THE INFLUENCE OF TYPE II CYTOKINES IN CENTRAL TOLERANCE

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LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
Ag	Antigen
AIRE	Autoimmune Regulator
APC	Antigen presenting cell
BGL	Blood glucose level
BM	Bone marrow
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Central nervous system
CVB	Coxsackievirus B
DCs	Dendritic Cells
DN	Double negative ($CD4^{-}CD8^{-}$)
DP	Double positive ($CD4^{+}CD8^{+}$)
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ETP	Early thymic progenitor
GAD	Glutamic acid decarboxylase
GM-CSF	Granulocyte macrophage-colony stimulating factor
GFP	Green florescent protein
HLA	Human leukocyte antigen
HR	Heteroreceptor
i.t	Intrathymic
IL-	Interleukin
LMPP	Lymphoid-primed multipotent progenitors
MHC	Major histocompatibility complex

MOG	Myelin oligodendrocyte glycoprotein
MPP	Multipotent progenitors
iNKT	Invariant natural killer T cells
NOD	Non-obese diabetic
NOD.scid	Non-obese diabetic severe combined immunodeficiency
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PLN	Pancreatic lymph node
PN	Pancreas
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SEM	Standard error mean
SP	Spleen
T1D	Type one diabetes
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
Tregs	T regulatory cells
β-cell	Beta cell

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ABSTRACT

While the genetic elements of many autoimmune conditions have been established, many environmental factors remain a mystery. Exposure to certain infections has been implicated as a possible explanation for the significantly different autoimmune disease rates in developing versus industrialized countries. In this manner, type II cytokines have been shown to play a role in peripheral tolerance. The full impact of these type II cytokines in central tolerance and autoimmunity has still yet to be explored. In chapter II, the data indicate that IL-4 and IL-13, dominant type II cytokines, can indeed be found within the thymic microenvironment of healthy C57BL/6 mice. IL-4/IL-13 signaling in ETPs that express the IL-4Rα/IL-13Rα1 heteroreceptor (HR) drives these progenitors to yield thymic resident DCs. These DCs aid in negative selection and the prevention of experimental allergic encephalomyelitis (EAE). In chapter III, the data presented shows that, in contrast to the C57BL/6 strain, HR^{+}ETPs from the type one diabetes (T1D) susceptible NOD strain give rise to T cells. Notably, the NOD thymus shows a dramatic reduction in the steady-state level of IL-4. Indeed, increasing the availability of this cytokine can rescue the lineage fate of HR^{+}ETPs and provide a new pool of thymic DCs. Restoration of NOD HR^{+}ETP lineage fate can improve negative selection, alter the TCRβ repertoire, and ultimately prevent T1D onset. Thus, microenviromental changes within the thymus may be implicated in fine-tuning the balance that dictates the negative selection of autoreactive cells. From this, one may envision that environmental induction of

IL-4 and IL-13 may play a role in altering that balance and shifting the tables towards autoimmunity prevention.

CHAPTER I: LITERATURE REVIEW

1. Overview of Autoimmune Diseases

A. General Epidemiology

The immune system's primary function is to protect the body from any damage incurred by invading pathogens. Autoimmunity arises when these protective measures incorrectly target self-tissues. This misdirection occurs when cells of the immune system cannot differentiate between self-antigen and foreign-antigen (1). In turn, these misguided responses can then lead to the destruction of various organs and tissues. Thus leaving the host unable to maintain normal homeostasis without intervention.

Under normal conditions, autoimmune responses are tightly controlled by two mechanisms: central and peripheral tolerance (2). However, neither of these processes are without flaw and may leave an individual susceptible to developing an autoimmune disease. Particular groups of people may be more likely to develop an autoimmune disease. For example, women represent over 78% of all cases of autoimmunity (2–4). While some autoimmune diseases show a strong gender bias (9 out of 10 systemic lupus erythematosus (SLE) patients are female), others exemplify an equal representation of genders, such as type one diabetes (T1D) (5). Additionally, individuals from industrialized countries are more susceptible to both allergic and autoimmune diseases (6). This imbalance continues to grow annually (7).

B. Disease Models

Identification of environmental circumstances and universal genetic factors that contribute to autoimmune disease is critical to advancing potential treatment

options. For this purpose, animal models have been used extensively to improve our understanding of disease pathogenesis mechanisms. This common use of animal models is valid for all immune-related conditions but especially so for autoimmune responses.

Murine models of disease have been extensively useful in immunology research, given the availability and ease with which model animals can be produced. Useful models often require the over-expression of a particular gene, complete knock out of a gene, or even the use of a cre-lox system to test the effects of a gene knock out in a specific cell subset (8, 9). Given the extensive utilization of mouse models in the history of research, murine-based reagents are in widespread use and, therefore, easier to come by than other species-based reagents (9). Additionally, the breeding and housing of mice is relatively undemanding when compared to animal models of greater size or with more extended reproduction intervals (10).

However, as with any animal model, there are significant differences between the immune system of a mouse and the immune system of a human. Because of the innate differences between organisms, translatability to human medicine should always be considered. Essential differences in the overall composition of the immune response are important to note, such as the balance between myeloid and lymphoid cells in the blood. For example, under homeostatic conditions, lymphocytes are the predominant immune cell found in murine blood, while human blood is rich in neutrophils (11–13). Significant innate immune response disparities have been reported. These include unique

mediators of iNOS induction in macrophages, mucosal defensin expression in murine neutrophils, and the absence of the Ly49 family of proteins in humans which marks murine NK and NKT cell (14–18). Differences in the adaptive immune response are vast and extend to variances in Ig subtypes, cytokine induction of class switching, Th1/Th2 phenotype skewing, MHC class II expression, and others (19).

Nevertheless, an ever-growing body of research indicates the critical role that mouse models play in improving our treatment of disease. The value of these models is further understood when comparing the therapeutic timeline of human diseases for which a mouse model is unavailable. As a result, little progress has been made in understanding the mechanism of diseases without mouse models, such as the neuropsychiatric manifestation of SLE (20). Additionally, the applicability of these animals is continually improving with the surge in the use of humanized mice (mice that have been genetically transplanted with a human immune system) (21). Thus, for the time being, murine models often provide the best method for studying autoimmunity.

There are three general types of mouse models available in terms of studying immune dysfunction: genetic models, inducible models, and spontaneous models. Genetic models of autoimmune diseases often include knocking out a specific gene or the insertion of transgenes, such as the amyloid P component knock out mouse and its use in SLE research (22). Models that require induction of disease have been useful (such as streptozotocin-induced diabetes) but often still require a particular genetic disposition, limiting what

mouse strains can be used (8). There is often predominant use of the SJL or C57BL/6 strains in experimental allergic encephalomyelitis (EAE), a mouse model for human multiple sclerosis (23). However, spontaneous autoimmune disease models are generally the first choice for researchers in autoimmunity. This preference for spontaneous models is because these models are the most reminiscent of human disease and thus serve as a better way to tease apart the cascade of events that leads to disease. Perhaps the best example of a spontaneous disease model is the commonly utilized non-obese diabetic (NOD) mouse, which is well-known for having a genetic predisposition to developing T1D (24–26). The NOD mouse's usefulness is further boosted by a strong environmental influence on disease penetrance, with individual research laboratories reporting widely different disease rates (27, 28).

2. Autoimmune Type One Diabetes (T1D)

A. Introduction to Type One Diabetes

a. Disease Manifestation

Type one diabetes, previously referred to as juvenile diabetes and additionally known as insulin-dependent diabetes mellitus, is an autoimmune disease that results from the targeted destruction of insulin-producing pancreatic β-cells by cells of the immune system (28). The disease is often diagnosed before adulthood and is one of the most common chronic conditions in children. However, the terminology has recently changed to acknowledge that T1D can be diagnosed at any age and with consideration to the fact that the condition is a

life-long diagnosis (29, 30). In contrast to the more prevalent type two diabetes (T2D), T1D accounts for less than 10% of worldwide cases of diabetes. However, the incidence of T1D is increasing annually (28).

Symptoms and complications of T1D occur when there is no longer enough insulin produced by β-cells within pancreatic islets to maintain normal blood glucose levels (normoglycemia). Increased blood glucose (hyperglycemia) leads to frequent urination, increased thirst, and diabetic ketoacidosis, among other symptoms. Long-term hyperglycemia can result in complications such as neuropathy, limb amputation, and blindness (31).

b. Key Immune Cells

The development of T1D is a complex multicellular inflammatory process. There is known involvement of T cells, B cells, and antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs). Studies have shown that individual depletion of any of these cell types can prevent or delay diabetes development in NOD mice, further proving the complexity of the inflammatory response within the pancreatic islets (32–34).

T cells are vital to the onset of autoimmune diabetes in terms of both initiating and maintaining inflammation in the pancreas. The use of anti-CD3 treatment to deplete T cells has shown to be effective in improving insulin production and delaying disease in NOD mice (34). Additionally, administration of anti-CD3 to relatives of T1D patients (deemed high-risk for developing diabetes by identification of two or more autoantibodies) can also significantly delay

disease onset (35). Interestingly, both $CD4^+$ and $CD8^+$ T cells are required for disease induction as the transfer of only one subset from a wild-type diabetic NOD into an immunocompromised host mouse may result in the recruitment of APCs to the pancreas, but full pancreatic infiltration is not induced (36, 37). It is currently thought that $CD8^+$ T cells play an effector role within the pancreatic islet while $CD4^+$ T cells serve to recruit APCs, support $CD8^+$ T cell function, and ultimately aid B cells in the production of autoantibodies (38–40). In an opposing fashion, invariant natural killer T cells (iNKT) may operate in an anti-inflammatory manner given their known production of a classically anti-inflammatory cytokine, IL-4 (41, 42). The overall impact of T cells in the development of autoimmune diabetes is fundamental to the disease process.

B cells and associated autoantibodies represent not only a contributing factor to islet destruction but are also a prominent predictor of future disease. A patient who presents with two or more autoantibodies (antibodies specific for islet-associated antigens) is nearly guaranteed to develop T1D when followed for a decade (43, 44). On top of the classical function of antibodies in the immune response, B cells have been shown to act as APCs that display islet-associated antigens for autoreactive T cells (45, 46). In addition, there is evidence to suggest that B cells produce pro-inflammatory cytokines, which, in turn, act in a feedback loop to activate T cells (47, 48). Studies using NOD mice deficient in B cells, and clinical trials with recently diagnosed patients treated long term with B cell depleting antibodies, have shown that removing B cells from the picture can delay the diabetogenic process (32, 49, 50).

Subsets of innate immune cells, such as macrophages and DCs, stage a unique but central function in the targeted destruction of β-cells. Macrophages are considered one of the earliest cells to infiltrate the pancreatic islets (51, 52). Within the pancreas, macrophages can produce large amounts of pro-inflammatory cytokines, such as IL-12, that prompt the activation of cytotoxic T cells (33). Circulating DCs may also produce large amounts of pro-inflammatory cytokines but, in contrast to macrophages, are vital to the insulitis stage of diabetes (53). Specifically, the $CD11c^{+}$ $CD11b^{+}$ $CD8\alpha^{-}$ cDC2 subset is exceptionally efficient at presenting β-cell antigens to T cells within the pancreatic draining lymph node (33, 53).

Interestingly, DCs from NOD mice are more sensitive to stimulation than strains not prone to autoimmune diseases, such as the C57BL/6 strain, due to an inherent hyperactivation of the IL-12-inducing transcription factor NF-ϰB (54). The picture is complicated further as other studies have suggested some DC subsets may be protective. For example, plasmacytoid DCs (pDCs), in conjunction with NKT cells, can act in a tolerogenic style and thwart T1D in NOD mice (55). In sum, the overall microenvironment of the pancreas and the associated lymph node is the crucial deciding factor that determines if APCs will promote or block the autoimmune process.

c. Antigen Targets

The identification of autoantibodies in insulin-dependent diabetic patients was crucial in determining that the condition was an overt autoimmune disease (56). This discovery eventually led to autoantibody presence/absence as a way to determine future disease risk in the relatives of T1D patients. Indeed, a patient with two or more diabetes-associated autoantibodies is in stage 1 of the disease (57–59). This knowledge is vital as those that test positive may be included in clinical trials to prevent or delay disease (60, 61).

On a larger scale, commonly detected autoantibodies represent shared antigen targets involved in disease development. These antigens are not only targeted by B cells but by both $CD4^+$ and $CD8^+$ T cells. The most pervasive of islet-associated autoantigens are crucial in both NOD mice and human patients (62). Some of the top antigens associated with T1D are derived from insulin, glutamic acid decarboxylase, and insulinoma antigen 2 (57, 63).

Insulin is often thought of as the foremost diabetes-associated antigen. The primary role of β-cells is to produce insulin, so it is no wonder why insulin-specific lymphocytes target β-cells. Tolerance to amino acids 9-23 of the insulin B chain (Insulin B:9-23) in NOD mice prevents diabetes, while the exclusion of other well-known autoantigens cannot always afford the same protection (64–67). Insulin is also unique as it is the only islet-associated autoantigen exclusively expressed in β-cells (68–70). An inflammatory response to insulin may often be the first antigenic trigger in β-cell autoimmunity because of these important

details. This idea is further supported by studies showing that T cells reactive to another autoantigen, islet-specific glucose-6-phosphatase catalytic subunit-related protein, cannot be found in the absence of insulin reactive T cells (66). However, while it is confirmed that insulin is an essential autoantigen, it is still unclear if treatments targeting insulin-reactive cells alone would be sufficient to prevent or cure T1D in humans.

Glutamic acid decarboxylase (GAD) is commonly known as a central nervous system enzyme that converts glutamic acid into the neurotransmitter gamma-amino butyric acid. GAD can also be found in the pancreatic islets where the functional role is less clear (71). GAD has both a 67 kDa (GAD_{67}) and 65 kDa (GAD_{65}) isoform, with GAD_{65} being a dominant autoantigen (72). While the exact role of GAD in the pancreas is unclear, it is important to note that increased insulin production is positively correlated with an increase in GAD expression by β-cells (73). While 8 out of 10 newly diagnosed patients will have GAD autoantibodies, 20% of T1D patients will remain negative; which indicates that it is unlikely reactivity to GAD alone that induces full insulitis (74, 75). However, the use of GAD in tolerance inducing treatments has shown success. In mice, Ig-GAD2 alone, which contains GAD amino acid sequences 206-220 incorporated into an immunoglobulin molecule, can successfully treat the disease in its early stages (76). GAD-alum, a GAD65 and aluminum hydroxide tolerogenic vaccine, has previously shown positive results in extending β-cell function in humans when used in early diagnosis (77, 78).

Like GAD, insulinoma antigen 2 (IA-2) is found in both the central nervous system and pancreatic islets (79). IA-2 in β-cells is thought to operate as an anchor for intracellular insulin secretory granules that aid in the release of the hormone (65, 79). Because IA-2 autoantibodies most often target the protein's intracellular domain, it is thought that reactivity to the antigen is most commonly induced after β-cell damage (80). Therefore, it is not clear if IA-2 represents a direct target or if the antigen is merely exposed due to cellular damage initiated by other autoreactive cells. Regardless of this, IA-2 autoantibodies are a crucial marker in determining T1D risk in first degree relatives (81).

B. Genetic Factors

a. HLA Genes

Among the genes linked to T1D, the human leukocyte antigen (HLA) region of chromosome 6 holds some of the strongest influence on an individual's likelihood of developing the condition (82). The HLA region contains over 200 identified genes, with only some being determiners for T1D. These determiners include HLA class I and HLA class II. Gene products of these loci lead to the cellular expression of major histocompatibility complex (MHC) that allows for the demonstration of antigen peptides on the cell surface. These peptides are presented to immune cells, including T cells known for having a major role in T1D induction (83).

Polymorphisms in the class II HLA genes, such as those which encode for DQ and DR, are considered the primary genetic determinate for T1D. The two

highest risk haplotypes include DRB1*04:01/02/04/05/08-DQA1*03:01-DQB1*03:02/04 and DRB1*03:01-DQA1*05:01-DQB1*02:01 (84, 85). When a person is heterozygous for these two haplotypes, they are at an even higher risk for developing the disease (86). Roughly 30% of T1D patients are heterozygous for these two high risk haplotypes (87).

Interestingly, this MHC-linked susceptibility to T1D is also found in the NOD mouse model. NOD mice express MHC class II molecules I-A^{g7} which contains a polymorphism similar to that in human diabetics (84, 88). Indeed, the substitution of a single amino acid in the beta chain of I-A^{g7} leads to resistance to diabetes (88).

While MHC haplotype is strongly tied to T1D incidence in both mice and humans alike, the mechanism behind this phenomenon is still being teased apart. It is currently thought that the specific peptide-binding pockets of T1D-associated MHC molecules are altered so that the size and composition of the binding pocket allow for altered interactions with self-peptide (89–91). It is important to note that MHC haplotype is not the only genetic component tied to the disease and, in fact, only makes up about 50% of the genetic risk (92).

b. Non-HLA Genes

Several other genes are known to play a role in diabetes susceptibility. These include both non-immune related genes, such as insulin, and those commonly associated with immune cell function, such as CTLA-4 and the IL-2 receptor (93, 94). Interestingly, variations in the IL-4/IL-13 signaling pathway have been

associated with both increasing and decreasing T1D incidence, both in humans and mice (95, 96). Additionally, other candidate genes continue to be exposed regularly.

Insulin, CTLA-4, and IL-2R are perhaps the most well studied non-HLA T1D-associated genes. For example, it is suggested that ~10% of genetic susceptibility originates from a polymorphic region flanking the gene for insulin (97). Additionally, as a negative regulator of T cell activation, it is not surprising that CTLA-4 polymorphisms are associated with T1D and other autoimmune diseases (98). The role of the IL-2 receptor in the development of T1D is more convoluted but thought to be due to the function of IL-2 in the normal development of T Regulatory (Tregs) cells, which are known to be anti-inflammatory and thus protective (89, 90). Together these genes make up a large part of non-HLA associated genetic susceptibility but certainly not all.

Studies analyzing the role of the IL-4/IL-13 signaling pathway in autoimmunity have identified the impact of over 13 SNPs in the IL-4R coding region alone. Many of these polymorphisms result in amino acid substitutions that impact receptor protein function (99). This not only alters IL-4 signaling but IL-13 signaling as well, given that IL-13 signaling occurs via the IL-4/IL-13 heteroreceptor (HR) (100). Specific haplotypes for these SNPs, as well as SNPs in the IL-4 and IL-13 coding regions themselves, have been linked to T1D (101). This link between the IL-4R/IL-4/IL-13 signaling pathways and T1D incidence is exciting as these cytokines are generally considered anti-inflammatory and therefore protective against autoimmune diseases (102).

C. Environmental Factors

a. Hygiene Hypothesis

While genetic factors have long been the established explanation behind T1D, it is becoming increasingly obvious that environmental factors, external to the individual, are equally important to consider. One of the best examples of environmental influence is found in the study of monozygotic twins in which one twin will develop the disease while the other is spared (103). Additionally, migration studies have shown that simply moving from one country to another can alter T1D rates to match that of the new host country (7, 103–106). Thus, one of the main goals of current research in T1D is to understand specific ways that the widely-defined environment impacts the development of the disease.

The hygiene hypothesis was initially developed in 1958 to explain allergic diseases when scientists first noticed that having older siblings was a protective factor in developing hay fever (107). Further characterization of the epidemiology of autoimmune diseases led to the extension of the hygiene hypothesis to include both allergic and autoimmune disorders (108–112). The basic idea behind the hypothesis is that humans have co-evolved with other potentially infectious organisms, such as bacteria and parasites, to the extent that they play a protective role in our inherent biology and serve to keep the immune response in check. This idea is supported by striking epidemiologic evidence that shows declining infections are inversely correlated to rising allergic and autoimmune diseases (108–112).

While prior studies showed purely correlative effects, recent epidemiologic studies have yielded a more direct link between hygiene and disease-specific individual risk. For example, studies have shown that having more siblings is positively correlated with a lower risk for T1D (108–112). Causal relationship studies have been done in varying faucets, including the use of parasitic infections and probiotics. These studies have consistently shown that these co-evolved organisms offer at least some protection from autoimmunity in various forms, including T1D (108–112).

b. Environmental Triggers and Protective Factors

Host circumstances, which may be considered environmental factors, appear in many forms. The common denominator is that these external factors hold influence over the immune response of the whole organism. This manipulation of the host may be directly impacting an autoreactive response or acting in early life to shift the immune repertoire completely. Environmental factors that have been shown to influence T1D incidence include viral infection, gut microbiota, and helminth infection (113–116).

Viral infection has long been considered a potential trigger of T1D. While many viruses have been investigated, well-known enteroviruses, such as coxsackievirus B (CVB), are considered a chief candidate (117). Enteroviral infection has been tied to T1D both prenatally, with mothers of future diabetic children more likely to have elevated levels of enterovirus antibodies during pregnancy, as well as during the postnatal period, with diabetic children more

likely to have experienced recent enteroviral infection than their unaffected sibling (118). Interestingly, a Finnish study noted that, in genetically susceptible children, the first appearance of autoantibodies coincided with the seasonal uptick of enterovirus infections (119). A common enterovirus, coxsackievirus B (CVB), has been demonstrated to be infectious to pancreatic islet cells (120, 121). Indeed, CVB infection of islet cells has been shown to increase these cells' susceptibility to apoptosis and to perhaps expose previously hidden diabetogenic antigens (122). A better understanding of how viruses, such as CVB, specifically aid or otherwise trigger immune cells in the targeted destruction of β-cells will be required before any possible prevention methods may be devised.

Microbial colonization is a universal aspect of life on earth and is a core feature of research aimed at understanding how the host environment impacts disease. In fact, it is estimated that 20% of all small molecules found in human blood are actually of microbiota origin (123). The immune system must regularly interact with these commensal microbes. The frequent cross-talk between microbe and host can train and modulate the immune system (123, 124). Research is still in the very early stages of deciphering the mechanisms involved in microbe influence over host and why some microbiomes, and the accompanying small molecules, may be protective while others increase the likelihood of disease. For example, mice housed under germ-free conditions remain resistant to EAE but become susceptible once again when recolonized with either diverse gut microflora or segmented filamentous bacteria alone (125–128). Interestingly, studies utilizing NOD mice have shown that the gender bias

associated with T1D in mice is nullified under germ-free conditions (129, 130). In contrast, the transfer of cecal microbiota from male mice into female NOD mice was protective by way of increased androgen signaling (129). Future research in this area will likely seek to understand the mechanisms by which various microbes influence T1D rates in humans.

In line with theories envisioned by the hygiene hypothesis, parasitic infection is often considered to be a protective factor. The idea that helminths may be protective is strongly supported by data tying the global increase in T1D diagnosis with improving hygiene and overall reduction in the exposure to agriculture and animals (131, 132). Studies utilizing the NOD mouse as a model of autoimmunity have shown some helminths can delay disease (*Schistosoma mansoni*) while others provide complete protection from T1D (*Heligmosmoides polygyrus*) (133–135). This protection is due Th2 skewing of the immune response with associated type II cytokines, such as IL-4 and IL-13 (136, 137). Regarding the mechanism of action, it is widely accepted that these cytokines serve an anti-inflammatory function in peripheral tolerance, but little is known about their role in central tolerance, and as such further studies are required.

c. Anti-Inflammatory IL-4 and IL-13 Cytokines

Type II cytokines IL-4 and IL-13 can be produced by many different cell types, including $CD4^+$ T cells, iNKT cells, innate lymphoid cells, and myeloid cells such as eosinophils (138–140). IL-4 and IL-13 are classically induced during the host response to intestinal parasites. IL-4 can signal via the IL-4Rα or the IL-

4/IL-13 heteroreceptor (HR), while IL-13 can only signal through the HR (141). Because the two cytokines share a receptor, many of their functions intersect. Both are considered anti-inflammatory as they can both suppress the function of typically pro-inflammatory cytokines and lead to the induction of IL-10, another anti-inflammatory cytokine (142–144).

Many studies have shown a role for these two cytokines in preventing autoimmune diseases, including T1D. Interestingly, long term treatment of NOD mice with either IL-4 or IL-13 can delay or even prevent T1D onset (145, 146). When young NOD mice are treated with a GAD65 immunogen protocol to delay disease, β-cell-targeted T cells maintained the expression of the HR (uncommon in mature T cells) and were therefore susceptible to signaling via either IL-4 or IL-13 (147). Additionally, HR expression on APCs has also been shown to affect disease outcome in NOD mice (148). While the entire picture is unclear, it is evident that IL-4 and IL-13 have a unique role in autoimmune diseases such as T1D.

3. Hematopoiesis & T cell Development

A. Multipotent Progenitors

Hematopoiesis, or the development of blood cells, requires self-renewing stem cells (149). This process is initiated by dividing hematopoietic stem cells (HSC) in the bone marrow (150). HSCs are capable of giving rise to all hematopoietic lineages and are self-renewable (151). The lack of Flt3 expression separates HSCs from multipotent progenitors (MPPs) (152). MPPs which are

$Flt3^{hi}$ are considered to have lost their potential for giving rise to erythrocytes (153, 154). Additionally, MPPs can express the *rag1* gene and are then considered primed for the lymphoid lineage and thus referred to as lymphoid-primed multipotent progenitors (LMPP) (155). However, LMPPs retain some myeloid potential and are thought to be the last stage before the split between exclusively myeloid or lymphoid lineage.

It was previously presumed that the first divide between myeloid and lymphoid progenitors occurs at the common myeloid progenitor (CMP) and common lymphoid progenitor stage (CLP). CMPs remain in the bone marrow and give rise to macrophages, DCs, and granulocytes (156). CLPs are flexible and may remain in the bone marrow where they give rise to B cells, or they may migrate to the thymus where they give rise to T cells and, to some extent, thymic B cells (157, 158). CLPs may also give rise to NK cells of either bone marrow or thymic origin (159, 160).

However, this black and white model of hematopoiesis has recently been brought to question. The presence of a thymic myeloid-T cell progenitor is supported by studies utilizing clonal expansion assays (161, 162). Additionally, $IL\text{-}7R^+$ cells, which were initially thought to be only lymphoid progenitors, retain significant myeloid potential when cultured *ex vivo* (163). These assays showed that early thymic progenitors (ETPs) do maintain myeloid potential. This evidence gave rise to a new hematopoiesis model, deemed the myeloid-based model, as defined by the concept that myeloid lineage fate serves as the default setting and the lymphoid fate as specialized types (164).

This concept is further supported with consideration to the source of thymically derived DCs. T cell progenitors in the thymus robustly maintain DC potential until the late DN3 stage, immediately prior to rearrangement of the TCR (165–167). Further evidence that many thymic DCs arise from T cell progenitors is supported by the presence of $CD8\alpha^{+}$ DCs which express mRNA for the α subunit of the pre-TCR (168). Additionally, one study showed that T cell progenitors could efficiently give rise to DCs under lymphopenia conditions or after DC-deletion (169). Altogether, these results demonstrate the plasticity of the development of thymic DCs and, as a consequence, ETPs.

B. T Cell Development

T cell development, from thymic settling progenitor to mature T cell, occurs in distinct stages. The first stage is the $CD4^{-}CD8^{-}$ double-negative (DN) 1 stage (170, 171). The DN1 stage is defined by the expression of CD44 and a distinct lack of CD25 expression (172). This subset also includes ETPs, which can be subdivided by their expression of the stem-like marker c-Kit (173). The ETP/DN1 subset is highly proliferative. It is estimated that one progenitor can produce around one million progeny cells (174, 175). Expression levels of CD24 and c-Kit can further subdivide the DN1 stages DN1a, DN1b, DN1c, DN1d, or DN1e (175, 176). Interestingly, it has been shown that thymically-derived DCs are sourced from the DN1c population (176, 177). However, this population does not give rise exclusively to DCs and has been shown to possess lymphoid potential as well (175, 177).

DN2 is the next stage of development and is defined by the co-expression of CD25 and CD44 (178). By the end of this stage, the cells have developed a high level of response to Notch1 signaling (179, 180). This Notch signaling is induced by near-constant contact with thymic stromal cells expressing Delta-like 1 (a Notch ligand) (180). In addition, late DN2 cells will have no longer express c-Kit and lose their capacity for the NK lineage fate (178).

By the early DN3 stage (defined as $CD44^-$ and $CD25^+$), the cells are no longer capable of rapid proliferation and are in cell cycle arrest (181). This stage marks the important step of RAG-mediated somatic recombination of the T cell receptor (TCR) (179). This step involves the expression of the constant (C) region, the selection of variable chain segments (V, D and J), and the insertion of random nucleotides within the TCR coding sequence (178, 179). This recombination results in a novel amino acid sequence that permits the TCR to bind MHC-bound antigen. In the case of the more common αβ TCR, cells can then be subdivided by pre- β (DN3a) and post- β (DN3b) selection (179, 182). This transition also coincides with the down-regulation for IL-7 signaling in exchange for an increase in Notch signaling (180, 184). CD27 expression can be used to define those cells which are post- β selection and can begin to divide rapidly once again (182, 183).

Following the successful rearrangement of the β chain, the TCR pairs with the germline pre-Tα and CD3 to form what is referred to as the pre-TCR complex (185). Signaling through the pre-TCR induces the downregulation of CD25, leading the cells to the DN4 stage ($CD44^-CD25^-$), and allows for the TCRα

chain's rearrangement, which only requires V and J segment rearrangement (186, 187). It is at this stage when cells may become double positive (DP) for CD4 and CD8 (187). These cells continue to express rag and rearrange the TCRα chain until the point of positive selection (188).

C. Central Tolerance `

a. Positive Selection

T cells must react to a cognate antigen and, as such, they must be able to bind MHC complexes. The process of positive selection ensures that mature T cells can bind MHC appropriately. Developing T cells require contact with cortical thymic epithelial cells (cTECs), which express both MHC class I and MHC class II. When cells cannot form sufficient TCR-MHC interactions, they will undergo a process known as “death by neglect.” Counterintuitively, death by neglect is a rather active process that involves signaling from glucocorticoid hormones and macrophages (189).

cTECs possess unique machinery that allows them to express an exclusive library of peptides (190). These peptides are partial TCR agonists or altered peptides that are unique but structurally similar to peptides that can activate peripheral T cells (190, 191). The generation of these peptides is likely due to multiple different mechanisms. For example, cTECs utilize special proteases and cathepsins, such as cathepsin L, to generate a unique peptide library (192, 193). In addition, cTECs are known to execute efficient macroautophagy, a process that sanctions the non-canonical delivery of

intracellular peptides and allows for their presentation on MHC class II molecules (192, 194).

The CD8 versus CD4 fate of developing T cells is determined by the recognition of antigen bound to either MHC class I or II, respectively. This TCR stimulation results in the upregulation of the activation marker CD69, which can be used to identify those cells that could successfully interact with positively-selecting cTECs (195, 196). However, the vast majority of cell death within the thymic cortex can be attributed to developing T cells that did not pass the positive selection test (197).

b. Negative Selection

The process of central tolerance, or selection, proceeds as the immature T cells travel to the thymic medulla. The thymic medulla is rich in APCs, including both medullary thymic epithelial cells (mTECs) and DCs (198, 199). These cells are the major mediators of the next selection process, known as negative selection. mTECs offer a unique ability to express antigens not typically found in the thymus (199, 200). These antigens include tissue-specific antigens, such as insulin or MOG. mTECs are able to produce these peptides given their near-exclusive production of the transcription factor AIRE (autoimmune regulator) (201, 202). The AIRE gene has proven to be vital to the process of negative selection. AIRE knock-out mouse models are susceptible to multiple autoimmune disorders (203). The same is true for human patients with AIRE gene mutations (204).

While there have been some reports of DCs expressing AIRE, most DCs can present these specific tissue-specific antigens by sourcing them from surface MHC on mTECs (205). The majority of the thymic DCs with this ability are of the $CD8\alpha^+$ variety and referred to as cDC1s. These are DCs are most frequently sourced from ETPs and are non-migratory as determined by their lack of SIRPα expression (206).

Together mTECs and DCs can process and negatively select up to 5×10^5 T cells in a single 24 hour period (207). T cells that too strongly interact with peptide-MHC complexes on these cells will undergo apoptosis, receptor rearrangement, or even diversion to the Treg linage fate (208). The determination of strong versus weak TCR signaling is due to TCR affinity, essentially for how long the interaction occurs. This increased affinity induces a signaling threshold involving enhanced concentrations of signaling mediators such as ZAP-70 and the translocation of these mediators to different organelles (209).

After the process of both negative and positive selection, only 5% of developing thymocytes survive. Ultimately, negative selection serves to limit the number of autoreactive cells that reach the periphery. This process is vital to creating a functional immune system and prevent autoimmunity.

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CHAPTER II: Type II Cytokines Fine-Tune Thymic T Cell Selection to Offset Murine Central Nervous System Autoimmunity

1. Abstract

Early thymic progenitors (ETPs) are bone marrow–derived hematopoietic stem cells that remain multipotent and give rise to a variety of lineage-specific cells. Recently, we discovered a subset of murine ETPs that expresses the IL-4Rα/IL-13Rα1 heteroreceptor (HR) and commits only to the myeloid lineage. This is because IL-4/IL-13 signaling through the HR inhibits their T cell potential and enacts commitment of HR^+ETPs to thymic resident $CD11c^+CD8\alpha^+$ dendritic cells (DCs). In this study, we discovered that HR^+ETP–derived DCs function as APCs in the thymus and promote deletion of myelin-reactive T cells. Furthermore, this negative T cell selection function of HR^+ETP–derived DCs sustains protection against experimental allergic encephalomyelitis, a mouse model for human multiple sclerosis. These findings, while shedding light on the intricacies underlying ETP lineage commitment, reveal a novel, to our knowledge, function by which IL-4 and IL-13 cytokines condition thymic microenvironment to rheostat T cell selection and fine-tune central tolerance.

2. Introduction

Early thymic progenitors (ETPs), the bone marrow (BM)–derived stem cells that settle in the thymus are pluripotent (1, 2) and give rise to myeloid as well as adaptive and innate lymphoid cells (3–6). Recently, we demonstrated that ETPs expressing the IL-4Rα/IL-13Rα1 heteroreceptor (HR) give rise only to myeloid cells (7). This is because endogenous IL-4 and IL-13 use the HR to activate STAT1 and STAT6 and inhibit the ETPs' T cell potential, leading to commitment to myeloid cells, the majority of which are dendritic cells (DCs) (8, 9). The biological significance of cytokine-driven maturation of HR^+ETPs to DCs remains a puzzle.

The thymus is the site for T cell development, a process that involves positive selection of maturing thymocytes and negative selection of self-reactive T cells (10). Both positive and negative selection of T cells require presentation of self-peptides by APCs (10). It is now clear that cortical thymic epithelial cells (cTEC) are the main APCs involved in positive selection, whereas medullary thymic epithelial cells (mTECs) and DCs are responsible for negative selection of self-reactive lymphocytes (11, 12). It is also known that mTECs sustain optimal expression of self-Ags in the thymus (13, 14), whereas DCs present self-peptides to the target T lymphocytes (15–17). This peptide presentation function has always been attributed to DCs generated from BM stem cells in sites peripheral to the thymus (18). The question that arises in this study is whether cytokines divert ETP maturation toward DCs to yield local APCs that would monitor developing thymocytes for self-reactivity and tighten negative selection and

elimination of potentially harmful autoreactive T cells. To test this postulate, we developed animal models defective for T cell selection and used these tools to interrogate cytokine-induced ETP-derived APCs for restoration of central tolerance. The findings indicate that ETP-derived APCs, although unable to contribute to positive selection of maturing T cells, are effective in carrying out negative selection of self-reactive T cells and lessening the clinical signs of experimental autoimmune encephalomyelitis (EAE) in an autoimmune regulator (aire) gene (13, 14)–dependent fashion. These previously unrecognized observations suggest that IL-4 and IL-13 serve as pillars for control of ETP commitment and fine-tuning of central tolerance.

3. Materials and Methods

Mice

All animal experiments were done according to protocols approved by the University of Missouri Animal Care and Use Committee. C57BL/6, Aire$^{-/-}$ C57BL/6 mice, and OT-1 and OT-II TCR-transgenic C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-13Rα1$^{+/+}$-GFP and IL-13Rα1$^{-/-}$ C57BL/6 mice were previously described (7). CT2Akd/IRES/Ly5.1 mice deficient for MHC class II (MHCII) expression in mTECs were previously described (19). MHCII$^{-/-}$ and MHC I$^{-/-}$/II$^{-/-}$ C57BL/6 mice were purchased from Taconic (Hudson, NY). 2D2 C57BL/6 mice carrying the transgenic TCR specific for myelin oligodendrocyte glycoprotein (MOG) were previously described (20). Only 6- to 8-wk-old mice were used throughout the study.

Ags

MOG peptide (MOGp) corresponding to MOG 35–55 peptide and MOGp tetramer (MOGtet) were previously described (21). Chicken OVA (OVA) aa 257–264 (SIINFEKL) and 323–339 (SQAVHAAHAEINEAGR) (OVAp) peptides were purchased from EZbiolab (Carmel, IN). Ig-OVA is an Ig chimera carrying OVAp (22), and Ig-p79 is carrying p79 peptide (23).

Flow cytometry

Abs were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA) BioLegend (San Diego, CA) or Vector Laboratory (Burlingame, CA) and used according to the manufacturer. Sample reading used a Beckman

Coulter CyAn (Brea, CA) and data were analyzed using FlowJo version 10 (Tree Star). Dead cells were excluded using 7-aminoactinomycin D (7AAD; EMD Biosciences) or Fixable Viability Dye (FVD) eFluor 780 (eBioscience).

Cell sorting

ETPs.

ETPs were isolated as previously described (7). In brief, thymic cells were depleted of Lin^+ thymic cells and the HR+ETPs ($cKit^+CD44^+CD25^-$) were sorted from IL-13Rα$1^{+/+}$-GFP reporter mice on the basis of GFP (IL-13Rα1) expression. HR−ETPs were sorted from Lin− thymic cells of IL-13Rα$1^{-/-}$ mice on the basis of CD44, cKit, and CD25 ($cKit^+CD44^+CD25^-$).

$CD4^+CD8^+$ thymocytes.

Thymi were harvested, and $CD3^+CD4^+CD8^+$ cells were sorted from either 2D2 TCR-transgenic or $MHCII^{-/-}$ C57BL/6 mice to isolate monoclonal and polyclonal double-positive (DP) thymocytes.

Thymic epithelial cells.

Thymic epithelial cells (TECs) were isolated from the thymus as described (24) with a slight modification. Briefly, thymi from 6- to 8-wk-old $MHCII^{+/+}$ C57BL/6 mice were treated with 0.005% (weight/volume) Liberase TH and 100 U/ml DNase I (Roche Diagnostics, Indianapolis, IN) to release epithelial cells from the thymi. cTECs were sorted as $CD45^-EpCAM^+MHCII^+UEA^-Ly51^+$ cells, whereas mTECs were isolated as $CD45^-EpCAM^+MHCII^+UEA^+Ly51^-$ cells.

HR^+ETP-derived $CD11c^+$ cells.

Sorted ETPs were cultured on OP9 stromal cells as previously described (25) with 10 ng of IL-4, and myeloid progeny were sorted on day 7 as $CD45^+CD11c^+$.

Sorting was performed on a Beckman Coulter MoFlo XDP (Brea, CA) cell sorter. Only sorts with a purity of >95% were used in this study.

Intrathymic injections

ETPs, DP thymocytes, cTECs, and mTECs were resuspended in 30 μl of PBS and injected into isoflurane-anesthetized mice through the skin between the third and fourth rib of the thoracic cavity using a 0.3-ml, 31-gauge, 8-mm insulin syringe.

ETP maturation in vivo

$HR^{+/+}$ C57BL/6 mice (CD45.1) were given (intrathymically [i.t.]) HR^+ETPs (5×10^4 cells per mouse) from $HR^{+/+}$ C57BL/6 donors (CD45.2), and thymic cells were harvested on day 12 or 16 posttransfer. The day 12 cells were used to analyze expression of CD11b, CD11c, and CD8α on CD45.2 gated cells, whereas the day 16 cells served to analyze expression of CD11b, CD11c, and CD3 markers.

Thymic-positive selection assay

$HR^{+/+}$ C57BL/6 mice deficient for MHCII ($MHCII^{-/-}$) or MHC class I and MHCII (MHC $I^{-/-}II^{-/-}$) were given (i.t.) HR^+ETPs (15×10^3 cells per mouse), or cTECs (10×10^3 cells per mouse) twice (7 d apart). The hosts were sacrificed at different time points, and their thymic and peripheral blood cells were assessed

for single-positive (SP) CD4 and CD8 T cells. Negative control mice received PBS with no cells (NIL).

Thymic-negative selection assay

Chimeric mice.

C2TAkd mice (CD45.1) were lethally irradiated (900 rad) and given BM cells (10×10^6 cells per mouse) from $MHCII^{-/-}$ mice. After 2 wk of reconstitution, the mice were injected (i.t.) with unselected DP $CD4^+CD8^+$ monoclonal 2D2 TCR-transgenic or polyclonal (CD45.2) $MHCII^{-/-}$ thymocytes. In parallel, the hosts were given HR^+ETPs (12 × 103 cells per mouse) from $MHCII^{+/+}$ mice and negative selection was measured at different time points by assessing the number of SP $CD4^+$ T cells by flow cytometry.

$Aire^{-/-}$ C57BL/6 mice.

The mice were given (i.t.) HR^+ETPs (15×10^3 cells per mouse) from $aire^{+/+}$ C57BL/6 mice twice, 7 d apart. Thymic cells were harvested at different time points, and the number of SP $CD4^+$ or $CD8^+$ T cells were analyzed by flow cytometry. Positive control mice were given (i.t.) mTECs (15×10^3 cell per mouse) from $aire^{+/+}$ C57BL/6 mice, and the number of SP $CD4^+$ and $CD8^+$ T cells in thymus were analyzed on day 6 posttransfer by flow cytometry.

Induction of EAE

Mice were induced for EAE with 60 μg of MOGp, as previously described (21). The mice were scored daily for clinical signs of EAE as follows: 0, no clinical

signs; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness; 5, forelimb paralysis; and 6, moribund or death. The cumulative disease score was calculated by adding the daily scores that the mice received during the monitoring period divided by the number of mice per group. The mean maximal disease score (mmds) represents the average of the highest score received by each mouse during the monitoring period.

Statistical analysis

Data were analyzed using either an unpaired, two-tailed Student t test, one-way ANOVA, or Mann–Whitney U test as indicated. All statistical analyses were performed using Prism software version 4.0c (GraphPad).

4. Results

HR^+ETPs give rise to myeloid cells in vivo, most of which belong to the CD8α$^+$ DC subset

In earlier studies, we have shown that HR^+ETPs cultured in vitro on OP9 stromal cells in the presence of IL-4 or IL-13 cytokines give rise to CD11c$^+$ DCs (9). In this study, we asked whether these HR^+ETP–derived DCs can function as APCs. The results show that DCs derived in vitro from HR+ETP by culture on OP9 stromal cells in the presence of IL-4 function present Ag on MHC class I and MHCII molecules (Fig. 1). Indeed, the DCs were able to present free Kb-restricted SIINFEKL peptide to OT-I CD8 T cells as measured by CFSE dilution (Fig. 1A). Moreover, the DCs are able to cross-present whole-OVA protein and induced CFSE dilution of OT-1 CD8 T cells when the native OVA was loaded into the DCs by osmotic shock in hypertonic but not isotonic media (Fig. 1A). Similarly, the HR^+ETP–derived DCs were able to present class II–restricted free OVAp to OT-II CD4 T cells (Fig. 1B). In addition, when OVAp was delivered to the DCs in the form of Ig-OVA, endocytic presentation was operative and the T cells were able to proliferate and dilute CFSE (Fig. 1B). The control p79 peptide and Ig-p79 did not induce proliferation of OT-II CD4 T cells. In all, DCs derived from HR^+ETPs by stimulation with IL-4 function as APCs in vitro.

The CD11c$^+$ DCs derived from ETPs comprise CD8α$^+$DCs that express IRF-8 but not SIRPα markers (9). These nonmigratory CD8α$^+$SIRPα$^-$ DCs are commonly found in mice sufficient for the HR (9) perhaps because IL-4 is readily available in the thymic environment (26). To ensure that HR^+ETP maturation is

restricted to the myeloid lineage and give rise to DCs in vivo, the cells were sorted from the thymi of CD45.2 IL-13Rα1$^{+/+}$GFP reporter donor C57BL/6 mice, transferred i.t. into CD45.1 IL-13Rα1$^{+/+}$ C57BL/6 hosts and their in vivo maturation to both myeloid and lymphoid lineages was analyzed. The results show that HR^{+}ETPs do not give rise to T cells but rather to myeloid CD11c^{+}cells that would include both CD11c^{+}CD11b^{+} and CD11c^{+}CD11b^{-} cells (Fig. 1C). Data compiled from several experiments show that the findings are statistically significant despite that the incubation period was extended to 16 d, an optimal time point for CD3 expression (27). Myeloid cells are diverse in nature and can be broadly classified into CD11b^{+} monocyte/macrophage cells or CD11c^{+} DCs. Because conventional DCs are specialized APCs and contribute to thymic T cell selection (10) we determined the cellular make-up of the in vivo ETP-derived myeloid population. The results show that 17.7 ± 1.2% of the CD45.2 cells expressed only CD11b, a profile for monocyte/macrophage cells (Fig. 1D). However, 61.9 ± 2.7% of the cells had CD11c but not CD11b phenotype, which represents a conventional DC phenotype (Fig. 1D). Interestingly, most of the DCs (78.7 ± 2.7) expressed the CD8α DC subset–specific marker. In terms of cell number, data collected from several experiments indicated that 50 × 10^{3} HR+ETPs gave rise to 400 × 10^{3} myeloid cells on average. Of these myeloid cells, 70.8 × 10^{3} (17.7%) were CD11b^{+} CD11c^{-} cells and 247.6 × 10^{3} (61.9%) were CD11b^{-} CD11c^{+} DCs. Of these CD11b^{-}CD11c^{+} cells, 193 × 10^{3} (78%) were CD8α^{+} DCs. Overall, HR+ETPs give rise to myeloid but not T cells in vivo and a significant number of these cells are CD8α^{+}CD11c^{+} DCs.

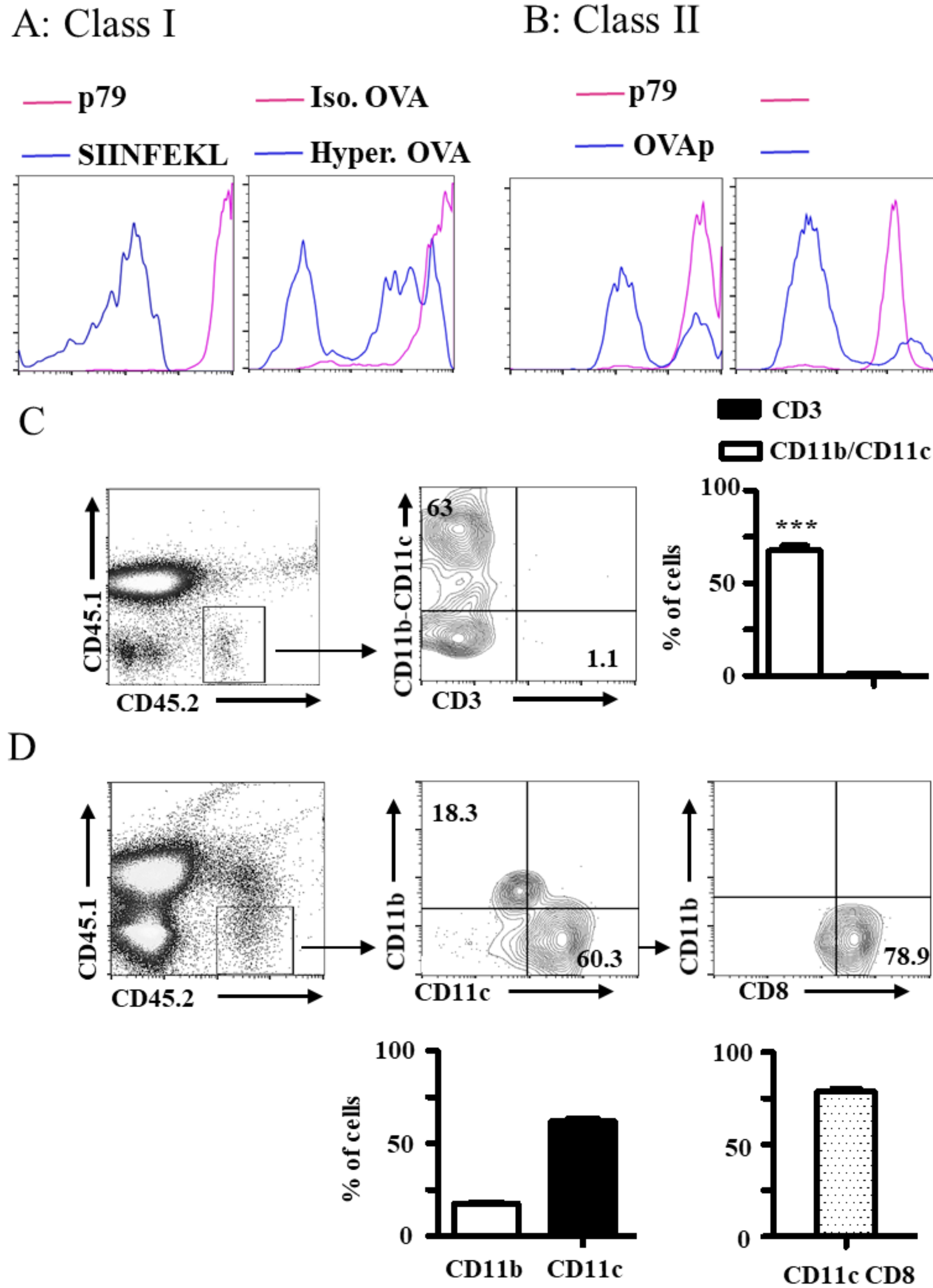

Figure 1. HR+ early thymic progenitors generate APCs which are predominately thymic resident dendritic cells

(A, B) Shows dilution of CFSE staining by either CD8 OT-I (A) and CD4 OT-II (B) T cells (1 x 10^5 cell/ well) upon stimulation with *in vitro* cultured IL-4-guided ETP-derived DCs that were pre-loaded with Ag. (A) For Class I classical presentation the DCs (5 x 10^3 cells/well) were loaded with free SIINFEKL (1μM) or control p79 (10 μM) peptide (left panel) and for class I cross-presentation, the DCs were loaded with soluble OVA (70 μM) after osmotic shock in hypertonic (Hyper.OVA) or isotonic (Iso.OVA) media (right panel) as described (40). (B) For class II classical presentation, the DCs were loaded with 10 μM free OVAp or negative control p79 peptide (left panel) and for endocytic presentation the DCs were loaded with 1 μM Ig-OVA or negative control Ig-p79 (right panel). This is representative of 3 experiments. (C) Thymic cells from CD45.2 IL-13Rα1-GFP reporter mice were depleted of Lin+ cells and the Lin CD4-CD8- cells were stained with antibodies to CD25, CD44, and c-Kit. The $CD25^-CD44^+c\text{-}Kit^+GFP^+$ (HR^+ETPs) were sorted and injected i.t. (50 x 10^3 cells/mouse) into congenic (CD45.1) hosts. The thymic cells were stained with anti-CD45.1, CD45.2, CD11b, CD11c, and CD3ε antibodies and evaluated for CD11b, CD11c, and CD3 expression by $CD45.2^+$ cells on day 16 after transfer. The contour plots show a representative experiment while the bar graph shows data compiled from 3 independent experiments. *** $p< 0.001$ as determined by a two-tailed unpaired Student t-test. (D) CD45.2 HR^+ETPs were injected i.t. (50 x 10^3 cells/ mouse) into CD45.1 hosts and the thymic cells harvested on day 12 post-transfer were stained with anti-CD45.1, CD45.2, CD11b, CD11c, and CD8α antibodies. The $CD45.2^+$ cells were evaluated for CD11b, CD11c, and CD8α expression. The

contour plots show a representative experiment while the bar graphs show the mean ± SD of cell percentage compiled from 3 independent experiments.

B. HR^+ETP–derived APCs contribute to thymic T cell selection

Because BM-derived DCs migrate to the thymus and participate in thymic selection of T cells (18, 28, 29), one would envision that HR^+ETPs give rise to $CD8\alpha^+SIRP\alpha^-$ thymic resident DCs to assist in T cell development. To test this premise, experimental models were set up to assess the contribution of HR^+ETP–derived APCs to positive, as well as negative, T cell selection. Accordingly, $MHCII^{-/-}$ mice, in which CD4 T cell selection is not operative, were given i.t. HR^+ETPs and analyzed for development of $CD4^+$ SP T cells in the thymus and the periphery. The result shows that the frequency of $CD4^+$ T cells did not increase in either the blood or thymus 15 d postintrathymic injection, as the numbers of cells were similar to mice that did not receive HR^+ETPs (Fig. 2A). In contrast, $MHCII^{-/-}$ mice recipient of $MHCII^+$ cTECs that support positive T cell selection (10) showed an increase in the percentage of CD4+ SP T cells in both the thymus and in the periphery (Fig. 2B, upper panels). Data compiled from several experiments show that the increases in $CD4^+$ SP T cells were statistically significant (Fig. 2B, lower panels). Furthermore, when $MHCI^{-/-}/II^{-/-}$ mice were given HR^+ETPs and tested for positive selection of both CD4 and CD8 T cells, there was no significant increase of either T cell type in the thymus (Fig. 3A). Again, mice recipient of $MHCI^+II^+$ cTECs had significantly increased percentages of both CD4 and CD8 T cells in the blood and thymus (Fig. 3B, 3C). Data compiled from several experiments show that the increases in $CD4^+$ and $CD8^+$ SP T cells were statistically significant (lower panels in Fig. 3B, 3C). Overall,

these findings indicate that HR^+ETP–derived APCs are unable to support thymic-positive selection of T cells.

The fact that HR^+ETPs do not contribute to positive selection of T cells does not necessarily preclude contribution to negative T cell selection. To test this premise, we set up a chimeric mouse model suitable for this investigation as illustrated in Fig. 4A. Accordingly, C2TAkd mice (19), which lack MHCII on mTECs but not cTECs, were lethally irradiated and reconstituted with BM from $MHCII^{-/-}$ mice yielding a chimeric host devoid of MHCII expression in APCs and mTECs but not cTECs. The thymic configuration in this host supports positive but not negative selection. This chimera was then given HR^+ETPs from $MHCII^{+/+}$ mice to provide thymic resident MHCII-sufficient APCs that could serve in negative selection. The hosts were then given unselected $CD4^+CD8^+$ DP 2D2 TCRMOGp monoclonal (20) or CD45.2 C57BL/6 polyclonal thymocytes to serve as targets for negative selection. After 7–21 d the thymi were analyzed for $CD4^+CD8^-$ SP T cells as a measure of thymic-negative T cell selection. The results show that both monoclonal ($MOGtet^+$) and polyclonal ($CD3^+$) $CD4^+$ SP T cells were significantly lower in number in mice recipient of DP thymocytes and HR^+ETPs relative to those given DP thymocytes alone (Fig. 4B, 4C). Furthermore, when the polyclonal CD45.2 $CD4^+$ SP T cells were stained with Nur77 Ab and FVD, there was a gradual increase over time in the number (Fig. 4D) and percentage (Fig. 4E) of cells binding FVD, a marker for apoptosis, and expressing Nur77, a marker for negatively selected thymocytes (30), in the mice recipient of DP thymocytes and HR^+ETPs relative to those given the DP

thymocytes alone. These results indicate that HR^+ETP–derived APCs support negative selection of CD4 T cells. HR^+ETP–derived APCs were not tested for negative selection of CD8 T cells because a similar animal model could not be devised because of the lack of mice with MHC class I deficiency in mTECs.

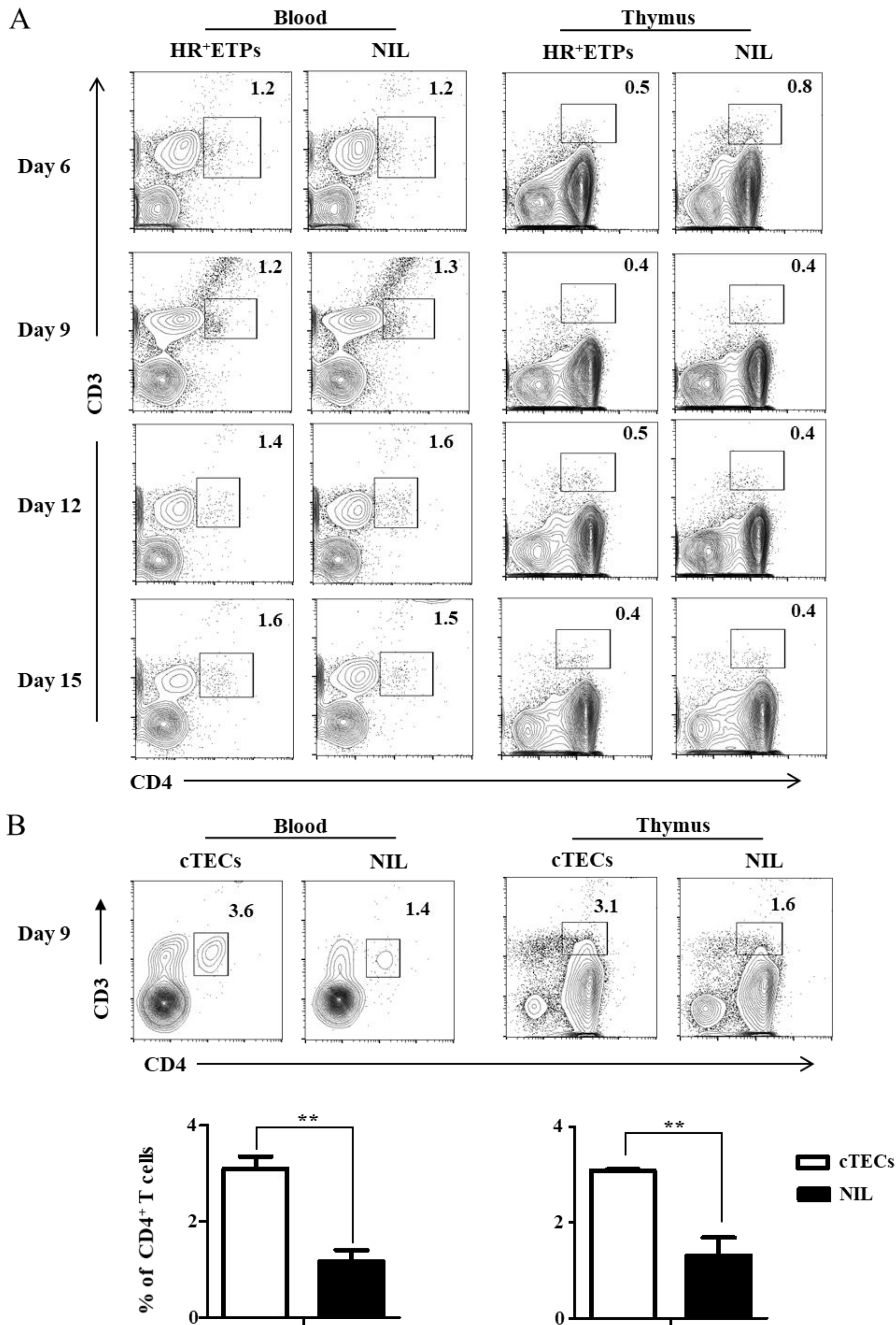

Figure 2. HR+ETPs do not support thymic positive selection of CD4 T cells.

(A, B) MHC Class $II^{-/-}$ C57BL/6 mice were given two i.t. injections (7 days apart) of (A) HR+ETPs (12 x 10^3 cells/mouse) or (B) cTECs (10 x 10^3 cells/mouse) from $MHCII^{+/+}$ C57BL/6 mice and the percentage of $CD3^+CD4^+$ T cells were determined in both the thymus and blood at the indicated time points. A group of mice that received PBS with no cells (NIL) was included in each experiment to serve as negative control. The contour plots show representative experiments for the indicated days. The bar graphs in (B) show the mean cell percentage ± SD compiled from 2 independent experiments. Each experiment included 5 mice per group that were tested individually. **$p<0.01$ as determined by two-tailed, unpaired Student's t-test.

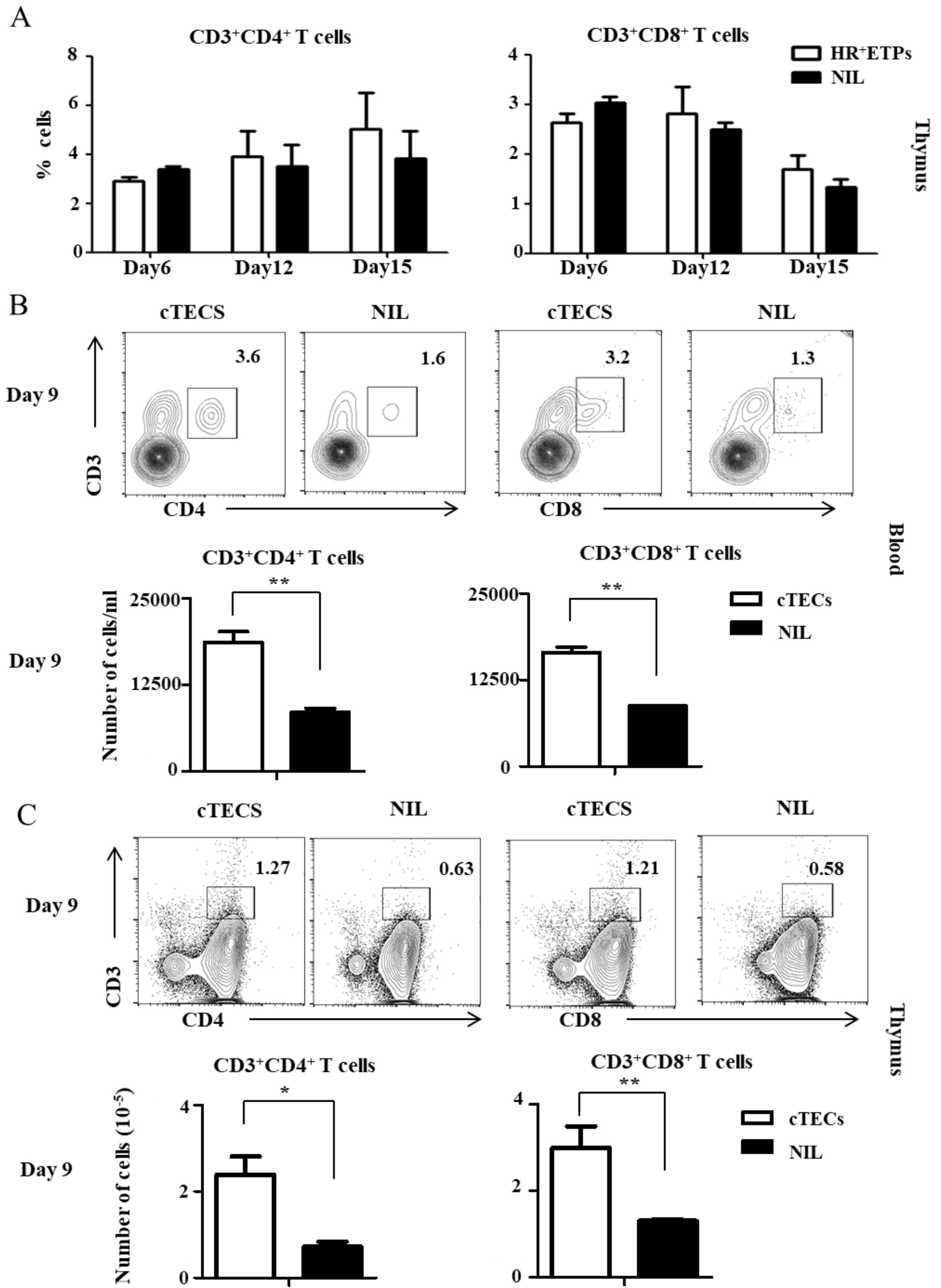

Figure 3. HR⁺ETPs do not support thymic positive selection of CD8 T cells.

(A) $MHCI^{-/-}$ $II^{-/-}$ C57BL/6 mice were given 2 i.t. injections (7 days apart) of HR+ETPs (12 x 10^3 cells/mouse) from $MHCI^{+/+}II^{+/+}$ C57BL/6 GFP reporter mice. A group of mice that received no cell transfer (NIL) was included in each experiment to serve as negative control. The bar graphs show the percentages of $CD3^+$ $CD4^+$ (left panel) and $CD3^+CD8^+$ (right panel) T cells in the thymus. In (B and C) the $MHCI^{-/-}$ $II^{-/-}$ C57BL/6 mice were given i.t. cTECs (10 x 10^3 cells/mouse) from $MHCI^{+/+}II^{+/+}$ C56BL/6 mice instead of HR+ETPs or PBS with no cell transfer (NIL) and the frequency of single positive T cells was measured on day 9 post transfer in the blood (B) and thymus (C). The contour plots are representative experiments showing the percentage of $CD3^+CD4^+$ and $CD3^+$ $CD8^+$ T cells while the bar graphs show the mean cell number ± SD compiled from 3 independent experiments. Each experiment included 3 mice that were tested individually. *$p<0.05$ and **$p<0.01$ as determined by two-tailed, unpaired Student's t-test.

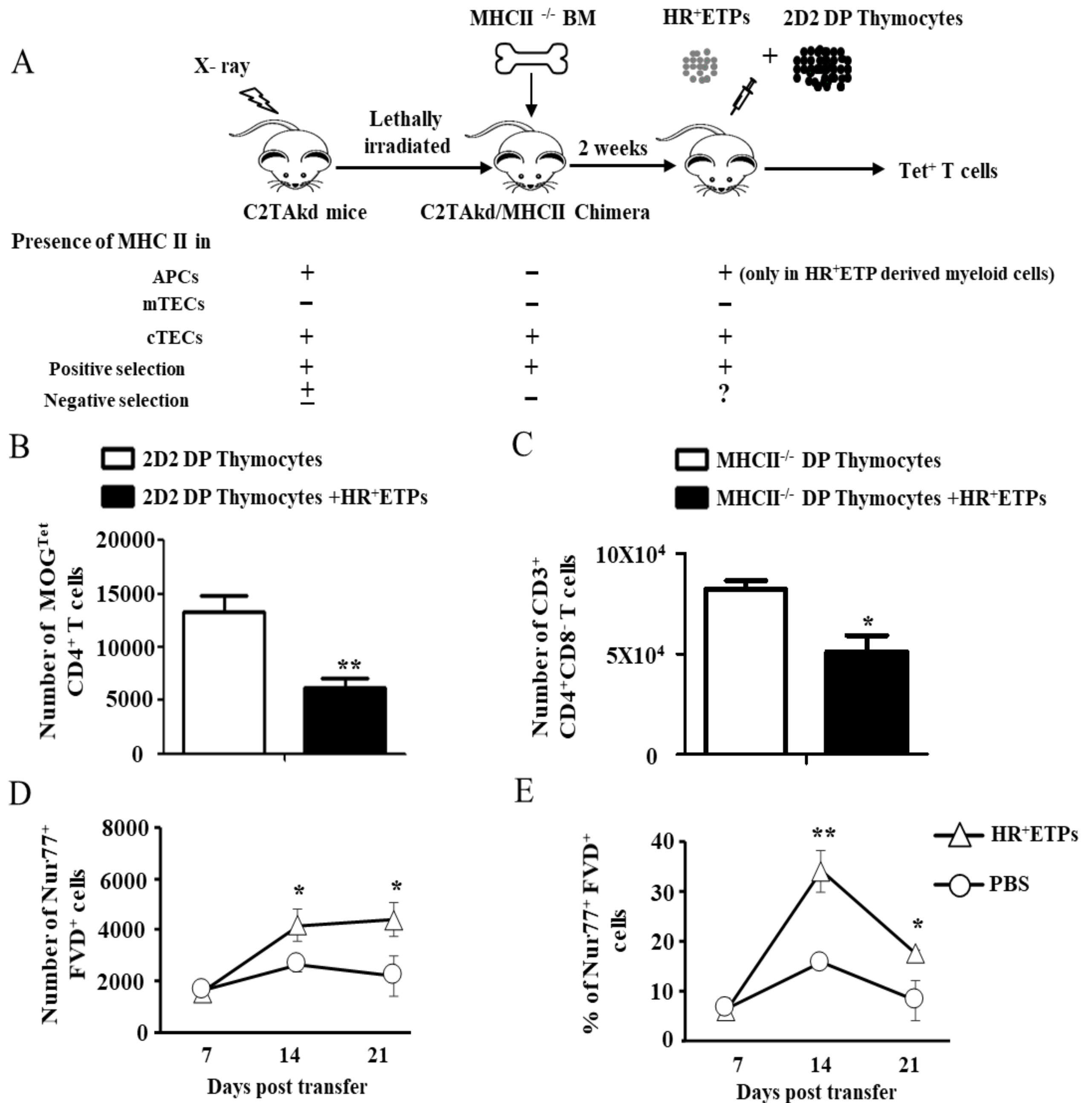

FIGURE 4. HR+ETP–derived thymic APCs support thymic-negative T cell selection.

(A) C2TAkd mice that specifically lack MHCII expression in mTECs but not cTECs, which can partially drive negative T cell selection, were lethally irradiated (990 rad) and given BM cells (10 x 10^6 cells) from $MHCII^{-/-}$ mice. Positive but not negative thymic T cell selection is functional in these chimeric mice. The chimeras were then given i.t. unselected DP monoclonal (B) or polyclonal (CD45.2) thymocytes from 2D2 TCR-MOG–transgenic and normal C57BL/6 mice, respectively (C–E), and HR^+ ETPs (from $MHCII^{+/+}$ mice) and used to measure thymic-negative T cell selection. (B and C) Thymi were harvested on day 21 and used to assess for negative T cell selection. (B) The bars represent the mean ± SD of the absolute number of MOG Tet^+ CD4 SP 2D2 T cells. Data are representative of two independent experiments in which seven mice were tested individually. (C) shows the absolute number of $CD45.2^+$ $CD3^+$ polyclonal CD4 SP T cells. (D) and (E) show the number (D) and percentages (E) of $CD45.2^+$ $CD3^+$ polyclonal CD4 SP FVD^+ $Nur77^+$ T cells undergoing negative selection. *$p<0.05$, **$p<0.01$ as determined by two-tailed, unpaired Student t test.

C. HR^+ETP–derived APCs support protection against EAE

HR^+ETP–derived APCs contribute negative selection of self-reactive T cells and reduce the frequency of myelin-specific T lymphocytes in the periphery. This process likely impacts autoimmunity and impedes the development of EAE. To test this premise, $HR^{-/-}$ mice in which the frequency of thymic resident $CD8\alpha^+SIRP\alpha^-$ DCs is diminished (9), and negative T cell selection would be less effective, were induced for EAE with MOGp and their daily disease severity scores were compared with $HR^{+/+}$ mice. Indeed, the results show that $HR^{-/-}$ mice begin to develop signs of clinical EAE on day 5 post-disease induction, whereas $HR^{+/+}$ mice had the initial disease scores on day 9 (Fig. 5A, left panel). Also, the pattern of paralysis was more severe in $HR^{-/-}$ relative to $HR^{+/+}$ mice (Fig. 5A, right panel). Furthermore, the mmds was 4.0 ± 0.0 in $HR^{-/-}$ mice compared with 2.8 ± 0.4 in $HR^{+/+}$ animals. The cumulative disease score was 43.8 ± 2.7 in $HR^{-/-}$ mice which is much higher than the 24.4 ± 1.8 in $HR^{+/+}$ mice. These observations suggest that the increased frequency of ETP-derived DCs plays a critical role in the development of EAE. This statement is supported by data showing that $HR^{-/-}$ recipients of HR^+ETPs develop milder EAE relative to $HR^{-/-}$ mice that did not receive HR^+ETPs prior to disease induction (Fig. 5B). Indeed, although the onset of EAE was similar in both experimental groups, the pattern of paralysis is milder in the mice recipient of HR^+ETPs. In fact, the mmds decreased from 4.0 ± 0.0 in $HR^{-/-}$ mice with no ETP transfer to 3.0 ± 0.0 in $HR^{-/-}$ mice recipient of HR^+ETPs. In addition, the cumulative disease score, which was 45.0 ± 2.4 in $HR^{-/-}$ mice, decreased to 30.3 ± 1.2 in the mice recipient of HR^+ETPs. In all, the data

indicates that HR^{+}ETP–derived APCs support thymic-negative selection of self-reactive T cells leading to reduced susceptibility to EAE induction.

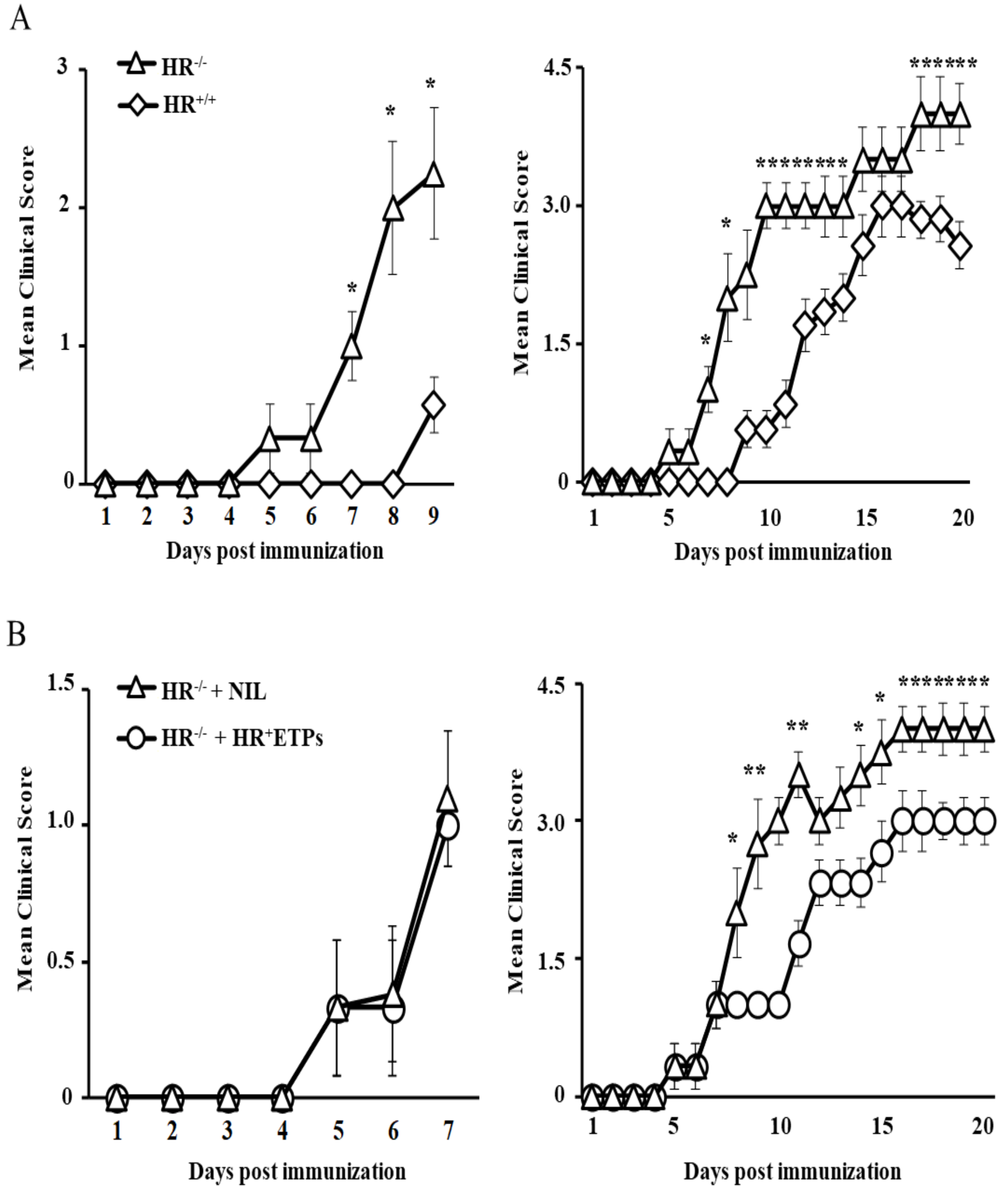

Figure 5. HR⁺ETP–derived APCs support protection against EAE.

(A) $HR^{+/+}$ and $HR^{-/-}$ C57BL/6 mice (six to eight per group) were induced for EAE with MOGp and monitored daily for disease severity for 20 d. The graphs show the mean ± SD clinical scores during disease onset (left panel) and progression (right panel). (B) $HR^{-/-}$ mice (six to eight per group) were given i.t. HR^{+} ETPs (15,000 cells per mouse) or saline with no cells (NIL) twice (7 d apart) and 2 wk later were induced for EAE with MOGp. The hosts were monitored daily for disease severity for 20 d. The graphs show the mean 6 SD clinical scores during disease onset (left panel) and progression (right panel). *$p<0.05$, **$p<0.01$ as determined by Mann–Whitney U test.

D. *Aire* gene function in mTECs is required for cooperation with HR^+ETP–derived APCs and protection against EAE

Thymic-negative T cell selection relies on the expression of the aire gene by mTECs (13, 14) and their cooperation with thymic APCs (10). Thus, we sought to determine whether HR^+ETP–derived APCs would require the function of aire gene to contribute negative T cell selection and protect against EAE. To this end, $aire^{-/-}HR^{+/+}$ hosts were given HR^+ETPs or mTECs from $aire^{+/+}$ mice and then tested for negative selection of T cells and resistance to EAE induction. The results show that the hosts, in which both endogenous mTEC and HR^+ETPs lack aire expression, had no significant decrease in numbers of either CD4 or CD8 SP T cells when given $aire^+HR^+ETPs$ or PBS (NIL) (Fig. 6A). In contrast, hosts recipient of $aire^+mTECs$ showed significant reduction in both frequency and numbers of CD4 or CD8 SP T cells relative to NIL hosts (Fig. 6B, 6C), indicating cooperation between endogenous $aire^-HR^+ETP$–derived APCs and exogenous $aire^+mTECs$. These findings parallel with data presented in Fig. 4 showing cooperation between HR^+ETP–derived APCs and $MHCII^-aire^+mTECs$ of the C2TAkd hosts. In addition, given that APCs derived from HR^+ETPs, whether in vitro or in vivo, have little aire mRNA relative to mTECs (Fig. 6D), these results suggest that negative selection of T cells mediated by HR+ETP–derived APCs require aire expression in mTECS. This statement is supported by findings showing that HR+ETPs are able to lessen the clinical signs of EAE in $HR^{-/-}$ mice sufficient for aire (Fig. 6E). Indeed, the clinical signs EAE in $HR^{-/-}Aire^{-/-}$ mice were similar whether the hosts are given HR^+ETP or PBS (NIL) control (Fig. 6E,

left panel). However, the severity of EAE was significantly lower in $HR^{-/-}Aire^{+/+}$ hosts given HR^{+}ETP in comparison with $HR^{-/-}Aire^{+/+}$ mice given PBS (NIL) (Fig. 6E, right panel). Given that the host mice are deficient for the HR and do not have endogenous HR^{+}ETPs, the differential patterns of EAE is because of cooperation between aire and the transferred HR^{+}ETPs. In all, HR^{+}ETP–derived APCs require the function of aire gene to contribute central tolerance and impact the development of autoimmunity.

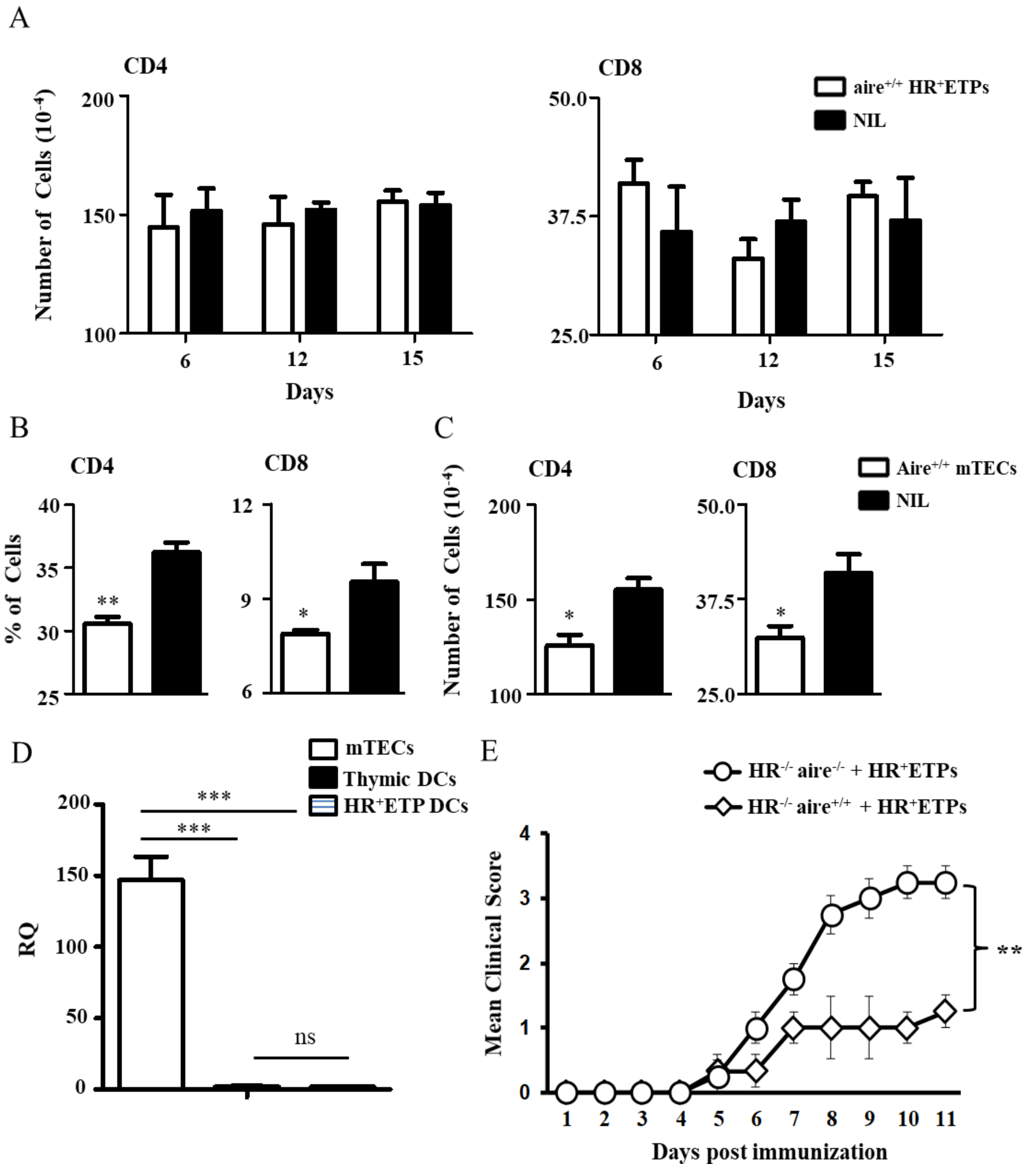

Figure 6. HR+ ETP-derived APCs rely on *aire* gene expression to support protection against EAE

(A) Aire$^{-/-}$ C57BL/6 hosts were given (i.t.) PBS (NIL) or HR^{+}ETPs (15 x 10^{3} cells/mouse) twice (7 days apart) and thymic SP CD4 and CD8 T cells were analysed at the indicated day post transfer. The bars show the mean absolute number of cells ± SD compiled from 3 independent experiments. Each experiment included 5 mice per group that were tested individually. (B and C) Aire$^{-/-}$ C57BL/6 hosts were given (i.t.) PBS (NIL) or aire-sufficient mTECs (15 x 10^{3} cells/mouse) once and thymic SP CD4 and CD8 T cells were analysed on day 6 post transfer. The bars show the mean percentage (B) and absolute number (**C**) of cells ± SD compiled from 3 independent experiments. *$p<0.05$ and **$p<0.01$ as determined by two-tailed unpaired Student t-test. (D) mTECs, thymic CD11c^{+}, and HR^{+}ETP derived CD11c^{+} cells were sorted and assessed for *aire* gene expression by RT-PCR. ***$p<0.001$ as determined by one-way ANOVA. (E) HR$^{-/-}$ Aire$^{-/-}$ and HR$^{-/-}$ *Aire*$^{+/+}$ C57BL/6 mice were given (i.t.) HR^{+}ETPs (15,000 cells/mouse) twice (7 days apart) and two weeks later induced for EAE with MOGp. The mice were monitored daily for disease severity for 11 days. The graphs show the mean ± SD clinical scores. **$p<0.01$ as determined by Mann-Whitney U test.

5. Discussion

To our knowledge, this study reports new insights on the function of IL-4 and IL-13 in ETP maturation and its significance to central T cell tolerance and the development of CNS autoimmunity. IL-4/IL-13 were primarily identified as major players in allergic reactions and parasite immunity (31). The cytokines can also play anti-inflammatory functions against autoimmunity by promoting peripheral tolerance (32, 33). Recently we reported that IL-4/IL-13 signaling through the HR guides ETP maturation toward myeloid cells, the majority of which belong to the DC population (9). This previously unappreciated function positions the cytokines as factors that assist ETP commitment to a specific lineage and reinforce the contribution of the thymic microenvironment to ETP fate decision (34, 35).

The other intriguing aspect in the process of cytokine guided ETP maturation relates to the biological significance associated with the shifting of ETP maturation toward myeloid cells, specifically DCs. It is well known that DCs are professional APCs specialized in induction of immunity as well as peripheral tolerance (36). Lately, it has been reported that a specific population of BM-derived DCs, namely the $CD8\alpha^{+}CD11c^{+}$ subset is specialized in thymic selection of T cells (18, 28). Interestingly, IL-4/IL-13 driven signaling supports HR^{+}ETPs to give rise to DCs that are able to function as APCs and present Ag via MHC class I and MHCII classical pathways. The ETP-derived DCs are also able to cross-present Ag to CD8 T cells, a functional attribute of $CD11c^{+}CD8\alpha^{+}$ DCs that contribute to thymic-negative T cell selection. These observations prompted us to test whether the APC function of HR^{+}ETP–derived DCs contribute to thymic T

cell selection (10). The findings indicate that HR^+ETP–derived APCs, although unable to support positive selection of T cells because this is a defined function for cTECs (10), were able to negatively select self-reactive T cells including 2D2 TCR-transgenic myelin-specific T lymphocytes. In fact, HR^+ETP–derived APCs were able to lessen severity of EAE as the mice had milder clinical signs of disease when compared with animals in which negative selection is not operative. In addition, both negative selection of T cells and lessening of EAE by HR^+-derived APCs were dependent on the function of the aire gene (14), suggesting that the DCs present self-peptide generated from mTECs as was previously defined (13). Although it has been reported that EAE severity is slightly reduced in young $aire^{-/-}$ mice compared with their wild-type counterparts (37), this was not the case in young $aire^{-/-}$ $HR^{-/-}$ mice, and the disease pattern was similar to young $aire^{+/+}$ $HR^{-/-}$ mice, further supporting our prior observation that HR deficiency increases susceptibility to EAE (32). These observations point to new attributes for IL-4 and IL-13 whereby signaling through the HR guides ETPs to give rise to APCs that tighten negative selection of self-reactive T cells and reduce susceptibility to autoimmunity. The significance of these findings is 2-fold. On one hand, stimulators of IL-4 and IL-13 secretion would be able to influence ETP maturation and impact central tolerance as well as autoimmunity. On the other hand, the environment would also control this process under circumstances that could favor or deter the development of autoimmunity. For instance, parasitic infections and allergens that stimulate type II cytokines would support the generation of ETP-derived APCs, which foster negative T cell

selection and limit the generation of self-reactive T cells yielding a lymphocyte repertoire devoid of self-reactivity and thus, beneficial against autoimmunity. From this perspective, a clean environment would foster susceptibility to autoimmunity (38, 39). In all, these observations add insight as to the environmental factors that would control the development of autoimmune diseases and assert the hygiene hypothesis (40).

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CHAPTER III: Diminished IL-4 Fitness Handicaps T Cell Selection in NOD mice

1. Abstract

From an autoimmunity perspective, type II cytokines, such as IL-4 and IL-13, are anti-inflammatory because of their ability to foster peripheral tolerance. The full impact of IL-4 and IL-13 is still yet to be explored in central tolerance. Recently it has been shown that IL-4/IL-13 signaling in ETPs that express the IL-4Rα/IL-13Rα1 heteroreceptor (HR) primes these progenitors to become thymic resident DCs. These DCs aid in negative selection and the prevention of autoimmunity. In chapter III, the data presented shows that, in contrast to the C57BL/6 strain, HR^+ETPs from the type one diabetes (T1D) susceptible NOD strain give rise to T cells. Notably, the NOD thymus shows a dramatic reduction in the steady-state level of IL-4, likely due to diminished iNKT cell populations. Indeed, increasing the availability of either cytokine can rescue the lineage fate of HR^+ETPs. Restoration of NOD HR^+ETP fate improves negative selection, alters the TCRβ repertoire, and prevents diabetes onset. Thus, IL-4 fitness within the thymus may be implicated in fine-tuning negative selection of T cells. From this, one may envision that the lack of IL-4/IL-13-inducing infections may tip that table in favor of autoimmunity in developed nations.

2. Introduction

Bone marrow (BM)-derived multipotent progenitors travel through the blood to the thymus where they proliferate at extraordinary rates. Upon reaching the thymus, these multipotent cells are referred to as early thymic progenitors (ETPs) and give rise to multiple cell fates. Recently, it has been shown that ETPs which express the IL-4Rα/IL-13Rα1 heteroreceptor (HR) yield exclusively myeloid cells (1, 2). IL-4 and IL-13 cytokines signal through the HR to induce STAT signaling, limiting the T cell lineage while simultaneously inducing the myeloid fate. HR^{+}ETPs, manipulated by cytokine exposure, give rise to DCs that can perform negative T cell selection (3). These ETP-derived DCs can tighten negative selection to such an extent that the severity of experimental allergic encephalomyelitis (EAE) is greatly dampened in animal recipients of HR^{+}ETPs. However, this phenomenon's biological significance is yet to be determined in other autoimmune diseases, such as type one diabetes (T1D).

The non-obese diabetic (NOD) mouse is the model of choice for studying the pathogenesis of T1D. Many defects in peripheral tolerance have been heavily investigated utilizing the NOD mouse. Type II cytokines, such as IL-4 and IL-13, are known modulators of peripheral tolerance in the NOD model. These cytokines reduce the effect of pro-inflammatory mediators and induce other anti-inflammatory cytokines, such as IL-10 (4, 5). However, the current understanding of NOD-specific defects in central tolerance likely reflects only the tip of the iceberg. While IL-4 and IL-13 are found in the thymus, their role in T cell selection in the NOD mouse is unknown. Herein, we show that there is a reduced

fitness of IL-4 in the NOD thymic microenvironment. As a consequence, NOD HR^{+}ETPs do not give rise to DCs but rather to T cells. The question that then arises in this study is whether a lack of thymic DCs results in a defective T cell selection process. The findings indicate that thymic enrichment with IL-4 induces HR^{+}ETP-derived DCs and blocks the T cell lineage fate. These HR^{+}ETP-derived DCs can perform negative selection and shift the TCR repertoire in IL-4-enriched NOD mice. This tightening of negative T cell selection results in the prevention of T1D in mice recipients of thymic IL-4. These previously unrecognized findings suggest that type II, or environmental cytokines, may sustain central tolerance and, as such, a deficit in their fitness may predispose the host to the development of an autoimmune disease.

3. Materials and Methods

Mice

All animal experiments were done according to protocols approved by the University of Missouri Animal Care and Use Committee. C57BL/6, NOD (H-2^{g7}), NOD BDC2.5, and NOD MHC $I^{-/-}II^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-13Rα$1^{+/+}$-GFP C57BL/6 were previously described (1). The IL-13Rα$1^{+/+}$-GFP NOD mice were generated by breeding IL-13Rα$1^{+/+}$-GFP C57BL/6 mice onto the NOD background via speed congenic technology based on 58 microsatellite markers on sequences between the C57BL/6 donor strain and NOD recipient strain. A total of eight backcrosses with wild-type NOD mice were performed to ensure homozygosity of NOD alleles. Only 6-8 week old female mice were used throughout the study unless otherwise noted. All animals were maintained under specific pathogen-free conditions in individually ventilated cages and kept on a 12 h light-dark cycle with access to food and water ad libitum.

Flow Cytometry

Antibodies and Tetramers. Anti-IL-4 (11B11), anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD25 (7D4), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD117 (2B8) were purchased from BD Biosciences (San Jose, CA). Anti-CD45.2 (104) was purchased from eBioscience (San Diego, CA). Anti-ICOS (C398.4A), anti-TCRβ

(H57-597), anti-CD122 (TMβ1) were purchased from Biolegend (San Diego, CA). PBS57-CD1d1 and diabetes-associated tetramers were obtained from the National Institutes of Health Tetramer Core Facility.

Lineage (Lin) depletion antibodies. Depleting antibodies were purchased from Miltenyi Biotech (San Diego, CA) as a kit that includes antibodies against CD8α (Ly-2), CD11b (Mac-1), CD11c, CD19, B220(CD45R), CD49b(DX5), CD105, $MHCII^+$, Ter-119^+, and TCRɣ/δ. Anti-CD4 microbead antibody (L3T4) was also used in the lineage depletion experiments.

Fluorochromes. Antibodies were directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy5, PE-Cy5.5, peridinin-chlorophyll-protein complex (PerCP)-Cy5.5, PE-Cy7, allophycocyanin, allophycocyanin-Cy7 (or allophycocyanin eFluor780), or biotin. Biotinylated antibodies were revealed with Streptavidin PE or allophycocyanin.

Sample reading. This used a Beckman Coulter CyAn (Brea, CA) and data were analyzed using FlowJo version 10 (Tree Star). Dead cells were excluded using 7-aminoactinomycin D (7AAD; EMD Biosciences).

Cell sorting

ETPs. ETPs were isolated as previously described (1). In brief, thymi were harvested from either IL-13Rα$1^{+/+}$-GFP NOD or C57BL/6 mice after perfusion with PBS, and the $CD4^+$ cells were eliminated by MACS using anti-CD4 microbeads. The ETPs were then isolated after depletion of Lin^+ ($CD8α^+$, $CD11b^+$, $CD11c^{+,}$

$CD19^+$, $B220^+$, $CD49b^{+,}$ $CD105^+$, $MHCII^+$, Ter-119^+, TCRɣ/$δ^+$) thymic cells. HR^+ETPs ($cKit^+CD44^+CD25^-GFP^+$) were sorted from Lin^- thymic cells of IL-13Rα$1^{+/+}$-GFP reporter mice on the basis of GFP (IL-13Rα1) expression. HR^+ ETPs represent the GFP^+ cells, and HR^- ETPs represent the GFP^- cells of the lin^- $cKit^+CD44^+CD25^-$ thymic cells.

αβ T cells. Cells were isolated from either the SP or PLN. Cells were isolated after perfusion with PBS. Cells were then stained with anti-TCRβ chain, sorted, and stored in RNAprotect Cell Reagent (Qiagen).

Sorting was performed on a Beckman Coulter MoFlo XDP (Brea, CA) cell sorter. Cell purity was routinely checked, and only sorts with a purity of >95% were used in this study.

Intrathymic Injections

ETPs or cytokine was diluted in 30µl PBS and injected into isoflurane-anesthetized mice through the skin between the 3rd and 4th rib of the thoracic cavity using a 0.3-ml, 31-gauge, 8-mm insulin syringe. Control mice received PBS injection alone. Intrathymic cytokine treated mice and PBS controls received injections once a week for two weeks between 4-6 weeks of age.

ELISA

IL-4 and IL-13 production was measured using anti-cytokine Abs and following standard BD Biosciences (San Jose, CA) protocol. The OD450 was read on a SpectraMax 190 counter (Molecular Devices, Sunnyvale, CA) and analyzed using

Softmax Pro 3.1.1 software. Cytokine concentrations were then extrapolated from the linear portion of a standard curve generated by graded amounts of the respective recombinant cytokine.

Sequencing of the αβ T cell repertoire

2 x 10^5 sorted TCRβ$^+$ cells per sample were stored RNAprotect Cell Reagent (Qiagen) and shipped overnight to iRepertoire (iRepertiore, Inc). RNA isolation was performed using an RNeasy Mini Kit (Qiagen). Multiplexed cDNA Libraries were created by iRepertoire by first using a set of nested primers for different variable and constant portions of the TCRβ chain and followed by a second amplification using communal primers according to the manufacturer's protocol. Sequencing was done on the Illumina Miseq system (250 PER). Samples included pooled (4 mice per group, 4 groups) SP and PLN T cells from both treated and control mice were from both "healthy" (9 weeks) and "pathogenic" (12 weeks) repertoires for a total of 8 cDNA libraries. The CDR3 of the TCRβ chain was sequenced at a read depth of 1 million per library.

RT-PCR

Bulk thymocytes were isolated by perfusion with PBS and ETPs were sorted as described above. These cells were then used to isolate RNA by Trizol extraction and isopropanol precipitation. RT-PCR was performed on a StepOnePlus Instrument cycler using Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was done with primers specific for:

GAPDH

Sense: 5’-AACTTTGGCATTGTGGAAGG-3’

Antisense: 5’-GGATGCAGGGATGATGTTCT-3’

C/EBPα

Sense:5′-AGCAACGAGTACCGGGTACG-3′

Antisense: 5′-GTTTGGCTTTATCTCGGCTC-3′

Notch1

Sense:5′-GGACATGCAGAACAACAAGG-3

Antisense: 5′-CAGTCTCATAGCTGCCCTCA-3′

IL-7Rα

Sense:5′-AGTCCGATCCATTCCCCATAA-3

Antisense: 5′-ATTCTTGGGTTCTGGAGTTTCG-3′

IL-4

Sense: 5’-GGAGATGGATGTGCCAAACG-3’

Antisense: 5’-GCACCTTGGAAGCCCTAC-3’

IL-13

Sense: 5’-GTGTCTCTCCCTCTGACCCT-3’

Antisense: 5'-GGGGAGTCTGGTCTTGTGTG-3'

Relative transcript abundance was determined by using the comparative threshold cycle method using the StepOne software (Applied Biosystems) normalization with GAPDH. All samples were run in triplicate.

OP9 and OP9-DL1 cell culture

OP9 and OP9-DL1 cultures were used as previously described (2), with slight modifications. Briefly, OP9 and OP9-DL1 stromal cells were plated 2d before initiation of cultures at a concentration of 20,000 cells/ml in 24-well plates. Progenitors were added at 3,000 per well. IL-7 was used at a final concentration of 1 ng/ml, Flt3 ligand (Flt3L) was used at 5 ng/ml, GM-CSF, and IL-4 were used at 10 ng/ml, and IL-13 was used at 20 ng/ml. Under these conditions, the lymphoid progeny was most evident at day 10 of OP9-DL1 cell culture and myeloid progeny was evident as early as day 3 of OP9 cell culture. Experiments designated as "co-culture" utilized a 1:1 seeding of OP9 and OP9-DL1 cells in combination with both pro-myeloid (GM-CSF) and pro-lymphoid (IL-7 and Flt3) growth factors.

Measurement of STAT activation

HR^+ETPs were sorted as previously indicated. ETPs were either immediately stained for pSTATs or pre-treated ex vivo with IL-4, IL-13, or both in media for 3 hours. Cells were fixed, permeabilized, and stained with anti-pSTAT1 (S727), anti-pSTAT1 (S701), or anti-pSTAT6 (Y641).

Detection of IL-4/IL-13 secretion

Thymic Cells. Thymic cells were harvested, by PBS perfusion, from the thymi of individual C57BL/6 or NOD mice (6-8 weeks of age). Thymic cells were stimulated with PMA and ionomycin for 6 hours. The supernatant was then used for ELISA.

iNKT cells. Thymi of individual mice were harvested and total thymic cells were isolated. Cells stained with allophycocyanin-labeled PBS57-CD1d1 tetramer (iNKT-tet). After thorough washing, the cells were incubated with anti-allophycocyanin microbeads, and the samples were run on MACS columns to isolate iNKT cells. iNKT cells were incubated for 72 hours with or without the presence of anti-CD3 (10µg/ml) and anti-CD28 (1µg/ml). BFA (10µg/ml) was added to the culture 2 hours prior to harvest. iNKT cells were then stained I.C for IL-4.

ETP maturation in vivo

C57BL/6 (CD45.1) or NOD (CD45.2) mice were given (i.t.) HR^+ETPs (5×10^4 cells/per mouse) from C57BL/6 (CD45.2) or NOD (CD45.1) donors, respectively, and thymic cells were harvested on days 12 or 16 post-transfer. The day 12 cells were used to analyze the expression of CD11b, CD11c, and CD8α on congenically marked cells, while the day 16 cells served to analyze the expression of CD11b, CD11c, and CD3 markers.

Thymic negative selection assay

NOD mice deficient for MHC I & II were given (i.t) either IL-4 pre-treated HR^+ ETPs or untreated HR^+ ETPs (12×10^3 cells/mouse). After two weeks, the hosts were given $CD45.2^+$ $CD69^+$ double positive (DP) $CD4^+CD8^+$ thymocytes. Negative selection was then measured by the number of SP transferred cells in the thymus at two weeks post-transfer, as well as the number and percent of FVD^+ $Nur77^+$ cells in the thymus.

Statistical Analysis

Data were analyzed using either an unpaired, two-tailed Students t-test, one-way ANOVA, or Mann–Whitney U test as indicated. All statistical analyses were performed using Prism software version 4.0c (GraphPad).

4. Results

A. NOD HR$^+$ETP potential is biased towards an early T cell lineage in contrast to myeloid-restricted C57BL/6 HR$^+$ETPs

Previous studies have shown that ETPs which express the HR are restricted to the myeloid lineage and give rise to primarily thymic resident DCs, which can serve as antigen-presenting cells (APCs) (3, 4) . Additionally, these ETP-derived APCs can aid in negative T cell selection and reduce the severity of EAE (5). Given the impact these cells have on the disease course of EAE, we sought to determine if this phenomenon was present and functional in a spontaneous model of autoimmunity, such as diabetes in the NOD mouse. To first address this question, the fate potential of HR$^+$ETPs from either the C57BL/6 mouse strain or the NOD mouse strain was compared. Using the *in vitro* OP9 culture system to test for myeloid fate potential, the results show that ETPs from the C57BL/6 or NOD strain can both give rise to myeloid cells (Fig. 1A). We next sought to utilize the OP9-DL1 culture system to test NOD HR$^+$ETPs for T cell potential. As expected, HR$^+$ETPs from the C57BL/6 strain could not yield T cell progenitors (Fig. 1B). Strikingly, however, HR$^+$ETPs from NOD mice efficiently yield T cells *in vitro* (Fig. 1B). While HR$^+$ETPs from both strains are more efficient than their HR$^-$ counterparts at generating myeloid cells (top panel), the number of T cells recovered from the OP9-DL1 culture was significantly, and dramatically, different between NOD and C57BL/6 HR$^+$ETPs (bottom panel) (Fig. 1C). This was particularly interesting as, like their C57BL/6 counterparts, NOD HR$^+$ETPs

are predominately found in the DN1c subset, which is known for being a major source of thymically-derived DCs (Fig. 2) (6). Since HR^+ETPs from the NOD strain appear to give rise to multiple lineage fates, we performed experiments utilizing a co-culture of OP9/OP9-DL1 cells to determine true lineage preference. The co-culture was comprised of a 1:1 ratio of OP9/OP9-DL1 cells and contained all growth factors and cytokines needed to stimulate the development of various myeloid cells (D6) or T cells (D10). The results show that the majority of the HR^+ETPs from the C57BL/6 strain give rise to $CD11c^+$ DCs both at D6 and by D10 of culture (Fig. 1D, left panel). However, while there was some live myeloid progeny present at D6 in the NOD culture, by D10 all detectable live cells are exclusively early T cells (Fig. 1D, right panel). These results were statistically significant (Fig. 1E) and indicated that, while C57BL/6 HR^+ETPs are restricted to the myeloid fate, HR^+ETPs from the NOD thymus preferentially give rise to T cells. This deviation of lineage fate was not merely due to reduced receptor expression, or increased expression of the competitive receptor IL-13Rα2, as HR expression on ETPs is significantly increased in the NOD strain, and expression of IL-13Rα2 is unchanged (Figure 3).

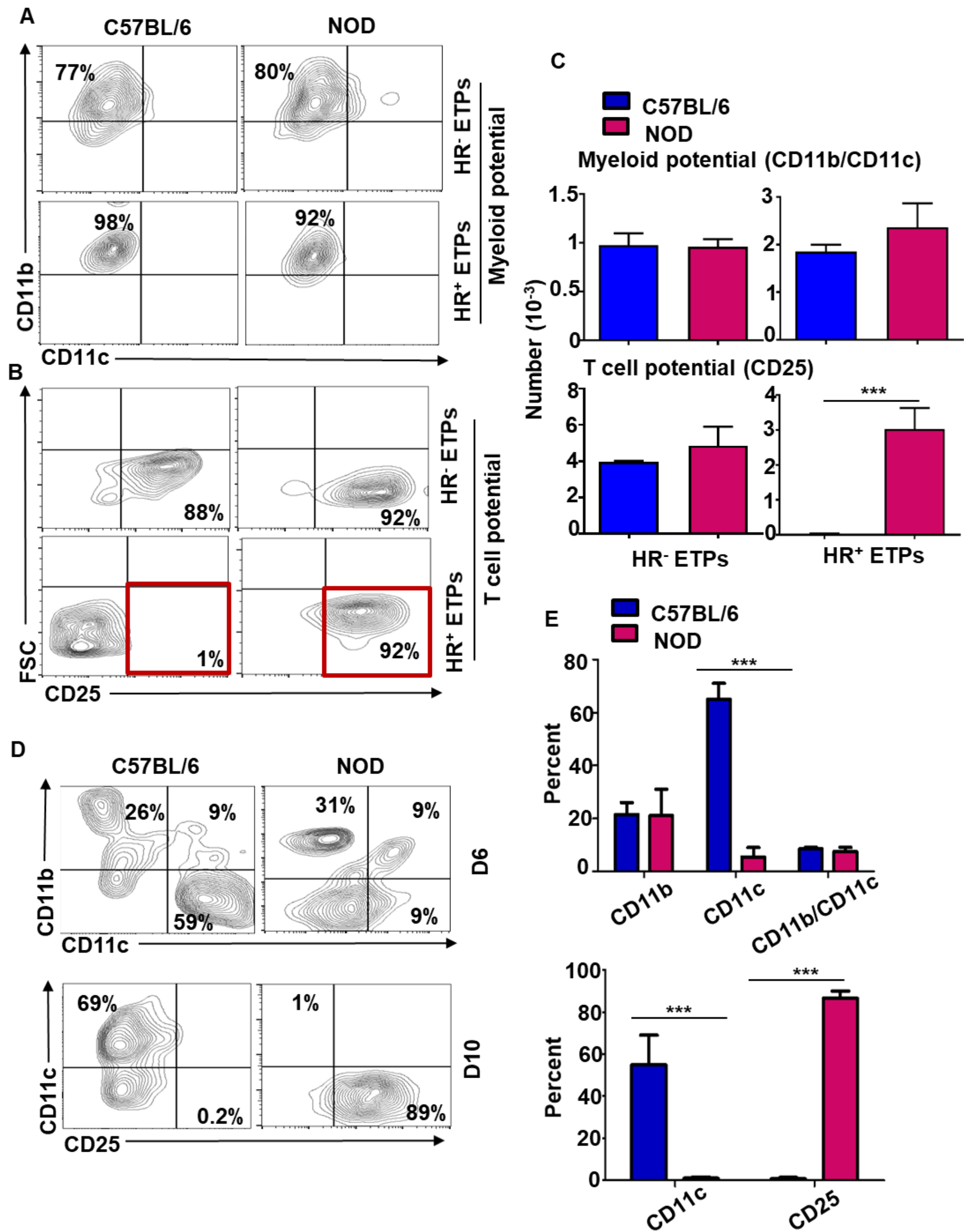
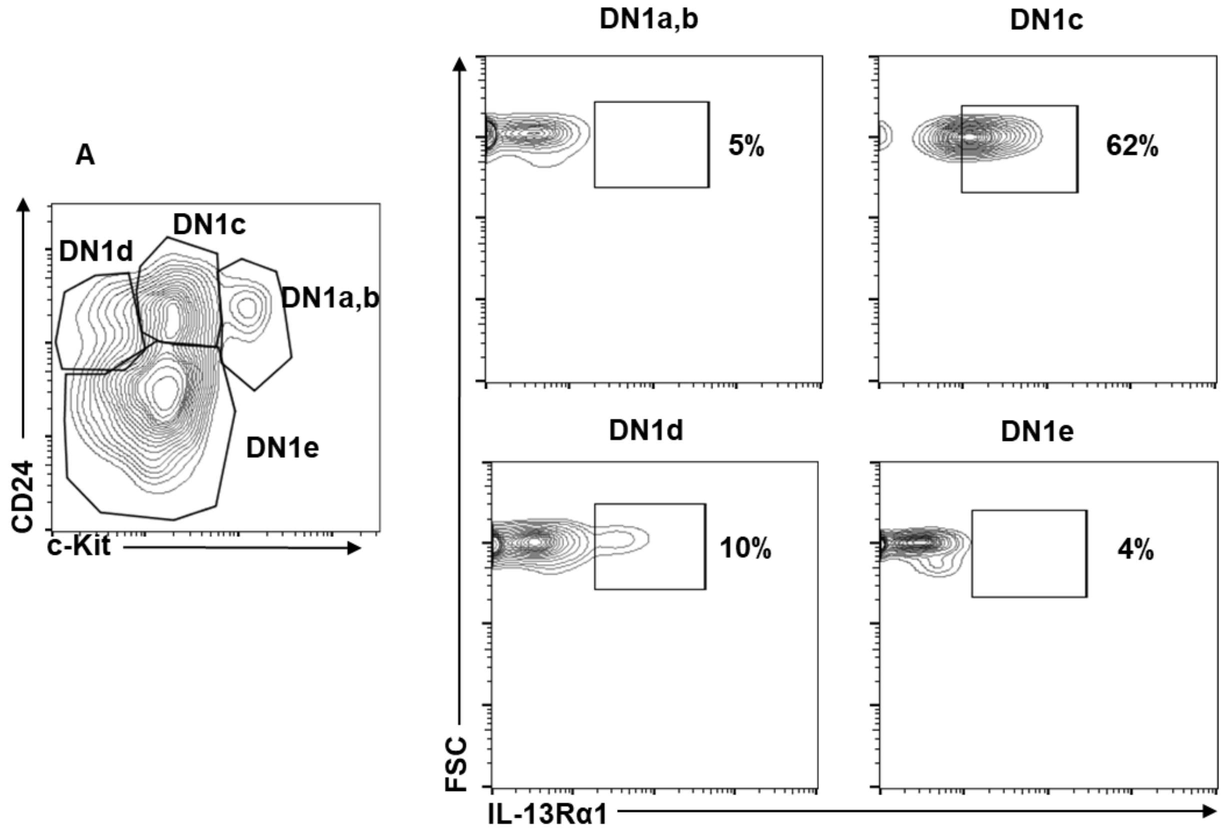


Figure 1. Differential fate decision among NOD and C57BL/6 HR+ETPs.

NOD and C57BL/6 HR^+ and HR^- ETPs were sorted from the thymus of either strain and cultured on OP9 **(A)** or OP9-DL1 **(B)** cells. The contour plots illustrate a representative experiment showing commitment to myeloid (CD11b/CD11c) and lymphoid (CD25) lineages as measured by flow cytometry. **(C)** Shows the number of myeloid (CD11b/CD11c, top panel) and lymphoid (CD25, bottom panel) cells. Each bar graph represents the mean ± SD of data compiled from four different experiments. **(D** and **E)** HR^+ETPs from both strains were cultured on mixtures of OP9/OP9-DL1 (1:1) stromal cells in the presence of GM-CSF, IL-7, and Flt3. **(D)** Shows a representative experiment illustrating commitment of the ETPs to myeloid (CD11b), DC (CD11c), and lymphoid (CD25) lineages at six (top panel) and ten (bottom panel) days of culture. **(E)** Shows the percentage of different cell lineages compiled from 4 experiments. ***$p < 0.001$ as determined by two-tailed, unpaired Student *t* test.



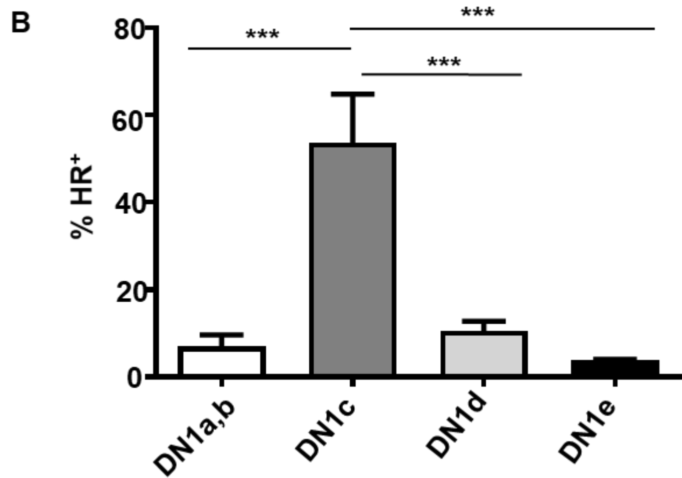


Figure 2. NOD HR^{+}ETPs belong to the DN1c subset.

Thymic cells from NOD IL-13Rα1–GFP reporter mice were depleted of Lin^+ cells, and $Lin^-CD4^-CD8^-$ cells were analyzed for CD25 and CD44 expression. $CD25^-CD44^+$ cells (DN1 cells) were further assessed for c-Kit and CD24 expression to distinguish the different subsets within the DN1 population. **(A)** The left panel shows the different DN1 subsets on the basis of expression of $CD24^+$c-Kit^{hi} (DN1a,b), $CD24^+$c-Kit^{int} (DN1c), $CD24^+$c-Kit^- (DN1d), and $CD24^-$c-Kit^- (DN1e). The right panel shows expression of IL-13Rα1 (HR) on the different subsets. **(B)** Shows HR expression data on the different subsets complied from four experiments. ***$p < 0.001$ as determined by two-tailed, unpaired Student t test.

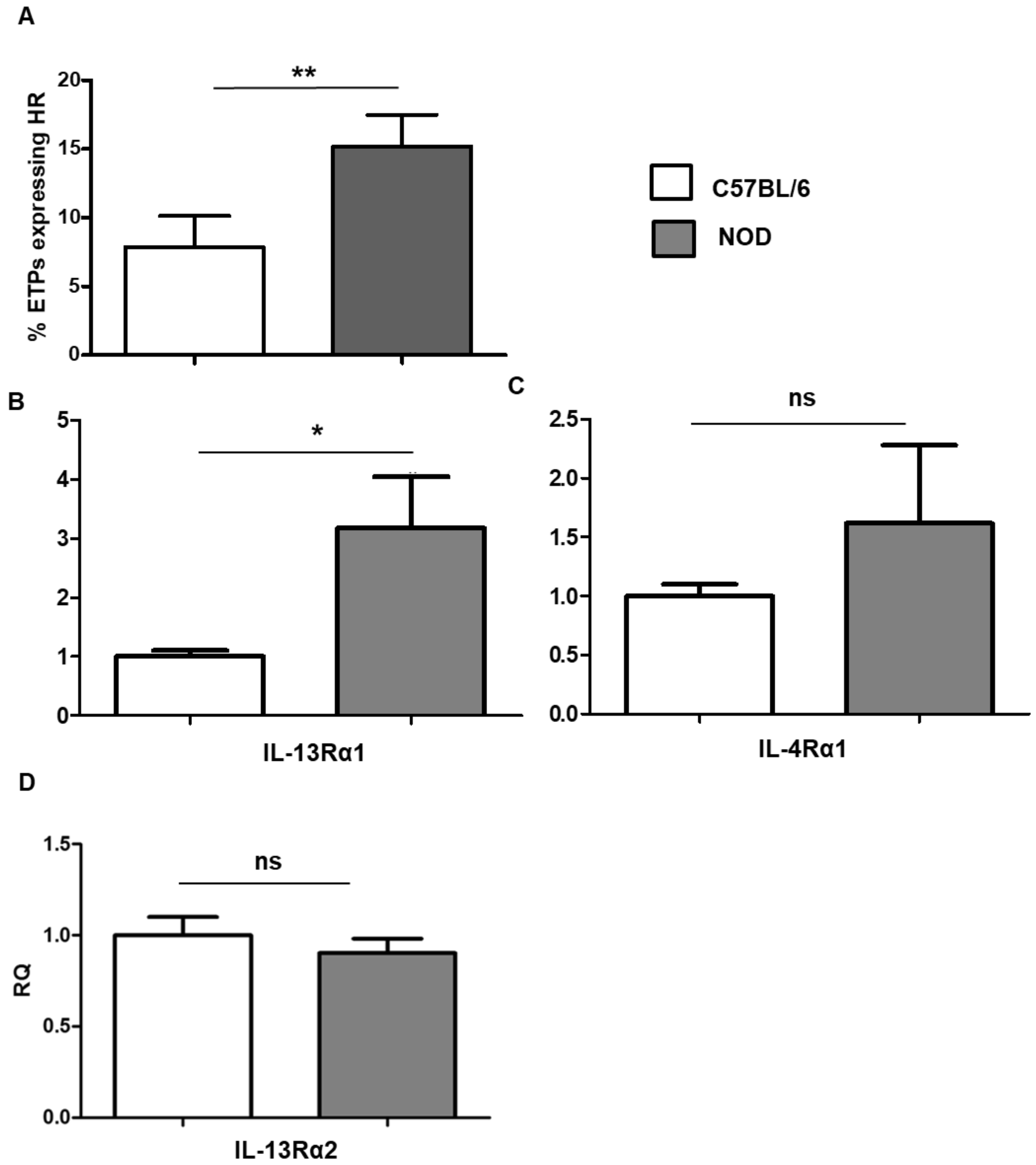


Figure 3. NOD ETPs express higher levels of the heteroreceptor in comparison to C57BL/6 ETPs.

HR^+ETPs from C57BL/6 and NOD mice were analyzed for receptors expression by both flow cytometry and RT-PCR. **(A)** Shows surface HR expression from data compiled from 5 experiments. *$p < 0.05$ as determined by two-tailed, unpaired Student t test. **(B)** Shows IL-13Rα1 gene expression while **(C)** shows IL-4Rα gene expression. **(D)** Shows IL-13Rα2 gene expression. **$p < 0.01$ as determined by two-tailed, unpaired Student t test.

B. NOD HR^+ETPs give rise to T cells *in vivo* at the expense of thymic resident DCs

To further expand on the fate of NOD HR^+ETPs in a more physiologically relevant *in vivo* system, HR^+ETPs were injected intrathymically (i.t) into respective CD45 congenic hosts from both strains, and their maturation potential was analyzed at 16 days post-transfer. The transferred cells were first probed for expression of CD11b, CD11c, or CD3 to resolve their general classification as either macrophage/monocytes ($CD11b^+CD11c^-$), conventional DCs ($CD11c^+$), or T cells ($CD3^+$). The results show that while C57BL/6 HR^+ETPs give rise to both $CD11b^+$ and $CD11c^+$ cell types, NOD HR^+ETPs exclusively give rise to $CD3^+$ T cells (Fig. 4A). Data combined from several experiments confirmed that these observations were statistically significant (Fig. 4B). Further examination of transferred cells from NOD mice revealed that NOD HR^+ETPs could give rise to either $CD4^+$ or $CD8^+$ T cells but not iNKT or B cells (Fig. 4C & D). Upon repeat experiments, $CD4^+$ T cells were more predominant among HR^+ ETP-derived T cells (Figure 4C, right bar graph). Together the results indicate that HR^+ETPs yield T cells when exposed to the native NOD thymic microenvironment.

Given that HR^+ETPs in the C57BL/6 strain provide a pool of thymic resident DCs, we hypothesized that NOD thymi might be deficient in this cell type compared to their non-autoimmune counterparts. Indeed, the frequency of $CD8\alpha^+$ $CD11c^+$ DCs, among total thymic APCs, was significantly reduced in 6-8 week old NOD mice when compared to C57BL/6 mice of the same age (Fig. 4E, left panel). This result is noteworthy as $CD8\alpha^+$ $CD11c^+$ DCs are specifically known

for their contribution to thymic negative selection. Additionally, the frequency of these DCs is diminished in older mice (12-14 weeks) of both strains, but still significantly higher in C57BL/6 mice (Fig. 4E, right panel). When younger NOD mice are compared to their older counterparts, the population of CD8α$^+$ CD11c$^+$ DCs is condensed further (Fig. 4F). Taken together, this data shows that NOD HR$^+$ETPs are diverted to the T cell lineage, rather than the myeloid fate, *in vivo*. In addition, CD8α$^+$ CD11c$^+$ DC numbers (cells which may be derived from HR$^+$ETPs in the C57BL/6 strain) are severely lacking in the NOD thymus. This skewing of the thymic APC population may be due to the divergence of NOD HR$^+$ETPs from the myeloid fate to the T cell lineage. This reconfirms the importance of investigating what drives the perplexing lineage fate of NOD HR$^+$ETPs.

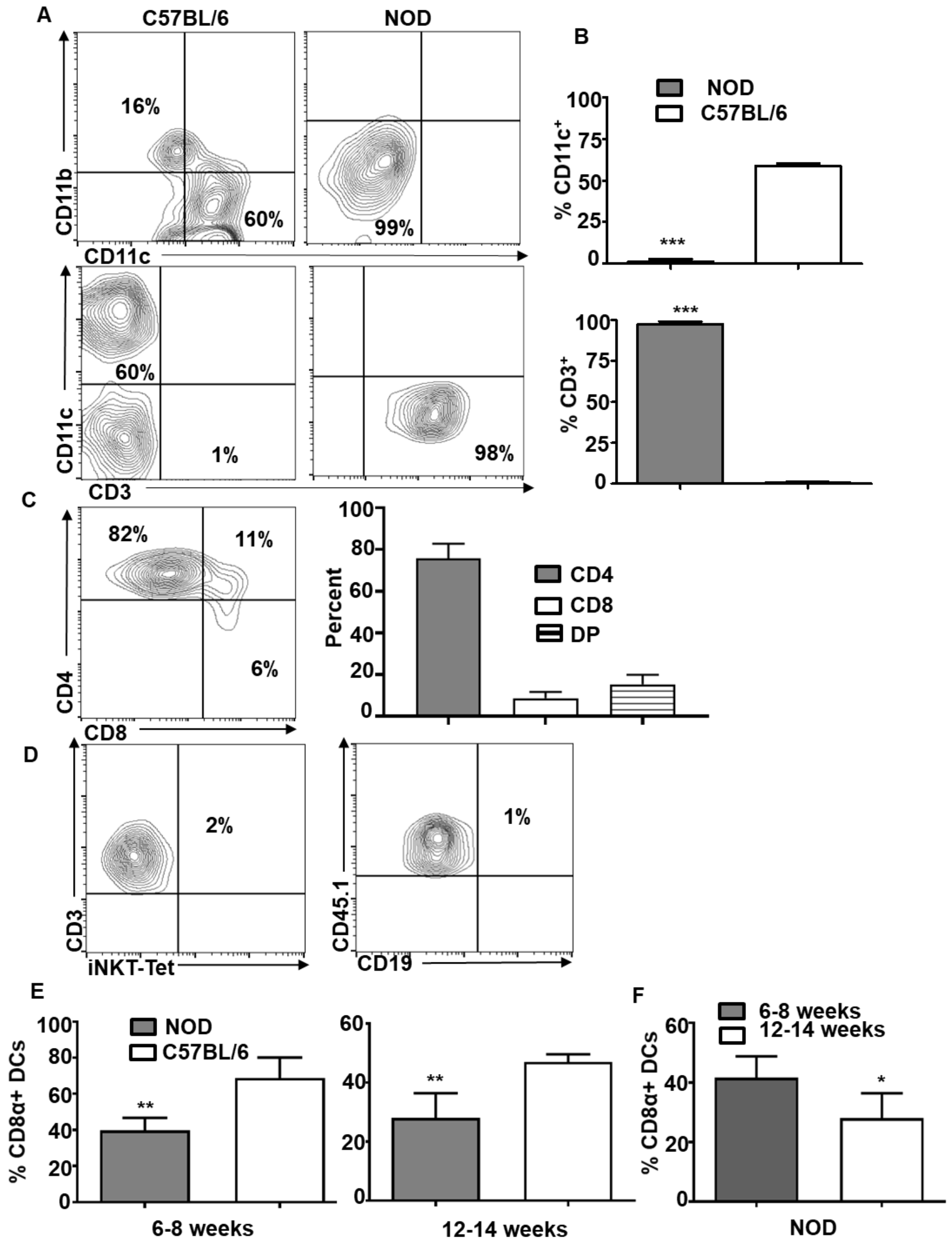


Figure 4. NOD HR⁺ETP fate decision is restricted to T cells *in vivo*.

CD45.1 NOD and CD45.2 C57BL/6 HR^+ETPs were injected intrathymically into CD45.2 NOD and CD45.1 C57BL/6 recipients, respectively. After 16 days, thymi were harvested and HR^+ETP-derived cells were analyzed for lineage phenotype. **(A)** Shows representative contour plots for expression of CD11b, CD11c, and CD3 markers. **(B)** Shows the frequency of $CD11c^+$ DCs (top panel) and $CD3^+$ T cells (bottom panel). Each bar graph represents the mean ± SD of data compiled from four different experiments. ***$p < 0.001$ as determined by two-tailed, unpaired Student t test. **(C)** Illustrates a representative experiment analyzing expression of other lymphoid lineage phenotypes including Tet^+ iNKT cells (left panel) and $CD19^+$ B cells (right panel). **(D)** Contour plot (left panel) shows a representative experiment for CD4 and CD8 expression on NOD HR^+ETP-derived T cells. The bar graph shows CD4 and CD8 expression results compiled from four experiments. **(E** and **F)** $CD3^-$ thymocytes from C57BL/6 and NOD mice were stained for CD11b, CD11c, CD8, and SIRPα at the age of 6-8 and 12-14 weeks. **(E)** Shows the frequency of $SIRP\alpha^-CD11c^+CD8^+$ thymic resident DCs from C57BL/6 relative to NOD mice. **(F)** Shows a comparison of the frequency of $SIRP\alpha^-CD11c^+CD8^+$ thymic resident DCs among 6-8 week versus 12-14 week old NOD mice. **$p < 0.01$ as determined by two-tailed, unpaired Student t test from five experiments.

C. Reduced cytokine in the NOD thymic microenvironment leads to inefficient STAT signaling

Previous studies in our lab have shown that HR^+ETPs from C57BL/6 mice require endogenous IL-4 or IL-13 signaling through the HR to induce STAT activation. These signaling mediators trigger the expression of pro-myeloid genes ($STAT1_{(S701)}$) or restrict the expression of pro-T cell lineage genes ($STAT6_{(Y641)}$) (3, 4). Given that NOD HR^+ETPs default to the T cell lineage, we sought to determine if signaling via the HR was inducing activation of STAT transcription factors. Indeed, both $pSTAT1_{(S701)}$ and $pSTAT6_{(Y641)}$ are significantly reduced in NOD HR^+ ETPs in comparison to their C57BL/6 counterparts (Fig. 5A, middle and right panel). Given that active $STAT1_{(S701)}$ and $STAT6_{(Y641)}$ are required to drive HR^+ETPs towards the myeloid lineage, and away from the T cell lineage, it was likely that downstream signaling mediators for these two opposing lineage fates would be inverted in NOD HR^+ETPs. Indeed, IL-7Rα and Notch1 transcripts, early T cell lineage markers, are significantly upregulated in NOD HR^+ETPs (Fig. 5B, top and middle graphs). Similarly, CEBP/α transcript, an early myeloid marker, was significantly reduced in NOD versus C57BL/6 HR^+ETPs (Fig. 5B, bottom graph). When taken together, these results indicate that cytokine signaling through the HR was insufficient and unable to induce STAT signaling. As a result, HR^+ETPs express markers that are consistent with the T cell lineage fate.

Given there is minimal signaling through the HR, there may be insufficient cytokine available in the thymus. We next sought to determine the steady-state

level of both intrathymic IL-4 and IL-13 in NOD mice. Transcriptional levels of both cytokines were compared using qPCR. The results show that IL-4 mRNA levels in the NOD thymus is significantly reduced compared to age-matched C57BL/6 mice (Fig. 5C). Protein expression of both cytokines was also analyzed by ELISA. Strikingly, NOD thymocytes produced dramatically low levels of IL-4 in comparison to C57BL/6 thymocytes (Fig. 5D, left panel). Interestingly, there was also a modest reduction in NOD thymocyte production of IL-13 (Fig 5D, right panel). Given that IL-4 mRNA and protein are severely reduced in the NOD thymus, while IL-13 is only moderately reduced upon stimulation, it is likely that insufficient IL-4 is the primary cause of reduced HR signaling. This idea aligns well with the fact that IL-4 is a predominant cytokine with many functions in the mouse thymus (7-9)

D. The scarcity of intrathymic IL-4 is likely due to thymic deficiency of IL-4-producing iNKT cells

Determining which cell type is responsible for the reduced thymic IL-4 fitness in the NOD strain is vital. Previous studies have shown that iNKT cells are a large source of thymic IL-4 (10, 11). Given this, we hypothesized that perhaps the NOD mouse has insufficient numbers of thymic iNKT cells, which results in low levels of IL-4 production. To test this premise, thymocytes were stained using the CD1d:αGlaCer tetramer (or iNKT tet) to mark iNKT cells. Strikingly, both the frequency (Fig. 5E) and number (Fig. 5F) of iNKT-tet^{+} cells were significantly reduced in the NOD thymus compared to the C57BL/6 thymus. In fact, C57BL/6 mice had, on average, more than double the number of iNKT cells when

compared to age-matched NOD mice (Fig. 5F). We next sought to determine if NOD thymic iNKT cells could be compensating for their reduction in population size by producing an excess of IL-4 upon stimulation. The results show that NOD iNKT cells are, in fact, even less efficient at producing IL-4 than their C57BL/6 counterparts (Fig 5G). Recently it has been shown that iNKT cells may be categorized into subsets, like $CD4^+$ helper T cells, with iNKT2 cells primarily producing IL-4 (12). Given that NOD thymic iNKT cells produce less IL-4 on a per cell basis, there is likely a specific reduction in the iNKT2 cell population. To test this notion, thymic iNKT cells were used to analyze the expression of markers associated with the three subsets. Indeed, while iNKT1 and iNKT17 cell frequency is increased, the iNKT2 frequency in NOD mice is drastically reduced (Fig. 5H). Indeed, the average frequency of iNKT2 cells among total thymic iNKT cells was 42% for the C57BL/6 stain but a mere 23% for the NOD strain (Fig 5H). These results indicate that iNKT cells are reduced in the NOD thymus and produce less IL-4. Together, these findings suggest that sufficient levels of IL-4 are not available to induce HR signaling in ETPs.

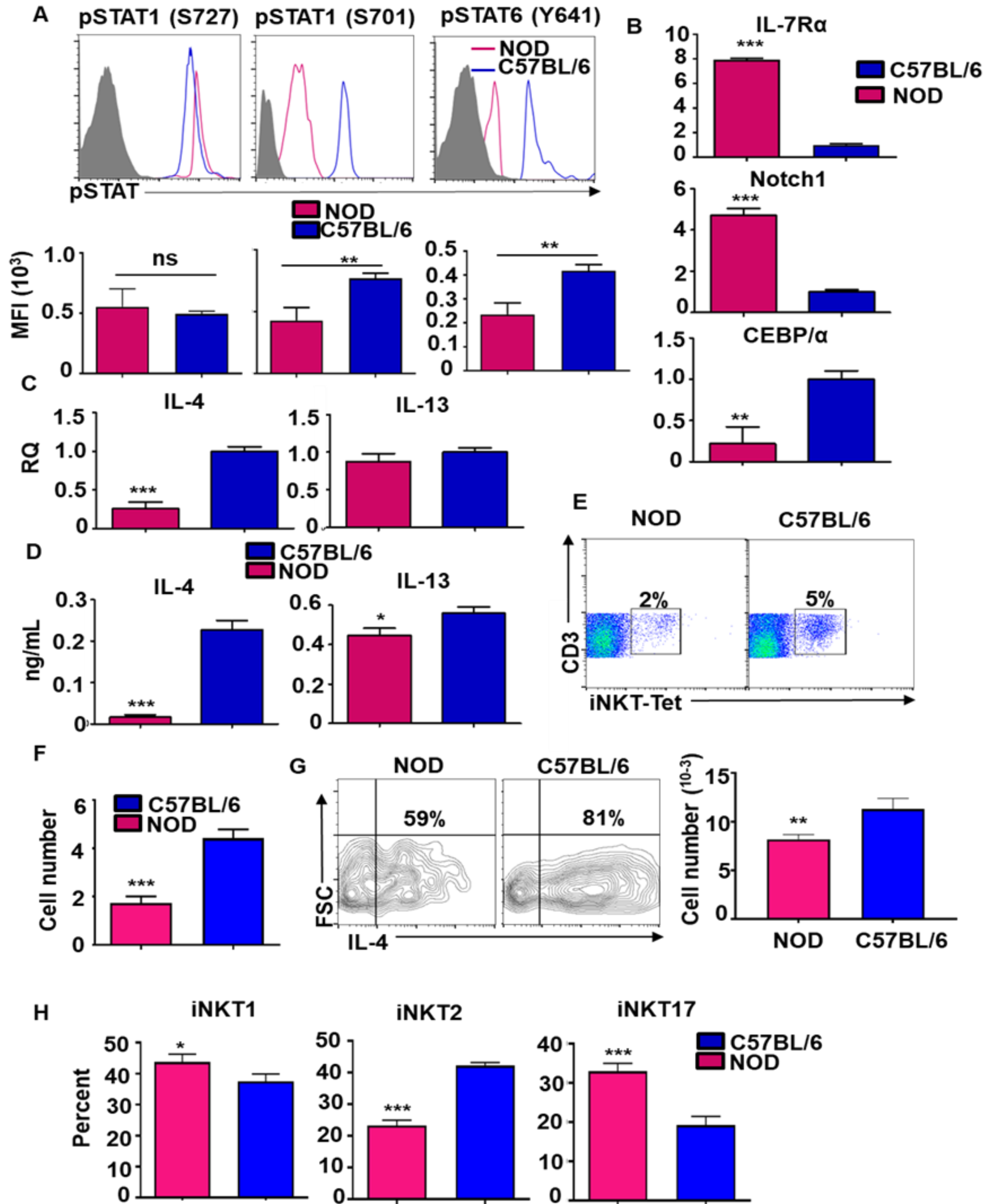


Figure 5. Diminished IL-4 in the NOD thymus parallels with a lower frequency of iNKT cells.

(A) HR^+ETPs from C57BL/6 and NOD mice were analyzed *ex vivo* for phosphorylation of different isoforms of STAT1 and STAT6 transcription factors. The histograms show a representative experiment, while the bar graphs show results compiled from three experiments. **(B)** Shows mRNA expression for IL-7Rα (top panel), Notch1 (median panel) and CEBP/α (bottom panel) in HR^+ETPs. **(C** and **D)** Total thymocytes from 6-8 week old mice were used to extract RNA **(C)** or stimulated with PMA/Ionmycin **(D)**. mRNA expression was analyzed by RT-PCR while IL-4 and IL-13 secretion were determined by ELISA. Each bar represents data compliled form three experiments. $^*p < 0.05$, $^{***}p < 0.001$ as determined by two-tailed, unpaired Student t test. **(E and F)** Show the percentage **(E)** and the number of cells **(F)** that stain for CD3 and αGlaCer tetramer (iNKT-tet) among fresh CD8 depleted thymocytes. $^{***}p < 0.001$ as determined by two-tailed, unpaired Student t test from four experiments. **(G)** The contour plots show intracellular IL-4 production by sorted iNKT cells that were stimulated with anti-CD3/anti-CD28. The bar graph shows the number of cells staining for intracellular IL-4. $^{**}p < 0.01$ as determined by two-tailed, unpaired Student t test from four experiments. **(H)** Shows the frequency of different subsets of iNKT cells including iNKT1 ($CD122^+$ $CD4^{+/-}$ $ICOS^-$), iNKT2 ($CD122^-$ $CD4^+$ $ICOS^+$), and iNKT17 ($CD122^-$ $CD4^-$ $ICOS^+$) cells. The bar graphs show data compiled from four experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by two-tailed, unpaired Student t test.

E. Cytokine treatment restores STAT signaling in HR^+ETPs and drives the development of thymic DCs

Since the NOD thymus is lacking in IL-4 and IL-13, we sought to determine if treatment with either cytokine may rescue ETP lineage fate. To test this, NOD HR^+ETPs were cultured on OP9-DL1 cells with the addition of cytokine treatment. Without cytokine treatment, 90% of HR^+ ETPs yield $CD25^+$ T cells (Fig. 6A). However, upon treatment with either cytokine, HR^+ETPs were less efficient in producing T cells (Fig. 6A). This influence of the cytokines on lineage fate was statistically significant for both IL-4 and IL-13 (Fig. 6B). However, it is interesting to note that IL-4 appeared to be much more efficient at diverting ETP lineage fate than IL-13. On average HR^+ETP affinity for the T cell fate was reduced by 20% for IL-13 treatment versus 39% for IL-4 treatment, compared to untreated controls (Fig 6B). We next wanted to confirm that the cytokines were diverting HR^+ETP fate by restoring STAT signaling. To test this premise, NOD HR^+ETPs were treated *ex vivo* with either IL-4, IL-13, or IL-4 + IL-13. Cells were then immediately analyzed for STAT activation. The results show that IL-4 induces $pSTAT6_{(Y641)}$ and $pSTAT1_{(S701)}$ expression (Fig. 6C & D). However, IL-13 treatment alone was less efficient and did not significantly increase the expression of $pSTAT6_{(Y641)}$ (Fig. 6D). This result further confirms the idea that IL-4 is likely the main cytokine responsible for inducing HR signaling in ETPs.

Given that IL-4 treatment *ex vivo* can induce STAT signaling and restore NOD HR^+ETP lineage fate, it is likely that treating NOD mice with intrathymic IL-4 will drive these cells toward the DC lineage fate. To this end, 4 week old NOD

mice received IL-4 (i.t) once per week for two weeks. Following treatment, HR^+ETPs were sorted and tested for lineage potential on OP9-DL1 cultures. The results show that, upon exposure to IL-4 *in vivo*, HR^+ETPs are sufficiently restricted from the T cell lineage (Fig. 6E). To confirm that this change was due to HR signaling in ETPs and not due to unanticipated effects of the intrathymic IL-4, we utilized the congenic transfer model. Prior to transfer, sorted HR^+ETPs were stimulated with IL-4 *ex vivo*. The results show that pre-treating HR^+ETPs with IL-4 enables these cells to readily give rise to myeloid cells, particularly $CD11c^+$ DCs (Fig. 6F). In contrast, IL-4 treatment strongly blocks T cell potential (Fig. 6G). These results were statistically significant (Bottom panels of Fig. 6F & 6G). Together these results indicate that intrathymic IL-4 treatment can specifically target HR^+ETPs and drive these cells towards the DC lineage fate.

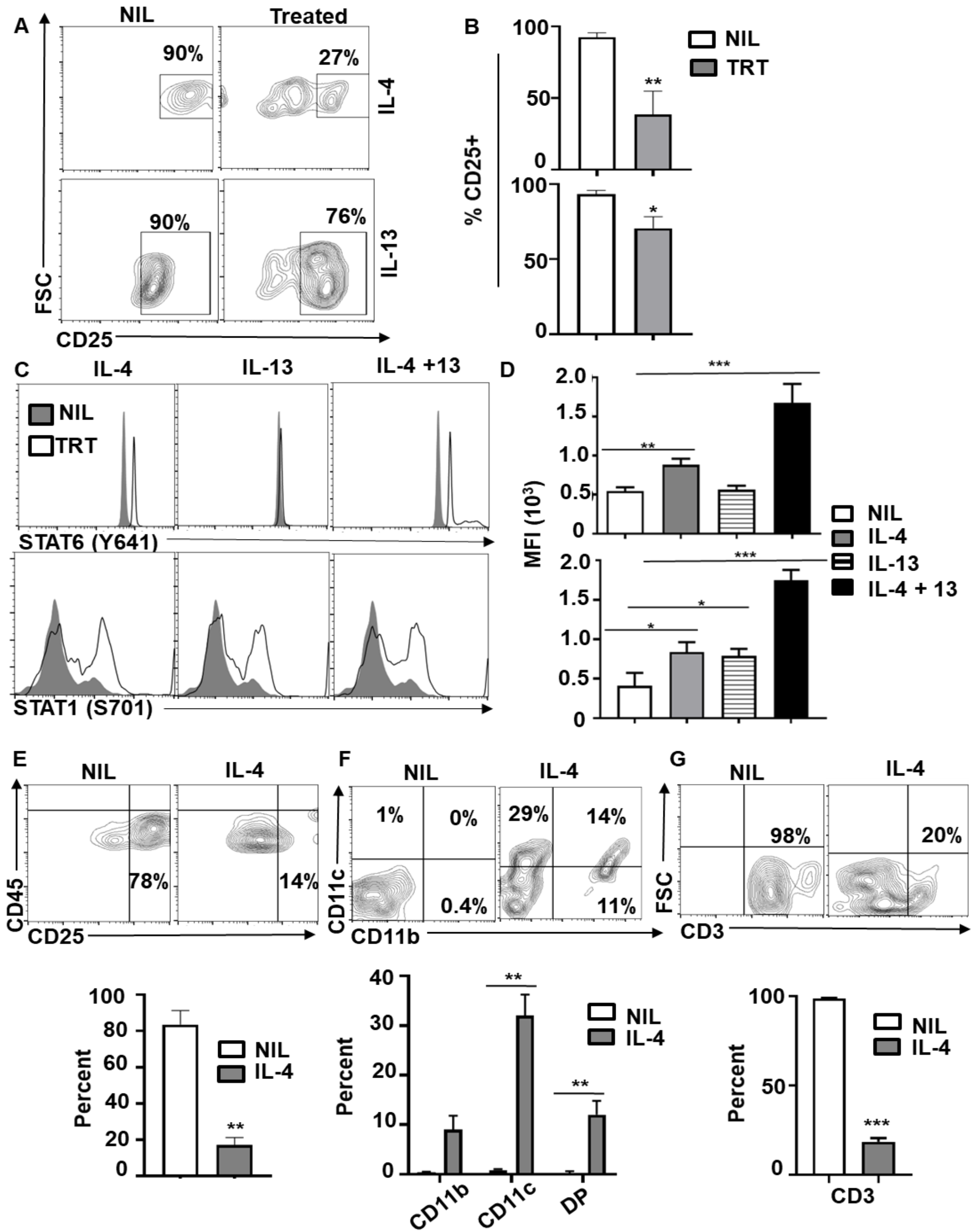


Figure 6. IL-4/IL-13 reverse NOD HR⁺ETP fate decision towards myeloid cells.

(A and **B)** NOD HR^+ETPs were cultured on OP9-DL1 + IL-7/Flt3 in the absence (NIL) or presence (treated) of IL-4 or IL-13 for 10d. **(A)** The contour plot shows a representative experiment illustrating reduction in fate decision towards the T cell lineage. **(B)** The bar graphs show the frequency of lymphoid lineage cells complied from four experiments. **(C** and **D)** The HR^+ETPs were stimulated with IL-4, IL-13, IL-4 + 13, or NIL (PBS) and phosphorylation of STAT6 and STAT1 transcription factors were analyzed by flow cytometry. **(C)** The histograms show a representative phosphorylation experiment. **(D)** The bar graphs show MFI data compiled from three experiments. **(E)** Four week old IL-13Rα1$^{+/+}$-GFP reporter NOD mice were given intrathymic IL-4 weekly for two weeks. Seven days later, HR^+ETPs were sorted and cultured on OP9-DL1 stromal cells for 10d. The contour plots show a representative experiment illustrating the frequency of $CD25^+$ lymphoid lineage cells. The bar graph shows compiled results from three experiments. **(F** and **G)** HR^+ETPs sorted from CD45.1 NOD mice were stimulated with IL-4 *ex vivo* and injected i.t into a congenic host (CD45.2). On day 16 after transfer, thymic cells were analyzed for lineage commitment. **(F)** The contour plots show a representative experiment illustrating expression of CD11b and CD11c markers on ETP-derived cells. The bar graphs shows the frequency of $CD11b^+$, $CD11c^+$, and CD11b/$CD11c^+$ cells of data compiled from three experiments. **(G)** The contour plots show a representative experiment illustrating expression of CD3 on ETP-derived cells. The bar graph represents the average frequency of $CD3^+$ cells as compiled from three different experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as determined by two-tailed, unpaired Student t test.

F. IL-4-Treated HR$^+$ETP-derived DCs can perform negative selection and delay diabetes onset

In the healthy C57BL/6 mouse strain, IL-4 is readily available in the thymus and serves to drive HR$^+$ETPs towards the DC lineage. Importantly, these ETP-derived DCs can perform negative selection and provide protection against experimental allergic encephalomyelitis (5). Given this, it is possible that upon treatment with IL-4 the NOD thymus is repopulated with ETP-derived DCs and, therefore, negative T cell selection will become more efficient. To test this premise, we devised an experimental model to test the contribution of IL-4-treated HR$^+$ETPs to negative selection (Fig. 7A). Accordingly, HR$^+$ETPs were sorted, briefly stimulated *ex vivo* with IL-4, and then injected (i.t) into hosts lacking MHC class I and II. This setup provided that only ETP-derived APCs would express MHC and were, therefore, the only cells that could induce negative selection. Two weeks later, host mice received (i.t) CD4$^+$CD8$^+$ (double positive) positively selected (CD69$^+$) thymocytes. The results show that mice recipients of IL-4-treated ETP-derived DCs had fewer thymic single positive (SP) T cells compared to control mice (Fig. 7B). In addition, SP donor cells were stained for Nur77, a marker for negative selection, and 7-AAD, a viability stain, to confirm that these cells are undergoing negative selection. Indeed, the percentage of SP cells from mice recipients of the IL-4-treated ETP-derived DCs that were undergoing negative selection was significantly increased compared to controls (Fig. 7C). The results indicate that intrathymic IL-4 increases the population of thymic DCs and allows for efficient negative selection of T cells.

As a result, fewer autoreactive clones would reach the periphery which could result in the delayed onset of T1D. To test this hypothesis, NOD mice were given intrathymic IL-4 or saline and monitored for disease progression. Strikingly, while control mice become diabetic by 14 weeks of age, none of the mice recipients of intrathymic IL-4 developed diabetes until after 18 weeks of age (Fig. 7D). Indeed, at peak disease, 80% of control mice are diabetic while only 28% of treated mice are diabetic (Fig.7D). The results indicate that intrathymic IL-4 delays T1D likely by increasing the population of thymic DCs. These DCs can induce negative selection of T cells which may lead to a less pathogenic TCR repertoire.

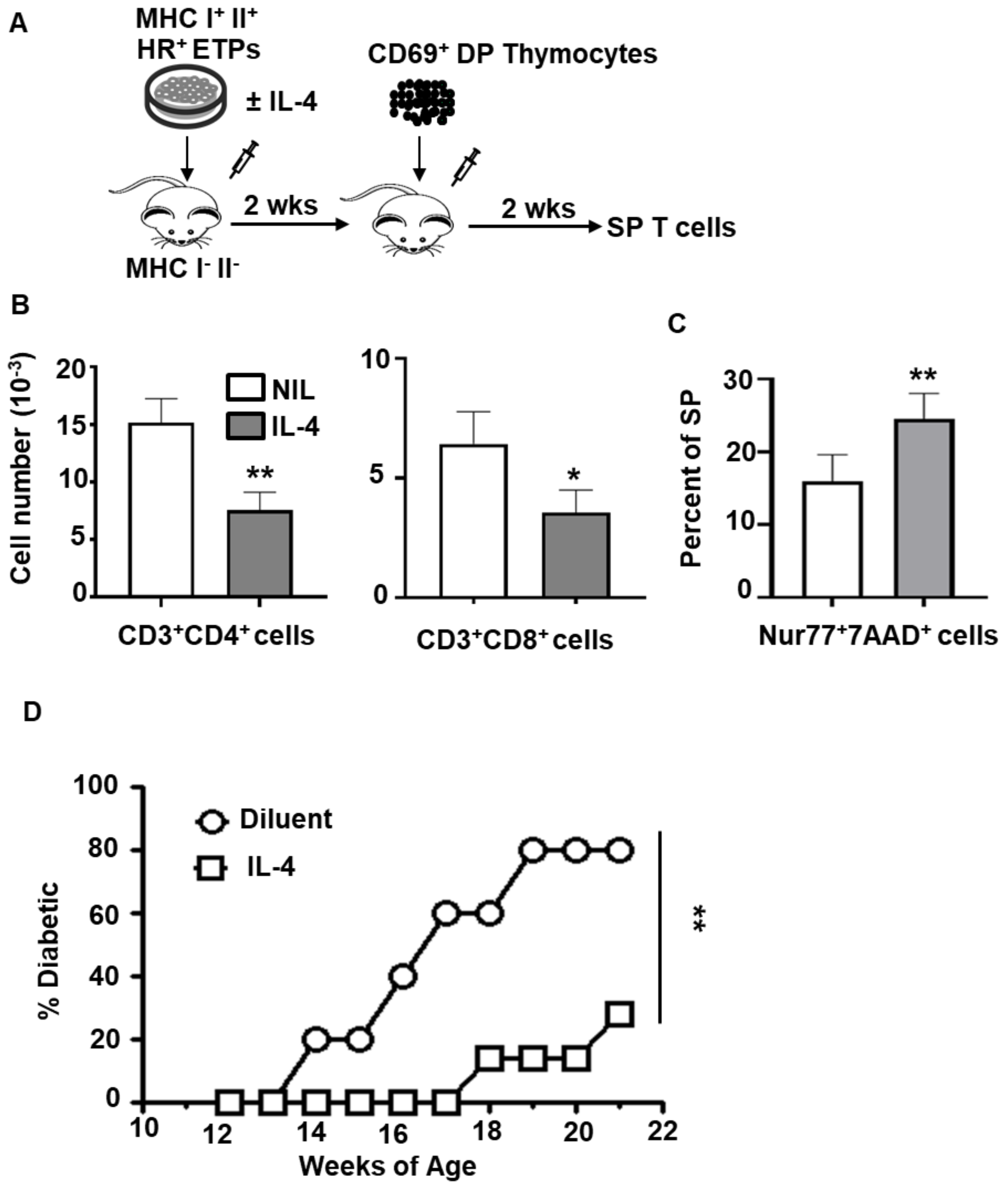


Figure 7. Intrathymic IL-4 tightens T cell negative selection and delays the onset of T1D.

(A) Shows a schematic representation of the animal model used to determine the effect of IL-4 on negative selection of T cells. In this model, NOD HR^+ETPs (50 x 10^3) were treated with IL-4 *ex vivo* and injected i.t NOD MHC I^-II^- mice. Two weeks later, the hosts were given i.t positively selected $CD69^+$ double positive ($CD4^+CD8^+$) thymocytes from MHC I^+II^+ congenic (CD45.2) mice. After two weeks, thymi were harvested and analyzed for live CD4 and CD8 single positive (SP) T cells. **(B)** Shows the total number of CD4 (left) and CD8 (right) SP $CD3^+$ T cells. **(C)** Shows the percentage of donor T cells undergoing apoptosis (7-AAD) and expressing Nur77, a marker for negative selection. *$p < 0.05$, **$p < 0.01$ as determined by two-tailed, unpaired Student t test from three experiments. **(D)** 4 week old NOD mice were given i.t IL-4 once a week for two weeks and then monitored for blood glucose level (BGL) starting at 12 weeks of age. **$p < 0.01$ as determined by Mann-Whitney *U* test.

G. Supplementation of the NOD thymus with IL-4 alters the T cell repertoire

An overabundance of peripheral T cells with a high affinity for self-antigens may result from a defective thymic negative selection. Thus, tightening the thymic negative T cell selection process may be directly reflected in the peripheral T cell receptor (TCR) repertoire. Given that IL-4 treatment delays T1D and improves negative selection, we sought to determine if intrathymic IL-4 treatment would alter the TCR repertoire. To first test this premise, NOD mice were treated with intrathymic IL-4 and sacrificed at 9 weeks of age (before developing overt disease). $TCR\beta^{+}$ T cells from the spleen and pancreatic lymph node were sent to iReperotire (iRepertiore, Inc) for TCRβ sequencing. The results showed that spleen samples from both treated and control mice had comparable usage of V-J genes (Fig. 8A, upper graphs). Strikingly, however, T cells from the PLN of IL-4 treated mice showed a vastly altered V-J gene usage (Fig. 8A, lower graphs). Indeed, PLN T cells from IL-4 treated mice utilized many V-J combinations that were unexpressed or only expressed at a low level in control mice (Fig. 8A, lower graphs). The usage of specific V genes can be enriched under various disease conditions. One such incidence is the overabundant usage of Vβ13 in T1D (13, 14). Interestingly, we found that there was indeed an overabundant use of Vβ13 among both SP and PLN T cells in untreated mice (Fig. 8B). However, when mice received intrathymic IL-4 treatment, the overall frequency of Vβ13 usage was dramatically lower in both organs (Fig. 8B).

The complementarity-determining region 3 (CDR3) of the TCRβ chain is critical for determining antigen specificity. In T1D, patient T cells have shorter

CDR3 regions with fewer random nucleotide insertions than healthy patients matched for MHC haplotype (15). Given this, we sought to determine if intrathymic IL-4 treatment would affect average CDR3 length and number of insertions. SP T cells from treatments showed a distinct pattern of longer CDR3 length than the untreated control (Fig. 8C, upper panel). However, the opposite is true of samples from the PLN (Fig. 8C, lower panel). This inverse relationship between SP and PLN samples was similar when analyzing the total number of random nucleotide insertions (Fig. 8D). We next sought to determine the overall diversity of the TCR repertoire by comparing the usage of the most common CDR3s among all CDR3s. This data acts as a readout for clonal expansion of diabetogenic T cells. Indeed, both treated and control samples from the SP lacked diversity and showed a high amount of clonal expansion (Fig. 8E, upper panel). However, PLN T cells from treated mice were highly diverse and the population sizes of the top clones were significantly reduced compared to PLN T cells from control mice (Fig. 8E, lower panel). This data indicates that intrathymic IL-4 treated mice have an altered TCR repertoire prior to disease onset. Specifically, SP T cells from treated mice have longer CDR3s and more random nucleotide insertions, while the opposite is true in the PLN. However, PLN T cells from treated mice are highly diverse and lack the signs of large clonal expansions.

Around 12-14 weeks of age NOD mice begin to become overt diabetic (BGL ≥ 300mg/dl). Given that recipients of the IL-4 treatment do not develop the disease until much later, we sought to determine how the pathogenic repertoire

of control mice might vary from treated mice of the same age. For these experiments, mice were allowed to age out to 12 weeks of age. The results showed a very diverse use of V-J genes in the SP samples from both the treated and control mice (Fig. 9A, upper graphs). Again, the V-J gene usage pattern was similar between the treatment and control PLN samples but was generally a more restrictive repertoire than was found in the SP (Fig. 9A, lower graphs). Interestingly, the IL-4 treatment group saw a drop in diabetes-associated Vβ13 usage, similar to the healthy repertoire (Fig. 9B). However, in this pathogenic-aged group, the effect was only seen in the PLN (Fig. 9B, bottom panel).

Given that, in the healthy repertoire control mice had shorter CDR3s with fewer random insertions, we hypothesized that this would also be the case in the pathogenic repertoire. Results show that, in the SP, CDR3 length is similar between treated and control mice (Fig. 9C, upper panel). Strikingly, however, the average CDR3 length from treated mice PLN samples was dramatically longer than that of controls (with all treated mouse T cells having a CDR3 length of 37 nucleotides or greater) (Fig. 9C, bottom panel). This trend was reflected in the number of random nucleotide insertions. Indeed, while the number of insertions in SP treated versus SP control samples were nearly identical, T cells from the PLN of treated mice had significantly more insertions (Fig. 9D). Interestingly, diversity in the SP of these aged mice was similar in both the control and treated groups (Fig. 9E, upper panel). However, in the PLN, diversity was limited in both groups, especially in the IL-4 treated group (Fig. 9E, lower panel). Together these results show that the intrathymic IL-4 treatment can noticeably alter the

TCR repertoire at the pathogenic stage. Interestingly, while the effects of IL-4 treatment on the CDR3 region were mostly seen in the SP at the healthy stage (Fig. 8), the opposite is true at the pathogenic stage (Fig. 9), where the significant effects of treatment can be seen within the PLN samples.

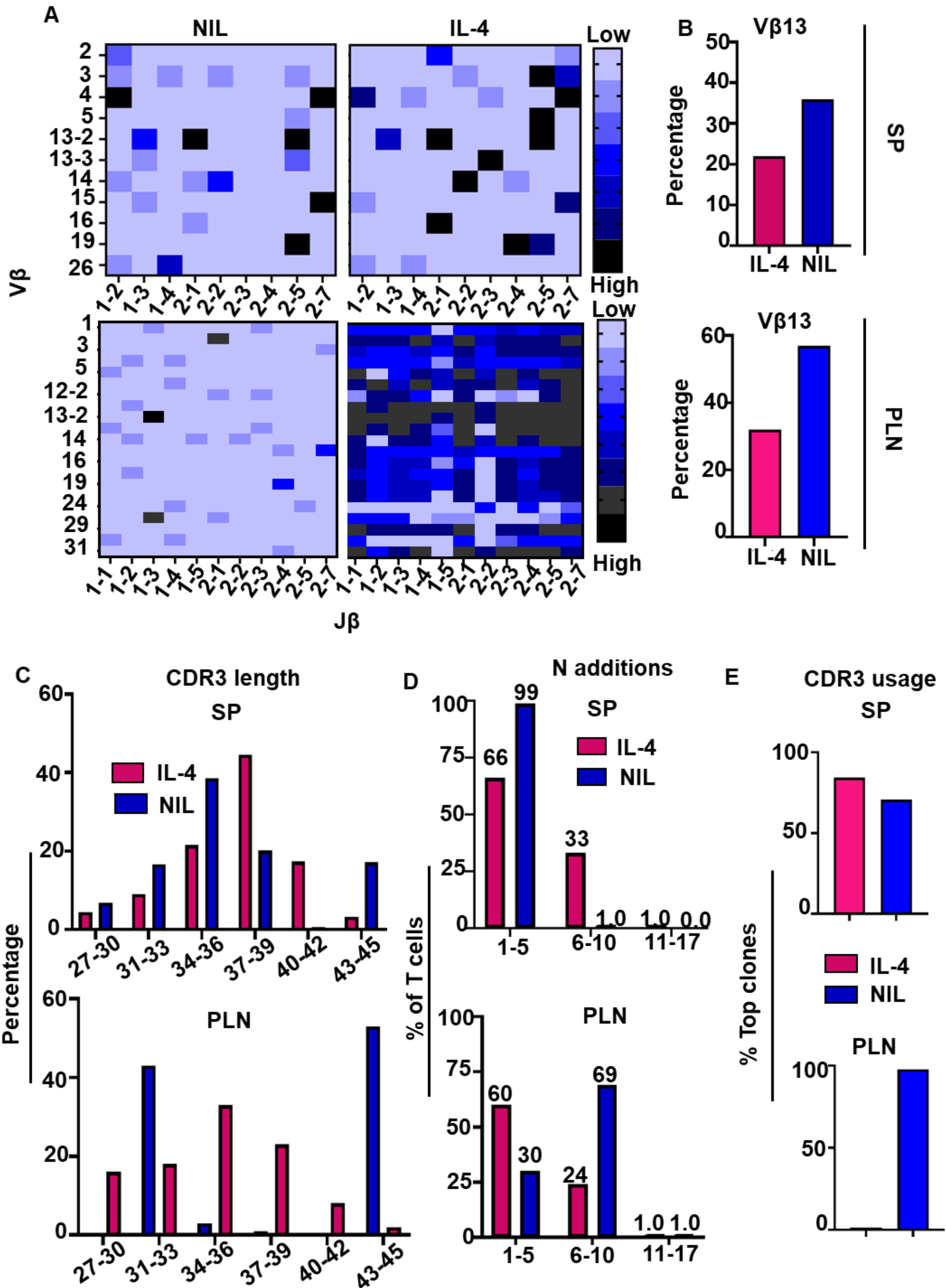
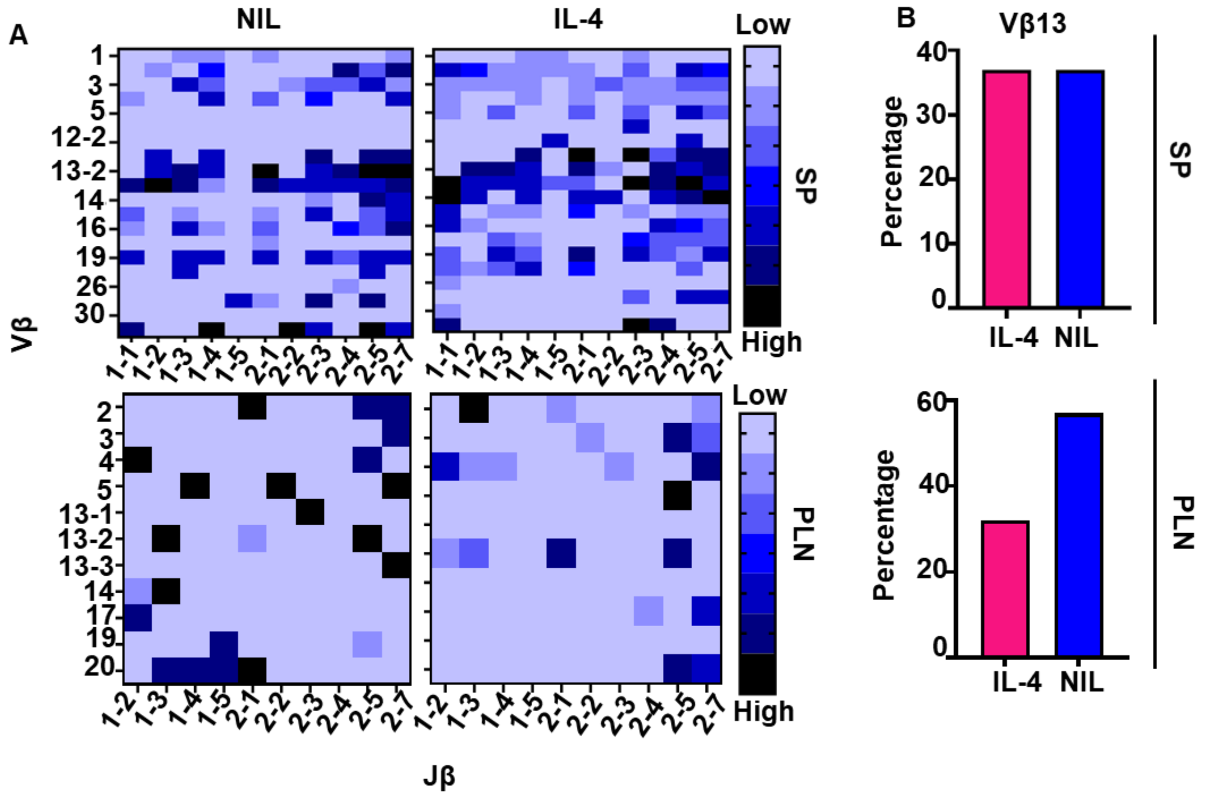


Figure 8. Intrathymic IL-4 influences the diversity of the TCRVβ repertoire prior to onset of T1D.

Four week old NOD mice (4 per group) were treated with intrathymic IL-4 or PBS once per week for two weeks. At 9 weeks of age priror to disease onset, the SP and PLN were harvested and used to sort TCRβ^{+} T cells. RNA isolated from T cells was utilized to generate cDNA libraries and the variable region of the TCRβ chain was sequenced by iRepertoire Inc. **(A)** Shows 2D heat maps of the relative frequency of V (y-axis) and J (x-axis) segments of TCRβ chain. V-J heat maps from the SP (top panels) and PLN (bottom panels) are illustrated. **(B)** Shows the frequency of Vβ13 usage in IL-4 versus PBS treated mice from SP and PLN T cells. **(C** and **D)** CDR3s were normalized such that each unique CDR3 is equal to a count of 1, regardless of total number of identical CDR3s. **(C)** Individual CDR3s were grouped on the basis of nucleotide numbers (length). The bars show the frequency of a particular CDR3 length among total number of CDR3s. **(D)** Shows the percentage of T cells with CDR3s encompassing different ranges of 'N' addtions. **(E)** Shows the percentage of cells with the 10 most frequent CDR3s among the total number of CDR3s read.



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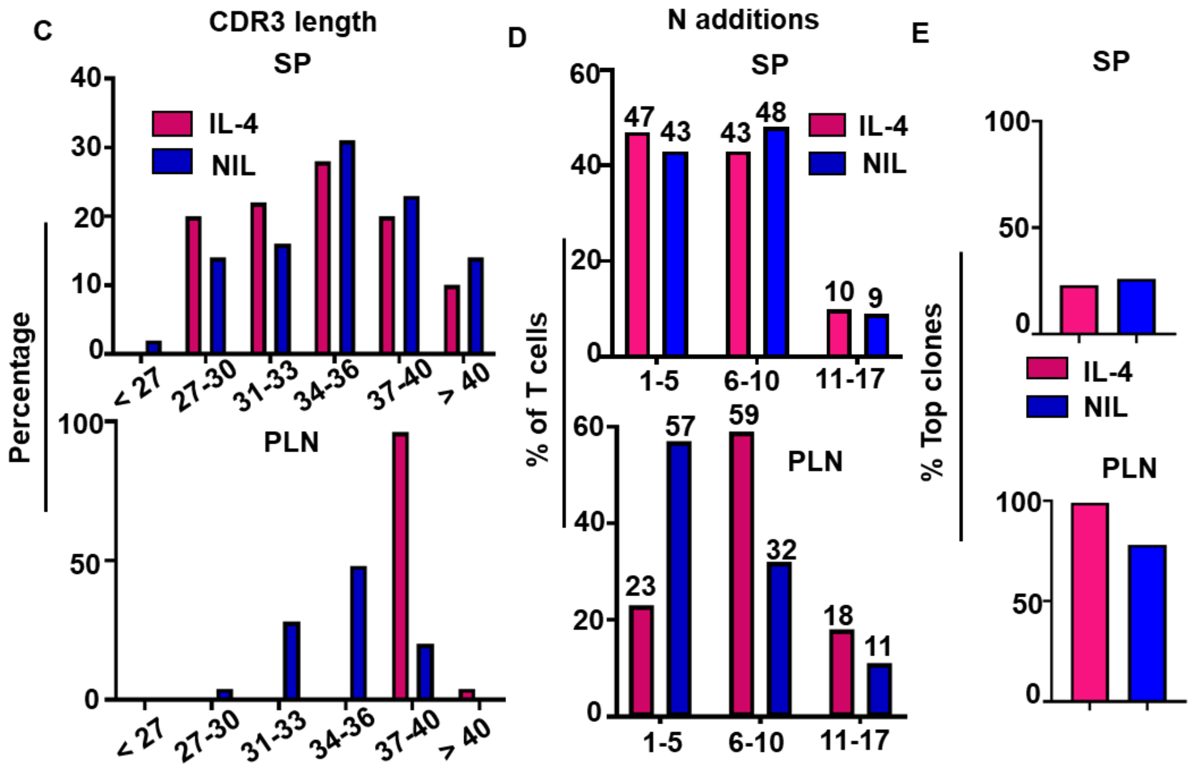

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Figure 9. Intrathymic IL-4 has a differential influence on the diversity of the TCRVβ repertoire at the onset of T1D.

Four week old NOD mice (4 per group) were treated with intrathymic IL-4 or PBS once per week for two weeks. At the onset of diabetes (12 weeks of age), the SP and PLN were harvested and used to sort $TCR\beta^{+}$ T cells. RNA isolated from T cells was utilized to generate cDNA libraries and the nucleotide sequence of variable region of the TCRβ chain was determined by iRepertoire Inc. **(A)** Shows 2D heat maps of the relative frequency of V (y-axis) and J (x-axis) segments of TCRβ chain. V-J heat maps from the SP (top panels) and PLN (bottom panels) are illustrated. **(B)** Shows the frequency of Vβ13 usage in IL-4 versus PBS treated mice from SP and PLN T cells. **(C** and **D)** CDR3s were normalized such that each unique CDR3 is equal to a count of 1, regardless of total number of identical CDR3s. **(C)** Individual CDR3s were grouped on the basis of nucleotide numbers (length). The bars show the frequency of a particular CDR3 length among total number of CDR3s. **(D)** Shows the percentage of T cells with CDR3s encompassing different ranges of 'N' additions. **(E)** Shows the percentage of cells with the 10 most frequent CDR3s among the total number of CDR3s read.

5. Discussion

In this chapter, the data shows that IL-4 fitness in the thymic microenvironment can have significant systemic effects on the host immune system. Specifically, in the diabetes-susceptible NOD mouse, scarcity of IL-4 can influence thymic multipotent progenitors, or ETP, lineage fate. The results herein indicate that it is the lack of thymic IL-4 in NOD mice that fails to drive HR signaling in ETPs, resulting in an increased thymic output of T cells progenitors at the expense of APCs.

The data indicates that this reduction in thymic IL-4 is likely due to a deficiency of iNKT2 cells in the NOD thymus. Interestingly, NOD-specific defects in the iNKT cell compartment is not a new concept. It has been shown that there is a systemic deficiency of this cell type near the onset of diabetes (20). In fact, stimulation of NOD iNKT cells with their cognate antigen can delay and reduce the incidence of T1D (21). Recent studies have shown that APCs are key cells in stimulating IL-4 production from thymic iNKT cells (22). This is an interesting concept as the presented study indicates that the NOD thymus is deprived of HR^{+}ETP-derived APCs. This phenomenon could represent a double-edged sword that leads to an endless cycle of fewer APCs and reduced functionality of iNKT cells.

An additional consequence of diminished thymic IL-4 fitness may be the reduced efficiency of negative T cell selection. Given that HR^{+}ETPs from the C57BL/6 strain yield myeloid cells with the capacity to drive negative selection, NOD mice, which lack this ETP-derived population, would likely have reduced selection

capabilities. The current interpretation of NOD-specific central tolerance deficits is ambiguous. Studies have shown that the NOD mouse thymus is profoundly disorganized and medullary thymic epithelial cells (mTECs) show phenotypic changes associated with diabetes onset (23, 24). Meanwhile, other studies suggest that NOD T cells can be successfully driven towards apoptosis during clonal deletion (25). Data presented herein supports both sides of the coin and indicates that the flaw in NOD central tolerance may be due to changes in the APC population that drives negative selection, rather than a defect in T cells themselves.

When the NOD thymus is supplemented with IL-4, HR^+ETPs give rise to myeloid cells which can contribute to negative selection, alter the T cell repertoire, and prevent the development of T1D. It is unlikely that this protective mechanism is due to the parallel reduction in T cell progenitors as HR^+ETPs only reflect a small portion of the total thymic progenitor pool. These observations lead to new conclusions about the role of the environment in central tolerance. Indeed, production of IL-4 likely plays a major role in shaping the T cell repertoire. This then leads to the question: how can environmental circumstances contribute to type II cytokine production within the thymus? Perhaps interplay between host microbiota and helminths can regulate a complex network cellular players that, in turn, alters the thymic microenvironment. Given this idea, one may envision that the influence of the host environment on central tolerance may represent newly exposed mechanism of environmental influence in autoimmunity.

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VITA

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