T CELLS IN THE PATHOGENESIS OF SPONTANEOUS AUTOIMMUNE PERIPHERAL POLYNEUROPATHY

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ABSTRACT

Collin-Jamal Smith: T cells and their cytokines are critical for pathogenesis in spontaneous autoimmune peripheral polyneuropathy.
(Under the direction of Maureen A. Su)

The immune system defends the body from pathogens, and its function is essential for life. Adaptive immunity protects the host through specific targeting and elimination of pathogens and toxins. However, due to the stochastic nature of adaptive immune cell antigen specificity, cells that attack the host are inadvertently generated. Autoimmunity, or immune attack against the host, causes severe morbidity and mortality in the population. Furthermore, treatments of autoimmune diseases often have limited efficacy and serious side-effects. To develop better treatments for autoimmunity, the underlying pathogenesis must be understood. For my thesis, I studied a mouse model of chronic inflammatory demyelinating polyneuropathy (CIDP) to understand the mechanisms behind disease. CIDP is a debilitating condition caused by autoimmune demyelination of peripheral nerves. In my studies, I researched the role of T lymphocytes, the immunosuppressive cytokine interleukin 10 (IL-10), and other immune cells and markers in *NOD.Aire*$^{GW/+}$ mice that develop spontaneous autoimmune peripheral polyneuropathy (SAPP) that resembles CIDP. I demonstrated that T cells are required for SAPP, and that IL-10 paradoxically exacerbates SAPP.

I delineated a novel mechanism in which IL-10-induced STAT3 increases *S1pr1* expression and CD4+ T cell migration to accelerate T cell-mediated destruction of peripheral nerves. My results suggest the increased IL-10 expression observed in CIDP patients may be a marker of disease activity and progression rather than immunosuppression.

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LIST OF ABBREVIATIONS

Ab	Antibody
AIRE	autoimmune regulator
ANOVA	analysis of variance
APC	antigen-presenting cell
APS-1	autoimmune polyglandular syndrome type 1
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
B6	C57BL6/J (mouse background)
CCL21	C-C motif chemokine ligand 21
CCR7	C-C chemokine receptor 7
CD	Cluster of Differentiation
CNS	central nervous system
cTEC	cortical thymic epithelial cell
CXCL10	C-X-C motif chemokine ligand 10
DAMP	damage-associated molecular pattern
EAE	experimental autoimmune encephalitis

EAN	experimental autoimmune neuritis
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
eTAC	extrathymic Aire-expressing cell
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FoxP3	forkhead box p3
GATA-3	GATA binding protein 3
GPCR	G-protein-coupled receptor
GW	G228W knockin mutation in Aire
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-10	interleukin 10
IFN-γ	interferon gamma
i.p.	intraperitoneal
ITAM	immunoreceptor tyrosine-based activation motif

iTreg	inducible regulatory T cell
i.v.	intravenous
JAK1	janus kinase 1
LN	lymph node
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MS	multiple sclerosis
mTEC	medullary thymic epithelial cell
NOD	nonobese diabetic (mouse background)
nTreg	natural regulatory T cell
P0T	P0 (myelin protein zero) T cell receptor transgenic mouse
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PRR	Pattern Recognition Receptor
PNS	peripheral nervous system
qPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
ROR-γt	retinoic acid receptor-related orphan receptor gamma t

S1p	sphingosine-1-phosphate
S1pr	sphingosine-1-phosphate receptor
SAPP	spontaneous autoimmune peripheral polyneuropathy
STAT3	signal transducer and activator of transcription 3
TdT	terminal deoxyribonucleotidyl transferase
TCR	T cell receptor
Teff	effector T cell
Tfh	T follicular helper cell
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
Th	T helper cell
TLR	toll-like receptor
Treg	regulatory T cell
Tyk2	tyrosine kinase 2
WT	wild-type

CHAPTER 1: INTRODUCTION

1.1 Immune system structure

The immune system defends the body from deleterious organisms, and its function is essential for life. Components of the immune system target and destroy potentially harmful entities by discriminating between "self", as in the host, and "non-self", as in of foreign origin. Non-self includes microbes, allografts, and xenografts. The immune system further protects the body by identifying and eliminating abnormal self, which occurs in cancer and viral infection. In order to manage the broad array of noxious insults the body may sustain, the immune system has evolved diverse and complex mechanisms. The first division of the immune system is into innate and adaptive immunity. Innate immunity is the first line of defense against an insult, and includes epithelial barriers, neutrophils, monocytes, macrophages, dendritic cells (DCs), mast cells, natural killer cells, innate lymphoid cells, and complement (1). Epithelial barriers cover the body's surfaces in contact with the external environment, and they provide physical and chemical protection from invading microbes. Examples include the skin, lung epithelium, and intestinal epithelium. When microbes live on or penetrate epithelia, cells of the innate immune system mount an immediate immune response by detecting the microbes non-specifically with pattern recognition receptors (PRRs) (1). PRRs detect conserved molecules broadly expressed by pathogens (pathogen-associated molecular patterns or PAMPs) or molecules that indicate cell damage (danger-associated molecular patterns or DAMPs). For example, Toll-like receptor 4 (TLR4) is a PRR that detects the PAMP lipopolysaccharide (LPS). LPS is a common component of bacterial cell walls, but is not made by mammalian cells. Thus, if an innate immune cell

detects LPS through TLR4, potentially pathogenic bacteria may be nearby and require eradication. Innate immune cells also remove damaged cells and induce tissue repair. Innate immunity may resolve infections, but some require a more targeted immune response. A key function of innate immunity is the recruitment and activation of adaptive immunity. Adaptive immunity is the second line of defense against pathogens, and can be separated into the humoral (B cell) and cellular (T cell) responses (1). In a primary infection, the adaptive immune system requires approximately a week to activate. Although the adaptive response is much slower than the innate response, adaptive immunity is highly specific. B cells and T cells express B cell receptors (BCR) and T cell receptors (TCR), respectively. BCRs bind to macromolecules, such as lipids, and can be membrane-bound or secreted. TCRs bind to specific peptide antigens and are membrane-bound. BCRs and TCRs allow for recognition of a specific pathogen by binding to antigens. After binding to cognate antigens, B cells and T cells undergo clonal expansion and differentiation into effector cells and memory cells. Effector cells mediate the adaptive immune response, and memory cells remain in the host to protect from future responses against the same pathogen.

B cell and T cell precursors develop from common lymphoid progenitor cells in the bone marrow (1). B cells also mature in the bone marrow; T cells, however, migrate to and mature in the thymus. Several broad lineages of T cells emerge from the thymus: αβ T cells bearing TCRs with α and β chains (1), natural killer T cells (NKT) bearing TCRs with an invariant α and limited β chain (2), αβ T cells that become intraepithelial lymphocytes (IELs) (3), and γδ T cells bearing TCRs with γ and δ chains (1). This thesis focuses on αβ T cells and their role in peripheral nervous system (PNS) autoimmunity.

1.2 T cell activation and differentiation

Although TCRs bind peptide antigens, they cannot bind to peptides alone. Rather, they bind to peptides bound to major histocompatibility complex (MHC) class I or II on antigen-presenting cells (APCs). Professional APCs include macrophages, dendritic cells, and B cells; other cell types may present antigen in certain contexts. TCRs also require the aid of the co-receptors CD4 and CD8 to stabilize their interaction with MHC-peptide complexes and initiate intracellular signaling. CD4 binds to MHC class II, and CD8 binds to MHC class I; thus, T cells expressing CD4 may only interact with cells expressing MHC class II (MHC class II-restricted), and CD8+ T cells interact with MHC class I (MHC class I-restricted). T cells that have not yet encountered their cognate antigen are called a naïve T cells. Naïve T cells are identified by high expression of CD62L (L-selectin), which promotes their trafficking to secondary lymphoid organs, and low expression of CD44, which is a cell adhesion molecule that promotes homing to and retention in peripheral tissues (1).

T cell activation requires additional signals beyond binding MHC-peptide complexes. T cells must also receive costimulatory signals. APCs express CD80 and CD86 after stimulation from microbes or other stimuli (4). CD80 and CD86 bind to CD28 on T cells, which promotes T cell activation (4). TCR stimulation without CD80 or CD86 co-stimulation or co-stimulation without TCR stimulation do not lead to T cell activation. Since APCs presenting antigens without costimulatory molecules are likely to be presenting self-antigens, it is important to preclude T cells responses against these antigens. To mitigate reaction to self-peptides, repeated TCR stimulation without co-stimulation causes the T cell to undergo programmed cell death (apoptosis) or enter a state of anergy. Anergic T cells cannot produce IL-2 and do not proliferate

even after TCR stimulation and co-stimulation (1). These mechanisms are part of peripheral tolerance, which will be discussed later.

In addition to TCR and CD28 stimulation, T cells respond to cytokines secreted by APCs and other cells in their environment. CD4+ T cells in particular may differentiate into distinct subsets (5). IL-12 from an APC promotes the generation of T helper type 1 (Th1) cells; Th1s are characterized by expression of the transcription factor T-bet and cytokine IFN-γ, and they fight intracellular pathogens. IL-4 promotes Th2 generation; Th2s are characterized by the transcription factor GATA-3 and cytokines IL4, IL-5, and IL-13, and they fight parasites. TGF-β plus IL-6 promote the generation of Th17s, which are characterized by the transcription factor ROR-γt and cytokines IL-17A, IL-17F, and IL-22. Th17s fight extracellular bacteria and fungi. Follicular helper T cell (Tfh) differentiation is promoted by IL-6 and IL-21. Tfhs express the transcription factor Bcl-6, secrete IL-6 and IL-21, and promote B cell development in germinal centers (5). Th9 T cells express PU.1, secrete IL-9 and IL-10, and fight parasites (6, 7). TGF-β promotes immunosuppressive induced Tregs (iTregs) that express the transcription factor FoxP3 and secrete TGF-β. Specific APC subsets and IL-10 promote the generation of immunosuppressive Tr1s (8); they do not express FoxP3 and secrete high levels of IL-10. Importantly, T helper subsets may express other cytokines; for example, Th1s may secrete tumour necrosis factor alpha (TNF-α) and IL-10 (9).

After TCR stimulation, the intracellular tails of the co-receptor CD3 trigger a complex signaling cascade that leads to activation of the T cell through their immunoreceptor tyrosine-based activation motifs (ITAM) (1). CD3 is expressed on all CD4+ and CD8+ T cells, and is composed of γ, δ, two ε chains, and two ζ chains. After binding cognate antigen, T cells become "activated" or "antigen-experienced" effector cells. Different markers are sequentially expressed

on the T cell's surface. Early activation markers appear within hours of T cell activation. These include CD69, which sequesters sphingosine-1-receptor 1 (S1pr1, discussed later). This promotes retention of the T cell in its current environment and gives it time to differentiate and proliferate. Another early activation marker is CD25, which is the α subunit of the IL-2 receptor. IL-2 is an important growth factor for T cells, and CD25 has a very high affinity for IL-2. CD40L on T cells binds CD40 on APCs and induces expression of co-stimulatory molecules CD80 and CD86. After proliferation and differentiation, activated effector T cells are identified by high expression of CD44, low expression of CD62L.

1.3 T cell development

As previously mentioned, T cells mature in the thymus. Immature T cells begin maturation in the outer cortex of the thymus. They are called double-negative T cells, which refers to their lack of CD4 and CD8 expression. The first stage of maturation in the αβ T cell lineage is recombination of one TCR β gene locus. If recombination is successful, a new TCR β chain is expressed on the T cell's surface along with a pre-Tα chain. The cell is now a pre-T cell with a pre-TCR complex. If the first TCR β recombination fails, recombination begins on the other locus. If both fail, the pro-T cell dies. The pre-TCR complex sends intracellular signals that promote recombination of the TCR α gene and prevent further recombination of the other TCR β locus. This prevention is termed allelic exclusion. Successful rearrangement of the TCR α chain leads to expression of a mature αβ TCR and concomitant expression of both CD4 and CD8 co-receptors. The T cell is now a double-positive T cell. If recombination of the TCR α chain fails, the T cell dies. TCR recombination is discussed in greater detail in the following section.

1.4 T cell receptor recombination and diversity

For a T cell to mount an immune response against a pathogen, the T cell must exist prior to encountering an antigen of the pathogen and express a TCR specific for that antigen. Thus, the host must generate a T cell expressing a TCR that binds to a previously unknown antigen. Furthermore, the innumerable diversity of pathogens in nature necessitates a similarly expansive repertoire of antigen specificities. The immune system has evolved an elegant solution to the conundrum of generating a vast library of αβ TCRs that recognize previously unencountered antigens. First, the α and β chain loci in the germline initially contain multiple genes (1). The α chain contains approximately 45 variable (V) gene segments, 50 joining (J) gene segments, and one constant (C) gene segment. The β chain contains approximately 48 V gene segments, two diversity (D) gene segments, two J gene segments, and two C gene segments. When constructing an α and β chain, one gene segment of each type is randomly selected and recombined. Thus, each unique combination of genes creates unique chains that are then ultimately assembled into a mature TCR heterodimer (combinatorial diversity). TCR recombination is performed by the lymphoid-specific enzyme VDJ recombinase and additional enzymes involved in DNA double-strand break repair. The VDJ recombinase contains the recombination activating genes 1 and 2 (RAG-1, RAG-2). RAG-1 and RAG-2 recognize sequences flanking V, D, and J segments, brings the segments close together, and allows for cleavage of intervening DNA. The double-strand breaks are then repaired by ligases, and a final recombined sequence is formed. In additional to combinatorial diversity, which is limited by the number of gene segments in the germline, the DNA sequence is further altered at the junctions between segments (junctional diversity). Nucleotides at junctions may be randomly removed by exonucleases or added by the lymphoid-specific enzyme terminal deoxyribonucleotidyl transferase (TdT). Finally, during

recombination, overhanging DNA sequences may be created and filled in. The nucleotides added under these conditions are called P-nucleotides. The three junctional regions that contribute most to antigen binding and diversity are called complementarity-determining regions (CDRs). CDR3 contains the greatest amount of diversity. Combinatorial diversity may produce approximately 6 x 10^6 unique TCRs, and junctional diversity may theoretically produce up to 10^{16} TCRs (humans are estimated to have 10^9 unique T cell clones). Thus, massive TCR diversity is generated from relatively few genes and among TCRs of similar structure. Given this diversity, an immunocompetent host's T cell repertoire is likely to contain TCRs that bind to antigens of a pathogen upon infection. These T cells then expand and mount a specific immune response against the pathogen.

1.5 Tolerance

1.5.1 Positive Selection

After expressing a TCR, T cells move to the inner cortex, where they attempt to interact with MHC-self-peptide complexes on cortical thymic epithelial cells (cTECs). Most of the recombined TCRs fail to bind MHC. Since binding to MHC-peptide complexes on APCs is required for T cell function in the periphery, T cells that cannot bind MHC would not be capable of mounting an immune response. These T cells do not receive survival signals from their TCRs and die by neglect. Approximately 90% of T cells generated die by this mechanism (10). T cells with TCRs that successfully bind self-peptide-MHC complexes with low to moderate affinity are selected to survive. This process is known as positive selection. TCRs that recognize MHC class I maintain CD8 expression and lose CD4 expression (1, 10). TCRs that recognize MHC class II preserve CD4 expression and cease CD8 expression. These changes create CD4 or CD8 single-positive T cells that are MHC class II or MHC class I-restricted, respectively.

The stochastic nature of TCR recombination allows the generation of an enormous T cell repertoire that can target many pathogens. However, TCR recombination also produces T cells that react to host antigens, or self-reactive T cells. Immune response against the body is known as autoimmunity. Self-reactive T cells must be eradicated to avoid serious damage to the host.

1.5.2 Negative Selection

Lack of immune activation against self-antigens is known as immunological tolerance. Tolerance is achieved through two main mechanisms: central tolerance and peripheral tolerance. Central tolerance occurs in the medulla of the thymus during T cell development. Peripheral tolerance occurs outside the thymus and acts on mature T cells.

Within the medulla of the thymus, medullary thymic epithelial cells (mTECs), DCs, and B cells trigger the deletion of self-reactive T cells in a process called negative selection (1, 11). mTECs, DCs, and B cells ectopically express self-antigens in the context of MHC. T cells that bind with high affinity or avidity to these self-antigens are forced to undergo apoptosis. Importantly, the self-antigens expressed here are expressed throughout the host. Thus, any T cells that reacts too strongly to an antigen expressed by a host tissue is eliminated before it can escape the thymus and mount an immune response. T cells that bind self-antigens with intermediate affinity or avidity may be redirected down the regulatory T cell (Treg) lineage. Tregs produced by this mechanism are “natural” or “thymic” Tregs (nTreg). Tregs exert immunosuppression, and are part of peripheral tolerance. Notably, negative selection may also occur during positive selection when developing T cells are interacting with cTECs (10).

1.5.3 Autoimmune Regulator

The ectopic expression of thousands of self-antigens in thymic cells is driven by at least two transcription factors: autoimmune regulator (AIRE) (12) and FEZF2 (13). AIRE and FEZF2 are necessary for negative selection and immune tolerance. AIRE also promotes the negative selection of autoreactive T cells in the periphery through extrathymic AIRE-expressing cells (eTACs) (14). Deficiency in AIRE or FEZF2 predisposes mice and humans to multi-organ autoimmunity. AIRE deficiency in humans, and resultant breakdown of central tolerance, causes a disease called autoimmune polyglandular syndrome type 1 (APS-1) or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (15). Patients with APS-1 develop autoimmunity against several organs, which frequently begins during childhood and teenage years. Affected organs include the parathyroid glands, adrenal glands, thyroid gland, pancreas, gonads, liver, stomach, hair, and skin. Interestingly, two unrelated children with APS-1 also developed autoimmunity against peripheral nerves that resembled chronic inflammatory demyelinating polyneuropathy (CIDP) (16). In support of a link between AIRE deficiency and CIDP, a strain of non-obese diabetic (NOD) mice harboring a dominant-negative G228W mutation in AIRE (*NOD.Aire*$^{GW/+}$; henceforth referred to as *Aire*$^{GW/+}$) develop spontaneous autoimmune peripheral polyneuropathy (SAPP) that resembles CIDP (17, 18). SAPP in *Aire*$^{GW/+}$ mice shares key features with CIDP, including infiltration of peripheral nerves by CD4+ T cells and F4/80+ macrophages (19–21), interferon gamma (IFN-γ) production by CD4+ T cells (22, 23), and loss of the myelin sheath (demyelination) of peripheral nerves (17, 24). Thus, peripheral nerves have been recognized as an additional target of autoimmune attack in APS-1 (25).

1.6 Chronic Inflammatory Demyelinating Polyneuropathy (CIDP)

CIDP is the most common acquired autoimmune peripheral neuropathy, affecting as many as 9 in 100,000 people (24). Sometimes considered the chronic form of Guillain-Barré Syndrome, CIDP is characterized by chronic, symmetric, relapsing-remitting or progressive sensory dysfunction, paresthesia, muscle weakness, impaired balance, and reduced/absent tendon reflexes due to autoimmune demyelination of nerves in the PNS (26). Symptoms typically arise between ages 30 and 60, but may also occur during childhood between five and 18 years old. Inflammation and demyelination in CIDP are multifocal, which causes disease manifestations and progression to vary widely among patients.

Electrodiagnostic testing is central to CIDP diagnosis; nerve conduction is quantified, and characteristics of demyelination can be detected. The myelin sheath on peripheral nerves is created by the wrapping of Schwann cell plasma membranes around axons of neurons. This insulates action potentials traveling along the axons, increasing their velocity and longevity. Demyelination leads to reduced conduction velocity, increased distal motor and sensory latencies, conduction block, and dispersion of compound muscle action potentials (CMAP) in electrodiagnostic testing.

First-line treatment options for CIDP include glucocorticoids, intravenous immunoglobulin (IVIg), and plasma exchange, which utilize nonspecific mechanisms of action against CIDP, do not improve disease symptoms in one-third of patients, and do not achieve remission or cure in over 70% of patients (27, 28). Thus, current treatments fail to address considerable CIDP disease burden. Better understanding of CIDP pathogenesis is an important step toward developing new mechanism-based therapies with greater efficacy.

Substantial evidence suggests T cells are critical for the development of CIDP (24). First, non-obese diabetic (NOD) mice develop spontaneous autoimmune peripheral polyneuropathy (SAPP) that resembles CIDP due to defective negative selection of T cells (17, 29). Second, T cell-deficient mice fail to develop experimental autoimmune neuritis (EAN), which is an induced model of CIDP (30). Third, CD4+ T cells are sufficient to transfer SAPP (17, 29, 31). Finally, T cells are present in sural nerve infiltrates of CIDP patients (24). Given the central role of T cells in CIDP pathogenesis, I investigated whether T cells were required for SAPP pathogenesis in *NOD.Aire*$^{GW/+}$ mice (Chapter 2). Notably, B cells and macrophages have also been implicated in CIDP pathogenesis (24, 32).

1.7 Interleukin 10 in spontaneous autoimmune peripheral polyneuropathy

A key function of T cells is the secretion of cytokines (24, 32), and CIDP has been associated with increased IFN-γ (22, 23) and tumor necrosis factor alpha (TNF-α) (33). Blockade of IFN-γ and TNF-α in SAPP mouse models have revealed disease-promoting roles for these cytokines (Bour-Jordan et al., 2005; Zeng et al., 2013; Zhang et al., 2012). Interestingly, increased IL-10 expression has also been associated with CIDP (22) (37). However, the role of IL-10 in CIDP pathogenesis is unclear.

IL-10 has multiple anti-inflammatory effects, which include suppressing production of pro-inflammatory cytokines, chemokines, and costimulatory molecules in macrophages and dendritic cells (38, 39). IL-10 dampens autoimmunity in multiple disease models, including rheumatoid arthritis (RA) (40, 41) and multiple sclerosis (MS) (42, 43). Recombinant human IL-10 is a 17-18 kDa homodimer (38). Its sequence is well conserved across multiple species; for example, mouse and human IL-10 have 73% sequence homology. IL-10 binds to and signals through IL-10 receptors 1 and 2 (IL-10R1, IL-10R2). IL-10R1 is primarily expressed on

hematopoietic cells, although its expression may be induced in other cell types (38). IL-10R1 is responsible for the immunosuppressive effects of IL-10, and it signals through janus kinase 1 (JAK1) and signal transducer and activator of transcription 1, 3, and 5 (STAT1, STAT3, STAT5 in non-macrophage cells). STAT3 mediates the immunosuppressive effects of IL-10. IL-10R2 is constitutively expressed on most cells, and it signals through tyrosine kinase 2 (Tyk2).

Although IL-10 typically suppresses inflammation, it has also been reported to promote immune responses. For example, IL-10 secretion by T helper 2 (Th2) cells promotes differentiation and antibody secretion in B cells (44, 45), which is strongly associated with inflammation in systemic lupus erythematosus (SLE) (46) and allergy (47). Furthermore, IL-10 stimulates the expansion and differentiation of effector CD8+ T cells, which boosts anti-tumor immunity in mice (48). Thus, while IL-10 is most commonly regarded as an anti-inflammatory cytokine, it is a pleiotropic cytokine that can also promote inflammation in certain immune contexts.

IL-10 signaling leads to phosphorylation of STAT3 (pSTAT3) (39). Along with increased IL-10, CD4+ T cells from CIDP patients with active disease have higher pSTAT3 (22). Separately, pSTAT3 has been shown to induce S1pr1 expression (49). The concurrent roles of pSTAT3 in IL-10 signaling and S1pr1 transcriptional induction suggest that IL-10 may contribute to S1PR1 expression and S1PR1-dependent lymphocyte migration. However, multiple cytokines can activate STAT3, and it is unclear whether IL-10-induced pSTAT3 upregulates S1PR1 expression.

1.8 Sphingosine-1-phosphate receptor 1 (S1PR1)

S1PR1 is a Gαi-coupled G-protein-coupled receptor (GPCR) important for lymphocyte migration (50); furthermore, it has been implicated in SAPP pathogenesis (51). S1PR1 is part of a family of five S1PR receptors. S1PR1 binds to its ligand sphingosine-1-phosphate (S1P) with picomolar affinity, enabling T cells to follow gradients of S1P (52). S1PR1-dependent migration allows T cells to exit the thymus after development, secondary lymphoid organs, and sites of inflammation. In lymph nodes, T cell migratory behaviour is driven by a balance between retention and egress signals. When naïve T cells first enter lymph nodes, their S1PR1 surface expression is very low because high S1P levels in the blood lead to S1PR1 internalization and degradation (52). Conversely, expression of the Gi GPCR C-C motif chemokine receptor 7 (CCR7) on the surface of T cells is high. CCR7 binds C-C motif chemokine ligand 19 and 21 (CCL19, CCL21) on lymph node stromal cells, which send retention signals to the cell. Thus, retention signals outweigh egress signals, leading to retention of the T cells in the node. Retention in lymph nodes grants time for T cells to sample antigens presented by local APCs and clonal expansion after binding cognate antigen (52). Over time, T cells become re-sensitized to S1P through re-expression of surface S1PR1. If T cells do not encounter cognate antigens or after clonal expansion is complete, S1PR1 signaling outweighs CCR7 signaling, leading to T cell egress from the lymph node.

Given the efficacy of S1PR1 as a therapeutic target (53, 54), there has been much effort in delineating factors controlling its expression. Regulators of S1pr1 expression in T cells include micro RNA 155 (miR-155), Forkhead box O1 (Foxo1), Krüppel-like factor 2 (KLF2), G protein-coupled receptor kinase-2 (GRK2), CD69, and Dynamin 2. miR-155 downregulates S1pr1 by binding the 3’-UTR (55). In the absence of TCR signaling, Foxo1 drives KLF2

expression (52, 56), and KLF2 binds and activates the promoter of S1pr1 (52, 57). After TCR stimulation, Akt phosphorylates and sequesters Foxo1 in the cytosol, preventing it from binding KLF2 and other downstream targets (52, 56). After S1P binds S1PR1, GRK2 phosphorylates and down-modulates S1PR1, allowing lymphocytes to escape circulation and enter lymph nodes (58). CD69 binds directly to S1PR1, which leads to S1PR1 internalization and degradation (52, 59, 60). Dynamin 2 is necessary for effective endocytosis and subsequent signaling of S1PR1 (61). Whether IL-10 also regulates S1pr1 through STAT3, however, is not known.

IL-10 and CIDP are correlated, but the effect of IL-10 in CIDP is unclear. Thus, I used *NOD.Aire*$^{GW/+}$ mice deficient in IL-10 to understand its role (Chapter 3). I found that IL-10 paradoxically promoted SAPP, and that this effect was due to the previously unappreciated transcriptional induction of S1pr1 by IL-10 through STAT3. While STAT3 has been reported to bind to S1pr1 promoter, a link between IL-10 and S1pr1 transcription was previously unknown. My secondary investigations of these mice yielded interesting results that may lead to additional research (Chapter 4).

CHAPTER 2: T CELLS IN NEUROPATHY

2.1 Introduction

Given that T cells are important in EAN and sufficient to transfer SAPP, we sought to determine whether they are required for SAPP development in *NOD.Aire*$^{GW/+}$ mice. To test this, we crossed *NOD.Aire*$^{GW/+}$ mice with TCRα-deficient *NOD* mice (62). Genetic ablation of the TCRα chain prevents immature T cells from interacting with cTECs and they die by neglect. Thus, no mature T cells are generated, and the periphery is devoid of T cells. B cells and other parts of the immune system are intact. I then monitored SAPP development in T cell-deficient *Aire*$^{GW/+}$ mice.

2.2 Materials and Methods

Mice

NOD.Aire$^{GW/+}$ mice were generated as previously described (63). *NOD.TCRα*$^{-/-}$ mice were purchased from Jackson Laboratory (62). Mice were housed in a specific-pathogen-free (SPF) barrier facility at the University of North Carolina, Chapel Hill. Clinical neuropathy, determined by hind limb weakness, and diabetes, determined by presence of glucose in urine, were assessed at least once per week as described previously (64). Diabetic mice were treated daily with intraperitoneal insulin until used in experiments or euthanasia (due to >20% weight loss). Only female mice were utilized due to higher SAPP incidence relative to males (17). Sciatic nerves of mice were utilized at 22 weeks of age unless otherwise noted. Due to the rapid progression of disease and death following initial symptoms, mice that developed SAPP prior to 22 weeks of age were harvested at SAPP onset. Experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research.

Histology

Harvested organs were fixed in 10% buffered formalin for at least 96 hours, washed in 30% ethanol for 20 minutes, and then stored in 70% ethanol. Organs were embedded in paraffin, sectioned, and stained with H&E by the UNC Animal Histopathology Core. Immune infiltration was scored while blinded to genotype as previously described (63). For sciatic nerves, scores of 0, 1, 2, 3, and 4 indicate 0%, 1-25%, 26-50%, 51-75%, and >75% infiltration, respectively.

Electrophysiology

Sciatic nerve conduction studies were performed as described (65), using a Teca Synergy T2X EMG system.

2.3 Results and Discussion

T cell-sufficient *Aire*$^{GW/+}$ mice developed SAPP between 15 and 25 weeks, whereas T cell-deficient *Aire*$^{GW/+}$ mice did not develop SAPP (Figure 2.1A). T cell-deficient *Aire*$^{GW/+}$ mice were free of SAPP out to 35 weeks of age. At 22 weeks of age, sciatic nerves of T cell-sufficient *Aire*$^{GW/+}$ mice were infiltrated with immune cells, whereas nerves from T cell-deficient mice were free of infiltration (Figure 2.1B, C). EMGs of 22-week-old T cell-sufficient *Aire*$^{GW/+}$ mice revealed signs of demyelination, including reduced peak amplitude, reduced conduction velocity, and increased action potential duration (Figure 2.1D, E). Conversely, T cell-deficient mice had no evidence of demyelination, and were indistinguishable from WT mice (Figure 2.1D, E). These data demonstrated that T cells are required for SAPP pathogenesis. Without T cells, *Aire*$^{GW/+}$ mice were free of any signs of disease. These results support the theory that T cells are central to CIDP pathogenesis, and suggest focusing on T cell biology in disease may yield efficacious therapeutic targets against CIDP.

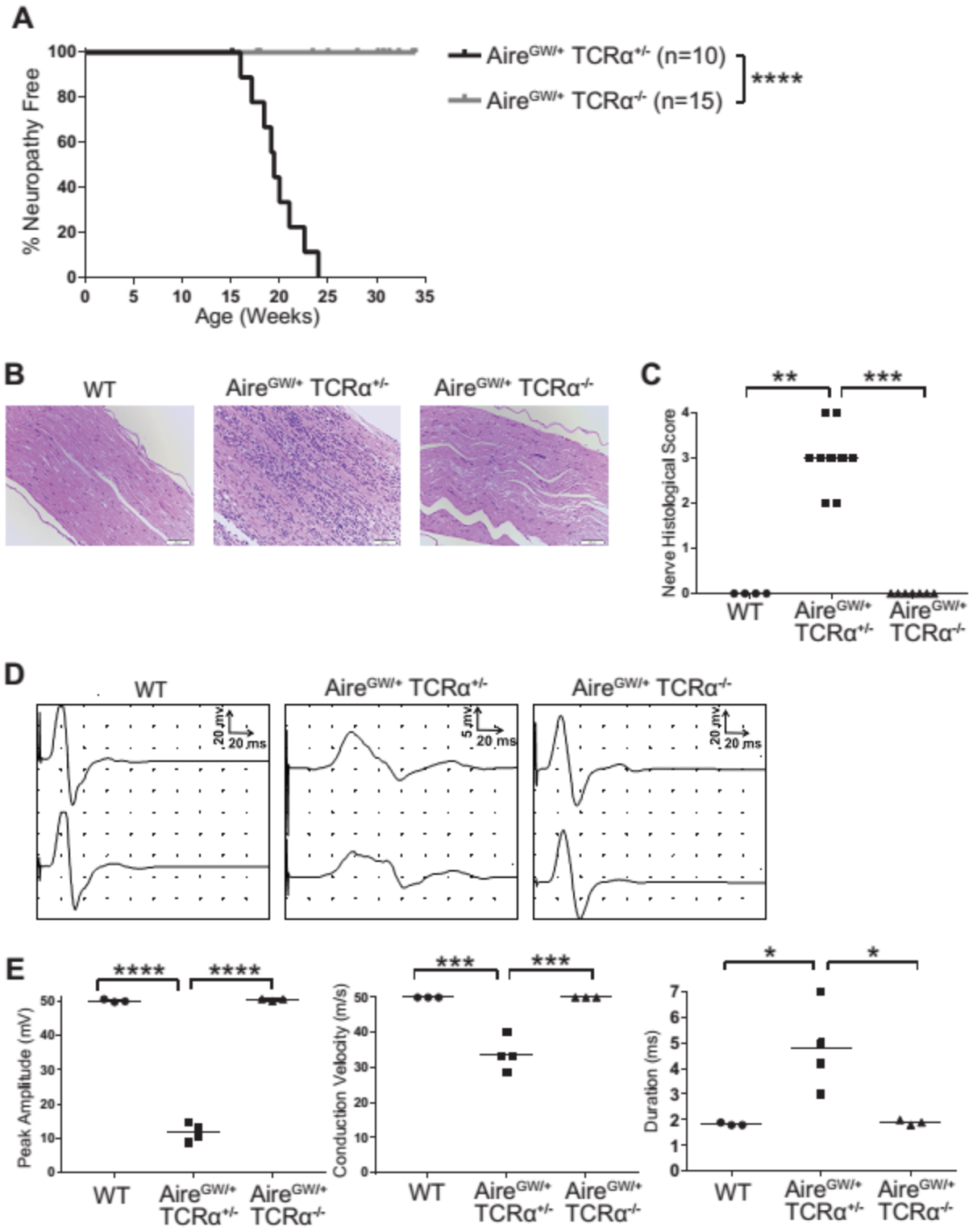


Figure 2.1. T cell deficiency prevents SAPP. *Aire*$^{GW/+}$ *TCRα*$^{+/-}$ and *Aire*$^{GW/+}$ *TCRα*$^{-/-}$ mice were monitored for SAPP. (A) SAPP incidence curves. p-value was calculated using Mantel-Cox log-

rank test; ****, $p < 0.0001$. (B) H&E-stained sciatic nerves from 22-wk-old mice (Magnification x200). (C) Cumulative sciatic nerve infiltration scores from 22-wk-old mice. p-value was calculated using Fisher's Exact test with Bonferroni's correction; **, $p < 0.01$, *** $p < 0.001$. (D) Representative proximal compound muscle action potentials from sciatic nerves of 22-wk-old mice. (E) Cumulative peak amplitude, conduction velocity, and duration of compound muscle action potentials from 22-wk-old mice. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; *, $p < 0.05$, ***, $p < 0.001$, ****$p < 0.0001$. Each symbol represents an individual mouse.

CHAPTER 3: CYTOKINES IN NEUROPATHY[1]

3.1 Introduction

IL-10 is increased in CIDP patients (22), but the functional significance of IL-10 in CIDP is unknown. In this study, we found that, like CIDP patients, *Aire*$^{GW/+}$ mice with SAPP have robust induction of IL-10. IL-10 expression was increased in inflamed peripheral nerves and T cells isolated from spleens and lymph nodes. To test the role of IL-10 in SAPP, we crossed *NOD.Aire*$^{GW/+}$ mice with *NOD.Il10*$^{-/-}$ mice to generate IL-10-sufficient and IL-10-deficient *Aire*$^{GW/+}$ mice (66). Unexpectedly, IL-10-deficient *Aire*$^{GW/+}$ mice were protected from SAPP development, suggesting IL-10 is a pathogenic cytokine in SAPP. IL-10 deficiency was associated with accumulation of activated CD4+ T cells in the draining lymph nodes and decreased PNS infiltration. These findings suggested that impaired T cell egress may underlie SAPP protection in IL-10 deficiency. Indeed, IL-10-deficient CD4+ T cells lacked *S1pr1* expression and displayed reduced migration to the S1PR1 ligand sphingosine-1-phosphate (S1P). Moreover, IL-10 induced S1PR1 mRNA expression, and IL-10-mediated *S1pr1* expression required STAT3. Together, these findings illustrate a previously unappreciated role of IL-10 in promoting S1PR1-dependent lymphocyte migration and autoimmune destruction of peripheral nerves.

[1]Originally published in *The Journal of Immunology*. Smith, C, D. Allard, Y. Wang, J. F. Howard, S. A. Montgomery, and M. A. Su. 2017. IL-10 paradoxically promotes autoimmune neuropathy through S1PR1-dependent CD4+ T cell migration. *J. Immunol*. © 2017 The American Association of Immunologists, Inc.

3.2 Materials and Methods

Mice

NOD.Aire$^{GW/+}$ mice were generated as previously described (63). *NOD.SCID* (JAX stock #001303), *NOD.µMT*$^{-/-}$ (JAX stock #004639), and *NOD.Il10*$^{-/-}$ (JAX stock #004266) mice were purchased from The Jackson Laboratory. Mice were housed in a specific-pathogen-free (SPF) barrier facility at the University of North Carolina, Chapel Hill. Clinical neuropathy, determined by hind limb weakness, and diabetes, determined by presence of glucose in urine, were assessed at least once per week as described previously (64). Diabetic mice were treated daily with intraperitoneal insulin until used in experiments or euthanasia (due to >20% weight loss). Only female mice were utilized due to higher SAPP incidence relative to males (17). Mice were utilized at 22 weeks of age unless otherwise noted. Due to the rapid progression of disease and death following initial symptoms, mice that developed SAPP prior to 22 weeks of age were harvested at SAPP onset. *NOD.Aire*$^{GW/+}$ *Il10*$^{+/-}$ or *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice were used as splenocyte donors. Experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research.

Immunohistochemistry

Mouse sciatic nerves were embedded in Optimal Cutting Temperature (OCT) compound, frozen at -20 °C for 2 hours, and then stored at -80 °C. Six micron-thick longitudinal sections were fixed with cold acetone, blocked with 2.5% goat serum, and then stained with anti-IL-10 antibody (Biolegend clone JES5-16E3) for 2 hours at room temperature. After washing with PBS + 0.1% Tween, sections were incubated with anti-rat-HRP antibody for 30 minutes at room temperature. DAB solution (Vector Laboratories) was applied to the sections as the chromogen.

Flow cytometry

Flow cytometry was performed as previously described (67). Briefly, single-cell suspensions were isolated from spleens or lymph nodes by crushing with forceps, or from sciatic nerves by mincing and digestion in 2 mg/ml collagenase. Cells were stained with live/dead fixable yellow dye (Life Technologies), anti-mouse CD4 (Biolegend clone GK1.5), anti-mouse CD44 (eBioscience clone IM7), anti-mouse CD62L (eBioscience clone MEL-14), anti-mouse S1PR1 (R&D clone 713412), anti-mouse IFN-γ (eBioscience clone XMG1.2), and anti-mouse IL-10 (BD clone JES5-16E3) antibodies. For intracellular cytokine staining, cells were stimulated with PMA/ionomycin for 4 hours at 37 ºC 5% CO_2, and permeabilized using BD Cytofix/Cytoperm according to manufacturer's instructions. Cells were analyzed on a CyAn ADP Analyzer (Beckman Coulter). Data were analyzed using FlowJo X.

Histology

Harvested organs were fixed in 10% buffered formalin for at least 96 hours, washed in 30% ethanol for 20 minutes, and then stored in 70% ethanol. Organs were embedded in paraffin, sectioned, and stained with H&E by the UNC Animal Histopathology Core. Immune infiltration was scored while blinded to genotype as previously described (63). For sciatic nerves, scores of 0, 1, 2, 3, and 4 indicate 0%, 1-25%, 26-50%, 51-75%, and >75% infiltration, respectively. Colon histology was scored in a blinded fashion by a board-certified veterinary pathologist as previously described (68). Briefly, H&E-stained distal colonic sections were scored based on an additive histological injury system that included mucosal ulceration, epithelial hyperplasia, and lamina propria mononuclear and neutrophil infiltrates. Pancreatic islet infiltration was scored in a blinded fashion as previously described (69). Briefly, H&E-stained pancreatic sections were scored based on average degree of islet infiltration.

Electrophysiology

Sciatic nerve conduction studies were performed as described (65), using a Teca Synergy T2X EMG system.

Antibody treatment

Anti–B7-1 Ab (clone 16.10A1) and anti–B7-2 Ab (clone GL1) were generous gifts of Greg Szot (UCSF) or purchased from BioXCell. To induce neuropathy, 14-day-old *NOD.Il10*$^{+/-}$ or *NOD.Il10*$^{-/-}$ mice were treated with 50 µg Ab or isotype controls (2A3 and Armenian Hamster IgG) every other day for seven treatments (36, 64).

Enzyme-linked Immunospot Assay (ELISpot)

ELISpot assays were performed according to manufacturer's protocol (BD). One million splenocytes or 8 x 10^5 lymph node cells were cultured alone, with synthetic ovalbumin (323-339) (Invivogen), synthetic myelin protein zero (180-199) (Genemed Synthesis), or PMA/ionomycin in DMEM + 10% FBS for 17 hours at 37 ºC 5% CO_2. Spots were enumerated using the AID iSpot Reader.

Adoptive Transfer

Adoptive transfer of CD4+ T cells from spleen was performed as previously described (17). Tissue culture plates were coated with 1 µg/mL anti-CD3ε (eBioscience clone 145-2C11) and 1 µg/mL anti-CD28 (BD Pharmingen clone 37.51) in sterile PBS overnight at 4 ºC. Plates were washed gently with sterile PBS. Whole spleen from 22-wk-old *NOD.Aire*$^{GW/+}$ *Il10*$^{+/-}$ or *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ donors were cultured with plate-bound anti-mouse CD3 and anti-mouse CD28 in DMEM (Gibco) + 10% FBS (Sigma-Aldrich) for 4 days at 37 ºC 5% CO_2. CD4+ T

cells were purified from cultures using the Magnisort CD4+ T cell Enrichment Kit (Invitrogen) and 1 x 10^6 CD4+ T cells were transferred to *NOD.SCID Il10*$^{+/-}$ or *NOD.SCID Il10*$^{-/-}$ recipients. Diabetic mice were excluded as donors.

FTY720 Treatment

Aire$^{GW/+}$ mice were treated with DMSO or 1 mg/kg FTY720 (Cayman Chemical). FTY720 was first dissolved in DMSO (25g in 1 mL DMSO), aliquoted, and frozen at -20 ºC. Mice were given intraperitoneal injections of DMSO or FTY720 dissolved 1:100 in 100 µL sterile water on every week day (5 days per week) from 14 weeks of age (before SAPP development) until utilization. Mice were harvested when they developed SAPP, or when all DMSO-treated controls developed SAPP.

Chemotaxis Assay

Chemotaxis was measured using transwell assays as described by (70). Migration of lymph node cells was measured using a 24-well transwell plate (Corning Life Sciences) with 6.5 mm polycarbonate filters and five µm pores. The lower chamber was coated overnight at 4 ºC with 600 µl of 100 µg/ml human collagen type IV (Sigma-Aldrich) in 0.5 M acetic acid, washed with PBS, and air-dried. Two million cells were resuspended in 100 µl RPMI 1640 medium with 0.1% fatty acid-free BSA (Sigma-Aldrich), 100 U/ml penicillin G, 2mM L-glutamine, and 25 mM HEPES buffer. Cells were placed on the Transwell inserts. S1P (20 nM in 600 µL) was added in the same medium to the lower chamber. Migration was performed for four hours at 37 ºC 5% CO_2. Migrated cells were counted with a hemocytometer. Migration assays without S1P were performed in parallel to assess baseline migration. Net migration to S1P was calculated by

subtracting the number of cells that migrated nonspecifically from the number of cells that migrated to S1P.

IL-10 Stimulation and Real Time RT-PCR

Five hundred thousand cells from fresh sciatic and lumbar lymph nodes of *NOD.Il10*$^{-/-}$ mice were stimulated with recombinant human IL-10 (100 ng/mL from Peprotech), stimulated with IL-10 and STA-21 (10 μM from Santa Cruz Biotech), or incubated without stimulation in XVIVO 15 + Transferrin serum-free media (Lonza) for 30 minutes at 37 ºC 5% CO_2. RNA was isolated from cells using the Zymo RNA MicroPrep kit. Superscript II (Invitrogen) reverse transcriptase was used to create cDNA. TaqMan universal PCR Master Mix (Applied Biosystems) was used for qPCR. Commercially available TaqMan primer-probe sets for IL-10 and S1Pr1 were used (Applied Biosystems). Cyclophilin A was used as an internal control and detected with the primer-probe set reported by (63). Reactions were run on a Quantstudio 6 Flex system (Life Technologies) and analyzed as described (63).

Statistics

Data were analyzed with Graphpad Prism 6 using one-sample two-tailed Student's *t* tests, unpaired two-tailed Student's *t* tests or one-way ANOVA. The Bonferroni or Tukey corrections for multiple comparisons were used when appropriate. Mantel-Cox log-rank tests were used to compare survival curves. R (v3.3.1) was used to perform Fisher's Exact test and false discovery rate (FDR) adjustments. $p<0.05$ was considered significant, unless the threshold was reduced for the Bonferroni correction.

3.3 Results

IL-10 promotes SAPP in two mouse models.

IL-10 mRNA expression was compared by qPCR in whole sciatic nerve from NOD wild-type (WT) vs. *Aire*$^{GW/+}$ mice with SAPP (Figure 3.1A). IL-10 mRNA was not detected in WT nerves, whereas *Aire*$^{GW/+}$ nerves expressed abundant IL-10. Additionally, immunohistochemical staining for IL-10 protein revealed increased protein expression in *Aire*$^{GW/+}$ nerves compared to WT (Figure 3.1B). Thus, similar to CIDP, SAPP in *Aire*$^{GW/+}$ mice is associated with increased IL-10 expression.

To determine IL-10's role in SAPP pathogenesis, we crossed *NOD.Il10*$^{-/-}$ mice (66) with *NOD.Aire*$^{GW/+}$ mice to generate *NOD.Aire*$^{GW/+}$ *Il10*$^{+/-}$ and *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice. Lack of IL-10 protein expression in *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice was confirmed by intracellular IL-10 staining and flow cytometry of CD4+ T cells (Figure 3.1C). On the C3H/HeJBir and 129/SvEv backgrounds, IL-10 deficient mice are susceptible to colitis, growth retardation, anemia, and early death (68, 71, 72). Such findings could potentially prevent our ability to observe SAPP in *NOD.Aire*$^{GW/+}$ mice. However, we did not observe colitis or its associated symptoms in *NOD.WT Il10*$^{-/-}$ or *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice (Figure 3.6 and data not shown). These results are consistent with prior reports that colitis is mild and transient on the NOD background (73), and suggest that *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice can be used to understand the impact of IL-10 on SAPP development.

Given their utility, *NOD.Aire*$^{GW/+}$ *Il10*$^{+/-}$ mice and *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ littermates were monitored for SAPP for 30 weeks. Consistent with our previous data, 80% of the IL-10-sufficient *Aire*$^{GW/+}$ mice developed SAPP by 22 weeks of age (*Aire*$^{GW/+}$ *Il10*$^{+/-}$, Figure 3.1D) (Su et al., 2012). Strikingly, compared to IL-10-sufficient *Aire*$^{GW/+}$ mice, IL-10-deficient *Aire*$^{GW/+}$

mice had a significant delay in SAPP (*Aire*$^{GW/+}$ *Il10*$^{-/-}$, Figure 3.1D), which suggests that IL-10 accelerates SAPP development. None of the IL-10-sufficient or deficient Aire WT mice developed SAPP during the study (*WT Il10*$^{+/-}$ or *Il10*$^{-/-}$, Figure 3.1D), indicating that IL-10 deficiency does not impair nerve function. Additionally, extensive cellular immune infiltrate was seen in H&E-stained sciatic nerves of IL-10-sufficient *Aire*$^{GW/+}$ mice, but was absent in IL-10-deficient *Aire*$^{GW/+}$ mice (Figure 3.1E, F). Finally, SAPP in IL-10-sufficient *Aire*$^{GW/+}$ sciatic nerves was associated with electrophysiological changes consistent with demyelination, including prolonged distal motor latencies, slowed conduction velocity, and increased compound muscle action potential (CMAP) duration due to temporal dispersion relative to WT nerves (Figure 3.1G, H). Conversely, abnormalities were not detected in sciatic nerves of IL-10-deficient *Aire*$^{GW/+}$ mice (Figure 3.1G, H). IL-10-deficiency therefore protects against SAPP development, nerve infiltration, and electrophysiological changes in *Aire*$^{GW/+}$ mice. These findings that suggest that IL-10 promotes SAPP were surprising, given IL-10's well-described functions as an immunoregulatory cytokine in a number of autoimmune diseases (38).

SAPP has been described in multiple mouse models, including NOD mice deficient in costimulatory molecules B7-1/2 (64). To determine whether the effect of IL-10 deficiency on SAPP was specific only to *Aire*-deficient mice or generalizable to other SAPP models, we treated IL-10-sufficient and IL-10-deficient WT mice with anti-mouse B7-1 and anti-mouse B7-2 to induce autoimmune peripheral neuropathy (36, 64). By approximately 12 weeks of age, 100% of the anti-B7-1/2 treated, IL-10-sufficient WT mice had SAPP compared to only 22% of IL-10-deficient WT mice (Figure 3.2A). At 12 weeks of age, sciatic nerves of IL-10-sufficient WT mice were heavily infiltrated, whereas sciatic nerves of IL-10-deficient WT mice had significantly less infiltration (Figure 3.2B, C). These data suggest IL-10's promotion of

autoimmunity against peripheral nerves is not specific to the Aire-deficient model, but is a more generalizable feature of SAPP pathogenesis.

It is unclear whether IL-10's effect in promoting SAPP reflects a more global, generalized role in promoting autoimmunity against all target tissues. In addition to SAPP, *Aire*$^{GW/+}$ mice also develop autoimmune diabetes (17), allowing us to concurrently assess the role of IL-10 in SAPP and diabetes in these mice. Although IL-10 deficiency delayed SAPP development, lack of IL-10 did not affect autoimmune diabetes development in *Aire*$^{GW/+}$ mice (Figure 3.7). This finding is consistent with previous reports that IL-10 does not alter diabetes incidence in *NOD.WT* mice (66). Thus, IL-10 appears to promote autoimmunity in a tissue-specific manner.

IL-10 produced by CD4+ T cells promotes SAPP development.

Many cell types in the innate and adaptive immune systems produce IL-10 (39). Therefore, it is not clear which IL-10-producing cell type is important in promoting SAPP. A key source of IL-10 is CD4+ T cells, and CD4+ T cells are sufficient to transfer SAPP in multiple models (29, 35, 36). Given the critical role for CD4+ T cells in SAPP, we examined IL-10 expression in CD4+ T cells from WT and neuropathic *Aire*$^{GW/+}$ mice. IL-10 expression in CD4+ T cells from the spleen and nerve-draining lymph node of neuropathic *Aire*$^{GW/+}$ mice was significantly increased compared to WT mice (Figure 3.3A, B). Furthermore, while CD4+ T cells were absent in WT sciatic nerve, >10% of infiltrating CD4+ T cells expressed IL-10 in *Aire*$^{GW/+}$ sciatic nerves. Thus, increased IL-10 expression within CD4+ T cells is associated with SAPP development.

Given increased CD4+ T cell IL-10 expression with SAPP, we next sought to determine whether IL-10 production by CD4+ T cells is sufficient to promote SAPP. Purified *Aire*$^{GW/+}$ CD4+ T cells that were IL-10 sufficient or deficient were transferred to immunodeficient *NOD.*Prkdc$^{SCID/SCID}$ (*SCID*) (74) recipients that were either IL-10 sufficient or deficient (outlined in Figure 3.3C, left). IL-10 sufficient donor CD4+ T cells induced SAPP in IL-10-sufficient and deficient recipients 6-7 weeks post-transfer (Figure 3.3C, right). Conversely, IL-10-deficient donor CD4+ T cells induced SAPP in IL-10-sufficient and deficient recipients 10 weeks post transfer, which was a significant delay relative to IL-10-sufficient donors (Figure 3.3C, right). At 7 weeks post-transfer, recipients of IL-10-deficient donor CD4+ T cells displayed significantly less sciatic nerve infiltration than recipients of IL-10-sufficient donor CD4+ T cells (Figure 3.3D, E). These data demonstrate that IL-10 production by CD4+ T cells is sufficient to promote SAPP development.

IL-10-deficient Aire$^{GW/+}$ *mice harbor highly activated CD4+ T cells.*

IL-10 has a well-described role in suppressing T cell activation (38). Since IL-10 unexpectedly promoted SAPP (Figure 3.1 and 3.2), whether IL-10 would suppress or enhance T cell activation was not clear. Thus, we characterized CD4+ T cell activation in IL-10-deficient *Aire*$^{GW/+}$ mice that were protected from SAPP. Frequencies of CD4+CD44^{hi}CD62L^{lo} activated T cells were compared in the spleens of WT, IL-10-sufficient *Aire*$^{GW/+}$, and IL-10-deficient *Aire*$^{GW/+}$ mice. IL-10-deficient *Aire*$^{GW/+}$ mice had significantly higher frequencies of activated CD4+ T cells relative to IL-10-sufficient WT mice and *Aire*$^{GW/+}$ mice (Figure 3.4A, B). We next test whether IL-10 would suppress or enhance CD4+ T cell expression of IFN-γ, a cytokine critical for SAPP development (35, 36). While IL-10-deficient *Aire*$^{GW/+}$ mice had significantly greater frequencies and absolute numbers of IFN-γ-producing CD4+ T cells in the spleen

compared to WT mice, numbers of IFN-γ-producing CD4+ T cells were not different compared to IL-10-sufficient *Aire*$^{GW/+}$ mice (Figure 3.4C, D). In the draining lymph node, IL-10-deficient *Aire*$^{GW/+}$ mice had greater absolute numbers of IFN-γ-producing CD4+ T cells compared to both WT and IL-10-sufficient *Aire*$^{GW/+}$ mice (Figure 3.4E, F). Thus, despite protecting from SAPP, IL-10 deficiency was associated with equivalent or increased CD4+ T cell activation and IFN-γ production.

In addition to suppressing T cell activation, IL-10 also downregulates expansion of self-antigen specific T cells (75). Since IL-10 unexpectedly promoted SAPP, however, we sought to determine how IL-10 deficiency would alter the precursor frequency of PNS-specific T cells. Pathogenic CD4+ T cells target the self-antigen myelin protein zero (P0) (17). For this reason, we assessed the expansion of P0-specific CD4+ T cells using an ELISpot detecting IFN-γ. One million splenocytes from WT, IL-10-sufficient *Aire*$^{GW/+}$, and IL-10-deficient *Aire*$^{GW/+}$ mice were left unstimulated (Media), stimulated with MHC II-restricted ovalbumin irrelevant peptide, stimulated with MHC II-restricted P0 peptide, or stimulated with PMA/ionomycin for a positive control (data not shown). IL-10-deficient *Aire*$^{GW/+}$ splenocytes stimulated with P0 elicited 119 IFN-γ spots on average, which was significantly higher than WT (11 spots) and trended higher than IL-10-sufficient *Aire*$^{GW/+}$ mice (84 spots) (Figure 3.4G, H). We also tested cells from the nerve-draining lymph nodes for response to P0. Similar to spleen, the number of P0-specific T cells was significantly higher in IL-10-deficient *Aire*$^{GW/+}$ lymph nodes (99 spots) relative to WT (6 spots) and similar to IL-10-sufficient *Aire*$^{GW/+}$ mice (111 spots) (Figure 3.4I, J). Thus, IL-10-deficiency protected from the development of SAPP, but does not decrease the frequency of nerve-specific CD4+ T cells in spleen or draining lymph nodes.

IL-10 deficiency is associated with enlarged lumbar lymph nodes, impaired lymphocyte migration, and reduced S1pr1 expression in CD4+ T cells.

IL-10 deficiency was associated with minimal immune infiltrate in the sciatic nerves (Figure 3.1 and 3.2) and, concurrently, activated T cells in the spleen and lymph nodes (Figure 3.4). One potential explanation for these disparate findings is that IL-10 is required for T cell egress from secondary lymphoid structures. Without IL-10, then, activated T cells may accumulate in the lymph nodes rather than migrating into peripheral nerves. Since reduced lymphocyte egress is associated with increased lymph node cellularity (76), we first tested whether IL-10 deficiency may affect migration by investigating lymph node size in IL-10-deficient mice. Indeed, the nerve-draining lumbar lymph nodes were enlarged in IL-10-deficient *Aire*$^{GW/+}$ mice relative to WT and IL-10-sufficient *Aire*$^{GW/+}$ mice (Figure 3.5A). On average, IL-10-deficient *Aire*$^{GW/+}$ lumbar lymph nodes had >3-fold more cells than WT lymph nodes and ~3-fold more cells than IL-10-sufficient *Aire*$^{GW/+}$ lymph nodes (Figure 3.5B). These findings lend support to the hypothesis that IL-10 may accelerate SAPP by promoting migration of T cells out of lymph nodes and into nerves.

T cells exit lymph nodes by expressing S1PR1 on the cell surface and following gradients of S1P (50). To test IL-10's effects on lymphocyte migration, we measured chemotaxis to S1P of lymph node cells from IL-10-sufficient and IL-10-deficient *Aire*$^{GW/+}$ mice in a transwell assay. IL-10-deficient *Aire*$^{GW/+}$ lymph node cells had significantly reduced net migration to S1P, with a >3-fold reduction in migrating cells relative to IL-10-sufficient *Aire*$^{GW/+}$ mice (Figure 3.6C). These findings suggest that IL-10 increases T cell migration to S1P.

We next sought to determine whether reduced chemotaxis to S1P by IL-10-deficient *Aire*$^{GW/+}$ lymphocytes may be due to reduced S1PR1 expression. To test this, we measured *S1pr1*

mRNA in CD4+ T cells purified from lumbar lymph nodes of IL-10-sufficient and deficient *Aire*$^{GW/+}$ mice. IL-10-deficient *Aire*$^{GW/+}$ CD4+ T cells expressed 12-fold lower levels of *S1pr1* mRNA relative to IL-10-sufficient *Aire*$^{GW/+}$ controls (Figure 3.5D). To determine whether reduced *S1pr1* mRNA correlated with reduced surface protein expression, we measured S1PR1 surface protein on CD4+ T cells from lumbar lymph nodes of IL-10-sufficient and deficient *Aire*$^{GW/+}$ mice. A significantly lower frequency of CD4+ T cells from lymph nodes of IL-10-deficient *Aire*$^{GW/+}$ mice expressed S1PR1 surface protein relative to IL-10-sufficient *Aire*$^{GW/+}$ mice (Figure 3.5E, F). Thus, *S1pr1* mRNA and protein were reduced in IL-10-deficient *Aire*$^{GW/+}$ CD4+ T cells. Together, these findings support a model in which IL-10 upregulates S1PR1 on CD4+ T cells to enhance their migration into the PNS and promote SAPP development.

IL-10 induces S1pr1 mRNA in a STAT3-dependent manner.

How IL-10 upregulates *S1pr1* expression is unclear. IL-10 signaling has been shown to activate STAT3, and, separately, STAT3 has been shown to upregulate S1PR1 transcription (38, 49). Based on these findings, we reasoned that IL-10 may induce *S1pr1* in T cells via STAT3 activation. To test this, we stimulated lymph node cells with recombinant IL-10 *in vitro* and measured relative expression of *S1pr1* by qPCR. Lymph node cells from IL-10 deficient (*WT Il10*$^{-/-}$) mice were used to avoid preexisting induction of *S1pr1* by IL-10. We stimulated whole lumbar lymph node instead of sorted CD4+ T cells because: 1. the lymph node is predominantly composed of T cells, and 2. the additional time required for sorting exposes the cells to an S1P-low environment, which may lead to *S1pr1* resensitization (52, 70). IL-10 induced *S1pr1* mRNA by ~ 1.8-fold compared to unstimulated cells (Figure 3.5G). Thus, addition of IL-10 is sufficient to upregulate *S1pr1* in lymph node cells. Notably, STA-21, a STAT3 inhibitor (77), abrogated

induction of *S1pr1* by IL-10 (Figure 3.5G). Together these data suggest that IL-10 activates STAT3 to induce *S1pr1* transcription.

S1pr functional antagonist FTY720 suppresses SAPP development

S1PR1 expression was reduced in IL-10-deficient *Aire*$^{GW/+}$ mice (Figure 3.5D-F), suggesting that decreased S1PR1 may underlie SAPP protection in IL-10-deficient *Aire*$^{GW/+}$ mice. S1PR1 blockade is being investigated for therapeutic potential in multiple autoimmune diseases (53), and is currently in clinical use for the treatment of MS (54), an autoimmune disease of the central nervous system (CNS). However, it is unclear whether loss of S1PR1 prevents SAPP development in *Aire*$^{GW/+}$ mice. To test this, we blocked S1PR1 activity in *Aire*$^{GW/+}$ mice using the S1PR functional antagonist FTY720 (78). FTY720-treated *Aire*$^{GW/+}$ mice were protected from SAPP development relative to DMSO-treated controls (Figure 3.5H). Additionally, on H&E stained sections of sciatic nerves, FTY720 treatment was associated with reduced infiltration (Figure 3.5I, J). Thus, S1PR1 plays a key role in promoting SAPP and supports a model in which IL-10-mediated upregulation of S1PR1 promotes SAPP development.

3.4 Discussion

Similar to other autoimmune conditions, such as RA, Sjogren's syndrome, and Grave's disease (38, 79–81), IL-10 expression is upregulated in PBMCs of patients with autoimmune demyelinating polyneuropathy. Unlike most other autoimmune conditions, however, we report here that IL-10 promotes autoimmune peripheral neuropathy rather than suppressing the autoimmune response. Moreover, IL-10 deficiency was associated with an accumulation of activated CD4+ T cells in the draining lymph nodes and a lack of immune cell infiltration in nerves, which suggests a defect in lymphocyte egress from lymph nodes. Indeed, IL-10 deficiency was associated with reduced S1PR1-mediated lymphocyte migration, and IL-10 was sufficient to induce *S1pr1* expression. These findings delineate a previously unappreciated mechanism by which IL-10 may function to promote autoimmune disease.

IL-10 has been reported to promote immune responses in other inflammatory contexts. For example, IL-10 enhances B cell-mediated immunity (44, 45). However, our data indicate that SAPP in *Aire*-deficient mice can occur independently of B cells. Thus, it is unlikely that IL-10 is functioning through B cell activation to promote SAPP. IL-10 also promotes CD8+ T cell-mediated immunity (82). Although it is possible that this IL-10-dependent mechanism may be contributing to SAPP development, our adoptive transfer experiments show that CD8+ T cells are dispensable for SAPP transfer since *NOD.SCID* mice lack CD8+ T cells (74). Instead, our data suggest IL-10 induces *S1pr1* expression, which promotes CD4+ T cell-mediated migration from lymph nodes and autoimmunity against the PNS.

On the surface, our findings may seem to contradict a recent report that IL-10 protected *NOD.*B7-2$^{-/-}$ mice from SAPP (83). Quan et al., 2015 show that transfer of dendritic cells (DCs) pretreated with IL-10 protects from neuropathy. These seemingly contradictory results, however,

may not be mutually exclusive. It is possible that IL-10 has distinct effects on different immune cell types in SAPP pathogenesis. While IL-10 may have anti-inflammatory effects on DC's, it may also promote CD4+ T cell egress from lymph nodes in SAPP. Overall, however, IL-10 appears to have a SAPP-accelerating effect, since mice with global IL-10 deficiency are protected from SAPP.

IL-10 protects against inflammation in multiple tissues, such as the colon, joints, and the CNS (38). What leads IL-10 to promote autoimmunity against the PNS, and not against other organs, is not clear. Peripheral nerves fundamentally differ from other tissues in several ways. For instance, a blood-nerve barrier surrounds the PNS, making it an immunologically privileged site (84). Furthermore, since nerve tissue and nerve antigens are widely dispersed throughout the body, the mechanisms behind nerve inflammation and tolerance may have unique characteristics. Also, tertiary lymphoid structures within target organs have been reported to be an important source of autoimmune T cells in autoimmune diabetes, MS, SLE, and RA (85), but are not seen in the PNS. Thus, differences in location (e.g., tertiary vs. secondary) of critical lymphoid structures may be key in accounting for differences between organs.

CIDP has been observed in several patients with mutations in *AIRE* (25); however, the majority of CIDP patients have no known *AIRE* deficiency. Thus, an important question is whether results obtained in the *Aire* deficiency model of SAPP can be generalized. We observed that IL-10 deficiency protected mice from SAPP not only in the *Aire* deficiency ($Aire^{GW/+}$) model, but also in the *Aire*-independent, anti-B7-1/2 antibody-induced model. Thus, our findings suggest that IL-10's promotion of SAPP pathogenesis is a common feature of autoimmune peripheral neuropathies. Since the *Aire*-deficient and anti-B7-1/2 antibody models are both on the NOD mouse background, it remains possible that IL-10's promotion of PNS autoimmunity is

specific to the NOD background. SAPP models reported to date are all on the NOD background (17, 29, 31, 86, 87), so it is not currently possible to test IL-10's effects on SAPP in other mouse strains.

Our results have multiple clinical implications. We show that IL-10 paradoxically promotes SAPP, which suggests increased IL-10 observed in CIDP patients may be a marker of disease progression rather than resolution. Furthermore, IL-10 is currently under investigation to treat autoimmune and inflammatory diseases, including RA (40, 41), MS (42, 43), and Crohn's disease (88). Our data suggest that IL-10 treatment may have previously unanticipated effects: IL-10 therapy may exacerbate disease in CIDP patients and/or unmask PNS autoimmunity in susceptible individuals. FTY720 is currently under investigation for clinical use to treat CIDP, and our data provide support for its use in treating autoimmune peripheral neuropathy (89). Separately, autoimmune peripheral neuropathies are an increasingly common side-effect of current treatments and infections. For example, immune checkpoint inhibitors are being extensively tested in skin, lung, and kidney cancers, but may cause PNS autoimmunity (90, 91). Furthermore, recent Zika virus outbreaks have been strongly linked to an acute autoimmune peripheral neuritis that resembles Guillain-Barré syndrome (92, 93). Thus, there is mounting need to understand the pathogenesis of autoimmune peripheral neuropathies. This study demonstrates a critical role for IL-10 in promoting lymphocyte migration and accelerating PNS autoimmunity, and has far-reaching implications for current treatment strategies in cancer and autoimmunity.

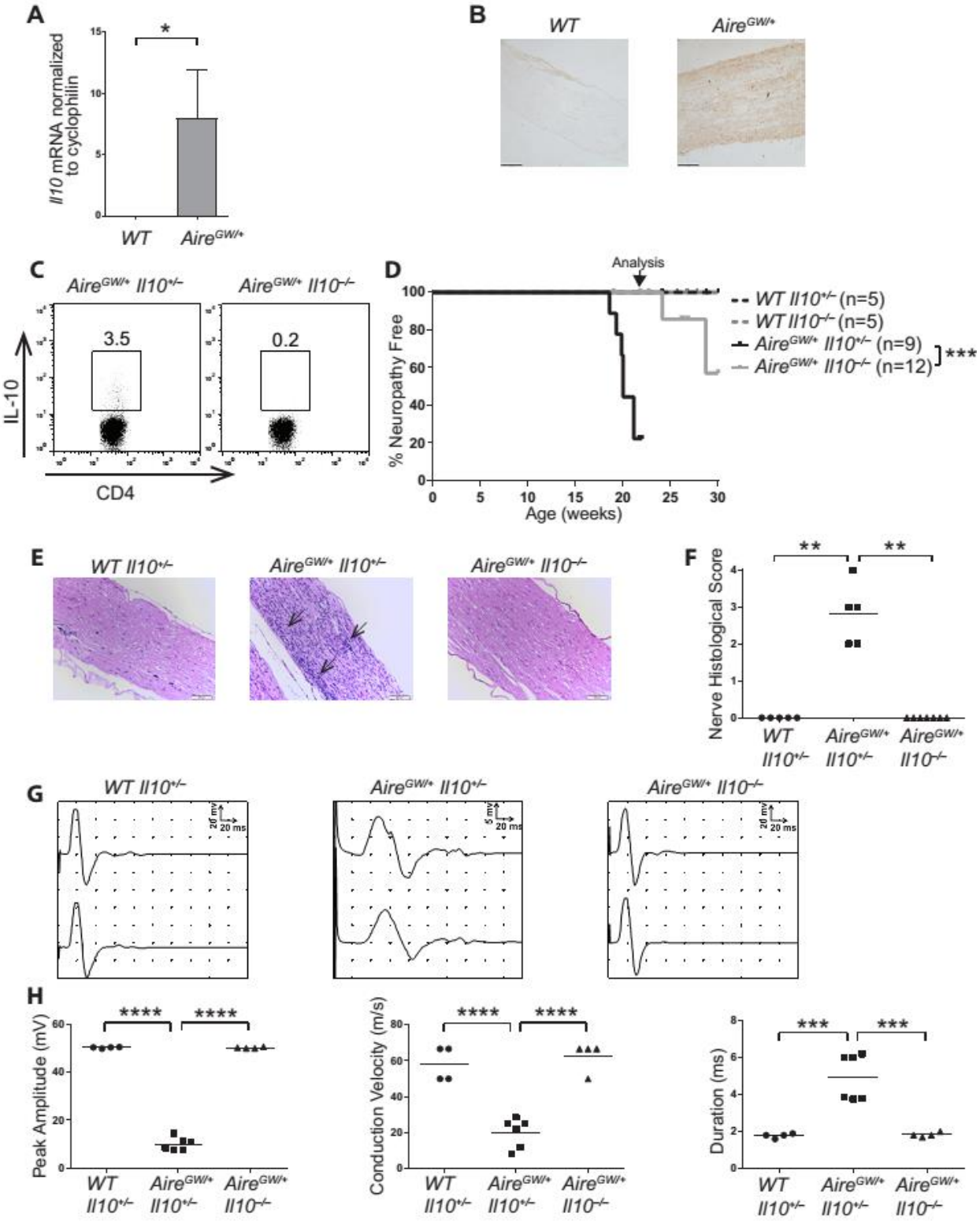


Figure 3.1. IL-10 deficiency is protective in SAPP. Relative IL-10 mRNA expression (A) and immunohistochemical stains of IL-10 (B) (magnification 200x) in sciatic nerves of NOD.WT

(WT, n=3) mice and *NOD.Aire*$^{GW/+}$ (*Aire*$^{GW/+}$, n=4) mice with SAPP. Scale bar = 100 µm. p-value was calculated using two-tailed unpaired Student's *t* test with Welch's correction; *, $p < 0.05$. (C) Representative flow cytometry plots of live, splenic CD4+ T cells from IL-10-sufficient (IL-10$^{+/-}$) and IL-10-deficient (IL-10$^{-/-}$) *Aire*$^{GW/+}$ mice stained for intracellular IL-10. Numbers on plots represent frequencies of IL-10-expressing cells. WT *Il10*$^{+/-}$, WT *Il10*$^{-/-}$, *Aire*$^{GW/+}$ *Il10*$^{+/-}$, and *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice were bred and monitored for SAPP. (D) SAPP incidence curves. Arrow points to age at which nerves were harvested for histology and EMG. p-value was calculated using Mantel-Cox log-rank test of *Aire*$^{GW/+}$ *Il10*$^{+/-}$ versus *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice; ***, $p < 0.001$. (E) H&E-stained sciatic nerves from 22-wk-old mice (magnification 200x). Arrow points to areas of dense immune cell infiltration. Scale bar = 200µm. (F) Cumulative sciatic nerve infiltration scores. p-values were calculated using Fisher's Exact test with Bonferroni's correction; **, $p < 0.01$. (G) Representative proximal and distal compound muscle action potentials from sciatic nerves of 22-wk-old mice. (H) Cumulative peak amplitude, conduction velocity, and duration of compound muscle action potentials from 22-wk-old mice. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; ***, $p < 0.001$, ****$p < 0.0001$. Each symbol represents an individual mouse.

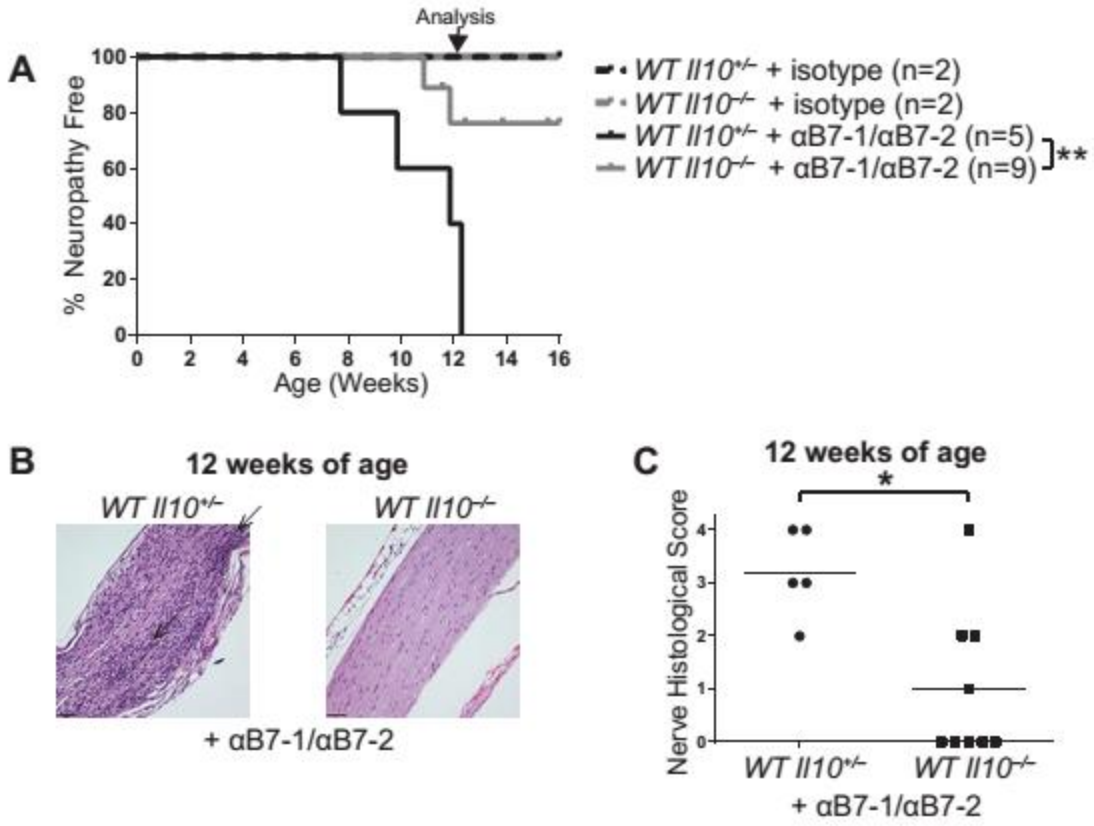


Figure 3.2. IL-10 deficiency is protective in anti-B7-1/anti-B7-2-induced autoimmune peripheral polyneuropathy. Anti-B7-1/anti-B7-2 or isotype treated WT or WT *Il10*$^{-/-}$ mice were monitored for SAPP. (A) SAPP incidence curves. p-value was calculated using Mantel-Cox log-rank test of anti-B7-1/anti-B7-2- treated WT *Il10*$^{+/-}$ versus WT *Il10*$^{-/-}$ mice; **, $p < 0.01$. Arrow points to age at which nerves were harvested for histology in B and C. (B) H&E-stained sciatic nerves from 12-week-old mice (magnification 200x). Arrows point to areas of dense immune cell infiltration. Scale bar = 60 µm. (C) Cumulative sciatic nerve infiltration scores from 12-week-old mice. p-value was calculated using Fisher's Exact test; *, $p < 0.05$. Each symbol represents an individual mouse.

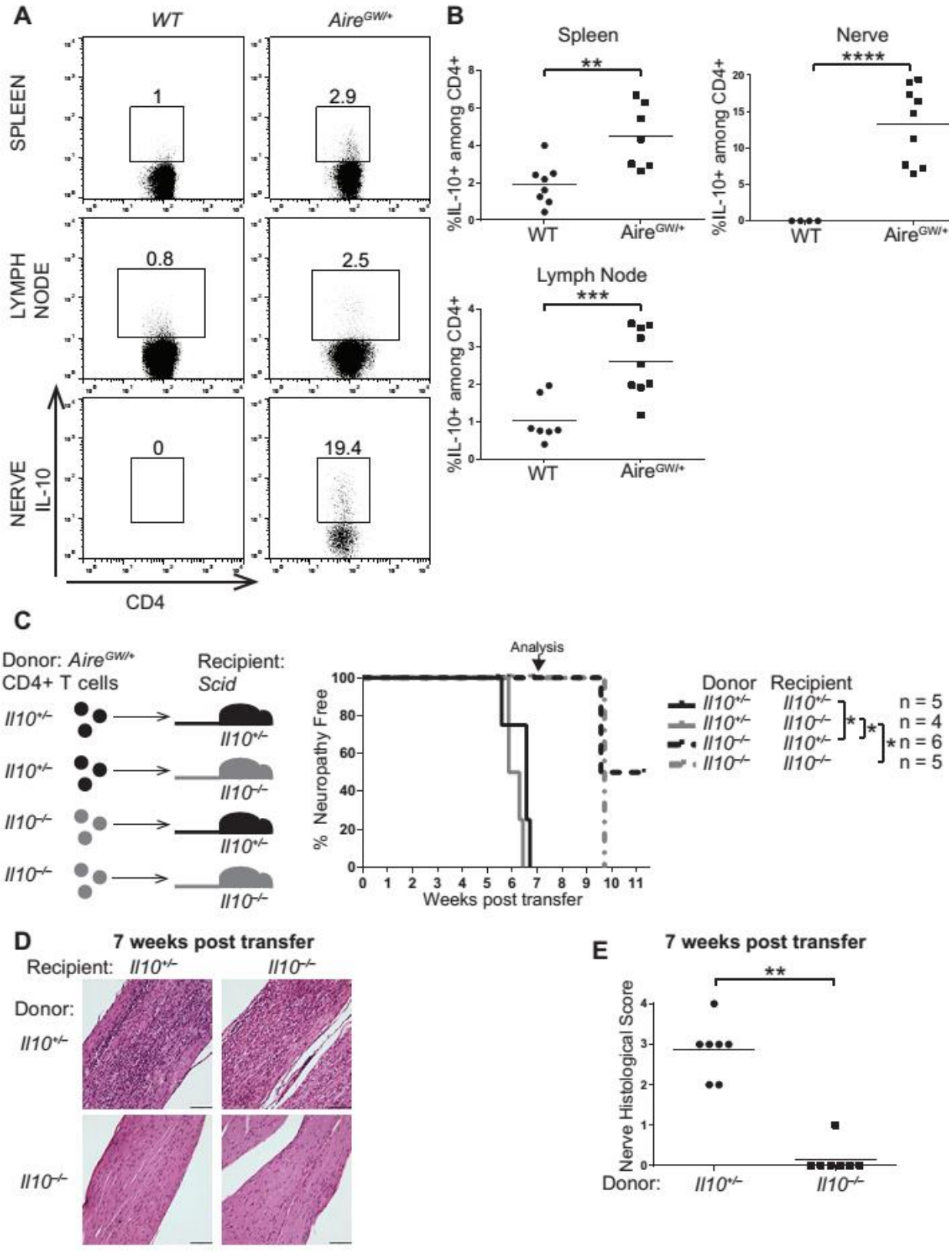

A
WT
Aire GW/+
SPLEEN
LYMPH NODE
NERVE
IL-10
CD4
1
2.9
0.8
2.5
0
19.4
B
Spleen
Nerve
Lymph Node
%IL-10+ among CD4+
WT
Aire GW/+
**
****
***
C
Donor: Aire GW/+ CD4+ T cells
Recipient: Scid
Il10+/-
Il10-/-
Analysis
% Neuropathy Free
Weeks post transfer
Donor Recipient
n = 5
n = 4
n = 6
n = 5
D
7 weeks post transfer
Recipient:
Donor:
E
7 weeks post transfer
Nerve Histological Score

Figure 3.3. IL-10 produced by CD4+ T cells promotes SAPP. Spleens, lumbar lymph nodes, and sciatic nerves of 22-wk-old WT mice and *Aire*$^{GW/+}$ mice stained for intracellular IL-10. (A) Flow cytometry plots of live CD4+ T cells. Numbers on plots represent frequencies of IL-10-expressing cells. (B) Cumulative frequencies of CD4+IL-10+ T cells. p-values were calculated using two-tailed unpaired Student's *t* test with Welch's correction; **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$. (C) Schematic (left) and SAPP incidence curve (right) of NOD.*SCID Il10*$^{+/-}$ or NOD.*SCID Il10*$^{-/-}$ recipient mice receiving purified CD4+ T cells adoptively transferred from 22-wk-old *Aire*$^{GW/+}$ *Il10*$^{+/-}$ or *Aire*$^{GW/+}$ *Il10*$^{-/-}$ donor mice. p-values were calculated using Mantel-Cox log-rank test with false discovery rate adjustment for multiple comparisons; *, $p < 0.05$. Arrow points to time when nerves were harvested for histology. (D) H&E-stained sciatic nerves harvested 7 weeks post CD4+ T cell adoptive transfer from the groups described in C. (magnification 200x). Scale bar = 98 μm. (E) Cumulative infiltration scores of sciatic nerves from recipient mice 7 weeks post transfer. Grouped by donor genotype. p-value was calculated using Fisher's Exact test; **, $p < 0.01$.

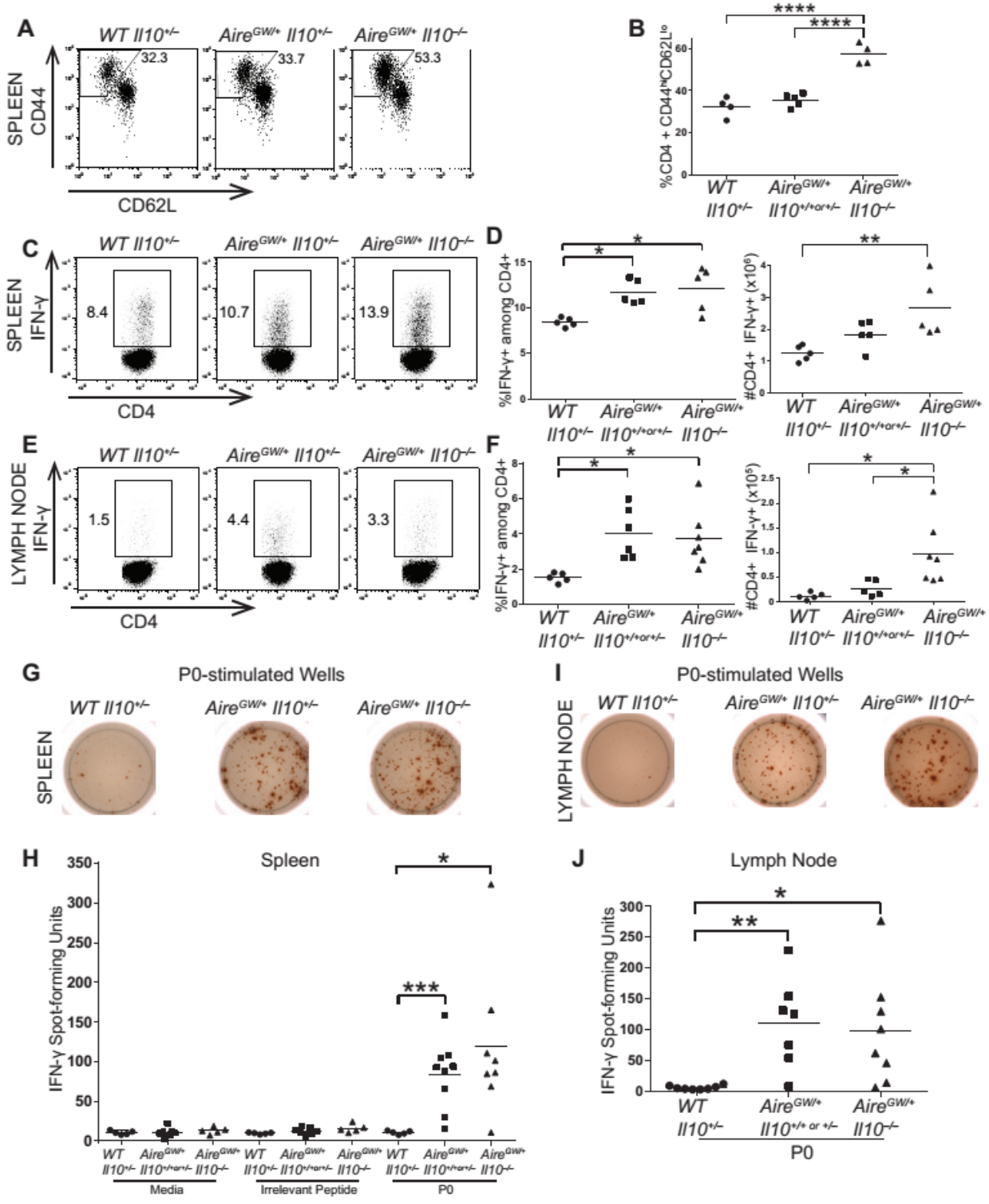


Figure 3.4. CD4+ T cells in IL-10-deficient *Aire*$^{GW/+}$ mice are highly activated. CD4+ T cells from spleens and nerve-draining lymph nodes of 22-wk-old WT, *Aire*$^{GW/+}$ *Il10*$^{+/+\ or\ +/-}$, and

Aire$^{GW/+}$ *Il10*$^{-/-}$ mice were analyzed. (A) Flow cytometry plots of live, splenic CD4+ T cells stained for CD44 and CD62L. Numbers on plots represent frequencies of CD44^{hi}CD62L^{lo} cells. (B) Cumulative frequencies of CD4+CD44^{hi}CD62L^{lo} activated T cells from spleens. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; ****, $p < 0.0001$. (C) Flow cytometry plots of live, splenic CD4+ T cells stained for intracellular IFN-γ. Numbers on plots represent frequencies of IFN-γ-expressing cells. (D) Cumulative frequencies and numbers of CD4+IFN-γ+ T cells from spleens. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; *, $p < 0.05$, **, $p < 0.01$. (E) Flow cytometry plots of live lymph node CD4+ T cells stained for intracellular IFN-γ. Numbers on plots represent frequencies of IFN-γ-expressing cells. (F) Cumulative frequencies and numbers of CD4+IFN-γ+ T cells from lymph nodes. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; *, $p < 0.05$. (G) ELISpot wells detecting IFN-γ from P0-stimulated splenocytes. (H) Cumulative numbers of IFN-γ spots from media only, ovalbumin-stimulated, or P0-stimulated splenocytes. p-values were calculated using two-tailed, unpaired Student's *t* test with Welch's correction and Bonferroni's correction; *, $p < 0.025$, *** $p < 0.001$. (I) ELISpot wells detecting IFN-γ from P0-stimulated lymph node cells. (J) Cumulative numbers of IFN-γ spots from P0-stimulated lymph node cells. p-values were calculated using two-tailed, unpaired Student's *t* test with Welch's correction and Bonferroni's correction; *, $p < 0.025$, ** $p < 0.01$. Each symbol represents an individual mouse.

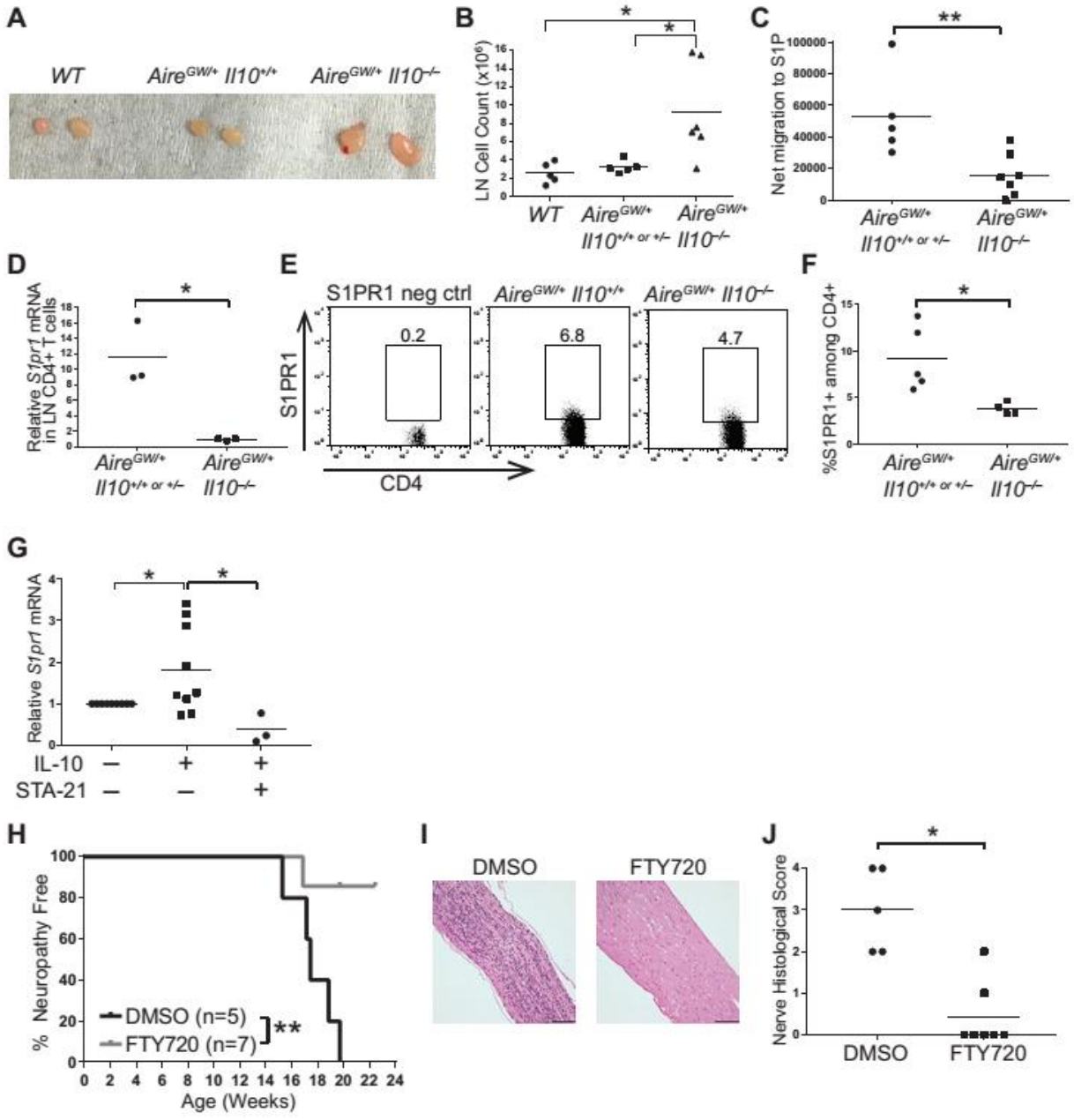


Figure 3.5. IL-10 promotes lymphocyte migration through STAT3-dependent *S1pr1* induction. Image (A) and cumulative cell counts (B) of lumbar lymph nodes from 22-wk-old WT, *Aire*$^{GW/+}$ *Il10*$^{+/+\ or\ +/-}$, and *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; *, $p < 0.05$. (C) Net migration *in vitro* to sphingosine-1-phosphate (S1P) of lymph node cells from 22-wk-old *Aire*$^{GW/+}$ *Il10*$^{+/+\ or}$

$^{+/-}$ and *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice. p-value was calculated using two-tailed unpaired Student's *t* test with Welch's correction; **, $p < 0.01$. CD4+ T cells from lumbar lymph nodes of 22-wk-old *Aire*$^{GW/+}$ *Il10*$^{+/+ \text{ or } +/-}$ and *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice were analyzed for S1PR1 expression. (D) Relative *S1pr1* mRNA expression. p-value was calculated using two-tailed unpaired Student's *t* test with Welch's correction; *, $p < 0.05$. (E) Flow cytometry plots of live, lumbar lymph node CD4+ T cells stained for extracellular S1PR1. Numbers on plots represent frequencies of S1PR1-expressing cells. "S1PR1 neg ctrl" indicates negative control for S1PR1 staining. (F) Cumulative frequencies of CD4+S1PR1+ T cells. p-value was calculated using two-tailed unpaired Student's *t* test with Welch's correction; *, $p < 0.05$. (G) Relative *S1pr1* mRNA expression of lumbar lymph node cells from WT *Il10*$^{-/-}$ mice stimulated with recombinant IL-10 or IL-10 plus STAT3 inhibitor (STA-21). p-values were calculated using one-sample *t* test (Unstimulated versus IL-10) and two-tailed unpaired Student's *t* test (IL-10 versus IL-10 + STA-21) with false discovery rate adjustment for multiple comparisons; * indicates $p < 0.05$. DMSO or FTY720-treated *Aire*$^{GW/+}$ mice were monitored for SAPP. (H) SAPP incidence curve. p-value was calculated using Mantel-Cox log-rank test; **, $p < 0.01$. (I) H&E-stained sciatic nerves (magnification 200x). Scale bar = 98 μm. (J) Cumulative sciatic nerve infiltration scores. p-value was calculated using Fisher's Exact test; *, $p < 0.05$.

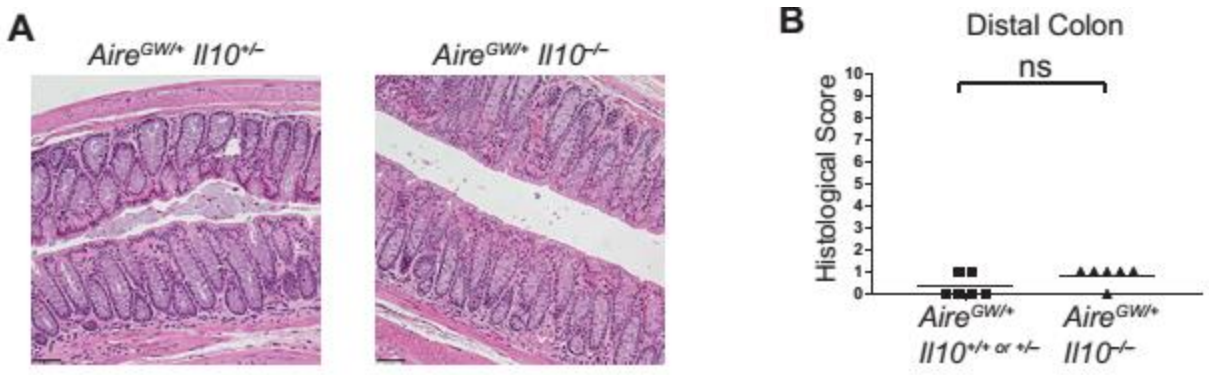


Figure 3.6. IL-10-deficient *Aire*$^{GW/+}$ mice have minimal colitis. Image (A) and cumulative histological scores (B) of H&E-stained distal colon from 22-wk-old *Aire*$^{GW/+}$ *Il10*$^{+/+ \text{ or } +/-}$ and *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice (magnification 200x). Scale bar = 60 µm. p-value was calculated using Fisher's Exact test. ns indicates no significant difference was observed. Each symbol represents an individual mouse.

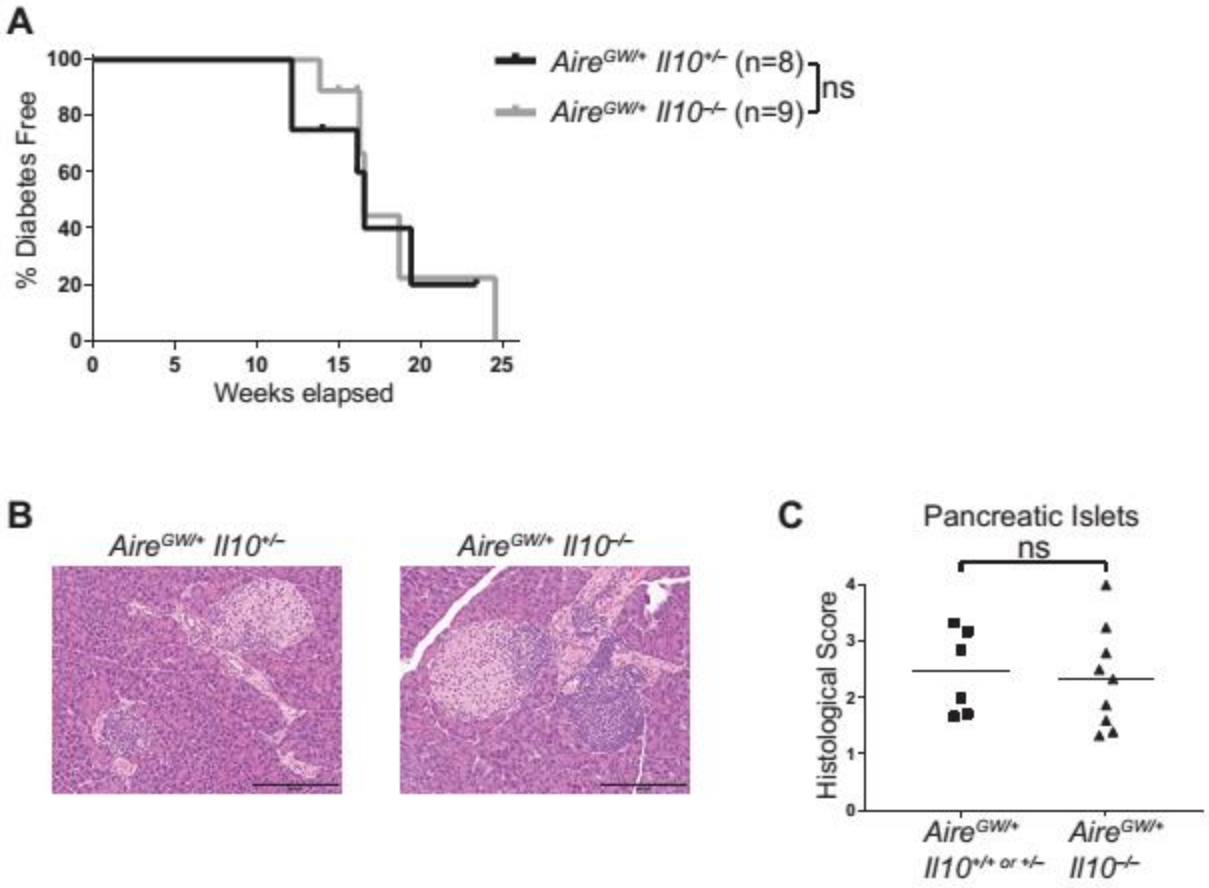


Figure 3.7. IL-10 deficiency does not affect diabetes in *Aire*$^{GW/+}$ mice. *Aire*$^{GW/+}$ *Il10*$^{+/–}$ and *Aire*$^{GW/+}$ *Il10*$^{–/–}$ mice were monitored for diabetes. (A) Diabetes incidence curve. p-value was calculated using Mantel-Cox log-rank test; ns indicates no significant difference was observed. Pancreatic islet images (B) and cumulative pancreatic islet infiltration scores (C) from 22-wk-old mice (magnification 200x). Scale bar = 200 µm. p-value was calculated using Fisher's Exact test. ns indicates no significant difference was observed. Each symbol represents an individual mouse.

CHAPTER 4: ADDITIONAL EXPERIMENTS ON IL-10-DEFICIENT *NOD.AIRE*$^{GW/+}$ MICE.

4.1 Introduction

While investigating the effects of IL-10 deficiency on SAPP in *NOD.Aire*$^{GW/+}$ mice, I performed various experiments that provide important insight into the immunology of the mice. The data are presented in this chapter.

4.2 Materials and methods

Mice

NOD.Aire$^{GW/+}$ mice were generated as previously described (63). *NOD.Il10*$^{-/-}$ (JAX stock #004266) mice were purchased from The Jackson Laboratory. C57BL/6 mice were purchased from The Jackson Laboratory. *BL/6.Il10*$^{-/-}$ mice were generous gifts from the R. Balfour Sartor lab. Mice were housed in a specific-pathogen-free (SPF) barrier facility at the University of North Carolina, Chapel Hill. Clinical neuropathy, determined by hind limb weakness, and diabetes, determined by presence of glucose in urine, were assessed at least once per week as described previously (64). Diabetic mice were treated daily with intraperitoneal insulin until used in experiments or euthanasia (due to >20% weight loss). Only female mice were utilized due to higher SAPP incidence relative to males (17). Mice were utilized at 22 weeks of age unless otherwise noted. Due to the rapid progression of disease and death following initial symptoms, mice that developed SAPP prior to 22 weeks of age were harvested at SAPP onset.

NOD.Aire$^{GW/+}$ *Il10*$^{+/-}$ or *NOD.Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice were used as splenocyte donors. Experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research.

Histology

Harvested organs were fixed in 10% buffered formalin for at least 96 hours, washed in 30% ethanol for 20 minutes, and then stored in 70% ethanol. Organs were embedded in paraffin, sectioned, and stained with H&E by the UNC Animal Histopathology Core. Immune infiltration was scored while blinded to genotype as previously described (63). For salivary glands, lacrimal glands, thyroid glands, and eyes, scores of 0, 1, 2, 3, and 4 indicate 0%, 1-25%, 26-50%, 51-75%, and >75% infiltration, respectively.

Flow cytometry

Flow cytometry was performed as previously described (67). Briefly, single-cell suspensions were isolated from spleens or lymph nodes by crushing with forceps in DMEM + 10% FBS, from sciatic nerves by mincing and digestion in 2 mg/ml collagenase + 1% FBS, or from pancreatic islet infiltrates by mashing whole pancreas into a 70 μm filters with the butt of a 5 mL syringe in DMEM + 10% FBS. Cells were stained with live/dead fixable yellow dye (Life Technologies), anti-mouse CD21, anti-mouse CD23, anti-mouse IL-17, anti-mouse CXCR3, anti-mouse S1PR1 (R&D clone 713412), anti-mouse IFN-γ (eBioscience clone XMG1.2), anti-mouse FoxP3, anti-mouse CD69, anti-mouse CD25 (clone PC61.5), and anti-mouse IL-10 (BD clone JES5-16E3) antibodies. For intracellular cytokine staining, cells were stimulated with PMA/ionomycin for 4 hours at 37 ºC 5% CO_2, and permeabilized using BD Cytofix/Cytoperm

according to manufacturer's instructions. Cells were analyzed on a CyAn ADP Analyzer (Beckman Coulter). Data were analyzed using FlowJo X.

Real Time RT-PCR

RNA was isolated from cells using the Qiagen mini prep kit or Zymo RNA MicroPrep kit. Superscript II (Invitrogen) reverse transcriptase was used to create cDNA. TaqMan universal PCR Master Mix (Applied Biosystems) was used for qPCR. Commercially available TaqMan primer-probe sets for IFN-γ, IL-10, Cxcl10, Icam1 and S1Pr1 were used (Applied Biosystems). Cyclophilin A was used as an internal control and detected with the primer-probe set reported by (63). Reactions were run on a Quantstudio 6 Flex system (Life Technologies) and analyzed as described (63).

4.3 CD8+ T cells are more activated and produce more IFN-γ in IL-10-deficient SAPP.

Summary

CD8+ cytotoxic T cells may play a role in SAPP, but are not required for disease since transfer of CD4+ T cells alone induces SAPP in *SCID* recipients (17, 36). It is possible that IL-10 promotes SAPP through CD8+ T cells since IL-10 promotes the growth, differentiation, and cytotoxicity of CD8+ T cells (38). Thus, CD8+ T cells were tested for activation markers and IFN-γ secretion alongside CD4+ T cells in IL-10-deficient *Aire*$^{GW/+}$ mice.

The frequencies of splenic CD8+CD44^{hi}CD62L^{lo} activated CD8 T cells were increased in IL-10-deficient *Aire*$^{GW/+}$ mice relative to IL-10-sufficient controls (Figure 4.1A, B). The numbers of IFN-γ-secreting CD8+ T cells in nerve-draining lymph nodes were increased in IL-10-deficient *Aire*$^{GW/+}$ mice relative to WT and IL-10-sufficient control mice (Figure 4.2C, D). These data suggested that, similarly to CD4+ T cells, CD8+ T cells were more activated in IL-10 deficiency. In contrast to CD4+ T cells, IFN-γ secretion was not globally increased in CD8+ T cells. However, the increased numbers of IFN-γ-secreting CD8+ T cells in nerve-draining lymph nodes supports the hypothesis that T cell emigration from lymph nodes is reduced.

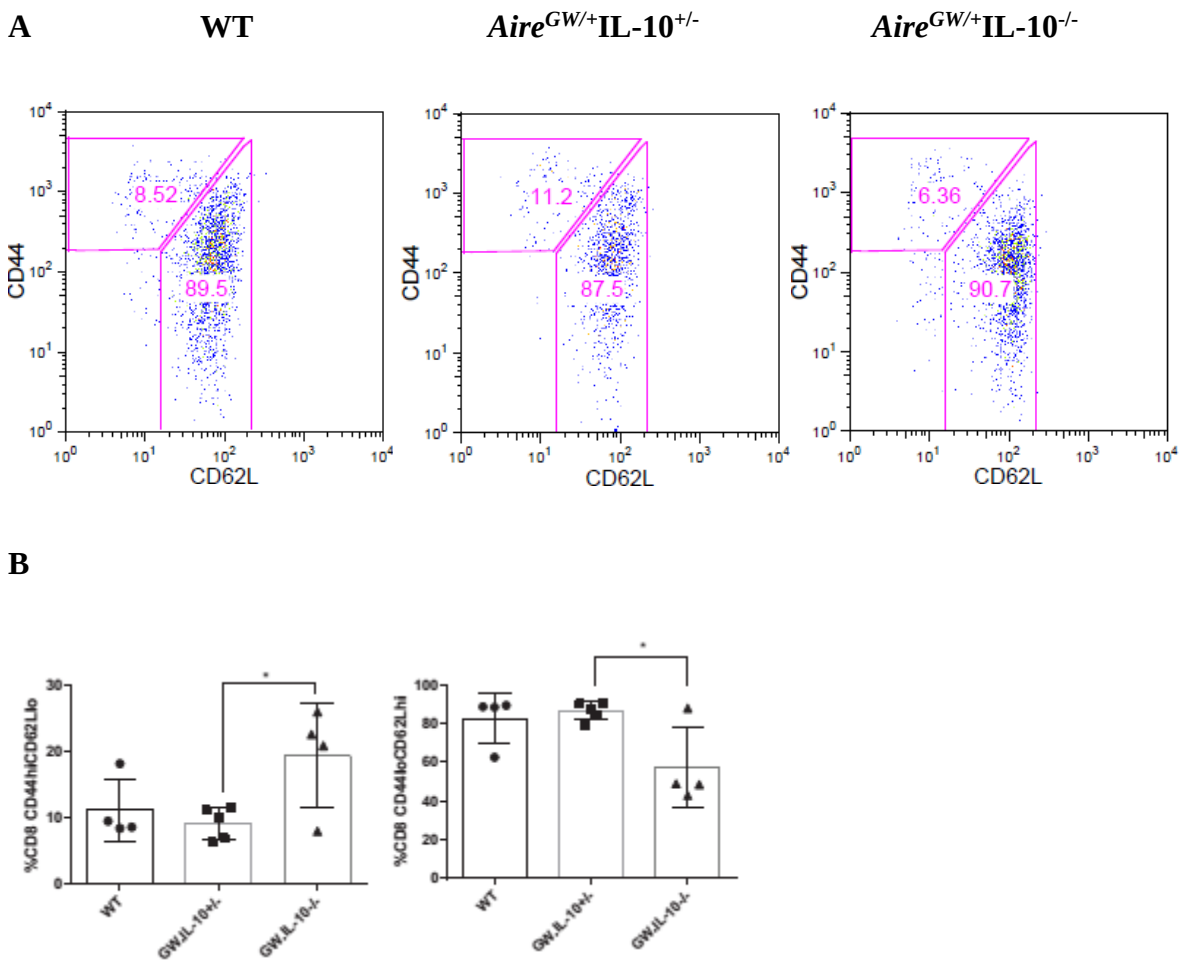
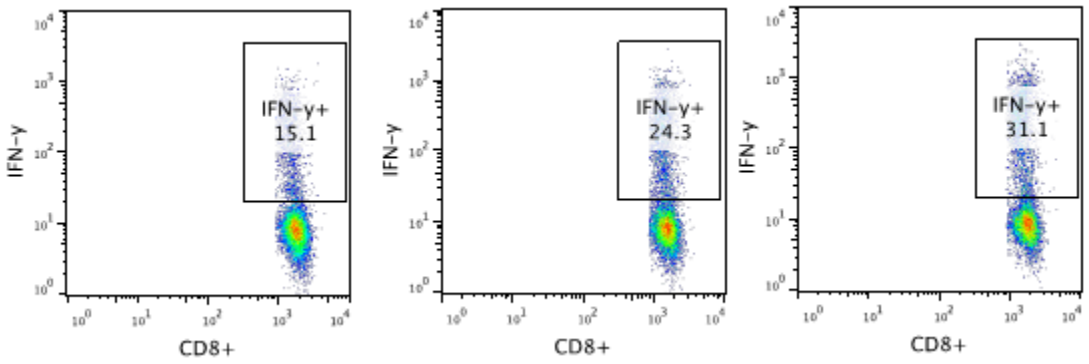


Figure 4.1. Activated CD8+ T cells are increased in frequency in IL-10-deficient *Aire*$^{GW/+}$ mice relative to IL-10-sufficient counterparts. CD8+ T cells from spleens and nerve-draining lymph nodes of 22-wk-old WT, *Aire*$^{GW/+}$ *Il10*$^{+/+ \text{ or } +/-}$, and *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice were analyzed. (A) Representative flow cytometry plots of live, splenic CD8+ T cells stained for CD44 and CD62L. Numbers on plots represent frequencies of CD44^{hi}CD62L^{lo} cells. (B) Cumulative frequencies of CD8+CD44^{hi}CD62L^{lo} activated T cells and CD8+CD44^{lo}CD62L^{hi} naïve T cells from spleens. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; ****, $p < 0.0001$.

A **WT** ***Aire*$^{GW/+}$IL-10$^{+/-}$** ***Aire*$^{GW/+}$IL-10$^{-/-}$**



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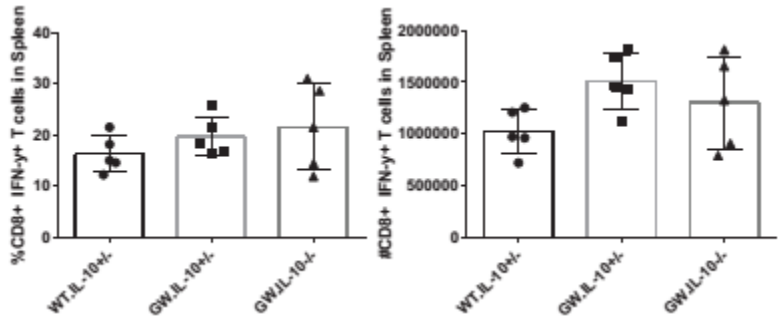
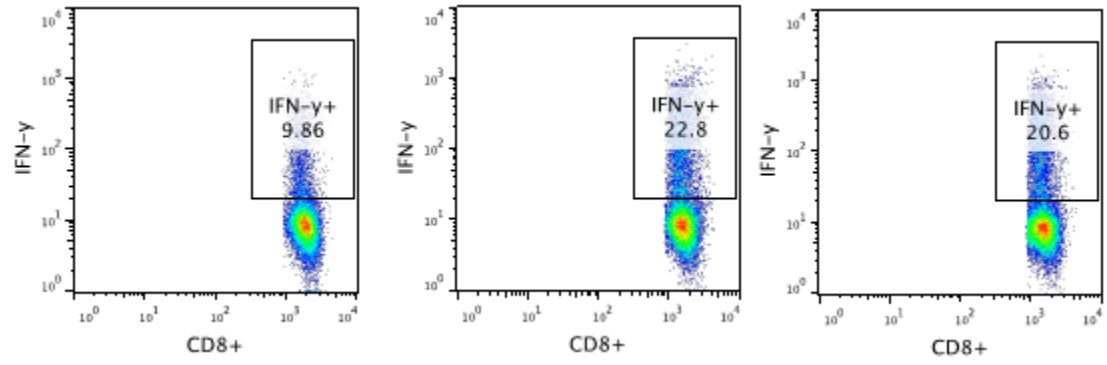


C **WT** ***Aire*$^{GW/+}$IL-10$^{+/-}$** ***Aire*$^{GW/+}$IL-10$^{-/-}$**



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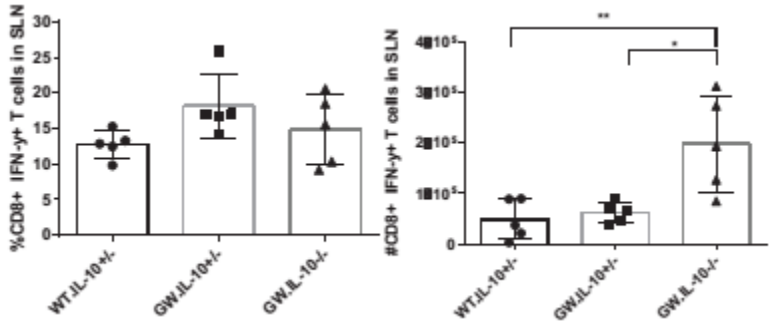


Figure 4.2. Numbers of IFN-γ-expressing CD8+ T cells are increased in the lymph nodes of IL-10-deficient *Aire*$^{GW/+}$ mice. CD8+ T cells from spleens and nerve-draining lymph nodes of 22-wk-old WT, *Aire*$^{GW/+}$ *Il10*$^{+/+\ or\ +/-}$, and *Aire*$^{GW/+}$ *Il10*$^{-/-}$ mice were analyzed. (A) Flow cytometry plots of live, splenic CD8+ T cells stained for intracellular IFN-γ. Numbers on plots represent frequencies of IFN-γ-expressing cells. (B) Cumulative frequencies and numbers of CD8+IFN-γ+ T cells from spleens. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; *, $p < 0.05$, **, $p < 0.01$. (C) Flow cytometry plots of live lymph node CD8+ T cells stained for intracellular IFN-γ. Numbers on plots represent frequencies of IFN-γ-expressing cells. (D) Cumulative frequencies and numbers of CD8+IFN-γ+ T cells from lymph nodes. p-values were calculated using one-way ANOVA with Tukey's correction for multiple comparisons; *, $p < 0.05$.

4.4 Marginal Zone B cells are increased in frequency in IL-10-deficient *Aire*$^{GW/+}$ mice.

Summary

We sought to identify the cellular mechanism by which IL-10 promoted SAPP development. IL-10 production by Th2 cells enhances B cell proliferation, differentiation, and antibody production (44, 45), and B cells have been shown to promote SAPP in B7-2-deficient mice (32). Hence, we tested the possibility that IL-10 promoted SAPP by activating B cells. The marginal zone subset of B cells expands (94) and secretes pathogenic autoantibodies in mouse models of the autoimmune disease systemic lupus erythematosus (95). Therefore, we investigated whether the marginal zone B cell population was expanded in *Aire*$^{GW/+}$ mice. Marginal zone B cells are identified by CD19+CD21^{hi}CD23^{lo} (96). Separately, follicular B cells are identified by CD19+CD21^{int}CD23+ (97). IL-10-sufficient *Aire*$^{GW/+}$ mice (15-22 wk old) had significantly increased frequencies of marginal zone B cells compared to WT (15-22 wk old) controls (Figure 4.3A, B). This increase in marginal zone B cells was absent in IL-10-deficient *Aire*$^{GW/+}$ mice (22 wk old), suggesting the increase was IL-10 dependent. Concomitantly, splenic follicular B cell frequencies were significantly reduced in IL-10-sufficient *Aire*$^{GW/+}$ mice compared to WT controls and IL-10-deficient *Aire*$^{GW/+}$ mice (Figure 4.3A, B). Increased marginal zone B cell and reduced follicular B cell frequencies correlate with SAPP in IL-10-sufficient *Aire*$^{GW/+}$ mice, which could indicate IL-10-dependent, marginal zone B cell-driven autoimmunity.

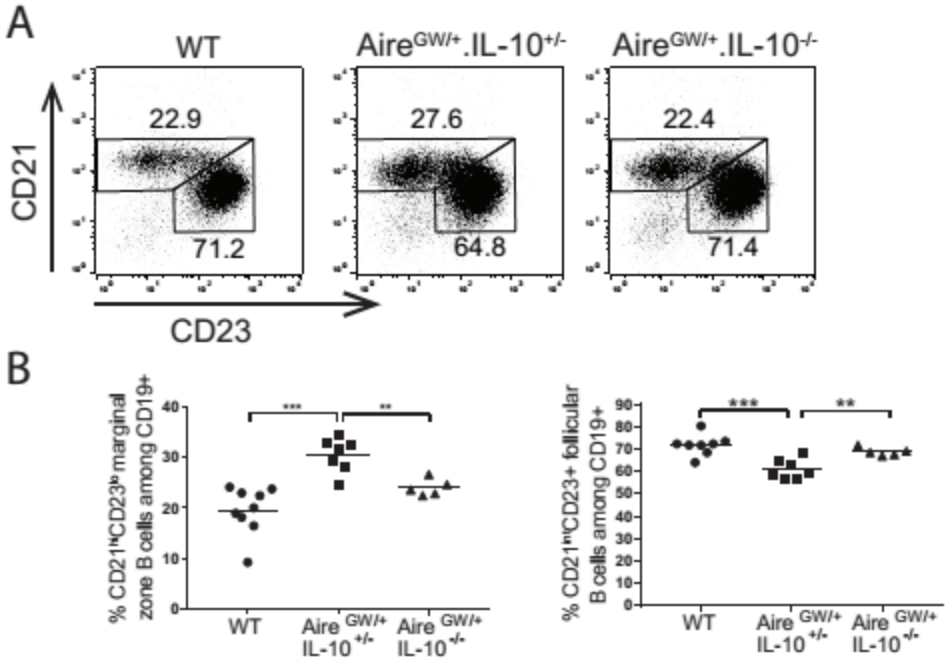


Figure 4.3. Marginal zone B cells are increased in IL-10-deficient *Aire*$^{GW/+}$ mice. A. Representative flow cytometry plots of live, singlet, CD19+ splenocytes from *WT*, *Aire*$^{GW/+}$*Il10*$^{+/-}$ and *Aire*$^{GW/+}$*Il10*$^{-/-}$ mice stained for CD21 and CD23 surface expression. Numbers represent frequency of CD21+CD23- marginal zone and CD21-CD23+ follicular B cells. B. Cumulative frequencies. Each symbol represents an individual mouse. p values were calculated using one-way ANOVA. **p<0.01, ***p<0.001.

4.5 Protection from SAPP in IL-10-deficient *Aire*$^{GW/+}$ mice was not mediated by B cells[1]

Summary

IL-10 production by CD4+ T helper 2 (Th2) cells can promote inflammation by enhancing B cell proliferation, differentiation, and antibody production (38, 44, 45). We therefore sought to test whether IL-10 promotion of SAPP development requires B cells.To test whether B cells were pathogenic in SAPP, we crossed *Aire*$^{GW/+}$ mice with NOD.μMT$^{-/-}$ mice that lack mature B cells (98), and monitored SAPP in a female cohort of *Aire*$^{GW/+}$.μMT$^{+/-}$ and *Aire*$^{GW/+}$.μMT$^{-/-}$ mice. B cell-deficient WT mice (WT.μMT$^{-/-}$) did not develop SAPP, and served as healthy controls (Smith *et al.*, 2017 Figure 3C-3F). SAPP occurred with the same incidence in B cell-sufficient and B cell-deficient *Aire*$^{GW/+}$ mice (Smith *et al.*, 2017 Figure 3C), and sciatic nerves exhibited similar infiltration (Smith *et al.*, 2017 Figure 3D). B cell-sufficient and B cell-deficient *Aire*$^{GW/+}$ mice displayed similar reductions in peak amplitude, reductions in conduction velocity, and increases in action potential duration, indicating equivalent demyelination (Smith *et al.*, 2017 Figure 3E, 3F). In summary, B cell deficiency does not protect *Aire*$^{GW/+}$ mice from SAPP, suggesting B cells are not pathogenic. Since B cells do not accelerate SAPP development, it is unlikely that B cell enhancement is the mechanism by which IL-10 promotes SAPP.

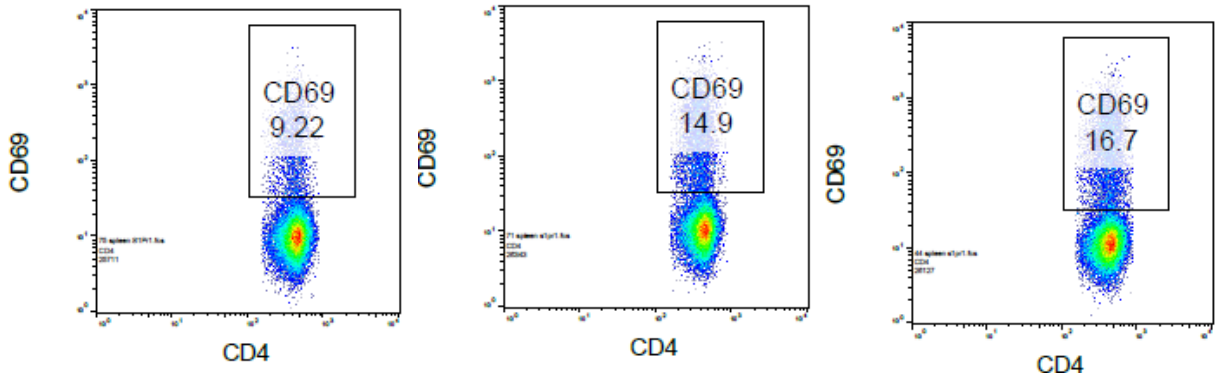
4.6 CD69 expression is not increased in IL-10-deficient *NOD.Aire$^{GW/+}$* mice.

Summary

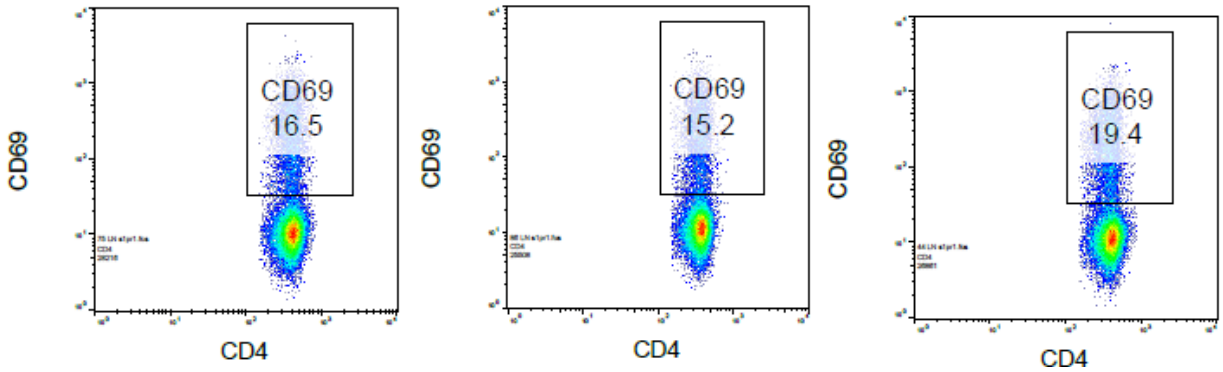
IL-10-deficient *Aire$^{GW/+}$* mice exhibited delayed SAPP and lymph node lymphocyte migration defects due to reduced S1PR1 expression in CD4+ T cells. IL-10 deficiency could indirectly lead to reduced S1PR1 expression through increased CD69 expression. CD69 is upregulated during the first few hours after T cell activation (1); it binds to S1PR1 and reduces its surface expression, suppressing emigration of the T cell from the lymph node (52). Furthermore, IL-10 deficiency promotes a pro-inflammatory phenotype in APCs (38) and correlates with increased T cell activation (Figure 3.4). Thus, loss of IL-10 could lead to increased CD69 expression which could then reduce S1PR1 in T cells. To investigate this possibility, I measured CD69 expression in CD4+ T cells of IL-10-sufficient versus IL-10-deficient *Aire$^{GW/+}$* mice.

CD69 expression was not different in CD4+ T cells from spleens and lymph nodes of IL-10-deficient *Aire$^{GW/+}$* mice compared to WT or IL-10-sufficient *Aire$^{GW/+}$* controls (Figure 4.4). These data suggested increased CD69 expression was unlikely to be the cause of reduced S1PR1 on CD4+ T cells. Although CD4+ T cells were more activated overall, CD69 expression is transient. Thus, it is possible that T cell activation is increased, but average CD69 expression is unchanged.

A **WT** ***Aire*$^{GW/+}$IL-10$^{+/-}$** ***Aire*$^{GW/+}$IL-10$^{-/-}$**



B **WT** ***Aire*$^{GW/+}$IL-10$^{+/-}$** ***Aire*$^{GW/+}$IL-10$^{-/-}$**



C

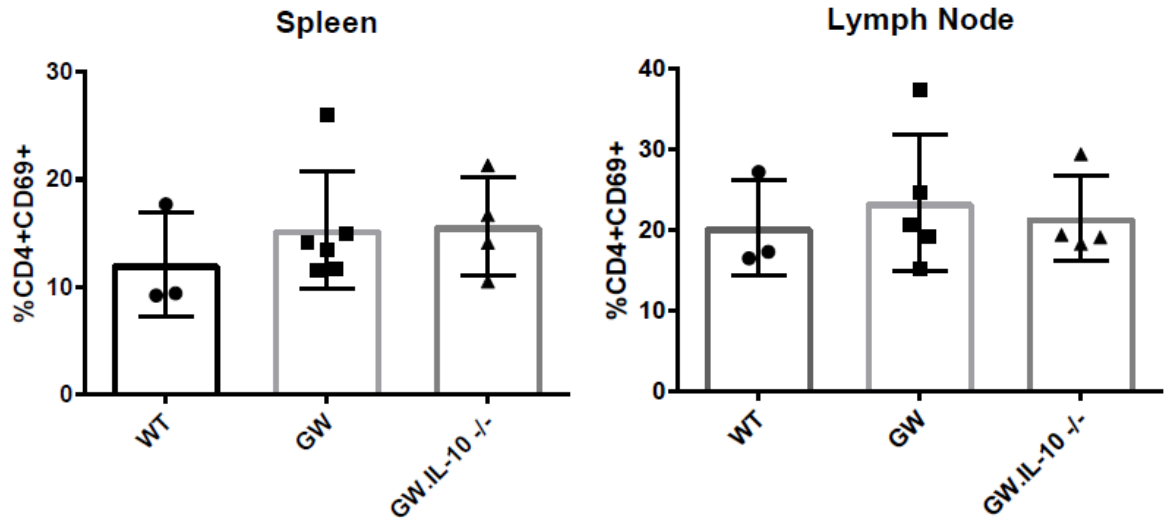


Figure 4.4. IL-10 deficiency does not affect CD69 expression in CD4+ T cells. CD4+ T cells from spleens and lymph nodes of 22-week-old *WT*, *Aire*$^{GW/+}$*Il10*$^{+/-}$ and *Aire*$^{GW/+}$*Il10*$^{-/-}$ were stained for CD69 expression using flow cytometry. A. Representative plots of CD4+ T cells from spleens. B. Representative plots of CD8+ T cells from lymph nodes. C. Cumulative frequencies of CD4+CD69+ T cells from spleens and lymph nodes. p values were calculated using one-way ANOVA and Tukey's correction for multiple comparisons. All comparisons were not significant.

4.7 S1pr1 mRNA is reduced in IL-10-deficient B6 mice.

Summary

IL-10 deficiency causes reduced T cell migration in *NOD.Aire*$^{GW/+}$ mice due to reduced S1pr1 expression in CD4+ T cells (Figure 3.5). IL-10's induction of S1pr1 may be restricted to the NOD mouse background or generalizable to other backgrounds. To determine whether the finding is generalizable, I tested S1pr1 mRNA in purified CD4+ T cells from lumbar lymph nodes of IL-10-sufficient and IL-10-deficient B6 mice.

CD4+ T cells from *B6.Il10*$^{-/-}$ mice expressed significantly less S1pr1 mRNA compared to B6 WT mice (Figure 4.5). These data suggest the association between IL-10 deficiency and reduced S1pr1 expression is generalizable to other mouse backgrounds. The difference between IL-10-sufficient and IL-10-deficient NOD mice is much larger than the same comparison in B6 mice (~12-fold in NOD versus 1.5-fold in B6). This difference may be due to the systemic autoimmunity in NOD mice, which creates an inflammatory milieu that boosts IL-10-secretion and S1pr1 expression. Conversely, B6 WT mice are uninflamed.

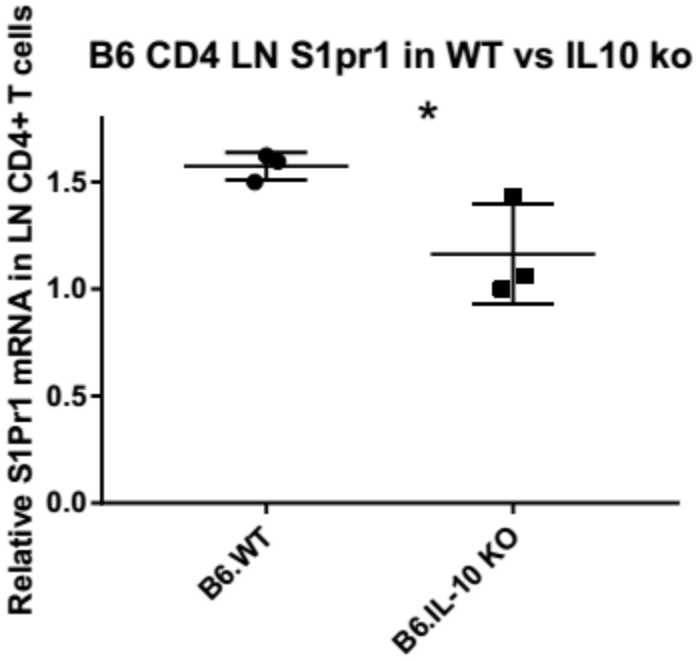


Figure 4.5. IL-10-deficient B6 mice have reduced S1pr1 mRNA in CD4+ T cells from lumbar lymph nodes. RNA was isolated from purified CD4+ T cells from lumbar lymph nodes of 12-week-old B6 female mice. S1pr1 mRNA was measured using qPCR with cyclophilin A as the control. Student's *t* test. *$p < 0.05$.

4.8 IL-10 deficiency does not affect autoimmune infiltration of organs beyond peripheral nerves.

Summary

APS-1 patients develop autoimmunity against various organs; endocrine organs are most frequently affected (15). Similarly, Aire-deficient *NOD* mice develop spontaneous autoimmunity against some of the same tissues (63). To assess whether IL-10 deficiency affects autoimmunity in other tissues, I measured tissue infiltration in the eye, thyroid gland, salivary gland, lacrimal gland, pancreatic islets, and distal colon.

Histological infiltration scores in the organs tested were not significantly different in IL-10-deficient $Aire^{GW/+}$ of mice versus IL-10-sufficient counterparts (Figures 3.6, 3.7, and 4.6). These data suggested IL-10 deficiency does not affect autoimmunity in organs beyond the PNS. Thyroid gland infiltration trended towards reduction with a near-significant p value ($p = 0.05303$). Thus, it is possible that IL-10 deficiency is also protective in thyroiditis.

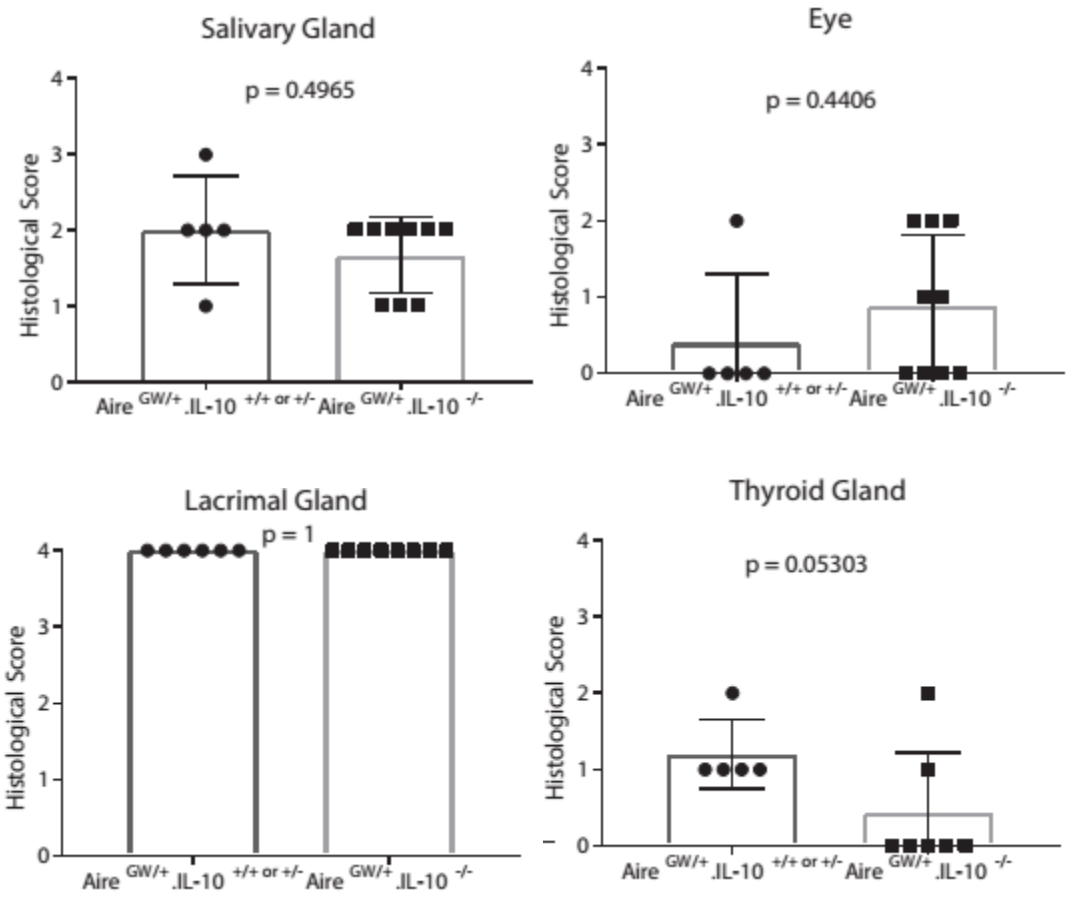


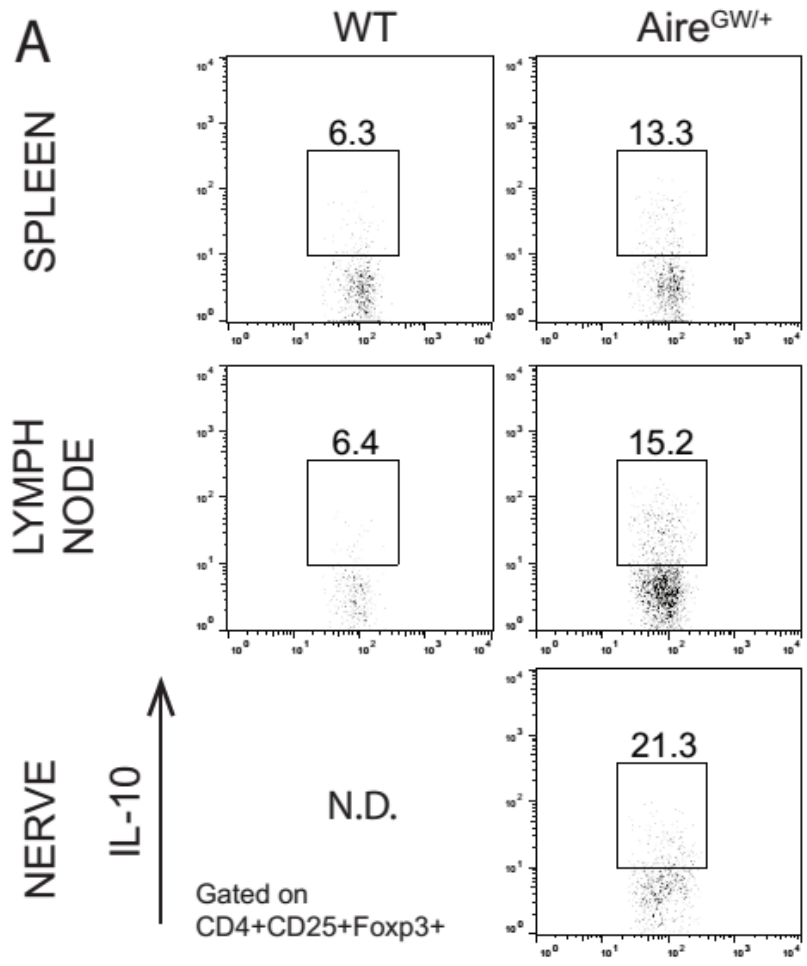
Figure 4.6. Autoimmune manifestations beyond SAPP are not significantly affected by IL-10 deficiency in *Aire*$^{GW/+}$ mice. Salivary glands (sialoadenitis), eyes (chorioretinitis), lacrimal glands (dacryoadenitis), and thyroid glands (thyroiditis) were harvested from 22-week-old *Aire*$^{GW/+}$*Il10*$^{+/+\ or\ +/-}$ and *Aire*$^{GW/+}$*Il10*$^{-/-}$ mice. p values were calculated using Student's *t* test. All p values were greater than 0.05 and therefore not significant.

4.9 IL-10 is induced in regulatory T cells and IFN-γ-expressing CD4+ T cells in SAPP.

Summary

Given that CD4+ T cells are sufficient to transfer SAPP (17, 29, 64), IL-10 was induced in CD4+ T cells in *Aire*$^{GW/+}$ mice (Figure 3.1B, C), and IL-10 produced by CD4+ T cells promoted SAPP (Figure 3.3), we further characterized the CD4+ T cell subsets expressing IL-10. FoxP3+ Tregs suppress T cells and APCs through production of IL-10 (39). IL-10 expression in CD4+CD25+FoxP3+ Tregs from the spleen, lumbar lymph node, and sciatic nerve infiltrates in neuropathic *Aire*$^{GW/+}$ mice was significantly increased compared to WT mice (Figure 4.7D, E). One would presume that the IL-10 produced by FoxP3 Tregs is anti-inflammatory since Tregs are immunosuppressive and they suppress SAPP development (unpublished data). However, given that IL-10 overall promotes SAPP (Chapter 3), it is possible that Treg IL-10 also contributes to S1pr1-mediated egress of nerve-reactive effector T cells. Further studies may determine the function of Treg IL-10 (see Chapter 5).

We also detected CD4+CD25-FoxP3- T cells that produced both IL-10 and IFN-γ. IL-10 and IFN-γ double-positive Th1 cells were significantly increased in spleen, lumbar lymph node, and sciatic nerve infiltrates in neuropathic *Aire*$^{GW/+}$ mice compared to WT mice (Figure 4.8F, G). Th1 cells that coexpress IL-10 are thought to be mediating their own immunosuppression (9). Additionally, it is possible that these cells contain Tr1 cells, which are FoxP3 negative immunosuppressive cells (99). However, results from Chapter 3 suggest these T cells may be promoting their egress from lymph nodes and subsequently promoting SAPP. Thus, either an immunosuppressive subset is exhibiting immune-activating properties, or this subset is proinflammatory despite expressing IL-10. In summary, IL-10 is induced in multiple CD4+ T cell subsets in SAPP, and the individual effects of IL-10 produced by these subsets remain to be determined (see Chapter 5).


A
WT
Aire^GW/+
SPLEEN
6.3
13.3
LYMPH NODE
6.4
15.2
NERVE
IL-10
N.D.
21.3
Gated on
CD4+CD25+Foxp3+

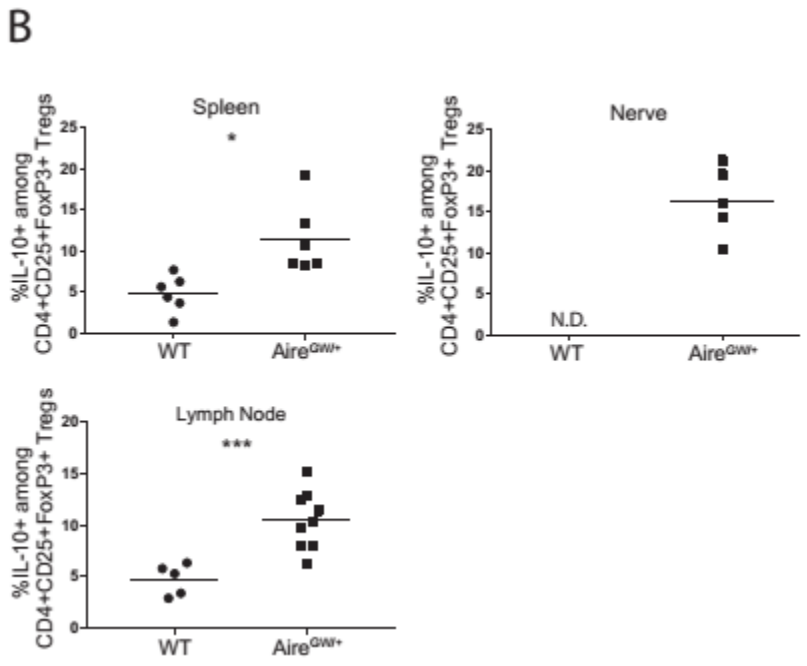
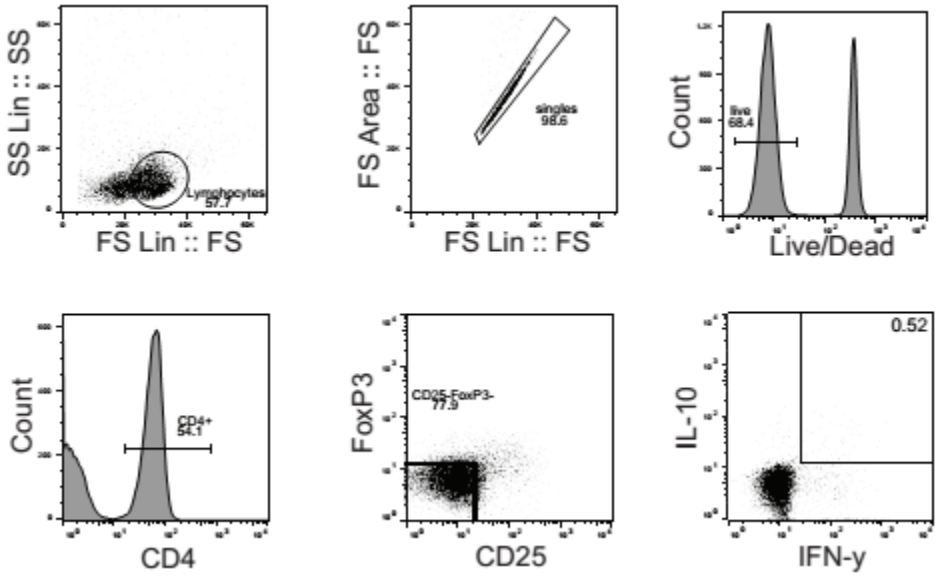
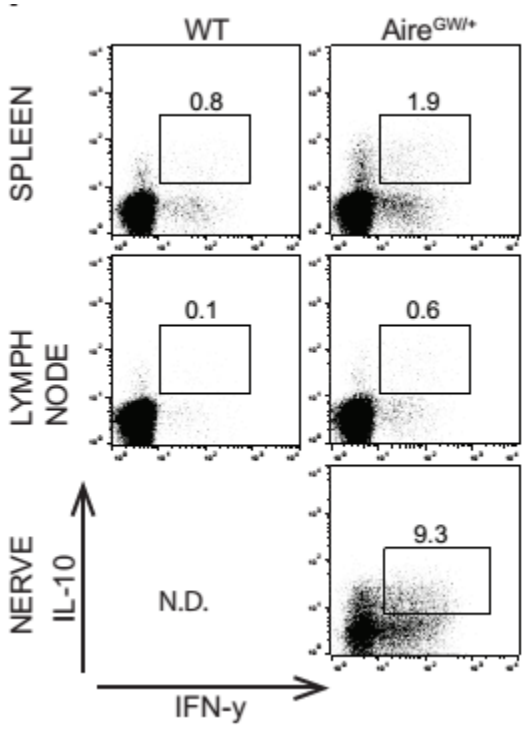

B
Spleen
*
%IL-10+ among CD4+CD25+FoxP3+ Tregs
WT
Aire^GW/+
Nerve
N.D.
Lymph Node
***

Figure 4.7. IL-10 production by FoxP3 Tregs is increased in SAPP. CD4+CD25+FoxP3+ Tregs from spleen, lumbar lymph nodes, and sciatic nerves of WT and *Aire*$^{GW/+}$ mice were stained for intracellular IL-10 using flow cytometry. A. Representative flow cytometry plots. Numbers on plots represent frequency of IL-10-expressing cells. B. Cumulative frequencies of IL-10 expressing CD4+CD25+FoxP3+ Tregs. p values calculated using *t* test. *** $p < 0.001$, * $p < 0.05$, N.D. none detected.

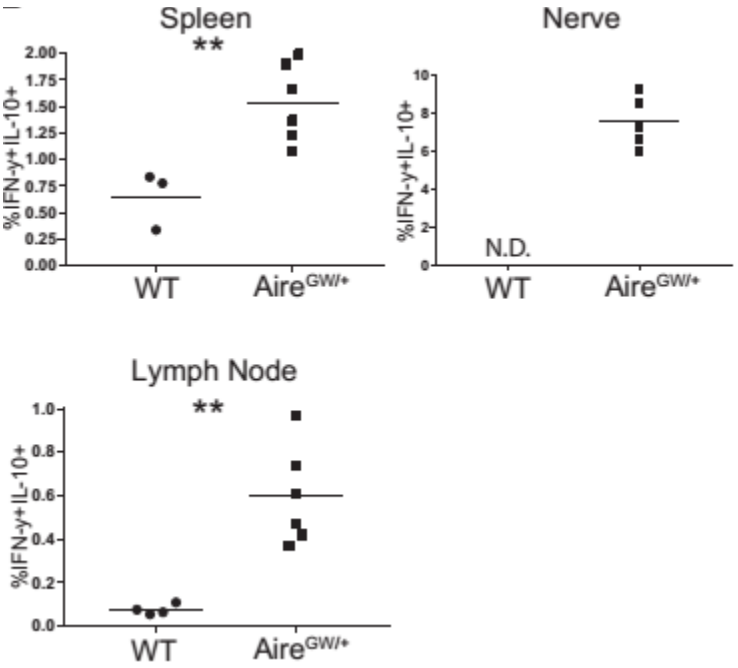
A


SS Lin :: SS
FS Lin :: FS
Lymphocytes
57.7
FS Area :: FS
FS Lin :: FS
singles
98.6
Count
live
68.4
Live/Dead
Count
CD4+
54.1
CD4
FoxP3
CD25-FoxP3-
77.9
CD25
IL-10
0.52
IFN-y

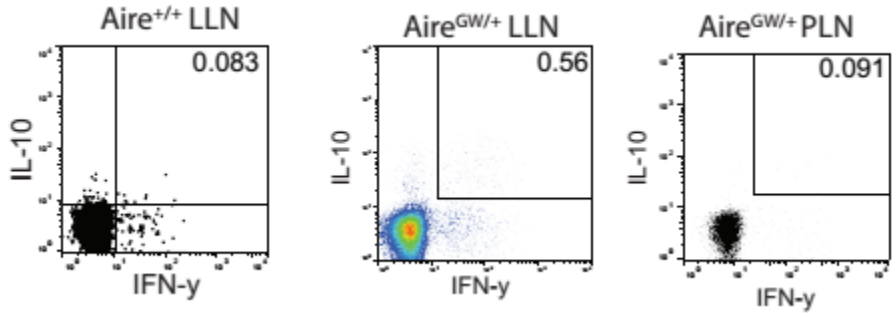
B


WT
Aire GW/+
SPLEEN
0.8
1.9
LYMPH NODE
0.1
0.6
NERVE
IL-10
N.D.
9.3
IFN-y

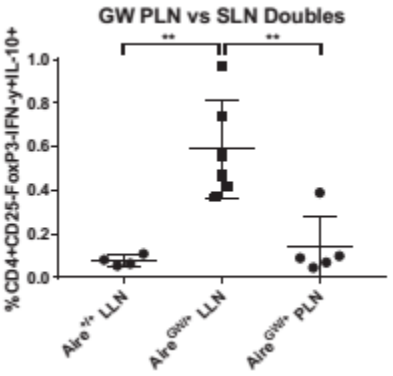
C


Spleen
**
%IFN-γ+IL-10+
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
WT
Aire^GW/+
Nerve
%IFN-γ+IL-10+
10
8
6
4
2
0
N.D.
WT
Aire^GW/+
Lymph Node
**
%IFN-γ+IL-10+
1.0
0.8
0.6
0.4
0.2
0.0
WT
Aire^GW/+

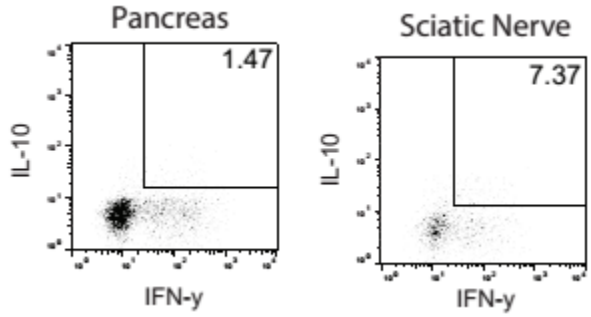
D


Aire+/+ LLN
0.083
IL-10
IFN-γ
Aire^GW/+ LLN
0.56
IL-10
IFN-γ
Aire^GW/+ PLN
0.091
IL-10
IFN-γ

E



F



G

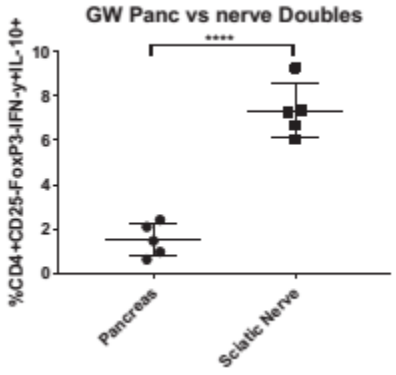


Figure 4.8. IFN-γ-IL-10-double-producing effector T cells are increased in frequency in *Aire*$^{GW/+}$ mice with SAPP. A. Gating strategy for CD4+CD25-FoxP3-IFN-γ+IL-10+ effector T

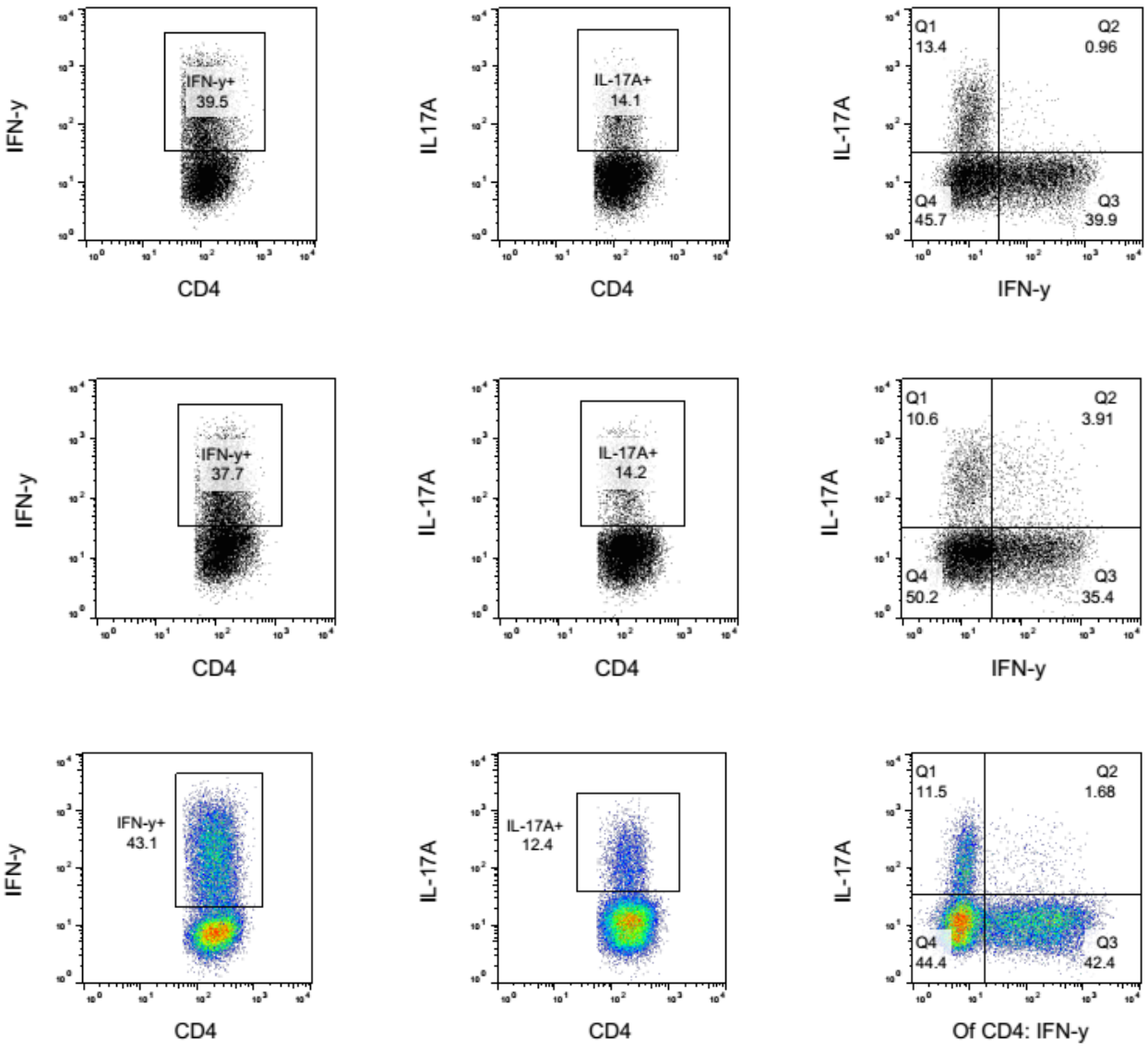
cells. Gating sequence proceeds from left-to-right. CD4+CD25-FoxP3- effector T cells from spleen, lumbar lymph nodes, and sciatic nerves of WT and *Aire*$^{GW/+}$ mice were stained for intracellular IFN-γ and IL-10 using flow cytometry. B. Representative flow cytometry plots. Numbers on plots represent frequency of IL-10-expressing cells. C. Cumulative frequencies of CD4+CD25-FoxP3-IFN-γ+IL-10+ effector T cells. CD4+CD25-FoxP3-IFN-γ+IL-10+ effector T cells were identified in lumbar lymph nodes and pancreatic lymph nodes of WT and *Aire*$^{GW/+}$ mice. D. Representative flow cytometry plots. E. Cumulative frequencies of CD4+CD25-FoxP3-IFN-γ+IL-10+ effector T cells. CD4+CD25-FoxP3-IFN-γ+IL-10+ effector T cells were identified in sciatic nerves and pancreatic islets *Aire*$^{GW/+}$ mice. F. Representative flow cytometry plots. G. Cumulative frequencies of CD4+CD25-FoxP3-IFN-γ+IL-10+ effector T cells. p values calculated using *t* test. **** $p < 0.0001$, ** $p < 0.01$, N.D. none detected.

4.10 Th17 cells are induced and recruited in IL-10-deficient SAPP.

Summary

To characterize SAPP in the context of IL-10 deficiency, I stained nerve infiltrates for IFN-γ in CD4+ and CD8+ T cells using flow cytometry. Approximately 40% of CD4+ T cells in infiltrated nerves of IL-10-deficient *Aire*$^{GW/+}$ mice expressed IFN-γ after stimulation (Fig 4.9A), which is similar to published results (17). As a negative control, I also stained for IL-17 in T cells. IL-17 is a pro-inflammatory cytokine that promotes EAE (100) and EAN (101); however, Th17 cells are not present in the infiltrated nerves of *Aire*$^{GW/+}$ with SAPP (17). Thus, I expected to see minimal IL-17 expression in nerve infiltrates of IL-10-deficient *Aire*$^{GW/+}$ mice. In contrast to expectation, 12 to 14 percent of CD4+ T cells in IL-10-deficient *Aire*$^{GW/+}$ sciatic nerves expressed IL-17 (Figure 4.9A). Furthermore, one to four percent of CD4+ T cells co-expressed IFN-γ and IL-17. Expansion of Th17 cells in IL-10 deficiency is likely a result of the lack of suppression of Th17 differentiation in the absence of IL-10 (102). Further work is needed to determine whether the Th17 T cells are affecting SAPP, or whether their involvement is simply an epiphenomenon. Around 90% of CD8+ T cells produced IFN-γ and around 1% produced IL-17 (Figure 4.9B).

A


IFN-y
CD4
IFN-y+
39.5
IL17A
CD4
IL-17A+
14.1
IL-17A
IFN-y
Q1
13.4
Q2
0.96
Q4
45.7
Q3
39.9
IFN-y
CD4
IFN-y+
37.7
IL-17A
CD4
IL-17A+
14.2
IL-17A
IFN-y
Q1
10.6
Q2
3.91
Q4
50.2
Q3
35.4
IFN-y
CD4
IFN-y+
43.1
IL-17A
CD4
IL-17A+
12.4
IL-17A
Of CD4: IFN-y
Q1
11.5
Q2
1.68
Q4
44.4
Q3
42.4

B

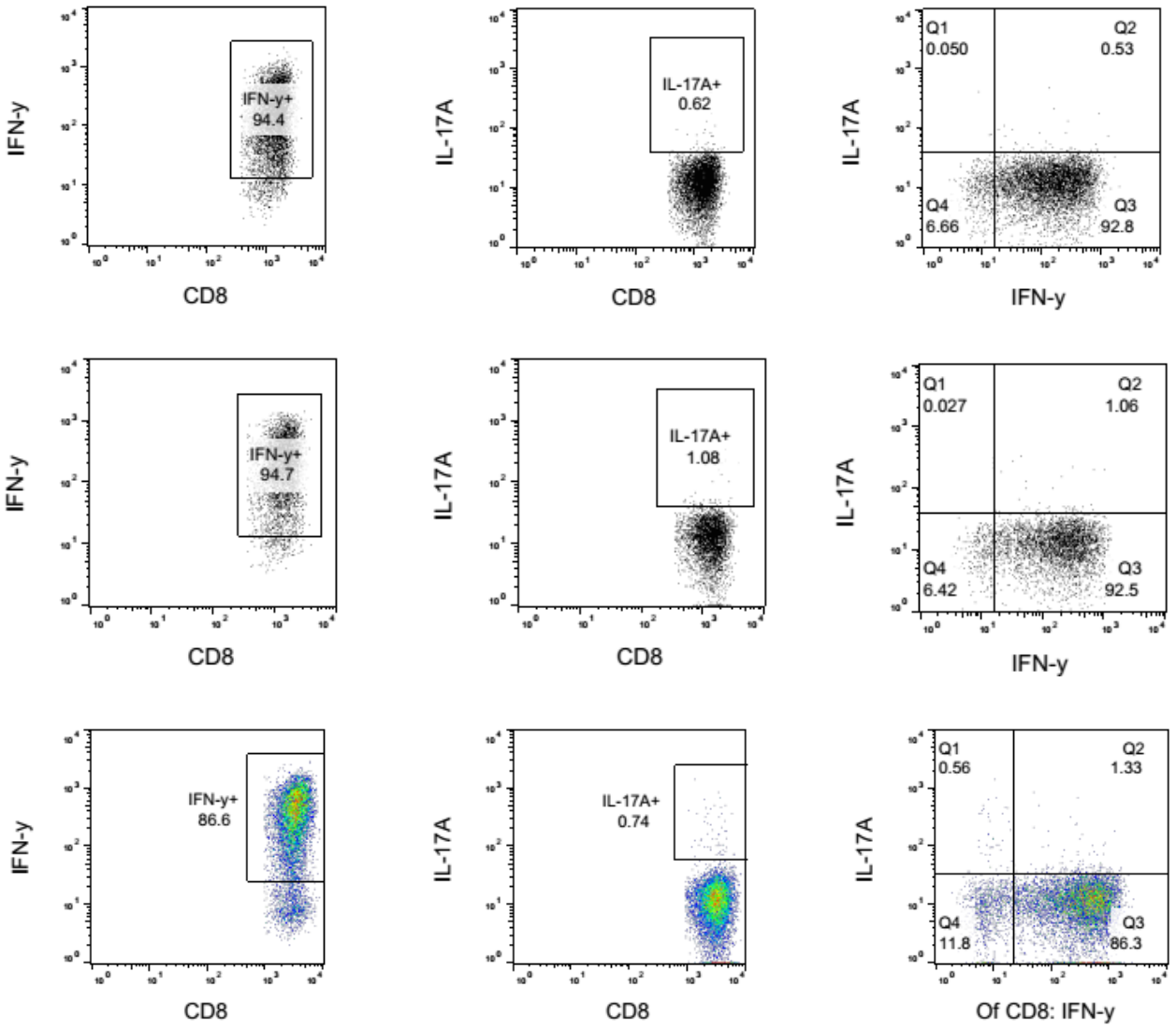


Figure 4.9. Th17 cells are present in infiltrated nerves of IL-10-deficient *Aire*$^{GW/+}$ mice with SAPP. Sciatic nerves from >30-week-old *Aire*$^{GW/+}$Il10$^{-/-}$ mice with SAPP were digested, stimulated with PMA/ionomycin, and stained for CD4 (A), CD8 (B), intracellular IFN-γ and intracellular IL-17A. Numbers represent frequencies of cytokine-expressing cells. Each row is an individual mouse. All plots are gated on live singlets, and the third column is gated on CD4 (A) or CD8 (B).

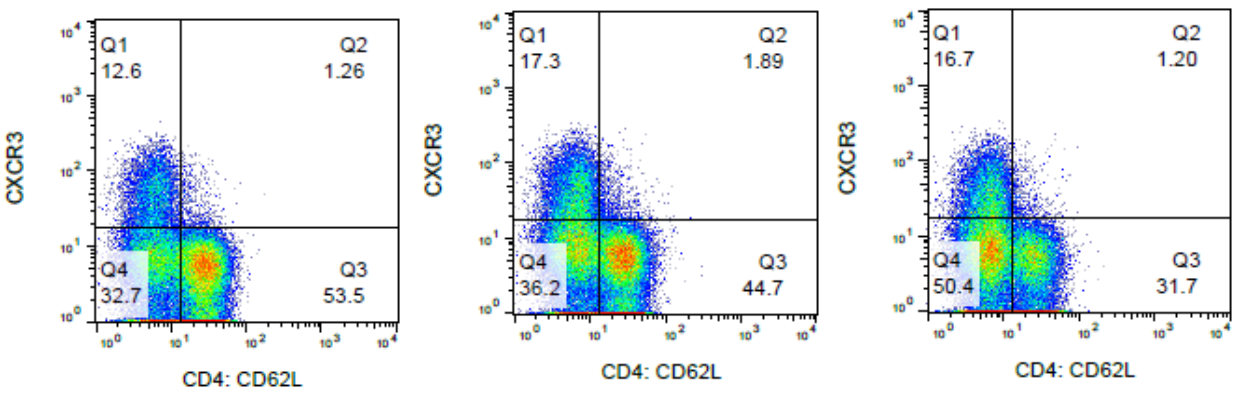
4.11 CXCR3 expression is unchanged in IL-10-deficient *NOD.Aire$^{GW/+}$* mice.

Summary

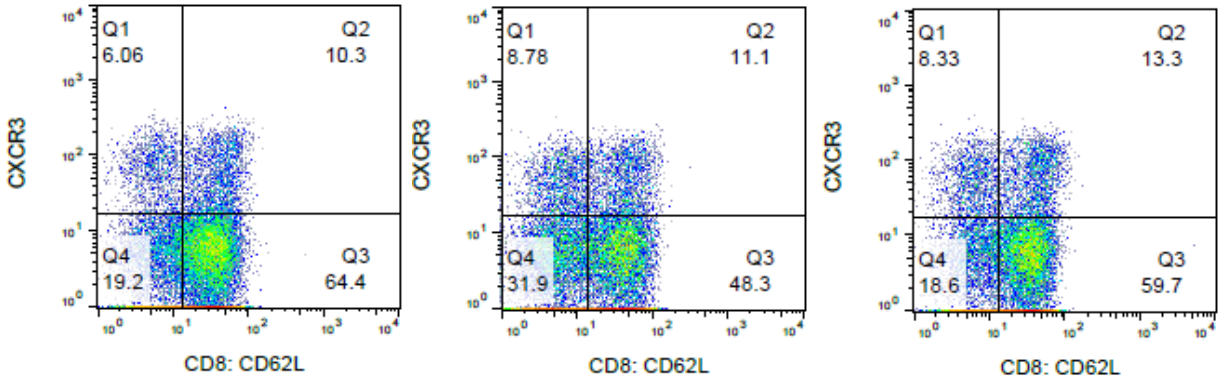
SAPP may be delayed in IL-10-deficient *Aire$^{GW/+}$* mice due to reduced T cell migration from the peripheral blood and into peripheral nerves. C-X-C motif chemokine ligand 10 (CXCL10) is upregulated in nerves with SAPP (36). CXCR3, which is expressed on Th1 cells, binds to CXCL10. CXCR3 may promote T cell infiltration of peripheral nerves through CXCL10. Thus, reduced CXCR3 expression could lead to reduced peripheral nerve infiltration and delayed SAPP in IL-10-deficient *Aire$^{GW/+}$* mice. To assess CXCR3 expression, I used flow cytometry on CD4+ and CD8+ splenic T cells from IL-10-sufficient versus IL-10-deficient mice.

CXCR3 expression was not different in CD4+ and CD8+ T cells from the spleens of IL-10-deficient *Aire$^{GW/+}$* mice compared to IL-10-sufficient controls (Figure 4.10). Preliminary data also show CXCR3 expression did not differ in the lymph nodes of these mice (data not shown). These data suggest IL-10 deficiency did not affect CXCR3 expression in T cells. Thus, it is unlikely that CXCR3-dependent migration into peripheral nerves is responsible for the delay in SAPP.

A **WT** ***Aire*$^{GW/+}$IL-10$^{+/-}$** ***Aire*$^{GW/+}$IL-10$^{-/-}$**



B **WT** ***Aire*$^{GW/+}$IL-10$^{+/-}$** ***Aire*$^{GW/+}$IL-10$^{-/-}$**



C

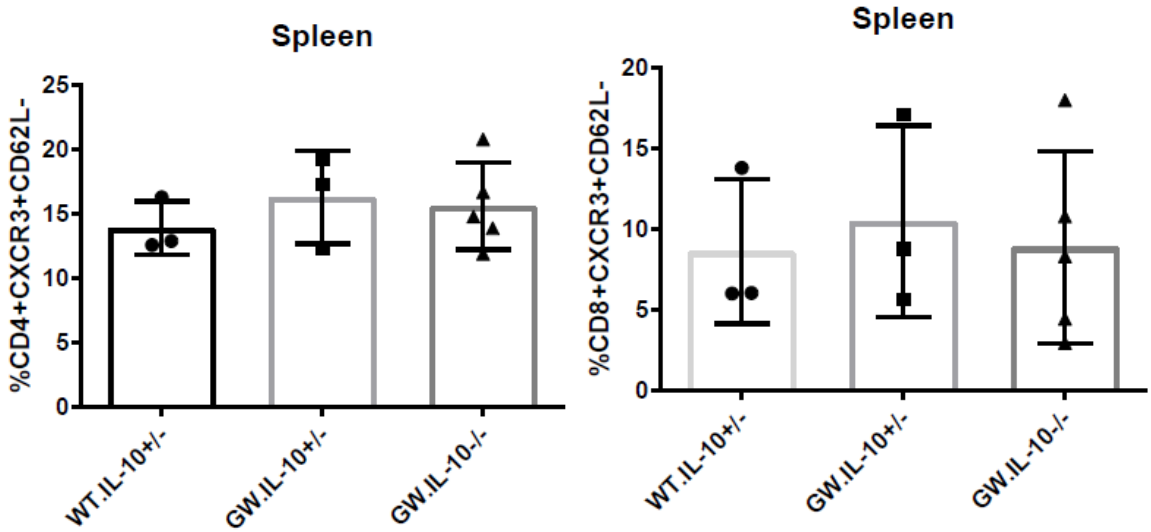


Figure 4.10. IL-10-deficiency does not affect CXCR3 expression in CD4+CD62L- and CD8+CD62L- T cells. T cells from splenocytes of 22-week-old *WT*, *Aire*$^{GW/+}$*Il10*$^{+/-}$ and *Aire*$^{GW/+}$*Il10*$^{-/-}$ were stained for CD62L and CXCR3 expression using flow cytometry. A. Representative plots of CD4+ T cells. B. Representative plots of CD8+ T cells. C. Cumulative frequencies of CD4+CD62L- and CD8+CD62L- T cells expressing CXCR3. p values were calculated using one-way ANOVA and Tukey's correction for multiple comparisons. All comparisons were not significant.

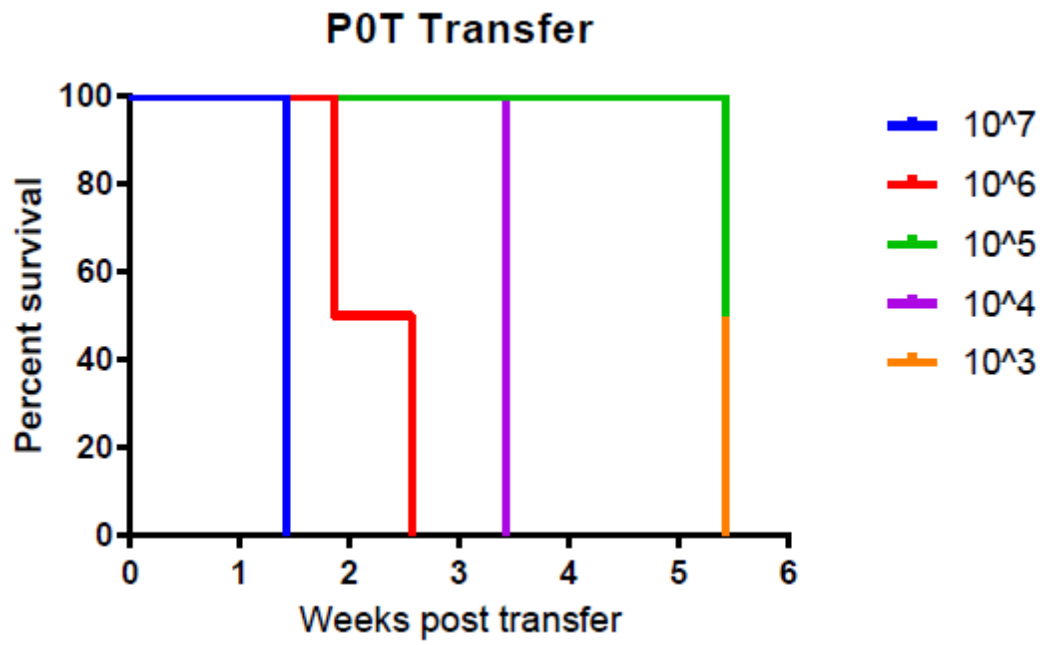
4.12 Optimization of P0 TCR transgenic splenocyte transfer

Summary

NOD.P0 TCR transgenic mice do not develop SAPP, but their splenocytes can induce SAPP in *SCID* recipients after *in vitro* anti-CD3/anti-CD28 stimulation or Treg depletion (103). To use these mice as an additional SAPP model, I transferred varying numbers of splenocytes to *SCID* recipients.

Transfers of 10^7, 10^6, and 10^4 splenocytes led to death of the recipients before SAPP development. Transfers of 10^5 and 10^3 splenocytes caused SAPP at 4 to 5 weeks post transfer (Figure 4.11). These transfers can be used to induce SAPP in large numbers of recipients using a single donor.

A


P0T Transfer
Percent survival
100
80
60
40
20
0
0
1
2
3
4
5
6
Weeks post transfer
10^7
10^6
10^5
10^4
10^3

B

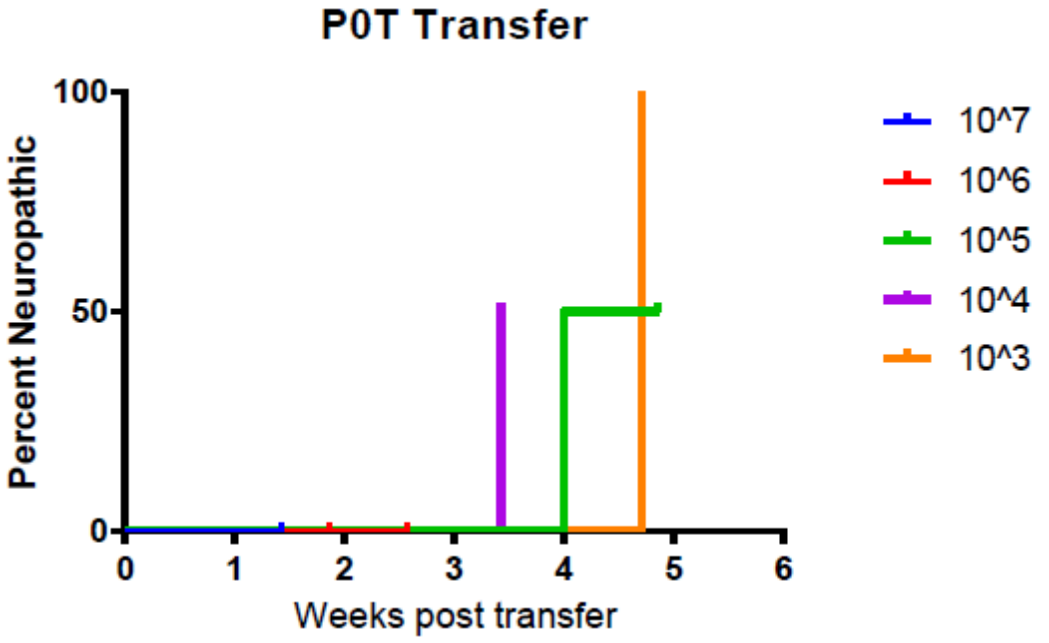

P0T Transfer
Percent Neuropathic
100
50
0
0
1
2
3
4
5
6
Weeks post transfer
10^7
10^6
10^5
10^4
10^3

Figure 4.11. *NOD.P0 TCR* Transgenic splenocytes induce neuropathy in *NOD.Scid* recipients. Splenocytes from NOD.P0 TCR Transgenic mice were stimulated for four days *in vitro* with plate-bound anti-CD3/anti-CD28. One thousand to Ten million stimulated splenocytes were adoptively transferred to *Scid* recipients. A. Percent survival for recipients of P0 TCR transgenic splenocytes. B. Percent neuropathic among recipients of P0 TCR transgenic splenocytes.

CHAPTER 5: DISCUSSION

CIDP is a chronic autoimmune disease marked by the targeting and destruction of peripheral nerve myelin. Mouse models of CIDP have demonstrated critical roles for T cells and their cytokines in pathogenesis. My research has found that in *NOD.Aire*$^{GW/+}$ mice: T cells are required for SAPP development (Chapter 2), and IL-10 produced by CD4+ T cells promotes SAPP through induction of S1pr1 and promotion of T cell migration (Chapter 3). My findings regarding IL-10 suggest that the increased IL-10 expression observed in CIDP patients may not be immunosuppressive. Instead, increased IL-10 may be a marker of inflammation and disease progression. In regards to CIDP therapy, my data suggest IL-10-based therapies may exacerbate or induce CIDP in susceptible patients. My data also suggest blocking or inhibiting IL-10 may alleviate autoimmunity in CIDP; however, this is highly inadvisable given IL-10's central role in general immunosuppression. Even if IL-10 blockade reduced CIDP disease activity, it may provoke autoimmunity or inflammation in other contexts. Notably, colitis could be induced (68). Modulation of IL-10 expression or signaling would have to specifically target peripheral nerve autoimmunity while preserving expression elsewhere.

Another strategy would be to inhibit T cell emigration from lymph nodes, which would be downstream of IL-10's disease-promoting effect. FTY720 has been tested in a CIDP clinical trial; however the trial was ended due to futility (89). There are a few reasons why this trial of FTY720 may have failed. First, given that FTY720 reduced SAPP in *NOD.Aire*$^{GW/+}$ mice when given before SAPP onset, it is possible that migration blockade is only effective when initiated before nerves become infiltrated. Second, the patients tested continued their normal treatments

(IVIg, etc.), so perhaps FTY720 alone is effective but it cannot provide benefit in addition to current treatment. Third, the patients in the study experienced less disease relapse than the researchers expected. Therefore, perhaps there was not enough disease activity to see an effect of FTY720. Given these caveats, blockade of lymphocyte migration may still be a viable treatment option for CIDP. S1PR1-blocking drugs beyond FTY720 may also have efficacy against CIDP (104).

My findings in Chapters 2 through 4 give rise to further questions about SAPP in *Aire*$^{GW/+}$ mice and CIDP pathogenesis.

5.1 Which IL-10-producing CD4+ T cell subset is responsible for promoting SAPP?

In Chapter 3, I demonstrate that IL-10 promotes SAPP in *NOD.Aire*$^{GW/+}$ mice, and that IL-10 produced by CD4+ T cells is sufficient to promote SAPP in the transfer model of disease. However, the specific CD4+ T cell subset or subsets that is or are promoting SAPP remains unknown. A multitude of CD4+ T cell subsets can produce IL-10, including Th1, Th2, Th9, Th17, FoxP3+ nTreg, FoxP3+ iTreg, and Tr1 (7, 9, 105). Several possibilities arise from this knowledge: a T effector subset produces pathogenic IL-10, an ordinarily immunosuppressive T cell subset is instead immune-activating in SAPP and produces pathogenic IL-10, an immunosuppressive T cell subset dampens autoimmunity except for production of pathogenic IL-10, or some combination of the three.

5.1.1 Pathogenic IL-10 is produced by a T effector subset

T effectors typically promote inflammation and immune responses. Different immune responses, and consequently autoimmune diseases, are often characterized by specific T effector subsets. SAPP pathogenesis is marked by significant expansion of Th1 T cells (17), and Th1-

derived IFN-γ is required for SAPP (36). Conversely, Th2 and Th17 subsets exist in *Aire*$^{GW/+}$ mice at low frequencies, do not expand with disease, and do not infiltrate nerves (17). Th9s are also absent in mice with SAPP (data not shown). Additional evidence against a role for Th2s in SAPP is that B cells, the targets of Th2-derived IL-10, do not contribute to SAPP pathogenesis (Chapter 4.5). Thus, these data suggest Th2, Th9, and Th17 subsets are unlikely candidates for promotion of SAPP. The increase in IL-10-producing Th1s in SAPP makes these cells strong candidates for SAPP promotion (Figure 4.6). This is also logical conceptually; the IFN-γ they produce is required for disease, and the IL-10 they produce may promote their escape from lymph nodes and subsequent infiltration of peripheral nerves. Whether Th1s producing IL-10 expand or whether pre-existing Th1s upregulate IL-10 in SAPP is an additional question. The latter seems more likely since IL-10 suppresses proliferation (38) and purified CD4+CD25-IL-10-GFP+ T cells did not proliferate after 4 days of stimulation *in vitro* with plate-bound anti-CD3 and anti-CD28 (observation after I performed this experiment).

To determine whether IL-10-producing Th1s promote SAPP, the adoptive transfer system could be used. IL-10+ Th1s could be purified and transferred to *SCID* recipients. As shown in (Figure 4.8A, B), CD4+ T cells that produce IFN-γ and IL-10 predominantly lack CD25 surface expression. Using *NOD.Aire*$^{GW/+}$ or *NOD.P0 TCR* transgenic mice expressing an enhanced green fluorescent protein (eGFP) reporter under IL-10, the cells can be purified with FACS up to around 70% purity (data not shown). The cells could be cultured *in vitro* with anti-CD3/anti-CD28 or transferred without manipulation. Recipients would be monitored for SAPP development. EMG would confirm the present of demyelination and decreased nerve function. Histology of sciatic nerves would be used to visualize global infiltration. Flow cytometry would characterize nerve-infiltrating T cells and T cells in draining lymph nodes. One caveat of this

experiment is whether T cells still express IL-10 post transfer. The lymphopenic environment in *SCID* recipients could change their phenotype. Thus, it would be important to assess what frequency and number of T cells maintain IL-10 expression post-transfer, if any. If recipient mice develop SAPP, but transferred cells do not express IL-10, then IL-10 may have an early role in promoting infiltration or may not have a role. If recipient mice do not develop SAPP, then IL-10-producing Th1s may not be sufficient to induce SAPP. The next step would be to co-transfer IL-10-producing Th1s with naïve IL-10-deficient CD4+ T cells. Controls for this experiment would be a transfer of naïve IL-10-sufficient CD4+ T cells alone and naïve IL-10-deficient CD4+ T cells alone. Naïve IL-10-sufficient CD4+ T cells would be the positive control for SAPP incidence. Naïve IL-10-deficient CD4+ T cells should induce SAPP with delayed incidence (Figure 3.3). If IL-10-producing Th1s promote SAPP, then co-transferring them with IL-10-deficient CD4+ T cells should accelerate SAPP incidence relative to IL-10-deficient CD4+ T cells alone and possibly be indistinguishable from IL-10-sufficient CD4+ T cells alone. Ratios and numbers of cells in co-transfers may require optimization. Alternative ways to assign a function to IL-10-producing Th1s is to do proliferation assays or suppression assays using co-cultures with fluorescently-labeled, IL-10-deficient CD4+ T cells. Such studies could find that IL-10-producing CD4+ T cells paradoxically promote proliferation or paradoxically lack immunosuppressive effects on IL-10-deficient CD4+ T cells. Such functions would be separate from the role of IL-10 in promoting migration, but may also be true.

5.1.2 Pathogenic IL-10 is produced by a T regulatory subset.

Immunosuppressive T cell subsets can produce IL-10, and IL-10 is thought to be immunosuppressive in this context. Furthermore, IL-10 is the primary mechanism of immunosuppression for iTregs and Tr1s. However, the fact that IL-10's net effect is promotion

of SAPP suggests IL-10 produced by regulatory T cell subsets may also promote SAPP. Preliminary studies suggest Tregs are immunosuppressive in SAPP. First, depletion of Tregs with anti-CD25 antibody exacerbates SAPP in male *Aire*$^{GW/+}$ mice (performed by Anil Nagavalli, data not shown). Second, FoxP3+ Treg transfer protects from SAPP (99 and performed by Annie Wang, data not shown). These data suggest it is unlikely that IL-10 produced by Tregs promotes SAPP since Tregs suppress SAPP overall. However, it remains possible that IL-10-negative FoxP3+ Tregs may suppress SAPP, whereas IL-10-expressing FoxP3+ Tregs promote SAPP. If IL-10 produced by regulatory T cell subsets promotes SAPP, then this effect is a paradoxical component of the overall immunosuppressive role of these cells. A related question is whether IL-10 is required for immunosuppression in these Treg subsets. Similarly to IL-10-producing Th1s, co-transfer experiments and suppression assays may answer these questions. Naïve IL-10-deficient CD4+ T cells could be co-transferred with IL-10-GFP positive Tregs or IL-10-deficient Tregs versus naïve IL-10-deficient CD4s alone to determine whether IL-10 produced by Tregs can promote SAPP. If recipients of IL-10-expressing Tregs develop SAPP faster than naïve IL-10-deficient CD4s alone, then IL-10 may promote SAPP even when expressed by Tregs. If recipients of IL-10-expressing Tregs develop SAPP at the same rate as naïve IL-10-deficient CD4s alone, then either Tregs have no role in SAPP in this system or SAPP-promotion by IL-10 may be balanced with SAPP suppression by the Treg overall. If recipients of IL-10-expressing Tregs develop SAPP slower than naïve IL-10-deficient CD4s alone, then SAPP incidence must be compared to IL-10-deficient Tregs. If IL-10-expressing Tregs permit faster SAPP than IL-10-deficient Tregs, then IL-10 in Tregs is pathogenic. If IL-10-expressing Tregs permit SAPP at the same rate as IL-10-deficient Tregs and both are slower than naïve IL-10-deficient CD4s alone, then Tregs protect from SAPP in an IL-

10-independent manner. If SAPP is slower in recipients of IL-10-expressing Tregs relative to IL-10-deficient Tregs, then IL-10 in Tregs is protective. Whether IL-10-expression is maintained in IL-10-GFP-positive Tregs after transfer is a caveat.

5.2 Does IL-10 therapy cause or accelerate neuropathy?

My data in Chapter 3 suggest IL-10 promotes SAPP. This corroborates another story where IL-10 overexpression in peripheral nerves induces spontaneous autoimmune neuropathy (107). The mechanism of IL-10's disease promotion in (107) is different; they propose that IL-10 recruits macrophages that kill Schwann cells through Fas-FasL interactions. They also see very few T cells infiltrating nerves. Despite these differences, both suggest IL-10 is an effector in SAPP. Whether IL-10 administration in NOD mice can induce or accelerate SAPP is not known. *NOD* mice could be injected with recombinant mouse IL-10 to assess whether SAPP occurs. *NOD.Aire*$^{GW/+}$ mice could be injected with IL-10 to assess whether SAPP occurs earlier or with greater severity. Treatment regimens may have to be optimized. IL-10 has a short half-life (108) and may not be adequately circulated after injection. Since CD4+ T cell production of IL-10 is sufficient to promote SAPP, it is possible that IL-10 must exert its effects specifically in the T cell niche of the lymph node (paracortex) in an autocrine and paracrine fashion. Thus, systemic delivery of IL-10 may have different effects. These studies may have clinical implications since IL-10 is being tested as a therapeutic for several autoimmune and inflammatory diseases (40–43, 88).

5.3 Is SAPP protection in IL-10-deficient *NOD.Aire$^{GW/+}$* mice due to differential gene expression beyond S1pr1?

IL-10 is a pleiotropic cytokine, and dysregulation or loss of IL-10 function changes the phenotype of immune cells and typically exacerbates disease (38, 68, 109, 110). In addition to reduced T cell migration (Chapter 3), IL-10-deficient *NOD.Aire$^{GW/+}$* mice exhibit increased frequencies of Th17 cells (Figure 4.9). Thus, it is likely that genes beyond S1pr1 are differentially expressed in *NOD.Aire$^{GW/+}$* mice. To screen for other gene expression changes that may affect SAPP pathogenesis, RNA sequencing of IL-10-sufficient versus IL-10-deficient CD4+ T cells from lumbar lymph nodes could be performed. Any immune-related genes with significantly different expression could be validated by qPCR and further tested for effects on SAPP.

5.4 Why does IL-10 only promote PNS autoimmunity in *NOD.Aire$^{GW/+}$* mice?

Interestingly, IL-10 deficiency protects against PNS autoimmunity, but it does not have a statistically significant difference in infiltration scores in the eye, thyroid, salivary glands, lacrimal glands, or islet beta cells (Figures 3.7 and 4.6). Infiltration scores in thyroid glands trended towards reduced infiltration in IL-10-deficient *Aire$^{GW/+}$* mice relative to IL-10-sufficient counterparts, and the p value was almost significant ($p = 0.05303$) (Figure 4.6). These data suggest IL-10 may also promote autoimmune thyroiditis. Why IL-10 promotes autoimmunity in the PNS, but not other tissues, is unknown. One possible explanation is that expansion of an IL-10-producing cell is specific to PNS autoimmunity. Indeed, Th1s that also produce IL-10 are expanded in infiltrated nerves and nerve draining lymph nodes, but not infiltrated islets and pancreatic lymph nodes (Figure 4.8D-G). If these Th1s are found to promote SAPP, they may be the mechanism by which IL-10 promotes migration of PNS-reactive T cells without affecting

other organs. Another possibility is that IL-10 selectively upregulates chemokines in peripheral nerves that recruit pathogenic effectors.

Many questions remain in the wake of my findings and about CIDP pathogenesis in general. Current treatment options are not sufficient to remedy disease in the majority of patients. Further study is required to discover new therapeutic targets, and mouse models of disease will continue to provide vital insight.

5.5 Summary of IL-10's function in the intestine and peripheral nerves.

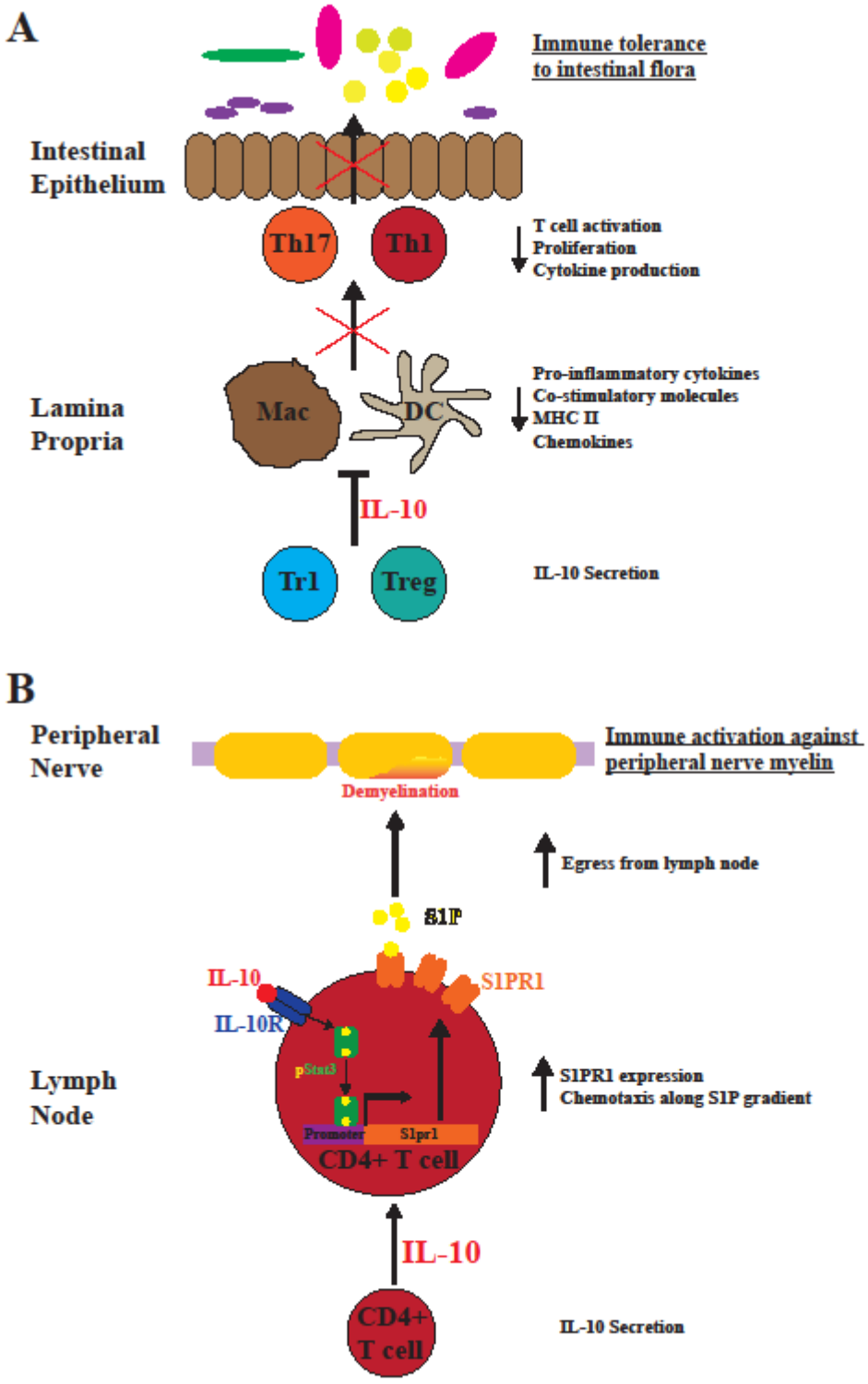


Figure 5.1. Graphical abstract of IL-10 function in the intestine versus peripheral nerves. Summary of the role of IL-10 in inflammation in the intestine (A) and peripheral nerves (B). In the intestine, IL-10 secreted by Treg and Tr1 cells promotes a resting/tolerigenic phenotype in APCs by downregulating pro-inflammatory cytokines, co-stimulatory molecules, MHC II, and chemokines. This leads to reduced activation of effector T cells and immune tolerance to intestinal flora. In contrast to the intestine, IL-10 promotes autoimmunity against peripheral nerves. In the nerve-draining lymph nodes, IL-10 secreted by T cells leads to STAT3-dependent upregulation of *S1pr1* in T cells in an autocrine and paracrine fashion. Increased S1PR1 expression in T cells leads to increased emigration from lymph nodes and subsequent infiltration of peripheral nerves. Immune infiltration of peripheral nerves leads to demyelination and compromised nerve function.

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