentation of exogenous antigens, I performed the OVA antigen presentation assay using APCs from AIRE-sufficient mice (wildtype) and AIRE-deficient mice (*Aire*^{-/-}) (Figure 4.7). Previous experiments showed the largest difference in the proliferation of T cells cultured in the presence of APCs loaded with low concentrations of SIINFEKL peptide (0.5 ng/mL), thus I chose this concentration increase the likelihood of observing subtle differences between the APC populations.

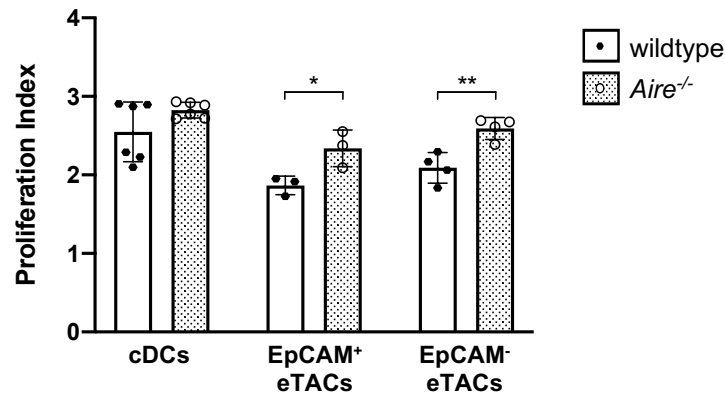


Figure 4.7. The contribution of AIRE to *in vitro* antigen-mediated CD8⁺ T cell proliferation by eTACs. The proliferation index was determined for OT.1 CD8⁺ T cells incubated for 3 days in the presence of SIINFEKL-loaded (0.5 ng/mL) APCs from 6- to 12-week-old wildtype or constitutively AIRE-deficient (*Aire*^{-/-}) mice. Graphs shown represent 2 independent experiments. Statistical validity was determined by multiple t tests where *p<0.05, **p<0.01.

AIRE-deficient eTACs, independent of their EpCAM phenotype, loaded with SIINFEKL (0.5 ng/mL) stimulate OT.1 CD8⁺ T cells more efficiently than their wildtype counterparts (EpCAM⁺ eTACs *p=0.035; EpCAM⁻ eTACs **p=0.0058). Differences in antigen presentation capacity were not observed between *Aire*^{-/-} and wildtype cDCs. There was evident clustering of data points by experiment within the wildtype cDC condition, and an F test revealed these variances were statistically significant (*p=0.0106). Although care was taken to treat all samples the same, this could potentially be explained by pipetting error when loading wildtype cDCs with SIINFEKL peptide, as all APCs were loaded with peptide simultaneously before being split into replicate wells for culture.

The experiments performed at NIH benefited from several technical advantages. APCs were sorted to high purity standards (>95%) that were confirmed by post-sort purity assessments. Total CD8⁺ T cells were isolated by gentle magnetic separation which decreased sort times and increased cell viability as evidenced by increased recovery of viable cells following CellTrace-labeling (83.0 ± 8.0% recovery using magnetic separation

versus $58.0 \pm 11.9\%$ using FACS Aria II; t test $*p=0.0394$). The use of OT.1 mice on a RAG1-deficient background ensured a CD8⁺ T cell population without contamination by T cells with endogenous receptor expression that could interfere with the assay, as only T cells with a transgenic SIINFEKL-specific TCR are able to recombine their receptors and mature in these mice.

In this chapter, I have shown evidence of *in vitro* antigen presentation by eTACs from non-transgenic, wildtype animals that is comparable to that by professional antigen presenting cells (cDCs). The antigen presentation capacity of eTACs sorted from AIRE-deficient mice was superior to that of eTACs sorted from wildtype mice. This could be due to AIRE-repressed genes that are involved in antigen presentation and cell signaling pathways, which would be upregulated in the absence of AIRE in eTACs from AIRE-deficient mice. RNA sequencing performed by Yamano *et al* on AIRE-reporter-positive lymph node cells from wildtype and AIRE-deficient mice revealed 373 AIRE-repressed genes (Yamano *et al.*, 2019). Biological process analysis (Gene Ontology Resource) of these 373 AIRE-repressed genes revealed an enrichment of genes associated with nucleic acid metabolic processes which would broadly regulate gene expression by globally affecting RNA synthesis. This could result in increased transcription of genes involved in antigen presentation and cell signaling pathways, although more studies are necessary to test this hypothesis. Based on my results, I conclude that eTACs are effective at presenting exogenous OVA antigen *in vitro* and that the antigen presentation capacity of eTACs is increased in the absence of AIRE, possibly due to the upregulation of AIRE-repressed genes in AIRE-deficient eTACs. Further studies are needed to determine whether the differences observed are physiologically relevant and to apply these findings more broadly to other model antigens.

5 Phenotypic analyses of constitutive and cell lineage-specific loss-of-AIRE mutant mice

To date, many groups have worked to assess *Aire*^{-/-} mice on various genetic backgrounds, however it is still incompletely known to what extent AIRE-expressing cell subsets contribute to T cell tolerance and the prevention of autoimmunity. Bone marrow chimera experiments have revealed that lethally irradiated AIRE-sufficient recipients do not develop organ damage while AIRE-deficient recipients do (Anderson *et al.*, 2002). This suggests that AIRE expression in radio-resistant stromal cells is necessary to prevent autoimmune organ damage, however these experiments do not directly implicate AIRE-expressing mTECs, although their involvement is implied. Although AIRE-sufficient bone marrow cells are not able to rescue lethally irradiated AIRE-deficient recipients, there has been some evidence of radio-resistance in eTACs (Gardner *et al.*, 2013), making it unclear whether eTACs play a role in preventing autoimmunity in the studies performed by Anderson *et al.*

Peripheral naïve B cells immigrate to thymus and receive CD40 signals upon interacting with CD4⁺ thymocytes, causing the B cells to express AIRE and upregulate their expression MHC class II and CD80 (Yamano *et al.*, 2015). Yamano *et al* also demonstrated that B cell-deficient mice had a higher frequency of CD4 SP thymocytes and hyperresponsive T cells, implying incomplete tolerization of thymocytes developing in the absence of B cells. However, the role of AIRE-expressing B cells in this process remains unknown, as B cells are known antigen presenting cells and efficiently present self-antigens to T cells during negative selection in the thymus (Perera *et al.*, 2013), independent of AIRE.

eTACs have been shown to express tissue-restricted genes in the periphery and to functionally inactivate T cells in an antigen-specific manner (Gardner *et al.*, 2008; Gardner *et al.*, 2013). Much work has been done to assess eTACs in artificial mouse models, but the true physiological significance of peripheral AIRE expression has not yet been fully elucidated. Overexpression of AIRE in bone marrow cells delayed the onset of experimental autoimmune encephalomyelitis in recipient mice (Ko *et al.*, 2010), suggesting that ectopic AIRE expression in hematopoietic cells, such as B cells and eTACs, can alter the development of autoimmune disease. While important, the biological relevance of this finding is unclear, given the exceedingly low number of AIRE-expressing B cells and eTACs detected in wildtype mice.

During the course of this project, the creator of the floxed *Aire* mice utilized here reported that mice rendered AIRE-deficient in FOXP3-expressing cells have increased mTEC^{hi}s and decreased tissue-restricted antigen expression, similar to what is seen in constitutively AIRE-deficient mice (Dobes *et al.*, 2018). The authors also generated mice that are AIRE-deficient in all hematopoietic cells with Cre recombinase activity driven by VAV1 expression. However, the authors did not perform extensive thymocyte/T cell phenotyping, nor did they address autoimmune manifestations in these mice.

To investigate the function of various AIRE-expressing cells, cell lineage-specific loss-of-AIRE mutant mice were generated using Cre-lox technology. Mice with a floxed *Aire* allele (*Aire*(fl/fl)) were crossed to *Foxp3*(Cre+), *Mb1*(Cre+), or *Rorc*(Cre+) mice to generate animals with an AIRE-deficiency in mTECs, B cells, or eTACs, respectively. Loss-of-AIRE

mouse strains were evaluated for thymocyte development, T cell maturation, and the presence of organ-specific autoimmunity compared to their wildtype or Cre-negative, littermate controls. Mice were aged to 5-months to increase the likelihood of identifying autoimmunity, which increases beyond 3-months of age in *Aire*^{-/-} mice on the C57BL/6 genetic background (Hubert *et al.*, 2009).

5.1 Generation of cell lineage-specific loss-of-AIRE mice

Mice with a floxed *Aire* allele were a generous gift from Dominik Filipp. These mice have *loxP* sites flanking exons 6 and 7, which lie within the Sp100, AIRE-1, NucP41/75, DEAF-1, or SAND, domain of *Aire*. When crossed to mice that express Cre recombinase, exons 6 and 7 are excised, resulting in the deletion of the majority of the SAND domain. This is important because the SAND domain is critical for DNA binding, appropriate protein localization, and multimolecular complex participation, all of which allow for the proper functioning of AIRE (Gibson *et al.*, 1998; Ilmarinen *et al.*, 2006; Purohit *et al.*, 2005; Ramsey *et al.*, 2002).

Additionally, one of the most common *AIRE* mutations in APECED patients lies within the SAND domain at arginine residue 257 (R257X) (Scott *et al.*, 1998).

To target *Aire* in mTECs, *Aire*(fl/fl) mice were crossed to *Foxn1*(Cre) mice (designated *Aire*(fl/fl):*Foxn1*(Cre)). *Foxn1*(Cre) mice were generated by the Holländer lab via the insertion of the iCre encoding sequence into a *Foxn1* P1 artificial chromosome (PAC), which was then injected into fertilized oocytes (Zuklys *et al.*, 2009). Targeting of *Aire* in B cells was accomplished by crossing *Aire*(fl/fl) mice to *Mb1*(Cre) mice (designated *Aire*(fl/fl):*Mb1*(Cre)). *Mb1*(Cre) mice were initially generated by Elias Hobeika and purchased from Jackson laboratories. These animals express Cre recombinase from the

Mb1 locus and demonstrate 99% recombination efficiency when crossed to a Cre-reporter strain (Hobeika *et al.*, 2006).

Unfortunately, two attempts to generate mice lacking AIRE in eTACs were unsuccessful. Initially, *Aire*(fl/fl) mice were crossed to *CD45*(Cre) mice (generated by Alexander Medvinsky; designated *Aire*(fl/fl):*CD45*(Cre)). Despite genotyping results that demonstrated homozygous floxed *Aire* alleles and the presence of Cre recombinase in progeny mice, sorted B cells did not show *Aire* excision at the DNA level. While it is possible that cells expressing low levels of CD45 (i.e. eTACs) could display incomplete Cre-recombination, I would have expected B cells, which express high levels of CD45, to demonstrate appropriate excision of *Aire*. Around this time, it was reported that eTACs express RAR-related orphan receptor gamma ($ROR\gamma_t$), the protein encoded by the *Rorc* gene (Yamano *et al.*, 2019). Thus, I chose to utilize *Rorc*(Cre) mice, which when crossed to *Aire*(fl/fl) mice should result in progeny rendered AIRE-deficient in eTACs. Given that only 9-16% of cells isolated according to my eTAC gating strategy (see Chapter 3) express AIRE, I sorted double positive (DP) thymocytes, which express $ROR\gamma_t$ at a critical stage in their maturation. PCR analysis of purified DNA from DP thymocytes from *Aire*(fl/fl):*Rorc*(Cre+) mice revealed amplification of both non-recombined (not excised) and recombined (excised) *Aire*, indicating incomplete Cre-mediated recombination. As confirmation, 5-6 lymph nodes from these and control mice were isolated, fixed, permeabilized, and stained for intranuclear AIRE expression.

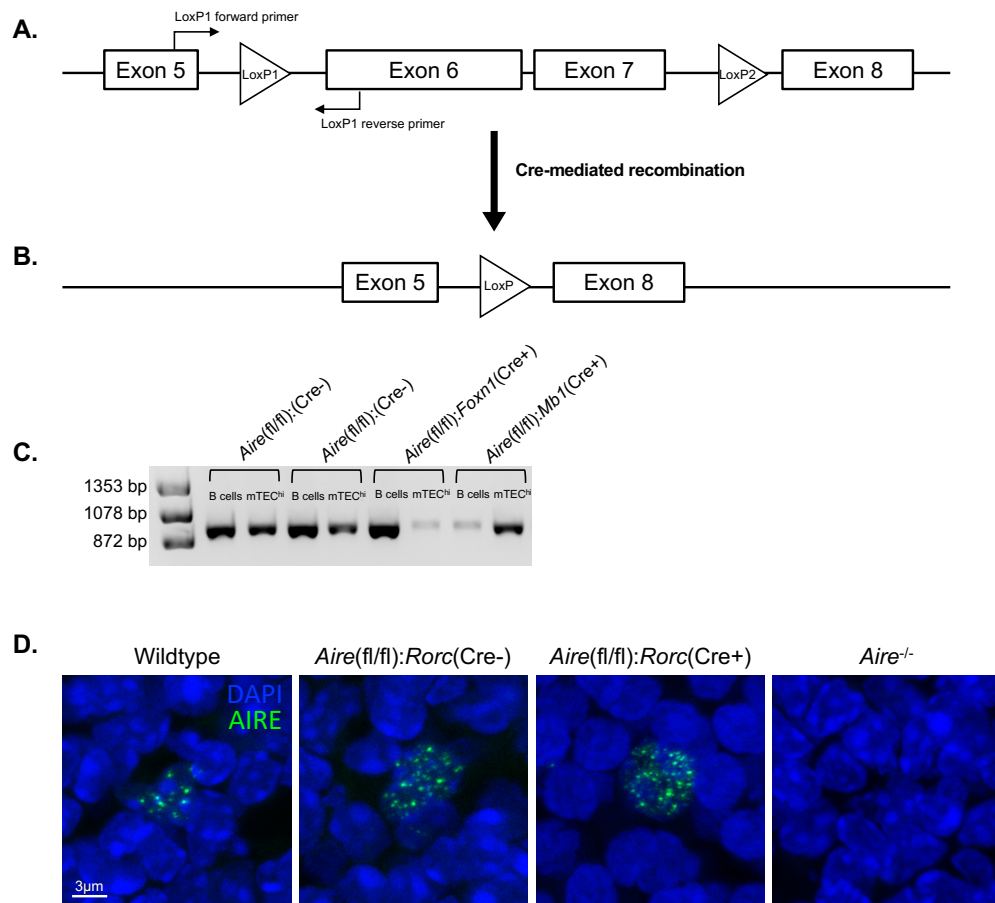


Figure 5.1. Cre-mediated recombination of *Aire*(fl/fl) mice. The *Aire*(fl/fl) genetic locus is shown A) before and B) after Cre-mediated recombination (schematic adapted from Dobeš *et al.*, 2017). C) DNA isolated from sorted B cells and mTEC^{hi}s from AIRE mutant mice was amplified using the LoxP1 primers set. The absence of Cre-mediated recombination resulted in an 891 bp band while Cre-mediated recombination resulted in no band. D) Confocal microscopy imaging of lymph nodes from 10-week-old wildtype, *Aire*(fl/fl):Rorc(Cre-), *Aire*(fl/fl):Rorc(Cre+), and *Aire*^{-/-} mice stained for the expression of intranuclear AIRE. Nuclei were counterstained using DAPI. AIRE⁺ cells were quantified in 5-6 lymph nodes per animal a blinded manner (AIRE⁺ cells per lymph node: wildtype 7.7 ± 2.1 AIRE⁺ cells; *Aire*(fl/fl):Rorc(Cre-) 6.4 ± 2.0 AIRE⁺ cells; *Aire*(fl/fl):Rorc(Cre+) 2.2 ± 0.8 AIRE⁺ cells; and *Aire*^{-/-} 0.0 ± 0.0 AIRE⁺ cells).

The appropriate excision of *Aire* was confirmed in sorted mTEC^{hi}s and B cells at the DNA level in *Aire*(fl/fl):*Foxn1*(Cre+) and *Aire*(fl/fl):*Mb1*(Cre+) mice, respectively. Cells that had not undergone Cre-mediated recombination have both exons 5 and 6 of *Aire* present, resulting in a band of 891 bp when amplified with the LoxP1 primer set (Figure 5.1A). Cells that had undergone Cre-mediated recombination lack exon 6 (and 7) of *Aire*, thus resulting in the absence of a band when amplified with the LoxP1 primer set (Figure 5.1B-C). For *Aire*(fl/fl):*Rorc*(Cre+) mice, AIRE⁺ cells were detected by immunofluorescence in the lymph

nodes of Cre-positive mice, albeit at a lower proportion than were observed in wildtype and Cre-negative controls (wildtype 7.7 ± 2.1 AIRE⁺ cells per lymph node; *Aire*(fl/fl):*Rorc*(Cre-) 6.4 ± 2.0 AIRE⁺ cells per lymph node; *Aire*(fl/fl):*Rorc*(Cre+) 2.2 ± 0.8 AIRE⁺ cells per lymph node; and *Aire*^{-/-} 0.0 ± 0.0 AIRE⁺ cells per lymph node). These findings imply either incomplete Cre-mediated excision efficiency (Bao *et al.*, 2013), or that a small proportion of eTACs do not express ROR γ t. Unfortunately, COVID-19-related restrictions coupled with the inadvertent appearance of a germline Cre in my *Aire*(fl/fl) colony (a rare occurrence that has been documented in *Mb1*(Cre) mice) precluded a third attempt at generating a strain of mice rendered AIRE-deficient in eTACs. As such, the analyses below were performed on constitutively AIRE-deficient mice (*Aire*^{-/-}), mice lacking AIRE in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)), and mice lacking AIRE in B cells (*Aire*(fl/fl):*Mb1*(Cre+)).

5.2 Loss of AIRE in the thymus affects thymocyte development

The development and education of maturing T cells, or thymocytes, occurs in the thymus, an organ critical to the formation of a diverse and tolerant T cell repertoire. Through the process of negative selection, thymocytes that react with high affinity against self-antigens are deleted from the developing T cell pool. The ectopic expression of antigens typically found only in tissues outside the thymus, or tissue-restricted antigens (TRAs), is mediated, in part, by AIRE. *Aire*^{-/-} mice have decreased TRA expression in mTECs (Anderson *et al.*, 2002), defective deletion of clonal T cell populations, and organ-specific autoimmunity (Anderson *et al.*, 2005; Liston *et al.*, 2004; Liston *et al.*, 2003). It is presumed that these manifestations in *Aire*^{-/-} mice are due to a lack of AIRE in mTECs, but the contribution of other AIRE-expressing cells has yet to be elucidated. Thus, I sought to assess the thymic compartments of cell-specific loss-of-AIRE mutant mice.

Thymi from mice with constitutive or cell lineage-specific loss-of-AIRE did not appear visually different from their wildtype or Cre-negative littermate controls. Thymic cellularity was comparable, both between 5-month-old AIRE-deficient and AIRE-sufficient mice, although some variability was observed (Figure 5.2).

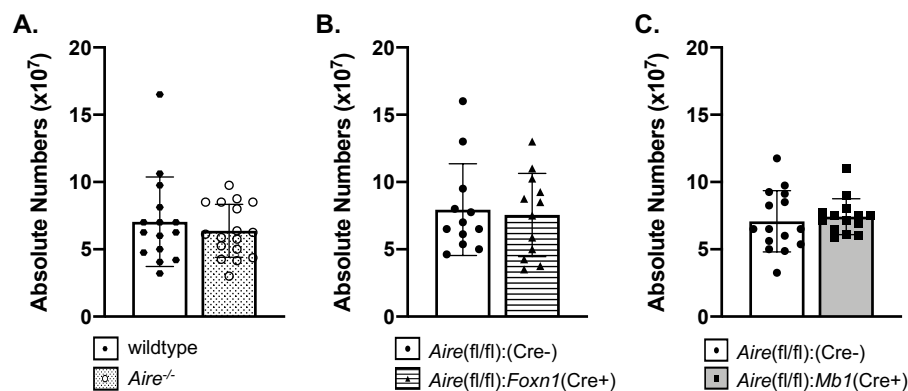


Figure 5.2. Thymic cellularity in constitutive and cell lineage-specific AIRE-deficient mice. Absolute thymic cell counts in A) wildtype (n=15) and *Aire*^{-/-} (n=17), B) *Aire*(fl/fl):*Foxn1*(Cre-) (n=12) and *Aire*(fl/fl):*Foxn1*(Cre+) (n=12), and C) *Aire*(fl/fl):*Mb1*(Cre-) (n=15) and *Aire*(fl/fl):*Mb1*(Cre+) (n=14) mice. Graphs shown represent ≥4 independent experiments per mouse strain. Statistical validity was determined by student's t test.

Aire^{-/-} mice on a C57BL/6 background have been shown to have normal thymus morphology, although thymic atrophy has been noted in some animals at 2- to 3-months of age (Ramsey *et al.*, 2002). Although I did not observe significant thymus atrophy in *Aire*^{-/-} mice when compared to wildtype controls, it is possible that differences could have been evident in younger mice and that naturally occurring age-related thymic atrophy diminished these changes by 5-months of age. Limited numbers of mice due to cage space constraints precluded the assessment of younger mice in addition to aged mice.

5.2.1 Normal early thymocyte development in cell-specific loss-of-AIRE mice

I next sought to assess early thymocyte maturation in cell lineage-specific loss-of-AIRE mutant mice (*Aire*(fl/fl):*Foxn1*(Cre+) and *Aire*(fl/fl):*Mb1*(Cre+)). A representative example of the gating strategy for early thymocyte maturation is shown in Figure 5.3. Gates were set using previously defined strategies established by the Holländer lab. Three to five million events were collected per sample.

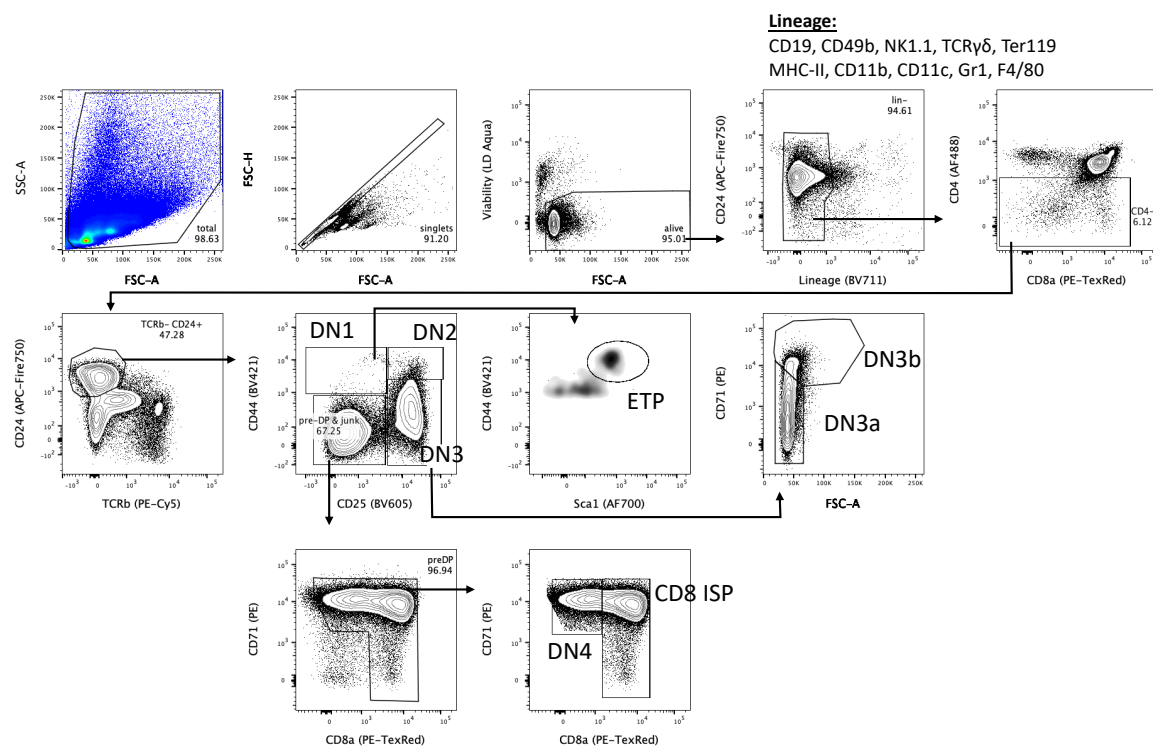


Figure 5.3. Gating strategy for early thymocyte maturation. The gating strategy is shown to identify various stages of early thymocyte maturation in 20-week-old AIRE mutant mice.

After gating on viable, single cells, a lineage channel was used to exclude B cells, NK cells, gamma-delta T cells, erythrocytes, granulocytes, and macrophages. Lineage-negative thymocytes were assessed for CD44 and CD25 expression, which delineates double negative (DN) stages 1 through 4. CD44⁺ CD25⁻ thymocytes are defined as DN1 and can be further categorized into early thymic progenitors (ETP), which express the stem cell marker Sca1 (Shortman & Wu, 1996). Upon gaining CD25 expression, thymocytes are classified as

DN2 (CD44⁺ CD25⁺). The decrease of CD44 expression defines the DN3 stage (CD44^{lo} CD25⁺), where immature thymocytes must undergo β -selection, a process by which thymocytes that have successfully rearranged their TCR β chain locus are selected. DN3 thymocytes can be further divided into the DN3a (pre- β -selection) and DN3b (post- β -selection) subpopulations based on the expression of the transferrin receptor CD71, which is required for the rapid proliferation of thymocytes having passed the β -selection checkpoint (Kelly *et al.*, 2007). The loss of CD25 expression defines the pre-double positive (pre-DP) developmental stage, which consists of cells at their final double negative stage of development (DN4) and thymocytes that are positive for CD8 but still lack the expression of the coupled $\alpha\beta$ TCR and thus represent a transient, immature stage of single CD8 positivity (CD8 ISP).

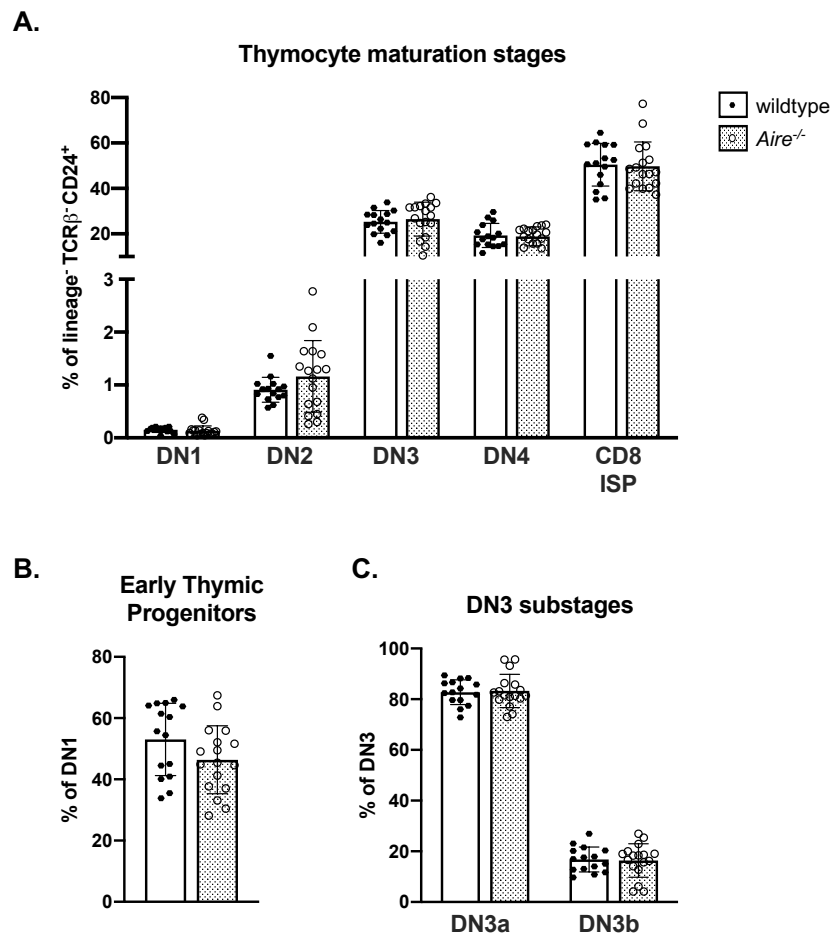


Figure 5.4. Early thymocyte maturation in constitutively AIRE-deficient mice. A) 20-week-old wildtype (n=15) and *Aire*^{-/-} (n=17) mice were assessed for the frequency of A) cells at various stages of early thymocyte maturation, B) early thymic progenitors (ETP) within the DN1 stage, and C) cells with DN3a and DN3b phenotypes. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test.

Two-tailed, unpaired t tests were used to compare *Aire*^{-/-} mice to their wildtype, littermate controls (Figure 5.4). Significant differences in early thymocyte maturation were not observed. This finding corroborates previous reports that demonstrated a lack of an impact of AIRE on early thymocyte development (Hubert *et al.*, 2009).

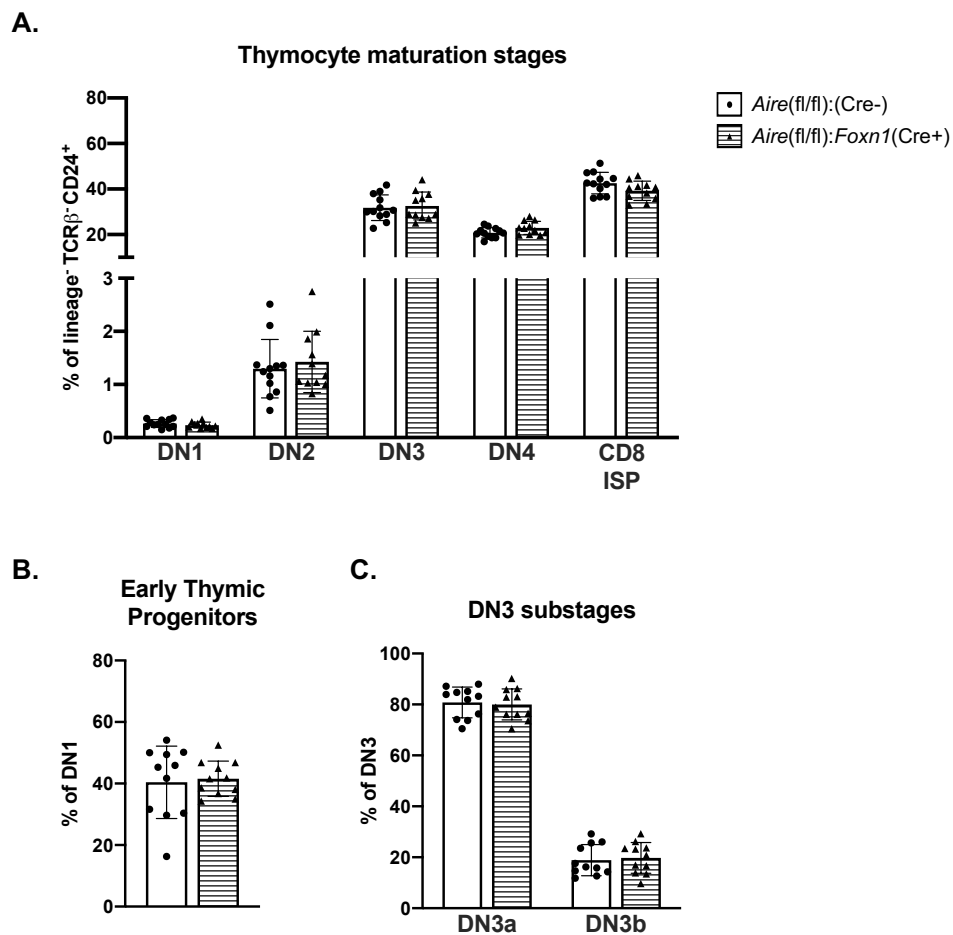


Figure 5.5. Early thymocyte maturation in mice rendered AIRE-deficient in mTECs. A) 20-week-old *Aire(fl/fl);(Cre-)* (n=12) and *Aire(fl/fl);Foxn1(Cre+)* (n=11) mice were assessed for the frequency of A) cells at various stages of early thymocyte maturation, B) early thymic progenitors (ETP) within the DN1 stage, and C) cells with DN3a and DN3b phenotypes. Graphs shown represent 4 independent experiments. Statistical validity was determined by student's t test.

Similar to what was observed in the constitutively AIRE-deficient mice, the frequencies of cells in the early stages of thymocyte maturation were uncompromised in mice rendered AIRE-deficient either only in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)) or in B cells (*Aire*(fl/fl):*Mb1*(Cre+)) when compared to AIRE-sufficient littermates (*Aire*(fl/fl):(Cre-)) (Figures 5.5 and 5.6). These findings reinforce the principle that AIRE does not play a role in early thymocyte development in mice (Hubert *et al.*, 2009).

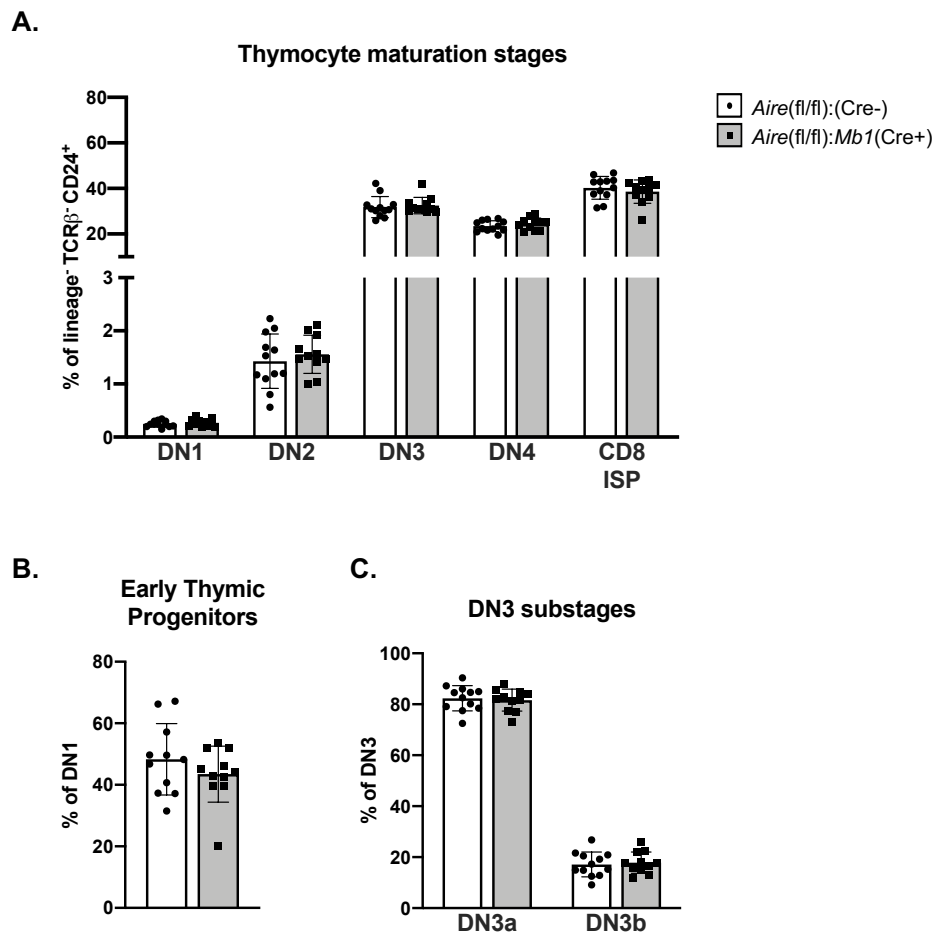


Figure 5.6. Early thymocyte maturation in mice rendered AIRE-deficient in B cells. A) 20-week-old *Aire*(fl/fl):(Cre-) (n=12) and *Aire*(fl/fl):*Mb1*(Cre+) (n=11) mice were assessed for the frequency of A) cells at various stages of early thymocyte maturation, B) early thymic progenitors (ETP) within the DN1 stage, and C) cells with DN3a and DN3b phenotypes. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test.

5.2.2 Loss of AIRE in the thymus affects thymocyte negative selection

Negative selection occurs in two waves, initially in the inner cortex, where double positive thymocytes with high affinity for self-antigens are deleted by cortical dendritic cells (Daley *et al.*, 2013; McCaughy *et al.*, 2008). Thymocytes that have survived this first wave of selection then acquire CCR7, which is necessary for entry into the thymic medulla (Nitta *et al.*, 2009). There, the second wave of negative selection occurs, mediated by mature mTECs via the promiscuous expression of peripheral antigens (Derbinski *et al.*, 2001). Given the role of AIRE for the promiscuous gene expression and thus thymocyte negative selection (Anderson *et al.*, 2005), I next sought to assess the waves of negative selection in mice constitutively and conditionally deficient for AIRE expression. The gating strategy used to assess the negative selection of double positive (DP) and CD4 single positive (CD4 SP) thymocytes is shown in Figure 5.7. Gates were set based on previously defined strategies from the Holländer lab. Five to ten million events were collected per sample.

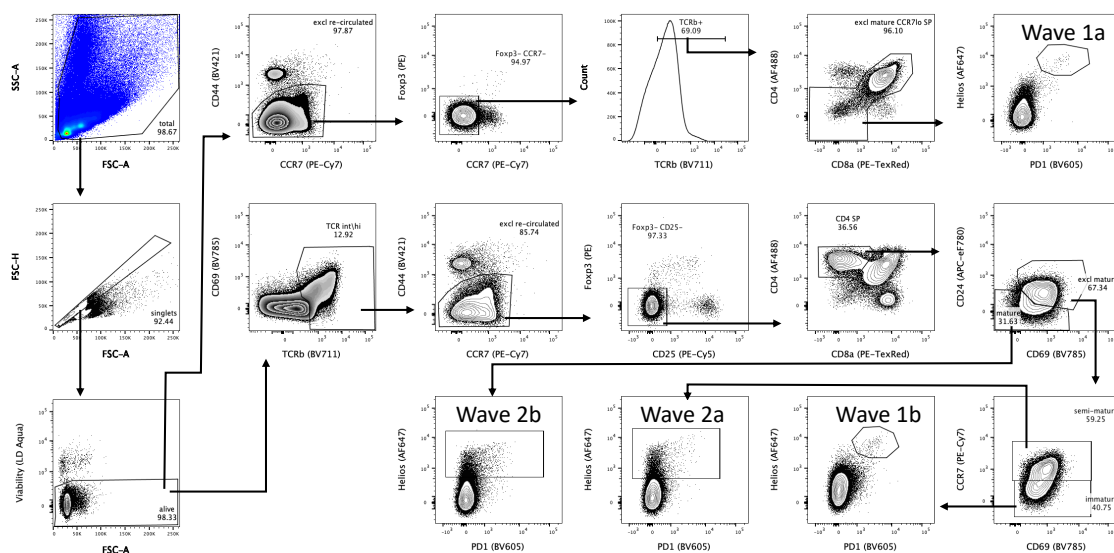


Figure 5.7. Gating strategy for negative selection of thymocytes. The gating strategy is shown to identify the four substages of thymocyte negative selection in 20-week-old AIRE mutant mice.

After gating on viable, single cells, re-circulating T cells and T_{reg} were excluded based on their CD44⁺ and FOXP3⁺ phenotypes, respectively. Cells with intermediate to high levels of TCR β expression were then assessed for CD4 and CD8a expression. Double positive (DP; i.e. CD4⁺ CD8⁺) thymocytes were then assessed for Helios and PD1 expression, a phenotype identifying the Wave 1a stage of negative selection (Daley *et al.*, 2013). For the remaining substages of negative selection, single positive CD4 (CD4 SP) cells were classified as either immature (CD24⁺ CCR7⁻), semi-mature (CD24⁺ CCR7⁺), or mature (CD24⁻ CCR7⁺). Immature CD4 SP thymocytes were then assessed for Helios and PD1 expression, with Helios⁺ PD1⁺ representing the Wave 1b stage of negative selection. Semi-mature and mature CD4 SP thymocytes expressing Helios constitute the Wave 2a and Wave 2b stages of negative selection, respectively.

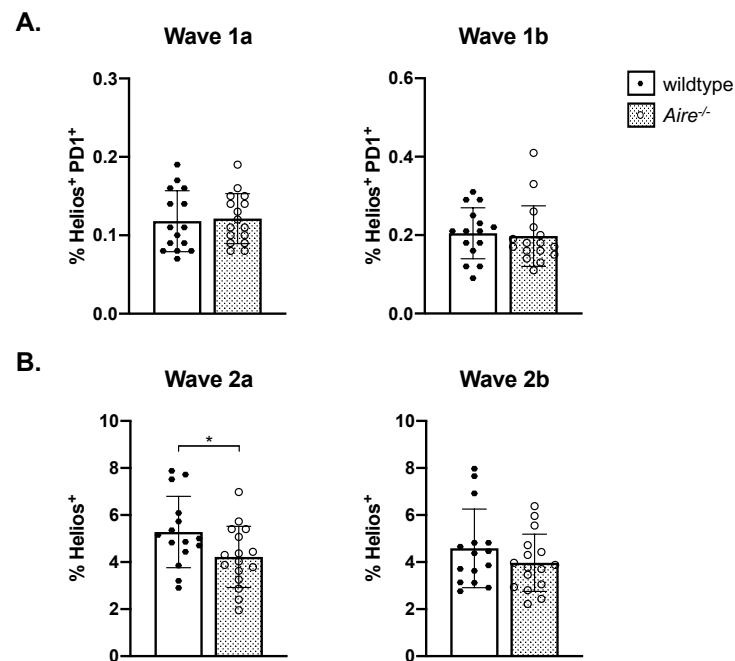


Figure 5.8. Negative selection of thymocytes in constitutively AIRE-deficient mice. 20-week-old wildtype (n=15) and *Aire*^{-/-} (n=16) mice were assessed for the frequency of cells at the A) Wave 1 and B) Wave 2 stages of negative selection. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test where *p<0.05.

Wave 1a and Wave 1b are defined as the percentage of Helios⁺ PD1⁺ cells among DP CD24⁺ CCR7⁻ and CD4 SP CD24⁺ CCR7⁻ thymocytes, respectively. Wave 2a and Wave 2b are defined as the percentage of Helios⁺ cells among CD4 SP CD24⁺ CCR7⁺ and CD4 SP CD24⁻ CCR7⁺ thymocytes, respectively. Constitutively AIRE-deficient (*Aire*^{-/-}) mice demonstrated a statistically significant decrease in the frequency of cells at the Wave 2a stage of negative selection when compared to their littermate controls (Figure 5.8; *p=0.0445). This finding confirms a similar observation that constitutively AIRE-deficient mice show an increase in the single positive 3 (SP3) stage of thymocyte development (see Figure 5.9) (J. Li *et al.*, 2007). Although these authors measured the number of cells that successfully avoided negative selection (SP1 through SP4), and I measured the frequency of cells marked for deletion between each of those stages (Wave 1a through Wave 2b), my findings of decreased cells in the Wave 2a stage are consistent with their findings of increased cells in the SP3 stage.

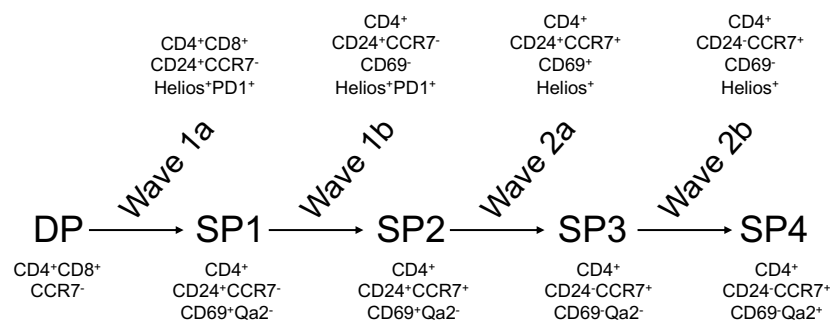


Figure 5.9. Comparison of phenotypes of thymocyte selection stages between SP thymocyte developmental stages and waves of negative selection. Between each single positive stage of thymocyte development, thymocytes must successfully avoid waves of negative selection.

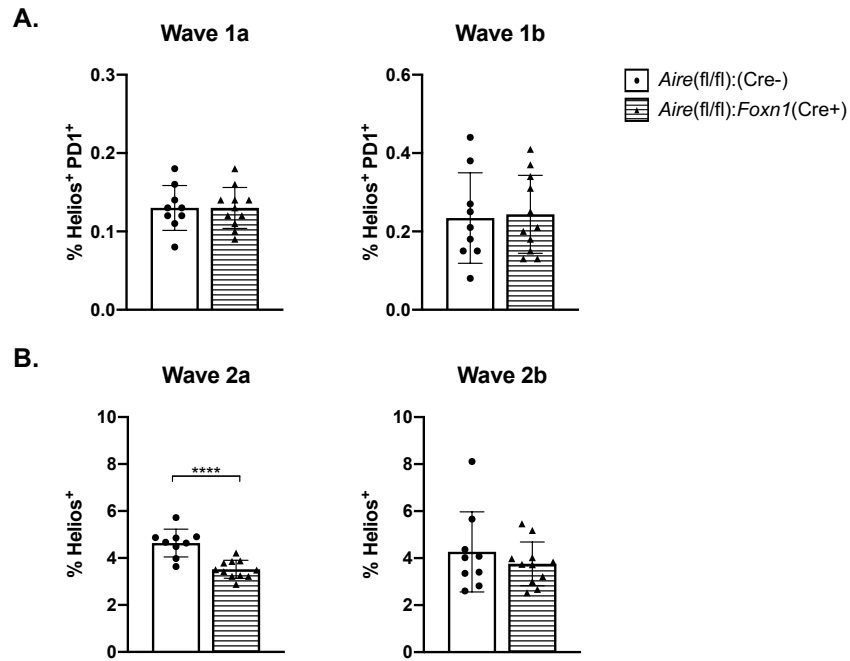


Figure 5.10. Negative selection of thymocytes in mice rendered AIRE-deficient in mTECs. 20-week-old *Aire(fl/fl):(Cre-)* (n=9) and *Aire(fl/fl):Foxn1(Cre+)* (n=11) mice were assessed for the frequency of cells at the A) Wave 1 and B) Wave 2 stages of negative selection. Graphs shown represent 4 independent experiments. Statistical validity was determined by student's t test where ****p<0.0001.

Mice lacking AIRE in mTECs (*Aire(fl/fl):Foxn1(Cre+)*) demonstrated a significant decrease in the frequency of cells at the Wave 2a negative selection stage compared to their littermate, Cre-negative controls (Figure 5.10; ****p<0.0001). This finding suggested that the lack of AIRE protein expression in mature mTECs impaired negative selection.

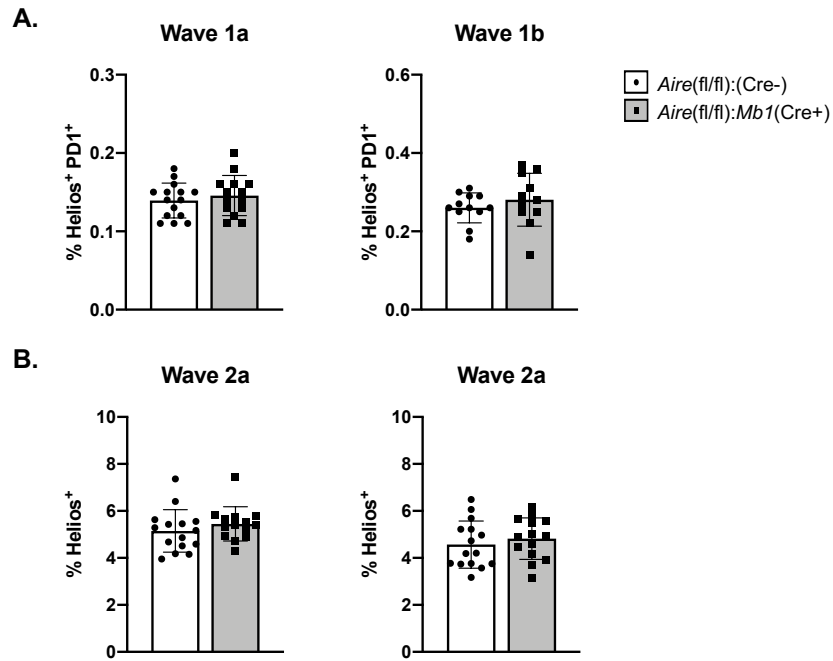


Figure 5.11. Negative selection of thymocytes in mice rendered AIRE-deficient in B cells. 20-week-old *Aire(fl/fl):Cre-* (n=15) and *Aire(fl/fl):Mb1(Cre+)* (n=14) mice were assessed for the frequency of cells at the A) Wave 1 and B) Wave 2 stages of negative selection. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test.

Differences in negative selection were not observed in mice lacking AIRE in B cells (*Aire(fl/fl):Mb1(Cre+)*) (Figure 5.11). This finding suggested that any contribution to negative selection made by AIRE-expressing B cells is either redundant, or so subtle it cannot be quantified by the methods used here.

5.2.3 Thymic T_{reg} and re-circulating T cells affected by loss of AIRE

A reduction of intrathymic T_{reg} cells has been observed in constitutively AIRE-deficient mice, implicating AIRE in the regulation of thymic T_{reg} cells (Lei *et al.*, 2011; Malchow *et al.*, 2013). Therefore I sought to assess thymic T_{reg} in mice rendered AIRE-deficient in mTECs and B cells (*Aire(fl/fl):Foxn1(Cre+)* and *Aire(fl/fl):Mb1(Cre+)*, respectively) to determine the contribution of AIRE in various cell subsets to this process. A representative example of the gating strategy for thymic T_{reg} is shown in Figure 5.12. Gates were set based on previously

defined strategies from the Holländer lab. Five to ten million events were collected per sample.

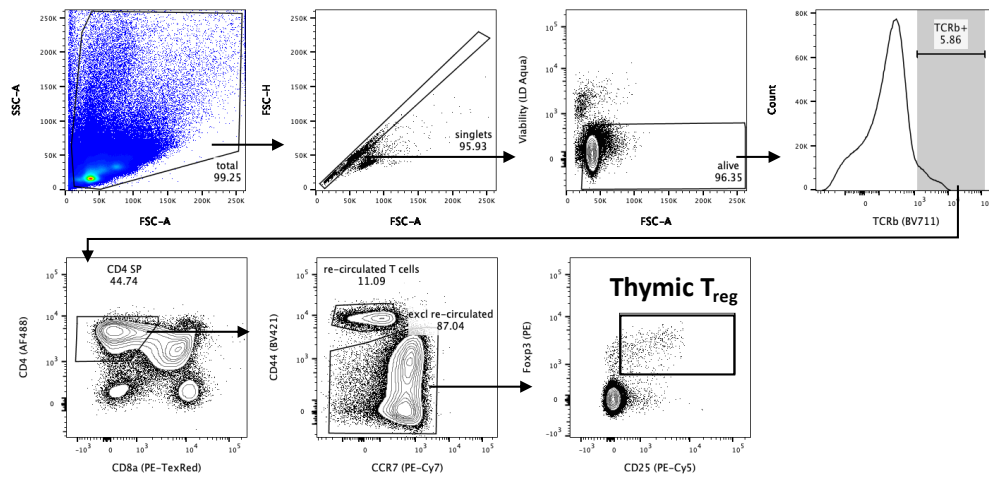


Figure 5.12. Gating strategy to identify thymic T_{reg}. The gating strategy is shown to identify thymic T_{reg} cells in 20-week-old AIRE mutant mice.

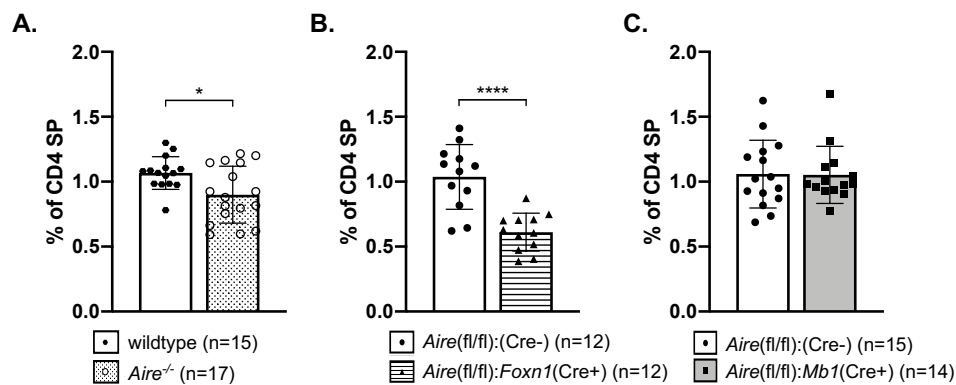


Figure 5.13. Thymic T_{reg} in different AIRE-deficient mouse strains. The frequency of thymic T_{reg} was assessed in mice lacking AIRE either A) constitutively (*Aire*^{-/-}), B) in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)), and C) in B cells (*Aire*(fl/fl):*Mb1*(Cre+)) compared to appropriate controls. Graphs shown represent ≥4 independent experiments per mouse strain. Statistical validity was determined by student's t test where *p<0.05, ****p<0.0001.

Thymic T_{reg} cells are defined as TCRβ⁺ CD44⁺ CD4 SP cells that express both FOXP3 and CD25. Mice lacking AIRE constitutively (*Aire*^{-/-}) and in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)) resulted in a statistically significant decrease in the frequency of thymic T_{reg} (Figure 5.13; *p=0.0144 and ****p<0.0001, respectively) compared to their respective controls. Such differences were not observed in mice lacking AIRE in B cells only ((*Aire*(fl/fl):*Mb1*(Cre+)).

These findings imply that the reduction in intrathymic T_{reg} in constitutively AIRE-deficient mice reported previously (Lei *et al.*, 2011; Malchow *et al.*, 2013) correlates with an absence of AIRE in mTECs. Indeed, double-transgenic mouse chimera experiments have shown that antigen presentation by AIRE-expressing mTECs is sufficient for the generation of antigen-specific T_{reg} (Aschenbrenner *et al.*, 2007), indicating that lack of antigen-specific T_{reg} may play a role in the development of autoimmune disease in the context of AIRE-deficiency.

Possible explanations for the more prominent decrease in thymic T_{reg} observed in mice rendered AIRE-deficient in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)) compared to constitutively AIRE-deficient mice (*Aire*^{-/-}) are technical and/or biological in nature. First, an F-test to compare the variances of wildtype and *Aire*^{-/-} animals revealed statistically significant differences in variation (*p=0.0417). In contrast, the variances observed in mice rendered AIRE-deficient in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)) or in B cells (*Aire*(fl/fl):*Mb1*(Cre+)) were not significantly different than their Cre-negative controls (p=0.0913 and p=0.5467, respectively). It is possible that increasing the sample size of constitutively AIRE-deficient mice could increase the significance. Alternatively, it is possible that other AIRE-expressing cells, such as thymic B cells, could be playing an opposing role in thymic T_{reg} commitment to that of AIRE-expressing mTECs (i.e. AIRE-expressing mTECs promote thymic T_{reg} commitment while AIRE-expressing B cells impede thymic T_{reg} commitment). In this scenario, the role of AIRE-expressing thymic B cells would have to be subtle, or I would expect to see an increase in thymic T_{reg} in mice lacking AIRE in B cells only, which was not the case. This could be further examined by overexpressing AIRE in B cells, either by treatment with anti-CD40 (Yamano *et al.*, 2015), or genetic targeting, followed by adoptive transfer into constitutively AIRE-deficient hosts.

I next assessed the frequency of T cells in the different mouse strains generated that have re-circulated from the periphery back to the thymus. The re-circulation of mature T cells from the periphery back to the thymus has been shown by the adoptive transfer of labeled mature T cells into recipient mice and through the use of transgenic mouse models (Agus *et al.*, 1991; McCaughy *et al.*, 2007; Michie *et al.*, 1988). Re-circulating T cells are thought to play a role in both positive (Kirberg *et al.*, 2008) and negative selection (Shimonkevitz & Bevan, 1988; Sprent *et al.*, 1990). A representative example of the gating strategy for re-circulating mature T cells in the thymus is shown in Figure 5.14. Gates were set based on previously defined strategies from the Holländer lab. Five to ten million events were collected per sample.

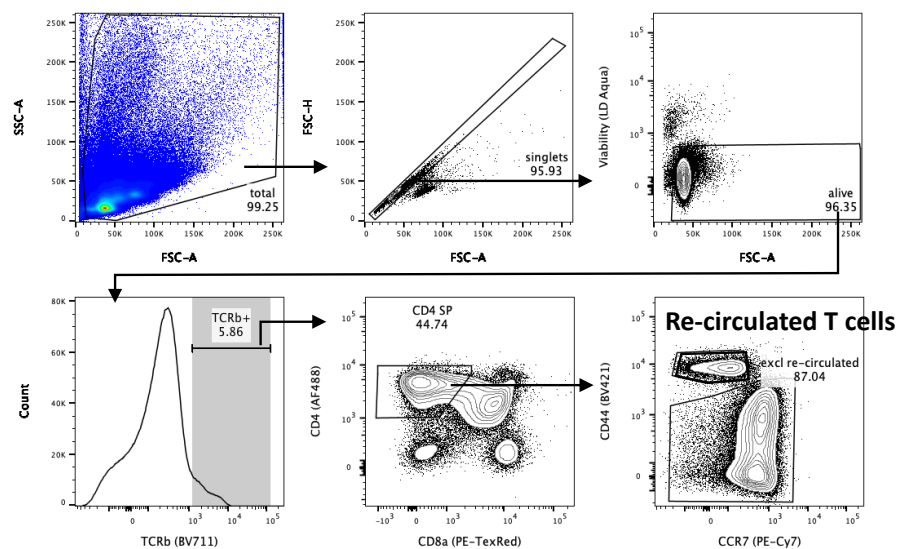


Figure 5.14. Gating strategy to identify re-circulating T cells in the thymus. The gating strategy is shown to identify T cells that have re-circulated from the periphery back to the thymus in 20-week-old AIRE mutant mice.

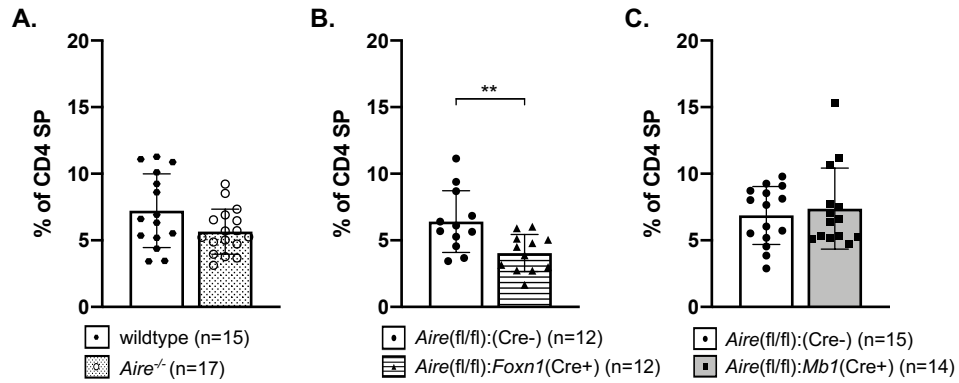


Figure 5.15. Detection of re-circulating T cells in the thymus of AIRE mutant mouse strains. The frequency of T cells that had re-circulated from the periphery back to the thymus was assessed in mice lacking AIRE A) constitutively (*Aire*^{-/-}), B) in mTECs (*Aire*(fl/fl):*Foxn1*(Cre⁺)), and C) in B cells (*Aire*(fl/fl):*Mb1*(Cre⁺)) compared to appropriate controls. Graphs shown represent >4 independent experiments per mouse strain. Statistical validity was determined by student's t test where **p<0.01

Re-circulating T cells are defined as TCR β ⁺ CCR7⁻ CD4 SP cells that express high levels of CD44. Lack of AIRE in mTECs (*Aire*(fl/fl):*Foxn1*(Cre⁺)) resulted in a statistically significant decrease in the frequency of re-circulating T cells (Figure 5.15; **p=0.0062) when compared to controls. Differences in re-circulating T cells were also observed in constitutively AIRE-deficient mice (*Aire*^{-/-}), but this was not statistically significant (p=0.0584). It is possible that significance could be reached by increasing the number of samples tested. These findings suggest that the absence of AIRE in mTECs limits the entry of peripheral T cells back into the thymus, further expanding on the role of re-circulating T cells in negative selection (Shimonkevitz & Bevan, 1988; Sprent *et al.*, 1990).

In the thymus, I have demonstrated a role for AIRE in mTECs in regulation of the Wave 2a stage of negative selection, thymic T_{reg}, and re-circulating T cells. Given the increase in SP3 thymocytes in *Aire*^{-/-} mice (J. Li *et al.*, 2007), my observed decrease in the frequency of thymocytes selected for deletion in the Wave 2a stage of negative selection is logical. The subsequently more abundant SP3 thymocytes are likely autoreactive in nature. It is unclear

whether AIRE-expressing mTECs are involved in the generation or maintenance of T_{reg}, although others have shown they are antigen-specific (Aschenbrenner *et al.*, 2007). Increased re-circulating T cells have not specifically be shown in *Aire*^{-/-} mice, but their role in negative selection has been reported (Shimonkevitz & Bevan, 1988; Sprent *et al.*, 1990).

5.3 Peripheral T cell compartment affected by loss of AIRE

It is the presence of autoreactive cells in the periphery that leads to autoimmune manifestations in AIRE-deficient mice and APECED patients (Anderson *et al.*, 2002; Kuroda *et al.*, 2005; Nagamine *et al.*, 1997; Perheentupa, 2006). Given the effect of AIRE-deficiency in mTECs on the Wave 2a stage of thymocyte negative selection and thymic T_{reg} generation and/or maintenance, I next sought to determine the impact of increased autoreactive thymocytes and decreased thymic T_{reg} on the peripheral T cell pool.

The most immature T cells to enter the periphery from the thymus are known as recent thymic emigrants (RTE), a population that is phenotypically distinct from that of SP thymocytes and mature naïve T cells (Boursalian *et al.*, 2004). It is thought that within the T cell (and B cell) repertoire, self-reactive ‘forbidden clones’ exist and must be deleted to prevent autoimmune disease (Burnet, 1972). RTE mature to become naïve T cells, which then acquire a memory phenotype upon interaction with their antigen. T cells that repeatedly encounter their antigen in the absence of strong CD28 costimulatory receptor ligation can be rendered functionally unresponsive, or anergic (Harding *et al.*, 1992; Mueller *et al.*, 1989). There are several independent pathways that can lead to anergy, at least one of which requires T_{reg} (Adler *et al.*, 1998; Martinez *et al.*, 2012). Given that *Aire*^{-/-} mice have defects in peripheral T cell populations, cell lineage-specific loss-of-AIRE mice

were assessed to elucidate the contribution of different AIRE-expressing cells to these findings. A representative example of the gating strategy for peripheral T cells is shown in Figure 5.16. Gates were based on previously defined strategies from the Holländer lab. Two million events were collected per sample.

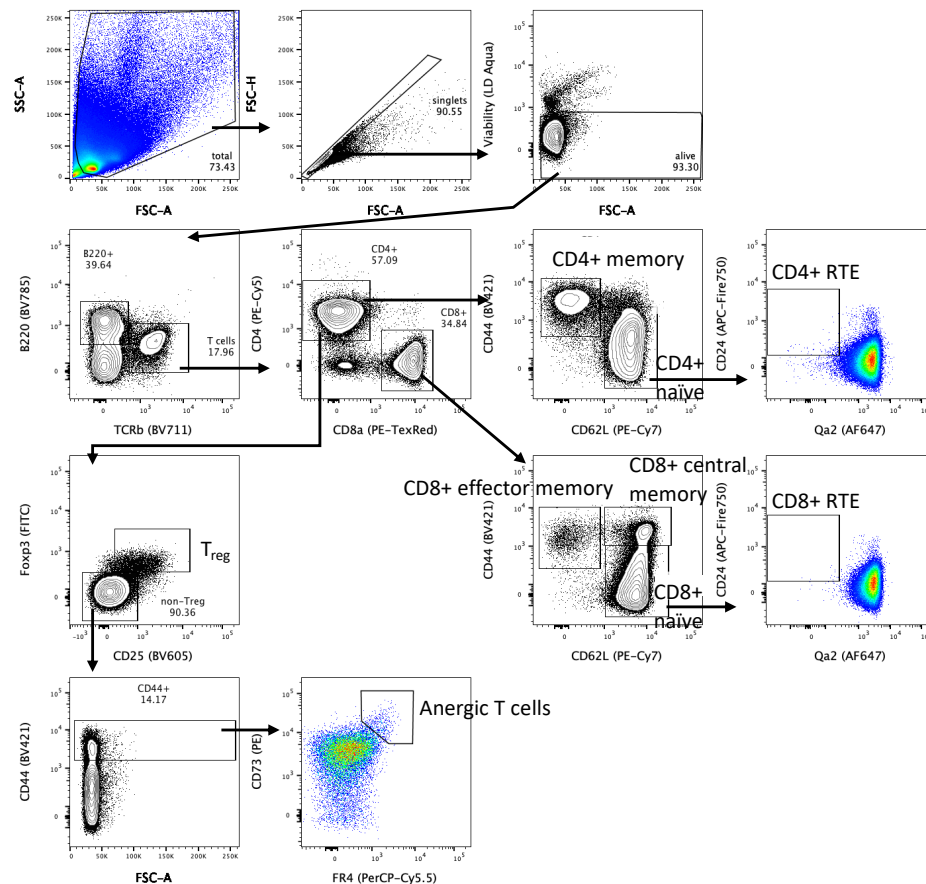


Figure 5.16. Gating strategy to identify peripheral T cells subsets in AIRE mutant mice. The gating strategy is shown to identify peripheral T cell subsets in 20-week-old constitutive and conditional loss-of-AIRE mutant mice.

After gating on viable, single cells, T cells (phenotypically defined as B220⁻ TCRβ⁺ in my analysis) were assessed for their CD4 and CD8a expression. Within CD4⁺ T cells, naïve and memory T cells were identified based on their CD44 and CD62L expression, identifying CD44^{lo/-} CD62L⁺ and CD44⁺ CD62L⁻ phenotypes, respectively. Within the naïve T cell gate, recent thymic emigrant (RTE) cells were identified as CD24⁺ Qa2⁻ (Xu & Ge, 2014). T_{reg} were identified as FOXP3⁺ CD25⁺, while the remaining, non-T_{reg} CD4⁺ cells were assessed for

CD44, CD73, and FR4 expression, with cells being positive for all three markers considered to be anergic CD4⁺ T cells (Kalekar *et al.*, 2016; Martinez *et al.*, 2012).

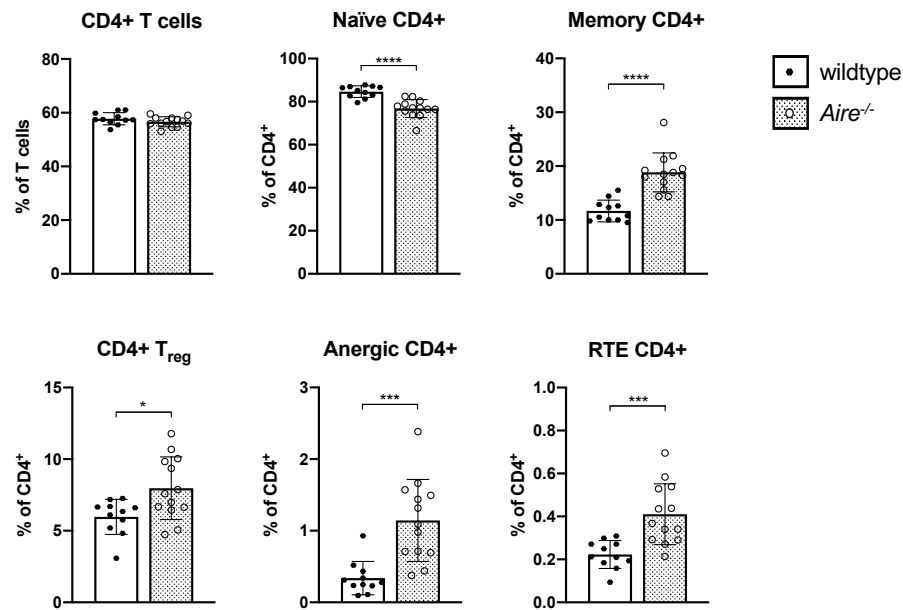


Figure 5.17. Peripheral CD4⁺ T cells in constitutively AIRE-deficient mice. Wildtype (n=11) and *Aire*^{-/-} (n=13) mice were assessed for the frequency of various CD4⁺ T cell subsets at 20-weeks of age. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test where *p<0.05, ***p<0.001, ****p<0.0001.

Mice lacking AIRE constitutively (*Aire*^{-/-}) demonstrated a statistically significant decrease in the frequency of splenic CD4⁺ naïve T cells (****p<0.0001) and, correspondingly, an increase in the frequency of CD4⁺ memory T cells (****p<0.0001). Moreover, the frequencies of T_{reg} (*p=0.0133), CD4⁺ anergic T cells (***p=0.0002), and recent thymic emigrant (RTE) CD4⁺ T cells (***p=0.0006) were increased compared to their littermate, wildtype controls (Figure 5.17). This is consistent with what has been shown in the literature (Kalekar *et al.*, 2016).

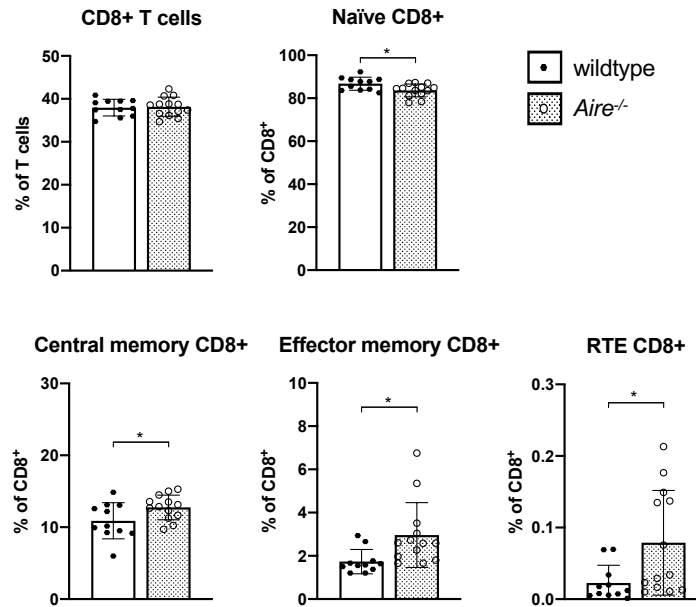


Figure 5.18. Peripheral CD8⁺ T cells in constitutively AIRE-deficient mice. Wildtype (n=11) and *Aire*^{-/-} (n=13) mice were assessed for the frequency of various CD8⁺ T cell subsets at 20-weeks of age. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test where *p<0.05.

Similar to what was seen for peripheral CD4⁺ T cells, constitutively AIRE-deficient mice (*Aire*^{-/-}) had decreased frequencies of CD8⁺ naïve T cells (*p=0.0183) and increased frequencies of CD8⁺ memory T cells (central memory *p=0.0435; effector memory *p=0.0179). The frequency of recently emigrated CD8⁺ T cells was also increased compared to controls (*p=0.0241) (Figure 5.18). It is important to note the high degree of variance observed within the RTE CD8⁺ population in *Aire*^{-/-} mice. An F-test to compare variances revealed a statistically significant difference in the variances of wildtype and *Aire*^{-/-} mice (**p=0.0018). There appear to be two distinct populations of RTE CD8⁺ cells, that do not segregate by experiment. This suggests heterogeneity within constitutively AIRE-deficient mice that does not seem to be technical in nature.

It follows logically that a decrease in negatively selected thymocytes at the Wave 2a stage would result in more autoreactive T cells leaving the thymus (increased recent thymic

emigrants). Similarly, autoreactive T cells have a better chance of seeing their cognate antigen in the periphery, thus more T cells would progress to a memory phenotype (increased memory T cells and decreased naïve T cells). Further, autoreactive T cells in the periphery can become anergic upon self-antigen recognition and anergic T cells can become anergy-derived T_{reg} through mechanisms of peripheral tolerance (Kalekar & Mueller, 2017).

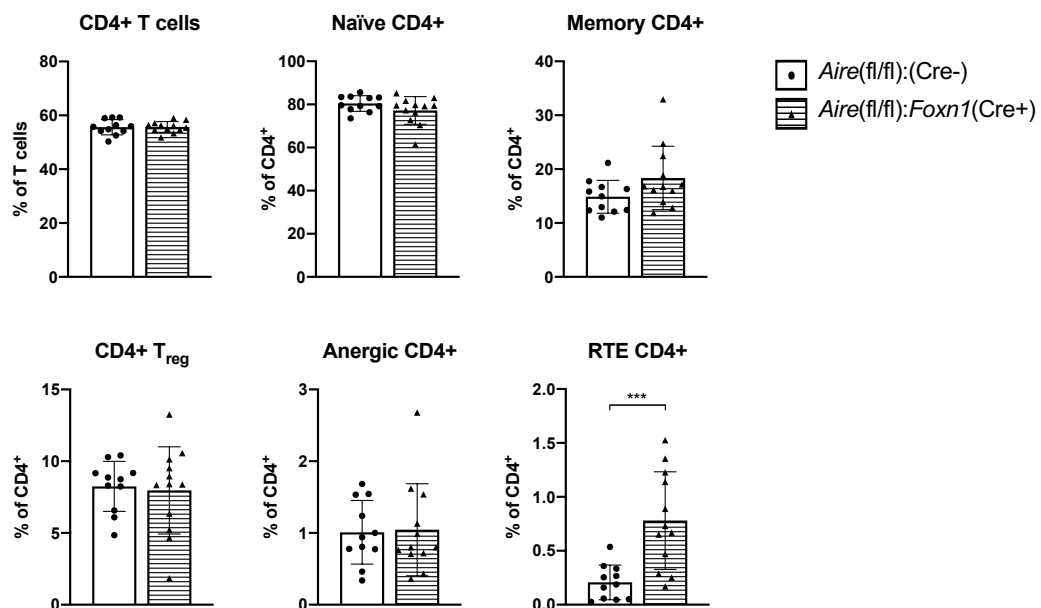


Figure 5.19. Peripheral CD4⁺ T cells in mice lacking AIRE in mTECs (*Aire(fl/fl):Foxn1(Cre+)*). *Aire(fl/fl):(Cre-)* (n=11) and *Aire(fl/fl):Foxn1(Cre+)* (n=12) mice were assessed for the frequency of various CD4⁺ T cell subsets at 20-weeks of age. Graphs shown represent 4 independent experiments. Statistical validity was determined by student's t test where ***p<0.001.

Of the peripheral CD4⁺ T cells subsets assessed, only the frequency of RTE CD4⁺ T cells was significantly increased in mice rendered AIRE-deficient in mTECs (*Aire(fl/fl):Foxn1(Cre+)*) compared to their littermate, Cre-negative controls (***p=0.0007) (Figure 5.19). These findings could suggest that cells immediately leaving the thymus with AIRE-deficient mTECs, which were not appropriately negatively selected and thus more numerous, were able to be removed from the repertoire elsewhere in the periphery. Given that, in contrast to *Aire(fl/fl):Foxn1(Cre+)* mice, abnormalities were observed in multiple peripheral CD4⁺ T

cell subsets in constitutively AIRE-deficient mice (*Aire*^{-/-}), it is possible that peripheral AIRE in eTACs plays a role in removing autoreactive CD4⁺ T cells from the repertoire.

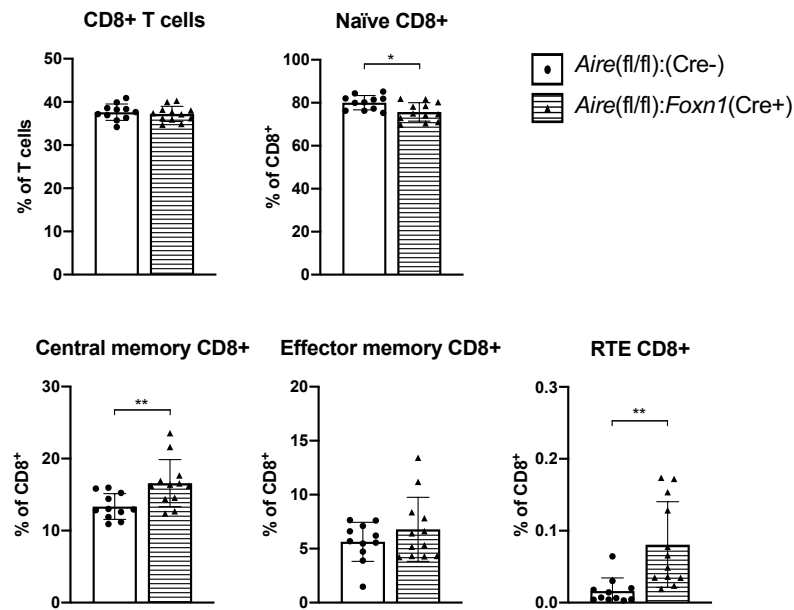


Figure 5.20. Peripheral CD8⁺ T cells in mice lacking AIRE in mTECs (*Aire(fl/fl):Foxn1(Cre+)*). *Aire(fl/fl):Cre-* (n=11) and *Aire(fl/fl):Foxn1(Cre+)* (n=12) mice were assessed for the frequency of various CD8⁺ T cell subsets at 20-weeks of age. Graphs shown represent 4 independent experiments. Statistical validity was determined by student's t test where *p<0.05, **p<0.01.

Within the CD8⁺ T cell compartment, *Aire(fl/fl):Foxn1(Cre+)* mice had decreased naïve CD8⁺ T cell frequencies (*p=0.0149) and increased central memory (**p=0.0080) and RTE (**p=0.0024) CD8⁺ T cell frequencies compared to their littermate, Cre-negative controls (Figure 5.20). These findings suggest that the abnormalities observed in peripheral CD8⁺ T cell subsets in *Aire*^{-/-} mice (increased RTE CD8⁺, decreased naïve CD8⁺, increased memory CD8⁺) are due to a lack of AIRE in mTECs, specifically. This leaves open the possibility that AIRE-mediated peripheral tolerance mechanisms might preferentially contribute to the deletion of CD4⁺ T cells over CD8⁺ T cells.

To determine whether AIRE-expressing B cells contribute to the maintenance of normal peripheral T cell populations, I next assessed mice rendered AIRE-deficient in B cells (*Aire*(fl/fl):*Mb1*(Cre+)) for splenic CD4⁺ and CD8⁺ T cell subsets.

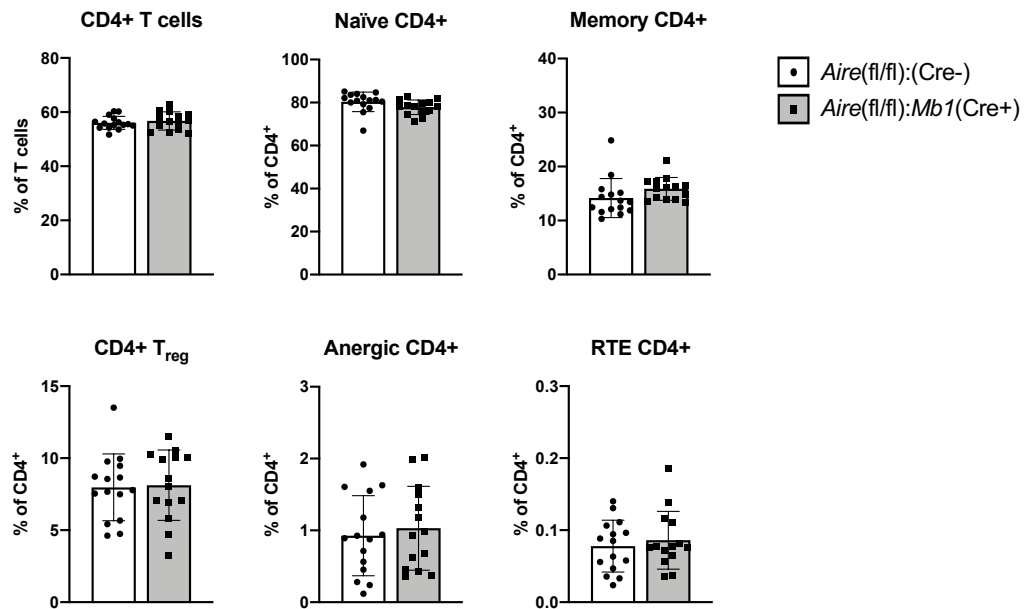


Figure 5.21. Peripheral CD4⁺ T cells in mice lacking AIRE in B cells (*Aire*(fl/fl):*Mb1*(Cre+)). *Aire*(fl/fl):Cre- (n=15) and *Aire*(fl/fl):*Mb1*(Cre+) (n=14) mice were assessed for the frequency of various CD4⁺ T cell subsets at 20-weeks of age. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test.

Significant differences in CD4⁺ and CD8⁺ T cell subset frequencies were not observed when comparing *Aire*(fl/fl):*Mb1*(Cre+) mice to their littermate, Cre-negative controls (Figures 5.21 and 5.22).

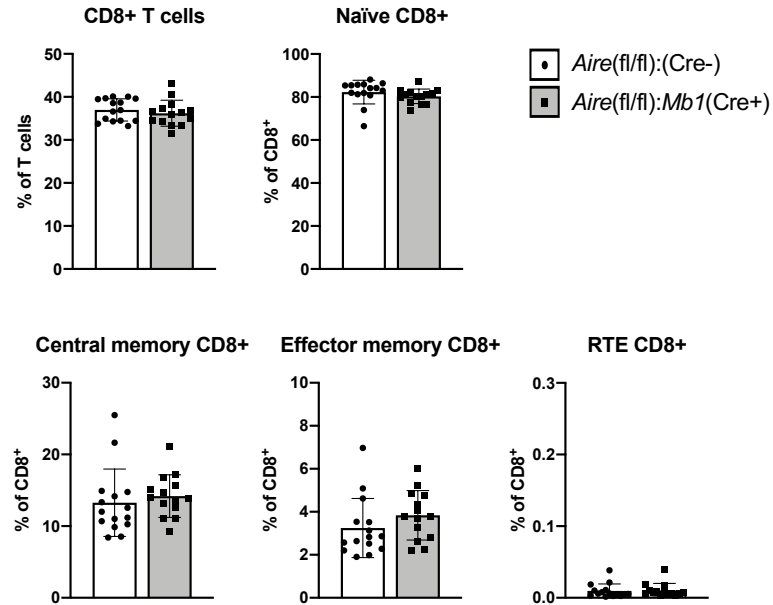


Figure 5.22 Peripheral CD8⁺ T cells in mice lacking AIRE in B cells (*Aire(fl/fl):Mb1(Cre+)*). *Aire(fl/fl):(Cre-)* (n=15) and *Aire(fl/fl):Mb1(Cre+)* (n=14) mice were assessed for the frequency of various CD8⁺ T cell subsets at 20-weeks of age. Graphs shown represent 5 independent experiments. Statistical validity was determined by student's t test.

Taken together, these results indicate that some, but not all, peripheral T cell abnormalities observed in *Aire*^{-/-} mice are explained by lack of AIRE in FOXP1-expressing mTECs (Table 5.1). Specifically, both constitutively AIRE-deficient mice (*Aire*^{-/-}) and mice rendered AIRE-deficient in mTECs (*Aire(fl/fl):Foxn1(Cre+)*) had increased frequencies of RTE CD4⁺ and RTE CD8⁺ T cells. This observation is logical given that constitutive AIRE-deficiency and AIRE-deficiency in mTECs (*Aire*^{-/-} and *Aire(fl/fl):Foxn1(Cre+)*, respectively) resulted in a decrease in the frequency of cells in the Wave 2a stage of negative selection. Fewer cells being deleted in the Wave 2a stage would lead to an increased number of thymocytes entering the periphery as RTE. Similarly, both constitutively AIRE-deficient mice (*Aire*^{-/-}) and mice rendered AIRE-deficient in mTECs (*Aire(fl/fl):Foxn1(Cre+)*) had decreased CD8⁺ naïve T cells and a corresponding increase in CD8⁺ memory T cells, presumably because the influx of RTE would contain autoreactive T cells that would encounter their antigen in the periphery, thus acquiring a memory phenotype.

Interestingly, differences observed between *Aire*^{-/-} and *Aire*(fl/fl):*Foxn1*(Cre+) mice, specifically in the peripheral CD4⁺ T cell compartment, might suggest a role for extra-thymic AIRE in peripheral tolerance. Although *Aire*(fl/fl):*Foxn1*(Cre+) mice have increased frequencies of RTE CD4⁺ T cells compared to controls, the frequencies of naïve, memory, T_{reg}, and anergic CD4⁺ T cells seem to normalize, unlike what was observed in constitutively AIRE-deficient mice (*Aire*^{-/-}). Given that differences were not observed in mice rendered AIRE-deficient in B cells (Figures 5.21 and 5.22), it is possible that eTACs are contributing to the deletion of autoreactive CD4⁺ T cells in the periphery. Indeed, it has been shown in various experimental mouse models that eTACs are able to delete autoreactive CD8⁺ and CD4⁺ T cells *in vivo* (Gardner *et al.*, 2008; Gardner *et al.*, 2013; Lee *et al.*, 2007; Yamano *et al.*, 2019).

5.4 Organ-specific autoantibodies more prevalent in the sera of mice lacking AIRE in mTECs

Aire^{-/-} mice have been shown to develop autoantibodies against various organ-specific antigens (Anderson *et al.*, 2002; Hubert *et al.*, 2009; Jiang *et al.*, 2005; Ramsey *et al.*, 2002). To assess the contribution of thymic and peripheral AIRE to this process, I assessed the sera of either mice with a constitutive or targeted loss of AIRE expression. I focused initially on the detection of retina-specific autoantibodies. The eye was chosen as a target organ because nearly all constitutively AIRE-deficient mice on a C57BL/6 background develop autoantibodies to the retinal proteins (Jiang *et al.*, 2005). To avoid the background signal by endogenous immunoglobulins in tissues, eye sections from *Rag1*^{-/-} mice were used, as these animals lack antibodies. Tissue sections (16µm) were incubated in the presence of

sera from 20-week-old constitutively AIRE-deficient mice (*Aire*^{-/-}; compared to wildtype sera) or mice rendered AIRE-deficient in mTECs or B cells ((*Aire*(fl/fl):*Foxn1*(Cre⁺) or *Aire*(fl/fl):*Mb1*(Cre⁺), respectively; compared to Cre-negative sera). Tissue sections were stained with DAPI and bound autoantibodies were revealed with AF488-conjugated anti-mouse IgG. Representative examples are shown in Figure 5.23.

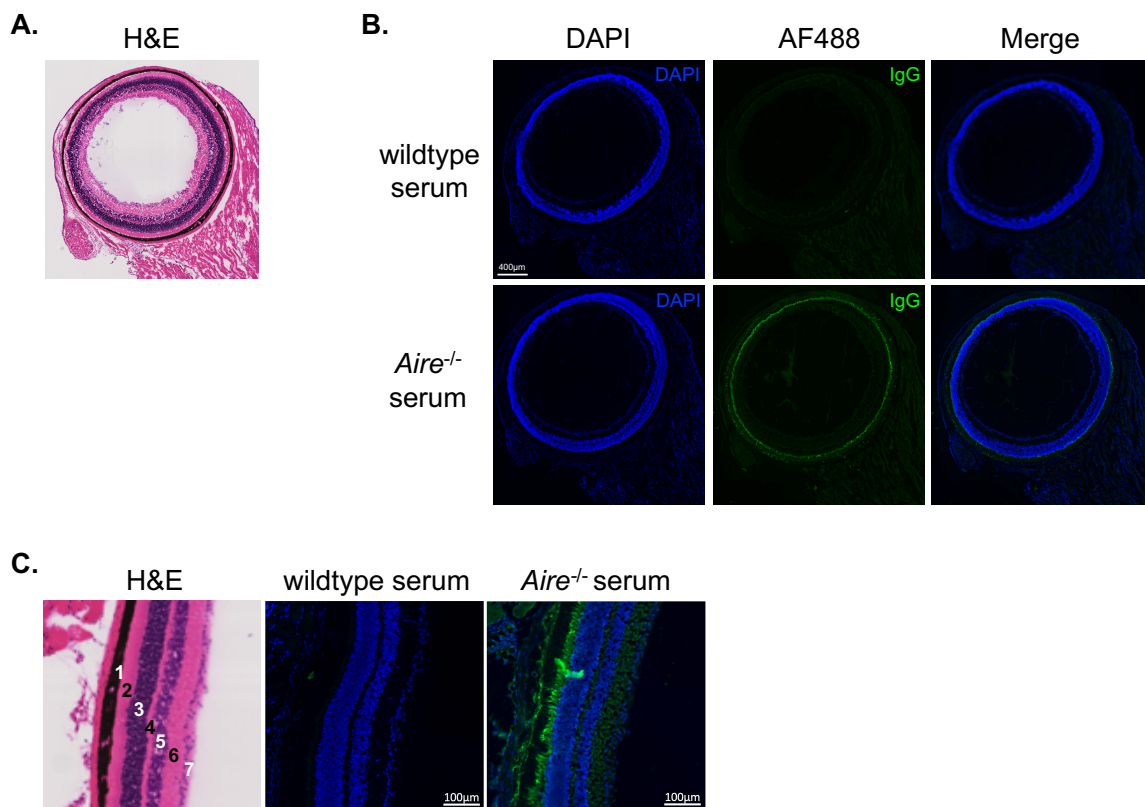


Figure 5.23. Eye-specific autoantibodies in the serum of *Aire*^{-/-} mice. Representative eye tissue sections from a 10-week-old *Rag1*^{-/-} mouse stained with A) H&E or B) DAPI and sera from 20-week-old wildtype (top panel) or *Aire*^{-/-} (bottom panel) mice revealed by AF488-conjugated anti-mouse IgG. C) High resolution images of 7 discrete retinal layers: 1) pigmented epithelial layer, 2) photoreceptor layer, 3) outer nuclear layer, 4) outer plexiform layer, 5) inner nuclear layer, 6) inner plexiform layer, and 7) ganglion cell/inner limiting membrane layer in tissue sections stained with H&E, DAPI and wildtype serum, or DAPI and *Aire*^{-/-} serum.

High resolution images (Figure 5.23C) reveal the presence of autoantibodies specific to the photoreceptor layer of the retina, consistent with what has been reported by others (Anderson *et al.*, 2002; DeVoss *et al.*, 2006; Hubert *et al.*, 2009). Post-processing analysis

was performed using Imaris software where fluorescence intensity mean was automatically quantified for the total image volume for DAPI and for AF488 using consistent settings across all images. The fluorescence intensity mean for the AF488 signal was normalized to the fluorescence intensity mean for the DAPI signal for that same sample. To compare this result to the results from wildtype mice and to decrease the impact of intra-assay variability, fluorescence intensity means were then normalized to the average fluorescence intensity mean for control samples within the same experiment. Normalized fluorescence intensity mean values are shown for each strain in Figure 5.24.

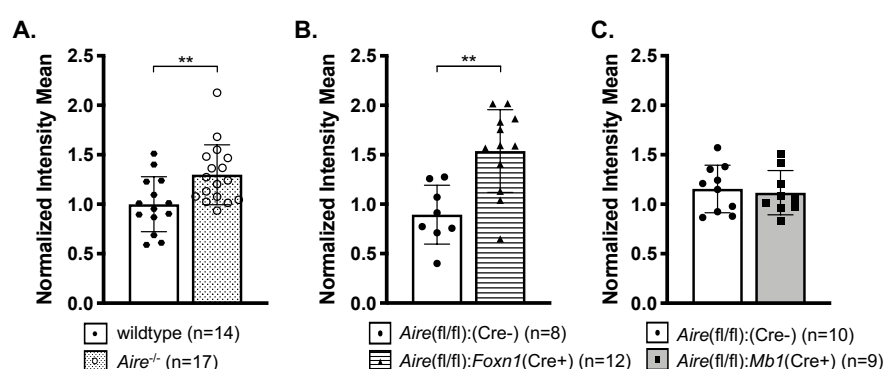


Figure 5.24. Retina-specific autoantibodies in the serum of constitutive and conditional loss-of-AIRE mutant mice. Normalized fluorescence intensity mean values indicating the presence of eye-reactive IgG in the sera of mice lacking AIRE A) constitutively (*Aire*^{-/-}), B) in mTECs (*Aire*(fl/fl):*Foxn1*(Cre⁺)), and C) in B cells (*Aire*(fl/fl):*Mb1*(Cre⁺)) compared to their respective littermate controls. Graphs shown represent 2 independent experiments per mouse strain. Statistical validity was determined by student's t test where **p<0.01.

Sera from *Aire*^{-/-} mice reacted more strongly with eye sections than did the sera from their wildtype, littermate controls (**p=0.0082). Similarly, sera from *Aire*(fl/fl):*Foxn1*(Cre⁺) mice were significantly more reactive to eye tissue than sera from their Cre-negative, littermate controls (**p=0.0015). *Aire*(fl/fl):*Mb1*(Cre⁺) mice did not demonstrate enhanced reactivity toward eye tissue compared to their Cre-negative, littermate controls (p=0.7185). These

findings indicate that autoreactivity to retinal tissues is the consequence of a loss of AIRE expression and subsequently an incomplete representation of TRAs by mTECs.

5.5 Thymic loss-of-AIRE affects inflammatory infiltration of organs

To assess the development of autoimmunity in constitutive and conditional loss-of-AIRE mutant mice, histological analyses were performed on the salivary glands and eyes of 20-week-old mice. It has been shown that mice lacking AIRE constitutively display extensive lymphocytic infiltration of salivary glands and develop spontaneous autoimmune uveitis due to retinal destruction by infiltrating lymphocytes (Hubert *et al.*, 2009). This autoimmune damage is evident by 3-months and increases with age.

5.5.1 Increased lymphocytic infiltration in the salivary glands of mice lacking AIRE in the thymus

Salivary glands were collected from 20-week-old mice, fixed in 10% formalin, stained with hematoxylin and eosin (H&E), and assessed for mononuclear cell infiltrates. Representative examples used to score the histological sections are shown in Figure 5.25A. Salivary glands without disease (that is, the absence of visible infiltration) were considered normal and used as a reference. In contrast, sections with sporadic infiltration (defined as 1 to 2 areas of infiltration, or foci) were considered to have mild disease. Moderate disease was defined as 3 to 6 foci of infiltrates and salivary glands with severe disease demonstrated ≥ 7 foci of extensive infiltration (Hubert *et al.*, 2009). Two H&E-stained salivary gland sections were assessed for each mouse in a blinded fashion and half of the slides were randomly selected and assessed by a second evaluator. Inter-evaluator reliability was 94% and discordance

related only to number of foci present (in one moderate and two severe samples) and did not change the severity score outcome.

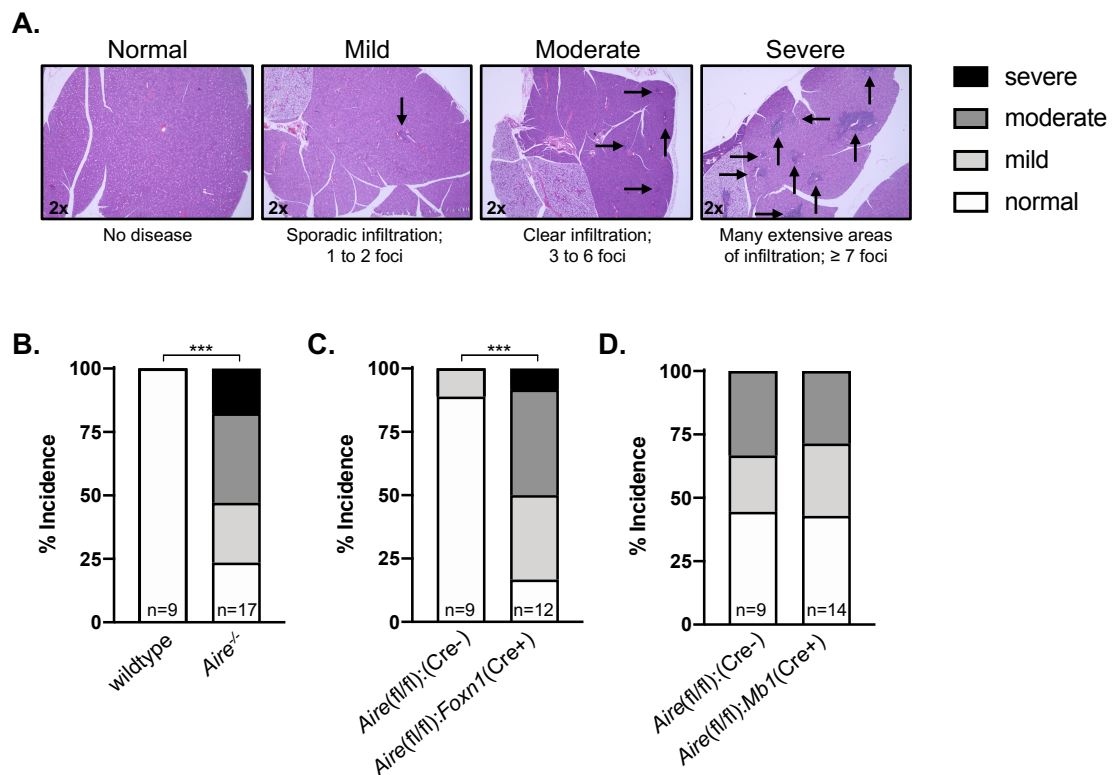


Figure 5.25. Lymphocytic infiltrates in the salivary glands of constitutive and conditional loss-of-AIRE mutant mice. A) Representative examples of the scoring method used to determine the severity of infiltration of salivary tissue in 20-week-old mice. The frequency of each disease severity is shown in mice lacking AIRE B) constitutively (*Aire*^{-/-}), C) in mTECs (*Aire*(fl/fl);*Foxn1*(Cre+)), and D) in B cells (*Aire*(fl/fl);*Mb1*(Cre+)) compared to controls. Statistical validity was determined by student's t test where ***p<0.001.

Mice lacking AIRE constitutively (*Aire*^{-/-}) and in mTECs (*Aire*(fl/fl);*Foxn1*(Cre+)) demonstrated a comparable degree of infiltrates and comparison of numerical scores (1=normal, 2=mild, 3=moderate, 4=severe) demonstrated a significant increase in disease severity in *Aire*^{-/-} (***p=0.0004) and *Aire*(fl/fl);*Foxn1*(Cre+) (***p=0.0006) mice compared to their respective littermate controls. Significant differences were not observed between *Aire*(fl/fl);*Mb1*(Cre+) mice and their Cre-negative controls (p=0.9342), although it is important to note that 55.6% of the Cre-negative, littermate controls (*Aire*(fl/fl);*Mb1*(Cre-)) demonstrated mild or moderate disease (22.2% mild, 33.3% moderate). Hubert *et al*

reported that approximately 30% of wildtype mice had mild salivary gland disease at 3-months of age, while 100% of wildtype mice had at least mild disease by 7-months of age (~60% mild, ~25% moderate, and ~15% severe) (Hubert *et al.*, 2009). Thus, mild or moderate disease in 55.6% of 20-week-old Cre-negative mice is a feasible outcome.

5.5.2 More severe retinal degeneration in mice lacking AIRE in mTECs

Eyes were collected from 20-week-old mice, fixed in 10% formalin, stained with hematoxylin and eosin (H&E), and assessed for degeneration of the retinal layers.

Representative examples of the scoring method used are shown in Figure 5.26A and reflect the number of retinal layers remaining intact and the level of disorganization observed. The 7 discrete anatomical layers are 1) pigmented epithelial layer, 2) photoreceptor layer, 3) outer nuclear layer, 4) outer plexiform layer, 5) inner nuclear layer, 6) inner plexiform layer, and 7) ganglion cell/inner limiting membrane layer. Eye sections with 7 intact retinal layers were deemed normal, while those with limited disorganization of retinal layers were considered to have mild disease. Moderate disease was defined by evident disorganization with a loss of 1 to 3 retinal layers. Severe disease was marked by ≤ 4 retinal layers still being present (Hubert *et al.*, 2009). Two H&E-stained sections per eye were visually scored for each mouse in a blinded fashion and the resulting scores (4 per mouse) were averaged. Half of the slides were randomly selected and assessed by a second evaluator. Inter-evaluator reliability was 92% and discordant samples were assessed by a third evaluator to determine the final severity score.

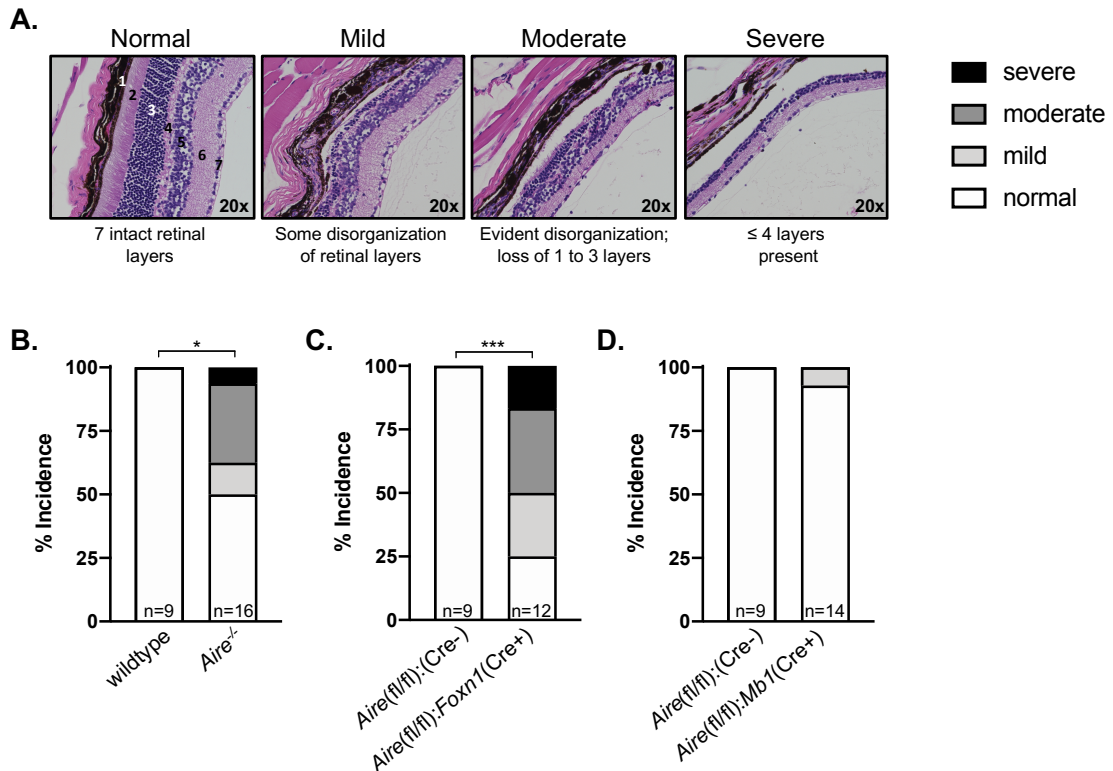


Figure 5.26. Spontaneously occurring retinal pathology in constitutive and conditional loss-of-AIRE mutant mice. A) Representative examples of the scoring method used to determine the severity of retinal disruption in 20-week-old mice. Disruption is shown as the average incidence of individual scores for the different classes of severity in mice lacking AIRE B) constitutively (*Aire*^{-/-}), C) in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)), and D) in B cells (*Aire*(fl/fl):*Mb1*(Cre+)) compared to controls. Statistical validity was determined by student's t test where *p<0.05, ***p<0.001.

Mice lacking AIRE constitutively (*Aire*^{-/-}) and in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+))

demonstrated a similar extent of disruption and comparison of numerical scores

(1=normal, 2=mild, 3=moderate, 4=severe) demonstrated a significant increase in disease severity in *Aire*^{-/-} (*p=0.0152) and *Aire*(fl/fl):*Foxn1*(Cre+) (***p=0.0010) compared to their respective littermate controls. While different from controls, significant differences were not observed between *Aire*^{-/-} and *Aire*(fl/fl):*Foxn1*(Cre+) mice (p=0.2522). Significant differences were also not observed between *Aire*(fl/fl):*Mb1*(Cre+) mice and their Cre-negative controls (p=0.4355). Taken together, these results suggest that salivary gland- and retinal-specific autoimmunity observed in *Aire*^{-/-} mice is due to lack of AIRE in mTECs and

that the expression of AIRE by peripheral cells does not further influence the extent of autoimmune disruption.

My findings in this chapter indicate that the majority of abnormalities observed in constitutively AIRE-deficient mice are explained by the lack of AIRE in mTECs, specifically. Discordant findings, specifically in the CD4⁺ peripheral T cell compartment, between constitutively AIRE-deficient mice (*Aire*^{-/-}) and mice rendered AIRE-deficient in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)) could potentially be explained by peripheral tolerance mechanisms mediated by eTACs. Further studies need to be performed using mice rendered AIRE-deficient in eTACs to elucidate the role of peripheral AIRE in the tolerization and deletion of CD4⁺ T cells.

6 Anti-cytokine autoantibodies

Autoantibodies against cytokines have been described in many contexts in which they lead to diseases that phenocopy those caused by genetic mutations in the cognate cytokine pathway. Neutralizing autoantibodies against granulocyte-macrophage colony-stimulating factor (GM-CSF) and genetic mutations in the GM-CSF receptor cause the rare lung disorder pulmonary alveolar proteinosis (Hamvas *et al.*, 2007; Kitamura *et al.*, 1999; S. H. Rosen *et al.*, 1958). Similarly, anti-interferon-gamma (IFN γ) autoantibodies lead to the same infection susceptibilities seen in patients with genetic mutations in the IFN γ -receptor (Browne, Burbelo, *et al.*, 2012; Doffinger *et al.*, 2004; Dorman & Holland, 2000; Hoflich *et al.*, 2004). Anti-cytokine autoantibodies have also been reported in patients with thymic defects, including APECED, thymoma, and RAG-deficiency (Burbelo *et al.*, 2010; Ferre *et al.*, 2016; Meager *et al.*, 1997; Meager *et al.*, 2006; Walter *et al.*, 2015). Autoantibodies against the type I interferons (IFN α , IFN β , IFN ω), which are a common feature of APECED, thymoma, and RAG-deficient patients, have also been identified in patients with severe viral infections (Ansari *et al.*, 2020; Bastard, Michailidis, *et al.*, 2021; Pozzetto *et al.*, 1984). In this chapter, I sought to assess the anti-cytokine autoantibody profiles of healthy individuals, APECED patients, and patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.

6.1 Anti-cytokine autoantibodies in healthy individuals

Reports of anti-cytokine autoantibodies in healthy individuals are variable and infrequent, likely due to the presence of cytokine-autoantibody complexes that impede their detection

(Watanabe *et al.*, 2007). Autoantibody levels and avidities may wax and wane over time, and limited access to samples from longitudinally followed healthy individuals challenges elucidation of their role in pathogenicity and disease. To establish a baseline anti-cytokine autoantibody profile, the serum/plasma of healthy individuals from the United States, Italy, and France were assessed for the presence of autoantibodies in a multiplex assay against 18 cytokine targets. Sera/plasmas were diluted 1 to 100 and incubated with differentially fluorescing magnetic beads that had been covalently coupled to recombinant human cytokines. Beads were washed and bound autoantibodies were detected using a PE-conjugated anti-human IgG antibody, in which fluorescence intensity (FI) is a function of autoantibody titer. Figure 6.1 depicts the direct binding of autoantibodies in the serum/plasma of healthy individuals to 18 cytokine targets. The average age of individuals was 55.6 years with a standard deviation of 15.9 years (18 to 89 years, n=239 total), with males accounting for 57.7% of the population.

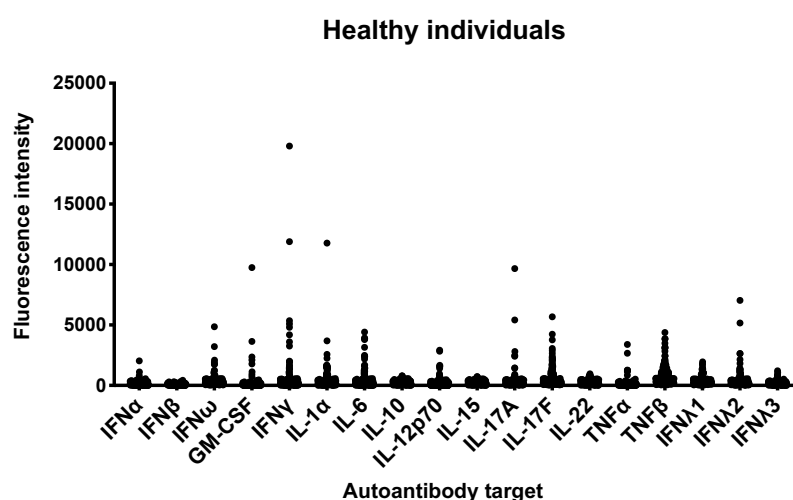


Figure 6.1. Anti-cytokine autoantibodies in healthy individuals. Serum/plasma from healthy individuals (n=239) were screened for the presence of autoantibodies against 18 cytokine targets.

In order to assess functionality, sera/plasmas demonstrating binding for cytokines (>5000 FI) were tested in vitro for their ability to block downstream signaling. Only 2 of 239

(0.84%) individuals were found to have blocking autoantibodies: one against IFN ω (Figure 6.2), and another against GM-CSF (Figure 6.3).

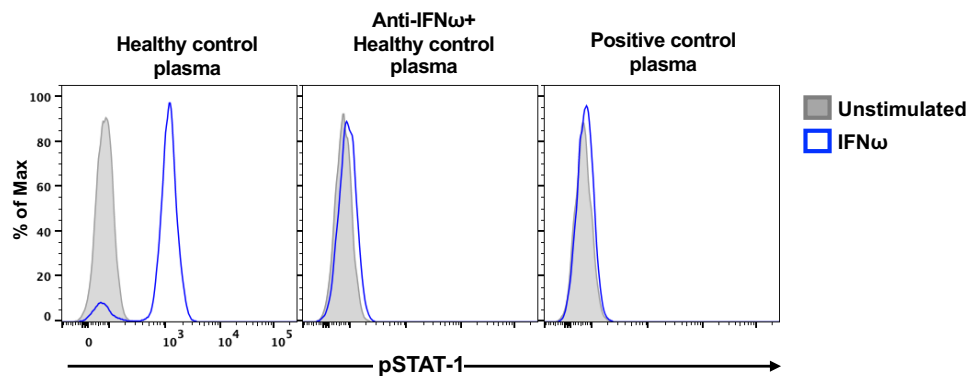


Figure 6.2. Blocking capacity of anti-IFN ω autoantibodies in a healthy control. Representative FACS plots depicting IFN ω -induced pSTAT1 levels in healthy control cells (gated on CD14⁺ monocytes) in the presence of autoantibody-negative healthy control plasma, anti-IFN ω + healthy control plasma, or positive control plasma.

The anti-IFN ω + healthy control was a 40-year-old Caucasian female whose sample was collected in France prior to the COVID-19 pandemic. The anti-GM-CSF+ healthy control was a 58-year-old Caucasian male, collected at the NIH Blood Bank in the United States in 2013.

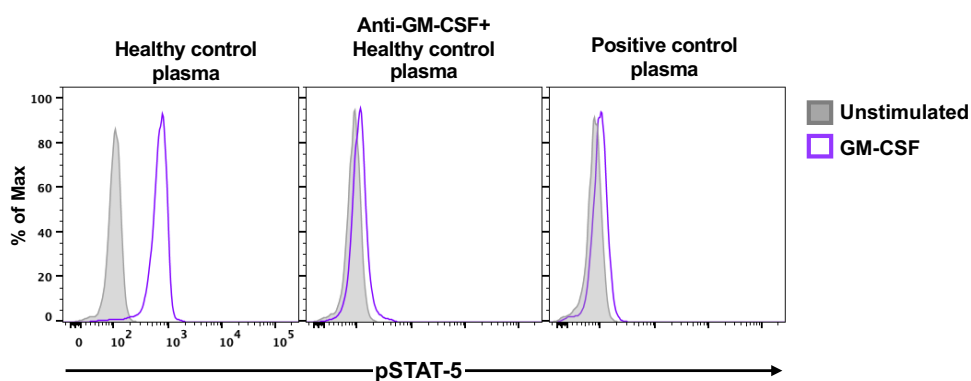


Figure 6.3. Blocking capacity of anti-GM-CSF autoantibodies in a healthy control. Representative FACS plots depicting GM-CSF-induced pSTAT5 levels in healthy control cells (gated on CD14⁺ monocytes) in the presence of autoantibody-negative healthy control plasma, anti-GM-CSF+ healthy control plasma, or positive control plasma.

Although all individuals were considered to be healthy at the time of blood donation, we have no way of knowing whether they remain healthy. The Holland lab has previously shown that autoantibodies to GM-CSF can occur >10 years prior to the development of symptoms of disseminated nocardiosis (L. B. Rosen *et al.*, 2015). In the case of the healthy control with blocking anti-IFN ω autoantibodies, it is possible she went on to develop COVID-19 (evidence for which will be presented in a later section). Similarly, it is possible the healthy control with blocking anti-GM-CSF autoantibodies went on to develop cryptococcal meningitis (L. B. Rosen *et al.*, 2013), disseminated nocardiosis (L. B. Rosen *et al.*, 2015), or pulmonary alveolar proteinosis (Kitamura *et al.*, 1999). Efforts were made to reach out to these individuals, but confidentiality agreements were prohibitive. The anti-GM-CSF+ individual has not donated blood at NIH in the last 7 years, possibly indicating the development of a disorder which rendered him unfit to donate, however specifics were not available.

6.2 APECED patients have a distinct anti-cytokine autoantibody profile

AIRE-deficiency induces organ-specific autoantibodies that cause tissue damage in both humans and mice (Anderson *et al.*, 2002). Additionally, APECED patients have been shown to develop autoantibodies to several cytokines, including the type I IFNs and those in the interleukin (IL)-17 family (Meager *et al.*, 2006; Meyer *et al.*, 2016; Puel *et al.*, 2010). I sought to assess NIH's cohort of APECED patients for autoantibodies against 10 cytokine targets. Analyses were limited to those anti-cytokine autoantibodies known to be prevalent in APECED patients (type I IFNs, IL-17A, IL-17F, IL-22), as well as control targets (GM-CSF, IFN γ , IL-1 α , and IL-12p70) that have been reported in contexts outside AIRE-deficiency.

Figure 6.4 shows the binding activity of APECED patient sera/plasmas to 10 cytokine targets.

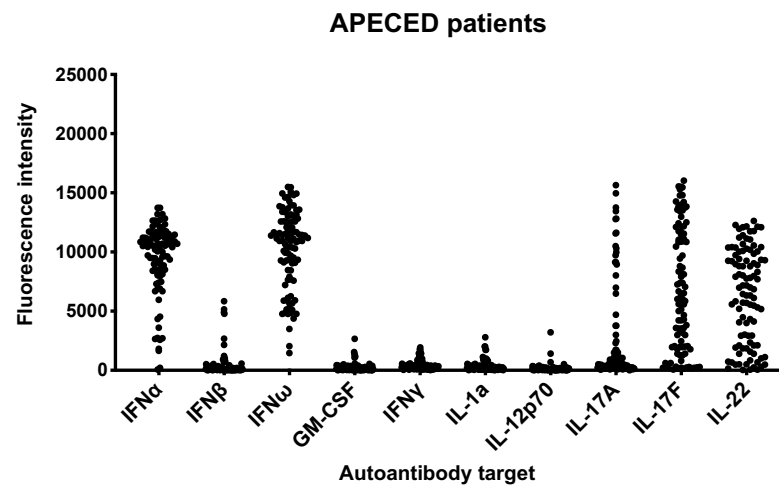


Figure 6.4. Anti-cytokine autoantibodies in APECED patients. APECED patients (n=99) were screened for the presence of autoantibodies against 10 cytokine targets.

APECED patient autoantibodies were not assessed for functionality here, as they have been shown previously to be blocking previously (Karner *et al.*, 2013; Kisand *et al.*, 2010; Puel *et al.*, 2010). Importantly, 97.0% of APECED patients were positive for autoantibodies against IFN ω using the stringent cutoff of >5000 FI, with 99.0% of APECED patients were positive with a cutoff of >1000 FI. Emphasizing the predictability of the anti-cytokine autoantibody profile in APECED patients as reflected in prior reports (Kisand *et al.*, 2008), our cohort was 97-99% positive for anti-IFN ω autoantibodies (depending on the threshold for positivity). The percent incidence of other autoantibodies commonly observed in APECED patients, including anti-type I IFN, -IL-17A, -IL-17F, and -IL-22, are listed in order of decreasing prevalence in Table 6.1.

Autoantibody target	Positivity cutoff	
	>5000 FI	>1000 FI
IFN ω	97.0%	99.0%
IFN α	88.9%	98.0%
IL-22	64.6%	85.9%
IL-17F	57.6%	83.8%
IL-17A	18.2%	36.3%
IFN β	2.0%	8.1%

Table 6.1. Anti-cytokine autoantibody incidence in APECED patients. The percentage of APECED patient sera/plasmas with direct binding activity against various cytokine targets using two FI cutoff values. Autoantibody targets are listed in order of decreasing prevalence.

In fact, due to their high prevalence among APECED patients, anti-IFN ω autoantibodies have been used as a diagnostic marker for the disease (Kisand *et al.*, 2008). Autoantibodies typically precede the development of clinical manifestations; thus, their identification allows for earlier diagnosis than conventional methods. While APECED patients also have organ-specific autoantibodies that correlate with autoimmune manifestations (Fishman *et al.*, 2017), a direct link between anti-cytokine autoantibodies and clinical symptoms is more tenuous. It was previously thought that the increased susceptibility to chronic mucocutaneous candidiasis (CMC) seen in APECED patients was the result of IL-17A/IL-17F/IL-22 blockade by neutralizing autoantibodies (Kisand *et al.*, 2010; Puel *et al.*, 2010). However, recent work has shown that increased local IFN γ production, rather than impaired Th17 responses, lead to mucosal fungal infection susceptibility (Break *et al.*, 2021). Despite their near 100% prevalence in APECED patients, the origin and importance of autoantibodies to type I IFNs remain unknown.

6.3 Autoantibodies against type I IFNs in COVID-19 patients

One of the major challenges of the coronavirus disease 2019 (COVID-19) pandemic has been the extreme interindividual clinical variability of those infected with SARS-CoV-2. While certain risk factors have been identified for life-threatening COVID-19 (advanced age, being male, and comorbid conditions), it remains unclear why individuals of similar demographic and epidemiological groups display such a wide range of clinical severity upon SARS-CoV-2 infection.

The Holland lab has previously shown that neutralizing anti-cytokine autoantibodies play a role in the susceptibility to various infectious diseases (Ansari *et al.*, 2020; Browne, Burbelo, *et al.*, 2012; L. B. Rosen *et al.*, 2013; L. B. Rosen *et al.*, 2015), and so I sought to identify autoantibodies against type I IFNs (IFN α , IFN β , and IFN ω), GM-CSF, IFN γ , IL-1 α , IL-6, IL-10, IL-12p70, IL-15, IL-17A, IL-17F, IL-22, TNF α , TNF β , IFN λ 1, IFN λ 2, and IFN λ 3 in COVID-19 patients. Given the established role of type I IFNs in the defense against viral pathogens (Schreiber, 2020; Teijaro, 2016), I hypothesized that neutralizing autoantibodies to type I IFNs might contribute to disease severity in some cases of COVID-19.

As part of the COVID Human Genetic Effort Consortium, I tested patients with proven SARS-CoV-2 infection (n=1696) and healthy controls (n=1252) for anti-cytokine autoantibodies. Disease severity was assessed for each patient according to the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (trial version 7), released by the National Health Commission & State Administration of Traditional Chinese Medicine on March 3, 2020. The criteria for disease severity classifications are outlined in Table 6.2.

Clinical Severity	Description
Asymptomatic	Tested positive by PCR but did not develop symptoms
Mild	Modest symptoms, no pneumonia
Moderate	Fever and respiratory symptoms PLUS radiological evidence of pneumonia. Use of low-flow oxygen is still part of this phenotype if O2 saturation is >93% at rest
Severe	Oxygen saturation at rest 93% or lower OR respiratory rate >30/min OR PaO ₂ /FiO ₂ <300. Use of low-flow oxygen is still part of this phenotype
Critical (life-threatening)	Any of the following: • Mechanical ventilation (CPAP, BiPAP, intubation, hi-flow oxygen) • Septic shock • Organ damage requiring admission in the ICU

Table 6.2. Clinical severity classifications in COVID-19. O₂= oxygen; PaO₂=partial pressure of oxygen; FiO₂= fraction of inspired oxygen; CPAP= continuous positive airway pressure; BiPAP= bilevel positive airway pressure.

Figure 6.5 shows the anti-cytokine autoantibody profiles of COVID-19 patients of varying severity while the anti-cytokine autoantibody profile of healthy controls can be found in Figure 6.1.

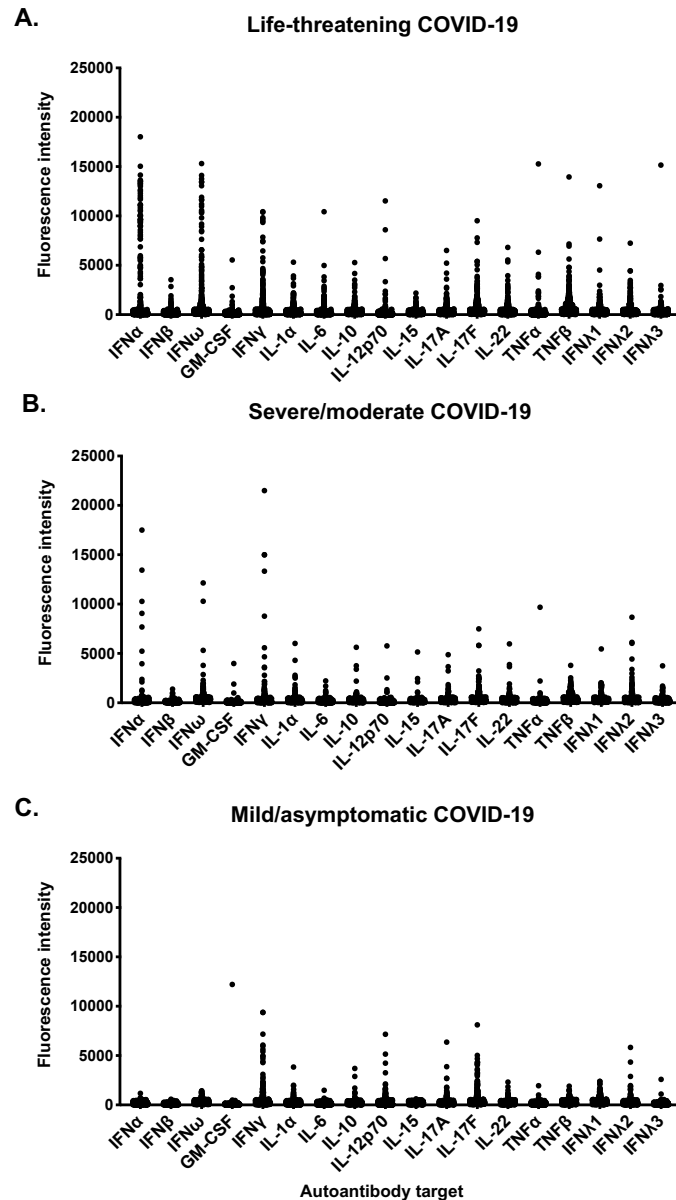


Figure 6.5. Anti-cytokine autoantibodies in COVID-19 patients. Patients with A) life-threatening (critical) COVID-19 (n=690), B) severe/moderate COVID-19 (n=302), C) mild/asymptomatic COVID-19 (n=385) were screened for the presence of autoantibodies against 18 cytokine targets.

An initial screen across 18 autoantibody targets revealed the plasmas of 11.7% of patients with life-threatening COVID-19 demonstrated direct binding activity of IgG against at least one type I IFN (81 out of 690) as compared to 0.42% of healthy controls (1 out of 239) (Figure 6.1). Of the 302 COVID-19 patients with severe/moderate disease, 0.99% (3 patients) were positive for binding activity against at least one type I IFN, while 0 of the 385 mild/asymptomatic COVID-19 patients initially screened were positive for type I IFN

autoantibodies. In order to determine the prevalence of these autoantibodies, I screened additional patients and healthy controls using a limited panel of cytokine targets (IFN α and IFN ω). Figure 6.6 depicts all individuals screened (including those shown in Figure 6.1).

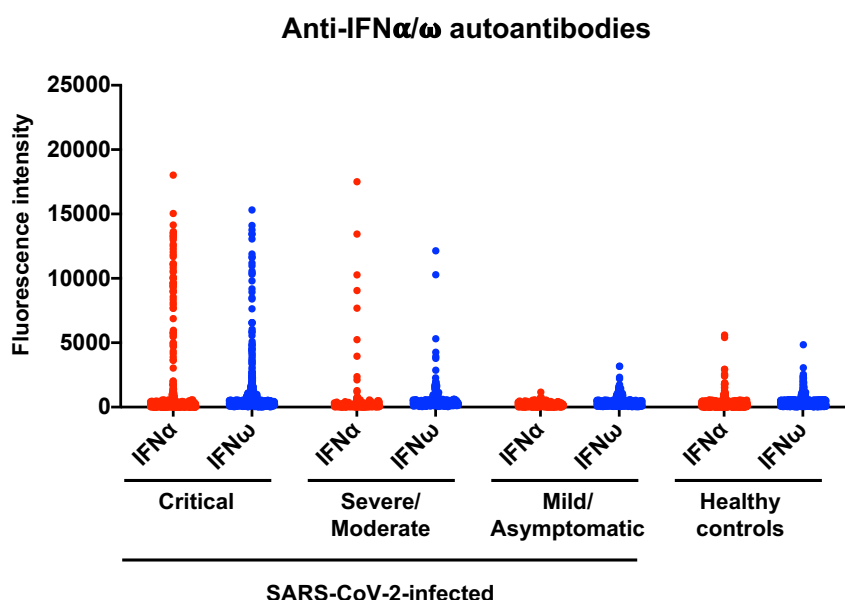


Figure 6.6. Anti-IFN α / ω autoantibodies in COVID-19 patients and healthy controls. Patients with A) life-threatening (critical) COVID-19 (n=782), B) severe/moderate COVID-19 (n=364), C) mild/asymptomatic COVID-19 (n=550), and D) healthy controls (n=1252) were screened for the presence of autoantibodies against IFN α and IFN ω .

For all individuals with a fluorescence intensity >1000 for IFN α , IFN β , or IFN ω binding, the pSTAT1 functional assay was performed to determine whether the autoantibodies detected were able to block downstream signaling. In this assay, healthy control PBMC were incubated in the presence of 10% patient or control plasma and either left unstimulated, or stimulated with 10 ng/mL IFN α , IFN β , or IFN ω for 15 minutes. The cells were then fixed, permeabilized, and stained for intranuclear phosphorylated STAT1. The stimulation index was calculated for each patient's plasma (the geometric mean fluorescence intensity for each stimulated condition divided by that of the unstimulated condition for the same patient) and was then normalized to the stimulation index of the healthy control plasma in that same experiment to yield a normalized stimulation index

value for each patient. Based on the normalized stimulation index, plasmas were classified as blocking (<20% pSTAT1 compared to healthy control plasma), partially blocking (20% to 64.99% pSTAT1 compared to healthy control plasma), or not blocking ($\geq 65\%$ pSTAT1 compared to healthy control plasma). Partially blocking and blocking were considered to be positive (<65% pSTAT1 compared to healthy control plasma) for the purposes of the following analyses.

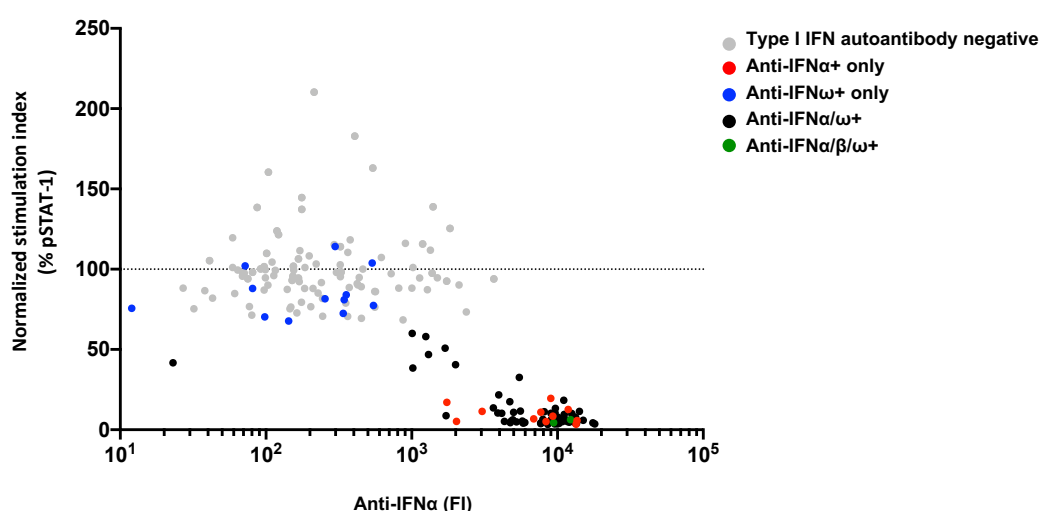


Figure 6.7. Neutralization capacity of anti-IFN α autoantibodies in COVID-19 patients. Plot of anti-IFN α autoantibody levels against their neutralization capacity. The stimulation index (stimulated over unstimulated condition) for each patient plasma was normalized against that of healthy control plasma from the same experiment. Spearman's rank correlation coefficient = -0.6805, ****p<0.0001.

When plotting anti-IFN α autoantibody levels against the normalized stimulation index for each anti-type I IFN autoantibody-positive patient and a random sampling of autoantibody-negative patients (Figure 6.7), the Spearman's correlation coefficient is -0.6805 (****p<0.0001), demonstrating a negative correlation between the two parameters. Of note, there was little cross-reactivity between anti-IFN α and -IFN ω autoantibodies, demonstrated by normalized stimulation indices near 100% seen in patients with only anti-IFN ω autoantibodies.

Of the 782 patients with critical (life-threatening) COVID-19, 81 (10.4%) were positive for blocking autoantibodies against at least one type I IFN. Of 364 patients with severe/moderate COVID-19, 8 (2.2%) were positive for blocking autoantibodies against at least one type I IFN. Of the 550 mild/asymptomatic patients, 1 was partially blocking for anti-IFN ω autoantibodies (0.18%). Of the 1252 healthy controls, 4 were found to have blocking autoantibodies against at least one type I IFN (0.32%). Of note, all healthy control samples were collected prior to the COVID-19 pandemic, and information on whether those 4 individuals developed life-threatening COVID-19 is not available due to confidentiality mandates.

I next sought to demonstrate that the blocking ability of patient plasma was limited to the IgG fraction by depleting the IgG on a protein G column. Figure 6.8 depicts healthy control plasma, anti-IFN α -containing plasma, and anti-IFN α /-IFN ω plasma that have been depleted of IgG and allow for full IFN-induced pSTAT1 signaling (bottom panel).

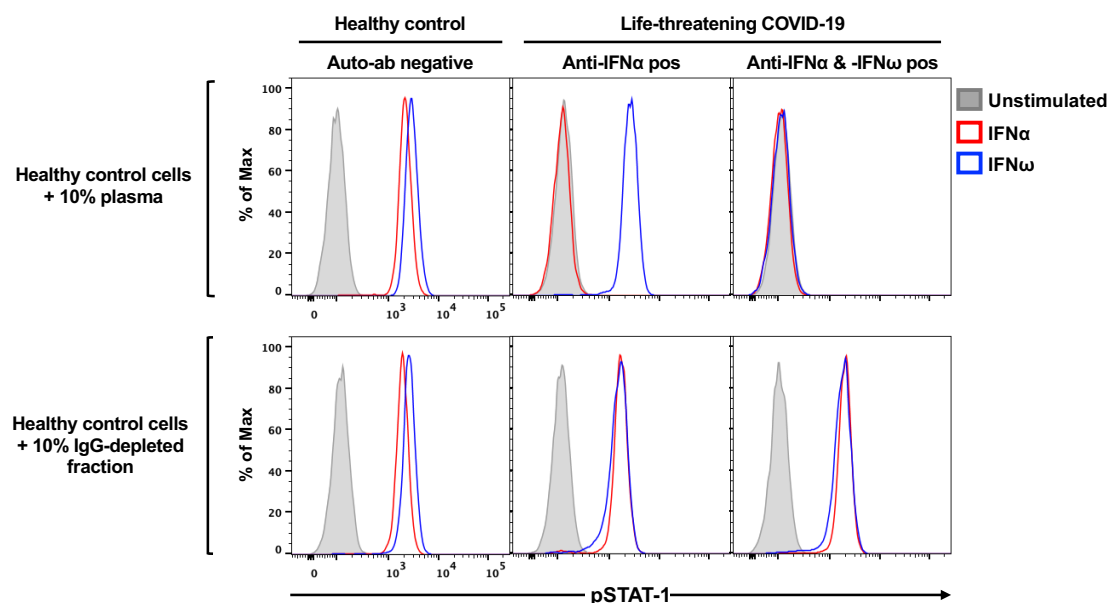
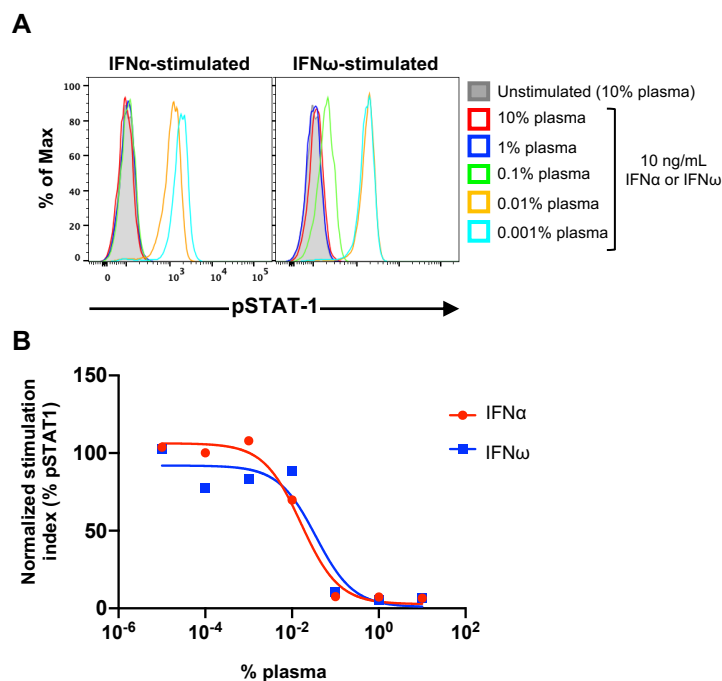


Figure 6.8. Neutralizing anti-IFN α / ω autoantibodies in COVID-19 patient plasma. Representative FACS plots depicting IFN α - or IFN ω -induced pSTAT1 in healthy control cells (gated on CD14⁺ monocytes) in the presence of 10% healthy control or anti-type I IFN autoantibody-containing patient plasma (top panel) or an IgG-depleted plasma fraction (bottom panel).

To evaluate the neutralizing ability of autoantibody-containing patient plasma, various dilutions of plasma from a patient positive for both anti-IFN α and anti-IFN ω autoantibodies were assessed using the pSTAT1 assay. The amount of plasma required to inhibit 50% of IFN α - and IFN ω -induced pSTAT1 (IC₅₀) was determined to be 0.016% and 0.0353%, respectively (Figure 6.9).



inhibited STAT1 phosphorylation up to 3.25 $\mu\text{g/mL}$ IFN α . Complete phosphorylation of STAT1 was only restored in the presence of 10% patient plasma at 6.5 $\mu\text{g/mL}$ IFN α .

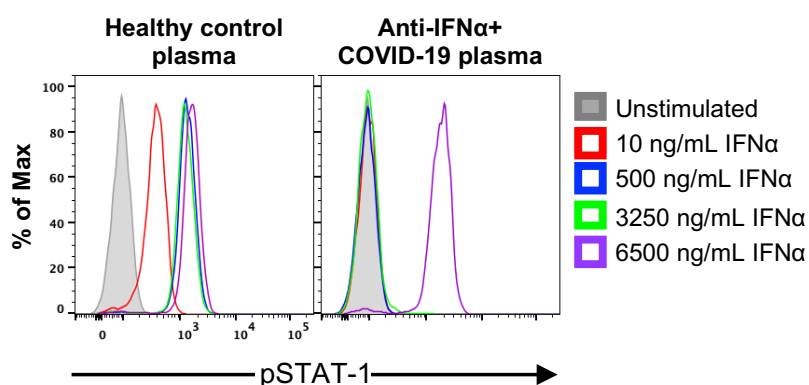


Figure 6.10. IFN α neutralizing ability of COVID-19 patient plasma. FACS plots showing the blocking activity against IFN α -induced pSTAT1 in healthy donor cells (gated on CD14 $^{+}$ monocytes) in the presence of 10% healthy control plasma or 10% plasma from a patient with autoantibodies against IFN α , with increasing doses of recombinant IFN α .

In addition to anti-IFN α and -IFN ω autoantibodies, two patients with critical COVID-19 also demonstrated blocking activity against IFN β (Figure 6.11). Patient 12 demonstrated partial blocking activity against IFN β (normalized stimulation index = 35.0% pSTAT1) while Patient 38 was found to be fully blocking of IFN β signaling (normalized stimulation index = 4.5% pSTAT1). Interestingly, Patient 12 did not demonstrate direct binding activity of IgG to IFN β (anti-IFN β 52 FI), despite showing partial blocking activity in the pSTAT1 assay, indicating some level of cross-reactivity between IFN β and the other type I IFN autoantibodies. For this reason, I tested all patients who were positive for either anti-IFN α or anti-IFN ω autoantibodies for blocking activity against IFN β , but did not find any additional patients with cross-reactivity.

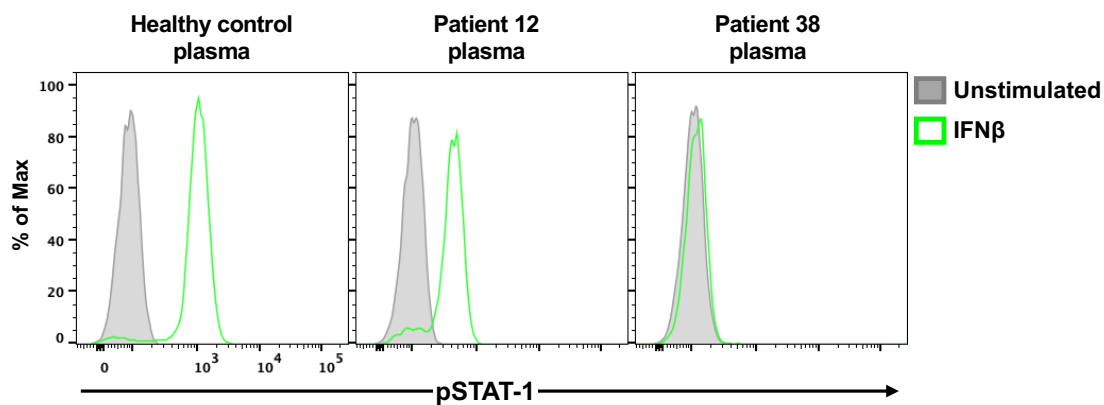


Figure 6.11. Neutralizing anti-IFN β autoantibodies in two patients with life-threatening COVID-19. Representative FACS plots showing the blocking activity against IFN β -induced pSTAT1 in healthy control cells (gated on CD14 $^{+}$ monocytes) in the presence of 10% healthy control plasma or plasmas from two patients with life-threatening COVID-19.

The clinical and epidemiological characteristics of the 94 individuals found to have autoantibodies to at least one type I IFN are listed in Table 6.3.

Number	Severity	Age	Gender	Ethnic origin	Comorbidities	Positive for anti-IFN α	Positive for anti-IFN ω	Outcome
1	Critical	61	M	EUR	HTN	Yes	Yes	Alive
2	Critical	74	M	EUR	COPD	Yes	Yes	Alive
3	Critical	58	M	EUR	T2D, HTN, obesity, dyslipidemia	Yes	Yes	Alive
4	Critical	53	M	EUR	s/p thyroid cancer	Yes	Yes	Alive
5	Critical	41	M	EUR	CVID	Yes	Yes	Alive
6	Critical	68	M	EUR	HTN, T2D, cardiomyopathy	Yes	Yes	Deceased
7	Critical	74	M	EUR	None	Yes	Yes	Deceased
8	Critical	69	M	EUR	HTN	Yes	Yes	Alive
9	Critical	60	M	EUR	Psoriatic arthritis	Yes	Yes	Alive
10	Critical	73	M	EUR	T2D	Yes	Yes	Alive
11	Critical	64	M	EUR	HTN, T2D	Yes	Yes	Deceased
12	Critical	69	M	EUR	Psoriasis	Yes	Yes	Deceased
13	Critical	64	M	EUR	HTN, HBV-related liver disease, hypercholesterolemia	Yes	Yes	Alive
14	Critical	67	M	EUR	Lymphoma, resolved TB infection, HTN	Yes	Yes	Alive
15	Critical	66	M	EUR	None	Yes	Yes	Alive
16	Critical	54	M	Hispanic/	None	Yes	Yes	Alive

				Latino				
17	Critical	71	F	EUR	Autoimmune disease, HTN	Yes	Yes	Deceased
18	Critical	67	M	EUR	T2D, HTN	Yes	Yes	Alive
19	Critical	73	M	EUR	T2D, HTN	Yes	Yes	Alive
20	Critical	68	M	EUR	None	Yes	Yes	Alive
21	Critical	61	F	EUR	None	Yes	Yes	Alive
22	Critical	62	M	EUR	HTN, obesity	Yes	Yes	Alive
23	Critical	70	M	EUR	T2D	Yes	Yes	Alive
24	Critical	52	M	N AFR	Lymphoma	Yes	Yes	Alive
25	Critical	80	M	EUR	T2D, HTN, peripheral vascular disease	Yes	Yes	Deceased
26	Critical	59	M	EUR	Obesity	Yes	Yes	Alive
27	Critical	70	M	EUR	Neurological disease, chronic renal insufficiency	Yes	Yes	Alive
28	Critical	65	M	EUR	None	Yes	Yes	Alive
29	Critical	68	M	EUR	COPD	Yes	Yes	Deceased
30	Critical	71	F	NA	Cardiomyopathy, HTN	Yes	Yes	Alive
31	Critical	70	M	EUR	T2D, HTN, obesity	Yes	Yes	Deceased
32	Critical	32	F	EUR	Incontinencia Pigmenti	Yes	Yes	Alive
33	Critical	25	M	AFR	NA	Yes	Yes	Alive
34	Critical	46	M	N AFR	None	Yes	Yes	Alive
35	Critical	44	M	South Asian	NA	Yes	Yes	Alive
36	Critical	25	M	South Asian	Obesity	Yes	Yes	Alive
37	Critical	25	M	AFR	Obesity	Yes	Yes	Alive
38	Critical	75	M	NA	Obesity, neoplasia	Yes	Yes	Deceased
39	Critical	70	M	EUR	Obesity, cardiomyopathy	Yes	Yes	Alive
40	Critical	70	F	EUR	Obesity, HTN	Yes	Yes	Deceased
41	Critical	58	M	EUR	Obesity, HTN	Yes	Yes	Alive
42	Critical	63	M	N AFR	Obesity, HTN, T2D, COPD	Yes	Yes	Deceased
43	Critical	48	M	NA	Chronic renal insufficiency, hypothyroidism, HTN, epilepsy	Yes	Yes	Alive
44	Critical	77	M	NA	Obesity, HTN	Yes	Yes	Alive
45	Critical	85	M	NA	HTN	Yes	Yes	Deceased
46	Critical	83	M	EUR	NA	Yes	Yes	Deceased
47	Critical	69	M	NA	NA	Yes	Yes	Deceased
48	Critical	55	M	NA	None	Yes	Yes	Alive
49	Critical	64	M	NA	None	Yes	Yes	Alive
50	Critical	76	M	NA	HTN	Yes	Yes	Alive
51	Critical	31	M	NA	None	Yes	Yes	Deceased

52	Critical	68	M	EUR	None	Yes	No	Alive
53	Critical	37	M	N AFR	Renal transplant, chronic renal failure, HBV-related liver disease, cardiomyopathy, thalassemia major	Yes	No	Alive
54	Critical	69	M	EUR	HTN	Yes	No	Alive
55	Critical	79	M	EUR	T2D, HTN	Yes	No	Deceased
56	Critical	63	M	EUR	HTN	Yes	No	Alive
57	Critical	76	M	EUR	T2D	Yes	No	Alive
58	Critical	67	F	EUR	T2D, COPD, HTN	Yes	No	Alive
59	Critical	59	M	EUR	Autoimmune disease, obesity	Yes	No	Alive
60	Critical	45	M	EUR	None	Yes	No	Alive
61	Critical	33	M	South Asian	None	Yes	No	Alive
62	Critical	65	M	N AFR	Obesity, HTN, T2D, renal disease	Yes	No	Alive
63	Critical	54	M	EUR	Obesity	Yes	No	Deceased
64	Critical	74	M	NA	HTN, coronary artery disease	Yes	No	Alive
65	Critical	58	M	NA	HTN	Yes	No	Deceased
66	Critical	47	M	Middle East	NA	Yes	No	Alive
67	Critical	80	M	NA	T2D, HTN, obesity	Yes	No	Deceased
68	Critical	70	M	NA	None	Yes	No	Alive
69	Critical	84	M	NA	HTN, lung cancer, psoriasis, T2D	Yes	No	Deceased
70	Critical	56	M	EUR	HIV	No	Yes	Alive
71	Critical	48	M	EUR	None	No	Yes	Alive
72	Critical	59	M	NA	HTN	No	Yes	Alive
73	Critical	54	M	EUR	Obesity	No	Yes	Alive
74	Critical	58	M	EUR	HTN	No	Yes	Alive
75	Critical	45	M	Middle East	None	No	Yes	Alive
76	Critical	36	M	AFR	NA	No	Yes	Alive
77	Critical	40	M	AFR	Obesity, HTN	No	Yes	Alive
78	Critical	35	M	NA	Obesity	No	Yes	Deceased
79	Critical	55	M	NA	None	No	Yes	Deceased
80	Critical	60	M	NA	Gastritis	No	Yes	NA
81	Critical	57	M	NA	Renal transplant, HBV infection	No	Yes	Deceased
82	Severe	88	F	EUR	HTN, candidiasis, hypothyroidism	Yes	Yes	Alive
83	Severe	52	M	NA	None	Yes	Yes	NA
84	Severe	64	M	NA	T2D	Yes	Yes	NA

85	Severe	83	M	NA	Ischemic cardiopathy, iatrogenic diabetes	Yes	Yes	NA
86	Severe	93	M	NA	Ischemic cardiopathy, hypercholesterolemia, T2D	Yes	Yes	NA
87	Severe	77	F	NA	NA	Yes	Yes	NA
88	Severe	44	M	NA	None	Yes	No	NA
89	Moderate	46	M	EUR	None	No	Yes	Alive
90	Asymptomatic	36	M	NA	None	No	Yes	Alive
91	Healthy control	46	F	NA	NA	Yes	No	NA
92	Healthy control	52	F	NA	NA	Yes	No	NA
93	Healthy control	40	F	NA	NA	No	Yes	NA
94	Healthy control	47	F	NA	NA	No	Yes	NA

Table 6.3. Clinical and epidemiological characteristics and outcomes of anti-type I IFN autoantibody-positive COVID-19 patients. HTN= hypertension; COPD= chronic obstructive pulmonary disease; T2D= type II diabetes mellitus; obese= body-mass index >30; CVID= common variable immunodeficiency; HBV= hepatitis B virus; TB= tuberculosis; EUR= European; N AFR= North African; AFR= African; NA= Not available.

Eleven anti-IFN α/ω autoantibody-positive COVID-19 patients were found to have direct binding activity against other cytokine targets. To assess the blocking activity of these anti-cytokine autoantibodies, various functional assays were employed using downstream signaling targets.

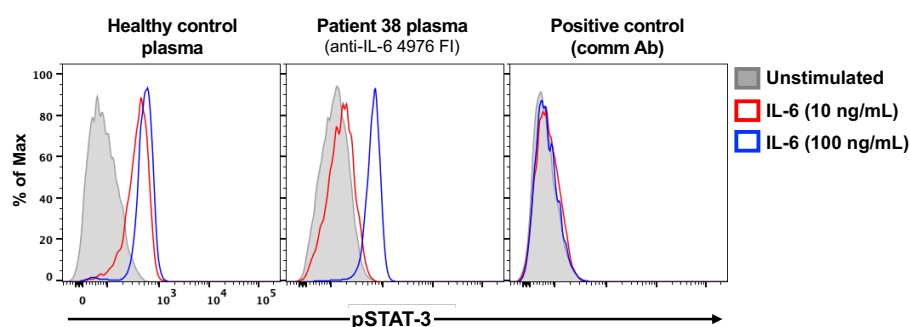


Figure 6.12. Partially blocking anti-IL-6 autoantibodies in a patient with COVID-19. Representative FACS plots showing the blocking activity against increasing doses of IL-6-induced pSTAT3 in healthy control cells (gated on CD14⁺ monocytes) in the presence of 10% healthy control plasma, COVID-19 patient plasma, or positive control anti-IL-6 commercial antibody spiked into healthy control plasma.

One critical COVID-19 patient (Patient 38) had partially blocking anti-IL-6 autoantibodies in addition to blocking anti-IFN α , -IFN ω , and -IFN β autoantibodies. These anti-IL-6 autoantibodies led to decreased downstream signaling (pSTAT3) in response to 10 ng/mL IL-6, but full STAT3 phosphorylation was restored by increasing the dose of IL-6 10-fold (100 ng/mL) (Figure 6.12). It is unclear the degree to which anti-IL-6 autoantibodies contributed to the patient's disease, however even partial blockade of IL-6 could result in a dampened inflammatory response and possibly more severe (less controlled) COVID-19 infection.

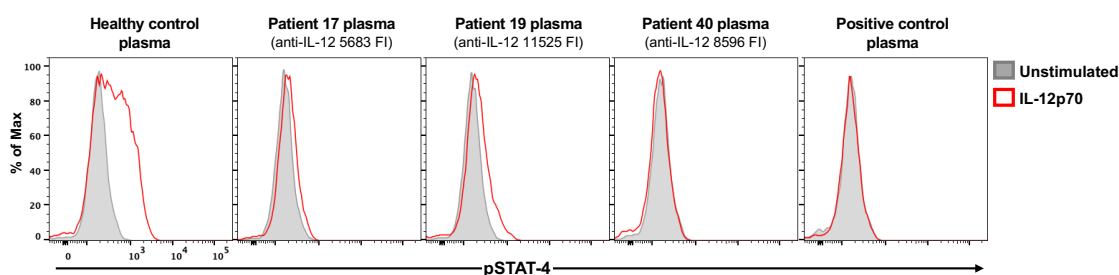


Figure 6.13. Partially blocking and blocking anti-IL-12p70 autoantibodies in three patients with COVID-19 and anti-type I IFN autoantibodies. Representative FACS plots showing the blocking activity against IL-12p70-induced pSTAT4 in activated healthy control cells (gated on CD4⁺ lymphocytes) in the presence of 10% healthy control plasma, COVID-19 patient plasmas, or positive control plasma.

Of the 94 individuals who were positive for at least one type I IFN autoantibody, three critical COVID-19 patients were also positive for anti-IL-12p70 autoantibodies (Figure 6.13). Plasmas from Patients 17 and 19 demonstrated partial blocking activity to 100 ng/mL IL-12p70 while Patient 40's plasma was able to block signaling fully, comparable to the positive control. Anti-IL-12p70 in combination with anti-type I IFN autoantibodies (sometimes anti-IL-22 autoantibodies) are a common finding in patients with thymoma, however the clinical information available for these patients did not indicate the presence of thymic malignancies. Further evidence that Patient 40 may have thymoma is the

presence of blocking anti-IL-22 autoantibodies in her plasma (Figure 6.14), however this remains unconfirmed from a clinical standpoint.

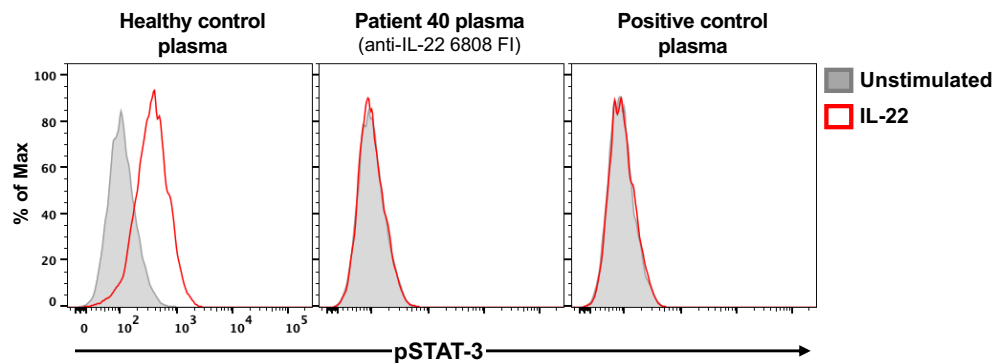


Figure 6.14. Blocking anti-IL-22 autoantibodies in a COVID-19 patient with anti-type I IFN and anti-IL-12p70 autoantibodies. Representative FACS plots showing the blocking activity against IL-22-induced pSTAT3 in A549 cells in the presence of 10% healthy control plasma, COVID-19 Patient 40 plasma, or positive control plasma.

Two patients demonstrated direct binding activity of IgG to TNF β (Patient 31 and Patient 42), however neither inhibited rat Thy1 expression in Jurkat 3T8 cells, which express rat Thy1 on their surface under the control of eight copies of the NF- κ B enhancers (Figure 6.15).

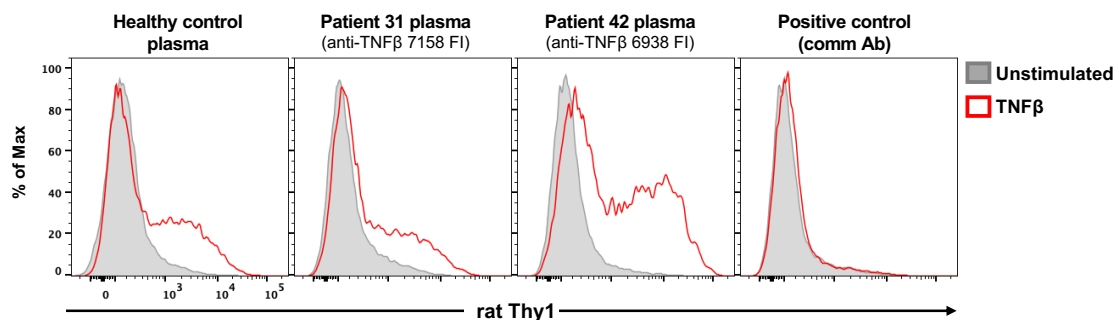


Figure 6.15. Non-neutralizing anti TNF β autoantibodies in two patients with COVID-19 and anti-type I IFN autoantibodies. Representative FACS plots showing the blocking activity against TNF β -induced rat Thy1 expression in Jurkat 3T8 cells in the presence of 10% healthy control plasma, COVID-19 patient plasmas, or positive control anti-TNF β commercial antibody spiked into healthy control plasma.

Three of the 94 individuals with anti-type I IFN autoantibodies had direct binding activity of IgG to IFN γ (Figure 6.16), although none were blocking when tested in the pSTAT1 functional assay. Anti-IFN γ autoantibodies typically occur in individuals of Southeast Asian descent with nontuberculous mycobacterial disease with or without opportunistic infections and are not seen in conjunction with anti-type I IFN autoantibodies (Browne, Burbelo, *et al.*, 2012). These three individuals were of European descent (or unknown for Patient 69) and did not have known clinical histories of bacterial or opportunistic infections.

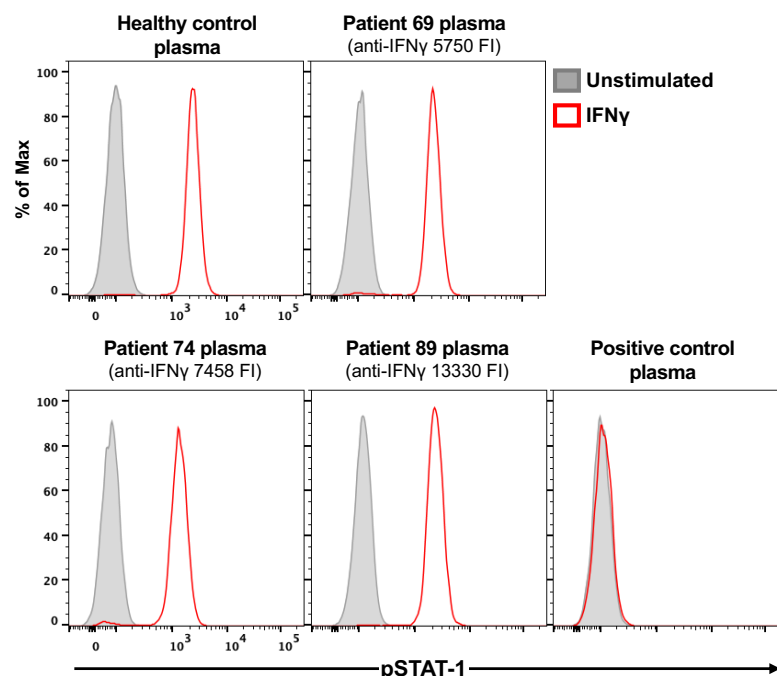


Figure 6.16. Non-neutralizing anti-IFN γ autoantibodies in three patients with COVID-19 and anti-type I IFN autoantibodies. Representative FACS plots showing the blocking activity against IFN γ -induced pSTAT1 in healthy control cells (gated on CD14⁺ monocytes) in the presence of 10% healthy control plasma, COVID-19 patient plasmas, or positive control plasma.

Patient 69 also demonstrated binding activity against GM-CSF (in addition to IFN α and IFN γ), however, these anti-GM-CSF autoantibodies did not inhibit GM-CSF-induced STAT5 phosphorylation in healthy donor cells (Figure 6.17). When blocking, anti-GM-CSF autoantibodies are associated with pulmonary alveolar proteinosis, cryptococcal

meningitis, and/or disseminated nocardiosis (Kitamura *et al.*, 1999; L. B. Rosen *et al.*, 2013; L. B. Rosen *et al.*, 2015). Patient 69 did not have clinical documentation of any of the ailments listed.

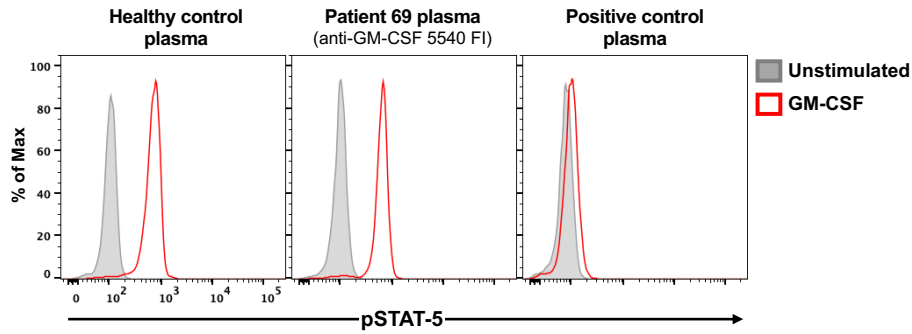


Figure 6.17. Non-neutralizing anti-GM-CSF autoantibodies in a COVID-19 patient with blocking anti-IFN α and non-blocking anti-IFN γ autoantibodies. Representative FACS plots showing the blocking activity against GM-CSF-induced pSTAT5 in healthy control cells (gated on CD14⁺ monocytes) in the presence of 10% healthy control plasma, COVID-19 patient plasma, or positive control plasma.

Finally, Patient 70 showed binding activity against IL-17F, in addition to IFN ω . When tested for its ability to inhibit IL-17F-induced IL-6 production in human foreskin fibroblast (HFF1) cells, Patient 70’s plasma was found to be non-neutralizing to IL-17F (Figure 6.18).

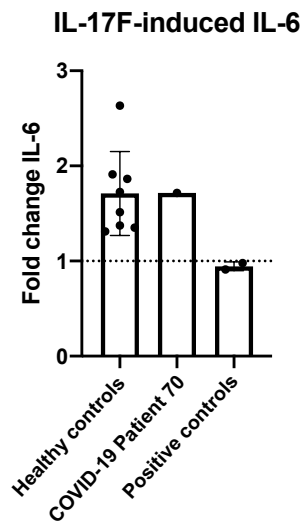


Figure 6.18. Non-neutralizing anti-IL-17F autoantibodies in a COVID-19 patient with blocking anti-IFN ω autoantibodies. Fold-change in IL-17F-induced IL-6 production in HFF1 cells in the presence of 10% healthy control, COVID-19 Patient 70, or positive control plasmas.

Anti-IFN α/ω autoantibody-positive COVID-19 patients found to have autoantibodies against other cytokines are summarized in Table 6.4. The direct binding activity for each cytokine target is listed as fluorescence intensity and blocking status is indicated for each patient's plasma.

Number	Severity	Additional autoantibodies	Binding activity (FI)	Blocking status
12	Critical	anti-IFN β	52	partially blocking
17	Critical	anti-IL-12p70	5683	partially blocking
19	Critical	anti-IL-12p70	11525	partially blocking
31	Critical	anti-TNF β	7158	NOT blocking
38	Critical	anti-IFN β	3538	blocking
		anti-IL-6	4976	partially blocking (at 10 ng/mL IL-6)
		anti-IL-12p70	8596	blocking
40	Critical	anti-IL-22	6808	blocking
		anti-TNF β	6938	NOT blocking
69	Critical	anti-IFN γ	5750	NOT blocking
		anti-GM-CSF	5540	NOT blocking
70	Critical	anti-IL-17F	5387	NOT blocking
74	Critical	anti-IFN γ	7458	NOT blocking
89	Moderate	anti-IFN γ	13330	NOT blocking

Table 6.4. Additional autoantibodies detected in the plasmas of anti-type I IFN autoantibody-positive COVID-19 patients. FI= Fluorescence intensity

The findings in this chapter present a look at the autoantibody profiles of healthy individuals, APECED patients, and patients with COVID-19 of varying severity. In healthy individuals, the rate of blocking anti-type I IFN autoantibodies is exceedingly low at 0.32%, however at least 10% of patients with life-threatening COVID-19 possess these autoantibodies. The presence of 10% plasma was able to inhibit IFN α -induced STAT1 phosphorylation up to 3.25 μ g/mL IFN α , while 10% healthy control plasma allowed for full

pSTAT1 signaling in response to 10 ng/mL IFN α . Blocking activity was limited to the IgG fraction of patient plasma, as IgG-deplete plasma allowed for full IFN α - and IFN ω -induced STAT1 phosphorylation.

Other anti-cytokine autoantibodies were detected in the plasmas of anti-IFN α/ω autoantibody-positive COVID-19 patients, however most were found to be non-neutralizing by functional testing. Interestingly, one patient's plasma was able to partially block IL-6 signaling (at 10 ng/mL IL-6). Anti-IL-6 autoantibodies have been reported in a child with recurrent staphylococcal cellulitis and chickenpox (Puel *et al.*, 2008), and more recently in a child with septic shock in the context of *Staphylococcus aureus* infection (Bloomfield *et al.*, 2019). Anti-IL-6 therapies exist for the treatment of rheumatologic disorders and Castleman's disease (Kang *et al.*, 2019), but most are targeted to the IL-6 receptor and would therefore not be detected by the screening assay used here. Siltuximab, however is an anti-IL-6 chimeric monoclonal with a human Fc region and could theoretically be detected using this assay. Efforts were made to gather additional clinical information on this patient but evidence of staphylococcal infection, rheumatological disorders, or Castleman's disease could not be obtained. Although the contribution of anti-IL-6 autoantibodies to this patient's disease is unclear, it is possible that partial blockade of IL-6 signaling could result in a diminished immune response early in the course of disease, leading to more severe COVID-19 infection.

Two anti-IFN α/ω autoantibody-positive COVID-19 patients demonstrated partial blocking activity to IL-12p70, while one patient was positive for blocking anti-IL-12p70 and anti-IL-22 autoantibodies. The presence of these autoantibodies in combination with anti-type I IFN

autoantibodies has been associated with thymoma (Burbelo *et al.*, 2010; Meager *et al.*, 1997), however clinical information available for these patients did not indicate the presence of thymic malignancies. Although two of the three patients are deceased, long term follow up on Patient 42 may provide insights into the origin and role of these autoantibodies.

Factors contributing to the predisposition of these individuals to develop autoreactivity to type I IFNs remain elusive. Whole exome analyses of anti-type I IFN autoantibody-positive COVID-19 patients has not yet revealed a genetic component, nor has a familial association been appreciated. Analyses of comorbidities and treatments between autoantibody-positive and autoantibody-negative COVID-19 patients did not reveal differences between the two groups (Abers *et al.*, submitted 2021). Environmental risk factors, which are thought to be important in the development of anti-IFN γ autoantibodies (C. H. Lin *et al.*, 2016), have not yet been ruled out as contributing to anti-type I IFN autoantibody predisposition.

When innate immune sensors recognize viral infections, the IFN response is the first major line of host defense (Park & Iwasaki, 2020). Upon binding to their receptor, IFNs induce the activation of interferon-stimulated genes (ISGs) which are able to interfere with viral replication at several stages of the viral life cycle through numerous mechanisms (Schoggins & Rice, 2011). Blockade of this host defense program through the neutralization of type I IFNs by autoantibodies leads to a dampened antiviral response and more severe disease. More work needs to be done to establish the natural history of these autoantibodies. Because individuals do not present until the onset of clinical symptoms, it

is unknown whether autoantibodies to type I IFNs preceded SARS-CoV-2 infection. The scarcity of these autoantibodies in healthy controls (0.32%, or 4 of 1252), along with preliminary data that demonstrate a decrease in the autoantibody levels of COVID-19 patients after recovery (to below the limit of detection in some cases), make it plausible that these autoantibodies are present at low/undetectable levels in ~10% of otherwise healthy individuals and that SARS-CoV-2 infection causes a rapid increase in autoantibody levels. This suggests that type I IFN-specific memory B cells are already present in these individuals at the time of infection and the rapid increase in type I IFN production causes activation of these memory B cells which become plasma cells and secrete large amounts of type I IFN-specific IgG.

The findings outlined in this section have direct clinical implications. Anti-IFN α/ω autoantibody screening assays could be used to identify patients at high risk for developing life-threatening COVID-19. When identified early in the course of infection, such individuals could be treated by plasmapheresis or with exogenous IFN β , as anti-IFN β autoantibodies were rare and only detected in two of the 1696 COVID-19 patients screened. Further research is needed to understand if the autoantibodies are pathogenic, by attenuating the inflammatory response needed to clear the viral infection, or protective, by mitigating cytokine storm. It is possible that type I IFN blockade can have dual effects, depending on where the patient is in the clinical course of the disease. This information could drive novel therapeutic interventions tailored to patients at various phases of COVID-19.

7 Discussion

AIRE is a transcription facilitator primarily expressed in mature medullary thymic epithelial cells (mTECs) where it modulates the promiscuous gene expression of tissue-restricted antigens (TRAs) typically detected in peripheral tissues. Aire expression in mTECs ensures that developing T cells in the thymus are exposed to a broad array of peptides derived from self-antigens and that strongly self-reactive thymocytes are deleted from the repertoire to establish central tolerance (Sansom *et al.*, 2014). Thymic negative selection is only 60-70% effective at removing autoreactive T cell clones, leaving a significant number of self-reactive T cells to enter the periphery (Bouneaud *et al.*, 2000; Gallegos & Bevan, 2006). Thus, peripheral tolerance mechanisms are necessary to remove these autoreactive T cell clones and prevent autoimmune tissue damage. Given the role of Aire-expressing mTECs in clearing autoreactive T cells and inducing T_{reg} production via the expression of TRAs, the presence of an Aire-expressing cell population with similar tolerogenic functions situated within lymph nodes is an attractive candidate in the regulation of peripheral tolerance.

The expression of Aire outside of the thymus has been documented by several groups and yet the precise phenotype of extra-thymic Aire-expressing cells (eTACs) remains controversial. In 1997, *Aire* (mRNA) transcript was found to be most prevalent in the thymus, but also present in human lymph nodes (Nagamine *et al.*, 1997). The first report of Aire protein in the periphery described Aire expression in peripheral blood mononuclear cells by immunocytochemical staining (Kogawa *et al.*, 2002). A fraction of human CD14⁺ monocytes stained positively for Aire protein, as did differentiated dendritic cells,

suggesting peripheral AIRE was limited to the myeloid lineage. Later studies concluded eTACs were stromal in nature and non-hematopoietic (Gardner *et al.*, 2008), although they were later clarified to be bone marrow-derived CD45⁺ cells (Gardner *et al.*, 2013). eTACs were described as dendritic cell (DC)-like, although they could not be definitively placed into any previously existing DC subset based on their transcriptomic profile. Comparisons between the tissue-restricted antigens expressed by AIRE⁺ mTECs and AIRE⁺ eTACs in mice have revealed that different genes are regulated by AIRE in mTECs and eTACs, with only 7 overlapping genes regulated by both (Gardner *et al.*, 2008). Later, eTACs were described as a novel subset of innate lymphoid cells (ILC3) (Yamano *et al.*, 2019) and were independently identified in human tonsils as a mature and migratory DC subset with transient AIRE expression (Fergusson *et al.*, 2018). Importantly, Fergusson *et al* found no evidence of enriched expression of tissue-restricted antigens in eTACs and attributed transient AIRE expression to a maturation process undergone by dendritic cells. Discrepancies observed between *Aire* (mRNA) transcript and AIRE protein (Yamano *et al.*, 2019) provides a biological reason for conflicting reports and difficulties associated with identifying eTACs.

AIRE is expressed in peripheral lymph node cells

My findings confirm the presence of extra-thymic AIRE expression in the lymph nodes of wildtype, non-transgenic mice. Immunofluorescence revealed eTACs reside in the interfollicular area of lymph nodes and express high levels of the costimulatory molecules CD86, MHC-II, and CD40, and are negative for EpCAM expression. This was corroborated by Yamano *et al*, who reported that while *Aire* (mRNA) transcript was detected in three eTAC populations (CD11c⁻ EpCAM⁻, CD11c⁺ EpCAM⁻, and CD11c⁺ EpCAM⁺), AIRE protein was only

observed by intracellular staining in the CD11c⁻ EpCAM⁻ population (Yamano *et al.*, 2019). My findings of AIRE expression in EpCAM⁺ eTACs sorted onto poly-L-lysine coated slides contradicts this work, as 15.7% of sorted EpCAM⁺ eTACs stained positive for AIRE protein by immunofluorescence. This could indicate that AIRE expression is transient in nature, further evidenced by the fact that intracellular AIRE protein was only detected in 10-20% of AIRE-reporter⁺ cells in transgenic mice with a modified *Aire* locus that drives green fluorescent protein expression (Yamano *et al.*, 2019).

My FACS gating strategy proved effective for identifying eTACs in murine lymph nodes, as the distinctive nuclear speckling pattern consistent with AIRE staining in mTEC^{hi}s was evident by confocal microscopy in eTACs sorted onto slides. However only in smaller fractions of EpCAM⁺ eTACs (15.7%) and EpCAM⁻ eTACs (9.3%) could AIRE protein be detected. This indicates the presence of AIRE-negative cells within my eTAC gates, likely to be dendritic cells based on the surface markers used, which exclude endothelial cells, B cells, macrophages, NK cells, neutrophils, erythrocytes, and T cells. Due to the likely transient nature of peripheral AIRE expression (Fergusson *et al.*, 2018), identification of a population of 100% AIRE-expressing cells is not possible, even when using transgenic AIRE-reporter mice (Yamano *et al.*, 2019).

eTACs are effective antigen presenting cells *in vitro*

Several studies have identified a role for eTACs in the presentation of antigen and deletion of antigen-specific T cells *in vivo*. In these transgenic mouse models, tissue-specific antigen expression was driven under the *Aire* promoter, and the proliferation and subsequent deletion of adoptively transferred antigen-specific T cells was assessed (Gardner *et al.*,

2008; Gardner *et al.*, 2013; Yamano *et al.*, 2019). Although these studies demonstrated the antigen presentation ability of eTACs in the context of both CD4⁺ and CD8⁺ T cells, they failed to demonstrate a direct role for AIRE expression in the presentation of antigens by eTACs. The expression of a neo-antigen under the control of *Aire* does not prove causality or that AIRE is responsible for the presentation of the antigen on the surface of AIRE-expressing cells. In fact, if AIRE is expressed transiently in eTACs, AIRE could be turned off by the time the neo-antigen is being processed and loaded on to MHC molecules for presentation on the cell surface. Additionally, these studies did not address the contribution of the two distinct eTAC populations identified (EpCAM⁺ eTACs and EpCAM⁻ eTACs) to this process, nor did they compare their functional capacity to that of professional APCs.

It has been shown that eTACs express high levels of costimulatory molecules, such as CD80, CD86, CD40, and MHC-II (Yamano *et al.*, 2019). This is important because the upregulation of costimulatory molecules on the surface of APCs is associated with a mature phenotype characterized by migration to lymph nodes, enhanced antigen presentation capacity, and chemokine secretion that attracts T cells for subsequent activation (Bachmann *et al.*, 2006; Fujii *et al.*, 2004; Murphy & Weaver, 2017). In this work I demonstrate increased MHC-II and CD40 expression in EpCAM⁺ eTACs, specifically, compared to conventional dendritic cells (cDCs), indicating the potential for superior antigen presentation capacity in EpCAM⁺ eTACs compared to professional APCs. I investigated the functional consequences of this observation by assessing the ability of eTACs and cDCs to present exogenous antigenic peptide to antigen-specific T cells *in vitro*. My findings provide the first evidence for antigen presentation by eTACs in wildtype, non-transgenic animals. Their ability to present

exogenous SIINFEKL peptide *in vitro* is comparable to that of conventional dendritic cells. The antigen presentation capacity of eTACs sorted from AIRE-deficient mice was superior to that of eTACs sorted from wildtype mice, potentially due to the upregulation of AIRE-repressed genes involved in antigen presentation and cell signaling pathways, although further studies are needed to test this hypothesis. Of note, Anderson *et al* demonstrated less effective antigen presentation of exogenously added OVA peptide to OT.1 T cells in culture by AIRE-deficient mTECs (Anderson *et al.*, 2005), implying different roles for AIRE in these distinct cell populations.

Mice rendered AIRE-deficient in mTECs recapitulate phenotypic findings in constitutively AIRE-deficient mice

Constitutively AIRE-deficient mice on several genetic backgrounds have been assessed in an attempt to recapitulate the autoimmune manifestations observed in APECED patients. *Aire*^{-/-} mice on the C57BL/6 background exhibit defective negative selection leading to increased autoreactive T cells in the periphery and organ-specific autoimmunity (Anderson *et al.*, 2005; Anderson *et al.*, 2002; Hassler *et al.*, 2006; Hubert *et al.*, 2009; Jiang *et al.*, 2005; Liston *et al.*, 2003; Ramsey *et al.*, 2002). However, the contribution of various AIRE-expressing cell populations to T cell tolerance and the prevention of autoimmunity has remained incompletely known.

Bone marrow chimera experiments suggest that AIRE expression in radio-resistant stromal cells is necessary to prevent autoimmune organ damage (Anderson *et al.*, 2002), however, there is evidence of radio-resistance in eTACs (Gardner *et al.*, 2013), so it is unclear whether eTACs play a role in preventing autoimmunity in these studies. Additionally,

thymic transplant experiments demonstrated autoimmune organ infiltration in athymic nude mice engrafted with AIRE-deficient thymic stroma while AIRE-sufficient thymic stroma prevented autoimmunity (Anderson *et al.*, 2005; Kuroda *et al.*, 2005). Thymic transplant experiments, however, do not address the contribution of AIRE-expressing B cells, which are thought to migrate to the thymus from the periphery and receive CD40 signals from developing thymocytes during thymic negative selection, resulting in AIRE expression. Although it is clear from these experiments that AIRE-expressing B cells are unable to overcome the effect of AIRE-deficient thymic stroma, the artificial system created by transplanting thymic lobes into athymic animals introduces new variables such as anomalies in the thymic architecture which could affect the recruitment of B cells to the thymus. In another study, the overexpression of AIRE in hematopoietic cells delayed the onset of experimental autoimmune encephalomyelitis in bone marrow chimera recipient mice (Ko *et al.*, 2010), although the physiological relevance of this artificial system is unknown given that AIRE-expressing B cells represent ~0.009% of thymic cellularity (Yamano *et al.*, 2015) and eTACs represent ~0.003% of lymph node cellularity.

By specifically ablating AIRE expression in mTECs and B cells, I demonstrate that lack of AIRE in mTECs is sufficient to recapitulate most of the phenotypic findings observed in constitutively AIRE-deficient mice. Table 7.1 summarizes my phenotypic findings in mice rendered AIRE-deficient constitutively (*Aire*^{-/-}), in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)), and in B cells (*Aire*(fl/fl):*Mb1*(Cre+)) compared to appropriate controls.

		<i>Aire</i> ^{-/-}	<i>Aire</i> (fl/fl): <i>Foxn1</i> (Cre+)	<i>Aire</i> (fl/fl): <i>Mb1</i> (Cre+)
Early thymocyte development	ETP	Normal	Normal	Normal
	DN1	Normal	Normal	Normal
	DN2	Normal	Normal	Normal
	DN3a	Normal	Normal	Normal
	DN3b	Normal	Normal	Normal
	DN4	Normal	Normal	Normal
	CD8 ISP	Normal	Normal	Normal
Negative selection	Wave 1a	Normal	Normal	Normal
	Wave 1b	Normal	Normal	Normal
	Wave 2a	Decreased (*)	Decreased (****)	Normal
	Wave 2b	Normal	Normal	Normal
Other thymic T cells	Thymic T _{reg}	Decreased (*)	Decreased (****)	Normal
	Re-circulating T cells	Normal	Decreased (**)	Normal
Peripheral T cells (CD4 ⁺)	Total CD4 ⁺	Normal	Normal	Normal
	Naïve CD4 ⁺	Decreased (****)	Normal	Normal
	Memory CD4 ⁺	Increased (****)	Normal	Normal
	T _{reg}	Increased (*)	Normal	Normal
	Anergic CD4 ⁺	Increased (***)	Normal	Normal
	RTE CD4 ⁺	Increased (****)	Increased (****)	Normal
Peripheral T cells (CD8 ⁺)	Total CD8 ⁺	Normal	Normal	Normal
	Naïve CD8 ⁺	Decreased (*)	Decreased (*)	Normal
	Central memory CD8 ⁺	Increased (*)	Increased (**)	Normal
	Effector memory CD8 ⁺	Increased (*)	Normal	Normal
	RTE CD8 ⁺	Increased (*)	Increased (**)	Normal
Organ-specific autoimmunity	Eye-specific autoantibodies	Increased (**)	Increased (**)	Normal
	Salivary gland infiltration	Increased (***)	Increased (***)	Normal
	Retinal degeneration	Increased (*)	Increased (***)	Normal

Table 7.1 Summary of findings in loss-of-AIRE mutant mice. The phenotypes are shown for each loss-of-AIRE mutant mouse strain compared to littermate controls. Statistical validity was determined by student's t test where *p<0.05, **p<0.01, ***p<0.001, and ****p<0.001.

My findings of normal early thymocyte development, which occurs in the outer cortex of the thymus, in AIRE-deficient mice are consistent with previous reports (Hubert *et al.*, 2009) and make sense from a temporospatial standpoint as AIRE expression is limited to the thymic medulla. Indeed, defects in thymocyte negative selection, which occurs in the inner cortex and medulla, have been observed in AIRE-deficient mice, resulting in a 70% increase in the number of SP3 thymocytes (CD4⁺ CD24⁻ CCR7⁺ CD69⁻ Qa2⁻) (J. Li *et al.*, 2007). This implies that more SP2 thymocytes (CD4⁺ CD24⁺ CCR7⁺ CD69⁺ Qa2⁻) successfully evade negative selection and progress to the SP3 phenotype in the absence of AIRE. Correspondingly, my findings reflect a decreased proportion of cells marked for deletion in

the Wave 2a stage of negative selection in constitutively AIRE-deficient mice (*Aire*^{-/-}) and mice rendered AIRE-deficient in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)). This implies that appropriate negative selection is mediated by AIRE-expressing mTECs, specifically at the Wave 2a stage. Given that the first wave of negative selection occurs in the cortex (Kurd & Robey, 2016), it is logical that AIRE expression in medullary thymic epithelial cells does not impact this stage from a spatial standpoint. Additional studies are required to determine whether another transcription regulator, such as FEZF2, could play a more critical (or at least redundant) role in the Wave 2b stage of negative selection, as differences were not observed in the frequency of cells marked for deletion at this stage.

In some of the measures assessed, my findings in mice rendered AIRE-deficient in mTECs were more prominent than those in constitutively AIRE-deficient mice (Table 7.1). Possible explanations for the disparities in the magnitude of differences observed for these parameters could be biological in nature. For instance, it is possible that another AIRE-expressing cell population (i.e., eTACs or B cells) could be playing an opposing role to AIRE-expressing mTECs. Given that I did not observe an increase in the frequency of thymocytes at the Wave 2a stage of negative selection, thymic T_{reg}, or re-circulating T cells in the thymi of mice rendered AIRE-deficient in B cells (*Aire*(fl/fl):*Mb1*(Cre+)), a role for AIRE-expressing B cells would have to be subtle enough to remain undetectable by the methods used here. In order to test this experimentally, mice could be chemically (via treatment with anti-CD40) (Yamano *et al.*, 2015) or genetically made to overexpress AIRE in B cells. Adoptive transfer of these cells into constitutively AIRE-deficient hosts could potentially provide insight into the role of AIRE-expressing B cells in these processes. Another possibility is an opposing role for eTACs, which could feasibly contribute to the recirculation of mature T

cells back to the thymus. If this were true, I would expect to see an increase in re-circulating mature T cells in the thymi of mice rendered AIRE-deficient in eTACs only. Unfortunately, two attempts to create such a mouse were unsuccessful during the course of this project necessitating additional studies in the future.

Yamano *et al* demonstrated that B cell-deficient mice had an increased frequency of CD4 SP thymocytes and hyperresponsive T cells, implying thymocytes that had developed in the absence of B cells were incompletely tolerized (Yamano *et al.*, 2015). My findings suggest these differences were not due to lack of AIRE-expressing B cells, as *Aire*(fl/fl):*Mb1*(Cre+) mice did not have increased CD4 SP thymocytes compared to Cre-negative controls.

Although total peripheral CD4⁺ and CD8⁺ T cell frequencies were unchanged between AIRE-deficient and control mice, constitutively AIRE-deficient mice demonstrated an increase in the frequency of recent thymic emigrant T cells, a decrease in the frequency of naïve T cells, and increased frequencies of memory T cells, anergic T cells, and peripheral T_{reg}. A decrease in the number of thymocytes marked for deletion at the Wave 2a stage of negative selection (observed in constitutively AIRE-deficient mice and mice rendered AIRE-deficient in mTECs) is therefore expected to lead to an increased number of potentially autoreactive T cells eventually emigrating from the thymus to the periphery. There, self-reactive recent thymic emigrants could encounter their cognate self-antigen, become activated and adopt an effector/memory phenotype. Compensatory peripheral tolerance mechanisms are enacted to incapacitate autoreactive T cells through anergy induction or T_{reg}-induced suppression (Kalekar & Mueller, 2017). It has been shown that T_{reg} suppressive functions are antigen-specific (Hori *et al.*, 2002; Takahashi *et al.*, 1998; Thornton &

Shevach, 1998), thus it is feasible that an increased frequency of autoreactive T cells could result in a higher likelihood of these cells being shunted toward a T_{reg} fate in the periphery.

Almost all of the observed changes in the peripheral CD8⁺ T cell compartment of constitutively AIRE-deficient mice could be explained by a loss of AIRE in mTECs. One notable exception concerns the increased frequency of effector memory CD8⁺ T cells observed in constitutively AIRE-deficient mice (*Aire*^{-/-}), but not in mice rendered AIRE-deficient in mTECs (*Aire*(fl/fl):*Foxn1*(Cre+)). This could indicate a role for eTACs in the deletion of autoreactive effector memory CD8⁺ T cells. It has been shown that eTACs can functionally inactivate CD8⁺ T cells in an antigen-specific manner (Gardner *et al.*, 2008; Lee *et al.*, 2007), and that memory CD8⁺ T cells are as susceptible to peripheral tolerance induction as naïve CD8⁺ T cells (Kreuwel *et al.*, 2002). It is possible that effector memory CD8⁺ T cells are being deleted in the periphery by eTACs, thus restoring normal frequencies of these cells in mice with a targeted loss of AIRE in mTECs. Further studies on eTACs and their interactions with naïve and memory T cells are necessary to further elucidate this hypothesis.

I observed several changes in peripheral CD4⁺ T cells in constitutively AIRE-deficient mice that were not explained by a targeted absence of AIRE expression in only mTECs (*Aire*(fl/fl):*Foxn1*(Cre+) mice). While CD4⁺ recent thymic emigrant cells were increased in these mice, other CD4⁺ T cell subsets (naïve, memory, T_{reg}, and anergic CD4⁺ T cells) remained unchanged compared to controls. Therefore, I propose that the expression of AIRE in eTACs plays a role in the deletion of CD4⁺ T cells in the periphery. Evidence for tolerance induction by eTACs has been shown through the antigen-specific deletion of CD4⁺

T cells *in vivo* (Gardner *et al.*, 2013; Yamano *et al.*, 2019), although this has yet to be demonstrated in non-transgenic mouse models.

Despite the evident differences observed in the peripheral T cell compartment of constitutively AIRE-deficient mice and mice lacking AIRE in mTECs, both strains exhibit the same organ-specific autoimmune manifestations in the eye and salivary glands. It is possible that, due to its immunological privilege, the eye is more susceptible to autoimmune manifestations because eye-specific antigens are not found in peripheral lymphoid organs where peripheral tolerance mechanisms occur (Caspi, 2008). It would be interesting to determine whether infiltrating cells observed in the salivary glands of *Aire(fl/fl):Foxn1(Cre+)* mice are preferentially CD8⁺ T cells given the aberrations observed in the CD8⁺ T cell compartment specifically in these mice.

Based on my findings in constitutive and cell lineage-specific AIRE-deficient mice, I conclude that the majority of the changes observed in constitutively AIRE-deficient are explained by a lack of AIRE in mTECs. Further studies need to be performed to assess mice lacking AIRE in eTACs only, which might explain discordant findings, specifically in the CD4⁺ T cell compartments of *Aire*^{-/-} and *Aire(fl/fl):Foxn1(Cre+)* mice. My work to assess the antigen presentation by AIRE-sufficient and AIRE-deficient eTACs implies that AIRE is not directly responsible for the exogenous antigen presentation capacity of eTACs, as SIINFEKL-loaded AIRE-deficient eTACs were at least comparable to AIRE-sufficient eTACs at activating OT.1 T cells to proliferate *in vitro*. Although AIRE is not required for the presentation of exogenous antigen, I cannot rule out the possibility that AIRE is mediating the expression of self-antigens in the periphery based on the methods used here. Work done by others on

this topic has been controversial. RNA sequencing demonstrated that eTACs were found to express a unique set of AIRE-regulated genes, with little overlap between those expressed by eTACs and those expressed by mTECs, with AIRE-induced genes found in eTACs significantly enriched for TRAs compared to a random gene set (Gardner *et al.*, 2008). More recent studies have agreed that eTACs express a unique set of gene transcripts compared to mTECs but found that the transcriptomic profile of eTACs in humans and mice is not enriched for TRAs (Fergusson *et al.*, 2018; Yamano *et al.*, 2019), arguing that AIRE in eTACs might perform an alternative role to AIRE in mTECs. Limitations of this work leave open-ended questions. Although I attempted twice to generate mice rendered AIRE-deficient in eTACs (*Aire*(fl/fl):*Cd45*(Cre+) and *Aire*(fl/fl):*Rorc*(Cre+)), neither strain resulted in effective excision of *Aire* in eTACs for reasons that remain unknown. Restrictions due to the COVID-19 pandemic and the inadvertent appearance of a germline recombination event in my colony of *Aire*(fl/fl) mice, a rare occurrence that has been documented in *Mb1*(Cre) mice, precluded a third attempt, although generation of an eTAC-specific loss-of-AIRE mouse will be critical in unraveling the role of eTACs in peripheral tolerance mechanisms.

Anti-type I IFN autoantibodies are associated with life-threatening COVID-19

Early in the COVID-19 pandemic, heterozygous mutations in *AIRE* were observed in a small proportion of patients with SARS-CoV-2 infection. Given the abundance of anti-cytokine autoantibodies in AIRE-deficient individuals and the role of type I interferons (IFN) in the control of viral infections, I sought to assess the sera/plasmas of healthy individuals, APECED patients, and patients with COVID-19 of varying severity for the presence of anti-cytokine autoantibodies. In healthy individuals, 0.32% (4 out of 1252) had anti-type I IFN autoantibodies with at least partial blocking activity. In COVID-19 patients, the incidence of

blocking autoantibodies against at least one type I IFN was 10.4% in critically ill patients and decreased with severity (2.2% of severe/moderate patients and 0.18% in mild/asymptomatic patients). The presence of anti-type I IFN autoantibodies did not correlate with the presence of heterozygous changes in *AIRE*. It should be noted that all of the healthy controls screened were collected prior to the COVID-19 pandemic and information on whether those individuals later became infected with SARS-CoV-2 is not available due to confidentiality restrictions.

Taken together, it is likely that the autoantibodies are present at low/undetectable levels in healthy individuals and that infection with SARS-CoV-2 causes a rapid increase in autoantibody levels. In order for this to occur in the short timeframe between SARS-CoV-2 exposure and clinical presentation of symptoms, one possibility is that auto-reactive, type I IFN-specific memory B cells are already present at the time of infection. Alternatively, while the timeline seems too fast for a break in tolerance given that autoantibodies are detectable in the sera/plasmas of COVID-19 patients upon hospital admission, it should be noted that the median incubation period of SARS-CoV-2 is estimated to be 5.1 days, with 97.5% of patients developing symptoms within 11.5 days of infection (Lauer *et al.*, 2020). Adaptive immune responses become effective within days of antigen interaction (Murphy & Weaver, 2017), making it feasible that a potential break in tolerance could occur between the time of infection and hospitalization of COVID-19 patients.

Another possibility is that anti-IFN α /IFN ω autoantibodies exist in healthy individuals at detectable levels, were they not bound to their cognate cytokine, rendering them undetectable by binding assays. In this scenario, increased type I IFN production in

response to SARS-CoV-2 infection might overwhelm the constitutive level of autoantibody production, activating pre-existing memory B cells, leading to plasma cell differentiation and IgG secretion. Increased IFN α/ω production in response to infection could then stimulate more IgG production which quickly neutralizes peripheral IFN α/ω while signaling for plasma cells to produce more IgG. If this were the case, I would expect to detect high levels of blocking anti-type I IFN autoantibodies in IVIg fractions, due to the acid treatment and fractionation process undergone by sera from healthy controls during the manufacturing of IVIg preparations. However, we have looked and did not find them (unpublished data by Holland lab).

If blockade in the type I IFN pathway were indeed a risk factor for developing life-threatening COVID-19, then APECED patients, nearly all of whom are positive for autoantibodies against at least one type I IFN, should be inherently and substantially more susceptible to developing severe COVID-19. However, confounding this assessment is the fact that APECED patients have had life-long health problems and are thus likely to take above-average precautions to protect themselves from SARS-CoV-2 infection. Interestingly, we have identified 22 APECED patients internationally who developed COVID-19, 86% of whom were hospitalized (Bastard, Orlova, *et al.*, 2021). Thus, work is ongoing to collect clinical information and perform SARS-CoV-2 serological testing on all APECED patients to calculate their estimated risk of severe COVID-19 relative to other populations.

Of note, only two COVID-19 patients were found to have anti-IFN β autoantibodies with partial or complete blocking activity. This is important because when identified early in the course of infection, patients with blocking anti-IFN α and/or -IFN ω autoantibodies could be

administered recombinant IFN β to potentially prevent the development of severe disease. In order to test this, I plan to test samples from patients enrolled on the Adaptive COVID-19 Treatment Trial 3 (ACTT3), a phase 3 clinical trial to evaluate the combination of IFN β -1a and the anti-viral medication remdesivir, compared to remdesivir alone. By testing critically ill COVID-19 patients in this trial, I would expect to detect anti-IFN α/ω autoantibodies in ~10%. Theoretically, half of those patients will have received IFN β -1a treatment and the other half will have received a placebo. By comparing clinical outcomes of autoantibody-positive patients in both arms, I should be able to draw conclusions about the effectiveness of early administration of IFN β to anti-IFN α/ω autoantibody-positive patients.

Another potential treatment for COVID-19 patients with blocking anti-IFN α/ω autoantibodies might be plasma exchange and Ig replacement. In a case series of 4 autoantibody-positive COVID-19 patients, only two survived, although it is difficult to deconvolute the contribution of other factors beyond the plasma exchange treatment (de Prost *et al.*, 2021). Autoantibody-depleting biologics, such as the anti-CD20 monoclonal rituximab, are probably not a viable treatment for anti-IFN α/ω autoantibody-positive COVID-19 patients given the lag observed in rituximab administration and plasma autoantibody levels, with clinical improvement only noted after 2-6 months of treatment (Browne, Zaman, *et al.*, 2012).

To study the natural history of anti-IFN α/ω autoantibodies in patients who go on to develop life-threatening COVID-19, it would be important to evaluate banked samples from SARS-CoV-2-infected patients that were collected prior to the COVID-19 pandemic. I plan to test plasma samples from military personnel who developed severe COVID-19 to identify

those with autoantibodies to type I IFNs. Previously banked samples could then be obtained from the Military Serum Repository to determine whether autoantibodies preceded SARS-CoV-2 infection. For those for whom peripheral blood mononuclear cells are available, the presence of circulating type I IFN-specific memory B cells could be assessed.

The contribution of type I IFNs to anti-viral immunity has been long appreciated but remains controversial in the context of COVID-19. Some data demonstrate beneficial effects of type I IFNs in COVID-19 treatment while others provide evidence for potential harm. It seems that the timing of therapeutic intervention is key in understanding the effects of type I IFN treatment. Clinical trials have shown that treatment with subcutaneous IFN β -1a has potentially protective effects early in the disease course but can be harmful when administered to patients already requiring high-flow oxygen (Consortium *et al.*, 2021; Hung *et al.*, 2020; Kalil *et al.*, 2021; Q. Zhang *et al.*, 2020). Finally, the presence of neutralizing anti-type I IFN autoantibodies in COVID-19 patients did not independently decrease patient survival in our cohort. This implies that anti-IFN $\alpha/\beta/\omega$ autoantibodies cause defective viral control early in the disease course, which leads to the development of critical COVID-19 pneumonia, but do not appear to independently drive patient mortality late in the course of infection (Abers *et al.*, submitted 2021). It is possible that early intervention with IFN β treatment could decrease disease progression in these patients.

In conclusion, the data shown in this thesis have demonstrated a direct role for AIRE-expressing mTECs in the negative selection of thymocytes and downstream autoimmune manifestations observed in the context of constitutive AIRE-deficiency. Additionally, due to

a redirection of research efforts to address the pandemic, I have also demonstrated a role for neutralizing autoantibodies to type I IFNs in the development of life-threatening COVID-19.

References

- Abramson, J., Giraud, M., Benoist, C., & Mathis, D. (2010). Aire's partners in the molecular control of immunological tolerance. *Cell*, 140(1), 123-135. doi:10.1016/j.cell.2009.12.030
- Aderem, A., & Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*, 17, 593-623. doi:10.1146/annurev.immunol.17.1.593
- Adler, A. J., Marsh, D. W., Yochum, G. S., Guzzo, J. L., Nigam, A., Nelson, W. G., & Pardoll, D. M. (1998). CD4+ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells. *J Exp Med*, 187(10), 1555-1564. doi:10.1084/jem.187.10.1555
- Agus, D. B., Surh, C. D., & Sprent, J. (1991). Reentry of T cells to the adult thymus is restricted to activated T cells. *J Exp Med*, 173(5), 1039-1046. doi:10.1084/jem.173.5.1039
- Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. *Nat Rev Immunol*, 4(7), 499-511. doi:10.1038/nri1391
- Anderson, M. S., Venanzi, E. S., Chen, Z., Berzins, S. P., Benoist, C., & Mathis, D. (2005). The cellular mechanism of Aire control of T cell tolerance. *Immunity*, 23(2), 227-239. doi:10.1016/j.immuni.2005.07.005
- Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., . . . Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science*, 298(5597), 1395-1401. doi:10.1126/science.1075958

- Ansari, R., Rosen, L. B., Lisco, A., Gilden, D., Holland, S. M., Zerbe, C. S., . . . Cohen, J. I. (2020). Primary and Acquired Immunodeficiencies Associated with Severe Varicella-Zoster Infections. *Clin Infect Dis*. doi:10.1093/cid/ciaa1274
- Aschenbrenner, K., D'Cruz, L. M., Vollmann, E. H., Hinterberger, M., Emmerich, J., Swee, L. K., . . . Klein, L. (2007). Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat Immunol*, 8(4), 351-358. doi:10.1038/ni1444
- Bachmann, M. F., Kopf, M., & Marsland, B. J. (2006). Chemokines: more than just road signs. *Nat Rev Immunol*, 6(2), 159-164. doi:10.1038/nri1776
- Bagnato, F., Clemenzi, A., Scagnolari, C., Strizzi, L., Di Pasquale, A., Bellomi, F., . . . Antonini, G. (2004). Neutralizing antibodies against endogenous interferon in myasthenia gravis patients. *Eur Cytokine Netw*, 15(1), 24-29.
- Bao, J., Ma, H. Y., Schuster, A., Lin, Y. M., & Yan, W. (2013). Incomplete cre-mediated excision leads to phenotypic differences between Stra8-iCre; Mov10l1(lox/lox) and Stra8-iCre; Mov10l1(lox/Delta) mice. *Genesis*, 51(7), 481-490. doi:10.1002/dvg.22389
- Bardhan, M., & Kaushik, R. (2021). Physiology, Complement Cascade. In *StatPearls*. Treasure Island (FL).
- Barker, C. F., & Billingham, R. E. (1977). Immunologically privileged sites. *Adv Immunol*, 25, 1-54.
- Bastard, P., Michailidis, E., Hoffmann, H. H., Chbihi, M., Le Voyer, T., Rosain, J., . . . Casanova, J. L. (2021). Auto-antibodies to type I IFNs can underlie adverse reactions to yellow fever live attenuated vaccine. *J Exp Med*, 218(4). doi:10.1084/jem.20202486

- Bastard, P., Orlova, E., Sozaeva, L., Levy, R., James, A., Schmitt, M. M., . . . Lionakis, M. S. (2021). Preexisting autoantibodies to type I IFNs underlie critical COVID-19 pneumonia in patients with APS-1. *J Exp Med*, 218(7). doi:10.1084/jem.20210554
- Bloomfield, M., Parackova, Z., Cabelova, T., Pospisilova, I., Kabicek, P., Houstkova, H., & Sediva, A. (2019). Anti-IL6 Autoantibodies in an Infant With CRP-Less Septic Shock. *Front Immunol*, 10, 2629. doi:10.3389/fimmu.2019.02629
- Bonfoco, E., Stuart, P. M., Brunner, T., Lin, T., Griffith, T. S., Gao, Y., . . . Green, D. R. (1998). Inducible nonlymphoid expression of Fas ligand is responsible for superantigen-induced peripheral deletion of T cells. *Immunity*, 9(5), 711-720. doi:10.1016/s1074-7613(00)80668-8
- Bouneaud, C., Kourilsky, P., & Bousso, P. (2000). Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity*, 13(6), 829-840. doi:10.1016/s1074-7613(00)00080-7
- Boursalian, T. E., Golob, J., Soper, D. M., Cooper, C. J., & Fink, P. J. (2004). Continued maturation of thymic emigrants in the periphery. *Nat Immunol*, 5(4), 418-425. doi:10.1038/ni1049
- Branda, C. S., & Dymecki, S. M. (2004). Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. *Dev Cell*, 6(1), 7-28. doi:10.1016/s1534-5807(03)00399-x
- Break, T. J., Oikonomou, V., Dutzan, N., Desai, J. V., Swidergall, M., Freiwald, T., . . . Lionakis, M. S. (2021). Aberrant type 1 immunity drives susceptibility to mucosal fungal infections. *Science*, 371(6526). doi:10.1126/science.aay5731

- Browne, S. K., Burbelo, P. D., Chetchotisakd, P., Suputtamongkol, Y., Kiertiburanakul, S., Shaw, P. A., . . . Holland, S. M. (2012). Adult-onset immunodeficiency in Thailand and Taiwan. *N Engl J Med*, 367(8), 725-734. doi:10.1056/NEJMoa1111160
- Browne, S. K., & Holland, S. M. (2010). Anticytokine autoantibodies in infectious diseases: pathogenesis and mechanisms. *Lancet Infect Dis*, 10(12), 875-885. doi:10.1016/S1473-3099(10)70196-1
- Browne, S. K., Zaman, R., Sampaio, E. P., Jutivorakool, K., Rosen, L. B., Ding, L., . . . Holland, S. M. (2012). Anti-CD20 (rituximab) therapy for anti-IFN-gamma autoantibody-associated nontuberculous mycobacterial infection. *Blood*, 119(17), 3933-3939. doi:10.1182/blood-2011-12-395707
- Burbelo, P. D., Browne, S. K., Sampaio, E. P., Giaccone, G., Zaman, R., Kristosturyan, E., . . . Holland, S. M. (2010). Anti-cytokine autoantibodies are associated with opportunistic infection in patients with thymic neoplasia. *Blood*, 116(23), 4848-4858. doi:10.1182/blood-2010-05-286161
- Burnet, F. M. (1972). A reassessment of the forbidden clone hypothesis of autoimmune disease. *Aust J Exp Biol Med Sci*, 50(1), 1-9. doi:10.1038/icb.1972.1
- Caspi, R. (2008). Autoimmunity in the immune privileged eye: pathogenic and regulatory T cells. *Immunol Res*, 42(1-3), 41-50. doi:10.1007/s12026-008-8031-3
- Cavadini, P., Vermi, W., Facchetti, F., Fontana, S., Nagafuchi, S., Mazzolari, E., . . . Badolato, R. (2005). AIRE deficiency in thymus of 2 patients with Omenn syndrome. *J Clin Invest*, 115(3), 728-732. doi:10.1172/JCI23087
- Ceredig, R., Dialynas, D. P., Fitch, F. W., & MacDonald, H. R. (1983). Precursors of T cell growth factor producing cells in the thymus: ontogeny, frequency, and quantitative recovery in a subpopulation of phenotypically mature thymocytes

- defined by monoclonal antibody GK-1.5. *J Exp Med*, 158(5), 1654-1671.
doi:10.1084/jem.158.5.1654
- Cheng, M. H., Fan, U., Grewal, N., Barnes, M., Mehta, A., Taylor, S., . . . Anderson, M. S. (2010). Acquired autoimmune polyglandular syndrome, thymoma, and an AIRE defect. *N Engl J Med*, 362(8), 764-766. doi:10.1056/NEJMc0909510
- Chentoufi, A. A., & Polychronakos, C. (2002). Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance: the mechanism by which the IDDM2 locus may predispose to diabetes. *Diabetes*, 51(5), 1383-1390. doi:10.2337/diabetes.51.5.1383
- Chi, C. Y., Chu, C. C., Liu, J. P., Lin, C. H., Ho, M. W., Lo, W. J., . . . Ku, C. L. (2013). Anti-IFN-gamma autoantibodies in adults with disseminated nontuberculous mycobacterial infections are associated with HLA-DRB1*16:02 and HLA-DQB1*05:02 and the reactivation of latent varicella-zoster virus infection. *Blood*, 121(8), 1357-1366. doi:10.1182/blood-2012-08-452482
- Choi, S., & Schwartz, R. H. (2007). Molecular mechanisms for adaptive tolerance and other T cell anergy models. *Semin Immunol*, 19(3), 140-152.
doi:10.1016/j.smim.2007.02.005
- Consortium, W. H. O. S. T., Pan, H., Peto, R., Henao-Restrepo, A. M., Preziosi, M. P., Sathiyamoorthy, V., . . . Swaminathan, S. (2021). Repurposed Antiviral Drugs for Covid-19 - Interim WHO Solidarity Trial Results. *N Engl J Med*, 384(6), 497-511. doi:10.1056/NEJMoa2023184
- Curotto de Lafaille, M. A., & Lafaille, J. J. (2009). Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity*, 30(5), 626-635. doi:10.1016/j.immuni.2009.05.002

- Daley, S. R., Hu, D. Y., & Goodnow, C. C. (2013). Helios marks strongly autoreactive CD4⁺ T cells in two major waves of thymic deletion distinguished by induction of PD-1 or NF-kappaB. *J Exp Med*, 210(2), 269-285. doi:10.1084/jem.20121458
- de Prost, N., Bastard, P., Arrestier, R., Fourati, S., Mahevas, M., Burrel, S., . . . Luyt, C. E. (2021). Plasma Exchange to Rescue Patients with Autoantibodies Against Type I Interferons and Life-Threatening COVID-19 Pneumonia. *J Clin Immunol*, 41(3), 536-544. doi:10.1007/s10875-021-00994-9
- Derbinski, J., Schulte, A., Kyewski, B., & Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol*, 2(11), 1032-1039. doi:10.1038/ni723
- Desanti, G. E., Cowan, J. E., Baik, S., Parnell, S. M., White, A. J., Penninger, J. M., . . . Anderson, G. (2012). Developmentally regulated availability of RANKL and CD40 ligand reveals distinct mechanisms of fetal and adult cross-talk in the thymus medulla. *J Immunol*, 189(12), 5519-5526. doi:10.4049/jimmunol.1201815
- DeVoss, J., Hou, Y., Johannes, K., Lu, W., Liou, G. I., Rinn, J., . . . Anderson, M. S. (2006). Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. *J Exp Med*, 203(12), 2727-2735. doi:10.1084/jem.20061864
- DiMeglio, L. A., Evans-Molina, C., & Oram, R. A. (2018). Type 1 diabetes. *Lancet*, 391(10138), 2449-2462. doi:10.1016/S0140-6736(18)31320-5
- Dobes, J., Edenhofer, F., Voboril, M., Brabec, T., Dobesova, M., Cepkova, A., . . . Filipp, D. (2018). A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire. *Eur J Immunol*, 48(3), 546-548. doi:10.1002/eji.201747267

- Doffinger, R., Helbert, M. R., Barcenas-Morales, G., Yang, K., Dupuis, S., Ceron-Gutierrez, L., . . . Kumararatne, D. S. (2004). Autoantibodies to interferon-gamma in a patient with selective susceptibility to mycobacterial infection and organ-specific autoimmunity. *Clin Infect Dis*, 38(1), e10-14. doi:10.1086/380453
- Dorman, S. E., & Holland, S. M. (2000). Interferon-gamma and interleukin-12 pathway defects and human disease. *Cytokine Growth Factor Rev*, 11(4), 321-333. doi:10.1016/s1359-6101(00)00010-1
- Dranoff, G., Crawford, A. D., Sadelain, M., Ream, B., Rashid, A., Bronson, R. T., . . . et al. (1994). Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science*, 264(5159), 713-716. doi:10.1126/science.8171324
- Eberl, G., & Littman, D. R. (2004). Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat+ cells. *Science*, 305(5681), 248-251. doi:10.1126/science.1096472
- Eldershaw, S. A., Sansom, D. M., & Narendran, P. (2011). Expression and function of the autoimmune regulator (Aire) gene in non-thymic tissue. *Clin Exp Immunol*, 163(3), 296-308. doi:10.1111/j.1365-2249.2010.04316.x
- Ema, H., Morita, Y., Yamazaki, S., Matsubara, A., Seita, J., Tadokoro, Y., . . . Nakauchi, H. (2006). Adult mouse hematopoietic stem cells: purification and single-cell assays. *Nat Protoc*, 1(6), 2979-2987. doi:10.1038/nprot.2006.447
- Ezzedine, K., Eleftheriadou, V., Whitton, M., & van Geel, N. (2015). Vitiligo. *Lancet*, 386(9988), 74-84. doi:10.1016/S0140-6736(14)60763-7
- Fan, Y., Rudert, W. A., Grupillo, M., He, J., Sisino, G., & Trucco, M. (2009). Thymus-specific deletion of insulin induces autoimmune diabetes. *EMBO J*, 28(18), 2812-2824. doi:10.1038/emboj.2009.212

- Ferber, I., Schonrich, G., Schenkel, J., Mellor, A. L., Hammerling, G. J., & Arnold, B. (1994). Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science*, 263(5147), 674-676. doi:10.1126/science.8303275
- Fergusson, J. R., Morgan, M. D., Bruchard, M., Huitema, L., Heesters, B. A., van Unen, V., . . . Spits, H. (2018). Maturing Human CD127+ CCR7+ PDL1+ Dendritic Cells Express AIRE in the Absence of Tissue Restricted Antigens. *Front Immunol*, 9, 2902. doi:10.3389/fimmu.2018.02902
- Ferre, E. M., Rose, S. R., Rosenzweig, S. D., Burbelo, P. D., Romito, K. R., Niemela, J. E., . . . Lionakis, M. S. (2016). Redefined clinical features and diagnostic criteria in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *JCI Insight*, 1(13). doi:10.1172/jci.insight.88782
- Fisher, G. H., Rosenberg, F. J., Straus, S. E., Dale, J. K., Middleton, L. A., Lin, A. Y., . . . Puck, J. M. (1995). Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*, 81(6), 935-946. doi:10.1016/0092-8674(95)90013-6
- Fishman, D., Kisand, K., Hertel, C., Rothe, M., Remm, A., Pihlap, M., . . . Peterson, P. (2017). Autoantibody Repertoire in APECED Patients Targets Two Distinct Subgroups of Proteins. *Front Immunol*, 8, 976. doi:10.3389/fimmu.2017.00976
- Fujii, S., Liu, K., Smith, C., Bonito, A. J., & Steinman, R. M. (2004). The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med*, 199(12), 1607-1618. doi:10.1084/jem.20040317
- Fuller, P. J., & Verity, K. (1989). Somatostatin gene expression in the thymus gland. *J Immunol*, 143(3), 1015-1017.

- Gallegos, A. M., & Bevan, M. J. (2006). Central tolerance: good but imperfect. *Immunol Rev*, 209, 290-296. doi:10.1111/j.0105-2896.2006.00348.x
- Gardner, J. M., Devoss, J. J., Friedman, R. S., Wong, D. J., Tan, Y. X., Zhou, X., . . . Anderson, M. S. (2008). Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science*, 321(5890), 843-847. doi:10.1126/science.1159407
- Gardner, J. M., Metzger, T. C., McMahon, E. J., Au-Yeung, B. B., Krawisz, A. K., Lu, W., . . . Anderson, M. S. (2013). Extrathymic Aire-expressing cells are a distinct bone marrow-derived population that induce functional inactivation of CD4(+) T cells. *Immunity*, 39(3), 560-572. doi:10.1016/j.immuni.2013.08.005
- Geenen, V., Legros, J. J., Franchimont, P., Baudrihay, M., Defresne, M. P., & Boniver, J. (1986). The neuroendocrine thymus: coexistence of oxytocin and neurophysin in the human thymus. *Science*, 232(4749), 508-511. doi:10.1126/science.3961493
- Gibson, T. J., Ramu, C., Gemund, C., & Aasland, R. (1998). The APECED polyglandular autoimmune syndrome protein, AIRE-1, contains the SAND domain and is probably a transcription factor. *Trends Biochem Sci*, 23(7), 242-244. doi:10.1016/s0968-0004(98)01231-6
- Godfrey, D. I., Kennedy, J., Suda, T., & Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol*, 150(10), 4244-4252.
- Godfrey, V. L., Wilkinson, J. E., & Russell, L. B. (1991). X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol*, 138(6), 1379-1387.

- Gourley, T. S., Wherry, E. J., Masopust, D., & Ahmed, R. (2004). Generation and maintenance of immunological memory. *Semin Immunol*, 16(5), 323-333. doi:10.1016/j.smim.2004.08.013
- Gray, D., Abramson, J., Benoist, C., & Mathis, D. (2007). Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J Exp Med*, 204(11), 2521-2528. doi:10.1084/jem.20070795
- Guerau-de-Arellano, M., Mathis, D., & Benoist, C. (2008). Transcriptional impact of Aire varies with cell type. *Proc Natl Acad Sci U S A*, 105(37), 14011-14016. doi:10.1073/pnas.0806616105
- Halonen, M., Pelto-Huikko, M., Eskelin, P., Peltonen, L., Ulmanen, I., & Kolmer, M. (2001). Subcellular location and expression pattern of autoimmune regulator (Aire), the mouse orthologue for human gene defective in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). *J Histochem Cytochem*, 49(2), 197-208. doi:10.1177/002215540104900207
- Hammerling, G. J., Schonrich, G., Momburg, F., Auphan, N., Malissen, M., Malissen, B., . . . Arnold, B. (1991). Non-deletional mechanisms of peripheral and central tolerance: studies with transgenic mice with tissue-specific expression of a foreign MHC class I antigen. *Immunol Rev*, 122, 47-67. doi:10.1111/j.1600-065x.1991.tb00596.x
- Hamvas, A., Cole, F. S., & Noguee, L. M. (2007). Genetic disorders of surfactant proteins. *Neonatology*, 91(4), 311-317. doi:10.1159/000101347
- Harding, F. A., McArthur, J. G., Gross, J. A., Raulet, D. H., & Allison, J. P. (1992). CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*, 356(6370), 607-609. doi:10.1038/356607a0

- Hassler, S., Ramsey, C., Karlsson, M. C., Larsson, D., Herrmann, B., Rozell, B., . . . Winqvist, O. (2006). Aire-deficient mice develop hematopoietic irregularities and marginal zone B-cell lymphoma. *Blood*, *108*(6), 1941-1948. doi:10.1182/blood-2006-04-019679
- Heino, M., Peterson, P., Sillanpaa, N., Guerin, S., Wu, L., Anderson, G., . . . Krohn, K. J. (2000). RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD mouse. *Eur J Immunol*, *30*(7), 1884-1893. doi:10.1002/1521-4141(200007)30:7<1884::AID-IMMU1884>3.0.CO;2-P
- Hildeman, D. A., Zhu, Y., Mitchell, T. C., Bouillet, P., Strasser, A., Kappler, J., & Marrack, P. (2002). Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity*, *16*(6), 759-767. doi:10.1016/s1074-7613(02)00322-9
- Hobeika, E., Thiemann, S., Storch, B., Jumaa, H., Nielsen, P. J., Pelanda, R., & Reth, M. (2006). Testing gene function early in the B cell lineage in mb1-cre mice. *Proc Natl Acad Sci U S A*, *103*(37), 13789-13794. doi:10.1073/pnas.0605944103
- Hoflich, C., Sabat, R., Rosseau, S., Temmesfeld, B., Slevogt, H., Docke, W. D., . . . Suttorp, N. (2004). Naturally occurring anti-IFN-gamma autoantibody and severe infections with *Mycobacterium chelonae* and *Burkholderia cenocepacia*. *Blood*, *103*(2), 673-675. doi:10.1182/blood-2003-04-1065
- Hogquist, K. A., Baldwin, T. A., & Jameson, S. C. (2005). Central tolerance: learning self-control in the thymus. *Nat Rev Immunol*, *5*(10), 772-782. doi:10.1038/nri1707
- Hori, S., Haury, M., Coutinho, A., & Demengeot, J. (2002). Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc Natl Acad Sci U S A*, *99*(12), 8213-8218. doi:10.1073/pnas.122224799

- Hozumi, K., Mailhos, C., Negishi, N., Hirano, K., Yahata, T., Ando, K., . . . Habu, S. (2008). Delta-like 4 is indispensable in thymic environment specific for T cell development. *J Exp Med*, 205(11), 2507-2513. doi:10.1084/jem.20080134
- Hubert, F. X., Kinkel, S. A., Crewther, P. E., Cannon, P. Z., Webster, K. E., Link, M., . . . Scott, H. S. (2009). Aire-deficient C57BL/6 mice mimicking the common human 13-base pair deletion mutation present with only a mild autoimmune phenotype. *J Immunol*, 182(6), 3902-3918. doi:10.4049/jimmunol.0802124
- Hung, I. F., Lung, K. C., Tso, E. Y., Liu, R., Chung, T. W., Chu, M. Y., . . . Yuen, K. Y. (2020). Triple combination of interferon beta-1b, lopinavir-ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. *Lancet*, 395(10238), 1695-1704. doi:10.1016/S0140-6736(20)31042-4
- Ilmarinen, T., Melen, K., Kangas, H., Julkunen, I., Ulmanen, I., & Eskelin, P. (2006). The monopartite nuclear localization signal of autoimmune regulator mediates its nuclear import and interaction with multiple importin alpha molecules. *FEBS J*, 273(2), 315-324. doi:10.1111/j.1742-4658.2005.05065.x
- Jameson, S. C., Hogquist, K. A., & Bevan, M. J. (1995). Positive selection of thymocytes. *Annu Rev Immunol*, 13, 93-126. doi:10.1146/annurev.iy.13.040195.000521
- Janeway, C. A., Jr., & Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol*, 20, 197-216. doi:10.1146/annurev.immunol.20.083001.084359
- Jiang, W., Anderson, M. S., Bronson, R., Mathis, D., & Benoist, C. (2005). Modifier loci condition autoimmunity provoked by Aire deficiency. *J Exp Med*, 202(6), 805-815. doi:10.1084/jem.20050693
- Kalekar, L. A., & Mueller, D. L. (2017). Relationship between CD4 Regulatory T Cells and Anergy In Vivo. *J Immunol*, 198(7), 2527-2533. doi:10.4049/jimmunol.1602031

- Kalekar, L. A., Schmiel, S. E., Nandiwada, S. L., Lam, W. Y., Barsness, L. O., Zhang, N., . . . Mueller, D. L. (2016). CD4(+) T cell anergy prevents autoimmunity and generates regulatory T cell precursors. *Nat Immunol*, 17(3), 304-314. doi:10.1038/ni.3331
- Kalil, A. C., Patterson, T. F., Mehta, A. K., Tomashek, K. M., Wolfe, C. R., Ghazaryan, V., . . . Members, A.-S. G. (2021). Baricitinib plus Remdesivir for Hospitalized Adults with Covid-19. *N Engl J Med*, 384(9), 795-807. doi:10.1056/NEJMoa2031994
- Kampmann, B., Hemingway, C., Stephens, A., Davidson, R., Goodsall, A., Anderson, S., . . . Levin, M. (2005). Acquired predisposition to mycobacterial disease due to autoantibodies to IFN-gamma. *J Clin Invest*, 115(9), 2480-2488. doi:10.1172/JCI19316
- Kamradt, T., & Mitchison, N. A. (2001). Tolerance and autoimmunity. *N Engl J Med*, 344(9), 655-664. doi:10.1056/NEJM200103013440907
- Kang, S., Tanaka, T., Narazaki, M., & Kishimoto, T. (2019). Targeting Interleukin-6 Signaling in Clinic. *Immunity*, 50(4), 1007-1023. doi:10.1016/j.immuni.2019.03.026
- Karner, J., Meager, A., Laan, M., Maslovskaia, J., Pihlap, M., Remm, A., . . . Kisand, K. (2013). Anti-cytokine autoantibodies suggest pathogenetic links with autoimmune regulator deficiency in humans and mice. *Clin Exp Immunol*, 171(3), 263-272. doi:10.1111/cei.12024
- Kawamoto, H., Ohmura, K., Fujimoto, S., Lu, M., Ikawa, T., & Katsura, Y. (2003). Extensive proliferation of T cell lineage-restricted progenitors in the thymus: an essential process for clonal expression of diverse T cell receptor beta chains. *Eur J Immunol*, 33(3), 606-615. doi:10.1002/eji.200323461

- Kelly, A. P., Finlay, D. K., Hinton, H. J., Clarke, R. G., Fiorini, E., Radtke, F., & Cantrell, D. A. (2007). Notch-induced T cell development requires phosphoinositide-dependent kinase 1. *EMBO J*, 26(14), 3441-3450. doi:10.1038/sj.emboj.7601761
- Khan, U., & Ghazanfar, H. (2018). T Lymphocytes and Autoimmunity. *Int Rev Cell Mol Biol*, 341, 125-168. doi:10.1016/bs.ircmb.2018.05.008
- Kirberg, J., Bosco, N., Deloulme, J. C., Ceredig, R., & Agenes, F. (2008). Peripheral T lymphocytes recirculating back into the thymus can mediate thymocyte positive selection. *J Immunol*, 181(2), 1207-1214. doi:10.4049/jimmunol.181.2.1207
- Kisand, K., Boe Wolff, A. S., Podkrajsek, K. T., Tserel, L., Link, M., Kisand, K. V., . . . Meager, A. (2010). Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. *J Exp Med*, 207(2), 299-308. doi:10.1084/jem.20091669
- Kisand, K., Link, M., Wolff, A. S., Meager, A., Tserel, L., Org, T., . . . Peterson, P. (2008). Interferon autoantibodies associated with AIRE deficiency decrease the expression of IFN-stimulated genes. *Blood*, 112(7), 2657-2666. doi:10.1182/blood-2008-03-144634
- Kitamura, T., Tanaka, N., Watanabe, J., Uchida, Kanegasaki, S., Yamada, Y., & Nakata, K. (1999). Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. *J Exp Med*, 190(6), 875-880. doi:10.1084/jem.190.6.875
- Ko, H. J., Kinkel, S. A., Hubert, F. X., Nasa, Z., Chan, J., Siatskas, C., . . . Alderuccio, F. (2010). Transplantation of autoimmune regulator-encoding bone marrow cells delays the onset of experimental autoimmune encephalomyelitis. *Eur J Immunol*, 40(12), 3499-3509. doi:10.1002/eji.201040679

- Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., . . . Radtke, F. (2008). Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J Exp Med*, 205(11), 2515-2523. doi:10.1084/jem.20080829
- Kogawa, K., Nagafuchi, S., Katsuta, H., Kudoh, J., Tamiya, S., Sakai, Y., . . . Harada, M. (2002). Expression of AIRE gene in peripheral monocyte/dendritic cell lineage. *Immunol Lett*, 80(3), 195-198. doi:10.1016/s0165-2478(01)00314-5
- Kreuwel, H. T., Aung, S., Silao, C., & Sherman, L. A. (2002). Memory CD8(+) T cells undergo peripheral tolerance. *Immunity*, 17(1), 73-81. doi:10.1016/s1074-7613(02)00337-0
- Ku, C. L., Lin, C. H., Chang, S. W., Chu, C. C., Chan, J. F., Kong, X. F., . . . Chi, C. Y. (2016). Anti-IFN-gamma autoantibodies are strongly associated with HLA-DR*15:02/16:02 and HLA-DQ*05:01/05:02 across Southeast Asia. *J Allergy Clin Immunol*, 137(3), 945-948 e948. doi:10.1016/j.jaci.2015.09.018
- Kuehn, H. S., Caminha, I., Niemela, J. E., Rao, V. K., Davis, J., Fleisher, T. A., & Oliveira, J. B. (2011). FAS haploinsufficiency is a common disease mechanism in the human autoimmune lymphoproliferative syndrome. *J Immunol*, 186(10), 6035-6043. doi:10.4049/jimmunol.1100021
- Kuehn, H. S., Ouyang, W., Lo, B., Deenick, E. K., Niemela, J. E., Avery, D. T., . . . Uzel, G. (2014). Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science*, 345(6204), 1623-1627. doi:10.1126/science.1255904
- Kumar, V., Abbas, A., Fausto, N., & Aster, J. (2010). *Diseases of the immune system* (Vol. 8).

- Kurd, N., & Robey, E. A. (2016). T-cell selection in the thymus: a spatial and temporal perspective. *Immunol Rev*, 271(1), 114-126. doi:10.1111/imr.12398
- Kuroda, N., Mitani, T., Takeda, N., Ishimaru, N., Arakaki, R., Hayashi, Y., . . . Matsumoto, M. (2005). Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. *J Immunol*, 174(4), 1862-1870. doi:10.4049/jimmunol.174.4.1862
- Kyburz, D., Aichele, P., Speiser, D. E., Hengartner, H., Zinkernagel, R. M., & Pircher, H. (1993). T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. *Eur J Immunol*, 23(8), 1956-1962. doi:10.1002/eji.1830230834
- Lauer, S. A., Grantz, K. H., Bi, Q., Jones, F. K., Zheng, Q., Meredith, H. R., . . . Lessler, J. (2020). The Incubation Period of Coronavirus Disease 2019 (COVID-19) From Publicly Reported Confirmed Cases: Estimation and Application. *Ann Intern Med*, 172(9), 577-582. doi:10.7326/M20-0504
- Lee, J. W., Epardaud, M., Sun, J., Becker, J. E., Cheng, A. C., Yonekura, A. R., . . . Turley, S. J. (2007). Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat Immunol*, 8(2), 181-190. doi:10.1038/ni1427
- Lei, Y., Ripen, A. M., Ishimaru, N., Ohigashi, I., Nagasawa, T., Jeker, L. T., . . . Takahama, Y. (2011). Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. *J Exp Med*, 208(2), 383-394. doi:10.1084/jem.20102327
- Li, J., Li, Y., Yao, J. Y., Jin, R., Zhu, M. Z., Qian, X. P., . . . Chen, W. F. (2007). Developmental pathway of CD4+CD8- medullary thymocytes during mouse ontogeny and its defect in Aire-/- mice. *Proc Natl Acad Sci U S A*, 104(46), 18175-18180. doi:10.1073/pnas.0708884104

- Li, Z., Woo, C. J., Iglesias-Ussel, M. D., Ronai, D., & Scharff, M. D. (2004). The generation of antibody diversity through somatic hypermutation and class switch recombination. *Genes Dev*, 18(1), 1-11. doi:10.1101/gad.1161904
- Liblau, R. S., Tisch, R., Shokat, K., Yang, X., Dumont, N., Goodnow, C. C., & McDevitt, H. O. (1996). Intravenous injection of soluble antigen induces thymic and peripheral T-cells apoptosis. *Proc Natl Acad Sci U S A*, 93(7), 3031-3036. doi:10.1073/pnas.93.7.3031
- Lin, C. H., Chi, C. Y., Shih, H. P., Ding, J. Y., Lo, C. C., Wang, S. Y., . . . Ku, C. L. (2016). Identification of a major epitope by anti-interferon-gamma autoantibodies in patients with mycobacterial disease. *Nat Med*, 22(9), 994-1001. doi:10.1038/nm.4158
- Lin, J., Yang, L., Silva, H. M., Trzeciak, A., Choi, Y., Schwab, S. R., . . . Lafaille, J. J. (2016). Increased generation of Foxp3(+) regulatory T cells by manipulating antigen presentation in the thymus. *Nat Commun*, 7, 10562. doi:10.1038/ncomms10562
- Liston, A., Gray, D. H., Lesage, S., Fletcher, A. L., Wilson, J., Webster, K. E., . . . Goodnow, C. C. (2004). Gene dosage--limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J Exp Med*, 200(8), 1015-1026. doi:10.1084/jem.20040581
- Liston, A., Lesage, S., Wilson, J., Peltonen, L., & Goodnow, C. C. (2003). Aire regulates negative selection of organ-specific T cells. *Nat Immunol*, 4(4), 350-354. doi:10.1038/ni906
- Malchow, S., Leventhal, D. S., Lee, V., Nishi, S., Socci, N. D., & Savage, P. A. (2016). Aire Enforces Immune Tolerance by Directing Autoreactive T Cells into the Regulatory T Cell Lineage. *Immunity*, 44(5), 1102-1113. doi:10.1016/j.immuni.2016.02.009

- Malchow, S., Leventhal, D. S., Nishi, S., Fischer, B. I., Shen, L., Paner, G. P., . . . Savage, P. A. (2013). Aire-dependent thymic development of tumor-associated regulatory T cells. *Science*, 339(6124), 1219-1224. doi:10.1126/science.1233913
- Martinez, R. J., Zhang, N., Thomas, S. R., Nandiwada, S. L., Jenkins, M. K., Binstadt, B. A., & Mueller, D. L. (2012). Arthritogenic self-reactive CD4⁺ T cells acquire an FR4hiCD73hi anergic state in the presence of Foxp3⁺ regulatory T cells. *J Immunol*, 188(1), 170-181. doi:10.4049/jimmunol.1101311
- Martinez-Moczygemba, M., Doan, M. L., Elidemir, O., Fan, L. L., Cheung, S. W., Lei, J. T., . . . Huston, D. P. (2008). Pulmonary alveolar proteinosis caused by deletion of the GM-CSFRalpha gene in the X chromosome pseudoautosomal region 1. *J Exp Med*, 205(12), 2711-2716. doi:10.1084/jem.20080759
- McCaughy, T. M., Baldwin, T. A., Wilken, M. S., & Hogquist, K. A. (2008). Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *J Exp Med*, 205(11), 2575-2584. doi:10.1084/jem.20080866
- McCaughy, T. M., Wilken, M. S., & Hogquist, K. A. (2007). Thymic emigration revisited. *J Exp Med*, 204(11), 2513-2520. doi:10.1084/jem.20070601
- McGargill, M. A., Derbinski, J. M., & Hogquist, K. A. (2000). Receptor editing in developing T cells. *Nat Immunol*, 1(4), 336-341. doi:10.1038/79790
- Meager, A., Vincent, A., Newsom-Davis, J., & Willcox, N. (1997). Spontaneous neutralising antibodies to interferon--alpha and interleukin-12 in thymoma-associated autoimmune disease. *Lancet*, 350(9091), 1596-1597. doi:10.1016/s0140-6736(05)64012-3
- Meager, A., Visvalingam, K., Peterson, P., Moll, K., Murumagi, A., Krohn, K., . . . Willcox, N. (2006). Anti-interferon autoantibodies in autoimmune polyendocrinopathy syndrome type 1. *PLoS Med*, 3(7), e289. doi:10.1371/journal.pmed.0030289

- Meager, A., Wadhwa, M., Dilger, P., Bird, C., Thorpe, R., Newsom-Davis, J., & Willcox, N. (2003). Anti-cytokine autoantibodies in autoimmunity: preponderance of neutralizing autoantibodies against interferon-alpha, interferon-omega and interleukin-12 in patients with thymoma and/or myasthenia gravis. *Clin Exp Immunol*, 132(1), 128-136. doi:10.1046/j.1365-2249.2003.02113.x
- Meyer, S., Woodward, M., Hertel, C., Vlaicu, P., Haque, Y., Karner, J., . . . Hayday, A. (2016). AIRE-Deficient Patients Harbor Unique High-Affinity Disease-Ameliorating Autoantibodies. *Cell*, 166(3), 582-595. doi:10.1016/j.cell.2016.06.024
- Michie, S. A., Kirkpatrick, E. A., & Rouse, R. V. (1988). Rare peripheral T cells migrate to and persist in normal mouse thymus. *J Exp Med*, 168(5), 1929-1934. doi:10.1084/jem.168.5.1929
- Milner, J. D., Fath, A., & Etzioni, A. (2008). Autoimmunity in severe combined immunodeficiency (SCID): lessons from patients and experimental models. *J Clin Immunol*, 28 Suppl 1, S29-33. doi:10.1007/s10875-007-9159-y
- Mouchess, M. L., & Anderson, M. (2014). Central tolerance induction. *Curr Top Microbiol Immunol*, 373, 69-86. doi:10.1007/82_2013_321
- Mueller, D. L., Jenkins, M. K., & Schwartz, R. H. (1989). An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J Immunol*, 142(8), 2617-2628.
- Murphy, K., & Weaver, C. (2017). *Janeway's Immunobiology, 9th Ed.* (9th ed.). New York, NY: Garland Science, Taylor & Francis Group LLC.

- Nagamine, K., Peterson, P., Scott, H. S., Kudoh, J., Minoshima, S., Heino, M., . . . Shimizu, N. (1997). Positional cloning of the APECED gene. *Nat Genet*, 17(4), 393-398.
doi:10.1038/ng1297-393
- Nitta, T., Nitta, S., Lei, Y., Lipp, M., & Takahama, Y. (2009). CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. *Proc Natl Acad Sci U S A*, 106(40), 17129-17133.
doi:10.1073/pnas.0906956106
- Palmer, E. (2003). Negative selection--clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol*, 3(5), 383-391. doi:10.1038/nri1085
- Papavasiliou, F. N., & Schatz, D. G. (2002). Somatic hypermutation of immunoglobulin genes: merging mechanisms for genetic diversity. *Cell*, 109 Suppl, S35-44.
doi:10.1016/s0092-8674(02)00706-7
- Pape, K. A., Merica, R., Mondino, A., Khoruts, A., & Jenkins, M. K. (1998). Direct evidence that functionally impaired CD4+ T cells persist in vivo following induction of peripheral tolerance. *J Immunol*, 160(10), 4719-4729.
- Park, A., & Iwasaki, A. (2020). Type I and Type III Interferons - Induction, Signaling, Evasion, and Application to Combat COVID-19. *Cell Host Microbe*, 27(6), 870-878. doi:10.1016/j.chom.2020.05.008
- Perera, J., Meng, L., Meng, F., & Huang, H. (2013). Autoreactive thymic B cells are efficient antigen-presenting cells of cognate self-antigens for T cell negative selection. *Proc Natl Acad Sci U S A*, 110(42), 17011-17016.
doi:10.1073/pnas.1313001110
- Perheentupa, J. (2006). Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J Clin Endocrinol Metab*, 91(8), 2843-2850. doi:10.1210/jc.2005-2611

- Peterson, P., Nagamine, K., Scott, H., Heino, M., Kudoh, J., Shimizu, N., . . . Krohn, K. J. (1998). APECED: a monogenic autoimmune disease providing new clues to self-tolerance. *Immunol Today*, 19(9), 384-386. doi:10.1016/s0167-5699(98)01293-6
- Pinkoski, M. J., Droin, N. M., Lin, T., Genestier, L., Ferguson, T. A., & Green, D. R. (2002). Nonlymphoid Fas ligand in peptide-induced peripheral lymphocyte deletion. *Proc Natl Acad Sci U S A*, 99(25), 16174-16179. doi:10.1073/pnas.262660999
- Poliani, P. L., Facchetti, F., Ravanini, M., Gennery, A. R., Villa, A., Roifman, C. M., & Notarangelo, L. D. (2009). Early defects in human T-cell development severely affect distribution and maturation of thymic stromal cells: possible implications for the pathophysiology of Omenn syndrome. *Blood*, 114(1), 105-108. doi:10.1182/blood-2009-03-211029
- Poliani, P. L., Kisand, K., Marrella, V., Ravanini, M., Notarangelo, L. D., Villa, A., . . . Facchetti, F. (2010). Human peripheral lymphoid tissues contain autoimmune regulator-expressing dendritic cells. *Am J Pathol*, 176(3), 1104-1112. doi:10.2353/ajpath.2010.090956
- Porritt, H. E., Rumfelt, L. L., Tabrizifard, S., Schmitt, T. M., Zuniga-Pflucker, J. C., & 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(6), 735-745. doi:10.1016/j.immuni.2004.05.004
- Pozzetto, B., Mogensen, K. E., Tovey, M. G., & Gresser, I. (1984). Characteristics of autoantibodies to human interferon in a patient with varicella-zoster disease. *J Infect Dis*, 150(5), 707-713. doi:10.1093/infdis/150.5.707
- Puel, A., Doffinger, R., Natividad, A., Chrabieh, M., Barcenas-Morales, G., Picard, C., . . . Casanova, J. L. (2010). Autoantibodies against IL-17A, IL-17F, and IL-22 in

- patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J Exp Med*, 207(2), 291-297.
doi:10.1084/jem.20091983
- Puel, A., Picard, C., Lorrot, M., Pons, C., Chrabieh, M., Lorenzo, L., . . . Casanova, J. L. (2008). Recurrent staphylococcal cellulitis and subcutaneous abscesses in a child with autoantibodies against IL-6. *J Immunol*, 180(1), 647-654.
doi:10.4049/jimmunol.180.1.647
- Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karnell, F. G., Bakkour, S., . . . Pear, W. S. (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity*, 11(3), 299-308. doi:10.1016/s1074-7613(00)80105-3
- Purohit, S., Kumar, P. G., Laloraya, M., & She, J. X. (2005). Mapping DNA-binding domains of the autoimmune regulator protein. *Biochem Biophys Res Commun*, 327(3), 939-944. doi:10.1016/j.bbrc.2004.12.093
- Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R., & Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity*, 10(5), 547-558. doi:10.1016/s1074-7613(00)80054-0
- Ramsey, C., Winqvist, O., Puhakka, L., Halonen, M., Moro, A., Kampe, O., . . . Peltonen, L. (2002). Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum Mol Genet*, 11(4), 397-409.
doi:10.1093/hmg/11.4.397
- Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I. A., Debatin, K. M., Fischer, A., & de Villartay, J. P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science*, 268(5215), 1347-1349. doi:10.1126/science.7539157

- Rinderle, C., Christensen, H. M., Schweiger, S., Lehrach, H., & Yaspo, M. L. (1999). AIRE encodes a nuclear protein co-localizing with cytoskeletal filaments: altered sub-cellular distribution of mutants lacking the PHD zinc fingers. *Hum Mol Genet*, 8(2), 277-290. doi:10.1093/hmg/8.2.277
- Rock, K. L., Reits, E., & Neefjes, J. (2016). Present Yourself! By MHC Class I and MHC Class II Molecules. *Trends Immunol*, 37(11), 724-737. doi:10.1016/j.it.2016.08.010
- Roederer, M. (2011). Interpretation of cellular proliferation data: avoid the panglossian. *Cytometry A*, 79(2), 95-101. doi:10.1002/cyto.a.21010
- Rosen, L. B., Freeman, A. F., Yang, L. M., Jutivorakool, K., Olivier, K. N., Angkasekwinai, N., . . . Browne, S. K. (2013). Anti-GM-CSF autoantibodies in patients with cryptococcal meningitis. *J Immunol*, 190(8), 3959-3966. doi:10.4049/jimmunol.1202526
- Rosen, L. B., Rocha Pereira, N., Figueiredo, C., Fiske, L. C., Ressler, R. A., Hong, J. C., . . . Browne, S. K. (2015). Nocardia-induced granulocyte macrophage colony-stimulating factor is neutralized by autoantibodies in disseminated/extrapulmonary nocardiosis. *Clin Infect Dis*, 60(7), 1017-1025. doi:10.1093/cid/ciu968
- Rosen, S. H., Castleman, B., & Liebow, A. A. (1958). Pulmonary alveolar proteinosis. *N Engl J Med*, 258(23), 1123-1142. doi:10.1056/NEJM195806052582301
- Rothenberg, E. V., Moore, J. E., & Yui, M. A. (2008). Launching the T-cell-lineage developmental programme. *Nat Rev Immunol*, 8(1), 9-21. doi:10.1038/nri2232
- Sakagami, T., Uchida, K., Suzuki, T., Carey, B. C., Wood, R. E., Wert, S. E., . . . Luisetti, M. (2009). Human GM-CSF autoantibodies and reproduction of pulmonary alveolar proteinosis. *N Engl J Med*, 361(27), 2679-2681. doi:10.1056/NEJMc0904077

- Sansom, S. N., Shikama-Dorn, N., Zhanybekova, S., Nusspaumer, G., Macaulay, I. C., Deadman, M. E., . . . Hollander, G. A. (2014). Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia. *Genome Res*, 24(12), 1918-1931. doi:10.1101/gr.171645.113
- Schalkwyk, L. C., Fernandes, C., Nash, M. W., Kurrikoff, K., Vasar, E., & Koks, S. (2007). Interpretation of knockout experiments: the congenic footprint. *Genes Brain Behav*, 6(3), 299-303. doi:10.1111/j.1601-183X.2007.00304.x
- Schnell, U., Cirulli, V., & Giepmans, B. N. (2013). EpCAM: structure and function in health and disease. *Biochim Biophys Acta*, 1828(8), 1989-2001. doi:10.1016/j.bbamem.2013.04.018
- Schoggins, J. W., & Rice, C. M. (2011). Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol*, 1(6), 519-525. doi:10.1016/j.coviro.2011.10.008
- Schreiber, G. (2020). The Role of Type I Interferons in the Pathogenesis and Treatment of COVID-19. *Front Immunol*, 11, 595739. doi:10.3389/fimmu.2020.595739
- Scott, H. S., Heino, M., Peterson, P., Mittaz, L., Lalioti, M. D., Betterle, C., . . . Antonarakis, S. E. (1998). Common mutations in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients of different origins. *Mol Endocrinol*, 12(8), 1112-1119. doi:10.1210/mend.12.8.0143
- Shevach, E. M. (2009). Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity*, 30(5), 636-645. doi:10.1016/j.immuni.2009.04.010
- Shimonkevitz, R. P., & Bevan, M. J. (1988). Split tolerance induced by the intrathymic adoptive transfer of thymocyte stem cells. *J Exp Med*, 168(1), 143-156. doi:10.1084/jem.168.1.143

- Shiono, H., Wong, Y. L., Matthews, I., Liu, J. L., Zhang, W., Sims, G., . . . Willcox, N. (2003). Spontaneous production of anti-IFN-alpha and anti-IL-12 autoantibodies by thymoma cells from myasthenia gravis patients suggests autoimmunization in the tumor. *Int Immunol*, 15(8), 903-913. doi:10.1093/intimm/dxg088
- Shortman, K., & Wu, L. (1996). Early T lymphocyte progenitors. *Annu Rev Immunol*, 14, 29-47. doi:10.1146/annurev.immunol.14.1.29
- Sprent, J., Gao, E. K., & Webb, S. R. (1990). T cell reactivity to MHC molecules: immunity versus tolerance. *Science*, 248(4961), 1357-1363. doi:10.1126/science.1694041
- Stanley, E., Lieschke, G. J., Grail, D., Metcalf, D., Hodgson, G., Gall, J. A., . . . Dunn, A. R. (1994). Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A*, 91(12), 5592-5596. doi:10.1073/pnas.91.12.5592
- Strobel, P., Murumagi, A., Klein, R., Luster, M., Lahti, M., Krohn, K., . . . Marx, A. (2007). Deficiency of the autoimmune regulator AIRE in thymomas is insufficient to elicit autoimmune polyendocrinopathy syndrome type 1 (APS-1). *J Pathol*, 211(5), 563-571. doi:10.1002/path.2141
- Suda, T., Takahashi, T., Golstein, P., & Nagata, S. (1993). Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell*, 75(6), 1169-1178. doi:10.1016/0092-8674(93)90326-l
- Suscovich, T. J., Perdue, N. R., & Campbell, D. J. (2012). Type-1 immunity drives early lethality in scurfy mice. *Eur J Immunol*, 42(9), 2305-2310. doi:10.1002/eji.201242391

- Suzuki, E., Kobayashi, Y., Kawano, O., Endo, K., Haneda, H., Yukiue, H., . . . Fujii, Y. (2008). Expression of AIRE in thymocytes and peripheral lymphocytes. *Autoimmunity*, 41(2), 133-139. doi:10.1080/08916930701773941
- Suzuki, T., Sakagami, T., Rubin, B. K., Noguee, L. M., Wood, R. E., Zimmerman, S. L., . . . Trapnell, B. C. (2008). Familial pulmonary alveolar proteinosis caused by mutations in CSF2RA. *J Exp Med*, 205(12), 2703-2710. doi:10.1084/jem.20080990
- Sytwu, H. K., Liblau, R. S., & McDevitt, H. O. (1996). The roles of Fas/APO-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice. *Immunity*, 5(1), 17-30. doi:10.1016/s1074-7613(00)80306-4
- Szondy, Z., Garabuczi, E., Toth, K., Kiss, B., & Koroskenyi, K. (2012). Thymocyte death by neglect: contribution of engulfing macrophages. *Eur J Immunol*, 42(7), 1662-1667. doi:10.1002/eji.201142338
- Takaba, H., Morishita, Y., Tomofuji, Y., Danks, L., Nitta, T., Komatsu, N., . . . Takayanagi, H. (2015). Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance. *Cell*, 163(4), 975-987. doi:10.1016/j.cell.2015.10.013
- Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., . . . Sakaguchi, S. (1998). Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol*, 10(12), 1969-1980. doi:10.1093/intimm/10.12.1969
- Teijaro, J. R. (2016). Type I interferons in viral control and immune regulation. *Curr Opin Virol*, 16, 31-40. doi:10.1016/j.coviro.2016.01.001
- Theofilopoulos, A. N., Kono, D. H., & Baccala, R. (2017). The multiple pathways to autoimmunity. *Nat Immunol*, 18(7), 716-724. doi:10.1038/ni.3731

- Thornton, A. M., & Shevach, E. M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med*, 188(2), 287-296. doi:10.1084/jem.188.2.287
- Torgerson, T. R., & Ochs, H. D. (2007). Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: forkhead box protein 3 mutations and lack of regulatory T cells. *J Allergy Clin Immunol*, 120(4), 744-750; quiz 751-742. doi:10.1016/j.jaci.2007.08.044
- Trapnell, B. C., Carey, B. C., Uchida, K., & Suzuki, T. (2009). Pulmonary alveolar proteinosis, a primary immunodeficiency of impaired GM-CSF stimulation of macrophages. *Curr Opin Immunol*, 21(5), 514-521. doi:10.1016/j.coi.2009.09.004
- Ueno, T., Saito, F., Gray, D. H., Kuse, S., Hieshima, K., Nakano, H., . . . Takahama, Y. (2004). CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J Exp Med*, 200(4), 493-505. doi:10.1084/jem.20040643
- Walter, J. E., Rosen, L. B., Csomos, K., Rosenberg, J. M., Mathew, D., Keszei, M., . . . Notarangelo, L. D. (2015). Broad-spectrum antibodies against self-antigens and cytokines in RAG deficiency. *J Clin Invest*, 125(11), 4135-4148. doi:10.1172/JCI80477
- Wang, F., Huang, C. Y., & Kanagawa, O. (1998). Rapid deletion of rearranged T cell antigen receptor (TCR) Valpha-Jalpha segment by secondary rearrangement in the thymus: role of continuous rearrangement of TCR alpha chain gene and positive selection in the T cell repertoire formation. *Proc Natl Acad Sci U S A*, 95(20), 11834-11839. doi:10.1073/pnas.95.20.11834

- Watanabe, M., Uchida, K., Nakagaki, K., Kanazawa, H., Trapnell, B. C., Hoshino, Y., . . . Nakata, K. (2007). Anti-cytokine autoantibodies are ubiquitous in healthy individuals. *FEBS Lett*, 581(10), 2017-2021. doi:10.1016/j.febslet.2007.04.029
- Wekerle, H., Ketelsen, U. P., & Ernst, M. (1980). Thymic nurse cells. Lymphoepithelial cell complexes in murine thymuses: morphological and serological characterization. *J Exp Med*, 151(4), 925-944. doi:10.1084/jem.151.4.925
- Wolff, A. S., Karner, J., Owe, J. F., Oftedal, B. E., Gilhus, N. E., Erichsen, M. M., . . . Husebye, E. S. (2014). Clinical and serologic parallels to APS-I in patients with thymomas and autoantigen transcripts in their tumors. *J Immunol*, 193(8), 3880-3890. doi:10.4049/jimmunol.1401068
- Wu, L., Antica, M., Johnson, G. R., Scollay, R., & Shortman, K. (1991). Developmental potential of the earliest precursor cells from the adult mouse thymus. *J Exp Med*, 174(6), 1617-1627. doi:10.1084/jem.174.6.1617
- Xing, Y., & Hogquist, K. A. (2012). T-cell tolerance: central and peripheral. *Cold Spring Harb Perspect Biol*, 4(6). doi:10.1101/cshperspect.a006957
- Xiong, J., Armato, M. A., & Yankee, T. M. (2011). Immature single-positive CD8⁺ thymocytes represent the transition from Notch-dependent to Notch-independent T-cell development. *Int Immunol*, 23(1), 55-64. doi:10.1093/intimm/dxq457
- Xu, X., & Ge, Q. (2014). Maturation and migration of murine CD4 single positive thymocytes and thymic emigrants. *Comput Struct Biotechnol J*, 9, e201403003. doi:10.5936/csbj.201403003
- Yamano, T., Dobes, J., Voboril, M., Steinert, M., Brabec, T., Zietara, N., . . . Filipp, D. (2019). Aire-expressing ILC3-like cells in the lymph node display potent APC features. *J Exp Med*, 216(5), 1027-1037. doi:10.1084/jem.20181430

- Yamano, T., Nedjic, J., Hinterberger, M., Steinert, M., Koser, S., Pinto, S., . . . Klein, L. (2015). Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction. *Immunity*, 42(6), 1048-1061. doi:10.1016/j.immuni.2015.05.013
- Yano, M., Kuroda, N., Han, H., Meguro-Horike, M., Nishikawa, Y., Kiyonari, H., . . . Matsumoto, M. (2008). Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. *J Exp Med*, 205(12), 2827-2838. doi:10.1084/jem.20080046
- Yoshida, M., Ikegami, M., Reed, J. A., Chroneos, Z. C., & Whitsett, J. A. (2001). GM-CSF regulates protein and lipid catabolism by alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol*, 280(3), L379-386. doi:10.1152/ajplung.2001.280.3.L379
- Yoshikawa, H., Sato, K., Edahiro, S., Furukawa, Y., Maruta, T., Iwasa, K., . . . Yamada, M. (2006). Elevation of IL-12 p40 and its antibody in myasthenia gravis with thymoma. *J Neuroimmunol*, 175(1-2), 169-175. doi:10.1016/j.jneuroim.2006.02.012
- Zhang, Q., Bastard, P., Bolze, A., Jouanguy, E., Zhang, S. Y., Effort, C. H. G., . . . Casanova, J. L. (2020). Life-Threatening COVID-19: Defective Interferons Unleash Excessive Inflammation. *Med (N Y)*, 1(1), 14-20. doi:10.1016/j.medj.2020.12.001
- Zhang, W., Liu, J. L., Meager, A., Newsom-Davis, J., & Willcox, N. (2003). Autoantibodies to IL-12 in myasthenia gravis patients with thymoma; effects on the IFN-gamma responses of healthy CD4+ T cells. *J Neuroimmunol*, 139(1-2), 102-108. doi:10.1016/s0165-5728(03)00136-x
- Zuklys, S., Gill, J., Keller, M. P., Hauri-Hohl, M., Zhanybekova, S., Balciunaite, G., . . . Hollander, G. A. (2009). Stabilized beta-catenin in thymic epithelial cells blocks

thymus development and function. *J Immunol*, 182(5), 2997-3007.

doi:10.4049/jimmunol.0713723