Title
Organ-Specific Autoimmunity Resulting from Combined Defects in Two Tolerance Checkpoints

Author
Proekt, Irina

Publication Date
2015

Peer reviewed|Thesis/dissertation

Organ-specific autoimmunity resulting from combined defects in two tolerance checkpoints

by

Irina Proekt

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

Dedication

I would like to dedicate my thesis to my husband, Leonid and to my parents, Dina and Edward. Your unconditional love and support made this possible.

Acknowledgements

First and foremost, I would like to thank my thesis advisor, Tony DeFranco, for his ongoing support and guidance. Tony has been an amazing mentor and an extraordinary source of encouragement and scientific wisdom. His door was always open, his advice was always plentiful and insightful. Tony's knowledge of immunology and professional integrity are unparalleled and have been a true inspiration. He helped instill in me a true passion for discovery and taught me to persevere in the face of the many scientific and personal challenges throughout my graduate career. I am honored to have been a part of his lab.

I would also like to thank members of my thesis committee, Jason Cyster and Michelle Hermiston. They provided continuous insight during our (almost) yearly committee meetings and gave me invaluable feedback about the direction of my project. I thank Jason for his incredibly insightful comments during our numerous meetings in preparation for journal clubs and in discussing data, and for his superhuman ability to read a full thesis in a day and find every single typo and grammatical error. I thank Michelle for being a source of scientific inspiration as well as for being a role model as a physician-scientist juggling a stellar career and a family. Her advice about organizing my time and managing expectations in the lab and at home helped me keep my sanity when I was trying to balance research and caring for my newborn twins.

I would also like to thank the many members of the DeFranco lab with whom I have been fortunate to work over the years. They have helped me transform from a clueless rotation student into a budding immunologist. In particular, I would like to thank Andy

Gross for teaching me about B-cell tolerance and passing on his knowledge about B-cell assays. A special thanks goes to my fellow grad students, Linda Lee, Matt Wheeler and Derek Rookhuizen, who have been a great source of advice, support and camaraderie. I thank Linda for always knowing where everything is and being a great presence and a shoulder to cry on during tough times. I would also like to thank our undergraduate student, Matt Dong, for always being enthusiastic and helpful during his time in the lab. Lastly, I would like to thank our lab manager, Ming Ji, for making sure that everything ran smoothly and the many, many hours she spent genotyping and running gels so that we could do our experiments on time.

Next, I would like to thank Mark Anderson, who has acted as a co-mentor in the last few years of my graduate career. Mark is a brilliant scientist who asks the really tough and relevant questions and has been a true inspiration. During our collaboration, he opened up his lab and resources to me, helping drive the project forward. I would also like to thank members of his lab, Ruth Taniguchi, Imran Khan and Maria Mouchess for help with T-cell assays and helpful discussions, and Kayla Fasano for help with histology and radioimmunoassays. I want to especially thank Corey Miller who has closely worked with me on the Aire Lyn project and whose hard work and support were essential to completing the project.

I would also like to thank Cliff Lowell and the past and present members of the Lowell lab, who have given me countless advice during joint lab meetings and countless mice for my numerous experiments. In particular, I would like to thank Patricia Scapini and Chrystelle Lamagna, my Lyn project buddies, with whom I had many discussions about

the biology of Lyn; Claire Abram, the ever-helpful source for the many mouse strains that I ended up using in my project, and Yongmei Hu for help with mouse husbandry and genotyping. I want to extend special thanks to Cliff Lowell who had been a great mentor and a source of constant good cheer and humor in our joint lab meetings.

Next, I would like to thank our lab neighbors on the tenth floor - members of the Cyster Lab, Lanier Lab, and more recently the Turnbaugh lab, for sharing reagents, helping with experiments and generally hanging our in the lunch room to take a break from science. I would like to give special thanks to Andrea Reboldi for many helpful discussions.

I would also like to thank the BMS staff, in particular Lisa Magargal and Monique Piazza who have been incredibly supportive during the first few years of my PhD. I would also like to thank Demian Sainz and Ned Molyneaux for a great transition, keeping everything running smoothly and staying on top of deadlines. Thank you for everything!

Lastly, I would like to thank my family. I thank my wonderful parents, Dina and Edward for the great love and support that they have given me over the years, always respecting my choices, even then the result was not as straightforward as they would like. You never stopped believing in me. Thank you for everything!

I thank my kids, Nora and Joseph, for teaching me what is means to live and to love. I thank my husband, Leonid for being there for me every second of every day. Without you, none of this would have been possible. Thank you, thank you, thank you!

Contibutions of Co-authors to Presented Work

Chapter two of this dissertation is based on the manuscript that is in preparation for submission. I designed and performed experiments. Corey N. Miller[2] helped with the design of the experiments and performed funduscopy and confocal microscopy. Marion Jeanne[3] performed funduscopy and provided helpful discussions. Kayla J. Fasano[2] performed H&E staining and IRBP antibody assays. Clifford A. Lowell[4] provided cell-specific Cre lines and Lyn Flox mice generated in his lab. Douglas B. Gould[3] provided the funduscope and helpful discussions. Anthony DeFranco[1] and Mark Anderson[2] acted as co-PIs and supervised the entirety of the work.

[1]Department of Microbiology and Immunology; [2]Diabetes Center; [3]Departments of Ophthalmology and Anatomy, Institute for Human Genetics, [4]Department of Laboratory Medicine, University of California, San Francisco, CA, 94143 USA

Organ-specific autoimmunity resulting from combined defects in two tolerance checkpoints

By Irina Proekt

ABSTRACT

Studies of genetic factors associated with human autoimmune disease suggest a multigenic origin of susceptibility, however, how these factors interact and through which tolerance pathways they operate generally remain to be defined. One key checkpoint occurs through the activity of the *Aire* gene which promotes central T cell tolerance. Recently, a variety of dominant-negative human *AIRE* mutations have been described which likely contribute to human autoimmunity more so than previously thought. The penetrance of autoimmunity is incomplete in these families suggesting that other checkpoints are playing a role in preventing autoimmunity. In Chapter two of this thesis, I describe studies testing if a defect in Lyn, an inhibitory protein tyrosine kinase implicated in systemic autoimmunity, could combine with an *Aire* mutation to provoke organ-specific autoimmunity. Indeed, mice with a dominant-negative allele of *Aire* and deficiency in *Lyn* spontaneously developed organ-specific autoimmunity in the eye. I further show that a small pool of retinal protein-specific T cells escaped thymic deletion due to the hypomorphic Aire function and escaped peripheral tolerance in the presence of Lyn-deficient dendritic cells, leading to highly destructive autoimmune attack. These findings demonstrate how two distinct tolerance pathways can synergize to unleash autoimmunity and have implications for the determinism of autoimmune disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2:

CHAPTER 3:

CHAPTER ONE:

Introduction

Multigenic susceptibility to autoimmune disease

The concept of tolerance towards self antigens was first proposed by Paul Ehrlich in the early 1900s, when he noticed that animals failed to develop autoantibodies when immunized with blood of their own species. He termed this effect "horror autotoxicus", referring to the inherent protective mechanisms that prevent an organism from mounting a destructive response against its own tissues (1). In the ensuing century, advances in our understanding of immunity have revealed that tolerance requires integrity of multiple checkpoints that govern development, activation and interactions of immune cells, but why such checkpoints fail in some individuals leading to particular autoimmune diseases remains poorly understood (2, 3).

Strikingly, most autoimmune diseases exhibit a high degree of genetic susceptibility, seen for example in studies of identical twins, which for most autoimmune diseases have a disease concordance of roughly 25-50% (4). In rare cases, autoimmunity is monogenic, revealing genes that control major mechanisms of immune tolerance, such as central tolerance of T cells (Aire) and regulatory T cells (FoxP3) (5-7). Much more frequently, however, autoimmune susceptibility is multigenic with incomplete penetrance (8). The biggest contributor to autoimmune susceptibility in most cases is particular alleles of MHC molecules, which present antigens to T cells, suggesting that a small number of self-antigens are particularly important for the initiation of autoimmune attack (8). Over the last decade, genome-wide association studies (GWAS) of human autoimmune diseases have uncovered a surprisingly large number of non-MHC genes, most of which individually have weak effects on disease risk, suggesting that they represent partial changes in function,

rather than the complete loss-of-function seen in the monogenic autoimmune diseases (8-10).

Interestingly, it appears that some autoimmune susceptibility genes can predispose to multiple diseases, the best studied example being the gene encoding protein phosphatase PTPN22, which is associated with increased risk for systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and type 1 diabetes (T1D) (11). A majority of shared susceptibility genes tend to cluster along several pathways governing immune cell signaling, cytokine production and innate cell activation (8). This indicates that genetic modifiers of common tolerance pathways, along with epigenetics and environmental factors, may determine the spectrum of an individual's autoimmune phenotype.

T-cell central tolerance

The immune system can be conceptually divided into innate and adaptive branches, although their actions are highly interdependent. The cells of the innate immune system include myeloid and dendritic cells (DCs) which recognize pathogens through a conserved set of pattern recognition receptors and mount a fast defensive response. B and T lymphocytes of the adaptive immune system respond to the cues from the innate cells to mount an antigen-specific response and develop immunological memory.

T lymphocytes are an integral part of the adaptive immune system. They rearrange gene segments of their TCRs into a large number of possible combinations that can

recognize a vast array of antigens from potential pathogens. The same recombination mechanism leads inevitably to generation of some specificities that recognize self components, with the potential to cause autoimmune disease. A diversity of tolerance mechanisms work together to limit the activity of self-reactive T lymphocytes (12). These mechanisms can be generally divided into central, or those acting during T cell development in the thymus, and peripheral, or those acting on mature autoreactive T cells.

T cell development in the thymus occurs through a two-step process that ensures production of a functional TCR that is also non-autoreactive (13). T cells originate from hematopoietic precursors in the bone marrow and migrate to the thymic cortex where they rearrange beta and alpha TCR chains and express CD4 and CD8 to become double positive (DP) thymocytes. Weakly self-reactive DP T cells are selected by peptide-MHC complexes expressed on stromal cells in the thymic cortex. Those thymocytes that fail to recognize peptide-MHC complexes die by apoptosis (14). Positively selected thymocytes then commit to the CD4 or CD8 single positive stage and migrate to the medulla where they encounter tissue-specific antigens (TSAs) directly presented by medullary thymic epithelial cells (mTECs) or indirectly presented by thymic DCs (15, 16). These TSAs are normally restricted to peripheral tissues, and thus their expression in the thymus ensures that most strongly autoreactive thymocytes either die by apoptosis or alternatively become regulatory T cells (Tregs) and enter the periphery to promote tolerance there. In the absence of negative selection, such as in mice with absent thymic medulla, or in models where mTECs are missing MHCs or co-stimulatory molecules, organ-specific autoimmunity ensues because of escape of autoreactive T cells to the periphery (14, 15, 17, 18).

Aire is required for central T-cell tolerance

Autoimmune regulator (Aire) was identified in 1997 as the causative gene behind Autoimmune polyglandular syndrome type 1 (APS1), or as it has also been called, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED) (19, 20). APS1 is a monogenic autoimmune disorder characterized by multi-organ autoimmunity, which most commonly manifests as hypoparathyroidism, adrenocortical failure and chronic mucocutaneous candidiasis (21, 22). Aire is a transcription factor primarily expressed in mTECs where it promotes expression of an array of tissue-specific antigens (TSAs) (23). Although the mechanisms by which Aire regulates gene transcription are not fully elucidated, sequence analysis revealed that it possesses domains involved in DNA-binding and protein-protein interactions and disease-inducing mutations cluster within these domains (24, 25).

Aire knockout mice were made by two different groups and found to have multi-organ autoimmunity closely resembling that seen in humans, mostly affecting glands and endocrine organs (7, 26). Further studies revealed that the autoimmunity was highly strain-dependent (27). On the autoimmune-resistant C57BL/6 background, salivary and lacrimal glands, and the retina were the most often affected. On the autoimmune-prone NOD background, more organs were affected, including exocrine pancreas, thyroid and sciatic nerve, and also the disease was of earlier onset and more severe. Although Aire is also expressed on stromally-derived cells in peripheral lymphoid tissues and can contribute to peripheral tolerance, autoimmunity is primarily prevented by expression of Aire in the thymus supporting its role as a major regulator of central T-cell tolerance(28).

A number of Aire-regulated TSAs were identified through comparison of mTEC gene-expression in wild type and *Aire*$^{-/-}$ mice, and subsequently the protein targets of Aire-regulated genes were shown to be targets of autoimmune attack in relevant organs (7, 29). A prototypical example of an Aire-regulated TSA is interphotoreceptor retinoid-binding protein (IRBP). IRBP has been identified as a major target of autoimmune retinal disease in *Aire*$^{-/-}$ mice (30). Transplants of *IRBP*$^{-/-}$ thymi into athymic Aire-sufficient nude mice revealed that absence of thymic IRBP expression is sufficient to induce spontaneous autoimmune uveitis, definitively linking thymic expression of a self-antigen to the maintenance of tolerance to that antigen in the periphery (30). In a further series of genetic and adoptive transfer experiments, it was determined that CD4 T cells are sufficient for the development of uveitis in the *Aire*$^{-/-}$ mice (31). An Aire-regulated epitope of IRBP had been identified in C56BL/6 mice, allowing the study of rare autoreactive T cells within the endogenous repertoire using the P2/I-A^b tetramer (32). In wild type mice, P2-specific T cells are subject to negative selection and cannot be expanded by immunization with the P2 peptide. However, they are detectable in Aire-deficient mice and expand during spontaneous disease or immunization (32).

Other Aire-regulated TSAs have also been recently linked to the autoimmunity in their respective tissues. They include vomeromodulin in lung (33), odorant binding protein 1a in lacrimal gland (34), myelin protein zero in peripheral nerves (35) and seminal vesicle secretory protein 2 (SVS2) in prostate (36). These studies are highly clinically relevant as they identify critical autontigens in human autoimmunity, like vomeromodulin in interstitial lung disease and SVS2 in prostatitis, providing critical insights into disease pathogenesis.

Regulation of T-cell peripheral tolerance by dendritic cells and B cells

Although negative selection is very efficient, not all autoreactive T cells are deleted in the thymus. This can be attributed to poor avidity of some self-reactive TCRs, insufficient expression of peripheral self-antigens or expression of new self-epitopes by inflamed tissues (37). In the periphery, autoreactive escapees can encounter cognate antigen on the surface of antigen-presenting cells (APCs). The outcome of this interaction is largely determined by the activation state of the APC and environmental cues. Under steady state conditions, self-reactive T cells are tolerized by functional unresponsiveness (anergy), clonal deletion, or conversion to antigen-specific regulatory T cells (Tregs). However, during infection or inflammation, activated APCs upregulate costimulatory molecules, such as B7 ligands, CD80 and CD86, which engage CD28 on the T cell surface leading to T cell priming. (12, 38).

Several cell types can act as APCs, including DCs, B cells, monocytes and macrophages, and they have all been implicated in T-cell tolerance. Classical dendritic cells (hereafter referred to as DCs) are the major antigen-presenting cells of the immune system. DCs reside at tissue interfaces, as well as within tissues and lymphoid organs, where they are exposed to a plethora of antigens, which they capture, process and present to T cells (38, 39). During infection or inflammation, activated DCs provide co-stimulatory signals and cytokines to cognate T-cells, resulting in priming and polarization. Conversely, during steady state, DCs present tissue self-antigens in the context of inhibitory signals like PD1 ligands and immunoregulatory cytokines such as TGF beta to promote anergy or Treg

induction (40-43). Thus, alterations in DC homeostasis and activation can have major effects on T-cell tolerance.

Dysregulation of DC function has been linked to many autoimmune diseases, including T1D, SLE, multiple sclerosis (MS), and inflammatory bowel disease (IBD) (38, 40). Insights into the roles of DCs in autoimmunity come from patients and mice with either total DC ablation or targeting of individual pathways in DCs. Patients with DC-deficiency syndromes suffer from autoimmunity, although it may be promoted by compensatory effects from other cell types (44). In mice, DC-ablation experiments using CD11c-DTR have resulted in worsening of T-cell mediated autoimmunity, however, this has been attributed mainly to regulating Treg homeostasis (45-47). As DCs are important for peripheral tolerance of T cells as well as for priming autoimmune effector T cells, effects of DC depletion are probably complex depending on which effect dominates.

More informative have been the studies of tolerance-related pathways specifically in DCs through ablation of individual genes using conditional gene targeting in mice. Numerous studies have shown that targeting of negative regulators of DC function that have known associations with human autoimmune diseases lead to autoimmunity. For example, deletion of A20, which negatively affects NFKB signaling downstream of tumor necrosis factor (TNF) and toll-like receptors (TLRs), caused colitis and spondyloarthritis (48); deletion of SHP1 phosphatase, which is involved in signaling downstream of integrins, TLRs and receptor tyrosine kinases, caused severe lupus-like autoimmunity (49); and deletion of STAT3, required for signaling downstream of anti-inflammatory cytokine IL-10, caused cervical lymphadenopathy and ileocolitis (50). Furthermore, the role of self-antigen

presentation by DCs has been extensively studied in mouse models of autoimmune diabetes, such as NOD mice, where DCs presenting insulin peptides in the pancreatic islets and draining lymph nodes promote localization and priming of diabetogenic T cells (40, 51) and in mouse models of multiple sclerosis, such as experimental autoimmune encephalomyelitis (EAE), where encephalitogenic T cells are primed and polarized by peripheral DCs and then restimulated by myelin-presenting DCs in the brain (52).

Additionally, B cells have been shown to be critical regulators of autoimmunity. B cell central and peripheral tolerance checkpoints are defective in patients with SLE, T1D and RA resulting in a greater proportion of autoreactive B cells in the periphery (53, 54). Dysregulation of B cell inhibitory signaling has been linked to lupus-like disease in mouse models, and to diseases like SLE and T1d in humans. The defects primarily involve mutations in inhibitory receptors like FCγRIIB and CD22, or in acetylation of CD22 ligands by sialyl acetyl esterase (SIAE), or in reduced function of downstream signaling mediators such as Lyn, SHP-1 and SHIP (55). B cells are efficient APCs and can contribute to activation and polarization of naïve T cells during autoimmune responses (56). B cells are one of the first islet-infiltrating cell types in NOD mice where they contribute to expansion of diabetogenic T cells, and B-cell depletion using anti-CD20 antibody in humanized NOD mice ameliorates disease (57, 58). Consistent with a pathogenic role of B cells, B-cell depletion therapies have shown some efficacy in treatment of RA and MS (59-61). However, B cell depletion actually exacerbates other conditions, like ulcerative colitis and psoriasis, indicating that they may have a significant role in dampening of harmful immune responses, and this could potentially account for the mixed results of B-cell depletion therapies (62). Indeed, a distinct IL-10 producing B cell population, termed B regulatory

cells (Bregs) has been recently recognized as able to suppress immune responses by curbing proliferation and proinflammatory cytokine production by Th1 and Th17 cells and by promoting Treg generation, both via production of soluble mediators and direct cell to cell contact (56). Furthermore, transfer of Bregs was effective in suppressing disease in mouse models of collagen-induced arthritis and lupus (63).

In sum, both DCs and B cells can regulate T-cell tolerance through a combination of cell-intrinsic and cell-extrinsic effects. The relative contribution of DCs and B cells to maintaining tolerance to tissue antigen is still unclear and is under active investigation.

Lyn is a negative regulator of signaling in immune cells other than T cells

Lyn is a gene that has been implicated in tolerance in mouse models of autoimmunity and in humans. Lyn is an Src-family kinase (SFK) that is expressed in all hematopoietic cells other than T cells and has a unique role as a negative regulator of signaling downstream of receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (64, 65). The role of Lyn in signaling has been best studied in B cells, where it regulates B-cell receptor (BCR) signaling thresholds by phosphorylating ITIMs of inhibitory receptors such as CD22 and FCγRIIB leading to recruitment of phosphatases such as SHP-1 and SHIP to attenuate B-cell activation (64). In myeloid cells, Lyn can function as both a positive and a negative regulator depending on the cell type, however, its precise signaling mechanisms are less well understood. Lyn has been implicated in regulating myelo- and erythropoiesis, and signaling downstream of integrins, and cytokine and growth factor receptors (65).

Consistent with its role as a key regulator of inhibitory pathways, *Lyn*$^{-/-}$ mice develop lupus-like disease characterized by production of anti-nuclear antibodies (ANAs), immune complex deposition and glomerulonephritis (66, 67). These mice have elevated levels of serum proinflammatory cytokines and exhibit progressive myeloproliferaton and splenomegaly (68, 69). On a cellular level, *Lyn*$^{-/-}$ mice exhibit increased activation of B cells, dendritic cells and myeloid cells. Although Lyn is not expressed in T cells, progressive activation of T cells also occurs in Lyn-deficient mice. This has been attributed to an inflammatory loop that is set up by hyperactive macrophages and DCs resulting in overproduction of B-cell activating factor (BAFF) and cytokines such as IL-6 and IL-12, which activate T cells and induce them to make IFNγ, which in turn enhances the proinflammatory nature of the macrophages and DCs (69).

Because Lyn is expressed in multiple cell types, there has been considerable interest in dissecting the contributions of individual cells to the *Lyn*$^{-/-}$ mouse phenotype. Mice carrying a conditional allele of *Lyn* were created by the Lowell group and used to study selective deficiency of Lyn in DCs or B cells (70, 71). Deletion of Lyn in B cells alone was sufficient for development of anti-nuclear antibodies and glomerulonephritis, and moderate activation of T cells and DCs compared to the complete knockout (71). Interestingly, deletion of Lyn only in DCs resulted in a much more severe autoimmune and inflammatory phenotype compared to the complete knockout and was accompanied by dramatic increase in production of proinflammatory cytokines and T-cell activation, indicating that loss of inhibitory pathways in dendritic cells alone can lead to autoimmunity (70).

Lyn has been associated with several human autoimmune diseases, such as SLE, asthma and psoriasis by GWAS (72). Because of its role in regulation of DC and B cell responses, perturbations in Lyn-mediated pathways are likely to have consequences for T-cell mediated autoimmunity, although organ-specific autoimmunity has not been observed in *Lyn*$^{-/-}$ mice to date. Given that human autoimmunity is multigenic in nature, I hypothesized that Lyn may act as a susceptibility factor in organ-specific autoimmunity when combined with another defect in immune tolerance. A model for such interaction is the subject of Chapter 2.

CHAPTER TWO:

Defects in the Lyn and Aire Pathways Cooperate to Promote Autoimmune Uveitis

INTRODUCTION

In most individuals, a diversity of tolerance mechanisms work together to prevent autoimmune disease by limiting the activity of self-reactive lymphocytes (2). Immune tolerance requires integrity of multiple checkpoints within both innate and adaptive branches of the immune system, but how such checkpoints fail in some individuals leading to particular autoimmune phenotypes remains poorly understood. Strikingly, most autoimmune diseases exhibit a high degree of genetic susceptibility, and in most cases this susceptibility is multigenic in origin (55, 73). In spite of this knowledge, little is known about how multiple genes interact to cause disease and which tolerance pathways are compromised.

Humans that harbor a complete defect in the *Autoimmune Regulator* (*AIRE*) gene develop a multi-organ specific autoimmune syndrome called Autoimmune Polyglandular Syndrome Type 1 (APS1) (19, 20). Aire promotes T cell tolerance by driving the expression of a wide array of tissue-specific self antigens (TSA's) in the thymus and therefore is critical for central tolerance of T cells recognizing these TSAs (74). In addition to rare individuals with autoimmunity and a complete absence of *AIRE*, a family was identified with an *AIRE* mutation and dominantly inherited autoimmune susceptibility (75, 76). A recent study indicates that individuals with dominantly acting alleles of *AIRE* may be more widespread than initially thought (75). Individuals with dominant *AIRE* mutations develop autoimmunity that is less penetrant and has later onset compared to individuals with complete *AIRE* deficiency. These clinical observations are consistent with hypomorphic Aire function in individuals with one dominant negative *AIRE* allele and one normal allele,

and suggest that additional genetic alterations, perhaps affecting other tolerance checkpoints, may contribute to disease onset in such individuals.

Whereas defects in Aire function lead to organ-specific autoimmune diseases in humans and mice, defects in *Lyn* lead to a severe lupus-like systemic autoimmunity in mice (64, 65). Lyn is a Src-family tyrosine kinase that is expressed by B cells, myeloid cells and dendritic cells (DCs), but not by T cells. It has a unique role of enabling function of inhibitory receptors that have Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) in their cytoplasmic domains by phosphorylating the ITIMs (64). Selective deletion in mice of Lyn in B cells or in DCs leads to systemic autoimmunity, the former being especially important for production of anti-nuclear antibodies and the latter being important for increased inflammation (66, 70, 71). Although single nucleotide polymorphisms (SNPs) near the *LYN* gene have been associated with systemic lupus erythematosus (SLE) in GWAS studies, it is unclear whether these SNPs affect Lyn expression or function (77, 78). More compelling are observations that loss of function mutations in sialic acid acetylesterase (SIAE), which is critical for the function of CD22, an inhibitory receptor whose function requires Lyn, have been found at elevated frequency in individuals with SLE, rheumatoid arthritis, and type 1 diabetes (79). While most individuals with these diseases have normal alleles of SIAE, individuals with loss of function of SIAE have an 8-fold increased risk of developing autoimmune disease. Interestingly, type 1 diabetes is a quintessential organ-specific autoimmune disease, suggesting that Lyn-dependent inhibitory pathways may be important for preventing organ-specific autoimmunity as well as systemic autoimmunity.

To test the hypothesis that a defect in a peripheral tolerance pathway can interact with a defect in central tolerance, we generated *Lyn* deficient mice also harboring a hypomorphic mutation in *Aire* on an autoimmune-resistant genetic background. Remarkably, these double mutant mice were found to develop an early onset, highly destructive autoimmune uveitis. Mechanistic analysis of this digenic autoimmune disease model demonstrated that Lyn deficiency in DCs results in increased priming in the immunologic periphery of T cells recognizing an Aire-regulated retinal self antigen. This altered priming permitted expansion of an organ-specific population of T cells that had escaped deletion during development in the thymus due to reduced function of Aire. Taken together these results demonstrate how defects in distinct genetic pathways that control immune tolerance can synergize to provoke autoimmunity.

RESULTS

Aire$^{GW/+}$ *Lyn*$^{-/-}$ double mutant mice develop spontaneous posterior uveitis.

A dominant-negative allele of *Aire* containing the G228W point mutation when combined with a wild type *Aire* allele results in hypomorphic Aire function and leads to a mild autoimmune susceptibility on the autoimmune-resistant C57BL/6 genetic background, limited to lacrimal and salivary gland infiltrates in Aire$^{GW/+}$ mice (80). To determine whether a defect in Lyn could enhance this mild susceptibility and affect the disease spectrum, we generated a line of double mutant *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice. Cohorts of double mutant mice and single mutant controls were aged for 8-10 months and analyzed for the presence of inflammatory infiltrates in organs known to be a target of autoimmune attack in mice completely deficient for Aire (7, 80).

Histological sections of lacrimal and salivary glands from *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ double mutants revealed lymphocytic infiltrates similar in incidence and severity to *Aire*$^{GW/+}$ mice (Fig. 1A). Furthermore, infiltrates in the stomach, pancreas, liver, lungs and the sciatic nerve of the double mutant mice were generally mild or absent (Fig. 1A). Two out of ten double mutants had moderate lung infiltrates, however, the sera from these mice were negative for antibodies to Bpifb1, a major lung autoantigen in *Aire*$^{-/-}$ mice (data not shown) and similar inflammation was seen in some single mutant mice (Fig. 1A) (33). Surprisingly, histological sections of eyes from four out of ten double mutant mice showed evidence of a severe retinal lymphocytic infiltration, which was absent from the single mutant mice. Thus, the eye was the only organ from our panel that appeared to be selectively affected in double mutant mice.

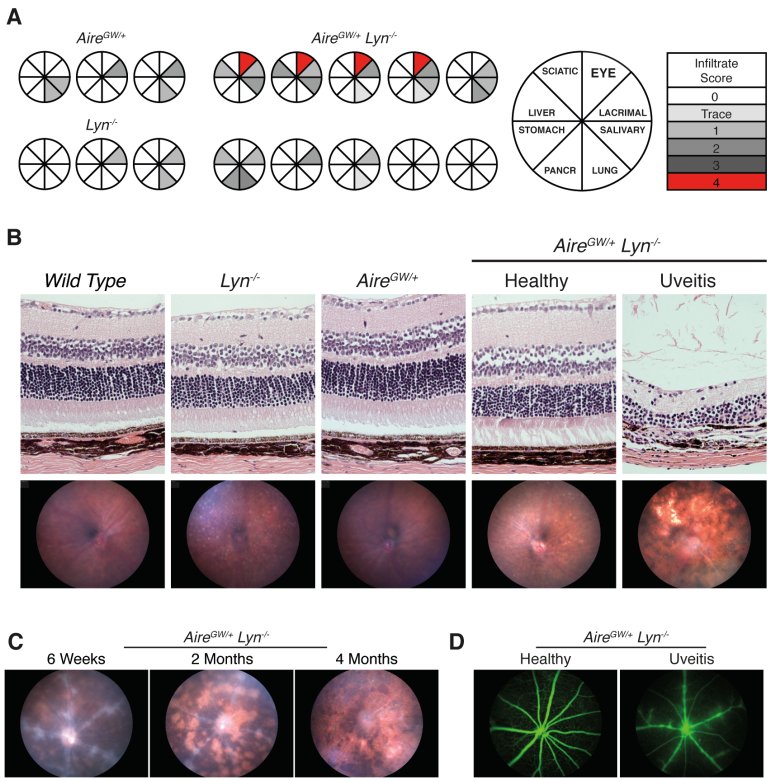

Figure 1. *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ double mutant mice develop progressive posterior uveitis.

A. 8-10 month old *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ (n=10), *Aire*$^{GW/+}$ (n=4) or *Lyn*$^{-/-}$ (n=6) mice were analyzed by scoring H&E-stained histological sections for presence of inflammatory infiltrates in a panel of organs consisting of eye, lacrimal gland, salivary gland, lung, pancreas, stomach, liver and sciatic nerve. Pie graphs represent individual mice, with shaded sections indicating the presence of mononuclear infiltrate in the designated organ. The degree of shading reflects the severity of autoimmune damage, as indicated. The characteristics of all ten double mutant mice are shown, whereas three representative examples of each single mutant mouse strain are shown.

B. Representative funduscopic images (bottom row) or H&E stained retinal sections (20x magnification, top row) from 5 month old *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice with and without uveitis and wild type, *Aire*$^{GW/+}$, and *Lyn*$^{-/-}$ mice.

C. Time course of funduscopic changes in an *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mouse with uveitis. Early changes consisted of swelling of retinal vessels and perivascular exudates and subsequent development of inflammatory lesions. Advanced disease led to extensive retinal destruction and scarring. (n=3 mice with uveitis analyzed longitudinally)

D. Representative fluorescein imaging of retinal vessels in healthy (left) and diseased (right) 7-week old *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice. (at least 3 mice per group were analyzed)

We therefore chose to focus our further investigation on retinal disease in the double mutant mice. The eyes of 5-month-old mice were analyzed by in vivo funduscopic imaging and subsequent histology (Fig. 1B). Funduscopy revealed severe inflammation, including multiple retinal lesions with areas of complete retinal destruction, characteristic of advanced uveitis. By histology, disease manifested as extensive mononuclear infiltrates in the retina and choroid, and destruction of photoreceptor outer segments and nuclei. Over the course of the study, a cohort of 100 mice were analyzed at three to six months of age, and the incidence of retinal disease was 48%. None of the *Lyn*$^{-/-}$ mice examined developed uveitis, and disease was seen in only 6% of *Aire*$^{GW/+}$ mice (Table 1). In addition, ten 4-6 month old *Aire*$^{GW/+}$ *Lyn*$^{+/-}$ mice were evaluated by funduscopy and histology and were all found to be free of uveitis, suggesting that haploinsufficiency of Lyn was not enough to drive Aire-dependent uveitis (Table 1).

Table 1.
Incidence of uveitis in *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice and controls

Genotype	Disease Observed	Incidence (%)
WT C57BL/6J	0/50	0
Aire$^{GW/+}$	3/50	6
Lyn$^{-/-}$	0/50	0
Aire$^{GW/+}$ *Lyn*$^{+/-}$	0/10	0
Aire$^{GW/+}$ *Lyn*$^{-/-}$	48/100	48

3-6 month old mice of indicated genotypes were screened for uveitis by funduscopy and/or histology. Shown are numbers of mice with disease per total number of mice and resulting incidences.

To gain a better understanding of the dynamics of eye disease, we monitored young double mutant mice by funduscopy for disease development and progression. Interestingly, the first signs of uveitis were typically seen at around 6 weeks of age, with some mice developing disease as early as 5 weeks. Early disease was characterized by swelling of retinal vessels and perivascular exudates potentially corresponding to early T-cell infiltration (Fig. 1C). In mice with early stage disease, leakiness of eye blood vessels was demonstrated by use of a fluorescein tracer (Fig. 1D). Subsequently, inflammation progressed to multiple lesions and then to complete retinal destruction, scarring and atrophy (Fig. 1C). Conversion to uveitis in *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice older than 10 weeks of age was almost never observed.

Uveitis in *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice is accompanied by an adaptive immune response to IRBP.

The uveitis seen in mice completely deficient for *Aire* is strongly linked to a T cell response to a retinal autoantigen, interphotoreceptor-retinoid binding protein (IRBP), the expression in the thymus of which is controlled by Aire (30). Therefore, we examined the adaptive immune responses to IRBP in *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ double mutant mice.

As in many organ specific autoimmune diseases, autoantibodies to ocular antigens correlate with uveitis in the Aire-deficient model (30, 31). Therefore, sera from mice with and without uveitis were immunoblotted against mouse whole eye extracts (Fig S1). Sera derived from *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice with uveitis but not from unaffected double mutants

reacted strongly with a single 150 kDa antigen, which corresponds to the molecular weight of IRBP (30).

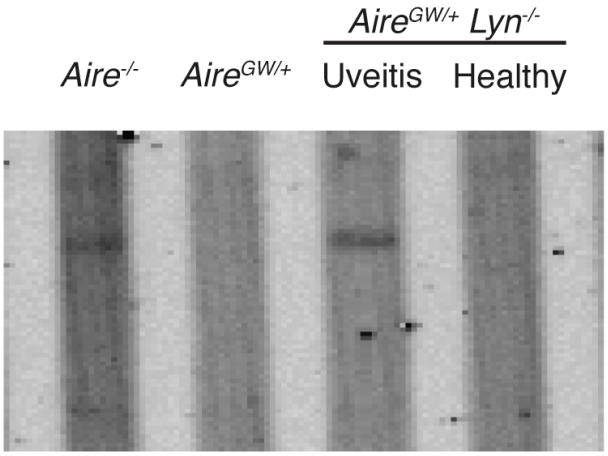

Figure S1.
Sera from double mutant mice predominantly recognize a single eye antigen, IRBP.
Whole mouse eye extracts were resolved by SDS-PAGE, transferred to filters and immunoblotted from sera collected from 3-8 month-old mice of indicated genotypes. Presence of uveitis was confirmed by histology. At least 5 mice for each genotype were analyzed and representative reactivities are shown.

No other reactivities were detected in this assay. To examine the magnitude of the anti-IRBP response in a cohort of 5-9 month old double mutant and control mice, we measured serum IgG binding to in-vitro synthesized radiolabeled mouse IRBP (Fig. 2A). All double mutant mice with uveitis had detectable titers of anti-IRBP antibodies. None of the healthy double mutant mice or control mice had these antibodies, with the exception of a single *Aire*$^{GW/+}$ mouse, which had disease. Therefore, the presence of anti-IRBP IgGs closely correlated with uveitis.

Uveitis in *Aire*$^{-/-}$ mice is also accompanied by an expansion of CD4 T-cells specific for the P2 peptide, corresponding to amino acids 271-290 of IRBP (32). Importantly, P2-specific T cells are negatively selected in Aire$^{+/+}$ mice and their escape from the thymus occurs in *Aire*$^{-/-}$ mice (32). We used a fluorescently-labeled I-A^{b} tetramer reagent to detect

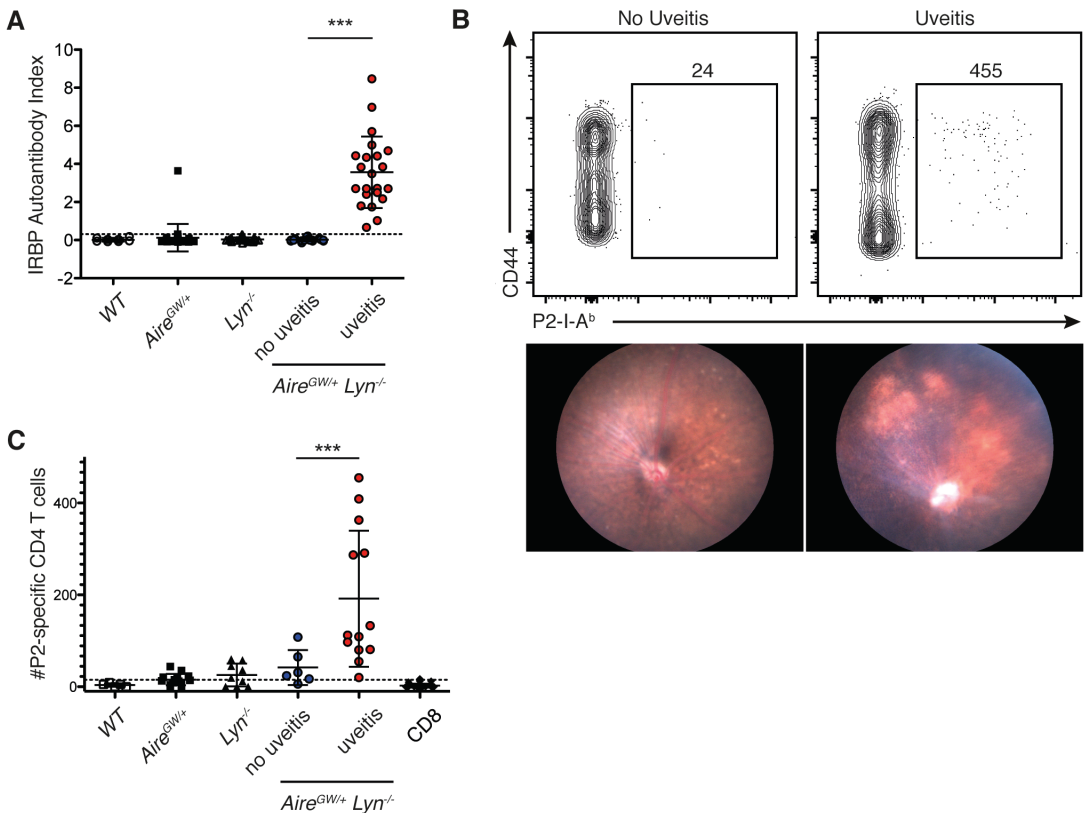

Figure 2. Presence of anti-IRBP antibodies and IRBP-reactive T cells in *Aire$^{GW/+}$ Lyn$^{-/-}$* mice with uveitis.

A. The sera of 2-6 month-old *Aire$^{GW/+}$ Lyn$^{-/-}$* mice with (n=14) and without (n=9) uveitis, or of wild type (n=8), *Aire$^{GW/+}$* (n=36) or *Lyn$^{-/-}$* (n=31) mice were analyzed for the presence of anti-IRBP antibodies with a radioligand-binding assay and normalized to a commercially available anti-IRBP antibody to determine autoantibody index (AI). Each dot represents an individual mouse and the horizontal lines show mean ± standard deviation Dashed line represents a limit of detection (LOD), calculated as an average of AI values for all wild type samples + 3 standard deviations. ***$P < 0.001$, 1-way ANOVA

B. Representative flow cytometric analysis of IRBP P2 tetramer-binding CD4 T cells in pooled spleen and cervical lymph nodes (top) and funduscopy (bottom) from 2-5 month old *Aire$^{GW/+}$ Lyn$^{-/-}$* mice with and without uveitis. Plots were pregated for CD4+ T cells as described in Methods. The calculated total number of tetramer-positive cells in the whole sample is shown on the plot.

C. Numbers of P2-specific CD3+ CD4 T cells gated as in (B) from individual *Aire$^{GW/+}$ Lyn$^{-/-}$* mice with and without uveitis, and from wild type and single mutant control mice. Each dot represents an individual mouse and the horizontal lines show mean ± standard deviation. Dashed line represents the limit of detection, calculated as average number of P2-binding CD3+CD8+ T cells + 3 standard deviations. Data are pooled from 3-5 independent experiments. ***$P < 0.001$, 1-way ANOVA

P2-specific CD4 T cells within the polyclonal T cell repertoire of double mutant mice with and without uveitis. Cells from the cervical and submandibular lymph nodes and the spleen of individual mice were pooled, incubated with P2-tetramer, enriched and analyzed by flow cytometry. A robust expansion of P2-specific CD4 T cells was detected in double mutant mice with uveitis (Fig. 2B,C). Moreover, a majority of tetramer-binding CD4 T cells expressed high levels of the activation marker CD44 (Fig. 2B), suggesting previous antigen encounter. These data demonstrate that uveitis in *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice is accompanied by an adaptive immune response to IRBP, including expansion of Aire-regulated IRBP-specific CD4 T cells in the periphery and production of anti-IRBP antibodies.

Uveitis in *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice is accompanied by retinal infiltration of IRBP-specific T cells.

We next examined the T cell response in the retinas of double mutant mice with uveitis. After disease status was ascertained by funduscopic examination, retinas were isolated and their cellular composition analyzed by flow cytometry. As expected, very few CD45+ TCRβ+ T cells were present in the retinas of control Aire$^{GW/+}$ mice, comprising less than 0.3% of total retinal cells, whereas diseased retinas manifested a massive influx of T cells, resulting in a 90-fold increase in total TCRβ+ T cell numbers (Fig. 3A). Consistent with these findings, confocal images of retinal sections from diseased, but not healthy, double mutant mice displayed significant TCRβ+ T cell infiltrates in the outer retina and choroid (Fig. 3C).

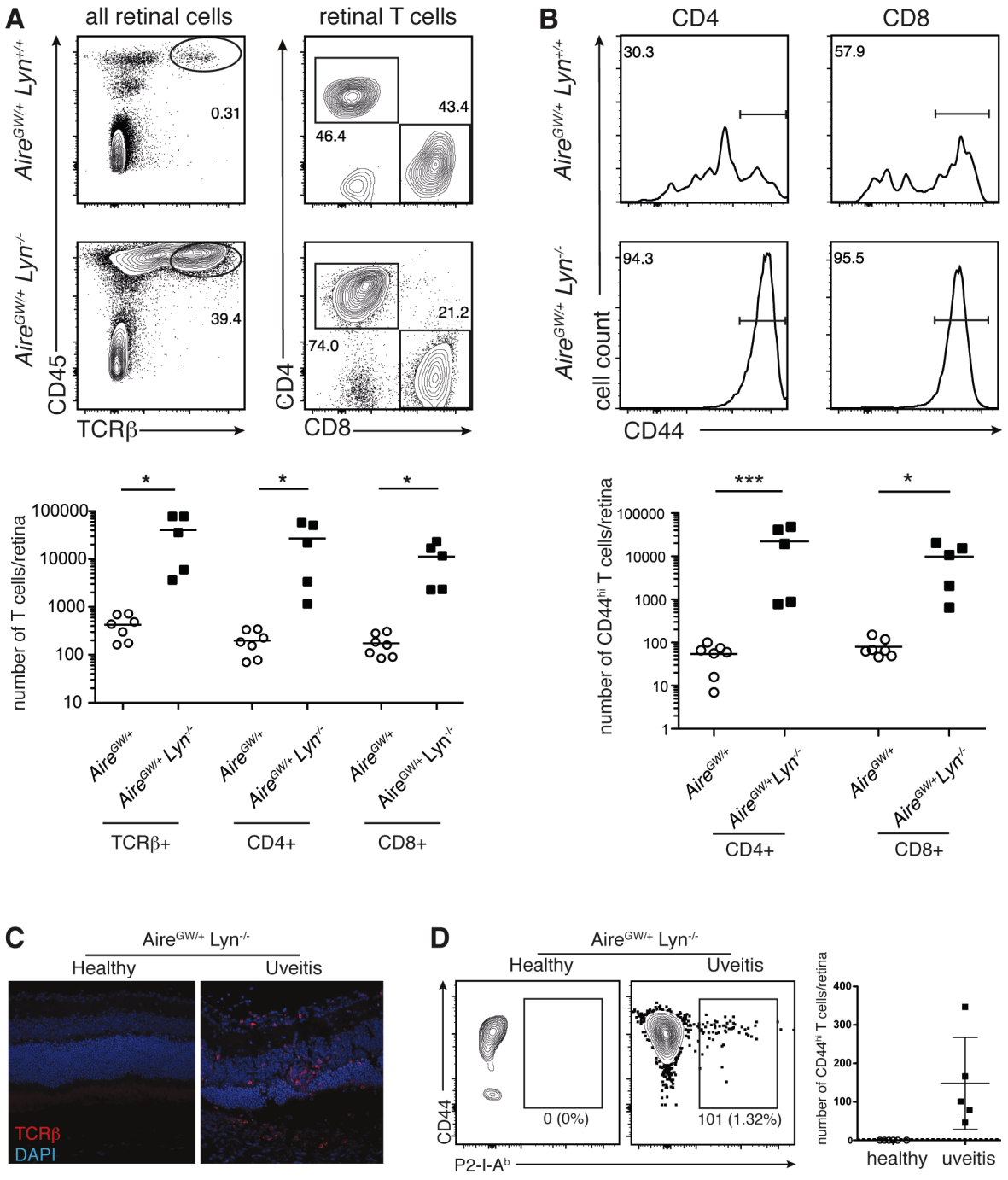

Figure 3. Activated IRBP-specific T cells infiltrate the retinas of *Aire$^{GW/+}$ Lyn$^{-/-}$* mice with uveitis.

Figure 3 (cont).

6-10 week old *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ and *Aire*$^{GW/+}$ *Lyn*$^{+/+}$ mice were screened by funduscopy and their retinal T cell populations analyzed by flow cytometry.

A. Upper: Representative flow cytometric plots pre-gated on total live retinal cells (left) or CD45+ TCRβ+ retinal T cells (right); numbers shown indicate percent of cells in gate. Lower: absolute numbers of the indicated cell type per retina of the indicated mouse strain. Each dot represents an individual mouse and the horizontal lines show mean.

B. Analysis of CD44 expression by retinal CD4 and CD8 T cells gated as in (A). Upper: Representative flow cytometric plots showing frequency of CD44^{hi} cells. Lower: The absolute numbers of CD44^{hi} CD4 and CD44^{hi} CD8 T cells per retina. Each dot represents an individual mouse and the horizontal lines show mean.

Data shown are representative of at least three independent experiments with n = 3-5 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired student's T test.

C. Confocal immunofluorescent imaging of retinal sections of *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice with or without uveitis showing infiltration of TCRβ+ T cells (red) in mice with disease. Data are representative of two independent experiments.

D. Flow cytometric analysis of IRBP P2 tetramer-specific CD4+ T cells in the retinas of *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice without and with uveitis (left and center panels, respectively); plots were gated on CD45+ TCRβ+ CD4+ T cells as described in Methods. Shown are total absolute numbers of tetramer-positive cells per retina (quantified on the right), followed by their frequency in parenthesis. Each dot represents an individual mouse and the horizontal lines show mean ± SD. Dashed line represents the limit of detection, calculated as in Figure 2. Data are pooled from 3 independent experiments.

Further analysis of the retinal infiltrates revealed that CD4 T cells outnumbered CD8 T cells approximately 2:1 in diseased retinas (Fig 3A). Moreover, over 80% of infiltrating CD4 and CD8 T cells expressed high levels of the activation marker CD44 as opposed to only 20-40% of the T cells in the retinas of *Aire*$^{GW/+}$ control mice (Fig. 3B). This translated to a 420-fold increase in absolute numbers of CD44^{hi} CD4 T cells and a 120-fold increase in absolute numbers of CD44^{hi} CD8 T cells, suggesting a massive influx of primed effector-memory T cells into the target tissue, consistent with a CD4 T-cell dependent autoimmune uveitis. Moreover, a significant fraction of retinal CD4 T cells from individual mice bound P2 tetramer, comprising 1-3% of total retinal CD4 T cells (Fig. 3D). Notably, no tetramer-specific cells could be detected in retinas from individual double mutant mice without disease (Fig. 3D). The absence of P2 tetramer-binding CD4 T cells in the retinas of control mice was verified by pooling retinas from five or more *Aire*$^{GW/+}$ or *Lyn*$^{-/-}$ mice or double mutant mice grouped by disease status (Fig. S2). Interestingly, the absolute numbers of P2 tetramer-specific CD4 T cells detected in the retinas of mice with disease were similar to the numbers detected in all of the pooled secondary lymphoid organs of comparable mice with disease (Fig 2C). Thus, it appears that IRBP-specific CD4 T cells were highly enriched in the retina during the active stages of uveitis.

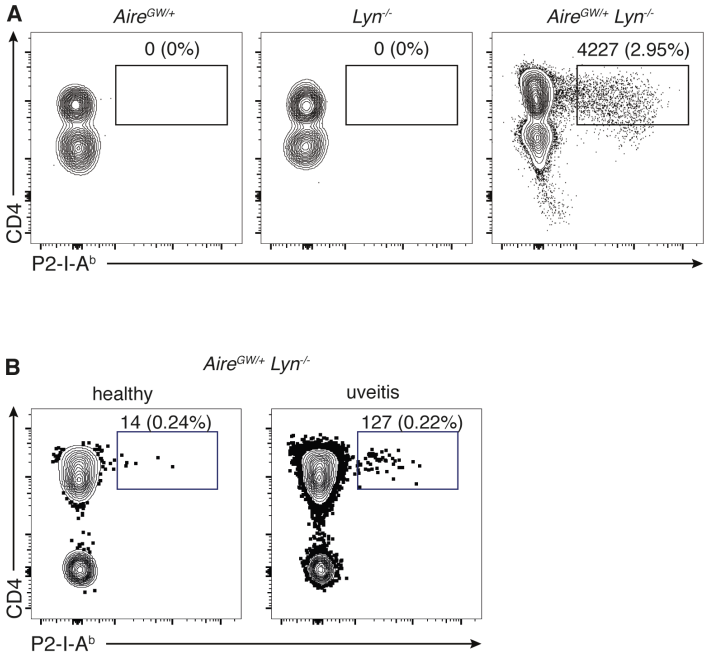

Figure S2. Absence of tetramer-specific events in pooled retinas from *Aire$^{GW/+}$* and *Lyn$^{-/-}$* mice.

Flow cytometric analysis of IRBP P2 tetramer-specific CD4+ T cells in pooled retinas from mice of indicated genotypes. Shown are plots that have been gated on CD45^{+} TCRβ^{+} CD4^{+} T cells as described in Methods. Calculated total numbers of tetramer-positive cells per retina are shown, followed by their frequency in the T cell population in parenthesis.

A. Retinal P2+ CD4 T cells in pooled *Aire$^{GW/+}$* (n-7), *Lyn$^{-/-}$* (n=5), and *Aire$^{GW/+}$ Lyn$^{-/-}$* mice (n=8; 5 mice with uveitis).

B. Retinal P2+ CD4 T cells in pooled *Aire$^{GW/+}$ Lyn$^{-/-}$* mice with or without uveitis (n=5 per group).

Deletion of Lyn in DCs combined with reduced Aire function is sufficient for uveitis.

Lyn is expressed in most immune cell types other than T cells, and its deficiency in either dendritic cells or B cells is sufficient to lead to spontaneous systemic autoimmunity (70, 71). To gain insight into which cell type promotes uveitis in *Aire*$^{GW/+}$ mice, we generated *Aire*$^{GW/+}$ mice carrying a floxed *Lyn* allele and crossed them to Cre-recombinase expressing lines that delete either in DCs (*CD11c-Cre*), macrophages and neutrophils (*LysM-Cre*) or B cells (*Mb1-Cre*).

Cohorts of mice were aged and evaluated for the presence of uveitis by funduscopic examination starting at 2 months of age until 6 months of age. Whereas deletion of Lyn in macrophages, neutrophils or B cells did not lead to disease, deletion of Lyn in DCs resulted in uveitis in 13/33 of mice (Table 2 and Fig. 4A). As with the *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ double mutant mice, uveitis in *Aire*$^{GW/+}$ *CD11c-cre Lyn*$^{F/F}$ mice (hereafter called *Aire*$^{GW/+}$ *Lyn-DC*$^{-/-}$) occurred at a young age, before overt systemic autoimmunity. The onset of uveitis in the *Aire*$^{GW/+}$ *Lyn-DC*$^{-/-}$ mice was slightly delayed, with first funduscopic changes evident 7-8 weeks as opposed to 5-6 weeks in the *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice (Fig. 4B), indicating that in addition to DCs, Lyn deficiency in another cell type may contribute to development of autoimmune uveitis.

Table 2.
Incidence of uveitis in *Aire*$^{GW/+}$ *Lyn-DC*$^{-/-}$ mice and controls

Genotype	Disease Observed	Incidence (%)
Lyn$^{F/F}$	0/22	0
Aire$^{GW/+}$ *Lyn*$^{F/F}$	2/34	6
CD11c-cre Lyn$^{F/F}$	0/30	0
Aire$^{GW/+}$ *Mb1-cre Lyn*$^{F/F}$	0/9	0
Aire$^{GW/+}$ *LysM-cre Lyn*$^{F/F}$	0/11	0
Aire$^{GW/+}$ *CD11c-cre Lyn*$^{F/F}$	13/33	39

3-6 month old mice of indicated genotypes were screened for uveitis by funduscopy and/or histology. Shown are numbers of mice with disease per total number of mice and resulting incidences.

Similarly to the *Aire*$^{GW/+}$ mice lacking Lyn in all cells, uveitis in the *Aire*$^{GW/+}$ *Lyn-DC*$^{-/-}$ mice was accompanied by high titers of anti-IRBP antibodies and retinal T cells infiltrates, including activated P2 tetramer-specific CD4 T cells (Fig. 4C-E).

These findings indicate that absence of Lyn in DCs is sufficient for induction of autoimmune uveitis in *Aire*$^{GW/+}$ mice.

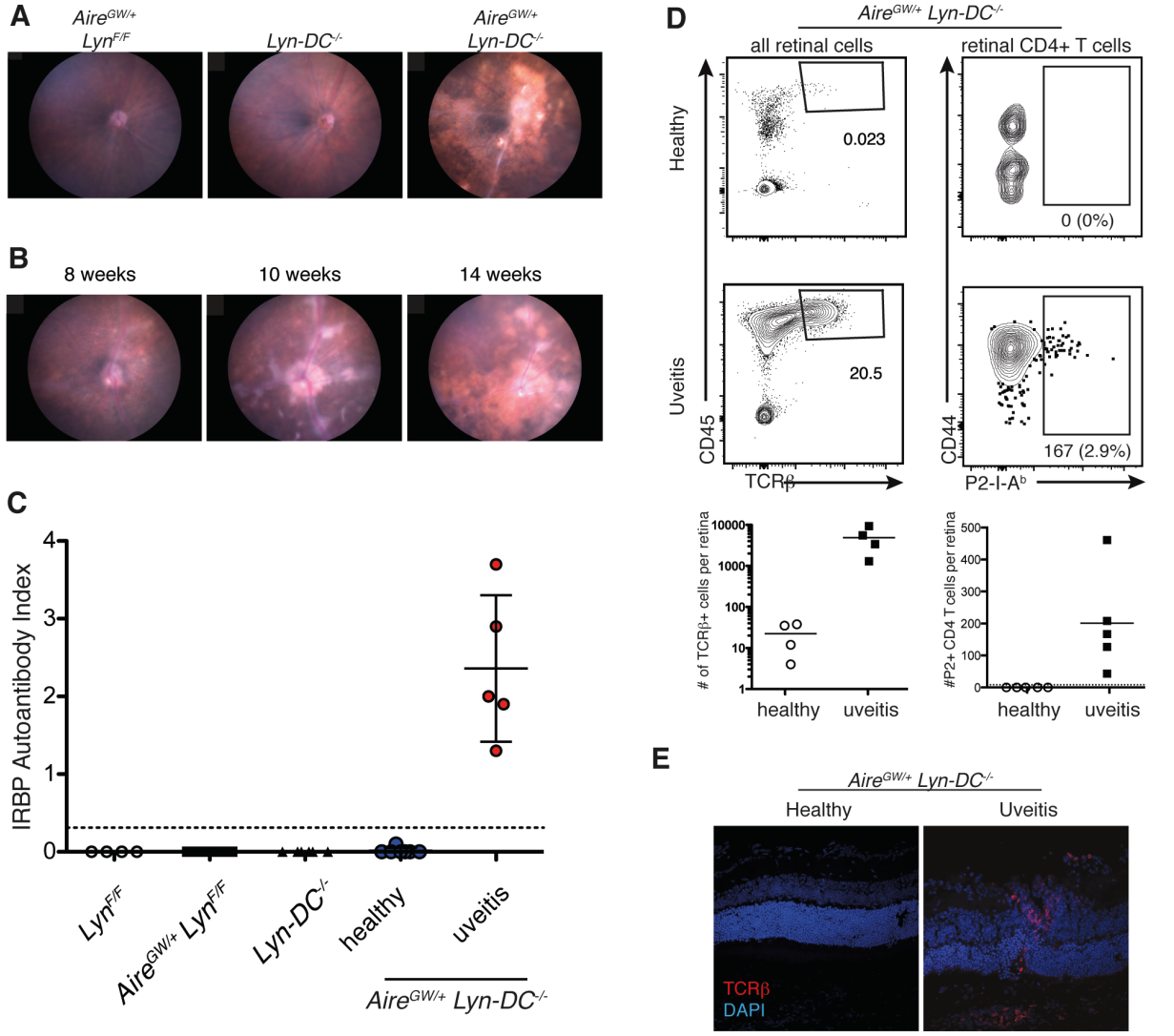

Figure 4. *Aire*$^{GW/+}$ *Lyn-DC*$^{-/-}$ mice develop uveitis that is accompanied by an anti-IRBP immune response.

Figure 4 (cont).

A. Representative funduscopic images of 3-6 month old mice of indicated genotypes showing retinal disease in a subset of the *Aire$^{GW/+}$ Lyn-DC$^{-/-}$* mice.
B. Representative time course of funduscopic changes in an *Aire$^{GW/+}$ Lyn-DC$^{-/-}$* mouse with uveitis. (representative of n=3 mice with uveitis analyzed longitudinally)
C. Anti-IRBP antibody levels in the serum of 2-6 month old Aire$^{GW/+}$ Lyn-DC$^{-/-}$ mice with (n=5) and without uveitis (n=7) were measured. Control mice analyzed consisted of *Lyn$^{F/F}$* (n=8), *Aire$^{GW/+}$ Lyn$^{F/F}$* (n=15) and *CD11c-cre Lyn$^{F/F}$* (n=10). Each dot represents an individual mouse, the horizontal lines show mean ± SD, dashed line represents limit of detection.
D. Upper: Representative flow cytometry plots of retinal cell populations from *Aire$^{GW/+}$ Lyn-DC$^{-/-}$* mice without uveitis (top) or with uveitis (bottom). Single cell suspensions from retinas of individual mice were analyzed for retinal CD45+ TCRβ+ T cells (left) and P2-tetramer specific CD4 T cells (right). Numbers shown indicate percent of cells in gate. Lower: calculated total numbers of the indicated cells per retina. Each circle or square represents an individual mouse and the horizontal lines show mean. Dashed line represents the limit of detection, as in Figure 2. Data is representative of at least three independent experiments.
E. Confocal imaging of retinal sections of *Aire$^{GW/+}$ Lyn-DC$^{-/-}$* mice with or without uveitis showing infiltration of TCRβ+ T cells (red) in mice with disease. Data are representative of two independent experiments.

Increased antigen presentation by Lyn-deficient DCs in the eye-draining lymph node leads to expansion of IRBP-specific T cells in *Aire$^{GW/+}$ Lyn$^{-/-}$* mice

As there were substantial numbers of IRBP P2-tetramer-binding CD4 T cells in the retinas of *Aire$^{GW/+}$ Lyn$^{-/-}$* mice with uveitis, we examined their numbers in the eye-draining (cervical and submandibular) or in other peripheral (axillary and inguinal) lymph nodes of individual animals. We found a significant expansion of P2-specific CD4 T cells in the eye-draining but not in the non-draining peripheral lymph nodes of double mutant mice with uveitis (Fig. 5A). Interestingly, a small but significant population of P2-specific CD4 T cells could also be seen in the draining lymph nodes of double mutant mice without uveitis, whereas there was no detectable expansion in either of the single mutant mice (Fig. 5A). This result suggested that either there was more IRBP reaching the draining lymph of double mutant mice without evident inflammation of the retina and/or the Lyn-deficient DCs that presented IRBP in the draining lymph were more stimulatory than wild type DCs.

To test whether eye-draining lymph node DCs from Lyn-deficient mice indeed presented more IRBP, we isolated DCs from either eye-draining or peripheral lymph nodes by anti-CD11c-magnetic-bead enrichment and examined the ability of DCs from these different sites to stimulate a T-cell hybridoma specific for the P2 peptide of IRBP (32). The DCs from eye-draining lymph nodes of Lyn$^{-/-}$ mice were significantly better at stimulating the hybridomas than were wild type DCs across a range of DCs numbers tested, indicating that more endogenous IRBP was presented (Fig. 5B,C). The stimulation of the P2-specific hybridoma was dependent on the presence of IRBP in the draining lymph node, since there was little

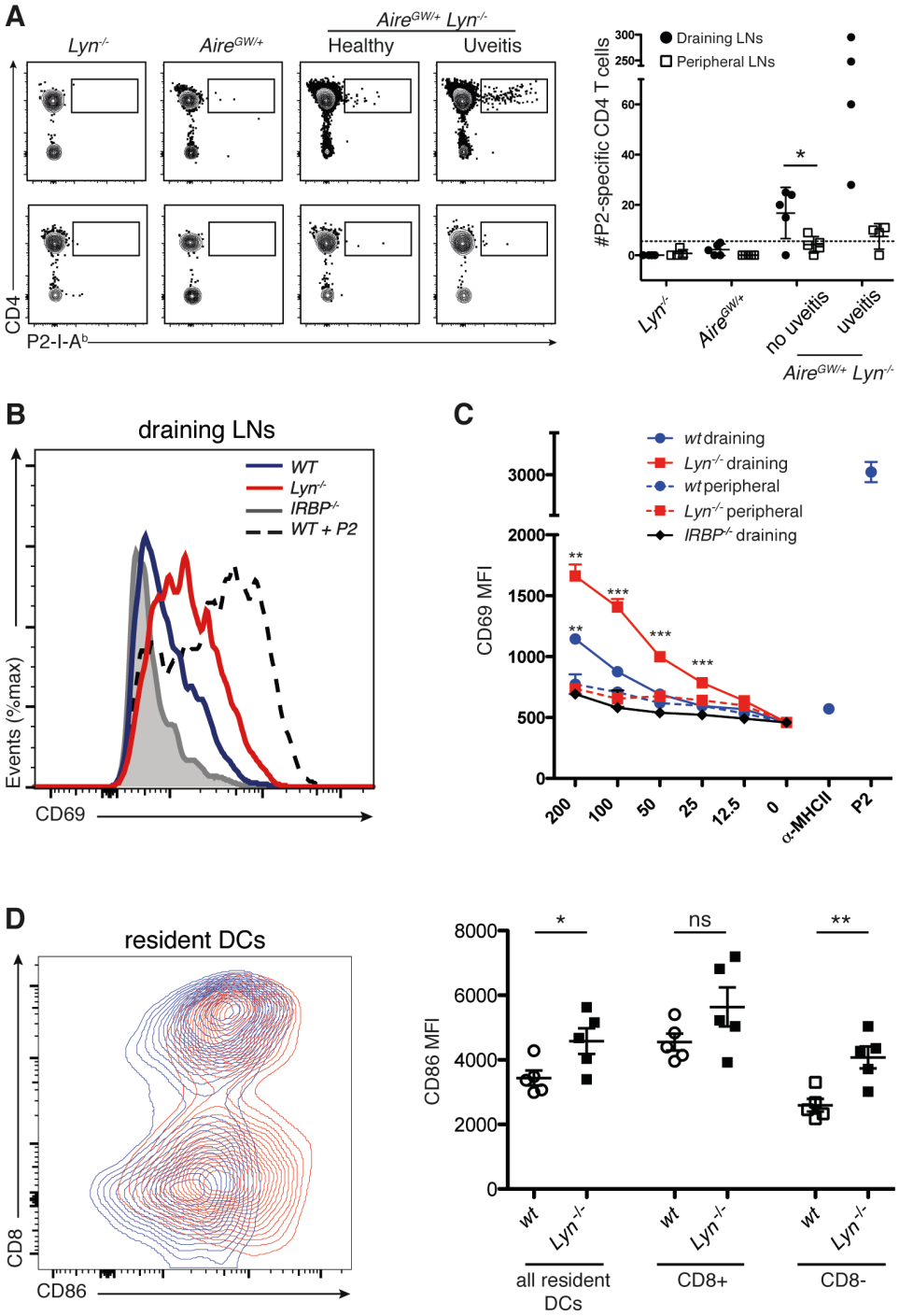

Figure 5. Increased priming of IRBP-specific CD4 T cells in eye-draining lymph nodes of *Aire$^{GW/+}$ Lyn$^{-/-}$* mice.

Figure 5 (cont).

A. Left set of panels: Representative flow cytometry plots measuring the frequency of P2-specific CD4 T cells in the eye-draining cervical LNs (top) or in non-draining pooled axillary and inguinal LNs (bottom) from 15-20 wk old individual mice of indicated genotypes. Right panel: the total numbers of P2-specific CD4 T cells from the eye-draining (solid circles) or other peripheral (open squares) LNs. Each dot represents an individual mouse and the horizontal lines show mean ±SD. Dashed line represents the limit of detection. Data are pooled from three independent experiments.

B-C. 50,000 P2-specific T-cell hybridoma cells were co-cultured with DCs from either eye-draining cervical LNs or non-draining pooled axillary and inguinal LNs. Cells were incubated overnight without addition of exogenous peptide, except where indicated. Hybridoma stimulation was measured by upregulation of CD69. (B) Flow cytometry plot showing representative CD69 levels on hybridoma cells co-cultured with 200,000 DCs from draining LNs of mice of indicated genotypes. Dashed histogram corresponds to added 0.1 ug/ml P2 peptide. (C). CD69 levels on hybridoma cells co-cultured with varying numbers of DCs from either eye-draining or peripheral LNs of wild type, *Lyn*$^{-/-}$ or *IRBP*$^{-/-}$ mice in the absence of exogenous peptide. As a positive control, hybridomas were co-cultured with 12,500 wt DCs with addition of 0.1 ug/ml P2 peptide. As a negative control, anti-MHCII blocking antibody was added to hybridomas co-cultured with 200,000 wt DCs.

D. Left: Representative flow cytometry plots showing CD86 and CD8 expression by resident (CD11c+ MHCII^{lo}) cervical LN DCs from wild type and *Lyn*$^{-/-}$ mice. Right: Quantification CD86 MFI of indicated DC populations. Each data point represents an individual mouse and the horizontal lines show mean. Data are representative of at least three independent experiments.

or no stimulation by DCs from non-draining lymph nodes from the same mice or by draining lymph node DCs from *IRBP*$^{-/-}$ mice (Fig 5B, C). Furthermore, there was no difference between *Lyn*$^{+/+}$ and *Lyn*$^{-/-}$ DCs in the ability to present added whole IRBP or P2 peptide, indicating that there is no effect on antigen processing (Fig. S3). Importantly, draining lymph node DCs from *Lyn-DC*$^{-/-}$ mice were also better at stimulating the hybridoma than control DCs, indicating that the effect of Lyn deficiency was DC-intrinsic (Fig. S4).

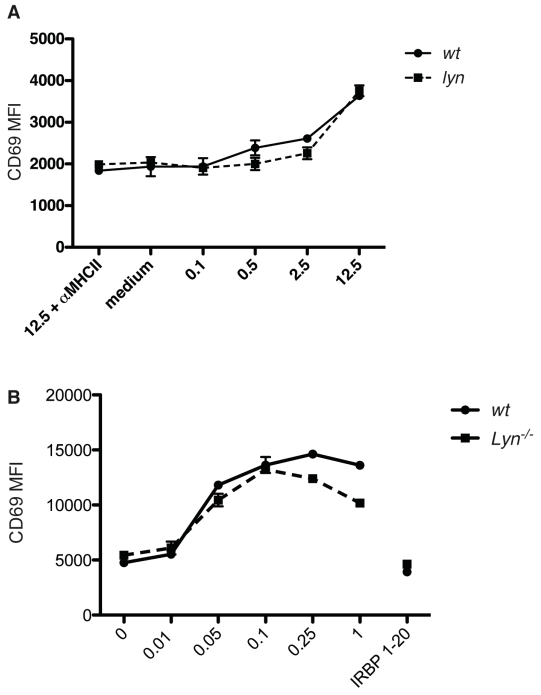

Figure S3. Similar abilities of wt and *Lyn*$^{-/-}$ DCs to present exogenous IRBP to P2-specific T-cell hybridoma

50,000 P2-specific hybridoma cells were co-cultured with either wild type or *Lyn*$^{-/-}$ DCs. Shown are CD69 levels on hybridoma cells after overnight incubation with DCs.

A. 30,000 splenic DCs from wild type or *Lyn*$^{-/-}$ mice were cultured for 24 hours in the presence of whole mouse IRBP at the indicated concentrations, followed by addition of hybridoma cells and subsequent overnight incubation.

B. 100,000 DCs pooled from spleen and cervical LNs of wild type or *Lyn*$^{-/-}$ mice were cultured with hybridoma cells in the presence of indicated concentrations of P2 peptide or IRBP 1-20 peptide as a negative control. Data are representative of two independent experiments.

We next tested whether there was a difference in activation status between *Lyn*$^{+/+}$ and *Lyn*$^{-/-}$ lymph node DC populations by examining surface levels of the CD86 activation marker. Lyn-deficient resident CD11b+CD8- DCs had significantly higher levels of CD86 expression than their wild type counterparts (Fig. 5D). Similar upregulation of CD86 was seen on the Lyn deficient CD11b+CD8- DCs in the non-draining lymph nodes and spleen, and CD86 levels were not significantly different for CD11b- CD8+ DCs or migratory DCs (Fig. 5D and data not shown). Upregulation of CD86 was also observed on CD11b+CD8- resident DCs from *Lyn-DC*$^{-/-}$ mice (Fig. S4).

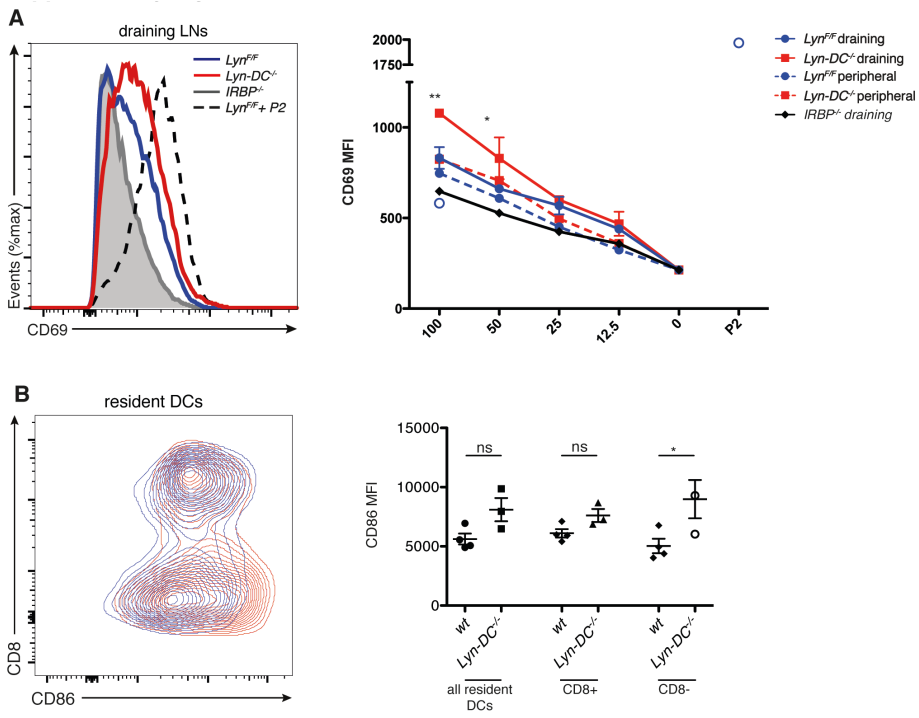

Figure S4. ***Lyn-DC*$^{-/-}$ DCs present more endogenous IRBP and are more activated**

Supplementary Figure 4 (cont). ***Lyn-DC*$^{-/-}$ DCs present more endogenous IRBP and are more activated**

A. 50,000 P2-specific T-cell hybridoma cells were co-cultured with DCs from either eye-draining cervical LNs or non-draining pooled axillary and inguinal LNs. Cells were incubated overnight without addition of exogenous peptide, except where indicated. Hybridoma stimulation was measured by upregulation of CD69. (Left) Flow cytometry plot showing representative CD69 levels on hybridoma cells co-cultured with 100,000 DCs from draining LNs of mice of indicated genotypes. Dashed histogram corresponds to added 0.1 ug/ml P2 peptide. (C). CD69 levels on hybridoma cells co-cultured with varying numbers of DCs from either eye-draining (solid lines) or peripheral (dashed lines) LNs *Lyn*$^{F/F}$, *Lyn-DC*$^{-/-}$ or *RBP*$^{-/-}$ mice in the absence of exogenous peptide. As a positive control, hybridomas were co-cultured with 12,500 *Lyn*$^{F/F}$ DCs with addition of 0.1 ug/ml P2 peptide. As a negative control, anti-MHCII blocking antibody was added to hybridomas co-cultured with 200,000 wt DCs (open circle). Data are representative of two independent experiments.
B. Left: Representative flow cytometry plots showing CD86 and CD8 expression by resident (CD11c+ MHCII^{lo}) cervical LN DCs from *Lyn*$^{F/F}$ and *Lyn-DC*$^{-/-}$ mice. Right: Quantification CD86 MFI of indicated DC populations from *Lyn*$^{F/F}$ and *Lyn-DC*$^{-/-}$ mice. Each circle or square represents an individual mouse and the horizontal lines show mean. Data are representative of two independent experiments.

DISCUSSION

Our results here have modeled how defects in genetic factors can synergize for the induction of organ-specific autoimmunity. Recent studies have suggested that there is genetic susceptibility to autoimmunity through mutations in *AIRE* that act in an autosomal dominant fashion (75, 76), but these patients often have a delay in their clinical presentation and fewer autoimmune features when compared to patients with the complete absence of *AIRE*. These clinical observations suggest that there is some residual Aire function in these individuals and that alterations in other tolerance checkpoints may contribute to the development of autoimmune disease in these individuals. In agreement with these interpretations, we report here that mice with one $Aire^{GW}$ allele and one $Aire^{+}$ allele, which are known to have some residual Aire function (35), develop a highly destructive autoimmune uveitis when this genetic defect is combined with a defect in *Lyn*. Remarkably, on the autoimmune-resistant C57BL/6 background, $Lyn^{-/-}$ mice did not exhibit any eye autoimmunity and $Aire^{GW/+}$ mice only rarely developed uveitis, so both genetic defects were required to abrogate immune tolerance to eye autoantigens. Previous work had established a critical role for Aire in central tolerance of T cells and here we have shown that Lyn deficiency promotes expansion of IRBP-specific T cells and a severe uveitis in mice with a partial defect in *Aire*. Unlike $Aire^{-/-}$ mice, which completely lack IRBP expression in the thymus, $Aire^{GW/+}$ mice express a small amount of IRBP, which is about one tenth of the wild type levels (80). Thus, we hypothesize that some P2-specific T cells escape negative selection in the thymus of these mice and can be activated and expanded when provided with an appropriate stimulus. Indeed, there was an expansion of P2-

specific T cells in eye-draining lymph nodes of all double mutant mice, with a greater expansion in both draining lymph nodes and retinas in mice with disease.

Deletion of *Lyn* in DCs but not in B or myeloid cells was sufficient for disease induction in combination with the dominant-negative allele of *Aire*. Lyn-deficient DCs were more activated and thus may be better able to prime autoreactive T cells. In addition, Lyn-deficient DCs from the eye-draining lymph nodes were better at stimulating IRBP-specific T cell hybridomas in the absence of exogenous antigen, indicating that more self-antigen was presented by the DCs in the draining lymph nodes if the DCs were deficient for Lyn. This latter observation likely explains why there was an expansion of IRBP-specific CD4 T cells in the eye-draining lymph nodes of the mice without disease. The increased expression of CD86 by a subset of lymph node DCs may have additionally contributed to this expansion. These results indicate that in the absence of Lyn-mediated inhibitory pathways, DCs in the eye-draining lymph nodes presented IRBP peptides to prime uveitogenic T cells that had escaped tolerance in the $Aire^{GW/+}$ mice, leading to their expansion and, in many mice, retinal infiltration and autoimmune tissue destruction.

It was surprising that the retina was the only organ severely affected in the $Aire^{GW/+}$ $Lyn^{-/-}$ double mutants and that no synergy was observed for other organs that can be subject of autoimmune attack in mice with complete Aire deficiency. In part, this restricted failure of immune tolerance may be due to the nature of the autoimmune-resistant C57BL/6 background. C57BL/6 $Aire^{GW/+}$ mice develop infiltrates in the salivary and lacrimal glands only, while on the autoimmune-prone NOD background a number of additional organs are affected despite similar levels of TSA expression in the thymus (80).

This suggests that peripheral tolerance mechanisms have a key role in determining whether or not autoreactive T cells that escape thymic deletion are activated and/or whether their expansion can proceed to autoimmune attack. Indeed, a previous study has shown that complete Aire deficiency combined with a defect in T cell anergy (*Cblb*) results in autoimmune exocrine pancreatitis (81). Another intriguing possibility is that Lyn may have a unique role in maintaining tolerance to retinal antigens, perhaps reflecting a role in promoting homeostasis in the retina. In any case, a limited breakdown of tolerance is a feature of the vast majority of patients with autoimmune disease so understanding the basis of this restriction is an important problem.

The development of uveitis in double mutant mice followed a time course that closely resembled that of the complete *Aire* knockout (7), with the earliest funduscopic findings appearing at 5-6 weeks of age (Fig. 1B). Thus, uveitis in $Aire^{GW/+}$ $Lyn^{-/-}$ mouse model precedes the onset of systemic autoimmune disease seen in $Lyn^{-/-}$ mice. In contrast, the first signs of autoimmunity in $Lyn^{-/-}$ mice manifest around 3-4 months of age and include progressive increases in anti-nuclear antibodies, proinflammatory cytokines and T-cell activation, culminating in severe lupus-like autoimmunity by 8-10 months of age (69). Notably, conversion to uveitis in older double mutant mice, which were aged up to a year, was almost never observed (data not shown) suggesting that the highly proinflammatory environment in aged $Lyn^{-/-}$ mice is not a major contributor to uveitis susceptibility.

Our data provide strong genetic evidence that Lyn-deficient DCs are the main cell type contributing to activation of uveitogenic T cells. Major Lyn-expressing cell types include DCs, B cells, and myeloid cells. All three are capable of acting as antigen-presenting

cells, however DCs are the most effective cell for priming naïve T cells. Deletion of Lyn in either B cells (with *Mb1-cre*) or myeloid cells (with *LysM-cre*) did not result in uveitis when combined with the $Aire^{GW/+}$ allele. This result was somewhat surprising, as deletion of Lyn in either B cells or DCs was sufficient for lupus-like disease manifestations, including production of anti-nuclear antibodies and elevated systemic T cell activation (80). DCs have emerged as key regulators of T-cell tolerance, however their role in mediating autoimmune disease is still unclear (82). There is considerable evidence that altering inhibitory or survival signaling in DCs results in systemic autoimmunity and/or inflammatory disease at epithelial barriers. Similar to Lyn, mice with DC-specific deletion of SHP-1 or A20 have severe lupus-like autoimmune diseases with a strong inflammatory component, whereas mice with a DC-specific deletion of Flip develop severe inflammatory arthritis (48, 49, 83). In any case, the results presented here highlight a critical role of Lyn signaling in DCs for promoting tolerance to retinal antigens.

How do Lyn-deficient DCs prime uveitogenic T cells? Important insights into the mechanism were obtained from co-cultures of lymph node DCs with an IRBP-specific hybridoma. Since no exogenous antigen was added to the cultures, the amount of hybridoma stimulation was a readout of the priming ability of lymph node DCs presenting endogenous IRBP. Significantly, DCs obtained from the eye-draining lymph node of wild type mice were better at stimulating the hybridomas than their peripheral lymph node counterparts (Fig 5C). This difference was abolished in the presence of an MHCII blocking antibody (Fig 5C), indicating that the stimulatory effect observed was due to the P2 presentation by the DC MHCII complexes. These findings indicate that some retinal antigen was draining to the cervical and submandibular lymph nodes where it was

presented by the DCs. We presume that in an *Aire*$^{+/+}$ *Lyn*$^{-/-}$ mouse, the T cells that are able to recognize the uveitogenic IRBP epitope are absent and therefore no retinal disease ensues. However, in an *Aire*-deficient mouse, there are circulating IRBP-reactive cells that can be primed by IRBP-presenting DCs to cause uveitis. *Aire*$^{GW/+}$ mice appear to lie in the middle of this spectrum, since they do not normally develop uveitis on the C57BL/6 background and require an additional "push" from Lyn-deficient DCs to develop disease. This interpretation is supported by the fact that DCs from the eye-draining lymph nodes of Lyn-deficient mice were significantly more potent at stimulating the IRBP-specific hybridoma than DCs from the eye-draining lymph nodes of wild type mice, while no difference was observed for stimulation by DCs from the peripheral lymph nodes. The IRBP-specific T cell hybridoma was apparently not sensitive to the elevated expression of CD86 by the *Lyn*$^{-/-}$ DCs, but probably in vivo priming of IRBP-specific CD4 T cells was enhanced by both the presence of more IRBP peptide/MHC II complexes and the higher CD86 levels on the DCs of the draining lymph nodes. Importantly, Lyn-deficient DCs did not differ from wild type DCs in their ability to present P2 peptide or whole IRBP (Fig. S3).

In summary, in order to gain insights into the mechanisms of multigenic autoimmune susceptibility, we created a novel model of digenic autoimmune susceptibility and found that a surprisingly restricted organ-specific autoimmunity emerge from synergy of defects in central T-cell tolerance and inhibitory signaling pathways in DCs. Autoimmune disease is often broadly segregated into organ-specific and systemic autoimmunity. Previous work has suggested that Lyn-deficiency results in a systemic autoimmune syndrome characterized by anti-DNA antibodies, immune complex deposition in the kidneys, and kidney disease (65). Here we report that Lyn-deficiency helps provoke

an organ-specific autoimmune disease when combined with a partial deficiency in central tolerance to tissue-specific antigens in the thymus. Frequently patients with systemic autoimmune disease like SLE, rheumatoid arthritis (RA), and ankylosing spondylitis will manifest organ specific autoimmune features like uveitis or thyroiditis (84, 85). Our findings here help shed further light on how this may occur through the interplay of innate and adaptive immune tolerance checkpoints. Refined analysis of Lyn-deficiency demonstrated that it can lead to increased activation of dendritic cells and enhanced antigen presentation, which results in aberrant priming of a small pool of autoreactive T cells that escape the thymus. Thus, one can envision a model whereby subjects with defects in innate tolerance checkpoints can also develop organ specific autoimmune features if there is an interaction with an underlying defect in a major adaptive immune tolerance checkpoint like that controlled by Aire.

MATERIALS AND METHODS

Mice. *Aire$^{GW/+}$, Lyn$^{-/-}$, Lyn$^{F/F}$, CD11c-cre, Mb1-cre*, and *LysM-cre* mice were previously described (70, 80, 86-88). All animals were backcrossed at least 10 generations onto the C57BL/6 background and housed in a specific pathogen-free facility at UCSF in accordance with University and National Institutes of Health (NIH) guidelines. All mice were screened for the *retinal degeneration 8* (*rd8*) mutation in the *crumbs 1 (Crb1)* gene using the primers and protocol previously described (data not shown) (89). All strains were negative for the rd8 mutation with the exception of the *Mb1-cre Lyn$^{F/F}$* line which was *Crb1$^{rd8/rd8}$.*

Western Blots. Extracts were made from eyes of wild type mice. To screen for the presence of IgG autoantibodies, eye proteins were resolved by SDS-PAGE, transferred to nitrocellulose filters and incubated with sera from individual mice as previously described (30).

Radioligand Binding Assay. Full-length mouse IRBP cDNA was transcribed, in vitro translated, and biosynthetically labeled with ^{35}S-methionine using the TNT Quick Coupled Transcription/Translation System (Promega). Radio-labeled protein was immunoprecipitated with serum samples loaded in triplicate in 96-well PVDF filtration plates (Millipore). Radioactivity retained in the filter was measured using a liquid scintillation counter (1450 MicroBeta Trilux, Perkin Elmer). A rabbit polyclonal anti-IRBP IgG antibody was used as a positive control (SC-25787, Santa Cruz Biotech). IRBP autoantibody indices for samples were calculated using the following formula: (cpm of

unknown – cpm of negative standard) ÷ (cpm of positive standard – cpm of negative standard) x 100.

Fundus Examination. Ocular funduscopy was performed using a Micron III camera (Phoenix Research Labs, Inc.). Mice were anesthetized with ketamine (100mg/kg)/xylazine (10mg/kg). Tropicamide ophthalmic 1% and phenylephrine hydrochloride 2.5% eye drops were applied to relax eye muscles and dilate the pupil. Goniovisc hypromellose 2.5% was used during imaging and to avoid dryness of the cornea. For the test of blood-retinal barrier integrity, fluorescein angiography was performed immediately after intraperitoneal injection of 20μL of fluorescein sodium (25 mg/mL in PBS, Altaire Pharmaceuticals, Inc.). Presence of uveitis and disease severity was determined using a previously described grading system (90).

Histology. Organs from mice were harvested and fixed overnight in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Immune infiltrates of organs were scored as previously described in a blinded fashion (80). Presence of uveitis was determined and scored based on the histological findings.

Immunfluorescence. Whole globes were dissected from the orbit by cutting the optic nerve and moved to PBS. The clear cornea was removed under a dissecting microscope and the lens and vitreous removed with fine forceps. Eyecups were fixed in 1% paraformaldehyde for 30 minutes at room temperature followed by washing in PBS three times for 15 minutes. Fixed eyecups were embedded in OCT and 20μm sections were cut

on a CM3050 S cryostat (Leica Microsystems). Sections were air-dried for 1 hour at room temperature and stained directly or stored at -80C. Dried sections were blocked with BlockAide (Life Technologies) for 1 hour followed by staining with anti-CD3 antibody (rabbit polyclonal, AbCam) diluted in BlockAide at 1:250 overnight in a humidified chamber at 4°C. Sections were washed with PBS and stained with goat anti-rabbit secondary antibody conjugated to Alexa555 (Life Technologies) diluted in BlockAide at 1:1000 and 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies). Stained retinal sections were then mounted in ProLong Diamond (Life Technologies) and imaged on an SP5 confocal microscope (Leica Microsystems).

Tissue Preparation and Flow Cytometry. For tetramer staining, spleen and lymph nodes were dissected, cleaned of fat and fasciae, and transferred to RPMI 1640 medium containing 2% fetal bovine serum (FBS). Single cells suspensions were prepared by mashing the cells through a 40-μm filter and blocked with anti-mouse CD16/CD32 (24G2) (UCSF Hybridoma Core Facility) and 5% normal rat serum.

For retinal cell staining, both retinas were dissected and transferred to RPMI 1640 medium containing 10% FBS. Retinas were minced and digested in the medium containing 0.5 mg/ml collagenase D (Roche) and 100 U/ml DNAse (Worthington Biochemicals) for 1 hr at 37°C. Tissue was dissociated by gentle pipetting and passed through a 70 μm strainer. The resulting single cell suspension was analyzed by flow cytometry using Accucheck counting beads (Invitrogen) to obtain absolute cell counts.

For dendritic cell staining, lymph nodes or spleen were digested in RPMI 1640-10% FBS, containing 1 mg/ml collagenase D and 100 u/ml DNAse for 1 hour at 37°C. Immediately

after digestion, collagenase was inactivated with 2mM EDTA, and cells were passed through a 100- μm filter and stained.

Antibodies used included CD45 (30-F11, BD Pharmigen), TCRβ (H57-597; eBiosciences), CD4 (GK1.5; Biolegend) CD8 (53-6.7; Biolegend), CD44 (IM7; Biolegend), B220 (RA3-6B2; eBiosciences), F4/80 (BM8; Biolegend), CD11b (M1/70; Biolegend), and CD11c (N418; Biolegend)

Flow cytometry was performed using a LSRII Flow Cytometer (BD Biosciences) at the Flow Cytometry Core at UCSF, and analyzed using FlowJo software (Tree Star Software).

Tetramer Analysis. APC-conjugated I-A^b P2 tetramer (QTWEGSGVLPCVG) corresponding to mouse IRBP amino acids 294-306 was obtained from the NIH tetramer facility, Atlanta, GA. Staining of endogenous tetramer-specific T cells was performed as described (32, 91). Briefly, single cell suspensions were incubated with tetramer for 1 hour at room temperature, followed by magnetic bead enrichment for tetramer positive cells. The positively selected cells were stained and tetramer-reactive cells were gated on CD3+CD4+CD8−B220-CD11b−CD11c−F4/80− lymphocytes and analyzed by flow cytometry. Counting beads (Accucheck, Invitrogen) were used to obtain absolute cell counts.

For tetramer analysis of infiltrating retinal T cells, retinal lymphocytes were enriched by centrifugation through a 30%/37%/70% Percoll step gradient and incubated with tetramer for 1 hour at room temperature. Because of the small number of total lymphocytes present in retinas, further enrichment with MACS beads was not necessary,

and tetramer-positive cells were analyzed following direct staining of the whole lymphocytic fraction.

Antigen Presentation Assays. Lymph node DCs were enriched by binding to anti-CD11c magnetic beads (Miltenyi), eluted, and plated at serial dilutions in 96-well round-bottom tissue culture plates (Costar) and incubated for 1 hour at 37°C. P2-binding T-cell hybridomas were labeled with 1 mM ester (CFSE SE) (Life Technologies) for 10 min at 37°C. The reaction was stopped by adding complete RPMI-1640. 50,000 hybridoma cells were added per well and co-cultured with DCs overnight. For whole IRBP presentation, DCs were incubated with IRBP for 24 hrs prior to addition of the hybridoma. Negative controls included addition of anti-MHC Class II (I-A/I-E) antibody (eBiosciences, clone M5/114.15.2), and culturing hybridomas in the absence of APCs.

Statistics. Statistical analyses were performed using Prism (Graph Pad). Statistical differences between two groups were calculated with unpaired 2-tailed Student's *t* test. Statistical differences between three groups or more were calculated with ANOVA. A P value of less than 0.05 was considered statistically significant. Data are presented as the mean ± SD.

Study Approval. All animal studies were approved by the UCSF Institutional Animal Care and Use Committee.

ACKNOWLEDGEMENTS

We thank R. Caspi and M. Mattapalil of National Eye Institute for assistance with Crb1 genotyping, C. Abram and C. Lamagna of UCSF for mice and helpful discussions, L. Lee and A. Reboldi of UCSF for helpful discussions, the NIH Tetramer Core Facility for providing tetramer reagent.

This work was supported by grants from the National Institutes of Health to A. L. D (AI020038, AI108684) and M. S. A. (EY16408, AI097457), and from the UCSF graduate division (T32AI007334) to I. P. I. P. was partially supported by a predoctoral training grant, T32EY007120. The UCSF Parnassus Flow Cytometry Core and the Diabetes and Endocrinology (DERC) Microscopy Core were supported by the DERC grant, NIH P30 DK063720.

CHAPTER THREE:

Discussion and Future Directions

Autoimmune susceptibility and tolerance thresholds

Human genetic studies have been essential in identifying genes involved in immune tolerance. Genetic linkage analysis of large families with penetrant phenotypes has been a powerful tool in identifying susceptibility genes with Mendelian inheritance, such as MHCII alleles (4). They also helped identify the genetic basis for several monogenic autoimmune disorders, such as APS1 (5). Although highly informative about key tolerance mechanisms, monogenic autoimmune disorders are exceedingly rare. On the other hand, most of the common autoimmune diseases exhibit multigenic susceptibilities, with most individual loci having small effect sizes. Over the last decade, large-scale GWAS studies have identified numerous susceptibility genes largely falling into pathways that are known to regulate development, activation and survival of both innate and adaptive immune cells (73). However, the GWAS approach is limited in that it's an association-based approach and thus necessitates mechanistic follow-up studies to prove relevance to autoimmune phenotypes. Furthermore, GWAS detect only the most common gene variants, and the disease risk ratios identified for most alleles are usually small, thus alleles that have strong effects but affect only a small subset of patients will be overlooked by this approach (10).

A key hypothesis that has emerged from human genetic studies to date is that complex autoimmune diseases arise through a cumulative effect, involving partial compromise of multiple tolerance checkpoints (2). This led to the idea that autoimmune disease pathogenesis involves a "threshold effect" by which independent defects in tolerance, each creating a small disturbance in the immune homeostasis, add up to overcome the threshold to activation and disease induction (8, 9). This may explain why

some individuals harboring risk alleles of tolerance-related genes have altered immune cell responses, yet don't develop disease, exemplified by a recent study finding hyperresponsiveness and altered negative selection of B cells in carriers of a SLE risk allele of phosphatase *Csk*, a binding partner for PTPN22 (92).

How can multiple genetic defects overcome tolerance thresholds and lead to autoimmunity? One possible mechanism is through alterations of multiple components in lymphocyte signaling pathways, lowering signaling thresholds and leading to lymphocyte hyperresponsiveness. For example, in the *PTPN22* signaling pathway, alleles with altered functions, like the Csk allele described above, augment signaling downstream of T-cells in B-cells in humans (92). In mice, cumulative defects in negative regulators of B-cell receptor signaling, Lyn, SHP1 and CD22 lead to B-cell hyperactivity in triple heterozygous mice (93). In that regard, it is worth noting that in our model, $Aire^{GW/+}$ $Lyn^{+/-}$ mice did not develop uveitis (Table 1), indicating that haploinsufficiency of Lyn is not enough to break the autoimmune threshold for activation of uveitogenic T cells.

Another possible mechanism to lower tolerance thresholds is cooperation between tolerance defects in distinct pathways regulating a particular cell type. This was modeled in mice deficient for both *Aire* and *Cbl-b*, which develop severe exocrine pancreatitis through inability to anergize autoreactive calls that have escaped central tolerance due to Aire deficiency (81). A third potential mechanism is cooperation between tolerance defects in different cell types, such as those affecting both innate cells and lymphocytes. This mechanism is likely to be a major factor in pathogenesis of human autoimmunity, as susceptibility genes affecting both arms of the immune system have been identified for

common autoimmune diseases, although how they cooperate is less clear and challenging to model.

Autoimmune-prone mouse strains, like the lupus-prone NZB x NZW mice and the diabetes-prone NOD mice, have been instrumental in mapping autoimmune susceptibility genes and dissecting their functions (94). They are presumed to be a close model of human autoimmunity and have potential to genetically deconvolute multigenic susceptibility and examine how different genes act in concert. In mouse models of lupus, chromosomal regions that confer increase risk of disease have been identified. In particular, studies from the Mohan group using congenic dissection identified susceptibility loci in the NZM2410 strain (Sle1, Sle2, and Sle3) which conferred autoimmunity when transferred into autoimmune-resistant strains (95). Interestingly, the disease phenotypes conferred by individual Sle loci were mild. Further studies linked individual Sle loci to distinct defects in B and T cell tolerance, supporting the idea that multiple susceptibilities are required to overcome tolerance thresholds (95). Other relevant examples illustrating this idea include the *Yaa* locus from the lupus-prone BXSB mouse which helped identify *Tlr7* as a gene responsible for production of anti-nucleolar antibodies by B cells (96) and the *Idd3* locus in diabetes-prone NOD mice, containing *Il2* (97).

Despite these advances in our understanding of mouse models of spontaneous autoimmunity, identifying the precise genetic variants that explains the effects of a locus and/or how a genetic variant acts mechanistically is challenging. To address effects of individual genes, reverse genetic approaches have been essential. These include complete knockouts, such as *Lyn* knockouts, and targeted mutations to replicate mutations found in

humans with autoimmunity, such as *Aire*$^{GW/+}$. Because of defined genetic defects, study of these models provides mechanistic insight into how a particular mutation affects tolerance. Furthermore, this approach allows for combination of defined genetic defects to study their effects on autoimmune susceptibility. Next, I will briefly address our use of such an approach to create a digenic model of autoimmune susceptibility.

Aire$^{GW/+}$ *Lyn*$^{-/-}$ digenic model of autoimmune susceptibility: summary

In chapter two, I describe a mouse model of digenic autoimmunity arising from defects in *Aire*, affecting T cell central tolerance, and *Lyn*, affecting peripheral tolerance. Lyn-deficient mice that also possessed a dominant negative *Aire* allele (*Aire*$^{GW/+}$ *Lyn*$^{-/-}$ double mutants*)* developed spontaneous autoimmune uveitis that was not observed in the parental strains, except in rare cases. The uveitis was accompanied by an adaptive immune response to a retinal antigen, IRBP, and involved an expansion of CD4 T cells specific for an Aire-regulated epitope of IRBP, P2. Genetic analysis of cell-specific knockouts of Lyn revealed that absence of Lyn in DCs, but not B cells or myeloid cells, was sufficient for uveitis. Importantly, P2-specific CD4 T cells were detected at a steady state in the eye-draining lymph nodes of uveitis-free *Aire*$^{GW/+}$*Lyn*$^{-/-}$ mice, but not in single mutant controls. This suggested that Lyn deficiency predisposed to priming of these self-reactive cells. Further mechanistic studies using a P2-specific T cell hybridoma revealed that Lyn-deficient DCs present more endogenous IRBP. Phenotypic examination of Lyn-deficient DCs revealed that CD11b-positive subset of DCs expressed higher levels of the activation marker CD86, suggesting that, in addition to presenting more self-antigen, they may

provide a better co-stimulatory signal *in vivo*. Based on these findings I propose a two-step model for development of retinal autoimmunity in the *Aire*$^{GW/+}$*Lyn*$^{-/-}$ mouse, involving consecutive failure of central and peripheral tolerance (Fig. 6).

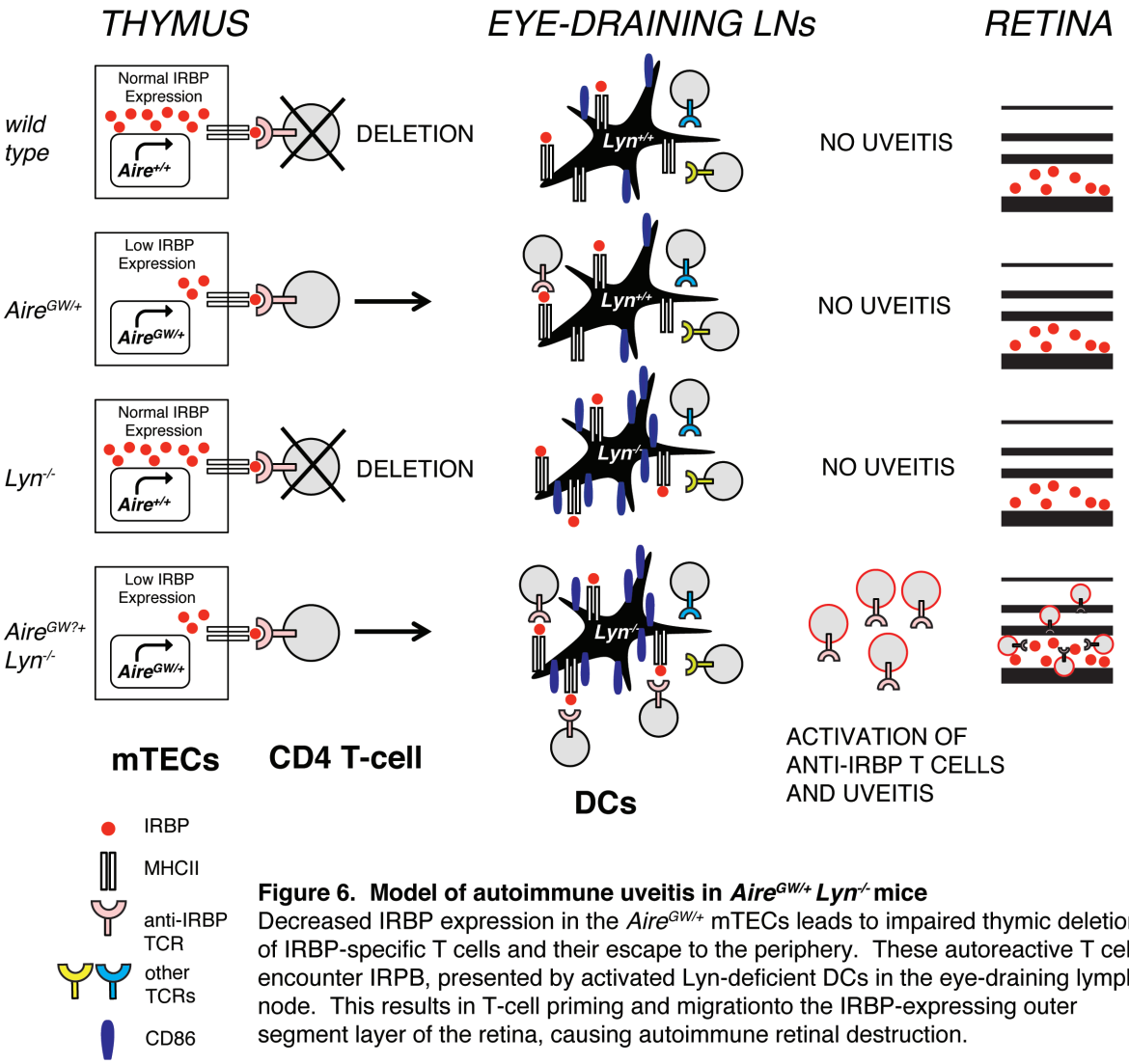

Figure 6. Model of autoimmune uveitis in *Aire*$^{GW/+}$ *Lyn*$^{-/-}$ mice
Decreased IRBP expression in the *Aire*$^{GW/+}$ mTECs leads to impaired thymic deletion of IRBP-specific T cells and their escape to the periphery. These autoreactive T cells encounter IRPB, presented by activated Lyn-deficient DCs in the eye-draining lymph node. This results in T-cell priming and migrationto the IRBP-expressing outer segment layer of the retina, causing autoimmune retinal destruction.

1. Loss of central tolerance to IRBP. In the *Aire*$^{GW/+}$ mouse, less IRBP is expressed in the thymus leading to inefficient negative selection of IRBP-reactive T cells and their escape to the periphery. In an *Aire*$^{GW/+}$*Lyn*$^{+/+}$ mouse these rare cells are efficiently tolerized in the periphery and no uveitis ensues.

2. Loss of peripheral DC tolerance. In the absence of Lyn, DCs express higher levels of the co-stimulatory molecule CD86. There is also more IRBP reaching the draining lymph nodes. Activated Lyn-deficient DCs present IRBP to cognate T cells, which get activated, migrate to the retina and cause uveitis.

I think that this is an attractive model to study digenic autoimmunity for several reasons. Firstly, both genetic defects are known and thus precise cell-intrinsic and cell-extrinsic consequences of these defects can be dissected. Secondly, the double mutants were generated on a pure, autoimmune-resistant C57BL/6 genetic background, eliminating potential confounding factors created by unmapped susceptibility loci that exist on mixed or autoimmune-prone backgrounds. Finally, conditional gene targeting enabled me to examine the role of individual Lyn-expressing cell types in promoting autoimmunity and could be expanded in follow-up studies to include ablation or genetic manipulation of Lyn- or Aire-expressing cells.

Next, I would like to discuss challenges presented by this model, including caveats relevant to interpreting the data described in Chapter 2, and cover future directions of our research.

Aire$^{GW/+}$ *Lyn*$^{-/-}$ digenic model of autoimmune susceptibility: challenges, caveats, and future directions

Addressing variability in spontaneous disease

While no single mouse model of autoimmunity can reproduce the whole spectrum of human disease, spontaneous and immunization-induced models of autoimmune uveitis have been instrumental in our understanding of relevant pathogenic mechanisms (98). Spontaneous disease models are highly informative because they are not confounded by adjuvants or pharmacologic treatment necessary to elicit a response in induced models of disease. In this study, *Aire*$^{GW/+}$*Lyn*$^{-/-}$ mice developed spontaneous uveitis that is very similar in progression and severity to that developed by the mice completely deficient for *Aire (7)*.

As with most spontaneous disease models, time of onset, disease progression and manifestations were somewhat variable between individual *Aire*$^{GW/+}$*Lyn*$^{-/-}$ mice. This variability likely accounts for the bimodal distribution of peripheral P2-positive CD4 T cells in 2-5 month old mice (Fig 2C), with the mice displaying highest numbers of tetramer-binding cells likely being at the peak of disease, and the mice with lower numbers being at later disease stages. This interpretation is supported by funduscopic findings: mice with fewer tetramer-reactive cells displayed more advanced retinal destruction, correlating with less antigen availability (data not shown). I also observed a spread in the numbers of T cells infiltrating the retina of mice with uveitis (Fig. 3A, B). Even though the mice analyzed in this series of experiments were at most 2-3 weeks apart in age, and all mice in the experimental group displayed funduscopic findings consistent with early disease, the numbers of retinal T cells differed by several thousands to tens of thousand of cells.

Despite this variability, there was a clear and significant difference between mice on the milder end of disease spectrum and disease-free controls in all parameters examined. Additionally, longitudinal studies of 2-8 month old mice monitored by funduscopy found a gradual decrease in the magnitude of the adaptive immune response to IRBP as the retina was being progressively destroyed (data not shown). Taken together, these observations suggest that this in vivo monitoring by funduscopy is effective at identifying relevant experimental cohorts and minimizing experimental variability in this model of spontaneous uveitis.

Evidence for priming of IRBP-specific T cells

Another challenge presented by spontaneous models of autoimmunity is dissecting events that contribute to priming of self-reactive T cells versus events that regulate their reactivation and/or effector functions. This is especially relevant for determining sites and cell types involved. For example, in models of EAE, DCs were shown to mediate initial priming and polarization of encephalitogenic T cells, but had protective effects at later stages of disease (38, 47). Since it is impossible to predict which mice will progress to disease, I used two approaches to obtain evidence for priming of P2-reactive CD4 cells.

Firstly, I used an *ex vivo* approach to examine P2-specific cells in the eye-draining LNs of mice with and without uveitis (Fig. 5A, B). As expected, mice with retinal disease had a significant expansion of uveitiogenic cells in the eye-draining, but not peripheral LNs, however at that stage it was unclear whether this was due to priming and/or reactivation. A more relevant finding was the presence of a small population of activated IRBP-reactive T cells in eye-draining, but not peripheral LNs of mice without disease, consistent with T cell

priming (Fig. 5A, B). I chose older double mutants (15-20 week old) for this experimental group, because I knew from previous studies that they will not progress to disease, thus the presence of activated IRBP-specific T cells in their draining LNs likely reflects continued priming. Similar expansion was observed in eye-draining LNs of younger, 8-12 week old mice without uveitis (data not shown). It may be informative to examine IRBP-specific T cells in draining lymph nodes of very young mice, at 3-5 weeks of age, before the onset of uveitis, to see if I can detect a greater expansion of IRPB-reactive cells in about half of the mice, consistent with priming and subsequent progression to disease.

Secondly, I used *in vitro* co-cultures of lymph node DCs with a P2-specific T cell hybridoma. The responsiveness of this hybridoma reflects amount of peptide presented by DCs as it not dependent on co-stimulation (32). However, there are other stimuli that could influence hybridoma activation, such as increased secretion of proinflammatory cytokines and/or TLR ligands from Lyn-deficient DCs. I do not think that this is a major contributor because there was no difference in hybridoma activation by Lyn-deficient and wild type DCs from the non-draining lymph nodes, or when exogenous antigen was added over a range of concentrations. Another caveat to these studies is the use of bead-enriched DCs, as opposed to sorted DCs. Co-cultures of sorted DC subsets could be especially informative in the light of the selective upregulation of CD86 by CD11b+ DC subset, as this subset in particular may be most efficient at presenting IRBP-derived peptides to self-reactive T cells.

Interestingly, in wild type mice, DCs from eye-draining lymph nodes were significantly better at stimulating the hybridoma than DCs from peripheral LNs. These

observations suggest that IRBP reaches the eye-draining lymph nodes under steady state conditions. It is not clear how that occurs, since the retina is not thought to possess lymphatic drainage. However, there have been recent reports describing lymphatic vessels in the brain (99), which may warrant a closer look at lymphatics in the eye. It is interesting to consider that presentation of P2 peptides in the periphery in the steady may be the underlying mechanism for development of uveitis in the *Aire*$^{-/-}$ mouse. In an Aire-sufficient mouse, T cells that can recognize this epitope are negatively selected. In an Aire-deficient mouse there may be enough P2+ CD4 T cells in circulation to encounter antigen and become activated. *Aire*$^{GW/+}$ mice, which normally don't develop uveitis, probably lie in the middle of this spectrum. Because these mice retain some IRBP expression in the thymus, it is possible that they have fewer uveitogenic T cells in the periphery compared to the *Aire*$^{-/-}$ mice, and therefore they may require hyperactivated Lyn-deficient DCs to overcome tolerance thresholds. This further illustrates the concept of how quantitative changes in tolerance thresholds may compound to induce autoimmunity.

Aire$^{GW/+}$ *Lyn*$^{-/-}$ digenic model of autoimmune susceptibility: future directions

Using IRBP TCR Transgenic to dissect mechanism of disease in *Aire*$^{GW/+}$*Lyn*$^{-/-}$ model of uveitis

In Chapter 2, I used a tetramer reagent to identify the rare IRBP-reactive CD4 T cells within endogenous repertoire. However, the limited numbers of tetramer-positive cells even during the peak of disease and the nature of the tetramer pulldown (91) precluded us from doing any detailed phenotypic or functional analysis of the isolated population. Thus,

it remains unclear whether IRBP-specific T cells are regulated differently on a Lyn-deficient background in terms their polarization, activation, and conversion into Tregs. To address these questions in future studies, it would be possible to create a TCR transgenic mouse specific for the Aire-regulated P2 epitope of IRBP (P2-Tg). This could be achieved using the TCR sequence of the P2-hybridoma that was made from a C56BL/7 Aire-deficient mouse immunized with P2 peptide of IRBP (32). A TCR-transgenic mouse specific for an epitope of IRBP that is used in experimental autoimmune uveitis (EAU) has been created on the EAU-susceptible B10.RIII background (100). These mice spontaneously develop progressive autoimmune uveitis, although the mechanism is still unclear. In this regard, it should be noted that uveitis can be induced in susceptible mouse strains by transfer of in-vitro expanded IRBP-reactive T cells from immunized mice, however, reports regarding efficacy of such transfers into C67BL/6 mice have been mixed (101, 102).

A P2-Tg mouse would be, to our knowledge, a first TCR transgenic specific for an Aire-regulated antigen and could prove highly informative in studying localization, phenotype and regulation of T cells that have escaped central tolerance. It would be especially interesting to determine whether P2-specific cells get spontaneously activated on a wild type background, consistent with our hypothesis that enough uveitiogenic T cells need to be present in circulation to be activated by P2-presenting DCs (outlined in the previous section). A TCR transgenic mouse would allow examination of how Lyn deficiency affects effector responses of uveitogenic T cells, such as their polarization and ability to convert to Tregs and potentially assess effects on disease progression.

Additionally, the P2-Tg would enable us to address priming in our double mutant model. This could be done in vitro in co-culture assays similar to the hybridoma studies, but perhaps a better approach would be to CFSE-label and adoptively transfer naïve or activated P2-specific T cells into *Lyn*$^{-/-}$ recipients and follow their activation and proliferation in the eye-draining lymph nodes of wild type or Lyn-deficient mice. When designing the TCR transgenic construct, addition of a fluorescent probe, such as tdTomato would permit tracking of the self-reactive cells and studying their interaction with DCs using immunofluorescence and/or live imaging.

Another fascinating area of study would be determining how Lyn deficiency influences uveitogenic T cells in the retina, allowing us to address where priming and reactivation happen. Studying effects of Lyn deficiency in the eye is highly relevant as the retina has resident immune cells, including microglia and CD11c-expressing APCs, which have been implicated in antigen presentation and priming of T cells during uveitis (103). The eye is a strongly immunomodulatory environment as injection of naïve antigen-specific T cells into the anterior chamber of the eye results in their conversion into Tregs (104). The regulatory aspect of the eye may be compromised in the absence of Lyn.

Future Directions: Examining environmental triggers in *Aire*$^{GW/+}$*Lyn*$^{-/-}$ model of uveitis

In human autoimmunity, penetrance of an autoimmune phenotype can be highly variable even between uniform populations or siblings with similar genetic susceptibilities, suggesting influence of genetic modifiers or the environment (73). Even in identical twin studies, disease concordance rate is only about 25-50%. Variable incidence in genetically

identical individuals could reflect environmental influences or stochastic events, like random VDJ recombination events generating autoreactive T cells that escape negative selection. Similarly, penetrance of autoimmunity is variable in mouse models. However, in the case of inbred mouse strains, there is minimal genetic variation, therefore differences in disease incidence can be mostly attributed to environmental triggers. The penetrance of uveitis in multiple cohorts of $Aire^{GW/+}Lyn^{-/-}$ mice ranged from 40% to 60% over the course of the study, with the average close to 50% (Table 1). Considered in the context of persistence of primed autoreactive T cells in mice that do not progress to disease, this incomplete penetrance suggests that another tolerance checkpoint exists post-priming and must be bypassed for uveitis development. What is this tolerance checkpoint and what are the triggers for breaking it?

The microbiome has recently emerged as a key regulator of autoimmunity, and has been implicated in driving pathogenic Th17 responses and influencing Treg homeostasis. In addition, alterations in the microbiome has been found in patients with autoimmune disorders T1D, Psoriasis and Crohn's disease (105). Microbial components can stimulate pattern recognition receptors on APCs and thus influence T-cell responses. Indeed, ablating Myd88-dependent signaling in either DC-specific or B-cell specific Lyn knockouts abrogated symptoms of lupus-like autoimmunity, although it is not clear whether TLR-mediated or IL1-mediated signaling is required (70, 71). There has also been some evidence that uveitogenic T cells may be primed in the gut by cross-reactive microbial peptides (R. Caspi, personal communication). Thus, it would be interesting to determine whether abolishing both Myd88 and Lyn signaling in DCs will prevent uveitis in our model.

Among environmental stimuli that may influence eye disease, light exposure is suspect number one (106). Retinal homeostasis is extremely sensitive to detrimental light exposure, which affects the turnover of retinal photoreceptors and results in their apoptosis. IRBP is involved in retinoid transport in the photoreceptor layer of the retina, thus it is possible that loss of photoreceptors after light exposure may influence levels of IRBP that is presented by APCs in the retina or in the periphery. It would be interesting to test whether in the case of retinal injury, by exposure to bright light or laser, more antigen would get to the draining LNs and promote uveitis in conjunction with Lyn-deficient DCs. This question could be addressed by exposing wild type mice to bright light at levels sufficient to induce photoreceptor loss, and using P2-specific hybridomas to determine whether DCs from the eye-draining lymph nodes present more IRBP shorty after exposure. I have performed preliminary experiments showing that brief (30 min) exposure to bright light induces retinal changes, as reported. Furthermore, I could test whether bright light exposure is a trigger to break tolerance in double mutant mice that have not progressed to disease and thus possibly create an inducible model of digenic autoimmunity.

There may be a plethora of other environmental triggers of autoimmunity, such as chemicals and bacterial metabolites that provide direct stimulus or induce epigenetic changes in critical immune pathways, and such possibilities could be addressed in follow up studies.

Concluding Remarks

Overcoming tolerance thresholds in human autoimmunity requires compound effects of multiple susceptibility genes, including common and rare alleles, as well as environmental factors. In this work, I have developed a model of digenic autoimmune susceptibility leading to spontaneous uveitis. This study can act as a platform for future mechanistic experiments. My results indicate that deregulation of DCs can drive activation and differentiation of autoreactive T cells and be a major contributor to breakdown of tolerance to tissue antigens in the context of defects in central tolerance.

REFERENCES

1. Silverstein AM. Autoimmunity versus horror autotoxicus: the struggle for recognition. *Nat Immunol.* 2001;2(4):279-81.
2. Goodnow CC. Multistep pathogenesis of autoimmune disease. *Cell.* 2007;130(1):25-35.
3. McGonagle D, and McDermott MF. A proposed classification of the immunological diseases. *PLoS Med.* 2006;3(8):e297.
4. Zenewicz LA, Abraham C, Flavell RA, and Cho JH. Unraveling the genetics of autoimmunity. *Cell.* 2010;140(6):791-7.
5. Cheng MH, and Anderson MS. Monogenic autoimmunity. *Annu Rev Immunol.* 2012;30(393-427.
6. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, and Ochs HD. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* 2001;27(1):20-1.
7. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, von Boehmer H, Bronson R, Dierich A, Benoist C, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science.* 2002;298(5597):1395-401.
8. Gregersen PK, and Olsson LM. Recent advances in the genetics of autoimmune disease. *Annu Rev Immunol.* 2009;27(363-91.
9. Andrews TD, Sjollema G, and Goodnow CC. Understanding the immunological impact of the human mutation explosion. *Trends Immunol.* 2013;34(3):99-106.
10. Xavier RJ, and Rioux JD. Genome-wide association studies: a new window into immune-mediated diseases. *Nat Rev Immunol.* 2008;8(8):631-43.
11. Vang T, Miletic AV, Arimura Y, Tautz L, Rickert RC, and Mustelin T. Protein tyrosine phosphatases in autoimmunity. *Annu Rev Immunol.* 2008;26(29-55.
12. Goodnow CC, Sprent J, Fazekas de St Groth B, and Vinuesa CG. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature.* 2005;435(7042):590-7.
13. Starr TK, Jameson SC, and Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol.* 2003;21(139-76.
14. Laufer TM, Glimcher LH, and Lo D. Using thymus anatomy to dissect T cell repertoire selection. *Semin Immunol.* 1999;11(1):65-70.
15. Hinterberger M, Aichinger M, Prazeres da Costa O, Voehringer D, Hoffmann R, and Klein L. Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. *Nat Immunol.* 2010;11(6):512-9.
16. Gallegos AM, and Bevan MJ. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med.* 2004;200(8):1039-49.
17. Akiyama T, Shimo Y, Yanai H, Qin J, Ohshima D, Maruyama Y, Asaumi Y, Kitazawa J, Takayanagi H, Penninger JM, et al. The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity.* 2008;29(3):423-37.
18. Gao JX, Zhang H, Bai XF, Wen J, Zheng X, Liu J, Zheng P, and Liu Y. Perinatal blockade of b7-1 and b7-2 inhibits clonal deletion of highly pathogenic autoreactive T cells. *J Exp Med.* 2002;195(8):959-71.

19. Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, Krohn KJ, Lalioti MD, Mullis PE, Antonarakis SE, et al. Positional cloning of the APECED gene. *Nat Genet.* 1997;17(4):393-8.
20. Finnish-German AC. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet.* 1997;17(4):399-403.
21. Peterson P, and Peltonen L. Autoimmune polyendocrinopathy syndrome type 1 (APS1) and AIRE gene: new views on molecular basis of autoimmunity. *J Autoimmun.* 2005;25 Suppl(49-55.
22. Waterfield M, and Anderson MS. Clues to immune tolerance: the monogenic autoimmune syndromes. *Ann N Y Acad Sci.* 2010;1214(138-55.
23. Mathis D, and Benoist C. Aire. *Annu Rev Immunol.* 2009;27(287-312.
24. Metzger TC, and Anderson MS. Control of central and peripheral tolerance by Aire. *Immunol Rev.* 2011;241(1):89-103.
25. Bjorses P, Halonen M, Palvimo JJ, Kolmer M, Aaltonen J, Ellonen P, Perheentupa J, Ulmanen I, and Peltonen L. Mutations in the AIRE gene: effects on subcellular location and transactivation function of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy protein. *Am J Hum Genet.* 2000;66(2):378-92.
26. Ramsey C, Winqvist O, Puhakka L, Halonen M, Moro A, Kampe O, Eskelin P, Pelto-Huikko M, and Peltonen L. Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. *Hum Mol Genet.* 2002;11(4):397-409.
27. Jiang W, Anderson MS, Bronson R, Mathis D, and Benoist C. Modifier loci condition autoimmunity provoked by Aire deficiency. *J Exp Med.* 2005;202(6):805-15.
28. Gardner JM, Devoss JJ, Friedman RS, Wong DJ, Tan YX, Zhou X, Johannes KP, Su MA, Chang HY, Krummel MF, et al. Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science.* 2008;321(5890):843-7.
29. Derbinski J, Gabler J, Brors B, Tierling S, Jonnakuty S, Hergenhahn M, Peltonen L, Walter J, and Kyewski B. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med.* 2005;202(1):33-45.
30. DeVoss J, Hou Y, Johannes K, Lu W, Liou GI, Rinn J, Chang H, Caspi RR, Fong L, and Anderson MS. Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. *J Exp Med.* 2006;203(12):2727-35.
31. Devoss JJ, Shum AK, Johannes KP, Lu W, Krawisz AK, Wang P, Yang T, Leclair NP, Austin C, Strauss EC, et al. Effector mechanisms of the autoimmune syndrome in the murine model of autoimmune polyglandular syndrome type 1. *J Immunol.* 2008;181(6):4072-9.
32. Taniguchi RT, DeVoss JJ, Moon JJ, Sidney J, Sette A, Jenkins MK, and Anderson MS. Detection of an autoreactive T-cell population within the polyclonal repertoire that undergoes distinct autoimmune regulator (Aire)-mediated selection. *Proc Natl Acad Sci U S A.* 2012;109(20):7847-52.
33. Shum AK, Alimohammadi M, Tan CL, Cheng MH, Metzger TC, Law CS, Lwin W, Perheentupa J, Bour-Jordan H, Carel JC, et al. BPIFB1 is a lung-specific autoantigen associated with interstitial lung disease. *Sci Transl Med.* 2013;5(206):206ra139.
34. DeVoss JJ, LeClair NP, Hou Y, Grewal NK, Johannes KP, Lu W, Yang T, Meagher C, Fong L, Strauss EC, et al. An autoimmune response to odorant binding protein 1a is

associated with dry eye in the Aire-deficient mouse. *J Immunol.* 2010;184(8):4236-46.
35. Su MA, Davini D, Cheng P, Giang K, Fan U, DeVoss JJ, Johannes KP, Taylor L, Shum AK, Valenzise M, et al. Defective autoimmune regulator-dependent central tolerance to myelin protein zero is linked to autoimmune peripheral neuropathy. *J Immunol.* 2012;188(10):4906-12.
36. Hou Y, DeVoss J, Dao V, Kwek S, Simko JP, McNeel DG, Anderson MS, and Fong L. An aberrant prostate antigen-specific immune response causes prostatitis in mice and is associated with chronic prostatitis in humans. *J Clin Invest.* 2009;119(7):2031-41.
37. Hogquist KA, and Jameson SC. The self-obsession of T cells: how TCR signaling thresholds affect fate 'decisions' and effector function. *Nat Immunol.* 2014;15(9):815-23.
38. Ganguly D, Haak S, Sisirak V, and Reizis B. The role of dendritic cells in autoimmunity. *Nat Rev Immunol.* 2013;13(8):566-77.
39. Hashimoto D, Miller J, and Merad M. Dendritic cell and macrophage heterogeneity in vivo. *Immunity.* 2011;35(3):323-35.
40. Price JD, and Tarbell KV. The Role of Dendritic Cell Subsets and Innate Immunity in the Pathogenesis of Type 1 Diabetes and Other Autoimmune Diseases. *Front Immunol.* 2015;6(288.
41. Hopp AK, Rupp A, and Lukacs-Kornek V. Self-antigen presentation by dendritic cells in autoimmunity. *Front Immunol.* 2014;5(55.
42. Steinman RM, Hawiger D, and Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol.* 2003;21(685-711.
43. Teichmann LL, Schenten D, Medzhitov R, Kashgarian M, and Shlomchik MJ. Signals via the adaptor MyD88 in B cells and DCs make distinct and synergistic contributions to immune activation and tissue damage in lupus. *Immunity.* 2013;38(3):528-40.
44. Collin M, Bigley V, Haniffa M, and Hambleton S. Human dendritic cell deficiency: the missing ID? *Nat Rev Immunol.* 2011;11(9):575-83.
45. Ohnmacht C, Pullner A, King SB, Drexler I, Meier S, Brocker T, and Voehringer D. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med.* 2009;206(3):549-59.
46. Darrasse-Jeze G, Deroubaix S, Mouquet H, Victora GD, Eisenreich T, Yao KH, Masilamani RF, Dustin ML, Rudensky A, Liu K, et al. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *J Exp Med.* 2009;206(9):1853-62.
47. Yogev N, Frommer F, Lukas D, Kautz-Neu K, Karram K, Ielo D, von Stebut E, Probst HC, van den Broek M, Riethmacher D, et al. Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor(+) regulatory T cells. *Immunity.* 2012;37(2):264-75.
48. Hammer GE, Turer EE, Taylor KE, Fang CJ, Advincula R, Oshima S, Barrera J, Huang EJ, Hou B, Malynn BA, et al. Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis. *Nat Immunol.* 2011;12(12):1184-93.
49. Abram CL, Roberge GL, Pao LI, Neel BG, and Lowell CA. Distinct roles for neutrophils and dendritic cells in inflammation and autoimmunity in motheaten mice. *Immunity.* 2013;38(3):489-501.

50. Melillo JA, Song L, Bhagat G, Blazquez AB, Plumlee CR, Lee C, Berin C, Reizis B, and Schindler C. Dendritic cell (DC)-specific targeting reveals Stat3 as a negative regulator of DC function. *J Immunol.* 2010;184(5):2638-45.
51. Calderon B, Carrero JA, and Unanue ER. The central role of antigen presentation in islets of Langerhans in autoimmune diabetes. *Curr Opin Immunol.* 2014;26(32-40.
52. Rangachari M, and Kuchroo VK. Using EAE to better understand principles of immune function and autoimmune pathology. *J Autoimmun.* 2013;45(31-9.
53. Meffre E, and Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. *Curr Opin Immunol.* 2008;20(6):632-8.
54. Kinnunen T, Chamberlain N, Morbach H, Cantaert T, Lynch M, Preston-Hurlburt P, Herold KC, Hafler DA, O'Connor KC, and Meffre E. Specific peripheral B cell tolerance defects in patients with multiple sclerosis. *J Clin Invest.* 2013;123(6):2737-41.
55. Pillai S. Rethinking mechanisms of autoimmune pathogenesis. *J Autoimmun.* 2013;45(97-103.
56. Mauri C, and Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol.* 2012;30(221-41.
57. Silveira PA, and Grey ST. B cells in the spotlight: innocent bystanders or major players in the pathogenesis of type 1 diabetes. *Trends Endocrinol Metab.* 2006;17(4):128-35.
58. Hu CY, Rodriguez-Pinto D, Du W, Ahuja A, Henegariu O, Wong FS, Shlomchik MJ, and Wen L. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *J Clin Invest.* 2007;117(12):3857-67.
59. Chatenoud L. Biotherapies targeting T and B cells: from immune suppression to immune tolerance. *Curr Opin Pharmacol.* 2015;23(92-7.
60. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, Bar-Or A, Panzara M, Sarkar N, Agarwal S, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med.* 2008;358(7):676-88.
61. von Budingen HC, Bar-Or A, and Zamvil SS. B cells in multiple sclerosis: connecting the dots. *Curr Opin Immunol.* 2011;23(6):713-20.
62. Yang M, Rui K, Wang S, and Lu L. Regulatory B cells in autoimmune diseases. *Cell Mol Immunol.* 2013;10(2):122-32.
63. Mauri C. Regulation of immunity and autoimmunity by B cells. *Curr Opin Immunol.* 2010;22(6):761-7.
64. Xu Y, Harder KW, Huntington ND, Hibbs ML, and Tarlinton DM. Lyn tyrosine kinase: accentuating the positive and the negative. *Immunity.* 2005;22(1):9-18.
65. Scapini P, Pereira S, Zhang H, and Lowell CA. Multiple roles of Lyn kinase in myeloid cell signaling and function. *Immunol Rev.* 2009;228(1):23-40.
66. Chan VW, Meng F, Soriano P, DeFranco AL, and Lowell CA. Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. *Immunity.* 1997;7(1):69-81.
67. Hibbs ML, Tarlinton DM, Armes J, Grail D, Hodgson G, Maglitto R, Stacker SA, and Dunn AR. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell.* 1995;83(2):301-11.
68. Harder KW, Quilici C, Naik E, Inglese M, Kountouri N, Turner A, Zlatic K, Tarlinton DM, and Hibbs ML. Perturbed myelo/erythropoiesis in Lyn-deficient mice is similar

to that in mice lacking the inhibitory phosphatases SHP-1 and SHIP-1. *Blood.* 2004;104(13):3901-10.
69. Scapini P, Hu Y, Chu CL, Migone TS, Defranco AL, Cassatella MA, and Lowell CA. Myeloid cells, BAFF, and IFN-gamma establish an inflammatory loop that exacerbates autoimmunity in Lyn-deficient mice. *J Exp Med.* 2010;207(8):1757-73.
70. Lamagna C, Scapini P, van Ziffle JA, DeFranco AL, and Lowell CA. Hyperactivated MyD88 signaling in dendritic cells, through specific deletion of Lyn kinase, causes severe autoimmunity and inflammation. *Proc Natl Acad Sci U S A.* 2013;110(35):E3311-20.
71. Lamagna C, Hu Y, DeFranco AL, and Lowell CA. B cell-specific loss of Lyn kinase leads to autoimmunity. *J Immunol.* 2014;192(3):919-28.
72. Ingley E. Functions of the Lyn tyrosine kinase in health and disease. *Cell Commun Signal.* 2012;10(1):21.
73. Cho JH, and Gregersen PK. Genomics and the multifactorial nature of human autoimmune disease. *N Engl J Med.* 2011;365(17):1612-23.
74. Anderson MS, and Su MA. Aire and T cell development. *Curr Opin Immunol.* 2011;23(2):198-206.
75. Oftedal BE, Hellesen A, Erichsen MM, Bratland E, Vardi A, Perheentupa J, Kemp EH, Fiskerstrand T, Viken MK, Weetman AP, et al. Dominant Mutations in the Autoimmune Regulator AIRE Are Associated with Common Organ-Specific Autoimmune Diseases. *Immunity.* 2015;42(6):1185-96.
76. Cetani F, Barbesino G, Borsari S, Pardi E, Cianferotti L, Pinchera A, and Marcocci C. A novel mutation of the autoimmune regulator gene in an Italian kindred with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, acting in a dominant fashion and strongly cosegregating with hypothyroid autoimmune thyroiditis. *J Clin Endocrinol Metab.* 2001;86(10):4747-52.
77. Lu R, Vidal GS, Kelly JA, Delgado-Vega AM, Howard XK, Macwana SR, Dominguez N, Klein W, Burrell C, Harley IT, et al. Genetic associations of LYN with systemic lupus erythematosus. *Genes Immun.* 2009;10(5):397-403.
78. Deng Y, and Tsao BP. Genetic susceptibility to systemic lupus erythematosus in the genomic era. *Nat Rev Rheumatol.* 2010;6(12):683-92.
79. Surolia I, Pirnie SP, Chellappa V, Taylor KN, Cariappa A, Moya J, Liu H, Bell DW, Driscoll DR, Diederichs S, et al. Functionally defective germline variants of sialic acid acetylesterase in autoimmunity. *Nature.* 2010;466(7303):243-7.
80. Su MA, Giang K, Zumer K, Jiang H, Oven I, Rinn JL, Devoss JJ, Johannes KP, Lu W, Gardner J, et al. Mechanisms of an autoimmunity syndrome in mice caused by a dominant mutation in Aire. *J Clin Invest.* 2008;118(5):1712-26.
81. Teh CE, Daley SR, Enders A, and Goodnow CC. T-cell regulation by casitas B-lineage lymphoma (Cblb) is a critical failsafe against autoimmune disease due to autoimmune regulator (Aire) deficiency. *Proc Natl Acad Sci U S A.* 2010;107(33):14709-14.
82. Platt AM, and Randolph GJ. Does deleting dendritic cells delete autoimmunity? *Immunity.* 2010;33(6):840-2.
83. Huang QQ, Perlman H, Birkett R, Doyle R, Fang D, Haines GK, Robinson W, Datta S, Huang Z, Li QZ, et al. CD11c-mediated deletion of Flip promotes autoreactivity and inflammatory arthritis. *Nat Commun.* 2015;6(7086.

84. Selmi C. Diagnosis and classification of autoimmune uveitis. *Autoimmun Rev.* 2014;13(4-5):591-4.
85. Peluso R, Lupoli GA, Del Puente A, Iervolino S, Bruner V, Lupoli R, Di Minno MN, Foglia F, Scarpa R, and Lupoli G. Prevalence of thyroid autoimmunity in patients with spondyloarthropathies. *J Rheumatol.* 2011;38(7):1371-7.
86. Clausen BE, Burkhardt C, Reith W, Renkawitz R, and Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Research.* 1999;8(4):265-77.
87. Caton ML, Smith-Raska MR, and Reizis B. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med.* 2007;204(7):1653-64.
88. Hobeika E, Thiemann S, Storch B, Jumaa H, Nielsen PJ, Pelanda R, and Reth M. Testing gene function early in the B cell lineage in mb1-cre mice. *Proc Natl Acad Sci U S A.* 2006;103(37):13789-94.
89. Mattapallil MJ, Wawrousek EF, Chan CC, Zhao H, Roychoudhury J, Ferguson TA, and Caspi RR. The Rd8 mutation of the Crb1 gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. *Invest Ophthalmol Vis Sci.* 2012;53(6):2921-7.
90. Xu H, Koch P, Chen M, Lau A, Reid DM, and Forrester JV. A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images. *Exp Eye Res.* 2008;87(4):319-26.
91. Moon JJ, Chu HH, Hataye J, Pagan AJ, Pepper M, McLachlan JB, Zell T, and Jenkins MK. Tracking epitope-specific T cells. *Nat Protoc.* 2009;4(4):565-81.
92. Manjarrez-Orduno N, Marasco E, Chung SA, Katz MS, Kiridly JF, Simpfendorfer KR, Freudenberg J, Ballard DH, Nashi E, Hopkins TJ, et al. CSK regulatory polymorphism is associated with systemic lupus erythematosus and influences B-cell signaling and activation. *Nat Genet.* 2012;44(11):1227-30.
93. Cornall RJ, Cyster JG, Hibbs ML, Dunn AR, Otipoby KL, Clark EA, and Goodnow CC. Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity.* 1998;8(4):497-508.
94. Ermann J, and Glimcher LH. After GWAS: mice to the rescue? *Curr Opin Immunol.* 2012;24(5):564-70.
95. Wakeland EK, Liu K, Graham RR, and Behrens TW. Delineating the genetic basis of systemic lupus erythematosus. *Immunity.* 2001;15(3):397-408.
96. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, and Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. *Science.* 2006;312(5780):1669-72.
97. Yamanouchi J, Rainbow D, Serra P, Howlett S, Hunter K, Garner VE, Gonzalez-Munoz A, Clark J, Veijola R, Cubbon R, et al. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet.* 2007;39(3):329-37.
98. Chen J, Qian H, Horai R, Chan CC, Falick Y, and Caspi RR. Comparative analysis of induced vs. spontaneous models of autoimmune uveitis targeting the interphotoreceptor retinoid binding protein. *PLoS One.* 2013;8(8):e72161.
99. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, Derecki NC, Castle D, Mandell JW, Lee KS, et al. Structural and functional features of central nervous system lymphatic vessels. *Nature.* 2015;523(7560):337-41.

100. Horai R, Silver PB, Chen J, Agarwal RK, Chong WP, Jittayasothorn Y, Mattapallil MJ, Nguyen S, Natarajan K, Villasmil R, et al. Breakdown of immune privilege and spontaneous autoimmunity in mice expressing a transgenic T cell receptor specific for a retinal autoantigen. *J Autoimmun.* 2013;44(21-33.
101. Shao H, Liao T, Ke Y, Shi H, Kaplan HJ, and Sun D. Severe chronic experimental autoimmune uveitis (EAU) of the C57BL/6 mouse induced by adoptive transfer of IRBP1-20-specific T cells. *Exp Eye Res.* 2006;82(2):323-31.
102. Caspi RR. Experimental autoimmune uveoretinitis in the rat and mouse. *Curr Protoc Immunol.* 2003;Chapter 15(Unit 15 6.
103. Heuss ND, Lehmann U, Norbury CC, McPherson SW, and Gregerson DS. Local activation of dendritic cells alters the pathogenesis of autoimmune disease in the retina. *J Immunol.* 2012;188(3):1191-200.
104. Zhou R, Horai R, Silver PB, Mattapallil MJ, Zarate-Blades CR, Chong WP, Chen J, Rigden RC, Villasmil R, and Caspi RR. The living eye "disarms" uncommitted autoreactive T cells by converting them to Foxp3(+) regulatory cells following local antigen recognition. *J Immunol.* 2012;188(4):1742-50.
105. Honda K, and Littman DR. The microbiome in infectious disease and inflammation. *Annu Rev Immunol.* 2012;30(759-95.
106. Wenzel A, Grimm C, Samardzija M, and Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog Retin Eye Res.* 2005;24(2):275-306.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature

08/24/15

Date